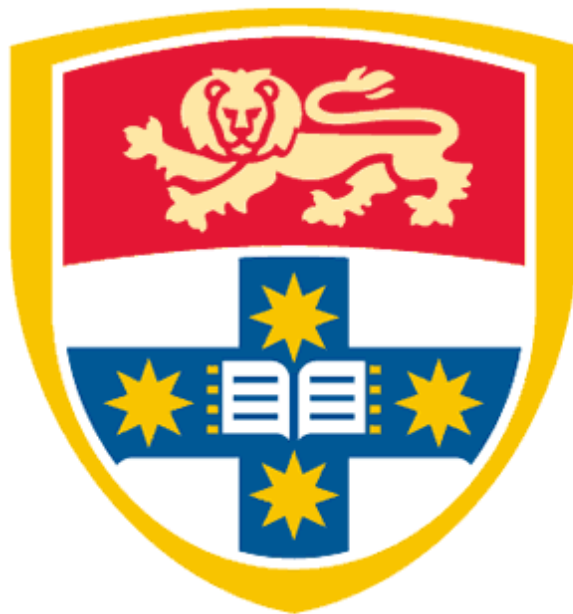


# **Neuronal Cell Death and Astrogliosis in the Developing Human Brain and Changes Seen in Sudden Infant Death Syndrome (SIDS)**



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Thesis submitted in fulfilment of the requirements for the  
degree of Doctor of Philosophy

September 2023

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## **DECLARATION**

I, Lauren Luijterink, hereby declare in accordance with the by-laws of the University of Sydney that all work in this thesis is entirely my own, unless otherwise acknowledged. This thesis was completed during my enrolment with the Department of Medicine from March 2020 to September 2023, under the supervision of A/Prof. Rita Machaalani and Prof. Karen A. Waters.

Human brain tissue was obtained from the Department of Forensic Medicine, Glebe, NSW, Australia, and Sheffield Children's Hospital, Sheffield, UK under ethics approval by the State Coroner, NSW Health and University of Sydney Ethics committees, and Health Research Authority (HRA), Health and Care Research Wales (HCRW) and Sheffield Children's NHS Foundation Trust respectively.

Tissue sectioning for the Australian based cohorts were performed prior to my joining the SIDS and Sleep Apnea Laboratory and were done so by Arunnjah Vivekanandarajah and Natalie Ambrose.

The piglet experimental protocols and subsequent brain tissue collection and embedment was also conducted prior to joining the SIDS and Sleep Apnea Laboratory.

This work has not been previously presented for any other degree or qualification, at this or any other university.

Lauren Luijterink.

### **Statement of originality**

This is to certify that to the best of my knowledge; the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Lauren Luijterink



## **AUTHORSHIP ATTRIBUTION STATEMENT**

Chapter 3 of this thesis is published as:

**Luijterink, L.L.M.**, Vivekanandarajah, A., Waters, K.A. and Machaalani, R., 2020. The  $\alpha 7$  and  $\beta 2$  nicotinic acetylcholine receptor subunits regulate apoptosis in the infant hippocampus, and in sudden infant death syndrome (SIDS). *Apoptosis*, 25, pp.574-589.

I co-designed and undertook experimental work for the study with the co-authors and undertook the analysis and drafting up of the manuscript.

Chapter 4 (section 4.1) is published as:

**Luijterink, L.**, Waters, K.A. and Machaalani, R., 2021. Immunostaining for NeuN does not show all mature and healthy neurons in the human and pig brain: focus on the hippocampus. *Applied Immunohistochemistry & Molecular Morphology*, 29(6), pp.e46-e56.

I co-designed the study with the co-authors, undertook the experimental work, analysis, and drafting up of the manuscript.

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author who in these cases is A/Prof Rita Machaalani.

Lauren Luijterink

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Rita Machaalani

## **ACKNOWLEDGMENTS**

I would first like to express my most sincere gratitude to my supervisor A/Prof Rita Machaalani for her continual support throughout my PhD journey. I am grateful for your immense patience, and the countless hours you have spent helping me navigate through the complexities of academia- I have been very fortunate to learn from your expertise. Your kindness in all areas of life and passion for research is something that has been continually inspiring.

To my co-supervisor, Prof. Karen Waters, thank you for your insightful feedback and ongoing support over the past few years. You have been an encouraging mentor throughout.

To Prof. Marta Cohen, my overseas co-supervisor during that time, thank you not only for your exceptional guidance, your hospitality and kindness. Your warm welcome and generosity is something that I will never forget.

To my co-authors, Arunnjah Vivekanandarajah and Michael Rodriguez, thank you for your valuable knowledge and input. Your feedback was very much appreciated and has helped me to improve my critical thinking and writing skills.

To Paul and his team at the Histopathology department at Sheffield Children's Hospital, thank you for your technical expertise and patience while training me. I gained valuable skills in tissue sectioning and immunohistochemistry during my time here.

To my fellow SIDS and Sleep Apnea Laboratory members, Natalie, Vanessa, Shannon, and Masarra- I don't know what I would have done without you and your support during these stressful times. I would like to extend my sincere gratitude to you and wish you all the best on your future endeavours.

I would like to extend my heartfelt appreciation to my family for their support, both in my academic pursuits and in every facet of my life. To my dad, thank you for your persistent belief in my academic potential and for instilling in me the values of hard work and perseverance. To my mum, thank you for your encouragement and endless kindness.

Finally, to Vekram, Monika, George, Lucy, Julia, Sarah, Margo, Shriya and Laura, I am extremely grateful for all of you during this time. Thank you for giving me the work-life balance I needed, and for always supporting me.

## **ABSTRACT**

**Introduction:** Sudden infant death syndrome (SIDS) is the sudden and unexplained death of a seemingly healthy infant. The mechanism of death is not known, although a major hypothesis is that these deaths are the result of abnormal brain control over cardiorespiratory, sleep, and arousal mechanisms in the face of a homeostatic stressor. Cell death is increased in the SIDS hippocampus; however, it is not known by which mechanism this occurs. Nicotinic acetylcholine receptors (nAChRs) are activated by nicotine, the major psychoactive component of cigarette smoke, which is a major risk factor for SIDS, and are known to have a role in regulating cell death. It is also not known whether SIDS infants also have a reduced number of hippocampal neurons, such as in the case of hippocampal sclerosis (HS), characterised by segmental neuron loss and astrogliosis. Astrogliosis is a broad marker of central nervous system (CNS) damage, involving broad molecular and functional changes (including characteristic astrocyte hypertrophy and proliferation), and has been studied in regions of the SIDS brain previously, including the hippocampus, with inconsistencies as to whether it occurs. Glial fibrillary Acidic Protein (GFAP) is a prototypical marker of astrogliosis given that its expression is proportionate to the degree of astrocyte hypertrophy and proliferation occurring and therefore allows for quantification that reflects the amount of activation. Quantitative standardised measures for diagnosing astrogliosis are lacking.

**Aims:** The first aim of this thesis was to investigate markers of cell death and nAChR subunits in the developing hippocampus, and determine whether there are changes associated with SIDS, or known SIDS risk factors. The second aim was to see if there is evidence of HS in SIDS. The third aim was to investigate astrogliosis in the hippocampus, but also extend to other regions in the developing human brain, including the medulla, cerebellum, and cortex, and determine whether there are any changes in SIDS or according to major SIDS risk factors. This was undertaken in two separate international cohorts characterised in the UK and Australia, after a sub-aim of standardising the method for assessing GFAP immunohistochemistry and identifying astrogliosis. Finally, this thesis compared the GFAP findings in the two cohorts to determine reproducibility and explored additional brain regions of the amygdala and basal ganglia in the Australian cohort, with the aim of determining any relationship between the GFAP findings and those for cell death and microglia previously studied in our laboratory using the exact same Australian cohort.

**Methods:** Both human (Chapters 3-4, 6-8) and piglet (Chapters 4-5) brain tissue samples were used for this thesis. The primary method used was immunohistochemistry, with the distribution of cell death markers (active caspase-3 and TUNEL), nAChR subunits ( $\alpha 7$  and  $\beta 2$ ), neurons (Neuronal Nuclei (NeuN)) and astrocytes (glial fibrillary acidic protein (GFAP)) explored in the hippocampus, and GFAP explored in a range of brain regions. Quantification was undertaken via microscopy image analyses, and statistics undertaken through SPSS.

**Results:** SIDS infants had increased markers of cell death throughout regions of the hippocampus compared to non-SIDS infants, with some changes in nAChR expression. Expression of cell death markers and nAChR correlated with one another and were also associated with the presence of various SIDS risk factors, most notably, the presence of an upper respiratory tract infection (URTI). HS could not be explored in the context of SIDS, given that preliminary studies in our laboratory found low immunostaining for the NeuN antibody. Parallel studies of NeuN in piglet brain tissue indicated lack of immunostaining to be due to tissue related parameters including fixation time, composition, and storage duration. An alternative means to study for cell loss utilising basic histochemical stain proved unreliable given that neuron counts differed widely between cases, as well as across serial sections within the same case. A novel method for assessing GFAP immunohistochemistry was devised and termed as Reactivity score (R-score) and was applied in subsequent chapters investigating astrogliosis. In the UK cohort, SIDS infants showed increased GFAP, indicative of astrogliosis in the internal granular layer of the cerebellum, CA4, temporal cortex and dorsal motor nucleus of the vagus (DMNV), with various changes according to major SIDS risk factors. These changes were not reproducible in the Australian cohort.

**Conclusion:** The specific mechanism for SIDS remains unknown, although this thesis supports the hypothesis that systems related to hypoxia and inflammation, due to the presence of the risk factors, are activated, leading to abnormal brain pathology in SIDS, including increased cell death, altered nAChRs, and astrogliosis.

## **PUBLICATIONS ARISING FROM THIS THESIS**

**Luijterink, L.L.M.**, Vivekanandarajah, A., Waters, K.A. and Machaalani, R., 2020. The  $\alpha 7$  and  $\beta 2$  nicotinic acetylcholine receptor subunits regulate apoptosis in the infant hippocampus, and in sudden infant death syndrome (SIDS). *Apoptosis*, 25, pp.574-589.

**Luijterink, L.**, Waters, K.A. and Machaalani, R., 2021. Immunostaining for NeuN does not show all mature and healthy neurons in the human and pig brain: focus on the hippocampus. *Applied Immunohistochemistry & Molecular Morphology*, 29(6), pp.e46-e56.

## **ABSTRACTS ARISING FROM THIS THESIS**

### **ORAL PRESENTATIONS**

Astrocytes (via GFAP expression) in the SIDS hippocampus; *International Stillbirth Alliance (ISA) and the International Society for the study and prevention of Perinatal and Infant Death (ISPID)*, Brisbane, Australia, November 2021.

Astrogliosis in the sudden infant death syndrome (SIDS) medulla- a comparison across 2 separate research cohorts; *ISA/ISPID*, Florence, Italy, October 2023.

### **POSTER ABSTRACTS**

Caveats to Use of the NeuN Antibody in Formalin-Fixed Human Infant Brain Tissue; *International Society for Neurochemistry (ISN)*, Honolulu, USA, October 2022.

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## **LIST OF ABBREVIATIONS**

|       |                                                                                         |
|-------|-----------------------------------------------------------------------------------------|
| AN    | Arcuate Nucleus                                                                         |
| CA    | Cornu Ammonis                                                                           |
| CNS   | Central Nervous System                                                                  |
| Cun   | Cuneate Nucleus                                                                         |
| DG    | Dentate Gyrus                                                                           |
| DMNV  | Dorsal Motor Nucleus of the Vagus                                                       |
| EC    | Entorhinal Cortex                                                                       |
| eSUDI | Explained Sudden Unexpected Death in Infancy                                            |
| FFPE  | Formalin Fixed Paraffin Embedded                                                        |
| GFAP  | Glial Fibrillary Acidic Protein                                                         |
| HS    | Hippocampal Sclerosis                                                                   |
| IGL   | Internal Granular Layer                                                                 |
| IHC   | Immunohistochemistry                                                                    |
| ION   | Inferior Olivary Nucleus                                                                |
| LRF   | Lateral Reticular Formation                                                             |
| ML    | Molecular Layer                                                                         |
| nAChR | Nicotinic Acetylcholine Receptor                                                        |
| NBF   | Neutral Buffered Formalin                                                               |
| NeuN  | Neuronal Nuclei                                                                         |
| NSTT  | Nucleus of the Spinal Trigeminal Tract                                                  |
| NTS   | Nucleus of the Solitary Tract                                                           |
| PCA   | Post-Conception Age                                                                     |
| PMI   | Post-mortem Interval                                                                    |
| SIDS  | Sudden Infant Death Syndrome                                                            |
| SUDI  | Sudden Unexpected Death in Infancy                                                      |
| TUNEL | Terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP-biotin Nick End-<br>Labelling |
| UD    | Undetermined/unascertained                                                              |
| URTI  | Upper Respiratory Tract Infection                                                       |
| uSUDI | Unexplained Sudden Unexpected Death in Infancy                                          |
| Vest  | Vestibular Nucleus                                                                      |

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# CHAPTER 1: INTRODUCTION

This thesis examines cell death in the hippocampus, and astrogliosis in the developing human brain, identifying variability amongst brain regions, and in sudden unexpected death in infancy.

The thesis begins with a review of SIDS, the relevant regions of the infant brain, neuronal apoptosis and hippocampal sclerosis (HS), and astrocytes and astrogliosis (Chapter 2).

The first study (Chapter 3) sought to investigate markers of cell death and nicotinic acetylcholine receptor (nAChR) subunits in the developing human hippocampus using immunohistochemical markers of active caspase-3, TUNEL,  $\alpha 7$  and  $\beta 2$  in 5 microscopically defined hippocampal regions. Given evidence of a role of nAChRs in regulating cell death, this study also sought to determine whether their expression was correlated, indicating a possible mechanism by which cell death occurs in the infant brain. Expression was further analysed according to the diagnosis of sudden infant death syndrome (SIDS) and the presence of SIDS risk factors. The findings suggest that increased cell death in the SIDS hippocampus occurs via nAChRs (Luijck et al., 2020).

The second study (Chapter 4) aimed to determine whether in addition to cell death, there was evidence of hippocampal sclerosis (HS) in SIDS, characterised by segmental neuron loss, using Neuronal-Nuclei (NeuN) immunohistochemistry. This study cautions against the use of NeuN in formalin fixed paraffin embedded (FFPE) tissue (Luijck et al., 2021).

The third study (Chapter 5) introduces a novel method to quantify GFAP immunohistochemistry, termed reactivity (R)-score, and tests the reproducibility of this method against other commonly used methods of quantification. The R-score aims to standardise typical subjective assessments used when diagnosing astrogliosis and its severity.

The fourth study (Chapter 6) aimed to investigate astrogliosis via GFAP immunohistochemistry in 21 microscopically defined regions in the developing human brain, including the cerebellum, hippocampus and medulla, and determine whether there are any changes in SIDS, or according to major SIDS risk factors. This study was the first to quantitatively investigate GFAP in as many regions of the infant brain, and identifies changes associated with postnatal development and SIDS.

The fifth study (Chapter 7) sought to determine whether the findings in Chapter 6 were reproducible in a separate research cohort, with investigation into additional brain regions of the amygdala and basal ganglia. Changes in expression with age and development were maintained, although changes according to diagnosis of SIDS and risk factors were not.

Chapter 8 combined and analysed data from the previous two chapters and re-ran analyses to compare the eSUDI and SIDS groups as one cohort. Further analyses included comparing the findings across the separate cohorts and found large variation within and between SUDI cohorts.

The thesis concludes with a summary chapter (Chapter 9) that discusses collective findings from these studies and suggests the future directions for research. After discussing patterns of cell death, nAChR and GFAP in the developing human brain, regions are discussed in light of SIDS and its major risk factors. While we saw many changes in the SIDS brain and according to the presence of risk factors, there are major inconsistencies between cohorts. The next step in research will be to stratify infants with astrogliosis by investigate additional markers to help identify the different mechanisms of death occurring (i.e., hypoxic vs infection/inflammation), and consider other means of measuring GFAP, including its expression in serum.

## **CHAPTER 2: LITERATURE REVIEW**

### **2.1: SUDDEN UNEXPECTED DEATH IN INFANCY (SUDI)**

#### **2.1.1: DEFINITIONS AND CLASSIFICATIONS**

Sudden unexpected death in infancy (SUDI) is a broad term that came to existence in 1953, applying to all deaths that occur suddenly and unexpectedly between 7 and 365 days of life (Werne and Garrow, 1953, Garrow and Werne, 1953). Following investigation, these deaths may either become explained (eSUDI), such as in the case of a cardiovascular anomaly, infection, or homicide, or can remain unexplained (uSUDI) such as in the case of sudden infant death syndrome (SIDS) or cases that remain undetermined/unclassified (UD).

##### **2.1.1.1: UNEXPLAINED SUDI SUB-CATEGORIES**

Unexplained SUDI cases account for approximately 80% of all SUDI deaths (Côté et al., 1999, Loughrey et al., 2005), although this varies by country, with SIDS on its own accounting for as low as 32.6% of SUDI cases in Japan and as high as 72.5% of SUDI cases in Germany (Taylor et al., 2015).

##### **2.1.1.1.1: SUDDEN INFANT DEATH SYNDROME (SIDS)**

SIDS was initially a term applied to replace “Cot Death”, with the first official definition of SIDS proposed in 1969, denoting overlapping clinical, epidemiological, and pathological features. In 1969, SIDS was defined as “the sudden death of any infant or young child, which is unexpected by history, and in which a thorough post-mortem examination fails to demonstrate an adequate cause for death,” (Beckwith, 1973). In 1991 the US National Institute of Child Health and Human Development (NICHD) specified that SIDS is limited to infants <1 year of age who undergo autopsy, death scene investigation and review of the clinical history (Willinger et al., 1991). This specification led to a growing body of research that associated SIDS with specific risk factors, such as ~95% of SIDS deaths occurring in the first six months of life, with a peak incidence between 2 to 4 months (Fleming et al., 2015).

In 2004, an expert panel met in San Diego to re-evaluate the definition of SIDS, attempting to standardise the definition for diagnostic and research purposes. The panel proposed that SIDS is “the sudden death of an infant under one year of age, with onset of the fatal episode occurring during sleep, that remains unexplained after a thorough case investigation, including



performance of a complete autopsy, examination of the death scene, and review of the clinical history,” (Krous et al., 2004). Thus, the proposed definition added an apparent association with sleep, and emphasised that the death scene investigation should review the entire circumstances of death. The San Diego panel further sub-categorised SUDI deaths of SIDS IA (classic features with a complete investigation), SIDS IB (classic features with an incomplete investigation), SIDS II (outside typical age range and/or genetic environmental conditions present that may have contributed to death) and unclassified. The purpose of this classification system is to improve the consistency and consensus in diagnosing cases for research and epidemiological purposes by ensuring comparability of groups between different studies and cohorts. The SIDS 1A group contains very narrow eligibility criteria, and due to the fact that SIDS 1B group differs only in that contains 1 incomplete test, research cohorts that have applied this diagnostic classification have combined the SIDS 1A and B categories ((e.g. (Ambrose et al., 2018)).

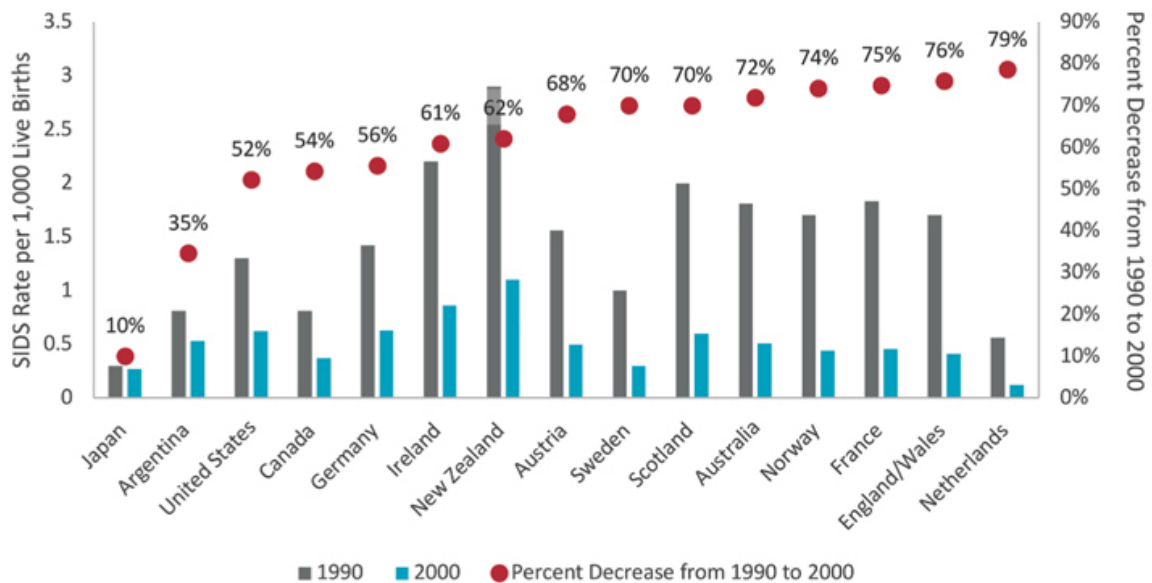
#### **2.1.1.1.2: UNDETERMINED/UNCLASSIFIED (UD)**

UD is a non-specific diagnosis and includes instances where the investigation remains incomplete, autopsies were not performed, or the circumstances of death are not well identified (Krous et al., 2004). UD classifications can be difficult for parents as they can lead to confusion about terminology, vulnerabilities to social stigma and self-blame (Byard, 2009, Crandall et al., 2017). Use of these terms provide further difficulties for organizations when providing bereavement support and education (Crandall et al., 2017), and inconsistencies in applying this diagnosis can limit comparability of results between research cohorts.

#### **2.1.2: SIDS EPIDEMIOLOGY**

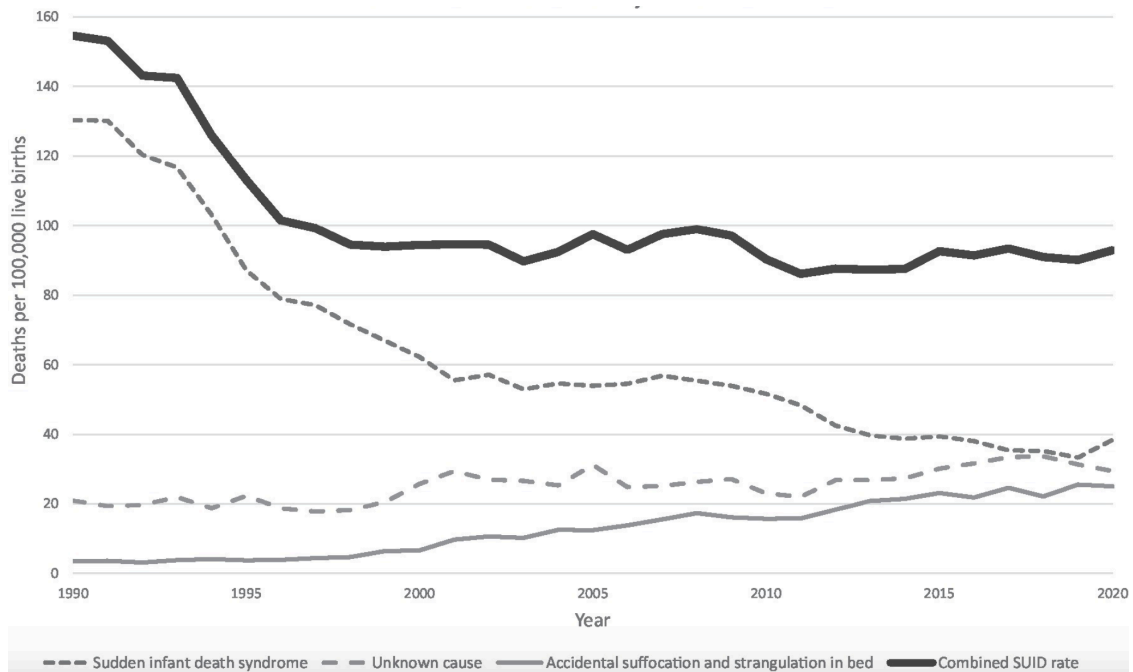
As of 2017, in Australia, the incidence of SUDI is 3.1 deaths per 1000 live births, of which 34% are SIDS or UD (or 1 death per 1000 live births) (Health and Welfare, 2022), a dramatic reduction from 2 deaths per 1000 live births prior to the implementation of national risk reduction campaigns such as the 1991 ‘Back to Sleep’ campaign. These campaigns advised safe-sleep practices for infants, including the recommendation that infants should not be placed prone to sleep (Mitchell et al., 1992), and should instead be placed on their back. Similar risk reduction campaigns were implemented across many other developed countries during the same period, with SIDS incidence ranging between 0.30 and 2.90 deaths per 1,000 live births in 1990 in Japan and New Zealand respectively and dropping to 0.12 to 1.10 deaths per 1000

live births in the Netherlands and New Zealand by 2000 (Figure 2.1). Following the 1994 ‘Back to Sleep’ campaign in the United States, the percentage of SIDS infants placed prone to sleep decreased by more than 50%, while those infants placed supine increased from 1.9% to 41.7% (Trachtenberg et al., 2012). SIDS incidence in the US subsequently declined by more than 50% (Syndrome, 2005).



**Figure 2.1: International SIDS rates from 1990-2000.** Percentage decrease annotated. Taken from (Shapiro-Mendoza et al., 2018).

However, a ‘diagnostic shift’ towards application of UD to cases that would have previously been diagnosed as SIDS may account at least in part for this reduction in incidence (Shapiro-Mendoza et al. 2018). This is supported by data that shows that uSUDI rates have been steady when considering the summation of UD and SIDS (Figure 2.2). As such, SIDS remains a leading cause of post-neonatal death in many developed countries.



**Figure 2.2: US mortality rates for SUID (aka SUDI) and its components in the “Back to Sleep” era.** Taken from (Wojcik et al., 2023).

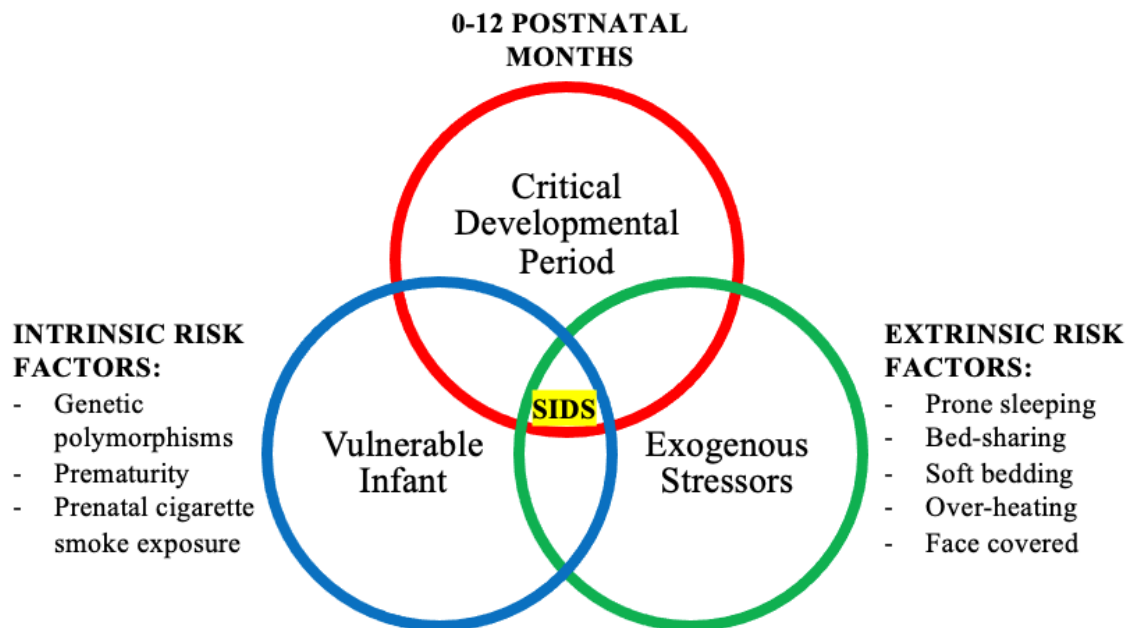
### 2.1.3: MECHANISM OF SIDS

A number of hypotheses have been proposed to explain the mechanism of death in SIDS, including: airway obstruction (Tonkin, 1975), thermal stress (Sawczenko and Fleming, 1996), infection/inflammation (Goldwater, 2017, Blackwell, 2004), apnea (Steinschneider, 1972) and cardiac arrhythmia/long QT syndrome (Schwartz, 1976, Schwartz et al., 1998). Many of these mechanisms remain hypothesis-driven and not supported by available epidemiological data (reviewed in (Mitchell, 2009)). Research continues to help find an explanation for these deaths. The fields of research within SIDS span epidemiology, pathology, physiology and education (Duncan and Byard, 2018). Through these fields, SIDS has come to be known to be associated with certain risk factors and characteristics which will be discussed below.

#### 2.1.3.1: TRIPLE RISK MODEL

A triple risk model was proposed initially in 1972 (Wedgwood, 1972), and subsequently added on in 1994 (Wedgwood, 1972, Filiano and Kinney, 1994), suggesting that SIDS occurs when three overlapping factors coincide; (1) a vulnerable infant with an underlying susceptibility (e.g. genetic polymorphisms, pre-natal exposure to cigarette smoke, prematurity (*intrinsic* risk factors)), (2) a critical developmental period (0-12 months of age), given that major changes in sleep and circadian functions, thermoregulation, and respiratory and cardiovascular function

occur during the first year of life (Gaultier, 1995), and (3) an exogenous stressor(s) (e.g. co-sleeping, sleeping prone, face-covered (*extrinsic* risk factors)) (Figure 2.3). This model incorporates epidemiological, physiological, and neuropathological data, and emphasises the multifactorial nature of SIDS. As such, SIDS is likely to be the end-point of a heterogeneous group of pathophysiologies, rather than the result of a single underlying cause (Byard and Jensen, 2008).



**Figure 2.3: The SIDS Triple Risk Model.** Adapted from Filiano and Kinney, 1994.

International risk-reduction campaigns throughout the past few decades have successfully reduced the incidence of SIDS by targeting extrinsic risk factors (see section 2.1.2.3). Filiano and Kinney (1994) suggested that the incidence of SIDS might further be avoided when the underlying vulnerability can be detected early and targeted through intervention strategies and therapeutic agents. However, there are currently no physiological biomarkers to assist with early pre-clinical diagnosis or assessment of individual susceptibility.

### **2.1.3.2: RISK FACTORS**

SIDS has been associated with several predominant characteristics and risk factors, including, a peak incidence at 2-4 postnatal months (Malloy and Hoffman, 1995), male gender (Mitchell and Stewart, 1997), ethnic background, and socioeconomic disadvantage (Blackwell et al., 2004).

Risk factors identified from epidemiological studies have been categorised as modifiable vs non-modifiable (Table 2.1). The main modifiable risk factors relate to parameters of sleep (i.e., prone sleeping, co-sleeping/bed-sharing, soft sleeping surfaces/soft bedding, and no pacifier use at bedtime), and exposure to cigarette smoke (Trachtenberg et al., 2012). Major non-modifiable risk factors include prematurity (Malloy and Hoffman, 1995) and recent infection (Blackwell, 2004).

**Table 2.1: Summary of modifiable and non-modifiable SIDS risk factors.**

| <b>Non-modifiable</b>                                                           | <b>Modifiable</b>                |
|---------------------------------------------------------------------------------|----------------------------------|
| Male sex                                                                        | Prone and side sleeping          |
| Age (2-4 months)                                                                | Bed-sharing and co sleeping      |
| Prematurity                                                                     | Cigarette smoke exposure         |
| Other genetic associations (i.e., cardiac channelopathy, serotonin transporter) | Not breastfeeding                |
| Low birth weight                                                                | Parental drug and/or alcohol use |
| Recent infection                                                                | Room temperature and overheating |
|                                                                                 | Soft sleep surface               |
|                                                                                 | Excessive bedding                |
|                                                                                 | Face covering                    |

#### **2.1.3.2.1: UNSAFE SLEEP ENVIRONMENT**

Prone sleeping has been identified as the main modifiable risk factor for SIDS (Mitchell et al., 1991) and parents are recommended to place infants to sleep on their back, rather than on their stomach. The proposed pathophysiological mechanism of death associated with sleeping prone is that it involves rebreathing of exhaled carbon dioxide, leading to an asphyxiating (hypoxic and hypercapnic) microenvironment (Campbell et al., 1997). Death as a result of this is normally unlikely due to regulation of appropriate arousal or ventilatory responses, however, during the first year of life, autonomic functions are still developing and infants are at an increased risk of cardio-respiratory dysregulation, particularly during sleep (Horne, 2014). Placing an infant to sleep on their side is also not recommended, given that this increases the risk that they will roll onto their stomach. Epidemiological data supports that the risk of sleeping prone is enhanced by thermal stress and excessive thermal insulation, which can occur during winter months, in cold latitudes, higher altitudes and in circumstances where the infant is unwell or has excessive bedding/bundling (Mitchell, 2009, Bach and Libert, 2022).

Bed-sharing has been associated with a significantly increased risk of SIDS (McIntosh et al., 2009, Scheers et al., 2003) and is hypothesised to be the result of accidental asphyxia, which may not be distinguishable at autopsy. The risk of suffocation is up to 40 times higher for infants sleeping in adult beds compared to those in cribs (Scheers et al., 2003), and the increased risk of SIDS when co-sleeping is likely compounded by other risk factors such as parental cigarette and alcohol usage (Carpenter et al., 2013).

#### **2.1.3.2.2: CIGARETTE SMOKE EXPOSURE**

Cigarette smoking is the largest modifiable risk-factor for pregnancy-related morbidity and mortality in developed countries (Benowitz and Dempsey, 2004) and the second major modifiable risk factor for SIDS (Mitchell and Milerad, 2006). Prenatal cigarette smoke exposure has been shown to lead to long-term alterations in blood pressure and heart rate response (for example during hypoxic conditions) in infants (Cohen et al., 2010, Søvik et al., 2001). Infants with pre-natal exposure to cigarette smoke are at a 4-times greater risk of SIDS than those without, and infants with postnatal exposure are 1.5-times more at risk (Mitchell and Milerad, 2006). This effect is dose-dependent, whereby the more cigarettes smoked, the greater the risk of SIDS (Mitchell and Milerad, 2006).

#### **2.1.3.2.3: INFECTION**

One hypothesis is that infection may have a causative association with SIDS. Viral and bacterial upper respiratory tract infections (URTI) have been noted in many SIDS cases (McKendrick et al., 1992, Morris, 1999), and the fact that SIDS incidence peaks during winter months was supportive of this relationship (Morris, 1999). Downstream inflammatory responses have been suggested to trigger the events leading to SIDS (Lundemose et al., 1993, Vege et al., 1995, Raza and Blackwell, 1999, Ferrante et al., 2008, Moscovis et al., 2015), and in a study over 6 decades ago, 84% of uSUDI cases showed microscopic inflammatory changes in at least 1 site of the respiratory tract, with many other organs also demonstrating evidence of inflammatory disease (Adelson and Kinney, 1956). It is subsequently suggested that an infant with an infection may be at heightened risk of cardiorespiratory and arousal dysfunction, particularly if other risk factors, such as prone sleeping, are present (Ponsonby et al., 1993). Detection of a virus at post-mortem examination does not necessarily indicate infection, however, nor does it indicate the role of the virus in the mechanism of death. In a more recent study that examined virological tests amongst 490 SUDI infants, there were no differences in

the frequency of virus detection between eSUDI due to non-infective causes and uSUDI (Weber et al., 2010). Viruses identified in SIDS infants with no evidence of viral infection or other clinical history may in fact represent normal/asymptomatic carriage that did not contribute to the cause of death. Thus, whether URTI has a causative association with SIDS is not known.

#### **2.1.4: SIDS NEUROPATHOLOGY**

SIDS deaths occur during a sleep period and a major hypothesis is that these deaths are the result of abnormal brain control over cardiorespiratory, sleep/arousal and homeostatic responses in the face of an exogenous homeostatic stressor (Hunt, 1992). The brainstem is the major brain site responsible for regulating these processes (Kandel et al., 2000, Gozal et al., 1994, Yuan and Silberstein, 2016, Ciriello and Calaresu, 1977, Cutsforth-Gregory and Benarroch, 2017), and brainstem impairments are known to result in sudden death (Talman and Lin, 2013, Jaster, 2001, Rhodes and Wightman, 2000). Sub-clinical defects in cardiorespiratory and/or arousal mechanisms consistent with brainstem dysfunction have been reported in infants who subsequently died of SIDS (Franco et al., 1999, Schechtman et al., 1996, Kahn et al., 1992, Southall, 1988), and as such, study of the SIDS brain has been undertaken with major focus on the brainstem. These studies have primarily investigated neurochemical abnormalities and neuronal cell death (reviewed in (Machaalani and Waters, 2014)).

Over the past two decades, the hippocampus (reviewed (Kinney et al., 2018)) and hypothalamus (reviewed (Waters et al., 2018)) have been included as regions of interest in SIDS; the hippocampus due to its ability to affect cardiorespiratory functions via projection to key brainstem nuclei (Ruit and Neafsey, 1988), and the hypothalamus due to its role in important functions such as maintaining energy homeostasis, circadian rhythms, stress response and growth/reproductive behaviours (Burbridge et al., 2011). Majority of studies to date have investigated neuronal cells within these brain regions. More recent data has examined microglia and astrocytes, and with their increasing relevance in neuroscience more broadly, it is now clear that studying these cells is imperative.

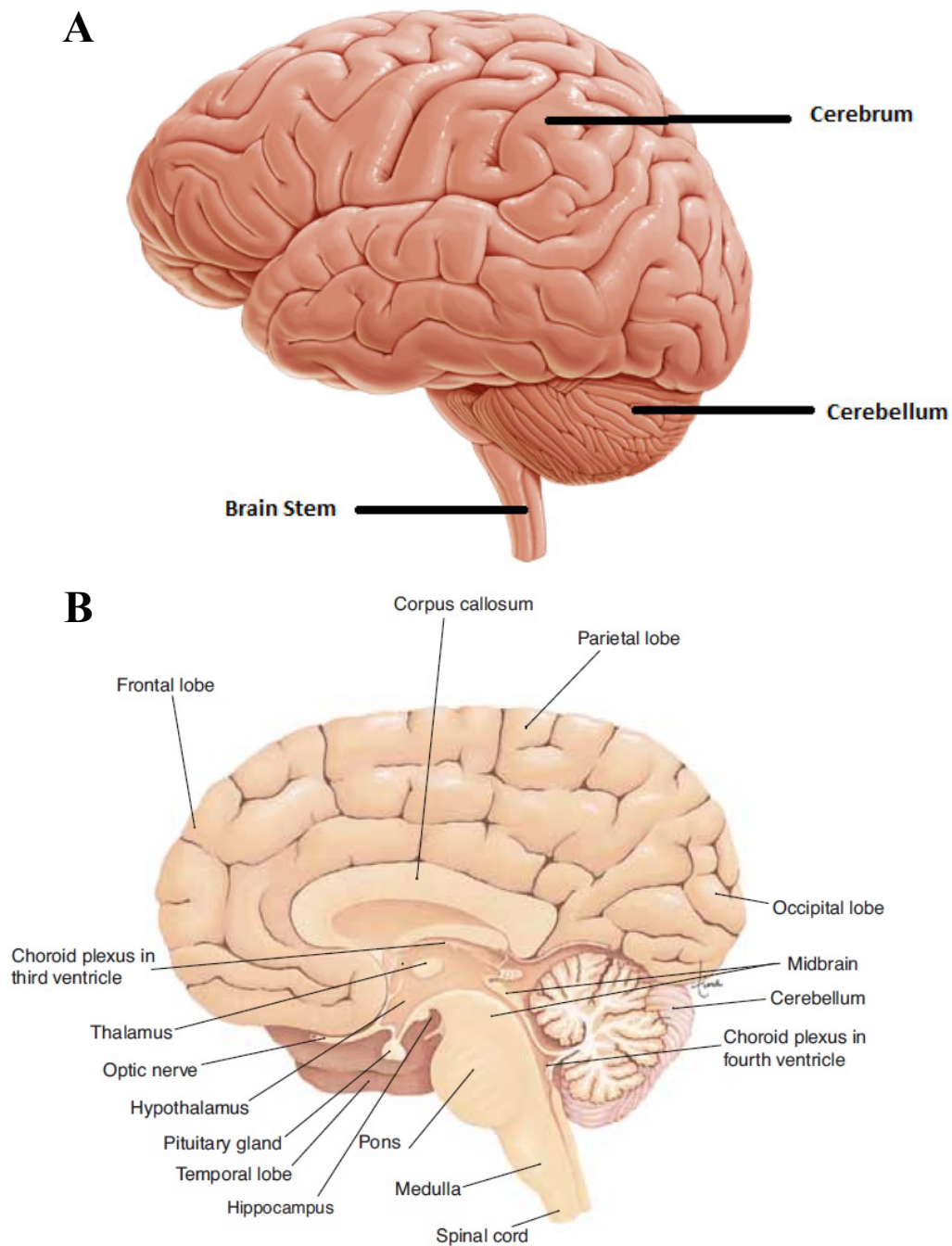
This thesis will examine neuronal cell death in the hippocampus and will look at astrocytes in a range of brain regions. Given methodologies for quantifying these cells is broad, an aspect of

this thesis will also aim to standardise methods for assessment. Brain regions that will be investigated in this thesis in the context of SIDS will be described (Section 2.2).

## **2.2: THE HUMAN BRAIN AND RELEVANT SUB-REGIONS IN SIDS**

The human brain broadly comprises 3 major divisions: the cerebrum, brainstem and cerebellum (Figure 2.4a). The cerebrum is the largest structure consisting of grey matter (cerebral cortex comprised of neuronal cell bodies), white matter (comprised of myelinated axons), and subcortical structures (deep grey and white-matter structures). The cerebrum is separated into (almost) symmetrical left and right hemispheres by the longitudinal fissure and connected via the corpus callosum, and can be further subdivided into 4 lobes: frontal, parietal, occipital and temporal. The subcortical structures sit beneath the cerebral cortex and include (although not limited to) the hippocampus, basal ganglia, amygdala, thalamus, hypothalamus, and pituitary gland. Surrounding the brain and spinal cord are the brain ventricles, cavities that store cerebral spinal fluid, which is produced in the choroid plexus (Figure 2.4b).

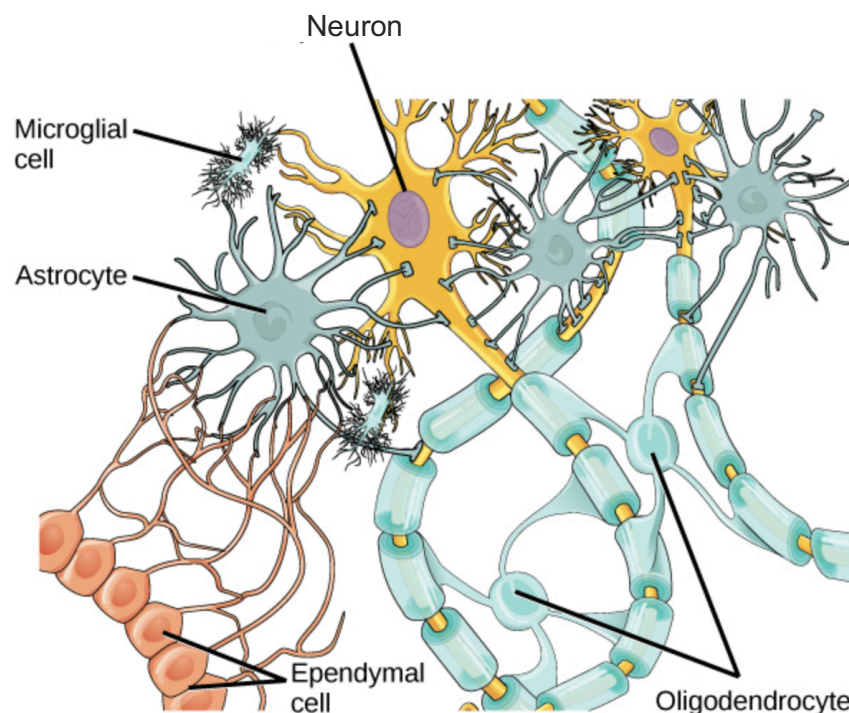




**Figure 2.4: Major structures of the human brain. (A)** Major brain sub-divisions, including the cerebrum brainstem and cerebellum, and **(B)**, several of its sub-structures. **A** taken from <https://www.simplypsychology.org/forebrain-midbrain-hindbrain.html>; **B** taken from [https://www.brainkart.com/article/The-Brain---Anatomy-and-Physiology\\_18710/](https://www.brainkart.com/article/The-Brain---Anatomy-and-Physiology_18710/).

The cells of the brain can be categorised as either neuronal or glial-type cells (Figure 2.5). Neurons, which tend to be larger in size, are electrically excitable and can generate action

potentials, allowing them to communicate with each other by sending and receiving chemical signals such as neurotransmitters (Meinertzhagen et al., 2009). Glial cells are non-neuronal cells of the brain, and include astrocytes, oligodendrocytes, microglia, and ependymal lineage cells. Broadly, the role of astrocytes and oligodendrocytes is to provide structural and functional support to neurons (Allen and Lyons, 2018). Microglia are the brain's resident immune cells, although also have other important roles in maintaining tissue homeostasis by regulating neuronal apoptosis, neurogenesis, synaptic pruning, axonal guidance and the formation and maintenance of neuronal circuits (reviewed in (Kierdorf and Prinz, 2017)). Further details on Neurons and Astrocytes will be provided in sections 2.3 and 2.4 respectively.



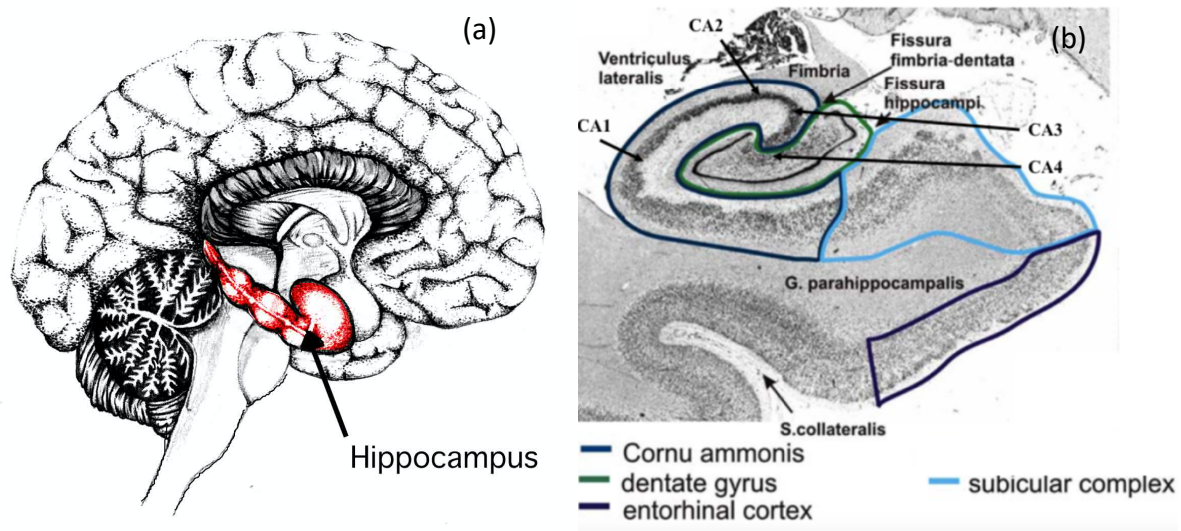
**Figure 2.5: Major cell-types in the brain.** Illustrations of neurons and glia (astrocytes, oligodendrocytes, microglia and ependymal cells) are denoted. Image adapted from <https://opentextbc.ca/biology/chapter/16-1-neurons-and-glial-cells/>.

The 3 brain regions to be focused on in this thesis are the hippocampus, cerebellum, and medulla oblongata, given all 3 were found to have abnormalities in SIDS (reviewed (Bright et al., 2018b)) as summarised below.

### 2.2.1: HIPPOCAMPUS

The hippocampal formation comprises a group of structures located deep within the temporal lobe of each cerebral cortex (Figure 2.6a), with the hippocampus being one of the major

structures within. There is a lack of consensus regarding which structures are included in the hippocampal formation, although the Dentate Gyrus (DG), Cornu Ammonis (CA)1, CA2, CA3, CA4, subicular complex (subiculum, presubiculum and parasubiculum), and entorhinal cortex (EC) are often included (Figure 2.6b) (Amunts et al., 2005, Duvernoy, 2013). The hippocampus refers specifically to the DG and the hippocampus proper (CA subfields), and forms part of the limbic system. The hippocampus will be a major area of interest throughout this thesis and thus, will be discussed in detail below.



**Figure 2.6: The human hippocampus.** (a) The human hippocampus, embedded deep within the temporal lobe of the cerebral cortex, taken from <https://fli.institute/2016/09/18/brains-hippocampus-helps-fill-in-the-blanks-of-language/>. (b) Subregions of the human hippocampal formation (Amunts et al., 2005).

The hippocampus is predominantly known for its role in learning, memory and spatial navigation (Scoville and Milner, 1957, Broadbent et al., 2004, O'Keefe and Dostrovsky, 1971), although may also have a role in regulating sympathetic activities, such as cardiorespiratory function, via projections to brainstem nuclei (Ruit and Neafsey, 1988, Zagon et al., 1994, Poe et al., 1996). Stimulation of hippocampal circuits was initially shown to lead to decreased heart rate, blood pressure, and slower respirations in anaesthetised rats (Ruit and Neafsey, 1988) and subsequent studies show that structures such as the amygdala, hippocampus and EC are connected via their projections to the medulla (Zagon et al., 1994, Edlow et al., 2016), the major brain anatomical site responsible for regulating cardiorespiratory control. The hippocampus has also been shown to contribute to the initiation of inspiratory effort during

transient respiratory events in both animal (Poe et al., 1996, Ajayi et al., 2018, Ajayi and Mills, 2017) and human studies (Kaada and Jasper, 1952, Herrero et al., 2018), with several regions of the hippocampus vulnerable to damage following hypoxia, including structural changes and a reduction in neurogenesis and synaptic plasticity in the DG (Cha et al., 2017, Khuu et al., 2019), and abnormal synaptic transmission, increased blood vessel density and cell death in the CA1 (Schneider Gasser et al., 2018, Yang et al., 2018).

Although the hippocampus is a highly integrated structure, there are regional variations in function. Additionally, different sub-regions are differentially targeted by ageing and disease. The main functions of each hippocampal region (and temporal cortex) are summarised in Table 2.2 below.

**Table 2.2: Summary of functions of the hippocampal formation and temporal cortex.**

| <b>Region</b>             | <b>Cytoarchitecture</b>                                                                                                                                                             | <b>Function</b>                                                                         | <b>Reference</b>                                                                                                               |
|---------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|
| <b>Dentate Gyrus (DG)</b> | Granule cells (immature & mature) with mossy fibre axons that project to CA3 pyramidal cells                                                                                        | Site of neurogenesis (ongoing into adulthood)                                           | (Ming and Song, 2005)                                                                                                          |
|                           |                                                                                                                                                                                     | Encoding of sensory inputs (vestibular, olfactory, visual, auditory, and somatosensory) | (Castle et al., 2005) (Morris et al., 2013)                                                                                    |
|                           |                                                                                                                                                                                     | Role in spatial/pattern separation                                                      | (O'reilly and McClelland, 1994) (Shapiro and Olton, 1994) (Gilbert et al., 2001); (Anderson and Jeffery, 2003); (Weeden, 2012) |
|                           |                                                                                                                                                                                     | Role in object recognition                                                              | (Spanswick and Sutherland, 2010)                                                                                               |
| <b>CA4</b>                | Large ovoid somata amongst myelinated mossy fibres                                                                                                                                  | Role in acquisition of declarative memories                                             | (Coras et al., 2014)                                                                                                           |
|                           |                                                                                                                                                                                     | Vulnerable to brain ageing process                                                      | (Jono et al., 2002)                                                                                                            |
|                           |                                                                                                                                                                                     | Hypoxia sensitive (affected within hours of hypoxic/ischemic insult)                    | (Kirino, 1982)                                                                                                                 |
| <b>CA3</b>                | Pyramidal cells here project to the CA1 region via the Schaffer Collateral Axons. Also contains fine, non-myelinated mossy fibres which form stratum lucidum, characteristic of CA3 | Role in spatial/pattern separation                                                      | (Barbosa et al., 2012)                                                                                                         |
|                           |                                                                                                                                                                                     | Memory coding and retrieval                                                             | (Seok and Cheong, 2020)                                                                                                        |
|                           |                                                                                                                                                                                     | Role in conditioned fear via connections to amygdala                                    | (Wen et al., 2015)                                                                                                             |
|                           |                                                                                                                                                                                     | Site of seizure propagation                                                             | (Lévesque et al., 2012)                                                                                                        |
| <b>CA2</b>                | Dense and narrow with large ovoid somata. Receive Schaffer collateral input from CA3 neurons                                                                                        | Less vulnerable to damage than other CA regions                                         | (Chevaleyre and Siegelbaum, 2010, Dudek et al., 2016)                                                                          |
|                           |                                                                                                                                                                                     | Role in social behaviour and episodic memory                                            | (DeVito et al., 2009)                                                                                                          |
|                           |                                                                                                                                                                                     | Memory coding and retrieval                                                             | (Seok and Cheong, 2020)                                                                                                        |
| <b>CA1</b>                |                                                                                                                                                                                     | Temporal context of events and detecting spatial novelty                                | (Barbosa et al., 2012)                                                                                                         |

|                               |                                                                                                                                                              |                                                                                                                      |                                                                 |
|-------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|
|                               | Small, scattered pyramidal cells with triangular somata that project excitatory input to subiculum                                                           | Regulation of cardio-respiratory functions                                                                           | (Ruit and Neafsey, 1988) (Ajayi et al., 2018, Poe et al., 1996) |
|                               |                                                                                                                                                              | Consolidating memories and episodic memory processing & retrieval                                                    | (Hunsaker et al., 2008, Seok and Cheong, 2020)                  |
| <b>Subiculum</b>              | Predominantly pyramidal cells that receive major input from CA1                                                                                              | Involved in spatial information processing, memory, motivation, and the temporal control of behaviour                | (reviewed in (O'Mara et al., 2009))                             |
|                               |                                                                                                                                                              | Memory retrieval                                                                                                     | (Seok and Cheong, 2020)                                         |
|                               |                                                                                                                                                              | Role in initiation and maintenance of epileptic discharges in temporal lobe epilepsy                                 | (Stafstrom, 2005)                                               |
| <b>Entorhinal Cortex (EC)</b> | Gives rise to axons that project to the DG, forming part of the 'perforant path' and receives major input from the hippocampus and output to the hippocampus | Principal source of to and from the hippocampus. Plays a role in working memory, episodic memory, and spatial memory | (Heys et al., 2012)                                             |
|                               |                                                                                                                                                              | Role in epilepsy due to reciprocal connections with the hippocampus                                                  | (Avoli et al., 2002, Bartolomei et al., 2005)                   |
|                               |                                                                                                                                                              | Selective vulnerability during ageing and Alzheimer's Disease                                                        | (Stranahan and Mattson, 2010)                                   |
| <b>Temporal cortex</b>        | Investigation of this region in this thesis will refer to temporal neocortex                                                                                 | Auditory, olfactory, vestibular, and visual senses<br>Formation of spoken and written language                       | (Perrett et al., 1992, Karnath, 2001, Sato et al., 1999)        |

### **2.2.1.1: HIPPOCAMPUS IN SIDS**

The hippocampus has been studied in the context of SIDS previously, predominantly in relation to cellular damage, neurochemical abnormalities, and its morphology (detailed below).

SIDS risk factor profiles have been shown to overlap with sudden unexpected death in epilepsy (SUDEP), and a common underlying mechanism has been proposed to occur in a proportion of SIDS cases (Coras et al., 2014, Devinsky et al., 2016, Buchanan, 2019). The morphology of the DG is of interest given that the DG has a known role in epileptogenesis (Pun et al., 2012, Scharfman et al., 2003) and recent studies have documented ‘focal granule cell bilamination’ (FGCB) in SIDS (Kinney et al., 2015, Machaalani et al., 2022), a pathological sub-classification of granule cell dispersion (GCD) (Blümcke et al., 2009). GCD refers to the widening or duplication of the DG’s granule cell layer and is thought to arise following seizures in early life that disturb the late migration of granule cells (Houser, 1990). To this end, and as reviewed in Table 1 by (Machaalani et al., 2022)), the incidence of FGCB has been reported in 41.2 % of uSUDI compared with 7.7 % of eSUDI in a cohort of 153 cases of SUDI from Boston, USA (Kinney et al., 2015), 1.6% of SIDS in a UK report analysis (Kon et al., 2020), 19.4% in SUDI in Seattle, USA (Roy et al., 2020) and 6.3% eSUDI vs 18.3% SIDS from our laboratory using an Australian cohort of 90 SUDI cases (Machaalani et al., 2022). Our laboratory’s study was unique in that it analysed serial hippocampal sections, and subsequently reported that where DG features were noted, they were highly localised and transient, appearing across several sections, disappearing, and then re-emerging later and in a different location of the DG. Thus, whether FGCB is of pathological significance in SIDS remains unknown.

Other findings related to the hippocampus in SIDS include: increased markers of cell death (Waters et al., 1999), a reduction in microtubule-associated protein (MAP-2) [a marker of dendrosomatic neuronal injury] positive neurons (Oehmichen et al., 2009), and downregulated microglia (Ambrose et al., 2020).

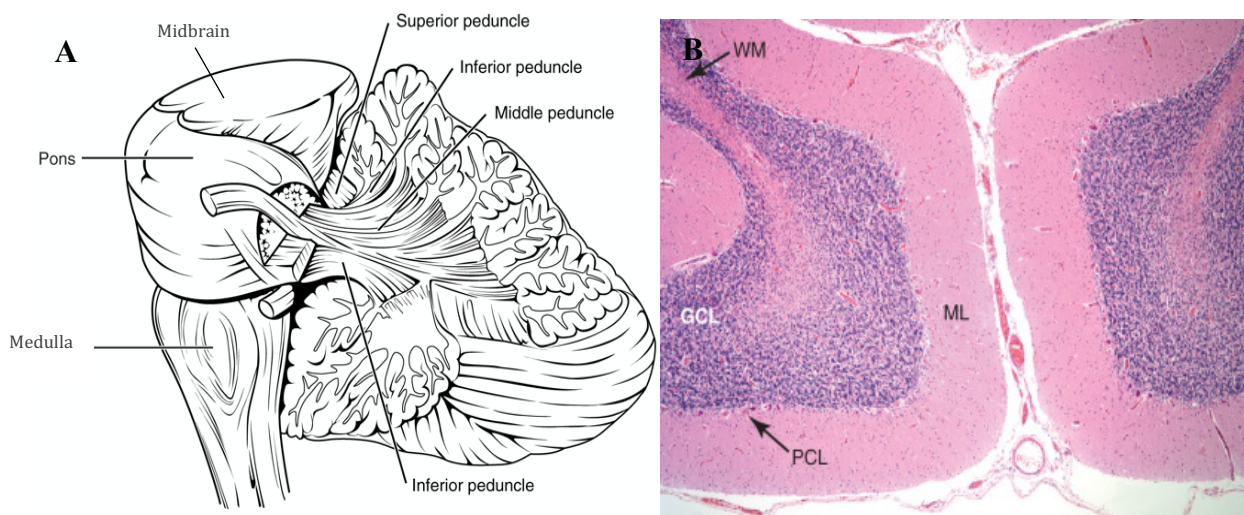
Neuronal cell death in the hippocampus will be discussed in detail in section 2.3.1.

### **2.2.2: CEREBELLUM**

The cerebellum sits under the posterior-inferior region of the cerebrum and connects to either side of the brainstem via inferior, superior and medial cerebellar peduncles (Figure 2.7a). The



cerebellum is known to have a role in regulation of: movement (coordination, balance, and fine motor control) (Manto et al., 2012), posture and gait (Thach and Bastian, 2004), motor skills (Thach, 1998), cognitive functions such as attention and emotion (Schmahmann and Caplan, 2006), and language and working memory (De Smet et al., 2013, Tomlinson et al., 2014). The cerebellum also plays a role in regulating breathing, heart rate, and other critical homeostatic functions via modulation of other brain structures (Calton et al., 2016, Calton et al., 2014, Harper et al., 2000), such as the brainstem. In particular, Purkinje cells are highly vulnerable to hypoxia and hypercapnia due to their high metabolic activity and oxygen demand (Hausmann et al., 2007, Brierley, 1984). A disruption in normal functioning of the cerebellum could therefore lead to autonomic dysregulation.



**Figure 2.7: The human cerebellum.** (A) Ventral view of the human brainstem and cerebellum; (B) Histological section of the cerebellar cortex stained with H&E, containing the molecular layer (ML), purkinje cell layer (PCL), granule cell layer (GCL) and white matter (WM). (Image A: [https://en.wikipedia.org/wiki/Cerebellar\\_peduncle](https://en.wikipedia.org/wiki/Cerebellar_peduncle) accessed 20/02/2023, Image B: (Brat, 2018).

The cerebellum consists of two cerebellar hemispheres which are connected by the vermis, a narrow midline area and contains both grey and white matter. The cerebellum contains several distinct histological regions (Figure 2.7b):

1. Molecular layer- the outermost layer of the cerebellum formed of tightly packed granule cells, dendrites of the Purkinje cells, stellate cells, mossy fibres originating from granule cells and parallel fibres.



2. Purkinje cell layer- the middle layer between the molecular and granule cell layer, containing ‘Purkinje’ neurons that are distinct due to their large cell bodies, long axons, and numerous dendritic arbours/synaptic contacts.
3. Granule cell layer- the inner cellular layer containing a high density of tightly packed granule cells.
4. White matter- containing myelinated axons which carry sensory information from the spinal cord and other regions of the brain to the cerebellum, and motor information from the cerebellum to the brainstem and spinal cord.

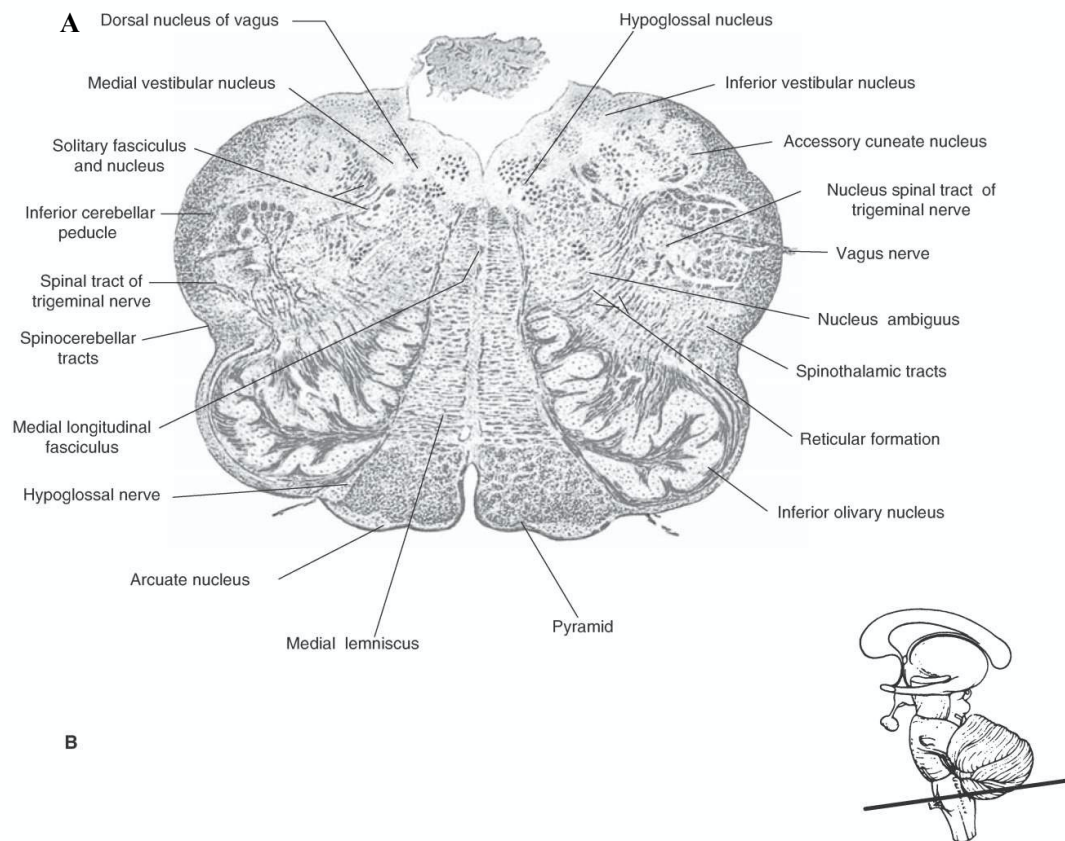
#### **2.2.2.1: CEREBELLUM IN SIDS**

Post-mortem studies of the SIDS cerebellum report: a higher incidence of delayed cerebellar cortex maturation (Cruz-Sánchez et al., 1997), increased Purkinje cell apoptosis (Lavezzi et al., 2006), reduced Purkinje cell transcriptional nucleoli activity (Lavezzi et al., 2016a), astrogliosis in the internal granular layer (Lavezzi et al., 2018) and increased unfolded protein markers in the dentate nucleus (Thomson et al., 2021). In a study that emulated cerebellar apoptosis in mice, developmental Purkinje cell loss significantly affected compensatory breathing patterns following mild CO<sub>2</sub> exposure (Calton et al., 2016). These findings support a potential mechanistic link between cerebellar abnormalities and increased difficulty when faced with a homeostatic challenge such as hypoxia. Cerebellar astrogliosis, as reported in SIDS (Lavezzi et al., 2018) may also be the result of hypoxia (Coimbra-Costa et al., 2021). Despite these findings, cytological and stereological investigations did not identify any differences in cerebellar cell number or volume in SIDS (Kiessling et al., 2013, Oehmichen et al., 1989b). Thus, whether the cerebellum has a role in SIDS, and its exact role remains unclear.

#### **2.2.3: BRAINSTEM**

The brainstem is located anterior to the cerebellum and is continuous with the spinal cord via ascending and descending fibres, connecting to deep subcortical structures. From rostral to caudal, the brainstem has three major subdivisions: the midbrain, pons, and medulla oblongata (medulla). The focus of this thesis will be on the medulla (Figure 2.8), given that it contains a number of nuclei involved in cardio-respiratory and arousal processes (Gozal et al., 1994, Yuan and Silberstein, 2016, Ciriello and Calaresu, 1977, Cutsforth-Gregory and Benarroch, 2017). Table 2.3 outlines the key nuclei to be studied in this thesis in the brainstem medulla, and provides an overview of their major function/s. These particular nuclei were chosen for

investigation throughout this thesis given that they are well defined anatomical regions that can be identified via the use of histochemical stain and microscopy (Paxinos and Huang, 2013), and also to ensure findings are directly comparable to previous studies undertaken in our laboratory utilising the same dataset (Ambrose et al., 2019, Ambrose et al., 2020).



**Figure 2.8: The human medulla.** (A) Cross-section diagram of the medulla with nuclei labelled, (B) taken at the level of the inferior olivary nucleus. Taken from <http://what-when-how.com/neuroscience/brainstem-i-the-medulla-organization-of-the-central-nervous-system-part-2/>.

**Table 2.3: Nuclei in the medulla oblongata to be studied in SIDS and their major functions.**

| <b>Nucleus</b>                                       | <b>Function(s)</b>                                                                                                                                       | <b>References</b>                                   |
|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------|
| <b>Arcuate Nucleus (AN)</b>                          | Chemoreception<br>Respiratory control                                                                                                                    | (Filiano et al., 1990)<br>(Gozal et al., 1994)      |
| <b>Cuneate Nucleus (CN)</b>                          | Processes sensory information from upper extremities, including arms and hands                                                                           | (Versteeg et al., 2021)                             |
| <b>Dorsal Motor Nucleus of the Vagus (DMNV)</b>      | Parasympathetic motor innervation of organs such heart, lungs, and gastrointestinal system                                                               | (Yuan and Silberstein, 2016)                        |
| <b>Hypoglossal Nucleus (XII)</b>                     | Motor innervation to muscles of the tongue                                                                                                               | (Watson, 2012)                                      |
| <b>Inferior Olivary Nucleus (ION)</b>                | Regulation of fine motor control and coordination                                                                                                        | (Paul, 2019)                                        |
| <b>Lateral Reticular Formation (LRF)</b>             | Receives afferent projections from cranial nerves<br>Modulation of motor function                                                                        | (Wang, 2009)                                        |
| <b>Nucleus of the Spinal Trigeminal Tract (NSTT)</b> | Relays somatosensory information such as temperature, touch, and pain from the face<br>Regulation of motor control in relation to chewing and swallowing | (Patel and Das, 2021)<br>(Westberg and Kolta, 2011) |
| <b>Nucleus of the Solitary Tract (NTS)</b>           | Relay station for visceral and taste afferents<br>Control of cardiorespiratory and gastrointestinal reflexes                                             | (Cutsforth-Gregory and Benarroch, 2017)             |
| <b>Vestibular Nucleus (Vest)</b>                     | Posture and balance, spatial orientation, Control of eye movements                                                                                       | (Hernandez, 2020)                                   |

### **2.2.3.1: BRAINSTEM IN SIDS**

As mentioned previously, the brainstem has been studied extensively in SIDS, and a number of abnormalities have been identified (reviewed in detail in (Machaalani and Waters, 2014, Bright et al., 2018a)), predominantly within regions of the medulla. This includes increased neuronal cell death (Machaalani and Waters, 2008, Ambrose et al., 2018, Ambrose et al., 2019, Stecco et al., 2005, Waters et al., 1999), cholinergic abnormalities (Kinney et al., 1995, Mallard et al., 1999, Machaalani et al., 2011), alterations in neurotransmitters such as serotonin (Kinney and Haynes, 2019), unfolded protein markers (Thomson et al., 2021), changes in microglia (Ambrose et al., 2020), and astrogliosis (Bruce and Becker, 1991, Storm et al., 1994, Obonai

et al., 1996, Storm et al., 1999, Sawaguchi et al., 2002, Matturri et al., 2006, Spinelli et al., 2018). A leading hypothesis in SIDS research is that infant brainstem defects lead to insufficient responses to homeostatic stressors thus resulting in death.

Currently no brainstem abnormality is specifically associated with the syndrome, given that not all SIDS cases have been found to have the abnormality reported and/or the findings have not been reproducible across cohorts. For example, across different cohorts examining cell death in the SIDS brainstem, different sub-nuclei were affected, and not all SIDS showed marked cell death compared to controls (Machaalani and Waters, 2008, Ambrose et al., 2018, Ambrose et al., 2019, Stecco et al., 2005, Waters et al., 1999). These contradictions may be due to different definitions and compositions of the control and SIDS groups, or the fact that only one tissue section was analysed per case. Despite this, the serotonergic (5-HT) and cholinergic findings seem to be the most reproducible.

5-HT is a neurotransmitter involved in many biological functions, and the 5-HT system of the medulla is critical for regulating autonomic and respiratory function and maintaining homeostasis in response to life-threatening challenges (Nalivaiko and Sgoifo, 2009). Findings of 5-HT abnormalities in the medulla of SIDS infants have been replicated across five separate research cohorts (Machaalani et al., 2009, Panigrahy et al., 2000, Kinney et al., 2003, Paterson et al., 2006, Duncan et al., 2010), and include decreased 5-HT neurotransmitter and receptor expression and binding, increased expression of immature 5-HT neurons, abnormalities in 5-HT synthesis and metabolism, and GABA<sub>A</sub> abnormalities in the 5-HT medullary system.

The cholinergic system and its major neurotransmitter acetylcholine (ACh) regulate important functions such as attention, memory, movement, heart rate and blood pressure (Everitt and Robbins, 1997, Tiwari et al., 2013). Brainstem abnormalities in the SIDS cholinergic system include decreased choline-acetyltransferase, the enzyme responsible for ACh synthesis (Mallard et al., 1999), and dysregulated expression of nicotinic acetylcholine receptor (nAChR) subunits (Aishah et al., 2019, Machaalani et al., 2011), receptors that are bound to and activated by endogenous ACh. Of note, decreased ACh hydrolysis enzymes, acetylcholinesterase (AChE) (blood and brain) (Mallard et al., 1999, Kalaria et al., 1993) and butyrylcholinesterase (BChE) (blood) (Harrington et al., 2022) have also been reported, with low levels of BChE in the blood being considered as a potential SIDS biomarker.

## **2.3: HIPPOCAMPAL NEURON DEATH (VIA APOPTOSIS AND NECROSIS) AND LOSS (HIPPOCAMPAL SCLEROSIS)**

### **2.3.1: NEURON DEATH**

Cell death in the brain can occur as result of developmental processes, as well as following central nervous system (CNS) damage/injury, via apoptosis or necrosis. Apoptosis is programmed cell death that occurs during normal development to eliminate unwanted cells (Brill et al., 1999, Voss and Strasser, 2020), as well as in response to certain types of cellular stress and DNA damage which may lead to neurogenerative diseases such as Alzheimer's Disease (Cotman and Anderson, 1995, Obulesu and Lakshmi, 2014, Verma et al., 2018) and ethanol-induced fetal alcohol syndrome (Ikonomidou et al., 2000, Olney et al., 2002). Apoptosis is primarily dependent on the activation of cysteine-dependent aspartate-specific proteases (caspases), a unique family of cysteine proteases that cleave various cellular substrates (Shi, 2004, Vitale et al., 2023). There are 14 known caspases found in mammalian species, which can be involved in either the initiation (caspases-1,2,4,5,8,9,10,11 and 12) or execution (caspases-3,6,7 and 14) of biochemical events that result in cell death (Hyman and Yuan, 2012). In response to different stimuli, initiator caspases activate executioner caspases to trigger apoptosis. Calcium signalling mediates many cellular functions, including apoptosis signalling via influences on free radical production, organelle disruption and proteolytic enzyme activation (Orrenius et al., 2003). Morphologically, apoptosis is characterised by features such as cytoplasmic shrinkage, chromatin condensation and nuclear fragmentation, membrane blebbing and formation of apoptotic bodies (Ziegler and Groscurth, 2004).

Necrosis is an unprogrammed form of cell death that occurs as a result of injury, trauma or ischemia, and is characterised by diverse morphological features, such as cell membrane permeability, dilated organelles, ribosomes dissociated from endoplasmic reticulum and in some cases, chromatin condensation (Ziegler and Groscurth, 2004).

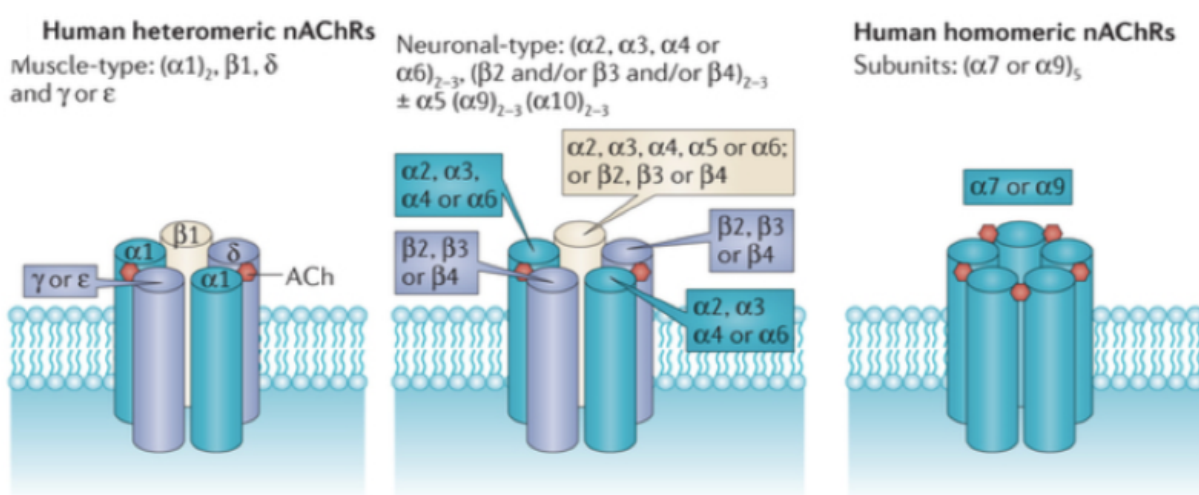
#### **2.3.1.1: NEURON DEATH IN THE SIDS HIPPOCAMPUS**

Alz-50, a marker of neurodegeneration (thus hypothesised to be an indirect marker of cell death) was shown to be increased throughout regions of the hippocampus (Sparks and Hunsaker, 1991, Sparks et al., 1996, Oehmichen et al., 1998). Alz-50 has since been recognised as a marker of phosphorylated (p)-Tau (Garcia-Sierra et al., 2003), and thus, is not indicative of cell death. Terminal deoxynucleotidyl transferase (TUNEL) (Waters et al., 1999), a marker

of late-stage cell death, has since been studied via IHC in the SIDS hippocampus, and was found to be present in both the hippocampus and subiculum. This data indicates that cell death does occur in the SIDS hippocampus.

### 2.3.2: NEURON DEATH VIA THE NICOTINIC ACETYLCHOLINE RECEPTORS (NACHR)

nAChR are family of ligand-gated ion channels that contain five transmembrane subunits organized around a central pore (Cooper et al., 1991). 17 different subunits ( $\alpha$ 1-10,  $\beta$ 1-4,  $\delta$ ,  $\gamma$  and  $\epsilon$ ) have been identified in vertebrate species to date (with the exception of  $\epsilon$  in mammalian species) which can co-assemble to form a variety of nAChR subtypes with different subunit combinations (Millar, 2003). These subunits are arranged as either heteropentamers or homopentamers throughout the CNS, autonomic ganglia and at skeletal muscular junction and are classified as either muscle or neuronal subtypes depending on where they are expressed.  $\alpha$ 1,  $\beta$ 1,  $\delta$ ,  $\gamma$  and  $\epsilon$  are classified as muscle-type subunits, while  $\alpha$ 2- $\alpha$ 10  $\beta$ 2-  $\beta$ 4 subunits comprise neuronal sub-types (reviewed in (Millar, 2003); Figure 2.9. The neuronal types contain a combination of  $\alpha$  and  $\beta$  subunits, whereby the presence of particular subunits determine ligand specificity and affinity, channel gating and cation permeability (Lindstrom, 2003). The two most common nAChR subtypes in the mammalian brain are the  $\alpha$ 4 $\beta$ 2 heteromeric and the  $\alpha$ 7 homomeric subunit combinations (Dani, 2015). The  $\alpha$ 4 $\beta$ 2 subtype has a high affinity for nicotine, while the  $\alpha$ 7 subtype has a low affinity for nicotine.



**Figure 2.9: Subunit composition of muscle type, neuronal type, heteromeric and homomeric nAChRs.** (Figure taken from (Grando, 2014).

Past literature supports a relationship between nAChR and cell death (Zoli et al., 1999) (Orr-Urtreger et al., 2000, Laudénbach et al., 2002, Hejmadi et al., 2003, Gergalova et al., 2012, Lykhmus et al., 2014). The underlying relationship between these two systems is not fully understood, however, these receptors may play a role in regulating processes of cell death via their ability to modulate calcium signalling (either having a pro or anti-apoptotic effect) (Berger et al., 1998, Dajas-Bailador et al., 2000, Ferchmin et al., 2003, Stevens et al., 2003, Resende et al., 2008, Wang et al., 2014).

Exposure of nAChR to nicotine and hypoxia can lead to changes in their function and expression (Dani, 2015, Buisson and Bertrand, 2001). As an example, our laboratory developed a piglet model of SIDS risk factors of postnatal cigarette exposure (via nicotine infusion) and hypoxia (intermittent hypercapnic hypoxia), and found that immunohistochemically, postnatal exposure to nicotine resulted in decreased expression of the  $\beta 2$  nAChR subunit in the CA3 region of the hippocampus, while exposure to hypoxia resulted in increased expression of the  $\beta 2$  subunit in the CA3, CA2 and DG (Vivekanandarajah et al., 2015). In human *infant* datasets, exposure to these risk factors can only be deduced following a review of the clinical history and death-scene information, and studies looking at nAChRs in the SIDS brain have only been undertaken in the brainstem (reviewed (Machaalani and Waters, 2014)). It remains to be determined what is happening to the nAChRs in the SIDS hippocampus, as well as the relationship between nAChR and neuronal death. This will be one aim of this thesis, herein.

### **2.3.3: NEURON LOSS IDENTIFIED AS HIPPOCAMPAL SCLEROSIS**

Hippocampal sclerosis (HS) (formerly Ammon's horn sclerosis) is a structural hippocampal lesion characterised by marked segmental neuronal loss and reactive gliosis, resulting in atrophy and tissue hardening (Sommer, 1880). HS is most commonly associated with drug-resistant medial temporal lobe epilepsy, but can also occur in other epilepsy syndromes (Margerison and Corsellis, 1966), and amongst nonepileptic populations in relation to ischemic injury and neurodegeneration (Zarow et al., 2008). Although HS is rare in infants <1 postnatal year, 50% of HS patients are reported to have suffered from an initial precipitating injury prior to the age of 4 (Mathern et al., 1995, MD et al., 2002), the most common of which being complex febrile seizures, while other antecedent injuries include birth trauma, head injury or meningitis. Following on from the limbic-seizure hypothesis, and the fact that there is increased cell death in the SIDS hippocampus (Waters et al., 1999), it is possible that a subset of SIDS

infants may have HS present, or otherwise, subtle patterns of cell loss. To date, there are no studies that specifically report on HS in SIDS.

HS is commonly a unilateral lesion, however, can involve sclerosis of adjacent medial temporal lobe structures (including the amygdala and parahippocampal gyrus) and subtle cortical pathologies. CA1 is highly vulnerable to HS regardless of the aetiology, although HS in epilepsy has the distinguishing feature of a sharp cut-off between the absent CA1 neurons and preserved subiculum neurons (Hatanpaa et al., 2014). This selective preservation is possibly due to the ability of the subiculum to sustain abnormal synchrony and hyperexcitability, allowing for the initiation and maintenance of epileptic discharges (reviewed in (Stafstrom, 2005)).

Additional pathological changes can include: GCD, FGCB and aberrant neurogenesis (as evidenced by abnormal migration of mature neurons) (Blümcke et al., 2009, Silva et al., 2007, Thom et al., 2010), reorganisation of hippocampal mossy fibres with axonal sprouting into the molecular layer of the DG (Cavazos et al., 2003), inflammation (Aronica and Crino, 2011, Vezzani et al., 2011), vascular abnormalities such as proliferation of microvasculature, angiopathy (bursting of small blood vessels), and/or potential blood-brain-barrier dysfunction that could promote/exacerbate epileptogenesis (Sommer, 1880, Van Vliet et al., 2007). Glial scar, as seen in HS, refers to a network of elongated astrocytes, immune cells and fibrotic tissue produced in response to cytokines and other inflammatory mediators (Sofroniew, 2009).

### **2.3.3.1: INTERNATIONAL CONSENSUS CLASSIFICATION**

In order to standardise data comparisons across research groups and integrate findings from emerging electroclinical correlations and magnetic resonance imaging (MRI) studies, the 2013 International League Against Epilepsy (ILAE) segregates HS into typical (type 1) and atypical variants (type 2 and 3), based on histological patterns of neuronal loss and astrogliosis (Figure 2.10) (Blümcke et al., 2013), thus not considering other features of HS (i.e. mossy fibre sprouting, inter-neuronal alterations).

Neuron loss is commonly visualised via NeuN (neuronal nuclei) immunohistochemistry (IHC). NeuN is an epitope of Rbfox3, which is a novel member of the Rbfox1 family splicing factor and is detected in post-mitotic neurons in nearly all parts of the vertebrate CNS (Duan et al., 2016). NeuN allows for cell loss to be easily visualised without the need for counting individual



cells. Other staining techniques, such as basic methods can be used, although require more rigorous cell counting procedures.

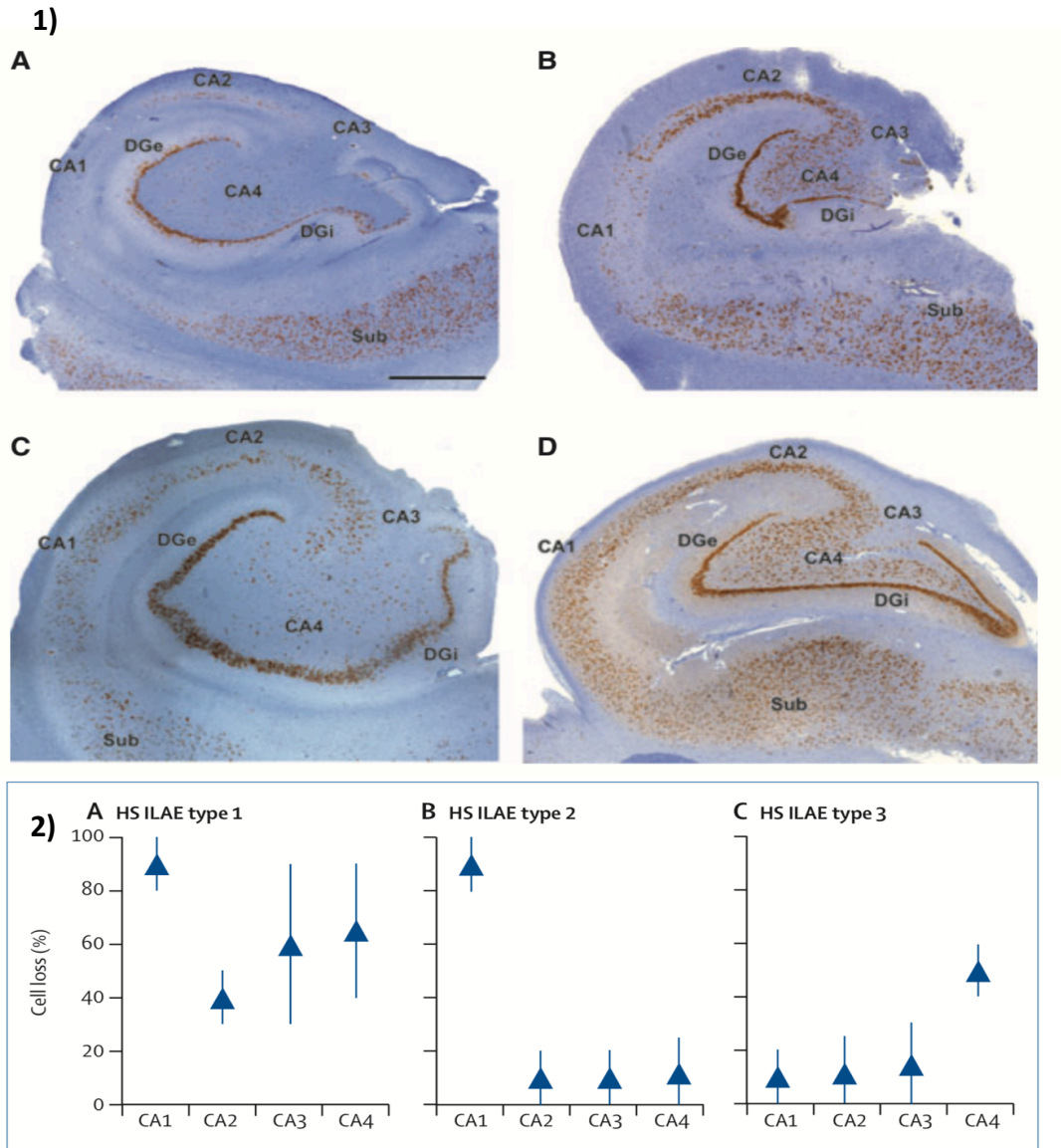
ILAE sub-types of HS include:

**Type 1** = CA1 is the most severely affected (> 80% cell loss), although all regions show significant neuronal cell loss (30-50% in the CA2, 30-90% in the CA3, and 40-90% in the CA4). Cell loss can also occur in the DG (50-60% granule cell loss), and it is acknowledged that any type of granule cell pathology (graded 0-2) can be present. This is the most common sub-type, representing 60-80% of cases.

**Type 2** = significant neuron loss in the CA1 (~80% of pyramidal cells), while CA2, CA3 and CA4 may only show mild decreases in cell numbers, almost undetectable by qualitative microscope inspection (< 20 – 25%). This subtype occurs in roughly 5-10% of TLE with HS cases. GCD can occur, although this pattern typically lacks severe granule cell loss.

**Type 3** = predominant cell loss in the CA4 (> 50% cell loss), and the DG (35%), while CA1, CA2 and CA3 are only moderately affected (< 30% cell loss). This subtype is rare, representing only 4-7% of all TLE with HS cases (Blümcke et al., 2007).

**Type 4** = only gliosis with no HS (no-HS). This subtype was characterised due to a growing body of literature supporting glial-mediated excitation and inflammation in modulating and triggering seizures (Devinsky et al., 2013).



**Figure 2.10: Histopathologic subtypes of HS based on ILAE consensus.** 1) Patterns of neuronal loss based on NeuN IHC: (A) Type 1, (B) Type 2 (C) Type 3 and (D) No HS, gliosis only. 2) Corresponding ILAE consensus classification of 3 HS subtypes. Triangles represent mean cell loss and bars represent SD (figures taken from (Blumcke et al., 2013)).

HS subtypes can be assessed using a semi-quantitative scoring system (graded 0-2) (Figure 2.11), which assesses neuronal cell loss via NeuN with: 0 = no obvious neuron loss or moderate astrogliosis (via glial fibrillary acidic protein (GFAP)) only; 1 = moderate neuron loss and gliosis; 2 = severe neuron loss (majority of neurons lost) and astrogliosis.

|                     | Subfield pathology patterns of neuronal cell loss and gliosis<br>(in <i>en bloc</i> resected samples) |                                        |                                        |                            |
|---------------------|-------------------------------------------------------------------------------------------------------|----------------------------------------|----------------------------------------|----------------------------|
| Class. <sup>a</sup> | HS ILAE<br>Type 1                                                                                     | HS ILAE<br>Type 2<br>"CA1 Predominant" | HS ILAE<br>Type 3<br>"CA4 Predominant" | No-HS /<br>Gliosis<br>only |
| CA1 <sup>b</sup>    | 2                                                                                                     | 1 - 2                                  | 0 - 1                                  | 0                          |
| CA2 <sup>b</sup>    | 0 - 2                                                                                                 | 0 - 1                                  | 0 - 1                                  | 0                          |
| CA3 <sup>b</sup>    | 0 - 2                                                                                                 | 0 - 1                                  | 0 - 1                                  | 0                          |
| CA4 <sup>b</sup>    | 2                                                                                                     | 0 - 1                                  | 1 - 2                                  | 0                          |
| DG <sup>c</sup>     | 0 - 2                                                                                                 | 0 - 1                                  | 0 - 2                                  | 0 - 1                      |

**Figure 2.11: ILAE consensus classification of hippocampal sclerosis.** Scoring of each hippocampal subregion is undertaken to determine HS subtype. Figure taken from (Blümcke et al., 2013).

### 2.3.3.2: HS IN INFANCY /PEDIATRICS

To date, only one study has reported HS in infancy (Mühlebner et al., 2016), although evidence of possible/probable HS has been reported in infancy via the use of imaging techniques (MRI) (Roulet Perez et al., 2000). Prolonged focal febrile convulsions have been shown to lead to hippocampal injury in infants, which can later develop into HS (Sokol et al., 2003) (VanLandingham et al., 1998), and several studies have presented examples of HS in early childhood (DeLong and Heinz, 1997, Harding and Thom, 2001, Siegler et al., 2005, Mühlebner et al., 2016). It is important to note that even modern, high-resolution MRI does not reveal significant pathology in 20–30% of patients with chronic focal epilepsy (Malmgren and Thom, 2012). Furthermore, investigation of specimens either post-mortem or following surgery are not common in infancy. Given that most studies investigating HS in infancy are based on imaging, the true incidence may be under-reported. Animal models support that even a few seizures in infancy can induce neuronal loss (Dunleavy et al., 2010) (Mohamed and Eltony, 2019), and in a rat model of epilepsy, seizures reduced cell numbers, increased caspase-3 positive cells, and changed the cellular appearance in the DG, indicative of injury (Mohamed

and Eltony, 2019). As such, another aim of this thesis is to determine whether HS is present in SIDS via NeuN IHC.

## **2.4: ASTROCYTES AND ASTROGLIOSIS**

Astrocytes are a specialised type of glial cell that play an integral role in the development, structure, and function of the CNS. Astrocytes are known to provide support for essential CNS activities such as: promotion of neuron survival, synapse formation and function, regulation of blood flow and maintenance of the blood-brain-barrier, provision of energy metabolites to neurons, and maintenance of the extracellular balance of ions, fluid, and neurotransmitters (Ullian et al., 2001, Simard and Nedergaard, 2004, Iadecola and Nedergaard, 2007, Pellerin et al., 2007, Zhang et al., 2016). Astrocytes interact extensively with one another forming their own network syncytium and interact at neuronal synapses and blood capillaries.

Astrocytes are constant surveyors of the CNS, and respond to disruptions in metabolic processes, such as hypoxia, hypercapnia, and reduced brain perfusion ((Angelova et al., 2015) & reviewed in (Marina et al., 2018)). Astrocytes are also known for their ability to become ‘reactive’ following almost any type of CNS injury or disease, a process that involves diverse morphological, transcriptional and functional changes (Sofroniew and Vinters, 2010). GFAP is the main component of astrocyte cytoplasmic intermediate filaments, and has become a prototypical marker of reactive astrocytes (reviewed in (Preston et al., 2019)) given its expression is significantly upregulated in response to physiological and pathological challenges. This review will outline the development and functions of astrocytes, focusing on astrogliosis, and its detection using GFAP IHC.

### **2.4.1: ASTROCYTE DEVELOPMENT**

During human development, astrocytes undergo significant changes in their number, morphology, function and location, and also guide migration and differentiation of neurons (Nowakowski et al., 2016). Following migration, astrocytes begin to express their common markers such as GFAP, S100 $\beta$ , Aldh1L1, Sex-determining Region Y (SRY) box 9 (SOX9), Adolase C (AldoC), Glutamate-transporter 1 (Glt1) and glutamine synthase (reviewed in (Molofsky et al., 2012, Preston et al., 2019)). GFAP is first detected in radial glia as early as embryonic day 14 in rats and mice (Teter et al., 1996, Fan et al., 2005), and 6 weeks post conception in humans (Holst et al., 2019). In humans, most astrocytes do not express GFAP in large quantities until the postnatal period (Holst et al., 2019), although there are inter and intra-

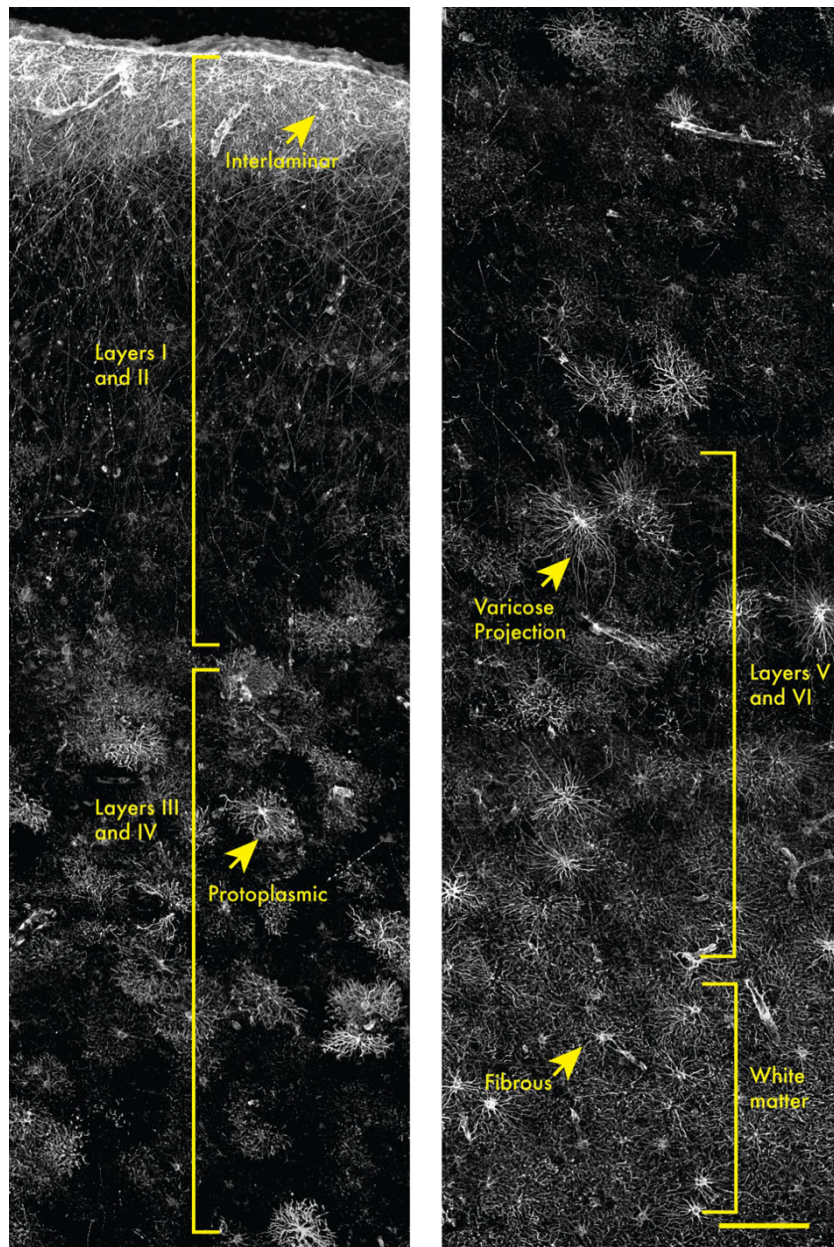
regional variations in expression at different stages of development (Holst et al., 2019). Astrocytes continue to mature postnatally in response to factors such as neural activity, exposure to cytokines and growth factors, and changes in the extracellular matrix (de Majo et al., 2020). The reason for delayed development of astrocytes in humans is not well understood, but is likely due to the increased complexity of human cerebral systems (Zhang et al., 2016).

#### **2.4.2: ASTROCYTE SUBTYPES**

Astrocytes are diverse cells that are often sub-categorised based on their morphological, functional, or molecular differences. 4 major astrocyte sub-types have been characterised (Figure 2.11) in the human cerebral cortex, including:

1. Protoplasmic astrocytes that predominate grey matter neuropil and have large, round somata with numerous short thick processes that overlap by as little as 5% at their boundaries. Protoplasmic astrocytes come into contact with synapses, blood vessels (forming part of the blood-brain-barrier), and other glial cells (Zhou et al., 2019).
2. Fibrous astrocytes which predominate white matter and have fewer processes that are long and thin, propagating among myelinated nerve fibre axons and associating with blood vessels (Elston et al., 2009).
3. Intralaminar astrocytes, present in layer I of the cerebral cortex in mammalian species, and have long, interlaminar processes that extend through several cortical layers (Verkhatsky et al., 2018).
4. Varicose projection astrocytes, which send several very long (up to 1mm) unbranched processes with varicosities that extend in all directions through the deep cortical layers, with the distinguishing feature that they are immunopositive for CD44 (Akiyama et al., 1993). Varicose projection astrocytes have not been detected in the neonatal brain, and appear to reflect age dependent changes and individual life experiences (Sosunov et al., 2014).





**Figure 2.12: Four major sub-categories of GFAP-positive cells in the human cortex.** Cell bodies of interlaminar astrocytes present in cortical layer I, with processes extending over millimetre lengths through layers 2–4. Protoplasmic astrocytes, the most common, reside in layers 2–6. Varicose projection astrocytes are found only in humans and are seen sparsely in layers 5–6. Fibrous astrocytes are found in the white matter and contain numerous overlapping processes. Yellow lines indicate areas in which the different classes of astrocytes reside. Scale bar = 150 $\mu$ m. Image taken from (Oberheim et al., 2009).

Additional region-specific astrocyte subtypes have also been characterised, including Müller glia in the retina (Reichenbach, 1989) and Bergmann glia in the cerebellum (Wang et al., 2012),

considered to be of astrocyte lineage due to the fact that they express overlapping molecules such as GFAP, S100 $\beta$ , and glutamine synthetase, and have the ability to become reactive under CNS insult.

Another method of sub-typing astrocytes is based on their 'reactive' state, with a recent model classifying them as either neurotoxic or neuroprotective (termed 'A1' and 'A2' respectively) (Zamanian et al., 2012, Chai et al., 2017). A1 astrocytes are associated with inflammation and are activated via activated microglia and downstream release of interleukin-1 alpha (IL-1 $\alpha$ ), tumour necrosis factor (TNF)- $\alpha$  and complement component C1q1 (Liddelow et al., 2017). A1 astrocytes release inflammatory and neurotoxic elements such as complement component 3 (C3) in high amounts, subsequently inducing the death of neurons and oligodendrocytes (Liddelow et al., 2017). A2 astrocytes are associated with hypoxia/ischemia and are activated via downregulation of the P2Y<sub>1</sub> purinergic receptor (Shinozaki et al., 2017), subsequently promoting neuron survival and axon regeneration (Anderson et al., 2016). Immunohistochemically, the most common and specific markers are C3 for A1 astrocytes, and S100 calcium binding protein A10 (S100a10) and pentraxin 3 (PTX3) for A2 astrocytes (reviewed (Fan and Huo, 2021)). Although there likely more diverse reactive astrocyte profiles (Chai et al., 2017, Batiuk et al., 2020), the A1/A2 model facilitates our understanding of diverse astrocyte changes under different types of injuries. Current emerging research is subtyping astrocytes utilising these terminologies; however, this will not be a focus of the study.

#### **2.4.3: ASTROGLIOSIS**

Broadly, astrogliosis refers to morphological, molecular and functional responses of astrocytes following CNS injury, disease, or other pathological conditions (Sofroniew, 2009). Non-reactive astrocytes constantly survey their environment for abnormalities and upon contact with pathogens, aggregated serum proteins, cytokines, or chemokines, activate receptor and signalling pathways which can then trigger astrocyte reactivity pathways (Kang and Hébert, 2011, Burda et al., 2016, Burda and Sofroniew, 2014). Specific states of astrocyte reactivity are highly heterogeneous and are driven by complex signalling cascades, with the Janus kinase-2/signal transducer and activator of transcription 3 (JAK-STAT3) pathway being a central regulator (Ceyzériat et al., 2016). Enhanced expression of proinflammatory cytokines help mediate JAK2-STAT3 pathways (i.e., TNF- $\alpha$  and IL-1 $\beta$ ) (Chen et al., 2013, Sriram et al., 2004). The specific activation pathway and changes that subsequently occur are dependent on the type of insult (Zamanian et al., 2012, Zhang et al., 2016), the brain region affected (Sun

and Jakobs, 2012, Emsley and Macklis, 2006, Messing and Brenner, 2020), and the time between injury and death (Hausmann et al., 2000). Astrocytes also receive signals from healthy neurons that actively suppress their activity, such as neuron-derived FGF-2 and astrocytic  $\beta$ 1-integrin (Kang et al., 2014, Robel et al., 2009).

Astrogliosis can result in beneficial or detrimental effects depending on the types of changes that occur (Anderson et al., 2016, Zamanian et al., 2012, Zhang et al., 2016). For example, astrogliosis may minimize the spread of damage and inflammation (Bush et al., 1999, Faulkner et al., 2004, Burda et al., 2016), although this can inhibit axonal and cellular regeneration that would otherwise allow for functional recovery of neural tissue (Bush et al., 1999, Faulkner et al., 2004). More severe CNS tissue damage can lead to astrocyte proliferation (Stringer, 1996, Li et al., 2010), and in the case of traumatic brain injury or multiple sclerosis, can lead to the formation of a glial scar (Figure 2.13 C), whereby astrocyte processes become elongated around the site of injury to minimise the spread of inflammation, infection and neurodegeneration to surrounding parenchyma (Faulkner et al., 2004, Adams and Gallo, 2018). The formation of a glial scar involves several other cell types, including stromal cells, NG2 cells and microglia (Bradbury and Burnside, 2019). Despite these findings, the complexity of astrocytic changes in response to injury is not fully understood, with a lack of consensus regarding specific features and definitions of astrogliosis amongst experts (Escartin et al., 2019).

#### **2.4.3.1: ASSESSMENT VIA GFAP IMMUNOHISTOCHEMISTRY**

GFAP is a type III intermediate filament protein present in most mature astrocytes (Eng et al., 2000), and was first isolated as a protein from old demyelinated plaques from multiple sclerosis patients (Eng et al., 1970, Eng et al., 2000). GFAP expression is regulated by many transcription factors (including JAK2-STAT3 and nuclear factor  $\kappa$ -light chain-enhancer of activated B cell signalling), protein kinase and signalling molecules via neuronal activity and hormones (O'Callaghan et al., 2014, Sriram et al., 2004) & reviewed in (Li et al., 2020)). Acute and diffuse trauma without tissue damage is sufficient to evoke a transient upregulation of GFAP (Wilhelmsson et al., 2006), while longer and more severe injuries lead to more significant GFAP upregulation, with hypertrophy of processes, and potentially distal processes extending into adjacent astrocyte domains (Myer et al., 2006).



GFAP has become a prototypical marker of astrogliosis given that its expression is proportionate to the degree of astrocyte hypertrophy, and therefore, its quantification is thought to reflect the amount of activation occurring (Sun and Jakobs, 2012, Zhu et al., 2004). Even so, the level of GFAP upregulation can differ between cells (Pekny et al., 2019), between brain regions (Sun and Jakobs, 2012, Emsley and Macklis, 2006, Messing and Brenner, 2020, Boos et al., 2021), and as a result of the type of injury, and the time between injury and death (Hausmann et al., 2000).

#### **2.4.3.1.1: METHODS OF ASSESSMENT**

When utilising GFAP IHC, injury increases the expression of GFAP so that previously immunonegative cells may become immunopositive, and astrocyte bodies and their processes become hypertrophied (Sun and Jakobs, 2012, Zhu et al., 2004). Quantification of GFAP IHC can be done via measurement of the intensity or distribution of the staining in tissue sections (Table 2.4). Commonly used methods of analysis for GFAP IHC in human studies include the application of:

1. Image analysis software: techniques such as mean optical density or binary image thresholding within a defined region of interest to make quantitative assessments on either the amount, or the intensity of staining.
2. Semi-quantitative scoring: scoring the intensity and/or distribution of staining via a graded scale. Based on GFAP expression, levels of gliosis are often categorised in 4 levels, ranging from no gliosis, mild to moderate gliosis, severe diffuse reactive gliosis, and severe gliosis that includes glial scar formation (Lyck et al., 2008, Wilhelmsson et al., 2006, Anderson et al., 2014); or sometimes 3 broad categories with severe gliosis that may or may not include glial scar formation (Blümcke et al., 2013) (Figure 2.13).
3. Point counting: counting the number of GFAP-positive cells in a defined area of the tissue section, thus expressed as the number of GFAP-positive cells per unit area. In many cases, this is done using image analysis software.

There remains a lack of standardised measures for assessing astrogliosis and its severity (Escartin et al., 2019). When utilising GFAP IHC, many research groups do not report sufficient detail pertaining to research methodology (quantification method, sequence of tissue sections, the number of sections analysed, and anatomical location of each section used)

(Ostergaard and Jensen, 2013), particularly with regard to criteria used for semi-quantitative methods that are based on subjective observer judgement. The type of measurement used has been shown to result in vastly different research findings within the same study (Weber et al., 2013, McNeal et al., 2016). For example, the study by Weber et al. (2013) found that both Western Blot and quantitative scoring methods did not show any significant differences in GFAP-immunoreactivity between groups sampled, while the semi-quantitative (score between 0 and 3, based on number of activated astrocytes) and qualitative (levels of immunoreactivity via graph representation of fibre lengths from immunofluorescence) methods did. These concerns pose major problems when comparing research findings between different research groups.

**Table 2.4: Features of commonly used techniques for quantifying GFAP immunohistochemistry in the context of astrogliosis.**

| Technique                                  | Method                                                                                                                                                                                                                                                    | Outcome measure                                                                   | Advantages                                                                           | Disadvantages                                                                                                                                                                                                      | Example of studies that have utilised method |
|--------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------|
| <b><i>Semi-quantitative techniques</i></b> |                                                                                                                                                                                                                                                           |                                                                                   |                                                                                      |                                                                                                                                                                                                                    |                                              |
| <b>Incidence method</b>                    | Pre-defined phenotype or boundary of what is considered “normal”                                                                                                                                                                                          | Pathological feature is either present or not e.g., yes vs no astrogliosis        | - Time and cost effective                                                            | - Subjective judgement bias<br>- Distinguishing ranges of phenotype can be limited                                                                                                                                 | (Kinney et al., 2015) (Kon et al., 2020)     |
| <b>Graded scoring system</b>               | Ordinal value indicating the level of severity of astrogliosis. This can be based on various criteria, such as intensity, distribution, morphology, and overall pattern of staining. Requires a trained observer utilising a self-standardised criterion. | Level of pathological severity, normally graded between no astrogliosis to severe | - Takes overall staining pattern into account<br>- Time-efficient and cost-effective | - Subjective judgement bias<br>- Less sensitive to subtle changes in pathological state<br>- GFAP expression differs between brain regions at baseline- may overlook changes in regions with lower GFAP expression | (Lyck et al., 2008, McNeal et al., 2016)     |

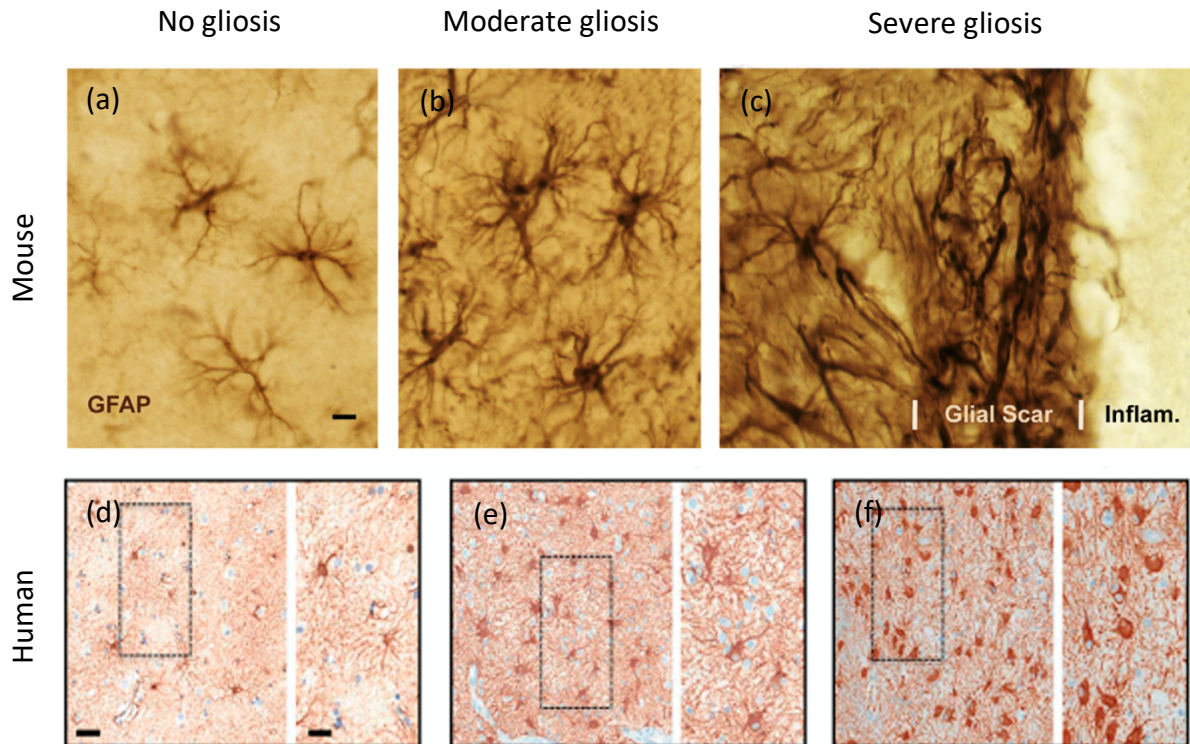
|                |                                                                                                                                                                                                                    |                                                                                                  |                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                        |                       |
|----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
|                | Ranks should reflect the range of phenotype present in the data to effectively distinguish group differences, although standardisation of methods will allow for comparability between studies and research groups |                                                                                                  |                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                        |                       |
| <b>H-score</b> | Determined by addition of the results of multiplication of the percentage of cells with staining intensity ordinal value                                                                                           | Value between 0-300 that represents intensity and proportion of staining at each intensity level | <ul style="list-style-type: none"> <li>- Standardisation of the semi-quantification method allowing for comparability of findings between studies/laboratories</li> <li>- Gradations can be adapted to fit specific study outcomes</li> </ul> | <ul style="list-style-type: none"> <li>- More time-consuming than traditional semi-quantitative methods</li> <li>- Subjective judgement required</li> <li>- Variations in formulas across research groups</li> <li>- Lack of standardised cut-offs when interpreting scores</li> </ul> | (Hazzaa et al., 2020) |

| <b><i>Quantitative techniques</i></b>                                        |                                                                                                                                                                                                                              |                                                            |                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                 |                                                                           |  |
|------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------|--|
| <b>Counts</b>                                                                | Number of immuno-positive astrocytes (or feature of interest e.g., astroglial projections, astrocytes of reactive phenotype) in a given field/area                                                                           | Counts per field/area                                      | <ul style="list-style-type: none"> <li>- May pick up subtle or early indications of reactive processes occurring as detecting upregulation of GFAP</li> <li>- A form of objective assessment and is therefore repeatable</li> <li>- Can be automated using imaging software</li> </ul> | <ul style="list-style-type: none"> <li>- Time consuming and may require specialised software to automate the process – different algorithms can produce different results affecting the outcome of the analysis</li> <li>- Requires high quality images</li> <li>- Does not detect cell hyperplasia/branching of GFAP-positive cells</li> </ul> | (Buser et al., 2012)<br>(Meadowcroft et al., 2015)                        |  |
| <b>Image segmentation- Binary image thresholding (pixel intensity level)</b> | ‘Signal’ (immunolabelled material) to ‘noise’ (non-specific material attributed to immunolabelling process) ratio. These parameters are defined manually and remain constant between groups. The number of pixels within the | Percentage of the image covered by positive immunostaining | <ul style="list-style-type: none"> <li>- Allows for a standardised comparison of the amount of staining</li> <li>- Accounts for changes in both the cell body and its processes</li> <li>- Can be automated</li> </ul>                                                                 | <ul style="list-style-type: none"> <li>- Threshold parameters rely on subjective judgement</li> <li>- The assumption that the threshold range is the same across groups needs to be met (including staining parameters that may affect signal/noise)</li> <li>- Pixel intensity histogram may complement standard thresholding</li> </ul>       | (Muñoz-Castro et al., 2022, Healy et al., 2018, Johnson and Walker, 2015) |  |

|                                                             |                                                                                                                                                                                                             |                                                                                                                            |                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                |                                               |
|-------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------|
|                                                             | signal range is then quantified.                                                                                                                                                                            |                                                                                                                            |                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                |                                               |
| <b>Image segmentation of individual cells or structures</b> | Requires image to have staining separated from background via thresholding, edge detection or region growing. Individual structures can then be labelled or quantified i.e., length of astrocyte processes. | Area, perimeter or other properties of staining in each object                                                             | <ul style="list-style-type: none"> <li>- Object based analysis rather than overall intensity of staining</li> <li>- Can be less affected by variations in stains and background, such as in the case of thresholding</li> <li>- Can provide spatial context to give context about staining distribution</li> <li>- Can be automated saving time and effort for researchers</li> <li>- Can provide multiple parameters</li> </ul> | <ul style="list-style-type: none"> <li>- Requires specialised software and hardware</li> <li>- Highly sensitive to image quality</li> <li>- Application of threshold introduces user bias and error</li> <li>- Over or under-segmentation can occur</li> </ul> | (Healy et al., 2018)                          |
| <b>Mean optical density (MOD)</b>                           | Average intensity of staining in the ROI (sum of intensity values divided by number of pixels). Can be done on a thresholded image                                                                          | Units of optical density (logarithmic scale representing the ratio of light transmitted through a sample to the light that | <ul style="list-style-type: none"> <li>- Reduced subjectivity methods</li> <li>- Can provide results that are reproducible, thus, can detect changes in staining over time/between different samples</li> </ul>                                                                                                                                                                                                                  | <ul style="list-style-type: none"> <li>- Subjectivity in defining the area of interest</li> <li>- Lack of sensitivity to spatial variations in staining intensity in the tissue</li> <li>- Lack of information on cell morphology</li> </ul>                   | (Eldomiaty et al., 2020, Petito et al., 1990) |

|                   |                                                                                                                                                                                                                         |                                                                                                                                                                            |   |                                                                                                                                                    |                                                                                                                                                                                                                                                                                                     |                       |
|-------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---|----------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
|                   | (optional). MOD values are normalised if necessary to correct for variations in image acquisition or staining protocol                                                                                                  | would have passed through if the sample were clear)                                                                                                                        |   |                                                                                                                                                    |                                                                                                                                                                                                                                                                                                     |                       |
| <b>Stereology</b> | Involves mathematical methods to estimate 3-dimensional properties (such as in relation to density, distribution, and morphology of GFAP) in structures such as tissue sections, based off multiple (8-10) 2D sections. | Wide range of possible outcome measures such as estimation of the number of GFAP-positive cells, astrocyte volume, astrocyte density or spatial distribution of astrocytes | - | Considered unbiased and higher precision in measurement can be obtained given all parts of the tissue are sampled- thus minimising sampling errors | - Laborious and complex approach often reserved for specialised labs.<br>- Requires specialist training and software<br>- Time consuming<br>- Still based on 2D images or sections which may lead to errors<br>- Accuracy can be affected by sample preparation and staining parameters<br>- costly | (McNeal et al., 2016) |

NA= Not applied



**Figure 2.13: Commonly applied semi-quantitative gradation of astrogliosis.** Based on GFAP IHC (a) normal; (b) moderate, and (c) severe glial pathology in the mouse cerebral cortex following injury (scale bar = 8 $\mu$ m. Taken from (Sofroniew, 2009)) and (d) normal; (e) moderate; and (f) severe glial pathology in the human cerebral cortex following injury (scale bar = 25 $\mu$ m with magnified regions (boxed areas) = 10 $\mu$ m. Taken from (Sofroniew and Vinters, 2010)).

#### 2.4.3.1.2: TIMING OF GFAP UPREGULATION AFTER INJURY

The time it takes for GFAP to upregulate following injury is highly variable and is dependent largely on the type and severity of the injury. Following an acute neurotoxin injection in a rat model, GFAP increased after 6-hours at the mRNA level, and 12-72 at the protein level (O'Callaghan et al., 2014). In the post-mortem human brain, the number of GFAP-positive astrocytes was increased 1-day following cortical contusion, and remained elevated for up to 4 weeks in tissue regions adjacent to the injury (Hausmann et al., 2000). Changes that do occur may persist over several weeks and can remain elevated for months (Neri et al., 2018).

The amount of GFAP expression present when investigating via IHC is largely dependent on survival time following brain injury. Following traumatic brain injury in humans, the number of GFAP positive astrocytes was highest in cases with survival times of 3 days or more (note:



this study did not investigate morphological changes) (Trautz et al., 2019). In human surgical resections that had undergone injury produced by intracranial electrodes (days post-injury ranged 4-301), microglial/macrophage injuries peaked at 28-30 days post-injury with subsequent decline in proliferating microglia, while GFAP remained relatively stable in density (GFAP-positive cells/mm<sup>2</sup>) across the different post-injury survival times studied (Goc et al., 2014). More research is still needed to determine the exact time course of GFAP upregulation following injury, particularly regarding morphological changes that occur.

#### **2.4.3.2: ASTROGLIOSIS IN SIDS**

Astrogliosis has been studied in the SIDS brain dating back to the 1970's, with most studies investigating brainstem nuclei. Many initial studies (summarised in Table 2.5) utilising basic histochemical stains reported the presence of brainstem gliosis (Naeye, 1976, Takashima et al., 1978, Kinney et al., 1983), although some did not (Ambler et al., 1981, Summers and Parker Jr, 1981). Reported astrogliosis seemingly occurred in brainstem nuclei involved in regulating cardio-respiratory control (Takashima et al., 1978, Takashima and Becker, 1985), supporting the hypothesis that SIDS involved chronic hypoventilation.

With the increase in use of IHC, subsequent studies (mid 1980's onwards) investigated astrogliosis via GFAP (summarised in Table 2.5). Contradictions in findings between these studies arose, and it remains unclear whether astrogliosis is a feature of the SIDS brain. Astrogliosis has been identified in nuclei of the brainstem (Bruce and Becker, 1991, Storm et al., 1994, Obonai et al., 1996, Storm et al., 1999, Sawaguchi et al., 2002, Matturri et al., 2006, Spinelli et al., 2018) and cerebellum (Lavezzi et al., 2018). Other studies however have reported no significant astrogliosis gliosis among SIDS infants compared to controls (Reske-Nielsen et al., 1987, Oehmichen et al., 1989a, Pamphlett and Treloar, 1996, Kinney et al., 2015).

When investigating for the presence of astrogliosis in the hippocampus, based on neuropathological analysis at the time of autopsy (thus, not specifically research based), one study indicated that incidence was higher amongst SIDS than non-SIDS (Kon et al., 2020), and another study reported a higher incidence of astrogliosis in eSUDI compared to uSUDI in both the CA3 of the hippocampus, and subiculum (Kinney et al., 2015). Discrepancies such as these highlight the issue with undertaking statistical analysis under the assumption that SIDS infants are one homogeneous group, and it is likely that SIDS is a diagnosis that incorporates infants

who died of different underlying causes (Byard, 2014). In support of this, (Storm et al., 1994) identified 2 distinctive subtypes of GFAP staining amongst SIDS infants, whereby half demonstrated upregulated GFAP in the ION, and the other half did not, similar to the control group.

Investigation of astrogliosis according to the presence of known SIDS risk factors may be more appropriate than correlating outcomes against diagnosis, given that unlike diagnosis, a particular risk factor may represent a common mechanism that is identifiable at death. In a study that correlated GFAP staining outcomes against major SIDS risk factors, there was a correlation between astrogliosis in the ION and pre-natal cigarette smoke exposure (Storm et al., 1999). In a separate study that measured the frequency and duration of sleep apnea 2-12 weeks in infants who happened to later die of SIDS, increased astrogliosis was reported in the raphe nuclei of the brainstem, and was associated with the frequency of episodes of obstructive sleep apnea and sleeping prone (Sawaguchi et al., 2002). The same association was not found in a follow up study investigating the DMNV (Sawaguchi et al., 2003), thus indicating region specific effects.

Contradictions in the research may also arise due to different IHC and laboratory protocols, definitions of SIDS and variations in the composition of control groups, gestational ages of the infants studied, anatomical regions investigated (which may have different age sensitivity for brain injuries), and methods for quantifying stains. This thesis will investigate GFAP further in the SIDS brain, expanding to regions of the hippocampus and cerebellum, and applying detailed analyses to determine whether astrogliosis is indeed a feature of SIDS pathogenesis.

**Table 2.5: Studies investigating astrogliosis in the SIDS brain in chronological order.**

| <b>Staining method</b> | <b>Controls used</b>                                                                                            | <b>Sample size</b>                                          | <b>Age range</b>           | <b>Brain regions investigated</b>                                          | <b>Quantification method</b>                                                                          | <b>Outcome in SIDS</b>                                    | <b>Reference</b>              |
|------------------------|-----------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------|----------------------------|----------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|-----------------------------------------------------------|-------------------------------|
| BS-Holzer              | Trauma, carbon monoxide poisoning, suffocation, drowning                                                        | SIDS- 28<br>Control- 18                                     | 1-12 postnatal months      | Brainstem reticular formation                                              | Point counting of astroglial fibers to produce a density score                                        | ↑                                                         | (Naeye, 1976)                 |
| BS-PTAH, Holzer        | Acute infection, drowning, trauma<br><br>Congenital heart disease analysed as a separate group for comparison   | SIDS- 46<br>Control- 19<br><br>Congenital heart disease- 24 | 0-12 postnatal months      | Brainstem nuclei of medulla, pons & midbrain                               | Number of astrocytes with fibres/ area measurement. 3-tier grading system for astrocyte proliferation | ↑ in NTS, DMNV & reticular formation compared to controls | (Takashima et al., 1978)      |
| BS-H&E                 | Asphyxia, acute infection, metabolic disease, trauma, congenital abnormalities with infection                   | SIDS- 32<br>Control- 26                                     | 18 postnatal days- 2 years | Brainstem nuclei, cerebellum & cerebrum                                    | 3-tier graded score based on astrocyte hypertrophy                                                    | No difference                                             | (Ambler et al., 1981)         |
| BS-PTAH, Cajal         | Heart failure, asphyxiation, suffocation, aspiration, infection                                                 | SIDS- 34<br>Control- 15                                     | 1-27 postnatal weeks       | Brainstem medulla & pons                                                   | 3 tier graded score                                                                                   | No difference                                             | (Summers and Parker Jr, 1981) |
| BS-H+E, LFB            | Infection myocarditis, sepsis, carbon monoxide poisoning, accidental asphyxia, trauma, Reye's syndrome, seizure | SIDS- 45<br>Control- 20                                     | 4-51 postnatal weeks       | Brainstem medulla nuclei: -median raphe of reticular formation, DMNV, NTS, | Counts/mm <sup>2</sup>                                                                                | ↑ in hilum of ION, lateral region of the medulla, and NTS | (Kinney et al., 1983)         |

|                                   |                                                                                                          |                                                                           |                                                                                          |                                                                                          |                                                                                              |                                                           |                                     |
|-----------------------------------|----------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------|------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------|-----------------------------------------------------------|-------------------------------------|
|                                   |                                                                                                          |                                                                           |                                                                                          | nucleus<br>ambiguous, &<br>hilum of ION                                                  |                                                                                              |                                                           |                                     |
| GFAP<br>(rabbit)                  | Spontaneous abortion,<br>respiratory distress<br>syndrome, congenital heart<br>disease, accidental death | SIDS- 15<br><br>Control- 23                                               | 1-8<br>postnatal<br>months<br>(some<br>controls fell<br>outside of<br>this age<br>range) | Brainstem<br>medulla nuclei:<br>magnocellular<br>reticular<br>nucleus, NTS,<br>& DMNV.   | Counts in centre of<br>regions                                                               | ↑ in<br>magnocellular<br>reticular<br>nucleus and<br>DMNV | (Takashima<br>and Becker,<br>1985)  |
| GFAP<br>(1:300)                   | Asphyxia, respiratory<br>distress, trauma,<br>myopathies, Ondine's<br>curse                              | SIDS- 20<br><br>Control- 15                                               | 28<br>gestational<br>weeks-10<br>years                                                   | Brainstem<br>medulla nuclei<br>of pyramid,<br>ION, reticular<br>formation,<br>DMNV & NTS | Cells per nuclear<br>subarea →<br>stratified into 6<br>groups according to<br>average counts | ↑ in reticular<br>formation and<br>DMNV                   | (Becker and<br>Takashima,<br>1985)  |
| GFAP                              | Homicide/accident                                                                                        | SIDS- 18<br><br>Control- 7<br>(only 2<br>controls<br>were <1<br>year old) | 0 postnatal<br>days-14<br>years                                                          | All cerebral<br>lobes, basal<br>ganglia,<br>cerebellum,<br>brainstem,<br>spinal cord     | Qualitative<br>observation<br>(descriptive)                                                  | No difference                                             | (Reske-<br>Nielsen et<br>al., 1987) |
| GFAP<br>(DAKO,<br>Z334,<br>1:1000 | Asphyxia, infection, drug<br>intoxication, homicide,<br>heart malformation                               | SIDS- 11<br><br>Control- 11                                               | 40-77 post<br>conception<br>weeks                                                        | Brainstem pons                                                                           | 3-tier semi-<br>quantitative score                                                           | No difference                                             | (Oehmichen<br>et al., 1989a)        |

|                                                       |                                                                                                                                                 |                             |                                   |                                                               |                                               |                                                            |                                     |
|-------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|-----------------------------------|---------------------------------------------------------------|-----------------------------------------------|------------------------------------------------------------|-------------------------------------|
| GFAP<br>(bovine,<br>Dakopatts)<br>(1:200)             | Congenital heart disease,<br>gastroenteritis, accidental<br>asphyxia, acute trauma                                                              | SIDS- 81<br><br>Control- 17 | 33-92 post<br>conception<br>weeks | Brainstem<br>medulla nuclei<br>of DMNV &<br>NTS               | Counts/mm <sup>2</sup>                        | ↑ DMNV<br>&NTS                                             | (Bruce and<br>Becker,<br>1991)      |
| GFAP<br>(GFA<br>m761,<br>Biotest,<br>DAKO)<br>(1:100) | Infection, hypoxia, sudden<br>accidents                                                                                                         | SIDS- 17<br><br>Control- 23 | 1-12<br>postnatal<br>months       | Brainstem ION                                                 | Counts/mm <sup>2</sup>                        | Half of SIDS<br>had reactive<br>astrocytes                 | (Storm et al.,<br>1994)             |
| GFAP<br>(DAKO)<br>(N/S)                               | Accidental trauma                                                                                                                               | SIDS- 15<br><br>Control- 8  | 8 hours – 28<br>postnatal<br>days | Brainstem<br>medulla<br>reticular<br>formation,<br>DMNV & NTS | Counts/mm <sup>2</sup>                        | ↑ all                                                      | (Obonai et<br>al., 1996)            |
| GFAP<br>(DAKO<br>Z334)<br>(1:750)                     | Acute hypoglycaemia,<br>peritonitis and pneumonia,<br>non-cyanotic congenital<br>heart disease, trauma<br>(smothering, aspiration,<br>poisoned) | SIDS- 12<br><br>Control- 8  | 42-88<br>postconception<br>weeks  | Brainstem<br>medulla<br>Hypoglossal<br>nucleus                | Density: No. of<br>astrocytes/mm <sup>3</sup> | No difference                                              | (Pamphlett<br>and Treloar,<br>1996) |
| GFAP<br>(GFA<br>m761,<br>Biotest,<br>DAKO)<br>(1:100) | Risk factor analysis                                                                                                                            | SIDS- 19                    | 2-8<br>postnatal<br>months        | Brainstem ION                                                 | Counts/mm <sup>2</sup>                        | ↑ in ION of<br>infants<br>exposed to<br>cigarette<br>smoke | (Storm et al.,<br>1999)             |

|                                                |                                                                                                                                            |                              |                             |                                                                                                                                                                                                      |                                             |                                                                                                                   |                             |
|------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|-----------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------|-------------------------------------------------------------------------------------------------------------------|-----------------------------|
| GFAP<br>(DAKO)<br>(N/S)                        | Congenital cardiac abnormalities, infected pulmonary dysplasia, septic shock, seizure, neonatal hypoxemia, meningitis and brain infarction | SIDS- 26<br><br>Control- 12  | 3-40<br>postnatal<br>weeks  | Brainstem<br>nuclei                                                                                                                                                                                  | Density:<br>reactive/total<br>astrocytes    | ↑ in raphe<br>nuclei                                                                                              | (Sawaguchi<br>et al., 2002) |
| GFAP<br>(DAKO)<br>(N/S)                        | Infection, myocarditis, cardiopathy with pulmonary hypertension, Opitz syndrome, general infection, infanticide and meningitis             | SIDS- 26<br><br>Control- 12  | 3-40 post-<br>natal weeks   | Brainstem<br>Midbrain<br>periaqueductal<br>grey matter,<br>MRF, Medulla<br>ambiguous<br>nucleus,<br>DMNV, NTS,<br>LRF, MRF,<br>LRT), & pons<br>nucleus raphe<br>magnus and<br>reticular<br>formation | Density:<br>reactive/total<br>astrocytes    | SIDS-specific<br>negative<br>correlation<br>between<br>frequent<br>obstructive<br>apnea and<br>density in<br>DMNV | (Sawaguchi<br>et al., 2003) |
| GFAP<br>(bovine,<br>Novocast<br>ra)<br>(1:200) | Cardiac abnormalities, infection, biliary atresia                                                                                          | SIDS- 102<br><br>Control- 20 | 1-12<br>postnatal<br>months | Brainstem<br>(pontomesence<br>phalic portion<br>to the lower<br>pole of the<br>ION),<br>cerebellum                                                                                                   | Density and ratio of<br>reactive astrocytes | ↑ brainstem                                                                                                       | (Matturri et<br>al., 2006)  |

|                                                                          |                                                                                                                                               |                           |                                                       |                                                                                                                                                                                                                    |                               |                                                                                              |                            |
|--------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|-------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|----------------------------------------------------------------------------------------------|----------------------------|
| GFAP<br>(Ventana<br>Medical<br>Systems,<br>760-<br>4345)<br>(prediluted) | Non-suffocation (trauma,<br>drug overdose, pulmonary<br>infection, fatty acid<br>oxidation disorder,<br>congenital myopathy) &<br>suffocation | uSUDI- 114<br>Control- 39 | 2-358<br>postnatal<br>days                            | Hippocampus,<br>subiculum,<br>entorhinal<br>cortex                                                                                                                                                                 | Qualitative- yes vs<br>no     | ↓ CA3 and<br>subiculum                                                                       | (Kinney et<br>al., 2015)   |
| NCL-<br>GFAP-<br>GA5<br>(Novoca<br>stra)<br>(1:300)                      | Congenital heart diseases,<br>pneumonia and<br>pericarditis.                                                                                  | SIDS- 10<br>Controls- 8   | 25<br>gestational<br>weeks – 6<br>postnatal<br>months | Cerebellum,<br>brainstem<br>medulla and<br>pons                                                                                                                                                                    | Yes vs no<br>immunopositivity | ↑ internal<br>granular layer<br>of the<br>cerebellum                                         | (Lavezzi et<br>al., 2018)  |
| GFAP<br>(DAKO<br>Z0334)<br>(1:40<br>0000)                                | Alone versus co-slept<br>SIDS                                                                                                                 | SIDS- 20                  | <1 year                                               | Brainstem<br>medulla ION,<br>medial<br>lemniscus,<br>arcuate<br>nucleus, dorsal<br>accessory<br>olivary nucleus,<br>reticular<br>formation,<br>posterior<br>spinocerebellar<br>tract, inferior/<br>medial/superior | Counts/mm <sup>2</sup>        | ↑ inferior +<br>medial<br>vestibular<br>nucleus,<br>cochlear<br>nucleus of<br>alone sleepers | (Spinelli et<br>al., 2018) |

|                         |                                          |                           |                                   |                                               |                                      |                                        |                       |
|-------------------------|------------------------------------------|---------------------------|-----------------------------------|-----------------------------------------------|--------------------------------------|----------------------------------------|-----------------------|
|                         |                                          |                           |                                   | vestibular<br>nucleus,<br>cochlear<br>nucleus |                                      |                                        |                       |
| Predomi<br>nantly<br>BS | SUDEP and SUDC (thus<br>not age matched) | SIDS- 130<br><br>SUDC- 48 | 3 postnatal<br>days – 16<br>years | Hippocampus                                   | Yes vs no<br>subjective<br>judgement | ↑ prevalence<br>in SIDS<br>hippocampus | (Kon et al.,<br>2020) |
| SUDEP- 18               |                                          |                           |                                   |                                               |                                      |                                        |                       |

*Abbreviations:* BS basic stain, DMNV dorsal motor nucleus of the vagus, eSUDI explained sudden unexpected death in infancy, GFAP glial fibrillary acidic protein, H&E haematoxylin and eosin, XII hypoglossal nucleus, ION inferior olivary nucleus, LFB luxol fast blue, SIDS sudden infant death syndrome SUDC sudden unexpected death in childhood SUDEP sudden unexpected death in epilepsy N/S not specified, NSTT nucleus of the spinal trigeminal tract, NTS nucleus of the solitary tract, PCA post conception age, PTAH phosphotungstic acid-hematoxylin, SIDS sudden infant death syndrome.



## **2.5: GAPS IN KNOWLEDGE**

In the context of neuron cell death and astrogliosis in the developing human brain and SIDS, a number of gaps in knowledge exist, including:

- The mechanism by which cell death occurs in the SUDI hippocampus, and whether the nAChRs may play a role in regulating this process
- The effects of known SIDS risk factors on nAChRs and cell death
- Whether hippocampal sclerosis, or early-stage neuron loss occurs in a subset of SIDS, consistent with the limbic seizure hypothesis
- The normal distribution of reactive astrocytes in the human postnatal brain (1-12 months)
- Characterisation of the differences, if any, in astrogliosis in the SIDS brain
- The effects of known SIDS risk factors on astrogliosis

## **2.6: AIMS AND HYPOTHESES**

### **2.6.1: AIMS OF THIS PROJECT**

The main aim of this thesis is to examine neuronal cell death and/or loss, and astrogliosis in the developing human brain, and to determine if there are any changes in these parameters in the SIDS brain, or for the presence of major SIDS risk factors.

This aim is comprised of the following sub-aims:

1. Use IHC to characterise markers of cell death (active caspase-3 and TUNEL) and nAChR subunits ( $\alpha 7$  and  $\beta 2$ ) in the hippocampus of the postnatal human brain (Chapter 3),
2. Compare and contrast markers of cell death (active caspase-3 and TUNEL) and nAChR subunits ( $\alpha 7$  and  $\beta 2$ ) between three SUDI subgroups: eSUDI, SIDS I and SIDS II, as well as for the presence of SIDS risk factors of male sex, exposure to cigarette smoke, recent URTI, bed-sharing/co/sleeping and found prone (Chapter 3),
3. Optimise protocol for immunohistochemical investigation of NeuN in formalin-fixed, paraffin-embedded brain tissue (Chapter 4),
4. Investigate whether there is HS present (NeuN) in the hippocampus of SUDI infants (Chapter 4),
5. Determine a reproducible method for quantifying GFAP IHC (Chapter 5),

6. Characterise density (based on number) and reactive status (based on morphology) of astrocytes present in multiple regions of the human infant brain (Chapter 6 and 7),
7. Compare and contrast astrocyte density and morphology between three SUDI subgroups: eSUDI, SIDS I and SIDS II, as well as for the presence of SIDS risk factors of male sex, exposure to cigarette smoke, recent URTI, bed-sharing/co/sleeping and found prone in a UK cohort (Chapter 6),
8. Determine whether findings in chapter 6 are replicated in an Australian research cohort, investigating three SUDI subgroups: eSUDI, SIDS I and SIDS II, as well as for the presence of SIDS risk factors of male sex, exposure to cigarette smoke, recent URTI, bed-sharing/co/sleeping and found prone (Chapter 7).

### **2.6.2: HYPOTHESES OF THIS PROJECT**

It was hypothesised that in the developing hippocampus, there will be regional heterogeneity in the expression of markers of cell death and nAChR subunits, and that there will be regional heterogeneity in the number and morphology of astrocytes. In the context of SIDS, expression of cell death markers is hypothesised to be directly related to expression of nAChR subunits in infants with exposure to cigarette smoke or hypoxia. An increase in the number and activation-status (based on morphology) of GFAP astrocytes is also expected in the SIDS hippocampus, but also to be a widespread phenomenon affecting other brain regions with connections to the hippocampus, including the brainstem medulla and temporal cortex.

## **CHAPTER 3: THE $\alpha 7$ AND $\beta 2$ NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNITS REGULATE APOPTOSIS IN THE INFANT HIPPOCAMPUS, AND IN SUDDEN INFANT DEATH SYNDROME (SIDS)**

Luijck, L.L.M., Vivekanandarajah, A., Waters, K.A. and Machaalani, R., 2020. The  $\alpha 7$  and  $\beta 2$  nicotinic acetylcholine receptor subunits regulate apoptosis in the infant hippocampus, and in sudden infant death syndrome (SIDS). *Apoptosis*, 25, pp.574-589.

### Summary:

Increased cell death, beyond what is required in normal physiological development has been demonstrated in the SIDS hippocampus previously, however, it is not known by which mechanism that this occurs. nAChRs are known to have a role in regulating cell death, and are activated by nicotine, the major psychoactive component of cigarette smoke. Pre- and post-natal exposure to cigarette smoke is a major risk factor for SIDS, and it is also not known by which mechanism this occurs. The aim of this chapter was therefore to explore markers of cell death and nAChR subunits in the SIDS hippocampus and correlate their expression to determine their relationship. Finally, we correlate changes found against major SIDS risk factors, including exposure to cigarette smoke. Diagnostic groups included eSUDI, SIDS I and SIDS II, in accordance with the San Diego Criteria (Krous et al., 2004).

This study is the first to investigated expression of cell death (caspase-3 and TUNEL) and nAChR subunits ( $\alpha 7$  and  $\beta 2$ ) simultaneously in the developing human hippocampus. SIDS II infants showed widespread increases in markers of cell death, as well as some changes in  $\alpha 7$ . Expressional changes were also seen according to the presence of major SIDS risk factors, with the most notable changes seen in infants with a recent URTI. Importantly, these changes were predominantly amongst infants who were not bed-shared. Finally, expressional correlations were seen amongst markers, indicating that nAChR play a role in regulating cell death in the infant brain, and may account for abnormalities in SIDS.

### Contribution statement:

Input to project design was by RM, AV and LL. Tissue sectioning was performed by AV, and experimentation was performed by AV and LL prior to commencement of this PhD. Analysis

and drafting of the manuscript were performed by LL. The final version of the manuscript was approved by all authors prior to publication, and they have accepted that this manuscript will be included in my thesis.

Human tissue used in this study and the accompanying autopsy and infant characteristic data were obtained from the NSW Forensic and Analytical Science Services (formerly known as the Department of Forensic Medicine). Antibodies used in this study were verified previously using positive controls in our laboratory, detailed for TUNEL (Machaalani and Waters, 2003b), Caspase-3 (Machaalani et al., 2007) and nAChR subunits in (Vivekanandarajah et al., 2015). Experimentation and image analysis was performed at the SIDS and Sleep Apnea Laboratory at the University of Sydney, Sydney, Australia.



# The $\alpha 7$ and $\beta 2$ nicotinic acetylcholine receptor subunits regulate apoptosis in the infant hippocampus, and in sudden infant death syndrome (SIDS)

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## Abstract

Apoptosis is increased in the hippocampus of infants who died of sudden infant death syndrome (SIDS), yet it is not known via which mechanism this has occurred. Following existing support for a role of the  $\alpha 7$  and  $\beta 2$  nicotinic acetylcholine receptor (nAChR) subunits in apoptotic regulation, we aimed to determine whether these subunits are altered in the SIDS hippocampus and if they are correlated with cell death markers of active caspase-3 (Casp-3) and TUNEL. Further analyses were run according to the presence of major SIDS risk factors related to hypoxia (bed-sharing and prone sleeping), infection (presence of an upper respiratory tract infection (URTI)), cigarette smoke exposure and gender. Immunohistochemical expression of the markers was studied in 4 regions of the hippocampus (Cornu Ammonis (CA)1, CA2, CA3, CA4) and subiculum amongst 52 infants (aged 1–7 months) who died suddenly and unexpectedly (SUDI) and for whom the cause of death was explained (eSUDI;  $n=9$ ), or not and characterised as SIDS I ( $n=8$ ) and SIDS II ( $n=35$ ) according to the San Diego diagnostic criteria. Results showed that SIDS II infants had widespread increases in TUNEL compared with eSUDI and SIDS I infants, as well as increased  $\alpha 7$  and Casp-3 in CA2 compared to eSUDI infants, although these changes were predominant amongst infants who did not bed-share. Cigarette smoke exposure had minimal effects on the markers, while an URTI was associated with changes in all markers (after accounting for bed-sharing). Our findings support the role of nAChRs in regulating apoptosis in the SIDS hippocampus, and highlight the need for separate analysis according to risk factors.

**Keywords** Alpha 7 · Beta 2 · Caspase · TUNEL · Cigarette · Nicotine

## Abbreviations

|        |                                              |
|--------|----------------------------------------------|
| ACh    | Acetylcholine                                |
| CA     | Cornu Ammonis                                |
| Casp-3 | Caspase 3                                    |
| ChAT   | Choline acetyltransferase                    |
| eSUDI  | Explained sudden unexpected death in infancy |
| HF     | Hippocampal formation                        |

|       |                                                                              |
|-------|------------------------------------------------------------------------------|
| IHC   | Immunohistochemistry                                                         |
| IHH   | Intermittent hypercapnic hypoxia                                             |
| nAChR | Nicotinic acetylcholine receptor                                             |
| SIDS  | Sudden infant death syndrome                                                 |
| SUDI  | Sudden unexpected death in infancy                                           |
| TUNEL | Terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick end labelling |
| uSUDI | Unexplained sudden unexpected death in infancy                               |
| URTI  | Upper respiratory tract infection                                            |

LLM Luijckink and A Vivekanandarajah are equal first co-authors.

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s10495-020-01618-0>) contains supplementary material, which is available to authorized users.

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## Introduction

Sudden unexpected death in infancy (SUDI) is a broad term that refers to all sudden and unexpected infant deaths. Following autopsy, the death becomes either explained (eSUDI) such as when a cardiovascular anomaly is found, or unexplained (uSUDI). Although uSUDI originally defined the sudden infant death syndrome (SIDS), some pathologists

now classify cases separately into categories of SIDS and undetermined. SIDS remains a diagnosis of exclusion, with the most recent definition being “the sudden death of an infant < 1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough case investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history,” [1].

A major hypothesis is that SIDS deaths are the result of abnormal functioning of brainstem control of cardiorespiratory, arousal and autonomic reflexes in the face of a homeostatic stressor [2]. Although the hippocampus is primarily regarded for its role in learning and memory, it has also been recognised for its ability to modulate cardiorespiratory control via projections to key brainstem nuclei that regulate heart rate, respiration, blood pressure and temperature [3–5]. Hippocampal abnormalities identified in SIDS infants include morphological differences in the dentate gyrus [6, 7], and increased markers of cell death/apoptosis in the Cornu Ammonis (CA) [8, 9]. Previous research also supports a relationship between hypoxic/ischemic events that precede SIDS deaths [10], with several regions of the hippocampus recognised for their hypoxia sensitive nature following findings of disruptions in neurogenesis and synaptic plasticity in the dentate gyrus [11, 12], and apoptosis, synaptic transmissions and inflammatory responses in the CA1 [13–15].

Cholinergic neurons are highly sensitive to hypoxia compared with other neurotransmitter systems [16]. The nicotinic acetylcholine receptors (nAChRs) belong to a diverse family of ligand gated cation channels that each contain 5 transmembrane subunits arranged around a central pore (reviewed in [17]). Genes encoding 17 different subunits have been identified in vertebrate species to date ( $\alpha$ 1–10,  $\beta$ 1–4,  $\delta$ ,  $\epsilon$  and  $\gamma$ ), present as either heteropentamers or homopentamers, whereby the presence of particular subunits determines ligand specificity and affinity, channel gating and cation permeability (reviewed in [17, 18]). Eleven of these subunits are found in the mammalian brain including  $\alpha$ 2– $\alpha$ 7,  $\alpha$ 9,  $\alpha$ 10,  $\beta$ 2–4, and they are divided into two main types;  $\alpha$ -bungarotoxin sensitive (homomeric or heteromeric made up of  $\alpha$ 7,  $\alpha$ 9,  $\alpha$ 10), or  $\alpha$ -bungarotoxin insensitive (heteromeric combinations of  $\alpha$ 2– $\alpha$ 6 and  $\beta$ 2–4 (reviewed in [18])). Of these, the  $\alpha$ 4 $\beta$ 2 heteropentameric and  $\alpha$ 7 homopentameric nAChRs are the main nAChR subtypes expressed in the mammalian brain (reviewed in [18]). Studies at the mRNA or protein level related to the nAChRs, are restricted to the subunits; thus, any change in the level of expression of a particular subunit (e.g. when comparing disease status to non-disease), cannot be translated to identify the exact receptor subtype that is affected. Regarding the  $\beta$ 2 and  $\alpha$ 7 subunits, given that their combinations as  $\alpha$ 4 $\beta$ 2 heteropentameric and  $\alpha$ 7 homopentameric predominate in the

brain, it is likely that expressional studies of these subunits would be indicative of these nAChR subtypes. It is these two subunits that will be the focus of this study.

The  $\alpha$ 7 and  $\beta$ 2 subunits are expressed in the human hippocampus, and their expression decreases with age [19]. There is extensive literature to support the role of both the  $\alpha$ 7- and  $\beta$ 2-containing nAChRs in regulating cell death processes, which is of interest to us given that apoptosis is increased in the SIDS hippocampus [8, 9]. Knockout of the  $\beta$ 2 subunit increased cell death in the hippocampus [20], and lead to a loss of neuroprotection in the face of nicotine exposure during the neonatal period [21, 22] which was also seen when specifically antagonising the  $\alpha$ 4 $\beta$ 2 receptor [21, 23]. For the  $\alpha$ 7 subunit however, there is evidence of both pro and anti-apoptotic outcomes [21, 23–26]. The differential outcomes of  $\alpha$ 7-containing nAChRs on influencing cell fate is largely dependent on age [27, 28], time and duration of the stimulus (reviewed in [29]), and cell type/brain region analysed (reviewed in [30]).

The expression of the  $\alpha$ 7 and  $\beta$ 2 nAChR subunits in the SIDS hippocampus has not yet been studied. Herein, we aimed to determine their expression and whether they are correlated with changes in the expression of apoptotic markers. The apoptotic markers measured were active caspase-3 (Casp-3), a marker of early apoptosis, and Terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick end labelling (TUNEL), which identifies DNA damage and is thus a marker of late stage cell death. Further analyses, performed for correlations with known SIDS risk factors, aimed to evaluate whether those associated with hypoxia (prone sleeping and bed-sharing), nicotine (cigarette smoke exposure) or infection (presence of an upper respiratory tract infection (URTI)), alternatively or additionally contribute to changes in marker expression. Combined, the findings help identify whether the  $\alpha$ 7 and  $\beta$ 2 nAChR subunits play a role in the process of increased cell death reported in the SIDS hippocampus, and thus highlight potential targets for intervention.

## Materials and methods

### Tissue collection

Tissue was sourced from infants who died aged 1–7 months between the years 2000–2012 from the Department of Forensic Medicine, Glebe, NSW following approval from the Human Ethics Committees of the University of Sydney (Approval No. 3013/235) and the NSW Central Sydney Area Health Service (Protocol No 3593 and X13-0038). Information from autopsy records, clinical history, and police death scene investigation reports were collated using a questionnaire to standardise the profile of information relevant to our

study, regarding SIDS risk factors, and clinical and autopsy details. This profile also included the sleep position found at death, sleep environment (alone or bed-shared) and cigarette smoke exposure status of household members.

Brain tissue is collected as part of the routine autopsy to help aid post-mortem investigation for cause of death. This tissue is fixed in 20% neutral buffered formalin (10% for cases sourced prior to 2003) and paraffin embedded. Tissue that remained after the completed autopsy was used in this study. Hippocampal tissue was sectioned at 7  $\mu$ m thickness using a rotary microtome, mounted on 2% 3-aminopropyltriethoxysilane-treated slides, dried overnight at 42 °C and stored in a dust free environment for a minimum of one week prior to immunohistochemical (IHC) staining.

### Immunohistochemistry

For each marker,  $\alpha$ 7,  $\beta$ 2, TUNEL, and Casp-3, one tissue section from each case was stained. Additionally, for 20% of cases an adjacent tissue section was also stained to provide duplicates that ensure reproducibility. IHC technique is as per our laboratories standard protocol [31, 32]. In brief, sections were deparaffinised, rehydrated through a graded series of ethanol to distilled water and then subjected to heat induced epitope retrieval by microwaving on high (Home-maker; EM925ENV; 900 W) in 10% Tris–EDTA buffer (1 mM EDTA, 1 mM Na Citrate, 2 mM Tris; pH 9.0) for 14 min. Slides were left to cool, washed in phosphate buffered saline (PBS) and quenched for endogenous hydrogen peroxidase activity by immersing in 50% PBS, 50% methanol and 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 25 min. Following washes in PBS, sections then underwent single staining for  $\alpha$ 7 and  $\beta$ 2, and double staining for TUNEL and Casp-3.

#### $\alpha$ 7 and $\beta$ 2 nAChRs

Sections were blocked with 10% normal horse serum (NHS) in PBS for 30 min, followed by incubation with the primary antibody;  $\alpha$ 7 (ab 10.096, Abcam, 1:200 dilution in 1% NHS) and  $\beta$ 2 (sc-11372, Santa Cruz, 1:100 in 1% NHS) overnight at room temperature. Specificity of these antibodies has been addressed in detail in our previous manuscript [19]. The following day, after PBS washes, sections were incubated in biotinylated secondary anti-rabbit made in horse antibody (BA-1100, Vector Laboratories Inc., 1:200 dilution in 1% NHS) for 45 min. Following PBS washes, sections were incubated with avidin–biotin horse radish peroxidase complex (VEPH-4000, Vector Stain ABC kit; Vector Laboratories Inc) for 45 min. Colour labelling was achieved with 3,3'-diaminobenzidine (DAB) (K346811, DAKO; USA). Sections were then washed in distilled water, counterstained with Harris' haematoxylin, cleared in acid/alcohol, and differentiated with Scott's blueing solution before being

dehydrated through a graded series of ethanol, cleared with xylene, and mounted and coverslipped with Dibutylphthalate Polystyrene Xylene (DPX).

#### Casp-3 and TUNEL double IHC

TUNEL labelling using a commercial kit (Millipore ApopTag Peroxidase in Situ Kit, #S7100) following the manufacturers' instructions preceded Casp-3 labelling. Tissue sections were incubated with equilibration buffer (30 s) followed by terminal deoxynucleotidyl transferase (TdT) enzyme (1:4 dilution in reaction buffer) for 60 min at 37 °C. Sections were then placed in stop/wash solutions for 10 min to stop the reaction, followed by two 3-min washes in PBS and then incubated for 40 min with anti-digoxigenin peroxidase. After PBS washes, colour was developed with DAB. Tissue sections were then thoroughly washed in PBS, incubated with 10% NHS in PBS for 30 min and then in Casp-3 (Cat no: 559565; BD Pharmigen, 1:300 dilution in 1% NHS) overnight at room temperature. Following PBS washes, tissue sections were incubated in biotinylated secondary anti-rabbit made in horse antibody (BA-1100, Vector Laboratories Inc., 1:200 dilution in 1% NHS) for 45 min, then washed in PBS and incubated in avidin–biotin alkaline phosphatase (AK 5000, Vectastain ABC kit; Vector Laboratories) in PBS for 45 min. Tissue sections were then rinsed in 0.05 M Tris buffered saline (TBS) pH 7.5, followed by the development of colour with nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution (K0598, Dako) in a dark environment for 45 min. After TBS and distilled H<sub>2</sub>O wash, tissue sections were taken quickly through a graded series of ethanol (70%, 95%, 100%, 100%) for 1 min each, cleared in histolene and mounted in Vectamount (H-5000, Vector Laboratories). Negative controls for TUNEL staining involved omitting the addition of TdT enzyme to the reaction buffer and negative controls for Casp-3 involved replacing the primary antibody with 1% NHS.

#### Hippocampus levels and quantitative analysis

Cases were only included in this study if the hippocampal tissue section was from the left-hand side of the brain and was at the level of the lateral geniculate nucleus spanning Figs. 39–45 from The Atlas of the Human Brain [33]. These criteria were set to reduce heterogeneity from a hippocampal functional perspective and up to 25% of our cases were excluded due to not meeting these criteria leaving 52 infant cases in our final dataset. The cause of death was determined by an expert panel as reported recently by our group [34] using the San Diego diagnostic criteria [1]. The diagnostic categories included eSUDI (n=9), SIDS I (n=8) and SIDS II (n=35). eSUDI included diagnoses of myocarditis (n=2), Waterhouse-Friderichsen

syndrome (n=2), congenital muscular dystrophy (n=1), mitral valve dysplasia (n=1), cardiac arrest (n=1), Tetralogy of Fallot (n=1) and meningoencephalitis (n=1).

Four regions of the hippocampus were studied (CA1, CA2, CA3, CA4), as well as the subiculum. These regions were defined with reference to Fig. 3.7 from *The Human Hippocampus: An Atlas of the Applied Anatomy* [35]. The dentate gyrus was not assessed because it was not analysed in our original study [8], and because recent studies showing it to have morphological abnormalities in SIDS [36] highlight that it needs more detailed study that is outside the scope of the current work.

Tissue sections were coded to ensure that the observer was blinded to diagnosis preventing quantitative analysis bias. Images of the staining were captured using a Leica Upright DM6000 Microscope (Leica Microsystems Ltd. Heerbrugg, Switzerland) at  $\times 10$  objective lens magnification. The quantitative analysis was performed manually using the cell counter function in ImageJ software (v2.00, National Institute of Health, USA) and was of neurons only. Counts were then converted to a percentage of positively stained neurons, which was obtained by counting the total number of positive neurons and dividing by the total number of neurons in that region.

## Statistical analysis

All data was exported to SPSS for Windows (version 25; SPSS (IBM) Inc., Illinois, USA) for statistical analysis, and categorical infant characteristics were assigned a numerical value prior to export (1 = yes, 2 = no). Chi square testing was used to compare nominal infant data characteristics between groups, where values were expressed as a percentage. ANOVA with post-hoc least significant difference (LSD) correction was used to compare continuous infant characteristic data and the mean percentage of positively stained neurons for IHC markers between diagnostic groups, where values were expressed as mean  $\pm$  standard error of the mean (SEM). Independent samples t-tests were used to compare mean scores of marker expression in the presence and absence of risk factors. Pearson correlations were used to determine the direction and strength of the relationship between each marker for each hippocampal region. Significance was taken at  $p \leq 0.05$ , and trends between 0.06 and 0.09 are noted given the small number of eSUDI, and cases when undertaking risk factor analyses.

## Results

### Group characteristics

Cases were matched across diagnostic groups on clinical and autopsy data, as well as risk factor profiles, although

the incidences of recent URTI, and bed-sharing were higher amongst SIDS II, consistent with diagnostic criteria [1] (Table 1). Tissue fixation time was higher in the eSUDI group due to the fact that 5 cases in the eSUDI group were from the early 2000s when the protocol at the forensics department was for a longer fixation time in a 10% buffered formalin solution as opposed to a 20% solution and shorter duration for cases post 2003. Despite this, we did not find staining quantification to differ based on fixation. Although not statistically significant, a trend towards a higher post-mortem interval was noted for the SIDS I group.

Following previously reported neuropathological differences between infants sleeping alone and those who were bed-sharing [37] and the hypothesised different mechanism of death [37, 38], analyses were undertaken in the SIDS II cohort comparing bed-sharing and non-bed sharing infants. Characteristically, these cohorts did not differ for clinical and autopsy data, tissue parameters and risk factor profiles (Table 1), although more bed-sharers had cigarette smoke exposure (48% vs 22%), yet this difference was not statistically significant ( $p = 0.19$ , Table 1).

### Staining pattern of $\alpha 7$ , $\beta 2$ nAChRs and Casp-3/TUNEL

$\alpha 7$  and  $\beta 2$  nAChR staining was visualised as brown staining in the cytoplasm of neurons (Fig. 1). Amongst the regions, expression of  $\alpha 7$  was highest in the CA1 and CA4 regions, while expression of  $\beta 2$  was highest in the CA3 and CA4 regions (Table 2).

Casp-3 staining was visualised as purple-blue staining in the cytoplasm of neurons, and TUNEL staining was visualised as dark brown in the nucleus of neurons (Fig. 1). Casp-3 and TUNEL staining did not differ greatly amongst regions, except in the SIDS II group (Table 2).

Correlation analyses were used to determine relationships between expression of the markers by hippocampal region. For the eSUDI group alone, no significant correlations were found (data not shown). For all cases (regardless of diagnosis), correlations were found between: total  $\beta 2$  and total  $\alpha 7$ ,  $r(47) = 0.29$ ,  $p = 0.05$ , total  $\beta 2$  and total TUNEL  $r(47) = 0.29$ ,  $p = 0.05$ , total  $\alpha 7$  and total Casp-3  $r(43) = 0.31$ ,  $p = 0.04$ , and  $\alpha 7$  and Casp-3 in the CA2 region  $r(41) = 0.62$ ,  $p < 0.01$  (data not shown).

To account for the possible contribution of nicotine to these correlations, the correlations were run separately for the cases without and with a history of cigarette smoke exposure. For those *without a history*, the correlation between the total  $\beta 2$  and total TUNEL was maintained  $r(22) = 0.51$ ,  $p = 0.01$  (data not shown), as was that of  $\alpha 7$  and Casp-3 in the CA2 region  $r(23) = 0.61$ ,  $p = 0.003$  (Fig. 2c). Additional correlations were noted for the CA2 region and were between  $\beta 2$  and TUNEL  $r(22) = 0.47$ ,  $p = 0.03$ , and  $\beta 2$  and



**Table 1** Clinical, autopsy and risk factor data of the study dataset amongst the 3 groups and then subdividing the SIDS II according to bed-sharing status

|                               | eSUDI (n = 9) | SIDS I (n = 8) | SIDS II (n = 35) | <i>p</i> -value* | Bed-sharers (n = 25) | Non-bed-sharers (n = 10) | <i>p</i> -value |
|-------------------------------|---------------|----------------|------------------|------------------|----------------------|--------------------------|-----------------|
| <i>Clinical data</i>          |               |                |                  |                  |                      |                          |                 |
| Males (%)                     | 5/9 (56)      | 5/8 (63)       | 23/35 (66)       | 0.85             | 15/25 (60)           | 8/10 (80)                | 0.26            |
| Birth weight (kg)             | 2.8 ± 2.4     | 3.0 ± 1.6      | 3.1 ± 1.4        | 0.69             | 3.3 ± 0.2            | 2.7 ± 0.3                | 0.67            |
| <i>Autopsy data</i>           |               |                |                  |                  |                      |                          |                 |
| Age at death (months)         | 4.5 ± 0.7     | 3.4 ± 0.6      | 3.3 ± 0.3        | 0.18             | 3.2 ± 0.3            | 3.6 ± 0.6                | 0.51            |
| PCA (weeks)                   | 57.2 ± 3.3    | 52.9 ± 2.2     | 51.7 ± 1.2       | 0.15             | 51.8 ± 1.3           | 51.2 ± 2.9               | 0.82            |
| Body weight (kg)              | 6.5 ± 0.8     | 6.1 ± 0.6      | 6.0 ± 0.3        | 0.80             | 6.1 ± 0.4            | 5.7 ± 0.6                | 0.58            |
| Brain weight (g)              | 715 ± 75      | 695 ± 52       | 722 ± 27         | 0.92             | 718 ± 28             | 732 ± 65                 | 0.82            |
| Body length (cm)              | 61.4 ± 2.5    | 62.4 ± 1.9     | 60.1 ± 1.3       | 0.70             | 60.4 ± 1.5           | 59.3 ± 2.9               | 0.70            |
| Head circumference (cm)       | 40.7 ± 1.6    | 40.3 ± 1.0     | 40.5 ± 0.6       | 0.98             | 40.4 ± 0.7           | 40.7 ± 1.0               | 0.86            |
| <i>Tissue parameters</i>      |               |                |                  |                  |                      |                          |                 |
| PMI (h)                       | 29.2 ± 8.2    | 38.5 ± 5.4     | 25.8 ± 1.2       | 0.06             | 26.4 ± 1.6           | 24.0 ± 1.1               | 0.40            |
| Fixation (weeks)              | 3.6 ± 1.2     | 1.7 ± 0.4      | 1.2 ± 0.1        | <b>&lt; 0.01</b> | 1.3 ± 0.1            | 0.8 ± 0.1                | 0.39            |
| <i>Risk factor prevalence</i> |               |                |                  |                  |                      |                          |                 |
| Found prone (%)               | NA            | 5/8 (63)       | 11/29 (38)       | 0.21             | 8/20 (40)            | 3/9 (33)                 | 0.73            |
| Bed-sharing (%)               | 0/9 (0)       | 0/8 (0)        | 25/35 (71)       | <b>&lt; 0.01</b> | N/A                  | N/A                      |                 |
| Cigarette smoke exposure (%)  | 1/5 (20)      | 5/8 (63)       | 12/30 (40)       | 0.30             | 10/21 (48)           | 2/9 (22)                 | 0.19            |
| Recent URTI                   | 1/9 (11)      | 1/7 (14)       | 19/33 (58)       | <b>0.01</b>      | 13/23 (57)           | 6/10 (60)                | 0.85            |
| Duration of URTI (days)       | 2             | 1              | 3–14             | N/A              | 3–14                 | 3–14                     |                 |

Results are presented as mean ± SEM. Fractions are provided where data was incomplete for all cases in that particular group

PCA post conception age, PMI post-mortem interval, N/A not applicable

\*Significance taken at  $p \leq 0.05$

Casp3  $r(22) = 0.47$ ,  $p = 0.03$  (Fig. 2a, b). Other correlations included  $\beta 2$  and TUNEL, and TUNEL and Casp3 for CA4 ( $r(23) = 0.75$ ,  $p < 0.01$  and  $r(23) = 0.43$ ,  $p = 0.04$  respectively) and  $\beta 2$  and  $\alpha 7$  for CA3  $r(25) = 0.57$ ,  $p < 0.01$  (data not shown). Analyses for the cases *with a history* of cigarette smoke exposure, revealed only one statistically significant correlation and this was between  $\alpha 7$  and Casp-3 in the CA2 region  $r(15) = 0.77$ ,  $p = 0.001$  (Fig. 2c).

### Comparison amongst diagnostic groups for cell death markers by proportion

For comparison with our previous data [8, 9], the proportion of cases with staining for Casp-3 and TUNEL per diagnostic group are reported in Supplementary Table 1. Amongst the eSUDI group, marker expression ranged from 2–10% for Casp-3 and 0–7% for TUNEL when averaged across the hippocampal regions studied. All 9 eSUDI infants had some positive staining of both Casp-3 and TUNEL in at least one brain region, however, the majority of cases only had between 2 and 9% expression, and no case showed greater than 20% staining for both markers. Of the SIDS I group, expressional levels for Casp-3 and TUNEL were relatively consistent with eSUDI, with expression ranging 3–13%

for Casp-3 and 0–4% for TUNEL in the total average, and no SIDS I infant showed greater than 20% staining. SIDS II were a more heterogenous group, with Casp-3 ranging between 1–23% expression, and TUNEL 0–39% in total average. The proportion of each marker varied greatly, and Casp-3 and TUNEL were expressed greater than 20% in at least one case for each brain region studied.

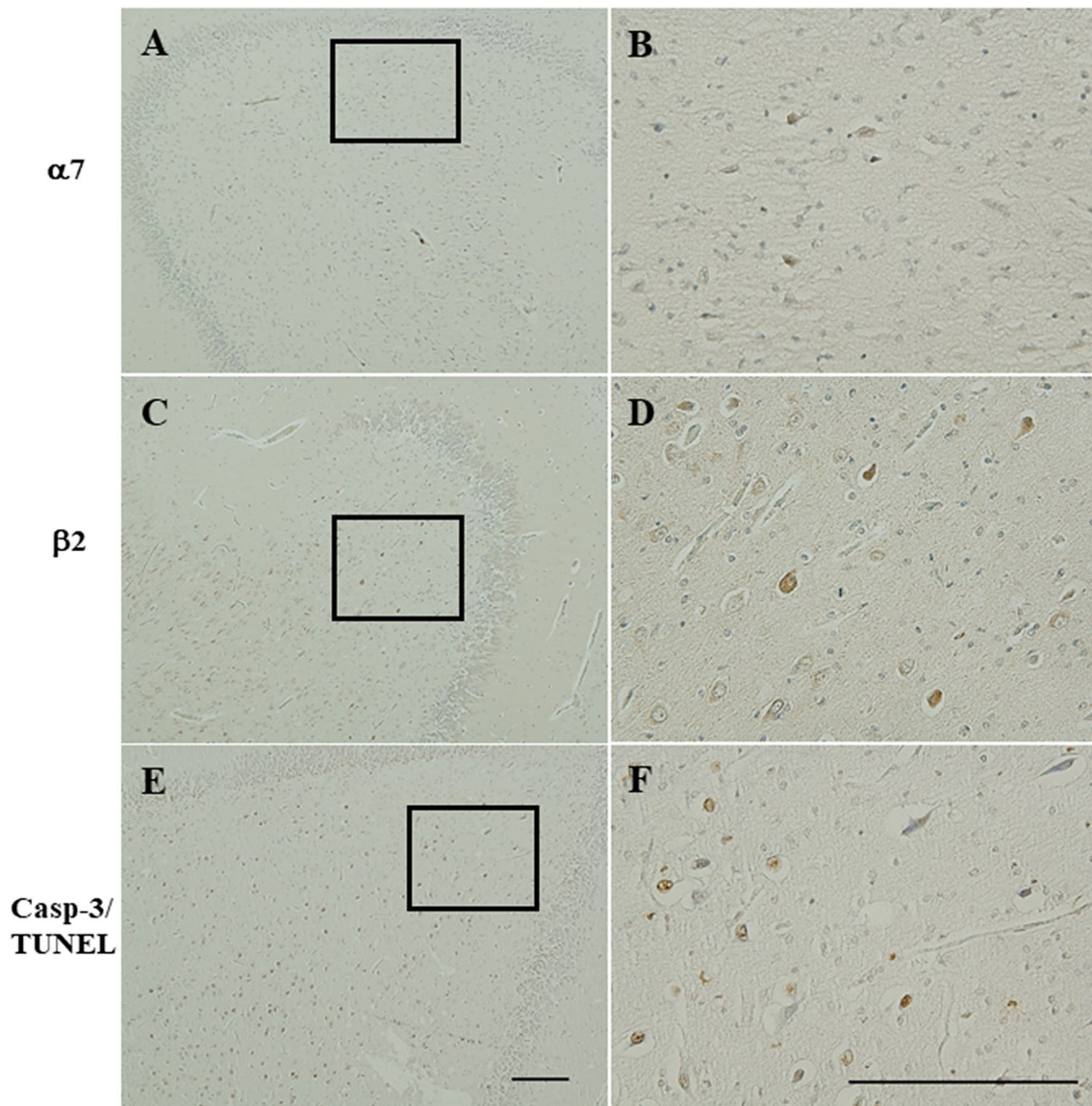
### Quantitative comparison amongst diagnostic groups

#### nAChR markers

Compared to the eSUDI group, SIDS II infants had significantly higher  $\alpha 7$  expression in CA2 ( $p = 0.05$ ) (Table 2). No significant differences were observed for  $\beta 2$  (Table 2).

#### Cell death markers

SIDS II infants had greater TUNEL expression in CA1 ( $p = 0.04$ ) and higher total expression ( $p = 0.05$ ), with a trend towards higher TUNEL in CA4 ( $p = 0.09$ ) compared to the SIDS I group. Compared with eSUDI, the differences were



**Fig. 1** Micrograph illustrating staining profile for  $\alpha 7$  (a, b),  $\beta 2$  (c, d) and Casp-3/TUNEL (e, f) in the CA4. Images shown at  $\times 10$  (a, c, e) and  $\times 40$  (b, d, f) magnification. Scale bars = 200  $\mu$ m

trends only; higher Casp-3 in CA2 ( $p=0.06$ ) and higher total TUNEL ( $p=0.08$ ) (Table 2).

#### Effects of the risk factors

**Risk factors independent of diagnostic group** Where data was available, the impact of a recent URTI, cigarette smoke exposure, and gender were analysed across all infants in our dataset, regardless of diagnosis (Table 3). No differences were found for any of the four markers (Table 3).

**Sleep related risk factor: SIDS only** As sleep-related risk factors were only pertinent to SIDS cases, analysis for prone sleeping and bed-sharing status was only under-

taken for SIDS I and SIDS II cases (Table 3). Infants who bed-shared had lower  $\beta 2$  in CA3 ( $p=0.05$ ) while infants found prone had lower  $\beta 2$  in the subiculum ( $p=0.04$ ). Bed-sharing infants showed a trend towards lower  $\alpha 7$  in CA2 ( $p=0.06$ ) (Table 3).

#### Excluding bed-sharers: comparison amongst diagnostic groups

Analyses were repeated after excluding infants who were bedsharing at the time of death (Table 4).

**Table 2** Hippocampal expression of  $\alpha 7$ ,  $\beta 2$ , Casp-3 and TUNEL for diagnostic categories and amongst SIDS II according to bed-sharing

|                                            | eSUDI (n=9)    | SIDS I (n=8)   | SIDS II (n=35)                               | SIDS II non bed-shared (n=10)                 | SIDS II bed-shared (n=25)                   |
|--------------------------------------------|----------------|----------------|----------------------------------------------|-----------------------------------------------|---------------------------------------------|
| <i><math>\alpha 7</math> nAChR subunit</i> |                |                |                                              |                                               |                                             |
| CA4                                        | 16.1 $\pm$ 6.8 | 20.4 $\pm$ 6.9 | 17.0 $\pm$ 2.7                               | 23.4 $\pm$ 4.5                                | 14.3 $\pm$ 3.3                              |
| CA3                                        | 6.5 $\pm$ 1.8  | 15.4 $\pm$ 5.0 | 12.1 $\pm$ 2.1                               | 17.1 $\pm$ 3.7*                               | 10.0 $\pm$ 2.5                              |
| CA2                                        | 2.4 $\pm$ 1.0  | 9.3 $\pm$ 2.5  | <b>10.3 <math>\pm</math> 1.8*</b>            | <b>17.0 <math>\pm</math> 4.7*</b>             | 7.8 $\pm$ 1.6                               |
| CA1                                        | 18.6 $\pm$ 6.2 | 16.4 $\pm$ 4.9 | 16.2 $\pm$ 2.6                               | 11.2 $\pm$ 2.4                                | 18.3 $\pm$ 3.5                              |
| SUB                                        | 7.7 $\pm$ 1.5  | 6.3 $\pm$ 1.7  | 8.8 $\pm$ 1.1                                | 9.7 $\pm$ 2.3                                 | 8.4 $\pm$ 1.2                               |
| Total average                              | 10.2 $\pm$ 1.9 | 13.6 $\pm$ 1.9 | 12.9 $\pm$ 1.3                               | <b>15.8 <math>\pm</math> 1.9*</b>             | 11.7 $\pm$ 1.6                              |
| <i><math>\beta 2</math> nAChR subunit</i>  |                |                |                                              |                                               |                                             |
| CA4                                        | 18.4 $\pm$ 3.7 | 18.1 $\pm$ 3.9 | 21.0 $\pm$ 3.2                               | 30.6 $\pm$ 8.5                                | 17.2 $\pm$ 2.6                              |
| CA3                                        | 21.9 $\pm$ 6.0 | 20.6 $\pm$ 8.6 | 19.0 $\pm$ 3.4                               | 33.1 $\pm$ 8.8                                | 13.3 $\pm$ 2.7                              |
| CA2                                        | 16.7 $\pm$ 6.9 | 9.6 $\pm$ 5.1  | 12.2 $\pm$ 2.8                               | 17.9 $\pm$ 4.7                                | 10.0 $\pm$ 3.4                              |
| CA1                                        | 6.8 $\pm$ 1.4  | 9.1 $\pm$ 2.7  | 10.6 $\pm$ 1.6                               | 15.8 $\pm$ 4.7                                | 8.5 $\pm$ 1.2                               |
| SUB                                        | 7.5 $\pm$ 2.9  | 5.9 $\pm$ 3.1  | 8.7 $\pm$ 1.6                                | 12.6 $\pm$ 4.9                                | 7.2 $\pm$ 1.2                               |
| Total average                              | 15.0 $\pm$ 3.6 | 12.6 $\pm$ 3.8 | 14.2 $\pm$ 2.0                               | 21.6 $\pm$ 5.2                                | 11.2 $\pm$ 1.6                              |
| <i>Caspase-3</i>                           |                |                |                                              |                                               |                                             |
| CA4                                        | 6.4 $\pm$ 1.4  | 10.4 $\pm$ 2.3 | 11.6 $\pm$ 1.8                               | 12.6 $\pm$ 2.7*                               | 11.2 $\pm$ 2.2                              |
| CA3                                        | 5.7 $\pm$ 1.7  | 6.6 $\pm$ 2.9  | 8.7 $\pm$ 1.7                                | 9.5 $\pm$ 2.7                                 | 8.4 $\pm$ 2.1                               |
| CA2                                        | 3.0 $\pm$ 0.9  | 8.6 $\pm$ 1.7  | 10.5 $\pm$ 2.2*                              | <b>13.8 <math>\pm</math> 4.5*</b>             | 9.3 $\pm$ 2.5                               |
| CA1                                        | 5.6 $\pm$ 1.7  | 4.8 $\pm$ 0.9  | 4.7 $\pm$ 1.0                                | 4.0 $\pm$ 1.1                                 | 5.0 $\pm$ 1.3                               |
| SUB                                        | 3.9 $\pm$ 1.3  | 7.0 $\pm$ 1.5  | 4.3 $\pm$ 0.7                                | <b>3.0 <math>\pm</math> 1.2<sup>^</sup></b>   | 4.8 $\pm$ 0.8                               |
| Total average                              | 4.9 $\pm$ 1.1  | 7.4 $\pm$ 1.3  | 8.0 $\pm$ 1.1                                | 8.6 $\pm$ 1.6*                                | 7.7 $\pm$ 1.4                               |
| <i>TUNEL</i>                               |                |                |                                              |                                               |                                             |
| CA4                                        | 4.5 $\pm$ 2.2  | 1.9 $\pm$ 0.9  | 10.2 $\pm$ 2.1 <sup>^</sup>                  | <b>19.3 <math>\pm</math> 4.7*<sup>^</sup></b> | <b>6.8 <math>\pm</math> 1.9<sup>#</sup></b> |
| CA3                                        | 1.8 $\pm$ 0.7  | 1.3 $\pm$ 0.8  | 4.7 $\pm$ 1.3                                | <b>8.2 <math>\pm</math> 2.8*<sup>^</sup></b>  | 3.4 $\pm$ 1.5                               |
| CA2                                        | 2.5 $\pm$ 0.9  | 2.3 $\pm$ 1.1  | 4.1 $\pm$ 1.2 <sup>^</sup>                   | 8.6 $\pm$ 3.5*                                | 2.3 $\pm$ 0.7                               |
| CA1                                        | 6.6 $\pm$ 1.6  | 2.4 $\pm$ 1.1  | <b>17.9 <math>\pm</math> 3.3<sup>^</sup></b> | <b>28.8 <math>\pm</math> 8.2*<sup>^</sup></b> | 13.8 $\pm$ 3.0                              |
| SUB                                        | 3.9 $\pm$ 1.4  | 2.7 $\pm$ 0.7  | 12.1 $\pm$ 2.8                               | <b>24.8 <math>\pm</math> 7.1*<sup>^</sup></b> | <b>7.3 <math>\pm</math> 2.2<sup>#</sup></b> |
| Total average                              | 3.6 $\pm$ 1.0  | 2.0 $\pm$ 0.6  | <b>9.8 <math>\pm</math> 1.7<sup>^</sup></b>  | <b>18.0 <math>\pm</math> 3.9*<sup>^</sup></b> | <b>6.7 <math>\pm</math> 1.5<sup>#</sup></b> |

Results presented as mean % positively stained neurons ( $\pm$ SEM) following one-way ANOVA with post-hoc LSD correction for comparison between groups, and two-tailed independent samples t-test to compare non-bed-shared with bed-shared infants. Significance taken  $p \leq 0.05$ . Bold used to highlight significance. Italicised indicate statistical trends with  $p = 0.06-0.09$

\*Significance compared to eSUDI, <sup>^</sup>Significance compared to SIDS I, <sup>#</sup>Significance compared to non-bed-sharing infants

### nAChR markers

Compared to the eSUDI group, non-bed-sharing SIDS II infants had significantly greater  $\alpha 7$  in CA2 ( $p < 0.01$ ), and greater total expression ( $p = 0.05$ ) while there were no differences in  $\beta 2$  expression. Compared with eSUDI, non-bed-sharing SIDS II infants trended towards increased  $\alpha 7$  in CA3 ( $p = 0.06$ ) and total average ( $p = 0.07$ ) (Table 4).

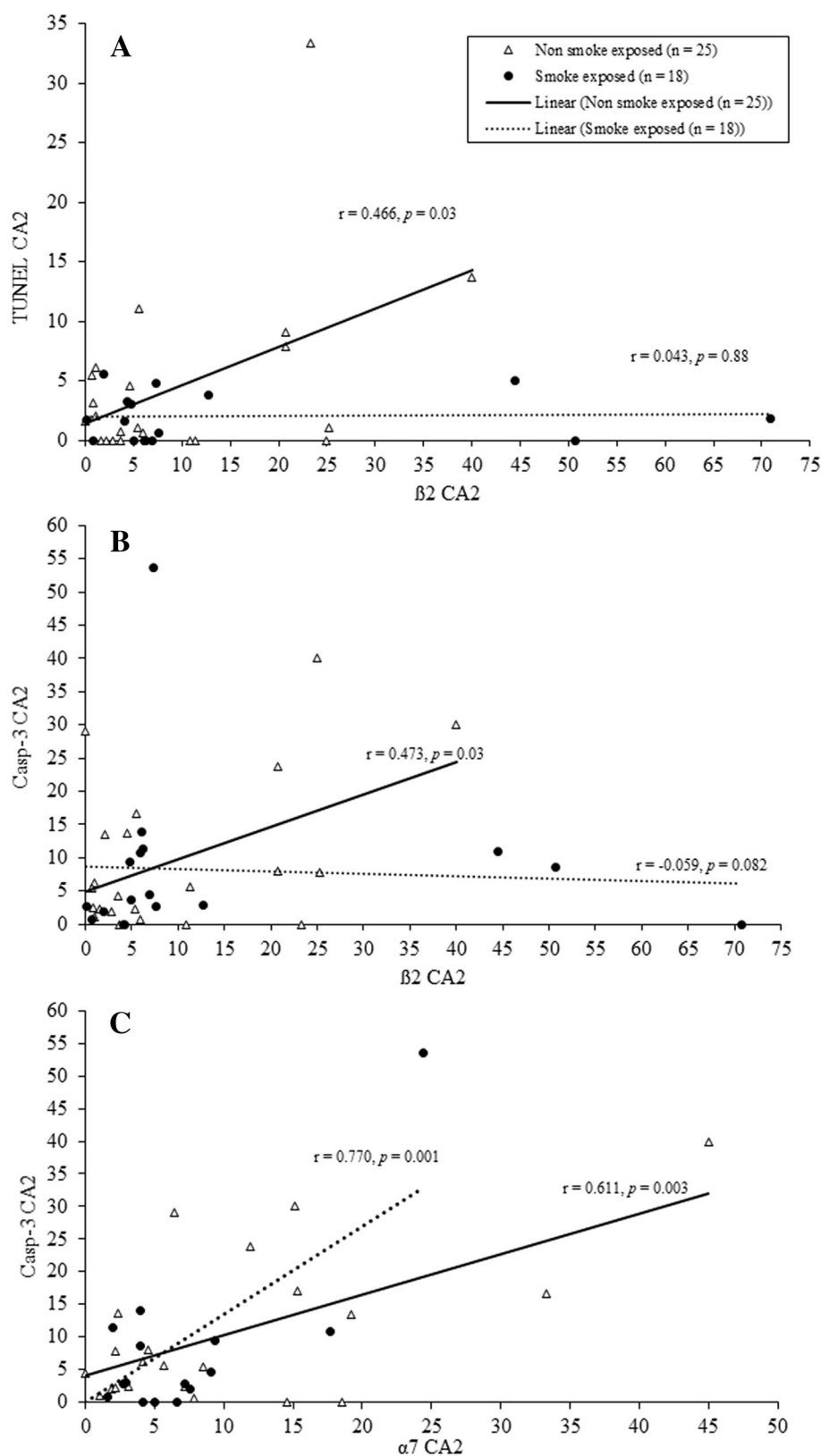
### Cell death markers

Compared to the eSUDI group, higher Casp-3 expression was seen in SIDS II infants in CA2 ( $p = 0.02$ ). SIDS I infants showed greater expression of Casp-3 in the subiculum ( $p = 0.05$ ) compared to non-bed-sharing SIDS II cases.

Compared with eSUDI, non-bed-sharing SIDS II infants tended to have higher Casp-3 in CA4 ( $p = 0.06$ ) and total average ( $p = 0.07$ ).

Compared to eSUDI, non-bed-shared SIDS II infants showed significantly greater TUNEL in CA4 ( $p < 0.01$ ), CA3 ( $p = 0.03$ ), CA1 ( $p = 0.01$ ) and the subiculum ( $p = 0.01$ ), as well as higher total expression ( $p < 0.01$ ). These increases in TUNEL amongst the non-bed-shared SIDS II group were also seen relative to SIDS I infants in CA4 ( $p < 0.01$ ), CA3 ( $p = 0.04$ ), CA1 ( $p < 0.01$ ) and the subiculum ( $p = 0.01$ ), as well as higher total expression ( $p < 0.01$ ). Non-bed-sharing SIDS II infants tended to have higher expression of TUNEL in CA2 compared with eSUDI ( $p = 0.09$ ) (Table 4).

**Fig. 2** Correlations between markers in the CA2 when analysing infants without (triangles) vs with (circles) a history of cigarette smoke exposure; **a** correlation between TUNEL &  $\beta 2$  in CA2, **b** correlation between Casp-3 &  $\beta 2$  in CA2, and **c** correlation between Casp-3 &  $\alpha 7$  in CA2



**Table 3** Effects of risk factors on hippocampal expression of  $\alpha 7$ ,  $\beta 2$ , Casp-3 and TUNEL

|                                            | UTRI all cases |                | Smoke exposure all cases |                | Gender All cases |                 | Bed-sharing (SIDS only) |                                  | Found prone (SIDS only) |                                 |
|--------------------------------------------|----------------|----------------|--------------------------|----------------|------------------|-----------------|-------------------------|----------------------------------|-------------------------|---------------------------------|
|                                            |                |                |                          |                |                  |                 |                         |                                  |                         |                                 |
|                                            | No (n = 27)    | Yes (n = 21)   | No (n = 25)              | Yes (n = 18)   | Male (n = 32)    | Female (n = 20) | No (n = 18)             | Yes (n = 25)                     | No (n = 21)             | Yes (n = 16)                    |
| <i><math>\alpha 7</math> nAChR subunit</i> |                |                |                          |                |                  |                 |                         |                                  |                         |                                 |
| CA4                                        | 14.3 $\pm$ 3.2 | 21.7 $\pm$ 3.6 | 20.2 $\pm$ 3.9           | 14.5 $\pm$ 2.5 | 16.6 $\pm$ 2.8   | 18.5 $\pm$ 4.3  | 22.1 $\pm$ 3.8          | 14.3 $\pm$ 3.3                   | 21.9 $\pm$ 4.0          | 15.7 $\pm$ 3.7                  |
| CA3                                        | 11.7 $\pm$ 2.8 | 11.2 $\pm$ 1.9 | 9.9 $\pm$ 1.7            | 13.2 $\pm$ 2.8 | 12.5 $\pm$ 2.4   | 10.6 $\pm$ 2.3  | 16.4 $\pm$ 2.9          | 10.0 $\pm$ 2.5                   | 12.2 $\pm$ 1.9          | 16.2 $\pm$ 4.5                  |
| CA2                                        | 8.0 $\pm$ 1.5  | 9.4 $\pm$ 2.6  | 10.8 $\pm$ 2.2           | 6.8 $\pm$ 1.6  | 9.1 $\pm$ 1.5    | 8.8 $\pm$ 2.7   | 13.7 $\pm$ 2.9          | 7.8 $\pm$ 1.6                    | 11.7 $\pm$ 2.7          | 11.0 $\pm$ 2.0                  |
| CA1                                        | 16.3 $\pm$ 2.9 | 16.0 $\pm$ 3.6 | 14.2 $\pm$ 2.7           | 21.4 $\pm$ 4.3 | 16.9 $\pm$ 2.8   | 15.9 $\pm$ 3.3  | 13.4 $\pm$ 2.4          | 18.3 $\pm$ 3.5                   | 17.8 $\pm$ 4.0          | 16.3 $\pm$ 3.3                  |
| SUB                                        | 7.9 $\pm$ 1.0  | 9.4 $\pm$ 1.5  | 7.8 $\pm$ 1.1            | 8.1 $\pm$ 1.2  | 9.2 $\pm$ 1.2    | 6.6 $\pm$ 0.8   | 8.2 $\pm$ 1.5           | 8.4 $\pm$ 1.2                    | 9.0 $\pm$ 1.5           | 7.5 $\pm$ 1.4                   |
| Total average                              | 11.7 $\pm$ 1.5 | 13.4 $\pm$ 1.5 | 12.6 $\pm$ 1.5           | 12.8 $\pm$ 1.3 | 13.0 $\pm$ 1.2   | 11.9 $\pm$ 1.9  | 14.9 $\pm$ 1.4          | 11.7 $\pm$ 1.6                   | 14.3 $\pm$ 1.7          | 13.7 $\pm$ 1.8                  |
| <i><math>\beta 2</math> nAChR subunit</i>  |                |                |                          |                |                  |                 |                         |                                  |                         |                                 |
| CA4                                        | 17.3 $\pm$ 2.3 | 25.0 $\pm$ 4.6 | 23.2 $\pm$ 4.0           | 18.7 $\pm$ 2.8 | 21.4 $\pm$ 2.9   | 18.1 $\pm$ 3.9  | 25.0 $\pm$ 5.1          | 17.2 $\pm$ 2.6                   | 22.7 $\pm$ 4.1          | 15.9 $\pm$ 3.0                  |
| CA3                                        | 18.9 $\pm$ 3.8 | 21.4 $\pm$ 4.9 | 19.7 $\pm$ 4.0           | 19.2 $\pm$ 4.9 | 20.2 $\pm$ 3.6   | 18.8 $\pm$ 4.6  | 27.5 $\pm$ 6.2          | <b>13.3 <math>\pm</math> 2.7</b> | 19.8 $\pm$ 4.6          | 21.9 $\pm$ 5.9                  |
| CA2                                        | 11.9 $\pm$ 3.3 | 14.2 $\pm$ 3.8 | 10.8 $\pm$ 2.4           | 13.5 $\pm$ 4.7 | 12.9 $\pm$ 3.3   | 11.8 $\pm$ 3.1  | 14.0 $\pm$ 3.5          | 10.0 $\pm$ 3.4                   | 12.6 $\pm$ 3.7          | 12.6 $\pm$ 4.4                  |
| CA1                                        | 9.0 $\pm$ 1.5  | 11.3 $\pm$ 2.3 | 10.6 $\pm$ 2.1           | 8.6 $\pm$ 1.4  | 10.4 $\pm$ 1.7   | 8.8 $\pm$ 1.7   | 12.8 $\pm$ 2.9          | 8.5 $\pm$ 1.2                    | 11.1 $\pm$ 2.5          | 9.4 $\pm$ 1.7                   |
| SUB                                        | 7.3 $\pm$ 1.4  | 9.4 $\pm$ 2.7  | 9.5 $\pm$ 2.3            | 7.1 $\pm$ 1.6  | 8.8 $\pm$ 2.0    | 7.2 $\pm$ 1.6   | 9.6 $\pm$ 3.1           | 7.2 $\pm$ 1.2                    | 11.0 $\pm$ 2.6          | <b>4.8 <math>\pm</math> 1.3</b> |
| Total average                              | 13.0 $\pm$ 1.8 | 16.2 $\pm$ 3.0 | 14.7 $\pm$ 2.4           | 13.4 $\pm$ 2.4 | 14.8 $\pm$ 2.1   | 12.9 $\pm$ 2.3  | 17.6 $\pm$ 3.4          | 11.2 $\pm$ 1.6                   | 15.4 $\pm$ 2.9          | 12.8 $\pm$ 2.6                  |
| <i>Caspase-3</i>                           |                |                |                          |                |                  |                 |                         |                                  |                         |                                 |
| CA4                                        | 9.9 $\pm$ 1.8  | 10 $\pm$ 1.9   | 10.8 $\pm$ 2.0           | 9.4 $\pm$ 1.7  | 10.1 $\pm$ 1.8   | 11.3 $\pm$ 1.8  | 11.6 $\pm$ 1.8          | 11.2 $\pm$ 2.2                   | 10.3 $\pm$ 2.0          | 11.8 $\pm$ 2.7                  |
| CA3                                        | 8.6 $\pm$ 2.1  | 6.8 $\pm$ 1.5  | 8.1 $\pm$ 2.1            | 6.2 $\pm$ 1.5  | 9.0 $\pm$ 1.9    | 6.2 $\pm$ 1.1   | 8.3 $\pm$ 2.0           | 8.4 $\pm$ 2.1                    | 7.1 $\pm$ 2.3           | 9.9 $\pm$ 2.4                   |
| CA2                                        | 6.2 $\pm$ 1.1  | 10.6 $\pm$ 2.7 | 10.1 $\pm$ 2.3           | 8.1 $\pm$ 3.1  | 7.8 $\pm$ 1.4    | 11.0 $\pm$ 3.5  | 11.5 $\pm$ 2.7          | 9.3 $\pm$ 2.5                    | 10.5 $\pm$ 3.3          | 10.1 $\pm$ 2.2                  |
| CA1                                        | 5.7 $\pm$ 1.2  | 3.4 $\pm$ 0.8  | 4.4 $\pm$ 0.8            | 4.7 $\pm$ 1.1  | 5.0 $\pm$ 1.1    | 4.6 $\pm$ 0.8   | 4.3 $\pm$ 0.7           | 5.0 $\pm$ 1.3                    | 3.4 $\pm$ 0.6           | 6.3 $\pm$ 1.8                   |
| SUB                                        | 4.3 $\pm$ 0.8  | 4.2 $\pm$ 0.9  | 4.7 $\pm$ 0.9            | 5.1 $\pm$ 1.0  | 4.6 $\pm$ 0.8    | 4.5 $\pm$ 0.9   | 4.4 $\pm$ 1.1           | 4.8 $\pm$ 0.8                    | 4.3 $\pm$ 0.8           | 5.1 $\pm$ 1.3                   |
| Total average                              | 6.9 $\pm$ 1.1  | 7.1 $\pm$ 1.2  | 7.5 $\pm$ 1.2            | 6.8 $\pm$ 1.3  | 7.3 $\pm$ 1.0    | 7.5 $\pm$ 1.3   | 8.1 $\pm$ 1.0           | 7.7 $\pm$ 1.4                    | 7.2 $\pm$ 1.4           | 8.5 $\pm$ 1.6                   |
| <i>TUNEL</i>                               |                |                |                          |                |                  |                 |                         |                                  |                         |                                 |
| CA4                                        | 6.7 $\pm$ 1.7  | 9.4 $\pm$ 2.9  | 8.6 $\pm$ 2.4            | 6.5 $\pm$ 2.4  | 8.3 $\pm$ 1.9    | 7.9 $\pm$ 3.0   | 12.4 $\pm$ 3.6          | 6.8 $\pm$ 1.9                    | 8.9 $\pm$ 2.4           | 8.0 $\pm$ 2.9                   |
| CA3                                        | 4.0 $\pm$ 1.4  | 3.9 $\pm$ 1.5  | 3.7 $\pm$ 1.3            | 1.6 $\pm$ 0.5  | 3.6 $\pm$ 0.9    | 4.2 $\pm$ 2.2   | 5.7 $\pm$ 2.0           | 3.4 $\pm$ 1.5                    | 2.7 $\pm$ 0.8           | 3.5 $\pm$ 1.7                   |
| CA2                                        | 2.6 $\pm$ 0.7  | 5.0 $\pm$ 1.8  | 4.5 $\pm$ 1.6            | 2.0 $\pm$ 0.5  | 4.1 $\pm$ 1.2    | 2.6 $\pm$ 1.0   | 6.1 $\pm$ 2.3           | 2.3 $\pm$ 0.7                    | 3.7 $\pm$ 3.7           | 2.7 $\pm$ 0.7                   |
| CA1                                        | 11.5 $\pm$ 3.4 | 18.9 $\pm$ 3.9 | 14.9 $\pm$ 4.1           | 11.5 $\pm$ 3.2 | 15.0 $\pm$ 3.0   | 12.7 $\pm$ 4.6  | 18.2 $\pm$ 5.9          | 13.8 $\pm$ 3.0                   | 14.4 $\pm$ 4.2          | 12.9 $\pm$ 3.5                  |
| SUB                                        | 7.2 $\pm$ 2.4  | 13.3 $\pm$ 3.9 | 11.7 $\pm$ 3.7           | 5.6 $\pm$ 1.4  | 8.1 $\pm$ 2.1    | 12.8 $\pm$ 4.7  | 16.9 $\pm$ 5.4          | 7.3 $\pm$ 2.2                    | 9.1 $\pm$ 2.8           | 8.0 $\pm$ 2.6                   |
| Total average                              | 6.2 $\pm$ 1.5  | 10.1 $\pm$ 2.4 | 8.4 $\pm$ 2.1            | 5.4 $\pm$ 1.1  | 7.7 $\pm$ 1.4    | 7.8 $\pm$ 2.7   | 11.6 $\pm$ 3.1          | 6.2 $\pm$ 1.5                    | 7.8 $\pm$ 1.9           | 6.9 $\pm$ 1.8                   |

Results presented as mean  $\pm$  SEM) following two-tailed independent samples t-test. Bold used to highlight significance when comparing with its counterpart. Italicised indicate statistical trends with  $p = 0.06-0.09$

**Table 4** Effects of risk factors on hippocampal expression of  $\alpha 7$ ,  $\beta 2$ , Casp-3 and TUNEL removing bed-sharers

|                                            | UTRI all groups |                                  | Smoke exposure all groups |                                 | Gender all groups |                 | Found prone (SIDS only) |                                  |
|--------------------------------------------|-----------------|----------------------------------|---------------------------|---------------------------------|-------------------|-----------------|-------------------------|----------------------------------|
|                                            | No (n = 17)     | Yes (n = 8)                      | No (n = 14)               | Yes (n = 8)                     | Male (n = 32)     | Female (n = 19) | No (n = 9)              | Yes (n = 8)                      |
| <i><math>\alpha 7</math> nAChR subunit</i> |                 |                                  |                           |                                 |                   |                 |                         |                                  |
| CA4                                        | 12.8 $\pm$ 3.4  | <b>35.5 <math>\pm</math> 3.7</b> | 25.6 $\pm$ 4.5            | 16.0 $\pm$ 4.8                  | 16.6 $\pm$ 2.8    | 18.5 $\pm$ 4.3  | 30.4 $\pm$ 5.2          | <b>11.8 <math>\pm</math> 3.5</b> |
| CA3                                        | 10.0 $\pm$ 2.6  | 17.1 $\pm$ 3.3                   | 12.6 $\pm$ 2.6            | 16.7 $\pm$ 5.3                  | 12.5 $\pm$ 2.4    | 10.6 $\pm$ 2.3  | 13.5 $\pm$ 3.4          | 20.0 $\pm$ 5.5                   |
| CA2                                        | 5.9 $\pm$ 1.4   | <i>18.4 <math>\pm</math> 6.0</i> | 13.0 $\pm$ 3.8            | 7.4 $\pm$ 2.0                   | 9.1 $\pm$ 1.5     | 8.8 $\pm$ 2.7   | 15.2 $\pm$ 5.5          | 13.3 $\pm$ 2.6                   |
| CA1                                        | 16.0 $\pm$ 3.5  | 10.7 $\pm$ 2.8                   | 14.6 $\pm$ 3.6            | 14.7 $\pm$ 4.0                  | 16.9 $\pm$ 2.8    | 15.9 $\pm$ 3.3  | 10.9 $\pm$ 2.3          | 17.5 $\pm$ 5.0                   |
| SUB                                        | 7.8 $\pm$ 1.0   | 9.2 $\pm$ 3.1                    | 8.6 $\pm$ 1.7             | 7.3 $\pm$ 1.6                   | 9.2 $\pm$ 1.2     | 6.6 $\pm$ 0.8   | 10.2 $\pm$ 2.3          | 5.6 $\pm$ 1.6                    |
| Total average                              | 10.6 $\pm$ 1.2  | <b>18.0 <math>\pm</math> 1.6</b> | 14.9 $\pm$ 1.6            | 12.4 $\pm$ 1.8                  | 13.0 $\pm$ 1.2    | 11.9 $\pm$ 1.9  | 15.7 $\pm$ 2.0          | 14.4 $\pm$ 2.1                   |
| <i><math>\beta 2</math> nAChR subunit</i>  |                 |                                  |                           |                                 |                   |                 |                         |                                  |
| CA4                                        | 16.6 $\pm$ 2.7  | <i>37.0 <math>\pm</math> 9.1</i> | 27.3 $\pm$ 6.2            | 15.3 $\pm$ 4.2                  | 21.4 $\pm$ 2.9    | 18.1 $\pm$ 3.9  | 28.2 $\pm$ 8.1          | 16.3 $\pm$ 3.9                   |
| CA3                                        | 20.9 $\pm$ 5.5  | 36.7 $\pm$ 8.9                   | 27.3 $\pm$ 6.4            | 18.4 $\pm$ 8.9                  | 20.2 $\pm$ 3.6    | 18.8 $\pm$ 4.6  | 25.4 $\pm$ 9.0          | 29.7 $\pm$ 10.3                  |
| CA2                                        | 13.1 $\pm$ 4.2  | 19.5 $\pm$ 5.0                   | 14.2 $\pm$ 3.6            | 9.2 $\pm$ 5.1                   | 12.9 $\pm$ 3.3    | 11.8 $\pm$ 3.1  | 13.7 $\pm$ 4.3          | 13.3 $\pm$ 6.9                   |
| CA1                                        | 8.2 $\pm$ 1.9   | 17.4 $\pm$ 5.2                   | 12.3 $\pm$ 3.4            | 8.5 $\pm$ 2.8                   | 10.4 $\pm$ 1.7    | 8.8 $\pm$ 1.7   | 15.6 $\pm$ 5.2          | 10.4 $\pm$ 2.9                   |
| SUB                                        | 7.4 $\pm$ 2.1   | 13.2 $\pm$ 7.0                   | 11.0 $\pm$ 4.0            | 6.0 $\pm$ 3.1                   | 8.8 $\pm$ 2.0     | 7.2 $\pm$ 1.6   | 14.7 $\pm$ 5.7          | 4.2 $\pm$ 1.3                    |
| Total average                              | 13.4 $\pm$ 2.6  | <b>24.7 <math>\pm</math> 5.8</b> | 18.3 $\pm$ 3.9            | 11.5 $\pm$ 4.0                  | 14.8 $\pm$ 2.1    | 12.9 $\pm$ 2.3  | 19.5 $\pm$ 5.7          | 14.4 $\pm$ 4.4                   |
| <i>Caspase-3</i>                           |                 |                                  |                           |                                 |                   |                 |                         |                                  |
| CA4                                        | 7.2 $\pm$ 1.2   | <b>15.1 <math>\pm</math> 2.8</b> | 9.7 $\pm$ 2.3             | 12.1 $\pm$ 1.6                  | 10.1 $\pm$ 1.8    | 11.3 $\pm$ 1.8  | 12.1 $\pm$ 2.9          | 10.1 $\pm$ 2.2                   |
| CA3                                        | 5.1 $\pm$ 1.4   | <b>11.3 <math>\pm</math> 2.9</b> | 7.4 $\pm$ 2.2             | 6.7 $\pm$ 2.2                   | 9.0 $\pm$ 1.9     | 6.2 $\pm$ 1.1   | 8.0 $\pm$ 2.9           | 8.3 $\pm$ 3.3                    |
| CA2                                        | 5.6 $\pm$ 1.2   | 15.5 $\pm$ 5.6                   | 10.3 $\pm$ 3.7            | 7.7 $\pm$ 1.9                   | 7.8 $\pm$ 1.4     | 11.0 $\pm$ 3.5  | 14.3 $\pm$ 5.1          | 8.9 $\pm$ 1.7                    |
| CA1                                        | 4.1 $\pm$ 0.8   | 5.7 $\pm$ 1.6                    | 4.7 $\pm$ 1.2             | 4.6 $\pm$ 0.9                   | 5.0 $\pm$ 1.1     | 4.6 $\pm$ 0.8   | 3.2 $\pm$ 1.0           | 4.7 $\pm$ 0.6                    |
| SUB                                        | 5.1 $\pm$ 1.2   | 2.7 $\pm$ 0.8                    | 4.2 $\pm$ 1.2             | 5.6 $\pm$ 1.3                   | 4.6 $\pm$ 0.8     | 4.5 $\pm$ 0.9   | 4.1 $\pm$ 1.5           | 5.2 $\pm$ 1.9                    |
| Total average                              | 5.3 $\pm$ 0.7   | <b>10.4 <math>\pm</math> 1.8</b> | 7.1 $\pm$ 1.4             | 7.5 $\pm$ 1.1                   | 7.3 $\pm$ 1.0     | 7.5 $\pm$ 1.3   | 8.6 $\pm$ 1.9           | 7.2 $\pm$ 1.0                    |
| <i>TUNEL</i>                               |                 |                                  |                           |                                 |                   |                 |                         |                                  |
| CA4                                        | 6.2 $\pm$ 2.4   | <b>17.6 <math>\pm</math> 5.6</b> | 12.4 $\pm$ 3.9            | 5.7 $\pm$ 2.7                   | 8.3 $\pm$ 1.9     | 7.9 $\pm$ 3.0   | 12.6 $\pm$ 3.9          | 7.1 $\pm$ 5.2                    |
| CA3                                        | 2.4 $\pm$ 0.9   | 8.2 $\pm$ 3.5                    | 5.9 $\pm$ 2.4             | 2.1 $\pm$ 0.9                   | 3.6 $\pm$ 0.9     | 4.2 $\pm$ 2.2   | 4.5 $\pm$ 1.8           | 3.5 $\pm$ 2.4                    |
| CA2                                        | 2.6 $\pm$ 0.7   | 10.1 $\pm$ 4.4                   | 6.5 $\pm$ 2.8             | 2.7 $\pm$ 0.8                   | 4.1 $\pm$ 1.2     | 2.6 $\pm$ 1.0   | 8.1 $\pm$ 4.1           | 3.0 $\pm$ 1.3                    |
| CA1                                        | 9.8 $\pm$ 4.6   | 24.8 $\pm$ 8.0                   | 21.7 $\pm$ 7.1            | <b>4.5 <math>\pm</math> 1.0</b> | 15.0 $\pm$ 3.0    | 12.7 $\pm$ 4.6  | 23.8 $\pm$ 9.1          | 4.9 $\pm$ 2.8                    |
| SUB                                        | 6.4 $\pm$ 2.5   | 24.9 $\pm$ 9.0                   | 20.7 $\pm$ 6.6            | <b>2.8 <math>\pm</math> 0.8</b> | 8.1 $\pm$ 2.1     | 12.8 $\pm$ 4.7  | 16.5 $\pm$ 5.8          | 8.1 $\pm$ 6.2                    |
| Total average                              | 5.2 $\pm$ 1.6   | <i>17.1 <math>\pm</math> 5.2</i> | 12.7 $\pm$ 3.6            | <b>3.5 <math>\pm</math> 0.8</b> | 7.7 $\pm$ 1.4     | 7.8 $\pm$ 2.7   | 13.1 $\pm$ 3.5          | 4.9 $\pm$ 3.2                    |

Results presented as mean % positively stained neurons ( $\pm$  SEM). Bold used to highlight significance. Italicised indicate statistical trends with  $p=0.06-0.09$

### Effects of the risk factors when excluding bed-sharers

**Independent of diagnostic group** The presence of a recent URTI was associated with higher  $\alpha 7$  in CA4 ( $p < 0.01$ ) and higher total  $\alpha 7$  expression ( $p < 0.01$ ), higher  $\beta 2$  total expression ( $p = 0.05$ ), higher Casp-3 in CA4 ( $p < 0.01$ ), CA3 ( $p = 0.04$ ) and higher total Casp-3 expression ( $p < 0.01$ ), as well as higher TUNEL in CA4 ( $p = 0.04$ ). A history of a recent URTI also resulted in trends towards higher  $\alpha 7$  in CA2 ( $p = 0.09$ ),  $\beta 2$  in CA4 ( $p = 0.06$ ), TUNEL in the subiculum ( $p = 0.09$ ) and total TUNEL expression ( $p = 0.06$ ) (Table 4).

Cigarette smoke exposure was associated with lower expression of TUNEL in CA1 ( $p = 0.03$ ), subiculum ( $p = 0.03$ ) and lower total expression ( $p = 0.03$ ), but had no associations with changes for  $\alpha 7$ ,  $\beta 2$  or Casp-3.

Gender was not associated with any significant differences in expression for any marker (Table 4).

**Sleep related risk factors** Being found prone was associated with decreased  $\alpha 7$  in CA4 ( $p = 0.02$ ), and a trend towards decreased TUNEL in CA1 ( $p = 0.08$ ) (Table 4).

### SIDS II cohort stratified according to Bed-sharing status

For the SIDS II cohort we separated bed-sharing infants from further risk factor analyses. Compared to infants who did not bed-share, the bed-sharing infants from the SIDS II group had lower TUNEL in CA4 ( $p < 0.01$ ), subiculum ( $p = 0.04$ ), and lower total TUNEL expression ( $p < 0.01$ ) (Table 2). Although not statistically significant, bed-sharing



infants also tended to have lower  $\alpha 7$  in CA2 ( $p=0.09$ ) and lower  $\beta 2$  in CA3 ( $p=0.06$ ) (Table 2).

When analysed according to the risk factors (Supplementary Table 2), the presence of an URTI was associated with many differences, while minimal differences were found for gender, smoking status and prone sleeping (Supplementary Table 2).

## Discussion

This is the first study to simultaneously examine the expression of cholinergic ( $\alpha 7$  and  $\beta 2$  nAChR) and apoptotic (TUNEL and Casp-3) markers in the human infant hippocampus. We also evaluated changes according to SUDI

subgroups, and major SIDS risk factors. Our main findings (summarised in Table 5, and in light of a history of bed-sharing greatly affecting result outcomes) were that in relation to SIDS: 1) corresponding increases were seen in Casp-3 and  $\alpha 7$  in CA2 of SIDS II while widespread increases in TUNEL were evident, and 2) when we narrowed this analysis to exclude bed-sharers, SIDS II infants had higher expression of  $\alpha 7$ , Casp-3 and TUNEL, suggesting a specific role of the  $\alpha 7$ , and not  $\beta 2$ , subunit in increased apoptosis in SIDS II. Considering the risk factors, cigarette smoke exposure, gender and, prone sleeping had minimal effects whereas the presence of an URTI was associated with increases in expression of both the  $\alpha 7$  and  $\beta 2$  subunits, and the apoptotic markers, suggesting a role of both these subunits in the face of an infection like stimulus within the infant hippocampus.

**Table 5** Summary of data

|                 | (A) Across diagnosis          |                           |                     |                                  |                             |                             |                     |                         |             |                           |                     |                         |
|-----------------|-------------------------------|---------------------------|---------------------|----------------------------------|-----------------------------|-----------------------------|---------------------|-------------------------|-------------|---------------------------|---------------------|-------------------------|
| Markers studied | All groups                    |                           |                     | All groups excluding bed-sharers |                             |                             |                     |                         |             |                           |                     |                         |
|                 | SIDS I vs eSUDI               | SIDS II vs eSUDI          | SIDS II VS SIDS I   | SIDS I vs eSUDI                  | SIDS II vs eSUDI            | SIDS II vs SIDS I           |                     |                         |             |                           |                     |                         |
| $\alpha 7$      | –                             | ↑ CA2                     | –                   | –                                | ↑ CA2, total                | –                           |                     |                         |             |                           |                     |                         |
| $\beta 2$       | –                             | –                         | –                   | –                                | –                           | –                           |                     |                         |             |                           |                     |                         |
| Casp-3          | –                             |                           | –                   | –                                | ↑ CA2                       | ↓ sub                       |                     |                         |             |                           |                     |                         |
| TUNEL           | –                             |                           | ↑ CA1 + total       | –                                | ↑ CA4, CA3, CA1, sub, total | ↑ CA4, CA3, CA1, sub, total |                     |                         |             |                           |                     |                         |
|                 | (B) According to risk factors |                           |                     |                                  |                             |                             |                     |                         |             |                           |                     |                         |
| Markers studied | URTI                          |                           |                     |                                  | Cigarette smoke exposure    |                             |                     |                         | Male gender |                           |                     |                         |
|                 | All                           | All excluding bed-sharers | SIDS II bed-sharers | SIDS II non-bed-sharers          | All                         | All excluding bed-sharers   | SIDS II bed-sharers | SIDS II non-bed-sharers | All         | All excluding bed-sharers | SIDS II bed-sharers | SIDS II non-bed-sharers |
| $\alpha 7$      | –                             | ↑ CA4, total,             | ↓ CA2               | ↑ CA4, total                     | –                           | –                           | –                   |                         | –           | –                         | –                   | –                       |
| $\beta 2$       | –                             | ↑ total                   | –                   | ↑ CA2, CA4, total                | –                           | –                           | –                   | ↓ CA4,                  | –           | –                         | ↑ CA4, CA3          | –                       |
| Casp-3          | –                             | ↑ CA4, CA3, total         | ↓ CA1               | –                                | –                           | –                           | –                   | –                       | –           | –                         | –                   | ↓ CA1,                  |
| TUNEL           | -                             | ↑ CA4                     | –                   | –                                | –                           | ↓ CA1, sub, total           | –                   |                         | –           | –                         | –                   | –                       |
|                 | (B) According to risk factors |                           |                     |                                  |                             |                             |                     |                         |             |                           |                     |                         |
| Markers studied | Found prone                   |                           |                     |                                  |                             |                             |                     |                         |             |                           |                     |                         |
|                 | All SIDS                      |                           |                     | SIDS excluding bed-sharers       |                             |                             | SIDS II bed-sharers |                         |             | SIDS II non-bed-sharers   |                     |                         |
| $\alpha 7$      |                               |                           |                     | ↓ CA4                            |                             |                             | –                   |                         |             | ↑ CA3                     |                     |                         |
| $\beta 2$       | ↓ sub                         |                           |                     | –                                |                             |                             | –                   |                         |             | –                         |                     |                         |
| Casp-3          | –                             |                           |                     | –                                |                             |                             | –                   |                         |             |                           |                     |                         |
| TUNEL           | –                             |                           |                     |                                  |                             |                             |                     |                         |             | –                         |                     |                         |

Significance taken at  $p \leq 0.05$

## Correlation between the markers

Amongst all SUDI cases, the total expression of  $\alpha 7$  and  $\beta 2$ , total  $\alpha 7$  with Casp-3, and total  $\beta 2$  with TUNEL correlated, as did  $\alpha 7$  with Casp3 in the CA2 region. However, when we ran this analysis accounting for the history of cigarette smoke exposure, it was only the correlation between total  $\beta 2$  with TUNEL that was maintained amongst the cases with *no-history*, with other more specific correlations by regions emerging for CA2 ( $\beta 2$  with TUNEL and Casp3) and CA4 (TUNEL with  $\beta 2$  and Casp3). The correlation between  $\alpha 7$  and Casp3 in the CA2 region was seen in both groups of infants (with and without history of cigarette smoke exposure). Combined, these findings indicate that the relationships are only present under ‘nicotine-free’ conditions in the human infant hippocampus, that they are due to endogenous acetylcholine (ACh), and  $\beta 2$ -containing nAChRs are involved in regulating both early (Casp-3) and late stages of cell death (TUNEL), while the  $\alpha 7$  nAChRs seem to only be involved in early stages of apoptotic pathways. These findings are in keeping with the fact that  $\alpha 7$ - and  $\beta 2$ -containing nAChRs have important different contributions to the regulation of cell death processes where  $\beta 2$ -containing nAChRs tend to be anti-apoptotic [21, 23], while  $\alpha 7$  nAChRs can be both pro-apoptotic and pro-survival, which is fitting given recent data showing the presence of cleaved caspase-3 in the cytoplasm of cells does not necessarily equate to the execution of apoptosis [39]. However, functional, as opposed to expression-based studies, would be warranted in order to confirm the underlying mechanism occurring, and within the context of the hippocampus, as well as consideration of mitochondrial versus cytosolic expression of these subunits. For e.g., under normal physiological conditions, it has been shown that mitochondrial  $\alpha 7$ -containing nAChRs can be activated by choline that is constantly present in the cytosol, while  $\beta 2$ -containing nAChRs (specifically  $\alpha 3\beta 2$  and  $\alpha 4\beta 2$ ) require ACh, produced by choline acetyltransferase (ChAT), and that the signalling pathways of apoptosis, via calcium regulation and cytochrome C, differ in efficacy; the  $\alpha 7$ -containing nAChRs affect mainly the phosphatidylinositol-3-kinase (PI3K)/Akt pathway while  $\alpha 3\beta 2$  and  $\alpha 4\beta 2$  additionally influence CaMKII- and Src-related pathways [26].

The finding that CA2 was the region to show the correlations for both  $\alpha 7$  and  $\beta 2$  is interesting. CA2 cells are less vulnerable to death following a range of insults compared with other CA regions [40] due to superior calcium-handling ability of CA2 pyramidal neurons [41]. Our data therefore suggest that these correlations within the CA2 are a defence mechanism as part of the normal developmental processes and could underlie the ability of the CA2

region to withstand hypoxic insults mediated by the protective role of  $\alpha 7$  and  $\beta 2$ -containing nAChRs.

## Comparative amongst diagnostic groups

We report increased cell death markers amongst the SIDS infants (SIDS II group) compared with eSUDI, consistent with previous literature showing increased cell death in the SIDS hippocampus [8, 9] and parahippocampal gyrus [42–44]. The novelty of the current study was our finding that the increased cell death was confined to the SIDS II cohort. Our additional analyses suggest that the presence of an URTI (discussed later on) may be a major contributor. The TUNEL expression found in the eSUDI group was not consistent with Waters et al. [8] who reported no apoptosis in the hippocampal regions of their control group. This is most likely due to differences between the control groups of the datasets; Waters et al. [8] included predominantly septicaemia and bronchopneumonia while those herein were predominantly cardiac-related deaths. In addition, differences in brain tissue fixation (the percentage and pH content of formalin used), and method of applying the TUNEL assay (microwave antigen retrieval was not employed in Waters et al. [8]) may also have contributed.

Expression of the cell death markers in the SIDS I group did not differ greatly from those in the eSUDI group, consistent with our findings in a subset of the same cohort [9]. Moreover, the expression of the  $\alpha 7$  and  $\beta 2$  nAChR subunits did not differ between these groups, similar to our finding that expression of the  $\alpha 3$  and  $\alpha 4$  nAChR subunits in the brainstem medulla did not differ [45]. Combined, these data support the SIDS I group being more homogenous than eSUDI and SIDS II due to the stringent inclusion criteria. Furthermore, the lack of changes in the SIDS I hippocampus, as opposed to SIDS II, suggests that in SIDS II, increased apoptosis is a consequence of other factors/stressors experienced by these infants and not the SIDS pathophysiology process itself.

The SIDS II diagnostic category includes infants where factors around the death or death scene suggest the possibility of mechanical asphyxia or suffocation from overlay, and a proportion of SIDS deaths that occur in the presence of soft-bedding and/or bed-sharing arrangements may well be the result of suffocation [38, 46]. Removing bed-sharers from our analyses strengthened some correlations (Casp-3 and TUNEL), and revealed new trends ( $\alpha 7$ , Casp-3, TUNEL) amongst the SIDS II group. This highlights concerns regarding the determination of cause of death in SIDS cases, likely resulting in infants with varying causes of death in the same dataset. Neuropathological differences have been found between infants who sleep alone and those who bed-share, with alone sleepers demonstrating: higher levels of  $\beta$ -amyloid precursor protein, a membrane protein known



to be upregulated in response to axonal injury [47], higher expression of glial fibrillary acidic protein in the brainstem medulla, which accumulates days to weeks following hypoxia or injury [48], and higher TUNEL in multiple brainstem nuclei [31]. These differences are hypothesised to be a result of a more rapid death occurring amongst bed-sharers, not allowing sufficient time for these markers to accumulate [37]. Our findings of widespread increases in TUNEL amongst the non-bed-shared SIDS II, not present amongst the bed-shared cohort, may be due to TUNEL being a marker of end-stage cell death supporting the assumption that non-bed-shared SIDS II infants may have experienced prolonged 'stressors' prior to the night of death. Moreover, that the CA2 in SIDS II (non-bed-sharers) was spared from increased TUNEL but was the only region where both Casp3 and the  $\alpha 7$  subunit were increased, is in line with the fact that CA2 neurons are less vulnerable to death following a range of insults compared with other CA regions as discussed above. In light of this, our data suggest that it was the last region affected in SIDS II infants.

### Cigarette smoke exposure

The lack of effect from cigarette smoke exposure on  $\alpha 7$  and  $\beta 2$  in the human infant hippocampus was not surprising given we previously reported no change in the hippocampus of a piglet model of post-natal exposure to nicotine, except for decreased  $\beta 2$  in CA3 [32]. Notably, however, altered expression of the same subunits in the brainstem medulla was linked to cigarette smoke exposure, of the human infant [49] and the piglet [50]. Combined, these findings suggest that the  $\alpha 7$  and  $\beta 2$  nAChRs are more sensitive to effects of nicotine in the developing brainstem than the hippocampus, and thus more indicative of the direct negative cardiorespiratory compromise attributed to nicotine during development (reviewed in [51]).

Decreased TUNEL in non-bed-sharing, smoke exposed infants contrasted in direction with our expectations based on previous findings of increased neuronal cell death in the offspring brain following maternal cigarette smoke exposure in both animal [52, 53] and human studies [31]. Although this may be attributable to the composition of the groups where only 22% of SIDS II were smoke exposed compared to 20% and 63% in eSUDI and SIDS I respectively, brain regional effects are also at play given all the above studies were predominantly of the brainstem, thus once again highlighting the differential effects of cigarette smoke exposure on apoptotic regulation according to brain region with evidence showing the brainstem more susceptible to increased apoptosis compared with hippocampus [54, 55].

An alternative hypothesis may be that nicotine as a consequence of cigarette smoke exposure, may have provided a pro-survival outcome in neurons of the infant hippocampus.

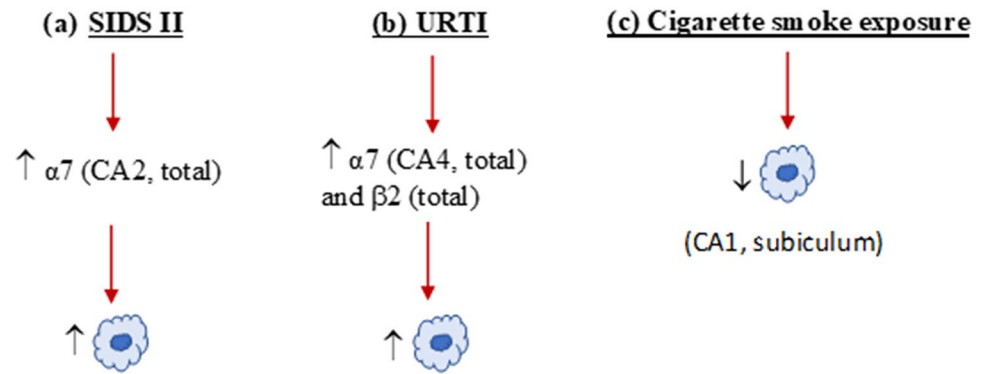
Although this is not supported by our data where there was a lack of change in  $\alpha 7$  and  $\beta 2$  nAChR subunit expression, this does not mean a change in receptor functionality is not occurring. For example, nicotine exposure during the early postnatal period in the rat was associated with fewer TUNEL expressing cells in the hippocampus strata oriens and radiatum, which correlated with increased  $\alpha 4\beta 2$  nicotine binding sites despite a lack of change in  $\alpha 7$  and  $\beta 2$  mRNA subunit expression [56]. Furthermore, pro-survival signalling of  $\alpha 7$  nAChRs expressed in the plasma membrane of certain cell types, as well as through their expression in the mitochondria have been reported [25, 30, 57]. Yet, whether this pro-survival is a good or bad thing remains in question given it could be associated with an alteration of normal processes of neuronal synaptic activity during this early stage of hippocampal development.

### Bed-sharing, prone sleeping and upper respiratory tract infection

Bed-sharing and being found prone were initially associated with decreased expression of the  $\beta 2$  subunit in CA3 and the subiculum, consistent with the role of this subunit in the organisation of sleep states [58], arousal from sleep, and respiration [59]. Expression of this subunit in hippocampal regions was affected by exposure to intermittent hypercapnic hypoxia (IHH) (a model for sleep-related risk factors) in our piglet model [60]. Hypoxia modulates nAChR expression by inhibition of ACh synthesis and of its pre-synaptic release [61]. ChAT, was decreased in SIDS infants in the hypothalamus and striatum [62], brain areas recently shown to have cholinergic projections to the hippocampus [63] and subiculum [64]. After excluding bed-sharers from the analyses, infants found prone had decreased  $\alpha 7$  expression in CA4, with no alterations in  $\beta 2$ . Decreased  $\alpha 7$  expression was found in a mouse model after prolonged exposure to hypoxia/ischemia (72 h) [65], and in our piglet model, repeated IHH induced more significant changes in the  $\alpha 7$  subunit in the hippocampus than acute IHH [60]. Thus, the alterations found in these cholinergic subunits determining whether they will induce pro- or anti- apoptotic downstream effects, appear dependent on the duration and type of hypoxic exposure [29].

Of all the risk factors analysed, the presence of an URTI 1–14 days prior to death had the most striking impact. Once the bed-sharing infants were excluded, total marker levels were increased, with specific increase in CA4 (summarised Table 5). The finding that all markers were affected suggests a strong interplay between them. Literature on effects of URTI on apoptosis or nAChRs is limited; recently we found that an URTI was associated with increased TUNEL in many brain regions (Ambrose et al. 2019), as well as increased  $\alpha 4$  nAChR subunit in the brainstem medulla [45] of the

**Fig. 3** Proposed pathway leading to apoptosis (blue shrunken cell) in hippocampal regions, via  $\alpha 7$  and  $\beta 2$  nAChR subunits in non-bed-shared infants when analysed for: **a** SIDS II, **b** an URTI and **c** cigarette smoke exposure



infant, but we could not find any animal studies pertaining to this directly. In a clinical study, an URTI induced cognitive, behavioural and physiological changes [66], suggesting the involvement of the hippocampus. Mechanistically, it is hypothesised that an URTI is associated with an inflammatory response following systemic immune challenge, that disrupts the blood–brain-barrier of the central nervous system [67], and leads to various signalling pathways [68], one being via the adenosine receptors [69] to exacerbate cell-death processes via mitochondrial apoptotic pathways, such as in the case of neurodegenerative diseases [70].

## Limitations

Factors for consideration include small size for eSUDI group, and swaying of the data by the heterogeneous SIDS II group as well. Moreover, our study is only from one level of the hippocampus given limited tissue availability; so our findings are based on a ‘snap-shot’ of the left hippocampus.

## Conclusion

Increased apoptotic markers (Casp-3 and TUNEL) in SIDS II infants in various hippocampal regions are associated with increased  $\alpha 7$  nAChR expression (Fig. 3a), thus supporting the hypothesis of a cholinergic mechanism (specifically via the  $\alpha 7$  nAChRs) for the previously observed [8, 9] neuronal apoptosis in the SIDS II hippocampus. Environmental stressors of a hypoxic or infectious nature independently contributed to changes in the SIDS hippocampus, supported by differences found between infants who bed-shared or not, as well as the impact of an URTI prior to death, where both  $\alpha 7$  and  $\beta 2$  are associated in the increased apoptosis in the same hippocampal regions (Fig. 3b), yet not for cigarette smoke exposure which was independent of changes in the expression of the  $\alpha 7$  and  $\beta 2$  nAChR subunits (Fig. 3c).

**Acknowledgements** The tissue used in this study was obtained from the NSW Forensic and Analytical Science Service.

**Funding** This study was funded by philanthropy; The Miranda Belshaw Foundation, Australia, and The SIDS Stampede, Australia.

## Compliance with ethical standards

**Conflict of interest** The authors declare they have no conflict of interest.

**Ethical approval** Ethical approval was from the NSW health RPAH zone (Protocol No 3593, X13-0038 and HREC/13/RPAH/54) and University of Sydney (Approval No. 3013/235) Ethics committees.

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### **3.1: APPENDIX**

This Appendix contains the supplementary material available for Luijckink et al, 2020. For ease of reference, the supplement tables retain their titles as referred to in the publication.

**Supplementary Table 1:** Proportion of cases with positive staining for Casp-3 and TUNEL according to diagnostic groups of eSUDI, SIDS I and SIDS II.

| eSUDI                    |                            |           |            |       |                                       | SIDS I                     |            |            |       |                                       | SIDS II                    |            |            |            |                                       | Fisher's<br>Exact | Kruskal<br>Wallice |
|--------------------------|----------------------------|-----------|------------|-------|---------------------------------------|----------------------------|------------|------------|-------|---------------------------------------|----------------------------|------------|------------|------------|---------------------------------------|-------------------|--------------------|
| Region                   | Apoptosis no. of cases (%) |           |            |       | Apoptosis<br>(%)<br>Median<br>(range) | Apoptosis no. of cases (%) |            |            |       | Apoptosis<br>(%)<br>Median<br>(range) | Apoptosis no. of cases (%) |            |            |            | Apoptosis<br>(%)<br>Median<br>(range) |                   |                    |
|                          | Positive<br>cases<br>(%)   | 2-<br>9%  | 10-<br>20% | >20%  |                                       | Positive<br>cases<br>(%)   | 2-<br>9%   | 10-<br>20% | >20%  |                                       | Positive<br>cases<br>(%)   | 2-<br>9%   | 10-<br>20% | >20%       |                                       |                   |                    |
| <i>Caspase-3</i>         |                            |           |            |       |                                       |                            |            |            |       |                                       |                            |            |            |            |                                       |                   |                    |
| CA4                      | 7 (88)                     | 5<br>(63) | 2<br>(25)  | 0 (0) | 5 (1-14)                              | 7 (100)                    | 3<br>(43)  | 4<br>(57)  | 0 (0) | 14 (1-17)                             | 29 (88)                    | 12<br>(36) | 11<br>(33) | 6 (18)     | 10 (0-38)                             | 0.61              | 0.55               |
| CA3                      | 6 (75)                     | 5<br>(63) | 1<br>(13)  | 0 (0) | 5 (0-14)                              | 5 (83)                     | 3<br>(50)  | 2<br>(33)  | 0 (0) | 5 (1-17)                              | 26 (79)                    | 14<br>(42) | 21<br>(70) | 5 (15)     | 5 (0-42)                              | 0.90              | 0.74               |
| CA2                      | 6 (75)                     | 6<br>(75) | 0 (0)      | 0 (0) | 2 (0-8)                               | 7 (100)                    | 3<br>(43)  | 4<br>(57)  | 0 (0) | 2 (2-14)                              | 26 (79)                    | 14<br>(42) | 7<br>(21)  | 5 (15)     | 5 (0-54)                              | 0.14              | 0.13               |
| CA1                      | 6 (86)                     | 4<br>(57) | 2<br>(29)  | 0 (0) | 3 (1-12)                              | 7 (100)                    | 7<br>(100) | 0 (0)      | 0 (0) | 3 (2-9)                               | 23 (70)                    | 18<br>(55) | 4<br>(12)  | 1 (3)      | 4 (0-26)                              | 0.30              | 0.51               |
| SUB                      | 4 (67)                     | 4<br>(67) | 0 (0)      | 0 (0) | 4 (0.9)                               | 5 (100)                    | 4<br>(80)  | 1<br>(20)  | 0 (0) | 4 (3-13)                              | 28 (85)                    | 25<br>(76) | 3 (9)      | 0 (0)      | 3 (0-15)                              | 0.53              | 0.20               |
| <i>Total<br/>average</i> | 8 (100)                    | 7<br>(88) | 1<br>(13)  | 0 (0) | 3 (2-10)                              | 7 (100)                    | 5<br>(71)  | 2<br>(29)  | 0 (0) | 3 (3-13)                              | 31 (94)                    | 19<br>(58) | 10<br>(30) | 2 (6)      | 6 (1-23)                              | 0.91              | 0.43               |
| <i>TUNEL</i>             |                            |           |            |       |                                       |                            |            |            |       |                                       |                            |            |            |            |                                       |                   |                    |
| CA4                      | 4 (50)                     | 3<br>(38) | 1<br>(13)  | 0 (0) | 2 (0-18)                              | 3 (50)                     | 3<br>(50)  | 0 (0)      | 0 (0) | 1 (0-5)                               | 23 (70)                    | 11<br>(33) | 2 (6)      | 10<br>(30) | 3 (0-42)                              | 0.35              | 0.15               |
| CA3                      | 3 (37.5)                   | 3<br>(38) | 0 (0)      | 0 (0) | 1 (0-6)                               | 2 (40)                     | 2<br>(40)  | 0 (0)      | 0 (0) | 0 (0-3)                               | 18 (55)                    | 13<br>(39) | 3 (9)      | 2<br>(6.1) | 2 (0-31)                              | 0.98              | 0.59               |
| CA2                      | 4 (50)                     | 4<br>(50) | 0 (0)      | 0 (0) | 2 (0.6)                               | 3 (50)                     | 3<br>(50)  | 0 (0)      | 0 (0) | 2 (0-6)                               | 17 (52)                    | 13<br>(39) | 3 (9)      | 1 (3)      | 2 (0-33)                              | 1.00              | 0.89               |
| CA1                      | 6 (86)                     | 4<br>(57) | 2<br>(29)  | 0 (0) | 6 (1-13)                              | 4 (67)                     | 4<br>(67)  | 0 (0)      | 0 (0) | 2 (0-7)                               | 30 (91)                    | 13<br>(39) | 5<br>(15)  | 12<br>(36) | 11 (0-71)                             | 0.09              | <b>0.03\$</b>      |
| SUB                      | 5 (71)                     | 4<br>(57) | 1<br>(14)  | 0 (0) | 4 (0-11)                              | 3 (60)                     | 3<br>(60)  | 0 (0)      | 0 (0) | 3 (1-5)                               | 25 (76)                    | 13<br>(39) | 7<br>(21)  | 5 (15)     | 4 (0-64)                              | 0.87              | 0.38               |
| <i>Total<br/>average</i> | 5 (63)                     | 5<br>(63) | 0 (0)      | 0 (0) | 4 (0-7)                               | 4 (67)                     | 4<br>(67)  | 0 (0)      | 0 (0) | 2 (0-4)                               | 26 (79)                    | 13<br>(39) | 8<br>(24)  | 5 (15)     | 5 (0-39)                              | 0.38              | <b>0.04\$</b>      |

Cases were considered negative if only rare neurons were positive (<1% positive cells). Kruskal Wallis was used for comparison between diagnostic groups. Fisher exact test was used to compare proportions between groups. Significance taken  $p \leq 0.05$ .

**Supplementary Table 2:** Expression of markers according to the presence of SIDS risk factors stratified according to bed-sharing SIDS II vs. non bed-sharing SIDS II.

| URTI                           |                    |              |                        |                |                         |                           |
|--------------------------------|--------------------|--------------|------------------------|----------------|-------------------------|---------------------------|
|                                | Bedsharing SIDS II |              | Non-Bedsharing SIDS II |                | <i>P value No vs No</i> | <i>P value Yes vs Yes</i> |
|                                | No (n = 10)        | Yes (n = 13) | No (n=4)               | Yes (n=6)      |                         |                           |
| <b><i>α7 nAChR subunit</i></b> |                    |              |                        |                |                         |                           |
| CA4                            | 17±6.7             | 13±3.9       | 10±1.2                 | <b>33±4.3</b>  | 0.51                    | <b>0.01</b>               |
| CA3                            | 15±6.2             | 8±1.8        | 16±7.9                 | 18±3.9         | 0.91                    | <b>0.01</b>               |
| CA2                            | 12±3.0             | <b>5±0.9</b> | 9±2.3                  | 24±7.0         | 0.54                    | <b>&lt; 0.01</b>          |
| CA1                            | 17±5.3             | 20±5.6       | 12±2.8                 | 11±3.7         | 0.58                    | 0.30                      |
| SUB                            | 8±2.2              | 9±1.5        | 7±2.3                  | 12±3.7         | 0.73                    | 0.44                      |
| Total average                  | 14±3.4             | 11±1.8       | 11±2.1                 | <b>19±2.0</b>  | 0.66                    | <b>0.01</b>               |
| <b><i>β2 nAChR subunit</i></b> |                    |              |                        |                |                         |                           |
| CA4                            | 19±4.6             | 17±3.7       | 9±3.3                  | <b>45±10.3</b> | 0.11                    | <b>0.04</b>               |
| CA3                            | 15±4.6             | 12±3.9       | 16±13.0                | 45±9.9         | 0.98                    | <b>&lt; 0.01</b>          |
| CA2                            | 10±5.2             | 11±5.3       | 4±2.0                  | <b>25±4.9</b>  | 0.56                    | 0.12                      |
| CA1                            | 10±2.4             | 8±1.2        | 8±5.5                  | 21±6.4         | 0.68                    | <b>0.01</b>               |
| SUB                            | 7±1.8              | 7±1.8        | 9±4.1                  | 15±7.9         | 0.68                    | <b>&lt; 0.01</b>          |
| Total average                  | 12±2.5             | 11±2.4       | 9±4.3                  | <b>30±6.2</b>  | 0.49                    | <b>&lt; 0.01</b>          |
| <b><i>Caspase-3</i></b>        |                    |              |                        |                |                         |                           |
| CA4                            | 14±4.2             | 7±2.2        | 10±3.3                 | 15±4.0         | 0.51                    | 0.08                      |
| CA3                            | 14±4.3             | 4±1.3        | 9±4.4                  | 10±3.9         | 0.56                    | 0.10                      |
| CA2                            | 7±2.1              | 8±2.8        | 7±3.4                  | 19±7.2         | 0.96                    | 0.09                      |
| CA1                            | 8±2.7              | <b>2±0.5</b> | 4±1.3                  | 4±1.9          | 0.33                    | 0.14                      |
| SUB                            | 3±0.8              | 5±1.3        | 4±2.7                  | 2±0.7          | 0.75                    | 0.17                      |
| Total average                  | 9±2.4              | 5±1.4        | 7±1.7                  | 10±2.5         | 0.54                    | 0.10                      |
| <b><i>TUNEL</i></b>            |                    |              |                        |                |                         |                           |
| CA4                            | 8±2.6              | 5±2.4        | 15±7.2                 | 23±6.5         | 0.23                    | <b>&lt; 0.01</b>          |
| CA3                            | 6±3.3              | 1±0.4        | 6±2.7                  | 10±4.6         | 0.89                    | <b>0.01</b>               |
| CA2                            | 3±1.5              | 2±0.7        | 3±1.8                  | 14±5.5         | 0.99                    | <b>0.01</b>               |
| CA1                            | 14±5.3             | 15±4.1       | 25±15.8                | 32±9.4         | 0.40                    | <b>0.08</b>               |
| SUB                            | 8±4.9              | 7±1.8        | 14±7.6                 | 34±10.3        | 0.55                    | <b>&lt; 0.01</b>          |
| Total average                  | 8±3.1              | 6±1.5        | 12±4.5                 | 22±5.6         | 0.43                    | <b>&lt; 0.01</b>          |

| Smoking                        |                    |              |                        |              |                         |                           |
|--------------------------------|--------------------|--------------|------------------------|--------------|-------------------------|---------------------------|
|                                | Bedsharing SIDS II |              | Non-Bedsharing SIDS II |              | <i>P value No vs No</i> | <i>P value Yes vs Yes</i> |
|                                | No (n = 11)        | Yes (n = 10) | No (n = 7)             | Yes (n = 2)  |                         |                           |
| <b><i>α7 nAChR subunit</i></b> |                    |              |                        |              |                         |                           |
| CA4                            | 13±6.4             | 13±2.6       | 29±5.0                 | 8±0.1        | 0.10                    | 0.40                      |
| CA3                            | 7±1.6              | 10±2.6       | 16±4.1                 | 18±14.4      | 0.07                    | 0.71                      |
| CA2                            | 8±1.8              | 6±2.4        | 21±6.3                 | 6±1.6        | 0.10                    | 0.91                      |
| CA1                            | 14±4.1             | 27±6.6       | 11±3.1                 | 15±5.5       | 0.65                    | 0.43                      |
| SUB                            | 7±1.3              | 9±1.9        | 12±3.0                 | 6±2.6        | 0.08                    | 0.50                      |
| Total average                  | 10±2.5             | 13±2.0       | 18±2.2                 | 10±3.8       | <b>0.05</b>             | 0.57                      |
| <b><i>β2 nAChR subunit</i></b> |                    |              |                        |              |                         |                           |
| CA4                            | 18±4.3             | 22±3.7       | 39±10.5                | <b>7±3.4</b> | 0.10                    | 0.12                      |
| CA3                            | 10±2.3             | 20±5.7       | 39±10.4                | 3±1.8        | <b>0.03</b>             | 0.25                      |
| CA2                            | 6±2.0              | 17±7.5       | 22±4.9                 | 3±3.0        | <b>&lt; 0.01</b>        | 0.45                      |
| CA1                            | 8±1.8              | 9±1.3        | 18±6.0                 | 3±0.4        | 0.16                    | 0.80                      |
| SUB                            | 8±2.0              | 8±1.7        | 16±6.7                 | 2±0.1        | 0.19                    | 0.17                      |
| Total average                  | 10±1.7             | 15±3.0       | 27±6.2                 | 4±0.3        | <b>0.01</b>             | 0.13                      |
| <b><i>Caspase-3</i></b>        |                    |              |                        |              |                         |                           |
| CA4                            | 12±3.4             | 7±2.7        | 13±4.1                 | 12±2.9       | 0.93                    | 0.39                      |
| CA3                            | 9±3.6              | 6±2.3        | 9±3.2                  | 5±4.5        | 0.94                    | 0.81                      |
| CA2                            | 10±3.0             | 9±5.7        | 16±6.7                 | 8±5.6        | 0.35                    | 0.99                      |
| CA1                            | 4±1.0              | 5±2.1        | 4±1.7                  | 5±1.0        | 0.78                    | 0.94                      |
| SUB                            | 5±1.4              | 5±1.5        | 2±0.6                  | 7±5.3        | 0.09                    | 0.62                      |
| Total average                  | 8±1.9              | 6±2.3        | 9±2.4                  | 7±0.5        | 0.82                    | 0.81                      |
| <b><i>TUNEL</i></b>            |                    |              |                        |              |                         |                           |
| CA4                            | 4±2.0              | 7±3.8        | 20±6.2                 | 12±9.0       | <b>0.01</b>             | 0.59                      |
| CA3                            | 2±0.6              | 1±0.5        | 9±4.1                  | 4±2.4        | 0.14                    | 0.08                      |
| CA2                            | 2±0.8              | 2±0.6        | 11±5.0                 | 1±0.9        | 0.13                    | 0.70                      |
| CA1                            | 7±2.2              | 17±5.0       | 38±10.1                | 5±2.4        | <b>0.03</b>             | 0.33                      |
| SUB                            | 4±1.3              | 8±2.2        | 31±8.7                 | 2±1.7        | 0.02                    | 0.25                      |
| Total average                  | 4±1.2              | 7±1.8        | 22±4.6                 | 5±2.9        | <b>&lt; 0.01</b>        | 0.63                      |



| Gender                  |                    |                 |                        |                |                             |                                 |
|-------------------------|--------------------|-----------------|------------------------|----------------|-----------------------------|---------------------------------|
|                         | Bedsharing SIDS II |                 | Non-Bedsharing SIDS II |                | <i>P value male vs male</i> | <i>P value female vs female</i> |
|                         | Male (n = 15)      | Female (n = 10) | Male (n = 8)           | Female (n = 2) |                             |                                 |
| <i>α7 nAChR subunit</i> |                    |                 |                        |                |                             |                                 |
| CA4                     | 14±4.2             | 15±5.5          | 21±4.3                 | 35±14.6        | 0.33                        | 0.17                            |
| CA3                     | 13±4.1             | 6±1.6           | 17±4.7                 | 18±1.0         | 0.57                        | <b>0.01</b>                     |
| CA2                     | 8±2.0              | 8±2.6           | 15±3.8                 | 25±20.2        | 0.10                        | 0.56                            |
| CA1                     | 17±4.8             | 20±5.3          | 12±2.9                 | 8±1.1          | 0.46                        | 0.32                            |
| SUB                     | 9±1.8              | 7±1.4           | 11±2.9                 | 6±0.5          | 0.70                        | 0.81                            |
| Total average           | 12±2.1             | 11±2.6          | 15±1.8                 | 18±7.5         | 0.40                        | 0.28                            |
| <i>β2 nAChR subunit</i> |                    |                 |                        |                |                             |                                 |
| CA4                     | 22±3.3             | <b>10±3.4</b>   | 28±9.4                 | 41±25.8        | 0.54                        | 0.44                            |
| CA3                     | 18±4.1             | <b>7±1.1</b>    | 31±10.9                | 41±10.9        | 0.28                        | 0.19                            |
| CA2                     | 14±5.6             | 4±1.1           | 16±6.0                 | 23±2.1         | 0.80                        | <b>&lt; 0.01</b>                |
| CA1                     | 9±1.1              | 8±2.6           | 17±5.8                 | 13±6.5         | 0.22                        | 0.49                            |
| SUB                     | 7±1.0              | 8±2.6           | 13±6.2                 | 12±4.8         | 0.35                        | 0.58                            |
| Total average           | 14±2.2             | 8±1.6           | 21±6.6                 | 26±0.3         | 0.35                        | <b>&lt; 0.01</b>                |
| <i>Caspase-3</i>        |                    |                 |                        |                |                             |                                 |
| CA4                     | 12±3.4             | 10±2.6          | 10±2.6                 | 21±2.8         | 0.75                        | 0.11                            |
| CA3                     | 11±3.4             | 5±1.3           | 9±3.6                  | 11±0.5         | 0.81                        | 0.13                            |
| CA2                     | 8±2.0              | 11±5.4          | 11±4.1                 | 24±16.0        | 0.44                        | 0.38                            |
| CA1                     | 6±2.1              | 4±0.9           | 3±0.9                  | <b>8±3.0</b>   | 0.33                        | 0.09                            |
| SUB                     | 5±1.1              | 5±1.3           | 3±1.6                  | 2±0.3          | 0.47                        | 0.35                            |
| Total average           | 8±2.0              | 7±2.0           | 7±1.6                  | 13±3.0         | 0.78                        | 0.25                            |
| <i>TUNEL</i>            |                    |                 |                        |                |                             |                                 |
| CA4                     | 7±2.5              | 6±3.2           | 19±4.3                 | 22±20.2        | <b>0.02</b>                 | 0.58                            |
| CA3                     | 3±1.4              | 4±3.0           | 7±2.1                  | 13±12.9        | 0.14                        | 0.30                            |
| CA2                     | 2±0.7              | 3±1.5           | 10±4.4                 | 5±4.5          | 0.13                        | 0.63                            |
| CA1                     | 15±3.3             | 13±5.8          | 29±9.1                 | 28±26.1        | 0.08                        | 0.38                            |
| SUB                     | 6±1.3              | 10±5.0          | 20±6.8                 | 42±22.0        | 0.08                        | <b>0.05</b>                     |
| Total average           | 7±1.5              | 7±3.1           | 17±3.3                 | 22±17.2        | <b>&lt; 0.01</b>            | 0.15                            |

| Found prone                    |                    |             |                        |               |                         |                           |
|--------------------------------|--------------------|-------------|------------------------|---------------|-------------------------|---------------------------|
|                                | Bedsharing SIDS II |             | Non-Bedsharing SIDS II |               | <i>P value No vs No</i> | <i>P value Yes vs Yes</i> |
|                                | No (n = 12)        | Yes (n = 8) | No (n = 6)             | Yes (n = 3)   |                         |                           |
| <b><i>α7 nAChR subunit</i></b> |                    |             |                        |               |                         |                           |
| CA4                            | 15±5.3             | 19±6.2      | 27±6.7                 | 18±7.0        | 0.19                    | 0.89                      |
| CA3                            | 11±2.2             | 13±6.9      | 11±4.2                 | <b>29±1.6</b> | 0.99                    | 0.19                      |
| CA2                            | 9±2.4              | 9±3.1       | 21±7.8                 | 15±4.1        | 0.21                    | 0.36                      |
| CA1                            | 24±6.9             | 15±4.7      | 13±3.2                 | 10±5.3        | 0.16                    | 0.54                      |
| SUB                            | 8±2.1              | 9±2.0       | 13±2.8                 | 3±0.2         | 0.19                    | 0.21                      |
| Total average                  | 13±2.7             | 13±2.9      | 16±3.0                 | 17±2.2        | 0.50                    | 0.46                      |
| <b><i>β2 nAChR subunit</i></b> |                    |             |                        |               |                         |                           |
| CA4                            | 19±3.7             | 16±4.8      | 32±12.1                | 16±3.1        | 0.34                    | 0.93                      |
| CA3                            | 16±4.5             | 14±5.0      | 31±13.2                | 39±16.6       | 0.31                    | 0.08                      |
| CA2                            | 12±6.0             | 12±6.0      | 18±5.8                 | 17±17.1       | 0.52                    | 0.73                      |
| CA1                            | 8±1.8              | 8±1.8       | 18±7.3                 | 14±6.5        | 0.21                    | 0.29                      |
| SUB                            | 8±1.7              | 6±2.2       | 17±7.7                 | 5±3.3         | 0.14                    | 0.88                      |
| Total average                  | 12±2.5             | 11±3.2      | 23±8.4                 | 17±6.6        | 0.26                    | 0.40                      |
| <b><i>Caspase-3</i></b>        |                    |             |                        |               |                         |                           |
| CA4                            | 9±2.8              | 13±4.7      | 11±3.9                 | 14±1.2        | 0.71                    | 0.93                      |
| CA3                            | 6±3.4              | 11±3.6      | 7±3.6                  | 15±6.2        | 0.86                    | 0.62                      |
| CA2                            | 8±4.4              | 11±3.9      | 17±6.5                 | 8±4.8         | 0.26                    | 0.67                      |
| CA1                            | 4±0.9              | 8±3.3       | 2±0.9                  | 5±0.8         | 0.45                    | 0.73                      |
| SUB                            | 4±1.0              | 5±1.9       | 4±1.7                  | 1±0.6         | 0.80                    | 0.30                      |
| Total average                  | 6±2.0              | 10±2.8      | 8±2.4                  | 9±1.7         | 0.55                    | 0.85                      |
| <b><i>TUNEL</i></b>            |                    |             |                        |               |                         |                           |
| CA4                            | 6±3.0              | 9±3.7       | 16±4.4                 | 18±14.7       | 0.09                    | 0.37                      |
| CA3                            | 2±0.6              | 4±2.4       | 6±2.2                  | 7±5.7         | 0.14                    | 0.52                      |
| CA2                            | 1±0.4              | 3±0.9       | 10±5.3                 | 5±3.1         | 0.15                    | 0.30                      |
| CA1                            | 8±2.9              | 19±5.0      | 31±10.7                | 10±7.3        | 0.09                    | 0.45                      |
| SUB                            | 4±1.5              | 8±2.2       | 21                     | 16±16.4       | 0.06                    | 0.70                      |
| Total average                  | 4±1.4              | 8±2.1       | 17±3.5                 | 11±9.4        | <b>&lt; 0.01</b>        | 0.80                      |

Results presented as mean % positively stained neurons (± SEM) following two-tailed independent samples t-tests. Significance taken  $p \leq 0.05$ . Bold used to highlight significance. Italicised indicate statistical trends with  $p = 0.06-0.09$ .

## **CHAPTER 4: CELL LOSS AND/OR HIPPOCAMPAL SCLEROSIS IN THE SIDS HIPPOCAMPUS**

Hippocampal sclerosis (HS) is a pathological lesion that is commonly present in surgical and post-mortem brain sections of patients with epilepsy (particularly of medial temporal lobe aetiology) (reviewed in (Thom, 2014)). HS is characterised by severe neuron loss (i.e. >50% in at least one hippocampal region) and reactive gliosis that predominates in the CA regions of the hippocampus, although can be present in surrounding structures (Sommer, 1880, Cavanagh and Meyer, 1956). The previous chapter showed cell death to be increased in the SIDS hippocampus, however, it is not known whether this was also associated with a reduced number of neurons. Given that previous data has identified a high prevalence of epilepsy-associated structural features in the SIDS DG (Kinney et al., 2015), supportive of the limbic-seizure hypothesis in a proportion of SIDS deaths (Harper, 1986, Duncan and Byard, 2018), the next aim was to determine whether HS was also present in accordance with cell death amongst the same dataset.

Although HS is uncommon in infancy, studies investigating autopsy or surgically derived tissue specimens from infants are limited (Fasulo et al., 2012, Kinney et al., 2015), with many investigations being on data obtained from MRI (Roulet Perez et al., 2000, Mühlebner et al., 2016). Studies that rely on a standardised MRI protocol for the diagnosis of HS can be less sensitive to detecting subtle neuron loss and morphometric changes (Von Oertzen et al., 2002), and pose an additional challenge in detection when pathology is both symmetrical and bilateral (reviewed in (Malmgren and Thom, 2012)). Animal models have suggested that even a few seizures in infancy are sufficient to induce significant neuronal loss (Dunleavy et al., 2010), and upregulate the expression of glial fibrillary acidic protein (GFAP) (Liu et al., 2012, Alese and Mabandla, 2019) a process which occurs following CNS injury (reviewed in (Eng et al., 2000)). As HS is reported to be more common in childhood than infancy (Mühlebner et al., 2016), a finding of neuron loss and/or gliosis could indicate a precursor to the development of HS.

The International League Against Epilepsy (ILAE) proposes a semi-quantitative scoring system (from 0-2) for the diagnosis of HS subtypes 1-4, based on patterns of segmental neuronal loss in the pyramidal cell layer of the CA, and astrogliosis (Blümcke et al., 2013) as follows: 0 = no obvious neuron loss or moderate astrogliosis (GFAP) only; 1 = moderate

neuron loss and gliosis; 2 = severe neuron loss (majority of neurons lost) and astrogliosis. The ILAE recommends the use of the NeuN antibody as the primary detector, given that it allows for recognition of HS subtypes visually without the need for laborious cell-counting (note: only recognisable when there is >50% loss in a given region), but also because it has the advantage of distinguishing neuronal cells from glia. Other means of detection include cell counts on basic histochemical stains, although this requires comparison to numbers seen in healthy controls (i.e., statistical comparisons between disease groups).

The aims of this chapter were to determine whether there was neuronal loss present in the infant hippocampus:

- 1- Utilising NeuN as the marker to identify major cell loss using the ILAE semi-quantitative classification system (section 4.1),
- 2- If marked cell loss is not detected visually, determining whether we can detect subtle differences in cell loss by applying cell counting techniques to a subset of our SIDS dataset (section 4.2).

#### **4.1: IMMUNOSTAINING FOR NEUN DOES NOT SHOW ALL MATURE AND HEALTHY NEURONS IN THE HUMAN AND PIG BRAIN - FOCUS ON THE HIPPOCAMPUS**

**Luijterink, L., Waters, K.A. and Machaalani, R., 2021.** Immunostaining for NeuN does not show all mature and healthy neurons in the human and pig brain: focus on the hippocampus. *Applied Immunohistochemistry & Molecular Morphology*, 29(6), pp.e46-e56.

##### Summary:

When NeuN was applied to our human infant formalin-fixed and paraffin-embedded (FFPE) brain tissue, IHC signal was low or even absent across sections. NeuN staining can be reduced as a result of antemortem factors or various tissue handling parameters, but modified pre-treatments can enhance immunostaining. We therefore aimed to optimise a laboratory protocol for the use of the NeuN antibody on autopsy tissue however, we found no technique was able to enhance staining to a level suitable for cell counting in our dataset. Signal improvement in past studies may not have been to a level suitable for cell counting, and the current study emphasises the need for caution when interpreting NeuN staining patterns in future studies utilising FFPE tissue.

##### Statement of contribution:

Input to project design was by RM and LL. Tissue sectioning was performed by AV for human cases and LL for piglet cases, and experimentation was performed by LL. Analysis and drafting of the manuscript were performed by LL. The final version of the manuscript was approved by all authors prior to publication, and they have accepted that this manuscript may be included in my thesis. Colour version of images included in the publication are provided in the Appendix.

Human tissue used in this study and the accompanying autopsy and infant characteristic data were obtained from the NSW Forensic and Analytical Science Services (formerly known as the Department of Forensic Medicine). Piglet tissue used in this study was obtained from an experimental model in our laboratory. Experimentation and image analysis was performed at the SIDS and Sleep Apnea Laboratory at the University of Sydney, Sydney, Australia.

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methods for enhancing the staining.<sup>16</sup> Our initial studies aimed to determine whether findings of increased apoptosis in the hippocampus of infants diagnosed with sudden infant death syndrome<sup>26</sup> is also associated with a reduced number of neurons, and found that well-defined neurons in the pyramidal cell layer of the CA did not stain positive for NeuN, while staining in other brain regions varied. Herein, we describe the methods used in an attempt to enhance NeuN staining in FFPE brain tissue. Validation was provided by parallel studies in piglet brain tissue where the fixation method was designed to emulate that used in the human tissue we access.<sup>27</sup> While some staining enhancement was achieved in the human FFPE tissue, it was not sufficient to identify cell loss. In contrast, the piglet tissue showed high levels of NeuN staining. The results are discussed in conjunction with a review of the literature.

## MATERIALS AND METHODS

### Tissue Sourcing

#### Human Brain Tissue

Tissue from 23 infants (aged 1 to 12 postnatal months) and 5 children (aged 4 to 10 y) who died between the years 2000 and 2012, was obtained from the Department of Forensic Medicine, Glebe, NSW, following approval from the Human Ethics Committees of the University of Sydney (approval no.3013/235) and the NSW Central Sydney Area Health Service (Protocol No 3593 and X13-0038). Brain tissue was collected as part of the autopsy to aid postmortem investigation for cause of death, and tissue that remained after completed autopsy was used in this study. Studies that have attempted to use NeuN in the infant brain are summarized in Table (Supplemental Digital Content 1, <http://links.lww.com/AIMM/A288>).

For the infant tissue, PMI ranged from 8.3 to 62.5 hours. At different timepoints over that period, the methods used for tissue fixation were altered. For cases sourced before 2002 ( $n=3$ ), brains were fixed suspended in 10% neutral buffered formalin (NBF) for a minimum of 2 weeks (maximum 12), while for cases sourced from 2002 onwards ( $n=20$ ), brains were fixed in 20% NBF for 2 to 17 days. The hippocampus and brainstem were cut into blocks, and some were returned to the fixative, while others were not (data we did not obtain), before being processed to paraffin blocks using an automated tissue processor.

Hippocampal and brainstem tissue used in this study were sectioned at 7  $\mu$ m using a rotary microtome and mounted on 2% 3-aminopropyltriethoxysilane-treated slides, dried overnight at 42°C and stored in a dust-free environment. The average duration of storage of the tissue used in this study was 12 months before immunohistochemical staining.

#### Piglet Brain Tissue

Piglet brain tissue collection was performed in the early 2000s (detailed in<sup>28</sup>), and was approved by the Animal Ethics committee of the University of Sydney. Piglets were aged 13 to 14 days, which corresponds to 2 to 4

postnatal months in humans from a brain development perspective.<sup>29</sup> The use of the NeuN antibody has been reported in the pig hippocampus.<sup>30–32</sup>

For the piglets, PMI did not exceed 30 minutes. The whole brain was removed down to the spinomedullary junction and fixed in 10% NBF for 14 days. The hippocampus and brainstem were subsequently sectioned into 4 mm slices and returned to 10% NBF for a further 5 days, before being processed to paraffin. Some brains were fixed in 10% NBF+concentrated glacial acetic acid (GAA) (detailed in<sup>27</sup>), or 20% NBF (whole brain fixed for 7 d, dissected in 4 mm blocks and returned to fixative for 3 d). Brain tissue from a total of 21 piglet cases was included in this study: 10 in 10% NBF, 8 in 20% NBF, and 3 in 10% GAA.

Tissue blocks at the rostral level of the medulla and the hippocampus, were sectioned at 7  $\mu$ m by a rotary microtome (Shandon Finesse 325; Thermo Fisher Scientific Inc., MA), mounted onto silanized slides, dried overnight at 42 to 45°C and stored at room temperature in a dust-free environment. The average duration of storage of the tissue used in this study was 10 months before immunohistochemical staining.

### Immunohistochemistry (IHC)

The IHC methodology is as per our laboratory's standard protocol, detailed in Luijck et al<sup>26</sup> and Machaalani and Waters<sup>33</sup> except where otherwise stated. Variations used included:

- (1) Comparing antigen retrieval method (microwave vs. pressure cooker), time and buffers
- (2) Trialing different antibody concentrations (1:250-1:1000).
- (3) Altering incubation temperature (room temperature overnight or 37°C for 2 h).
- (4) Trialing the NeuN antibody of different batch numbers.
- (5) Use of biotin conjugated versus horse radish peroxidase (HRP) conjugated secondary antibodies.
- (6) Use or exclusion of 0.1% Triton X-100 in the phosphate buffered saline (PBS).

Briefly, sections were deparaffinised in 2×30-minute xylene washes and rehydrated through a graded series of ethanol to distilled water. Slides were then subjected to heat induced epitope retrieval by microwaving on high for either 14 or 20 minutes, in either Tris-EDTA buffer (pH 9.0), or citrate buffer (pH 6.0), or using the pressure cooker protocol (Aptum Biologics Ltd, UK) with the R-universal buffer (EMS, 62719-20) at 121°C. Slides were left to cool, washed in PBS or 0.1% Triton X-100 in PBS, and quenched for endogenous hydrogen peroxidase activity.

Following PBS washes, sections were blocked with 10% normal horse serum (NHS) in PBS, followed by overnight incubation with Mouse Monoclonal NeuN antibody [MAB377 (A60 clone); Millipore, MA], at 1:1000 dilution in 1% NHS (this dilution produced optimal signal-to-noise ratio following a trial of different dilutions between 1:250 and 1:1000).

Following PBS washes, sections were incubated in a secondary antibody of either anti-rabbit made in goat HRP (ab205718; Abcam, Cambridge, UK at 1:200 dilution in 1%

**TABLE 1.** Studies Investigating the Effect of Different Methodological Parameters on NeuN Staining Outcomes

| Variable Studied                                           | Species and Brain Region(s)                                                                                                               | Tissue Type<br>(Frozen or<br>Fixed) | NeuN Outcome                                                                                                                                                                                                                                           | References |
|------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| PMI                                                        |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| 4-50 h                                                     | Human hippocampal autopsy tissue                                                                                                          | Both                                | Altering PMI had different outcomes between cases on Western Blot (bands at 50-75 kD), suggesting antemortem factors may be more at play                                                                                                               | 14         |
| 1-168 h                                                    | Pig brain (white matter vs. grey matter tissue micro arrays)                                                                              | Fixed                               | No effect                                                                                                                                                                                                                                              | 13         |
| 1-4 d                                                      | Human surgical and autopsy specimens from various brain regions and tumors                                                                | Fixed                               | No effect                                                                                                                                                                                                                                              | 4          |
| 0-5 h                                                      | Mouse hippocampal tissue (DG, CA3, CA2, CA1)                                                                                              | Frozen                              | From 30 min PMI, there was a decrease in the CA3 pyramidal cell layer that was more pronounced at 5 h (regional vulnerability)                                                                                                                         | 15         |
| Fixation                                                   |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| Fixative (adults)                                          | Human autopsy tissue from temporal lobe and hippocampus                                                                                   | Fixed                               | Formic acid superior protocol in adult brains for automated immunostaining                                                                                                                                                                             | 18         |
| Formalin                                                   |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| Formic acid                                                |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| Formalin/acetic acid+70% ethanol                           |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| Formalin/acetic acid+formic acid+70% ethanol               |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| GAA-10% NBF (infants)                                      |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| Fixative used                                              | Rat hippocampus                                                                                                                           | Fixed                               | Zinc formalin (<1 d) was optimal. Bouin's fluid (<1 d) with rapid dehydration before paraffin embedding also satisfactory                                                                                                                              | 11         |
| 10% NBF                                                    |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| Zinc formalin                                              |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| Ethanol                                                    |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| formaldehyde                                               |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| Bouin's fluid without thermal demasking                    |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| Fixation time: long (1-10 y) versus short (6-49 d) 10% NBF | Human autopsy cortical specimens                                                                                                          | Fixed                               | Short-fixed resembled what is reported for surgical specimens                                                                                                                                                                                          | 17         |
| Fixation time: formalin 18-94 d                            | Pig brain (white matter vs. grey matter tissue micro arrays)                                                                              | Fixed                               | Long fixed only a few cells were immunoreactive<br>Decreased from 66 d in fixative and completely lost by 94 d                                                                                                                                         | 13         |
| Immersion versus perfusion in 4% paraformaldehyde          | Mouse hippocampus                                                                                                                         | Frozen                              | Best in perfused brains (mediated by PMI, 0 min PMI for immersion similar to perfused brains)                                                                                                                                                          | 15         |
| Fixation time: 24 h, 4 mo or 10 y                          | Human autopsy neocortex, white matter and pons tissue                                                                                     | Fixed (+1 frozen sample)            | > 4 mo showed no staining even with heat induced epitope retrieval                                                                                                                                                                                     | 16         |
| 8 h-3 d (surgical)                                         |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| 8 h-4 wk (autopsy)                                         | Human surgical and autopsy specimens. Array of central and peripheral nervous system neurons (including hippocampus)+ganglion cell tumors | Fixed                               | Only 8 d and 3 wk immersion in 4% Lillies phosphate buffered formalin solution showed staining (note: scored 1.7 and 2.6 of 4, respectively, thus not suitable for neuron counting)<br>Optimal staining seen at 8 h, but still satisfactory up to 2 wk | 4          |
| Antigen retrieval and pretreatments                        |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| 1. None                                                    | Human and rat autopsy spinal cord                                                                                                         | Fixed (rat frozen and fixed)        | Alkaline pH buffer+heat was most effective (citrate→absence of staining)                                                                                                                                                                               | 12         |
| 2. Enzymatic                                               |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |
| 3. Heat induced                                            |                                                                                                                                           |                                     |                                                                                                                                                                                                                                                        |            |



|                                                                                                                                                                                                                                                              |                                                              |                                 |                                                                                                                                                                                                                                                                                                  |    |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------|---------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <ol style="list-style-type: none"> <li>1. CC1 Ventana pretreatment</li> <li>2. Microwave with kit (Unmasker Diapath)</li> <li>3. Formic acid pretreatment</li> <li>4. Triton X-100 permeabilization</li> <li>5. Combination of 1+3 or 2+4</li> </ol>         | Human autopsy tissue from temporal lobe and hippocampus      | Fixed                           | <p>Dependent on what formalin procedure was used</p> <p>Best was formic acid with CC1 Ventana for automated immunostaining</p> <p>Microwave irradiation and Unmasker Diapath for manual detection using only 10% NBF</p> <p>Reported no unmasking procedure necessary in the postnatal brain</p> | 18 |
| <p>Citrate or ethylene and microwave heat 15 min</p> <ol style="list-style-type: none"> <li>1. Extended microwave</li> <li>2. 1+catalyzed signal amplification (CSA)</li> <li>3. 98% formic acid 30 min before 20 min microwave</li> <li>4. 3+CSA</li> </ol> | Human autopsy cortical specimens                             | Fixed for a long period of time | <p>Only (4) partially recovered immunostaining</p> <p>Note: CSA based on peroxidase catalyzed deposition of biotinylated tyramide</p>                                                                                                                                                            | 17 |
| <ol style="list-style-type: none"> <li>1. 4 different buffers compared</li> <li>2. Heat versus enzymatic antigen retrieval</li> </ol>                                                                                                                        | Human autopsy neocortex, white matter and pons tissue        | Fixed (+1 frozen sample)        | <p>Heat induced antigen retrieval better than enzymatic</p> <p>Tris-EGTA buffer or target retrieval solution in combination with heat most effective</p>                                                                                                                                         | 16 |
| <p>Buffers</p> <p>Citric acid (pH 6.0)</p> <p>Tris (pH 7.0)</p> <p>Tris-buffered saline (pH 9.0)</p>                                                                                                                                                         | Human autopsy corpus callosum specimens                      | Fixed                           | <p>No difference between microwave and pressure cooker</p> <p>Citric acid was superior</p>                                                                                                                                                                                                       | 19 |
| <p>Buffers</p> <p>TRIS</p> <p>Citrate</p> <p>Protease XXIV</p> <p>Proteinase K</p> <p>Formic acid</p>                                                                                                                                                        | Pig brain (white matter vs. grey matter tissue micro arrays) | Fixed                           | Microarray-Autoclave as 120°C in citrate buffer (pH 6.0) for 10 min (optimized for 1:2000 dilution)                                                                                                                                                                                              | 13 |
| <p>Antibodies</p> <ol style="list-style-type: none"> <li>1. 18-7373 (mouse) (discontinued)</li> <li>2. MAB377 (mouse)</li> <li>3. Ab177487 (rabbit)</li> </ol>                                                                                               | Mouse hippocampus                                            | Fresh fixed                     | MAB377: labeling of mossy fiber terminals in CA3 and adjacent stratum lucidum that co-labeled with Synapsin I antibody. This did not happen using the rabbit clone                                                                                                                               | 20 |
| <p>Staining method</p> <p>IHC versus immunofluorescence</p>                                                                                                                                                                                                  | Human autopsy spinal cord specimens and rat                  | Fixed (rat frozen and fixed)    | DAB more effective than immunofluorescence, likely because of signal amplification step                                                                                                                                                                                                          | 12 |

CA indicates cornu ammonis; CSA, catalyzed signal amplification; DAB, 3,3'-diaminobenzidine; DG, dentate gyrus; GAA, glacial acetic acid; IHC, immunohistochemistry; NBF, neutral buffered formalin; NeuN, neuronal nuclei; PMI, postmortem interval.

**TABLE 2.** Qualitative Analysis of NeuN in the Hippocampus and Cortex

| Species   | 10% NBF   |           |           |           | 20% NBF   |            |           |           | GAA in 10% NBF |           |           |           |
|-----------|-----------|-----------|-----------|-----------|-----------|------------|-----------|-----------|----------------|-----------|-----------|-----------|
|           | 0 (%)     | 1 (%)     | 2 (%)     | 3 (%)     | 0 (%)     | 1 (%)      | 2 (%)     | 3 (%)     | 0 (%)          | 1 (%)     | 2 (%)     | 3 (%)     |
| Human     |           |           |           |           |           |            |           |           | NA             | NA        | NA        | NA        |
| Cortex    | 1/3 (33)  | 2/3 (67)  | 0         | 0         | 1/15 (7)  | 7/15 (47)  | 6/15 (40) | 1/15 (7)  |                |           |           |           |
| DG        | 0         | 0         | 3/3 (100) | 0         | 3/15 (20) | 7/15 (47)  | 7/15 (47) | 2/15 (13) |                |           |           |           |
| CA4       | 1/3 (33)  | 2/3 (67)  | 0         | 0         | 3/15 (20) | 10/15 (67) | 2/15 (13) | 0         |                |           |           |           |
| CA3       | 1/3 (33)  | 2/3 (67)  | 0         | 0         | 7/15 (47) | 7/15 (47)  | 1/15 (7)  | 0         |                |           |           |           |
| CA2       | 3/3 (100) | 0         | 0         | 0         | 6/15 (40) | 7/15 (47)  | 2/15 (13) | 0         |                |           |           |           |
| CA1       | 0         | 2/3 (67)  | 0         | 1/3 (33)  | 3/14 (21) | 7/14 (50)  | 4/14 (29) | 0         |                |           |           |           |
| Subiculum | 0         | 1/3 (33)  | 0         | 0         | 5/13 (38) | 7/13 (54)  | 1/13 (8)  | 0         |                |           |           |           |
| Pig       |           |           |           |           |           |            |           |           |                |           |           |           |
| Cortex    | 0         | 1/10 (10) | 2/10 (20) | 7/10 (70) | 0         | 0          | 3/8 (38)  | 5/8 (63)  | 0              | 0         | 1/3 (33)  | 2/3 (67)  |
| DG        | 0         | 0         | 2/9 (22)  | 7/9 (78)  | 0         | 0          | 1/6 (17)  | 5/6 (83)  | 0              | 0         | 1/2 (50)  | 1/2 (50)  |
| CA4       | 0         | 0         | 2/9 (22)  | 7/9 (78)  | 0         | 0          | 1/6 (17)  | 5/6 (83)  | 0              | 2/2 (100) | 0         | 0         |
| CA3       | 0         | 0         | 1/8 (13)  | 7/8 (88)  | 0         | 0          | 1/5 (20)  | 4/5 (80)  | 0              | 0         | 2/2 (100) | 0         |
| CA2       | 0         | 0         | 0         | 8/8 (100) | 0         | 0          | 1/5 (20)  | 4/5 (80)  | 0              | 1/2 (50)  | 1/2 (50)  | 0         |
| CA1       | 0         | 0         | 1/7 (14)  | 6/7 (86)  | 0         | 1/6 (17)   | 2/6 (33)  | 3/6 (50)  | 0              | 1/2 (50)  | 1/2 (50)  | 0         |
| Subiculum | 0         | 1/7 (14)  | 0         | 6/7 (86)  | 0         | 1/6 (17)   | 1/6 (17)  | 4/6 (67)  | 0              | 0         | 0         | 1/1 (100) |

0 = no staining present.

1 = 1% to 20% of neurons stained.

2 = 21% to 80% of neurons stained.

3 &gt; 80% of neurons stained.

Values expressed as proportion of cases expressing NeuN in that scoring category rounded to the nearest integer.

CA indicates cornu ammonis; DG, dentate gyrus; GAA, glacial acetic acid; NBF, neutral buffered formalin; NeuN, neuronal nuclei.

normal goat serum, note: HRP method required all other IHC steps involving NHS to be substituted with normal goat serum), or biotinylated anti-mouse/rabbit made in horse (BA-1400-2.1; Vector Laboratories Inc., Burlingame, CA, 1:200 dilution in 1% NHS) antibody for 45 minutes. Following PBS washes, sections treated with biotinylated secondary antibody were incubated with avidin-biotin HRP complex (VEPH-4000/PK-4000, Vector Stain ABC kit; Vector Laboratories Inc.) for 45 minutes.

Color labeling was achieved with 3,3'-diaminobenzidine (K346811, DAKO; USA). Sections were then washed in distilled water, counterstained with Harris' hematoxylin, cleared in acid/alcohol, and differentiated with the Scott blueing solution before being dehydrated through a graded series of ethanol, cleared with xylene, and mounted and cover slipped with dibutylphthalate polystyrene xylene.

### Addressing Antibody Specificity

Specificity of the primary antibody was determined by running a Western Blot in both human and piglet tissue (see Figure, Supplemental Digital Content 2, panel a, <http://links.lww.com/AIMM/A289>). Tissue homogenization was performed as detailed in<sup>34</sup> and western blot method as per.<sup>35</sup>

Negative controls had the primary antibody replaced with 1% NHS in order to control for nonspecific binding (see Figure, Supplemental Digital Content 2, panel b, <http://links.lww.com/AIMM/A289>), and isotype control was performed in our piglet tissue by replacing the primary antibody with Mouse; Monoclonal IgG<sub>1</sub> k Isotype (MOPC-21) (ab18443 at 1:5000 dilution) (see Figure, Supplemental Digital Content 2, panel c, <http://links.lww.com/AIMM/A289>).

Our tissue internal controls were neurons of the ION within the brainstem, and Purkinje neurons of the cerebellum given reports of lack of NeuN within these regions.<sup>1,4,5</sup>

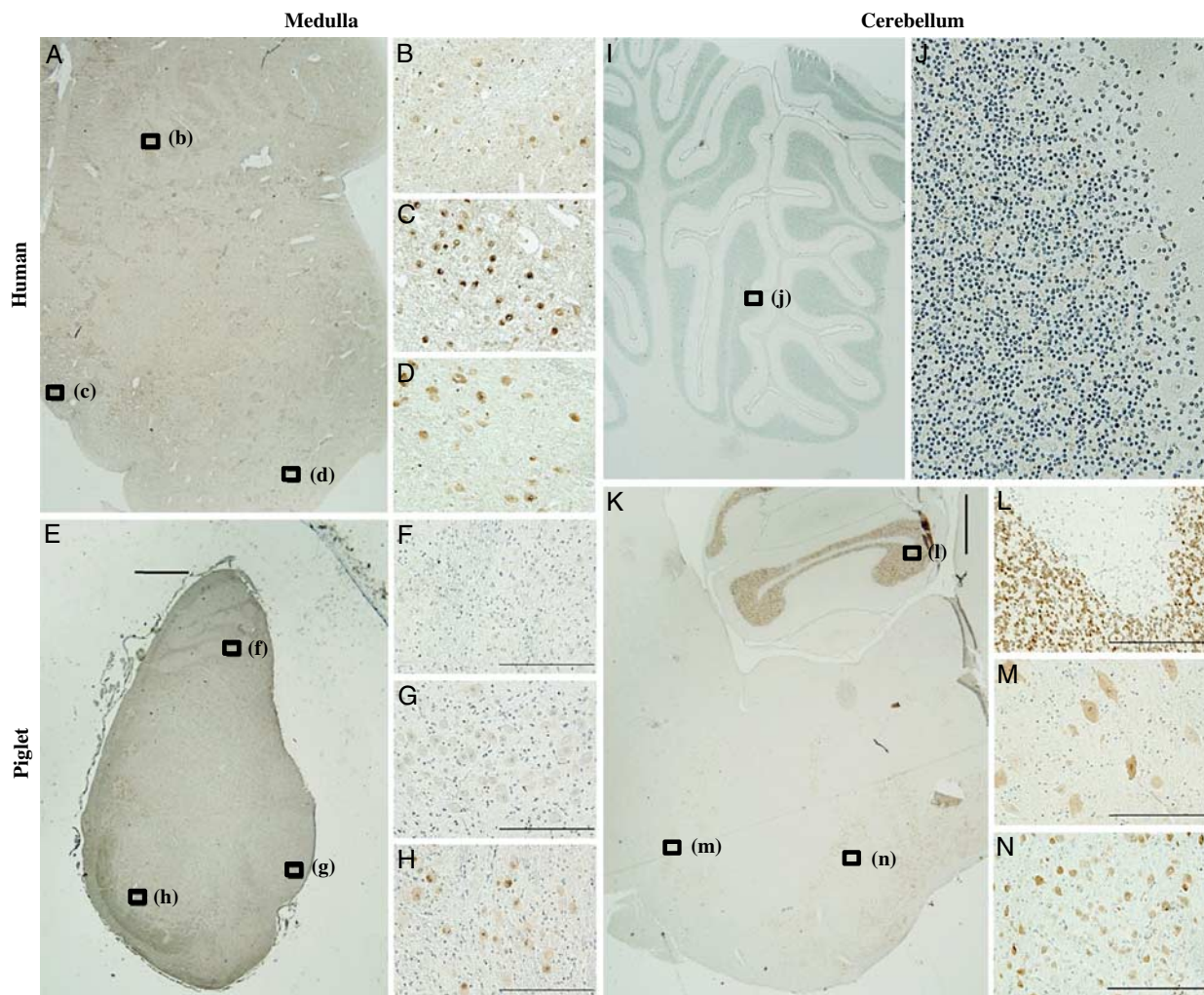
### Semiquantitative Analysis

Five regions of the hippocampus were studied, including the dentate gyrus (DG), CA1, CA2, CA3, CA4/hilus, as well as the subiculum. We also investigated the cortical tissue present in these sections. Where tissue was available, we also studied the medulla [ION, dorsal motor nucleus of the vagus nerve (DMNV), cuneate nucleus, and hypoglossal neurons (XII)], and the cerebellum (Purkinje neurons, granule neurons, pontine neurons, and facial nerve neurons) to evaluate regional variability in staining. Stained sections were analyzed using a Leica Upright DM6000 Microscope (Leica Microsystems Ltd, Heerbrugg, Switzerland) and each region was given a semiquantitative score of either 0 (no staining), 1 (< 20% of cells stained), 2 (21% to 80% of cells stained), or 3 (> 80% cells stained and suitable for cell counting). As our attempts to enhance staining did not result in vast differences in scoring, counts of individual tissue sections were averaged to produce a single score for each brain region per case (Table 2).

## RESULTS

### Staining Pattern of NeuN

NeuN staining, when present, was visualized as dark brown in the nucleus of neurons (Fig. 1C), and was often accompanied with light brown staining in the cytoplasm (Fig. 2). We also saw staining outside the cell bodies in the surrounding parenchyma, and along the stratum lucidum layer of the CA3 region in both human (Fig. 3C black arrow) and piglet tissue (Fig. 2C black arrow). In the human infant hippocampus, NeuN expression was highest in the DG and minimal throughout the CA regions (Table 2). The cortex also had some staining (Table 2), while among brainstem regions, staining was variable (see Table, Supplemental Digital



**FIGURE 1.** Micrograph illustrating neuronal nuclei staining of the human (A) and piglet (E) medulla fixed in 20% neutral buffered formalin at  $\times 2$  objective magnification with  $\times 40$  illustrations of the inferior olivary nucleus (B, F), dorsal motor nucleus of the vagus nerve (C, G), and cuneate (D, H). Also shown is the human cerebellum fixed in 20% neutral buffered formalin (I) at  $\times 2$ , with granule cell layer at  $\times 40$  (J), and piglet pons and cerebellum at  $\times 2$  (K), and  $\times 40$  illustrations of the granule cell layer of the cerebellum (L), facial nucleus (M) and pontine nucleus (N). Note, scale bar for images shown at  $\times 2$  objective lens = 1 mm, and  $\times 40$  objective lens = 200  $\mu\text{m}$ .

Content 3, <http://links.lww.com/AIMM/A290>). In the piglet hippocampus, staining was seen in all cases and was consistent from region to region, as well as in the cortex (Table 2), and cerebellum (see Table, Supplemental Digital Content 3, <http://links.lww.com/AIMM/A290>), but not among regions of the brainstem (see Table, Supplemental Digital Content 3, <http://links.lww.com/AIMM/A290>).

## Effect of Fixative

### Human

For tissue fixed in 10% NBF, NeuN staining was low to negligible in the pyramidal cells of the CA regions, subiculum, and cortex, although some staining was seen within the DG (Fig. 3, Table 2). For tissue fixed in 20% NBF, staining in the hippocampus varied markedly between cases and regions. We found  $>20\%$  positive staining in the cortex ( $n=7$ ) and DG ( $n=9$ ), while staining in the CA regions generally remained low

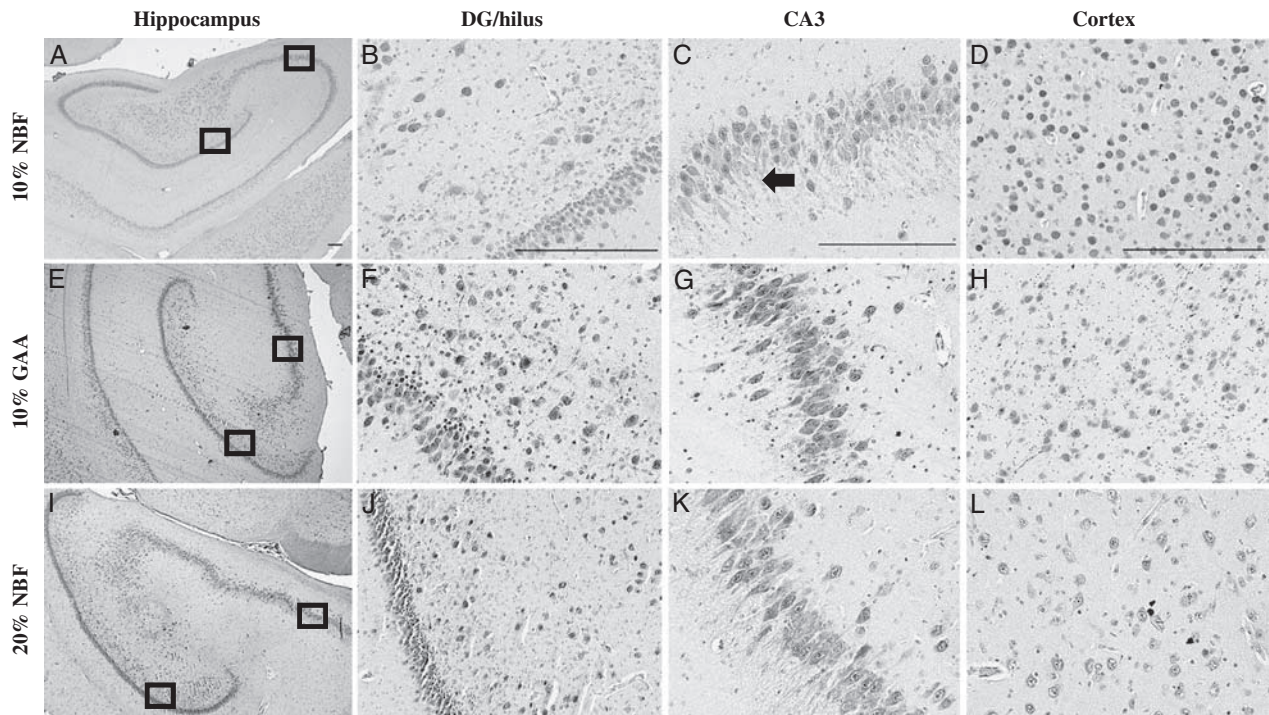
with a score of  $<20\%$  in up to 94% of the cases (Table 2). This high level of variation was also seen in sections from the medulla, with the DMNV being the only region with  $>80\%$  NeuN expression in 40% of cases, and all cases showing some positive NeuN staining in parts of the ION (Fig. 1, and Table, Supplemental Digital Content 3, <http://links.lww.com/AIMM/A290>).

To evaluate any effect of maturation (age) we stained hippocampal sections from 5 cases aged 4 to 10 years (2 in 10% NBF and 3 in 20% NBF). Some positive staining was seen in the DG and cortex, but minimal staining in the CA regions of the hippocampus (Fig. 3), with no difference observed between the fixatives.

### Piglet

For 10% NBF brains, 70% to 100% of piglet cases showed staining in  $>80\%$  of neurons in the cortex, hippocampus





**FIGURE 2.** Neuronal nuclei staining in the piglet hippocampus and cortex showing the effects of different fixatives. Panels (B), (F), and (J) are magnified of the DG and CA4/hilus region, as outlined in panels (A), (E), and (I), respectively. Panels (C), (G), and (K) are magnified of CA3, as outlined in panels (A), (E), and (I), respectively. Panels (D), (H), and (L) are magnified cortical images. Note both neuronal and parenchymal staining is evident (C) (black arrow). Scale bars = 200  $\mu$ m. Boxes represent the location of the magnified subregions. DG indicates dentate gyrus; GAA, glacial acidic acid; NBF, neutral buffered formalin.

and subiculum (Table 2). The morphological integrity of tissue was compromised in those fixed in GAA, resulting in greater variability. Notably, positive neurons in these cases had darker brown staining that clearly predominated in the nucleus compared with those fixed in 10% NBF (Fig. 2). For tissue fixed in 20%, although many cells stained positive, the strength of the staining was generally weaker (Fig. 2).

In the brainstem and cerebellum, positive staining was variable among nuclei regardless of whether the tissue had been fixed in 10% or 20% NBF. For cases fixed in 10% NBF, staining was absent in the ION (as expected), and the DMNV (unexpectedly) (Figure 1 and Table, Supplemental Digital Content 3, <http://links.lww.com/AIMM/A290>), while strong positive staining was seen in the granule cells, pontine neurons, and facial nucleus (Fig. 1). For cases fixed in 20% NBF, positive staining was seen in the ION in 1 case, and strong positive staining was seen in the granule cells, pontine neurons, and facial nucleus (see Table, Supplemental Digital Content 3, <http://links.lww.com/AIMM/A290>).

## Effect of IHC Altered Parameters

### Antigen Retrieval Time and Buffers

Use of different buffers, or varying antigen retrieval by varying the duration in the microwave, or using pressure cooker compared with microwave, had no effect on staining outcomes (Fig. 4).

### Utilization of 0.1% Triton X-100 in the PBS Versus its Absence

Addition of Triton X-100 in the PBS and subsequently carried over into the antibody incubation steps, did not enhance staining (Fig. 4 panels g-i).

### Antibody Concentration/incubation Heat, and HRP Versus ABC

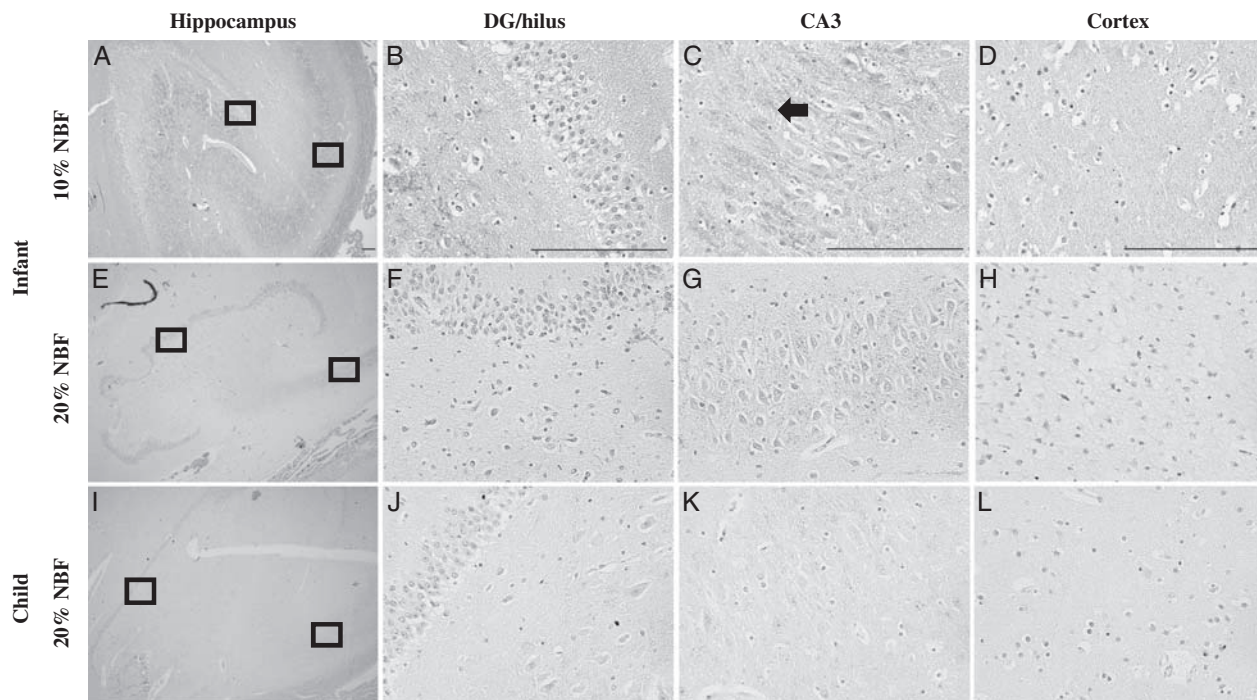
Concentrations of 1:250, 1:500, and 1:1000 were used in 1% NHS. In the piglet tissue and in the cortical regions of the infant tissue, a 1:1000 dilution produced sufficient signal with reduced background noise. Highly concentrated antibody, and incubation with primary antibody at 37°C, did not alter the proportion of positive staining (Fig. 4 panels j-l). No difference was observed using HRP or the ABC method.

### Antibody of Different Batch Numbers

There was no difference in staining outcomes when using different antibody batches (data not shown).

## DISCUSSION

Postmortem human brain tissue is not easily obtained for research, and the methods used to fix and store this tissue are generally not able to be controlled by the researcher, yet they can greatly affect the availability of target proteins and epitopes, particularly in FFPE



**FIGURE 3.** Neuronal nuclei Immunohistochemistry staining pattern in the human infant (A-H) hippocampus according to fixative of 10% neutral buffered formalin (NBF) versus 20% NBF, and child with 20% NBF (I-L) with magnification of the boxed areas representing the dentate gyrus and hilus (B, F, J) and CA3 (C, G, K). Also shown is the cortex (D, H, L). Note non-neuronal staining in the CA3 but positive parenchymal staining in the adjacent stratum lacunosum highlighted in the human infant (C) (black arrow). Scale bars = 200  $\mu$ m.

specimens (reviewed in<sup>36</sup>). As NeuN is known to be sensitive to certain tissue related parameters (summarized in Table 1), the current study investigated the use of this marker on archival FFPE human brain tissue, and reports on methodological attempts undertaken to enhance the staining. We also undertook a parallel study in piglet tissue where tissue parameters were tightly controlled, thus serving as our internal positive control. The main findings were that, in humans, (1) staining was often absent, even from neurons appearing morphologically normal; (2) staining showed marked variability between regions of the same tissue section, as well as between cases, with the DG and cortex showing the most variability between cases, while the CA regions consistently showed low staining; and (3) these outcomes remained consistent across the pediatric age range (thus not because of lack of maturation). No alterations in our IHC protocol enhanced immunostaining to a level suitable for cell counting. In piglets, (1) the composition of fixative (10% NBF vs. 20% NBF) did not notably affect the number of cells positive for NeuN, while the addition of GAA produced more variable staining throughout the same tissue section; and (2) regional variations in staining of the same case were minimal. Put together, our findings support suggestions that fixation parameters,<sup>18</sup> and/or antemortem factors<sup>14</sup> have marked effects on the antigenicity of the NeuN target protein. We conclude that NeuN is not a reliable marker of neurons in archival FFPE human brain tissue.

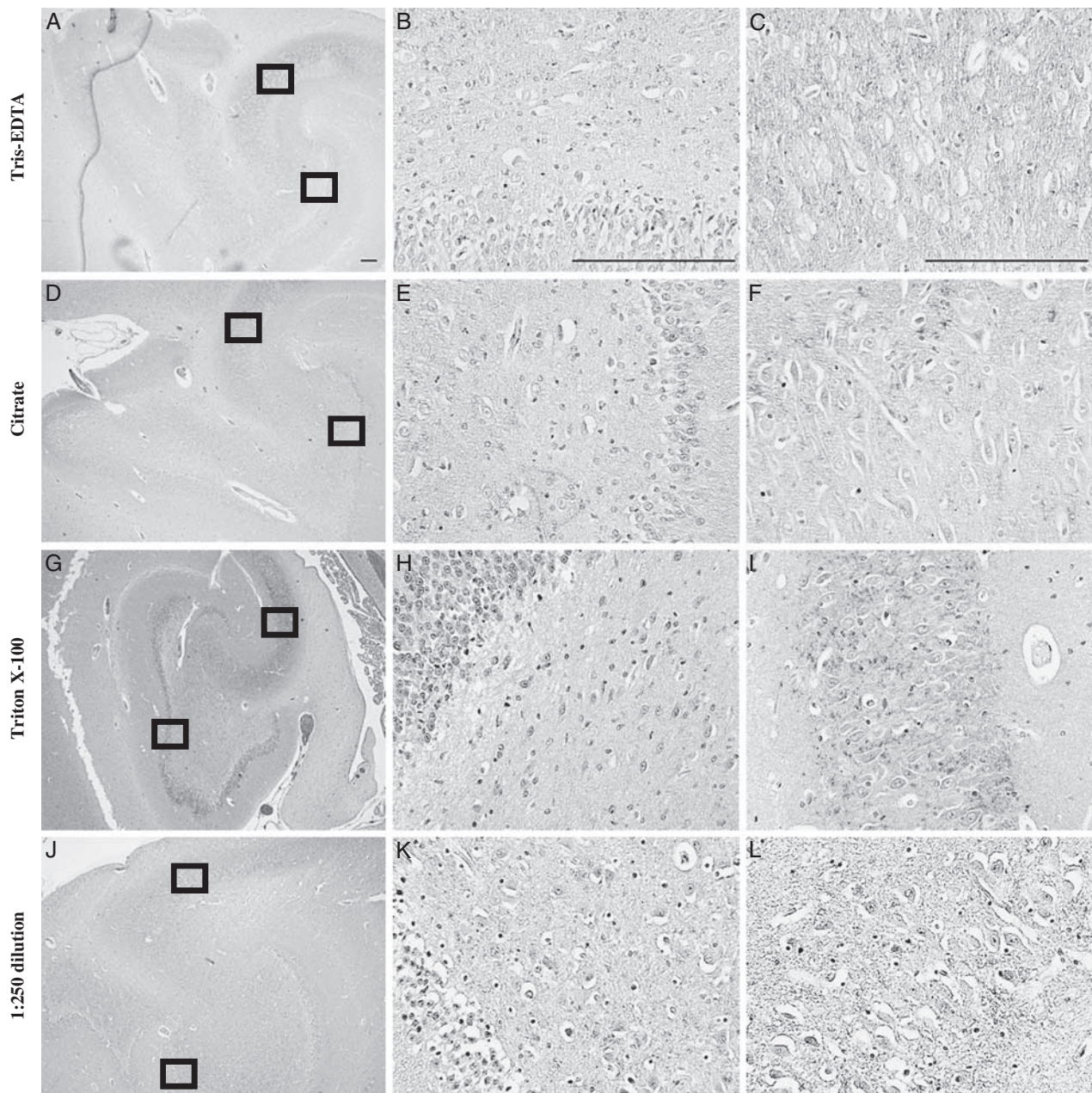
### Validation of Antibody Penetration and Attempts to Enhance Immunoreactivity

Other studies in our laboratory have identified cytoplasmic staining for active caspase-3, and  $\alpha$ 7 and  $\beta$ 2 nicotinic acetylcholine receptor subunits, nuclear staining of fragmented DNA using TUNEL<sup>26</sup> (see Figure, Supplemental Digital Content 4, <http://links.lww.com/AIMM/A291>), microglial markers<sup>37</sup> and astrocytes [glial fibrillary acidic protein (GFAP)] in these same cases (see Figure, Supplemental Digital Content 4, <http://links.lww.com/AIMM/A291>). The lack of immunoreactivity for NeuN therefore appears specific to either the NeuN antibody, or loss of antigenicity of the target epitope. Consistent with previous reports (summarized in Table 1), our use of various antigen retrieval methods [acidic and alkaline buffers, or addition of detergents (Triton X) in combination with various heat processes] were not effective at restoring staining to levels sufficient for cell counting. It is possible that harsher methods would have more success, although these can still fail.<sup>17</sup>

### Effect of Fixation

Formalin can eventually degrade DNA and mask protein epitopes by forming covalent bonds between neighboring proteins or nucleic acids,<sup>38</sup> and excessive fixation length can cause excessive cross-linking. NeuN is particularly vulnerable to this effect since longer fixation





**FIGURE 4.** Neuronal nuclei staining determining the effect of different buffers for Antigen retrieval on infant hippocampus: (A–C) Tris-EDTA buffer (pH 9.5) versus (D–F) citrate buffer (pH 6.0), addition of 1% Triton X-100 in phosphate buffered saline (G–I), and increased neuronal nuclei antibody dilution of 1:250 (J–L). Scale bars = 200  $\mu$ m. Boxes represent the location of the magnified subregions.

times greatly reduce staining,<sup>13</sup> but not for GFAP.<sup>13</sup> In accordance, we also demonstrate GFAP immunoreactivity throughout the hippocampus, even in sections where staining for NeuN was weak or absent (see Figure, Supplemental Digital Content 4, <http://links.lww.com/AIMM/A291>). The low level of immunoreactivity seen in our human dataset, despite our attempts to enhance it, is consistent with another laboratory that reported NeuN staining in only 2 cases of their dataset (fixed for 8 d or 3 wk in 4% Lillies phosphate buffered formalin), yet not to

a level suitable for cell counting,<sup>16</sup> where longer fixation times were associated with absent staining.<sup>16</sup>

The degree of formalin penetration, in combination with displacement of water, could account for the variable region to region NeuN staining we observed, both within and between cases. Formalin penetration is estimated at 2.4 mm after 24 hours,<sup>39</sup> and varies depending on fixation method, method of quantifying penetration, and type of tissue used.<sup>40</sup> Better staining outcomes have been reported for NeuN in brains fixed by perfusion before immersion,<sup>15,16</sup> although this

is not feasible for postmortem human brains. Other elements of the fixative that can affect the quality of the immunostaining include formalin concentration, pH, and storage temperature.<sup>36,41</sup> Dehydrating agents such as formic/acetic acid can reduce cross-linking and therefore enhance immunostaining, even with prolonged fixation times in adult brain tissue.<sup>17,21</sup> Although GAA is reported to enhance NeuN staining in infant brain tissue because of the high water content,<sup>18</sup> in piglet tissue we found that GAA increased the variability of staining between cases, and reduced the morphological integrity of the tissue. We were unable to study the effects of this fixative in our human infant cohort given the tissue we had from cases fixed in this way were sectioned over 10 years ago, which is known to reduce antigenicity.<sup>42</sup> Only tissue stored on slides for up to a year were included for analysis in our study, although it should be noted that some markers may show reduced antigenicity as early as 3 months,<sup>42</sup> not to mention the reduced antigenicity of nuclear antigens when tissue blocks are stored for a long period of time, even before sectioning<sup>43</sup> which was the case for our study. But since this applied to both the human and piglet tissue, and NeuN staining was of reasonable level in the piglet tissue, we do not believe this to have contributed to loss of antigenicity in our human tissue. Furthermore, our tissues were fixed at room temperature, although it is recommended that formalin fixation be carried out at 4°C to minimize nucleic acid degradation (reviewed in<sup>44</sup>).

## PMI

Multiple studies suggest that NeuN is not sensitive to PMI (summarized Table 1),<sup>4,13</sup> yet one of the major differences between our piglet and infant tissue was PMI. The PMI is known to affect the quality of immunostaining because of its impact on biochemical factors, which in turn influence many target proteins and epitopes.<sup>45</sup> Piglet brains in our laboratory are placed into fixative within 30 minutes and animal studies have shown reduced NeuN immunoreactivity as early as 30 minutes postmortem.<sup>15</sup> Another study evaluating NeuN immunoreactivity on Western Blot reported that cases may have different vulnerabilities to PMI, possibly because of antemortem factors.<sup>14</sup> Certain pathologic conditions appear to reduce the antigenicity of the NeuN epitope,<sup>10–12</sup> possibly because of the phosphorylation status of the target epitope.<sup>46</sup> It appears that changes in the NeuN epitope may occur rapidly after death, with additional impact from certain physiologic or antemortem factors.

## Unexpected NeuN immunostaining Patterns

An interesting finding was parenchymal staining in the CA3, also extending into the inner stratum lucidum in both human and piglet samples. This has been reported in the mouse brain<sup>20,46</sup> and was hypothesized to represent the localization of NeuN-immunoreactive material into distal dendrites of neurons, or antigen localization to granule cell axons that form mossy fibers. This pattern of staining was subsequently co-localized with the marker Synapsin I,<sup>20</sup> which supported the findings in western blotting for the band of 75 kDa,<sup>2,47</sup> yet this extracellular staining pattern was specific to the mouse monoclonal form of NeuN, and was not seen when

using a rabbit polyclonal (Ab177487; Abcam).<sup>20</sup> These findings highlight a potential cautioning when using the NeuN antibody, because of cross reactivity<sup>2,6</sup> which we verified in both human and piglet brain tissue on western blot (see Figure, Supplemental Digital Content 2, panel a, <http://links.lww.com/AJMM/A289>), detecting multiple bands ranging between 45 and 75 kDa (including the 46 and 48 kDa bands), similar to what has been reported previously.<sup>2,47</sup>

Absent NeuN staining in the human ION in FFPE tissue<sup>5</sup> is thought to be because of activity of the neuron-restrictive silencing factor (NRSF; REST) inhibiting NeuN expression.<sup>48–50</sup> We unexpectedly saw positive staining in the ION in some cases, and others have reported this in sudden infant death syndrome infants<sup>51</sup> as well as in rats.<sup>52,53</sup> It remains unclear why some studies see staining in the ION while others do not.

## CONCLUSION

Despite the demonstrated effectiveness of NeuN staining in surgical specimens or tissue from experimental models, our study highlights the shortcomings of the use of the NeuN antibody in retrospective studies that use archived FFPE human brain tissue, where lack of staining of the NeuN antibody in the nucleus of healthy appearing neurons predominated. In contrast, the NeuN antibody identified abundant staining in the piglet hippocampus, where the greatest factor of difference compared with the postmortem human tissue was PMI.

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#### **4.1.1: APPENDIX**

This Appendix contains the supplementary material available for Luijck et al, 2021, as well as colour versions of images included in the publication. For ease of reference, the supplement tables and images retain their titles as referred to in the publication.

**Supplemental Digital Content 1.** Summary of IHC studies for NeuN conducted on human infant brain tissue (>1week age) comparing whole brain fixed to surgical specimens (note, only studies that included autopsy specimens were included).

| Disease/D<br>isorder                                                   | Region<br>analysed                        | Fixation                                                                                                                                | Antibody<br>Dilution<br>factor | Tissu<br>e | Age of<br>cases                                 | Antigen<br>retrieval                                                                                       | PMI           | Quantificat<br>ion                                | Outcome                                                                                                                | Referenc<br>e                              |
|------------------------------------------------------------------------|-------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|------------|-------------------------------------------------|------------------------------------------------------------------------------------------------------------|---------------|---------------------------------------------------|------------------------------------------------------------------------------------------------------------------------|--------------------------------------------|
| <i>Autopsy Specimens</i>                                               |                                           |                                                                                                                                         |                                |            |                                                 |                                                                                                            |               |                                                   |                                                                                                                        |                                            |
| Non-<br>neurologic<br>al causes<br>of death                            | Corpus<br>callosum                        | 10% formalin<br>24 hours & in<br>paraffin (some<br>frozen and cut<br>in 60 µm<br>sections)                                              | 1:1000*                        | 12 µm      | 15 post<br>concepti<br>on<br>weeks–<br>59 years | None                                                                                                       | 4-24<br>hours | No. of<br>neurons per<br>section, and<br>per area | Distinguished<br>mature neurons<br>as early as 20<br>post conception<br>weeks                                          | (Jovanov-<br>Milošević<br>et al.,<br>2010) |
| Non-<br>neurologic<br>al causes<br>of death                            | Neocortex,<br>white<br>matter,<br>pons    | Varied (4%<br>Lillies most<br>common).<br>Fixation varied<br>i.e. 24 hours,<br>4mths, 10<br>years-)                                     | 1:400*                         | 5 µm       | 20 gest<br>weeks-<br>93 years                   | Heat<br>induced<br>epitope<br>retrieval<br>(Tris-<br>EGTA<br>buffer or<br>target<br>retrieval<br>solution) | 1-4<br>days   | 6-tier<br>graded<br>qualitative<br>assessment     | Better NeuN<br>with perfusion-<br>fixation.<br>Signal lost in<br>fixation >24hrs                                       | (Lyck et<br>al., 2008)                     |
| Bi-polar,<br>schizophre<br>nia (cause<br>of death<br>not<br>specified) | Corpus<br>callosum,<br>cingulate<br>gyrus | Tissue blocks<br>(1.5-2cm <sup>3</sup> )<br>10% NBF for<br>4 days, then<br>cryoprotected<br>for 1 week in<br>phosphate-<br>buffered 30% | 1:500 (not<br>specified)       | 25 µm      | 40 gest<br>weeks to<br>78 years                 | None                                                                                                       | 8-28<br>hours | Cells/mm <sup>2</sup>                             | Strong nuclear<br>and weak<br>cytoplasmic<br>staining. Decline<br>in NeuN density<br>during the first<br>year of life. | (Connor<br>et al.,<br>2009)                |

|                                              |                                                                                  |                                                                                                    |                          |            |                                      |               |               |                                                           |                                                                                                                                                                                                                                                                |                        |
|----------------------------------------------|----------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|--------------------------|------------|--------------------------------------|---------------|---------------|-----------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------|
|                                              |                                                                                  | sucrose before frozen, or 10% NBF for 20 hours then cut and immersed for 5 minutes in 100% acetone |                          |            |                                      |               |               |                                                           | No reported difference between the 2 fixation methods.                                                                                                                                                                                                         |                        |
| Sudden infant deaths                         | Leptomeningeal neurons                                                           | Whole brain 10% NBF for 14 days, then paraffin embedded                                            | 1:100 (Dako)             | 2 $\mu$ m  | 18-365 days                          | Not specified | 6-72 hours    | Cells/mm <sup>2</sup>                                     | NeuN positive cell adjacent to leptomeningeal blood vessel                                                                                                                                                                                                     | (Rickert et al., 2009) |
| Developmental (cause of death not specified) | Occipital lobe at the level of the calcarine fissure and parieto-occipital sulci | 10% formalin and Bouin fixative and paraffin embedded                                              | Not specified (Chemicon) | 10 $\mu$ m | 14-40 gest weeks + 10 month + adults | Not specified | Not specified | Cells per ( $\times 10^{-3}$ cell/ $\mu$ m <sup>2</sup> ) | NeuN expression reported as early as 16 weeks gestation. NeuN redistributed throughout different cortical layers throughout gestation, but stably expressed in the adult throughout all gyri and sulci of the occipital medial area and all neocortical layers | (Godovalova, 2010)     |

|                        |                                                 |                                                                           |        |      |                                          |                   |               |                                                                                                           |                                                                                                                                              |                         |
|------------------------|-------------------------------------------------|---------------------------------------------------------------------------|--------|------|------------------------------------------|-------------------|---------------|-----------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|
| Sudden infant deaths   | Brainstem/spinal cord                           | 10% NBF, paraffin embedded                                                | 1:100* | 5 µm | 17 – 40 weeks gest – 10 months postnatal | Citrate/microwave | < 24 hours    | 4 scoring quartiles (0, ≤ 30%, ≤ 30 (weak), > 30% (strong))<br><br>(took staining intensity into account) | Many cases did not exhibit > 30% staining (none amongst the SIDS or Sudden Intrauterine Unexplained Death groups)                            | (Lavezzi et al., 2013a) |
| Sudden perinatal death | Cerebellum/brainstem (including ION)            | 10% NBF + paraffin embedded                                               | 1:100* | 5 µm | 25 gest weeks – 12 post-natal months     | Citrate/microwave | 24-48 hours   | Only took intense brown staining as +                                                                     | Purkinje neurons negative in controls, but many sudden infant deaths resulted in positives (association noted with cigarette smoke exposure) | (Lavezzi et al., 2013b) |
| Ependymomas            | Supratentorial, posterior fossa and spinal cord | Formalin fixed and paraffin embedded (concentration of NBF not specified) | 1:50*  | 4 µm | 0.4-18.6 years                           | EDTA/microwave    | Not specified | % per 0.1 mm <sup>2</sup>                                                                                 | NeuN expressed significantly higher in supratentorial tumours compared to infratentorial tumours                                             | (Hagel et al., 2013)    |

|                                                           |                  |                                                                                                           |                            |      |                         |                                                               |               |                                                                                                                                    |                                                                                                                  |                         |
|-----------------------------------------------------------|------------------|-----------------------------------------------------------------------------------------------------------|----------------------------|------|-------------------------|---------------------------------------------------------------|---------------|------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------|-------------------------|
| Developmental study                                       | Spinal cord      | 4% NBF, + paraffin embedded                                                                               | 1:500*                     | 4 µm | 3 weeks – 8 gest months | None                                                          | Not specified | Average no. of cells per area                                                                                                      | NeuN immunoreactivity in spinal cord noted at different developmental time points                                | (Guo et al., 2015)      |
| Sudden infant deaths                                      | Pons and medulla | 10% NBF + paraffin embedded                                                                               | 1:1500*                    | 4 µm | 1-6 months              | Citrate/microwave                                             | Not specified | 5 scoring quartiles (no staining, ≤30% (weak), ≤30% (strong), >30% (weak), >30% (strong)). Staining based on cells/mm <sup>2</sup> | NeuN expressed in almost all brainstem nuclei studied (including the ION), most nuclei showed staining above 30% | (Lavezzi et al., 2016b) |
| Sudden infant death and adults of non-neurological deaths | Hippocampus      | Infant brains 10x glacial acetic acid solution in 10% NBF 8 days, transferred to 70% ethanol, followed by | Range from 1:25 to 1:200\$ | 7 µm | 1 month - adult brains  | 1. Ventana, 2. microwave with Unmasker, Diapath kit 3. formic | 40 hours      | 4-tier graded score                                                                                                                | Formalin protocol optimised for the infant brain due to known high water content in the post-natal brain.        | (Paradiso et al., 2018) |

|                                              |                            |                                                                                                                        |                                          |       |                                                                                        |                                                   |                                       |                                                                                         |                                                                           |                        |
|----------------------------------------------|----------------------------|------------------------------------------------------------------------------------------------------------------------|------------------------------------------|-------|----------------------------------------------------------------------------------------|---------------------------------------------------|---------------------------------------|-----------------------------------------------------------------------------------------|---------------------------------------------------------------------------|------------------------|
|                                              |                            | 80% ethanol (4 days each) and paraffin embedded                                                                        |                                          |       |                                                                                        | acid, 4. triton, 5. Combination of 1 + 3 or 2 + 4 |                                       |                                                                                         |                                                                           |                        |
| <b><i>Autopsy and surgical specimens</i></b> |                            |                                                                                                                        |                                          |       |                                                                                        |                                                   |                                       |                                                                                         |                                                                           |                        |
| Medial temporal lobe epilepsy                | Hippocampal granule cells  | a) autopsy specimens; 4% formalin <sup>3</sup> 2 weeks and paraffin embedded, b) surgical specimens; 10% NBF overnight | 1:500 (Clone A60, not further specified) | 60 µm | a) autopsy specimens; 4 gest weeks - 15 years b) surgical specimens; 8 months - 46 yrs | None                                              | 12 hours-3 days for autopsy specimens | Ammon's Horn Sclerosis classification. Dentate gyrus analysed as cells/ mm <sup>2</sup> | No comparison of staining outcomes between autopsy and surgical specimens | (Blümcke et al., 2001) |
| Epilepsy (frozen)                            | Neocortex and white matter | Immunocytochemistry blocks for both surgical and autopsy specimens immersed in                                         | 1:2000*                                  | 30 µm | 0-38 years                                                                             | None                                              | 3-11 hours autopsy samples            | Density per 10 000 µm <sup>2</sup>                                                      |                                                                           | (Andres et al., 2005)  |

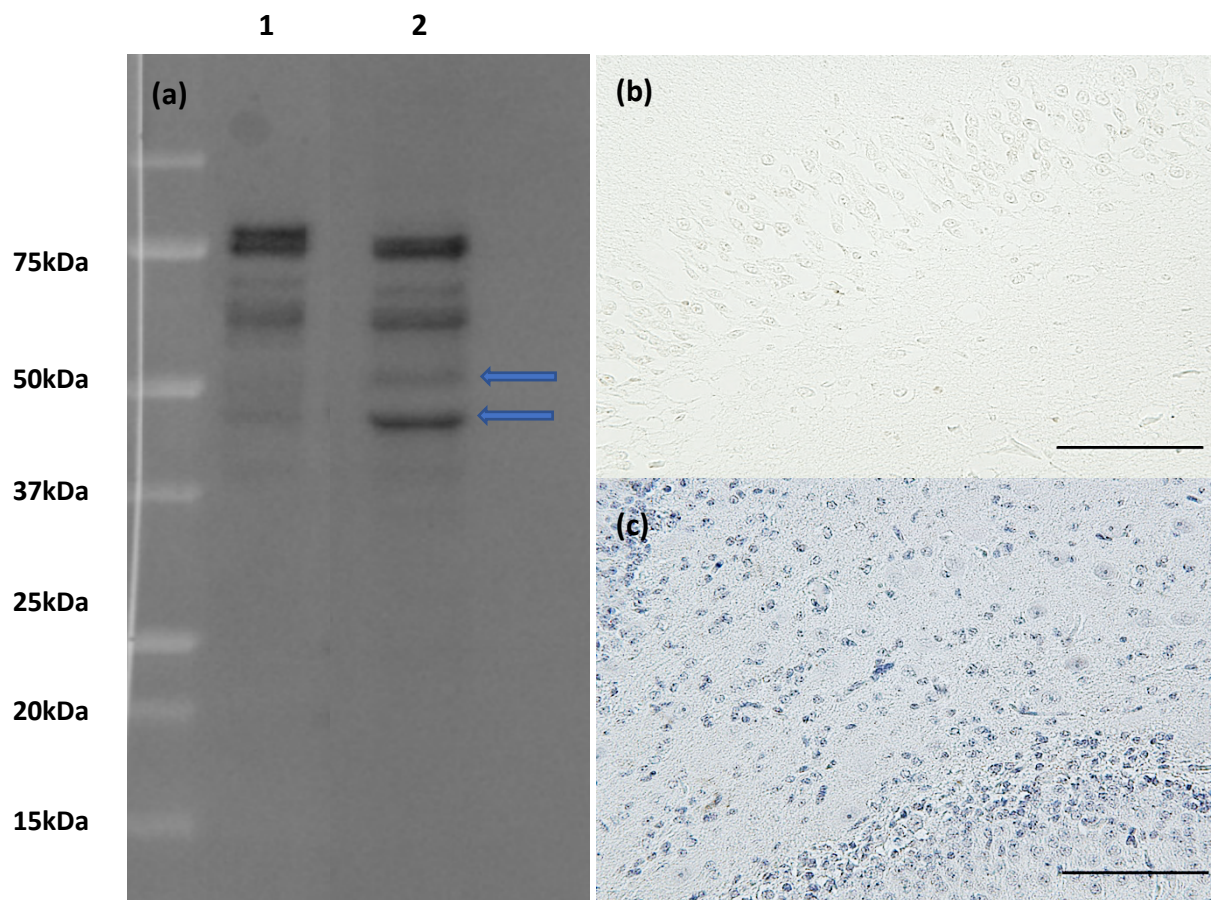
|                    |                     |                                                                                                                             |         |            |             |      |                                |                                                      |                                                                                                                                        |                        |
|--------------------|---------------------|-----------------------------------------------------------------------------------------------------------------------------|---------|------------|-------------|------|--------------------------------|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------|------------------------|
|                    |                     | 10% NBF 24-48hrs (frozen)                                                                                                   |         |            |             |      |                                |                                                      |                                                                                                                                        |                        |
| Hemimegalencephaly | Neocortex           | 1.5-2cm blocks of autopsy and surgical specimens prepared for immunocytochemistry and immersed in 10% NBF24-48 hrs (frozen) | 1:2000* | 30 $\mu$ m | 0.2-4 years | None | 3-11 hours for autopsy samples | Cells/10 000 $\mu$ m <sup>2</sup>                    | Subjects with hemimegalencephaly had alterations in NeuN density that correlated with various clinical and histopathological variables | (Salamon et al., 2006) |
| Hemimegalencephaly | Frontal lobe cortex | Not specified (paraffin embedded)                                                                                           | 1:1000* | 7 $\mu$ m  | 1-24 months | None | Not specified                  | Quantification from 1cm <sup>2</sup> for each region |                                                                                                                                        | (Aronica et al., 2007) |

\*MAB377

#ABN78C3

\$ MAB-90228. Antibody was raised against the N-terminal 100 amino acids of Human Fox3 as expressed in and purified from E. coli.

*NBF*= neutral buffered formalin, *Gest*= gestational, *ION*= inferior olivary nucleus.



**Supplemental Digital Content 2.** Western Blot for NeuN (a) in human (1) and piglet (2) brain tissue showing as expected in relation to the literature, the bands at 46 and 48kDA (arrows), but additional bands at 60 and around 75kDA also consistent with literature (Kim et al., 2009, Dredge and Jensen, 2011). No positive staining is present in the negative control where the primary antibody was replaced with 1% NHS in the human DG/hilar region (b), nor in the Isotype IgG<sub>1</sub> control antibody in the piglet DG/hilar region(c). Scale bars = 100  $\mu$ m.

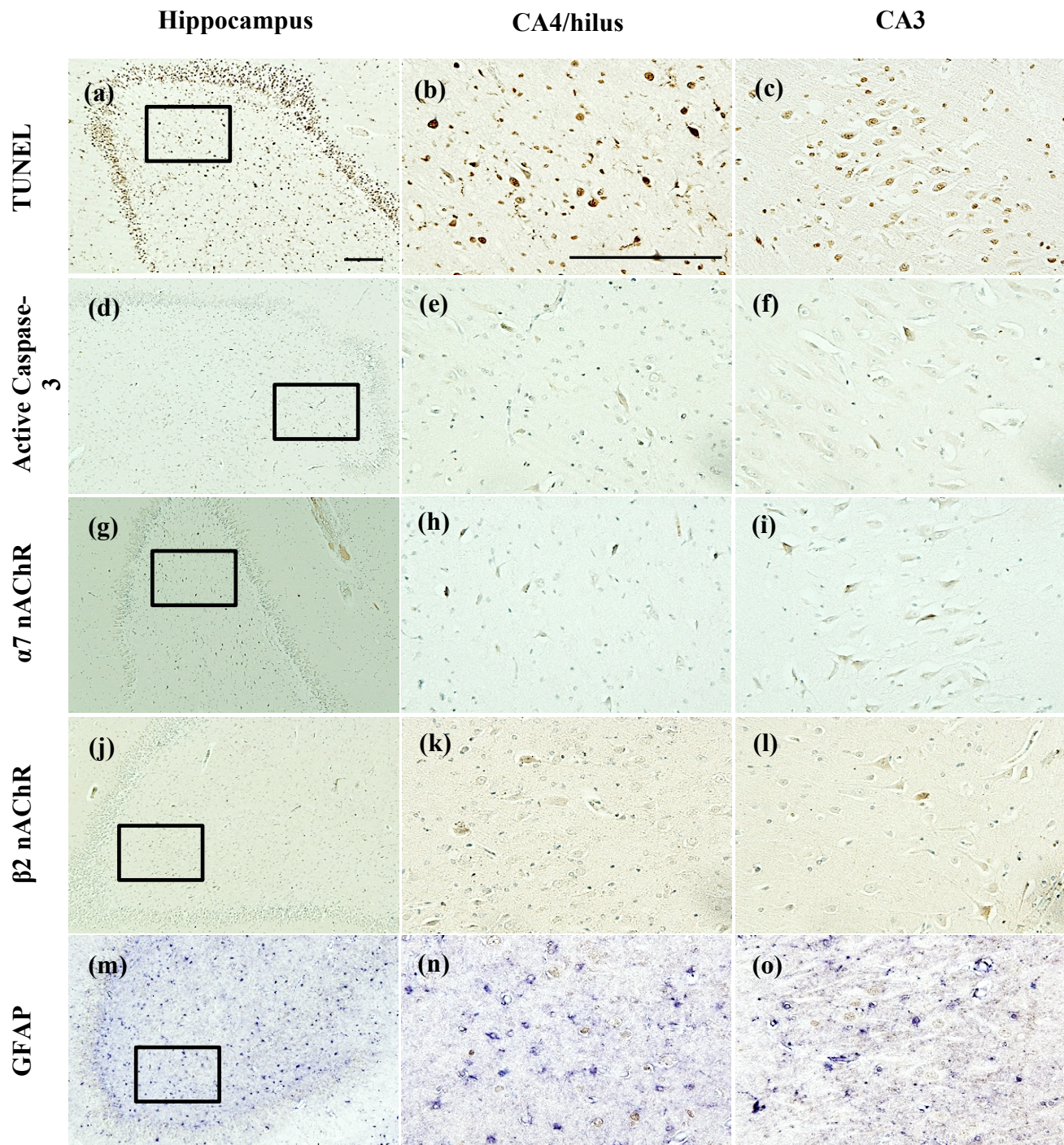


**Supplemental Digital Content 3.** Qualitative analysis of NeuN in various regions of the medulla and cerebellum

| <i>Species</i>          | <i>10% NBF</i> |              |              |              | <i>20% NBF</i> |              |              |              |
|-------------------------|----------------|--------------|--------------|--------------|----------------|--------------|--------------|--------------|
|                         | <i>0 (%)</i>   | <i>1 (%)</i> | <i>2 (%)</i> | <i>3 (%)</i> | <i>0 (%)</i>   | <i>1 (%)</i> | <i>2 (%)</i> | <i>3 (%)</i> |
| <u><i>Human</i></u>     | <i>NA</i>      | <i>NA</i>    | <i>NA</i>    | <i>NA</i>    |                |              |              |              |
| <i>ION</i>              |                |              |              |              | (0)            | 4/6 (67)     | 2/6 (33)     | (0)          |
| <i>DMNV</i>             |                |              |              |              | (0)            | 3/5 (60)     | (0)          | 2/5 (40)     |
| <i>Hypoglossal</i>      |                |              |              |              | 1/6 (17)       | 4/6 (67)     | 1/6 (17)     | (0)          |
| <i>Cuneate nucleus</i>  |                |              |              |              | (0)            | 2/4 (50)     | 2/4 (50)     | (0)          |
| <i>Purkinje neurons</i> |                |              |              |              | 3/3 (100)      | (0)          | (0)          | (0)          |
| <i>Granule neurons</i>  |                |              |              |              | 2/3 (67)       | 1/3 (33)     | (0)          | (0)          |
| <u><i>Pig</i></u>       |                |              |              |              |                |              |              |              |
| <i>ION</i>              | 1/1 (100)      | (0)          | (0)          | (0)          | 1/2 (50)       | 1/2 (50)     | (0)          | (0)          |
| <i>DMNV</i>             | 1/1 (100)      | (0)          | (0)          | (0)          | 2/2 (100)      | (0)          | (0)          | (0)          |
| <i>Hypoglossal</i>      | <i>NA</i>      | <i>NA</i>    | <i>NA</i>    | <i>NA</i>    | 1/2 (50)       | 1/2 (50)     | (0)          | (0)          |
| <i>Cuneate nucleus</i>  | <i>NA</i>      | <i>NA</i>    | <i>NA</i>    | <i>NA</i>    | (0)            | (0)          | 1/2 (50)     | 1/2 (50)     |
| <i>Purkinje neurons</i> | 3/3 (100)      | (0)          | (0)          | (0)          | 2/2 (100)      | (0)          | (0)          | (0)          |
| <i>Granule neurons</i>  | (0)            | (0)          | (0)          | 3/3 (100)    | (0)            | (0)          | (0)          | 2/2 (100)    |
| <i>Pontine neurons</i>  | (0)            | (0)          | (0)          | 3/3 (100)    | (0)            | (0)          | (0)          | 2/2 (100)    |
| <i>Facial nucleus</i>   | (0)            | (0)          | 1/2 (50)     | 1/2 (50)     |                |              | 1/3 (33)     | 2/3 (67)     |

0 = no staining present; 1 = 1- 20% of neurons stained; 2 = 21-80% of neurons stained; 3 > 80% of neurons stained. Values expressed as proportion of cases expressing NeuN in that scoring category rounded to the nearest integer.

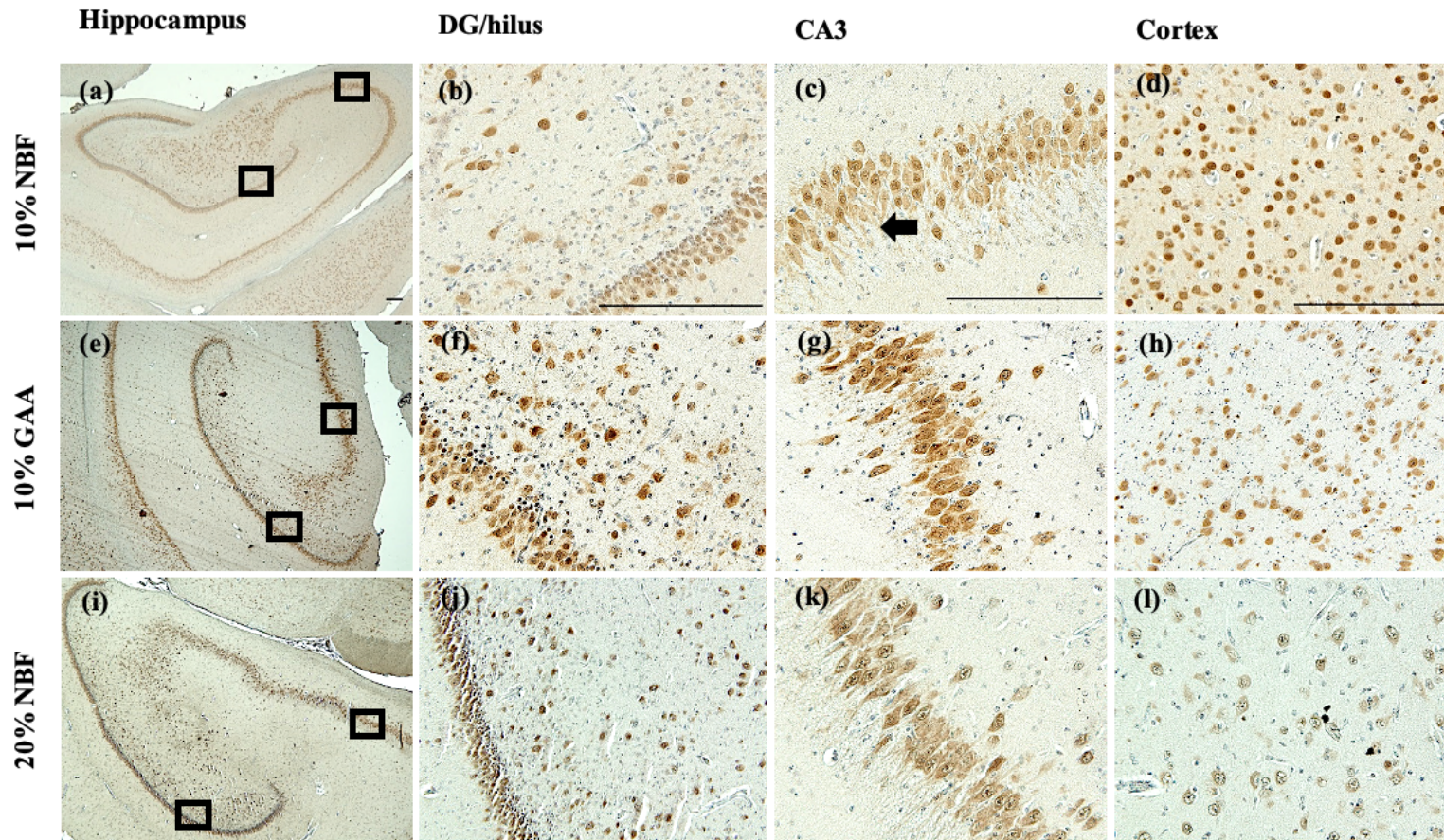
*NBF*= neutral buffered formalin, *ION*= inferior olivary nucleus, *DMNV*= dorsal motor nucleus of the vagus nerve.



**Supplemental Digital Content 4.** Human infant hippocampus showing staining for different markers indicating retained antigenicity for other antibodies. Panels b, e, h, k and n are magnified of the CA4/hilus region, and panels c, f, i, l and o are magnified of the CA3 (all at 40X objective magnification). Note TUNEL is visualised as dark brown in the nucleus of neurons,  $\alpha 7$  and  $\beta 2$  nicotinic acetylcholine receptors (nAChR) as brown in the cytoplasm of neurons, and glial fibrillary acidic protein (GFAP) as purple expressed extracellularly. Scale bars = 200  $\mu$ m.

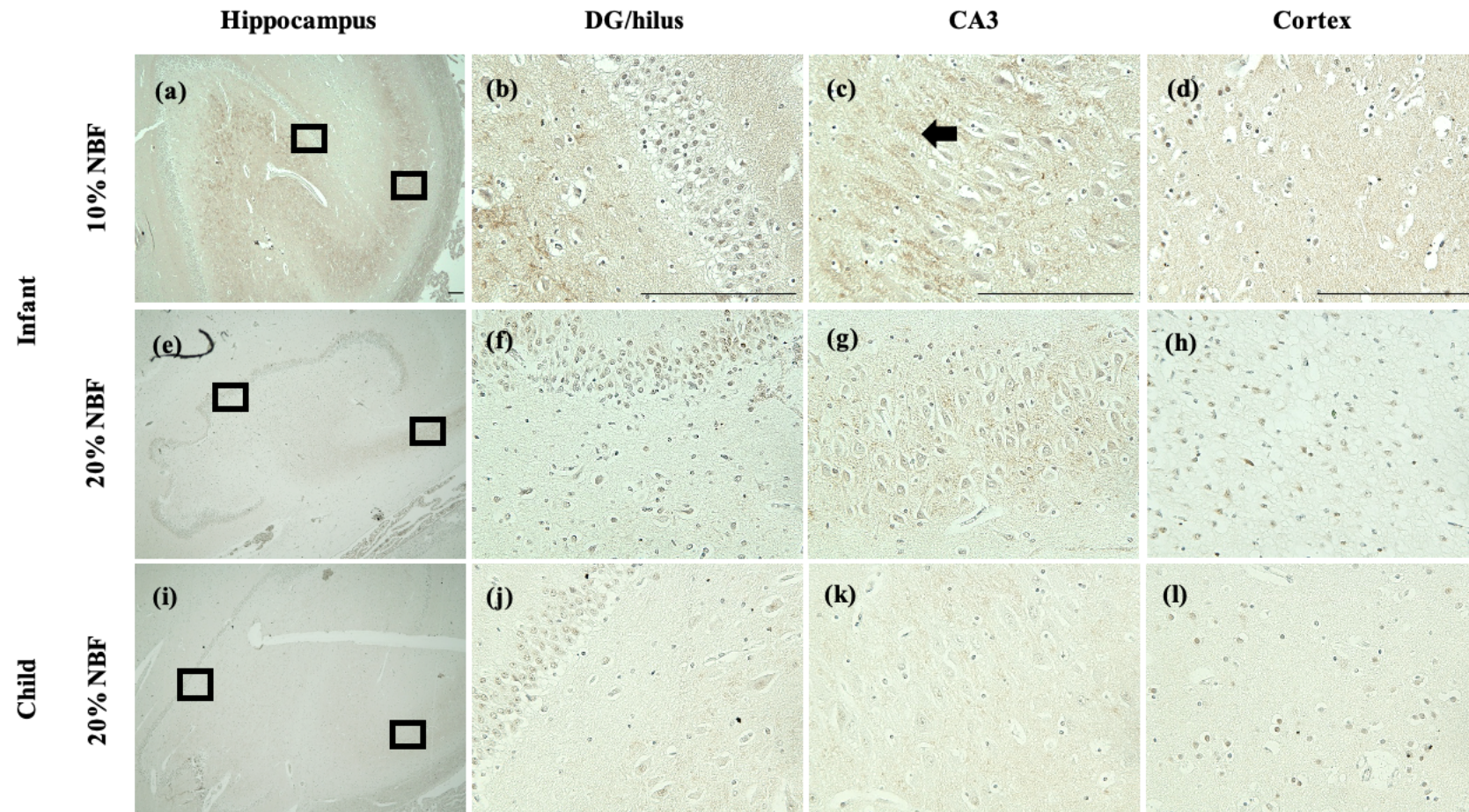


Colour version of Figures 2-4 as presented in publication:



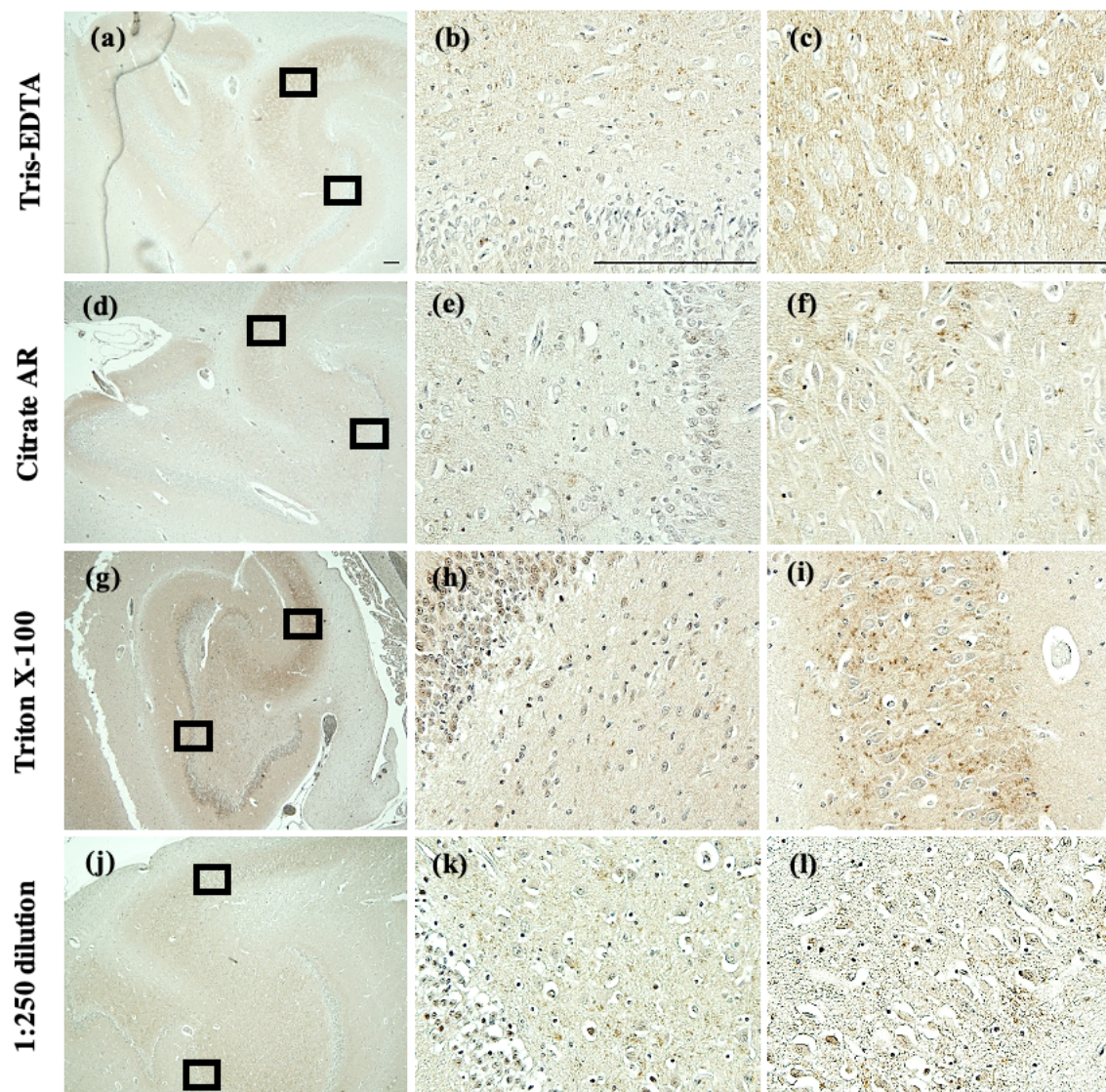
**Figure 2:** NeuN staining in the piglet hippocampus and cortex showing the effects of different fixatives. Panels b,f and j are magnified of the DG and CA4/hilus region, as outlined in panels a,e & i, respectively. Panels c, g and k are magnified of CA3, as outlined in panels a,e & i respectively. Panels d, h and l are magnified cortical images. Note both neuronal and parenchymal staining is evident (c) (black arrow). Scale bars = 200  $\mu$ m.





**Figure 3.** NeuN IHC staining pattern in the human infant (a-h) hippocampus according to fixative of 10% NBF vs 20% NBF, and child with 20% NBF (i-l) with magnification of the boxed areas representing the DG and hilus (b, f, j) and CA3 (c, g, k). Also shown is the cortex (d, h, l). Note non-neuronal staining in the CA3 but positive parenchymal staining in the adjacent stratum lacunosum highlighted in the human infant (c) (black arrow). Scale bars = 200  $\mu$ m.





**Figure 4.** NeuN staining determining the effect of different buffers for Antigen retrieval on infant hippocampus: (a-c) Tris-EDTA buffer (pH 9.5) vs (d-f) citrate buffer (pH 6.0), addition of 1% Triton X-100 in PBS (g-i), and increased NeuN antibody dilution of 1:250 (j-l). Scale bars = 200  $\mu$ m.

## 4.2: NEURON DENSITY IN THE SIDS HIPPOCAMPUS

### 4.2.1: INTRODUCTION

The use of NeuN on FFPE autopsy tissue did not produce immunostaining that was suitable for diagnosing HS ((Luijckx et al., 2021)- Chapter 4.1), however, the assessment of neuron loss can alternatively be undertaken utilising basic histological stains such as Hematoxylin and Eosin (H&E) and Cresyl Violet. Unlike IHC using NeuN, basic histological stains do not distinguish neurons from glia. As such this method is supported by the ILAE for diagnosing HS when employed with *quantitative* cell counting methods (Blümcke et al., 2013), i.e., by an experienced observer who can differentiate cell types based on morphological features (García-Cabezas et al., 2016). Manual counting has been applied successfully in previous studies related to adult HS (Na et al., 2015, Coras et al., 2014, Prada Jardim et al., 2018).

A preliminary study into neuronal density was initially carried out on a subset of the current dataset to assess the reliability of neuronal cell counts (utilising basic stains) across serial tissue sections at one level of the hippocampus in the developing human brain. Initial visual inspection of hippocampal tissue sections did not indicate that significant neuron loss was present. For more rigorous assessment, and as per ILAE recommendation, manual cell counts were subsequently undertaken to confirm the absence of HS. A secondary aim of this study was to determine if in the absence of HS, more subtle/early-stage cell loss is identifiable.

### 4.2.2: METHODS

24 tissue sections from 6 SUDI infants were analysed, ranging between 40-80 PCA. Diagnoses included SIDS (n=3), myocarditis (n=1), undetermined (n=1) and cardiac arrest due to thrombotic occlusion (n=1). PMI ranged 23.5-80 hours. Brains were fixed in 20% neutral buffered formalin for 3-17 days, before being processed to paraffin and sectioned at 7µm. All hippocampal tissue sections included for this study were sectioned coronally at the level of the lateral geniculate nucleus, spanning Figs. 39–45 from The Atlas of the Human Brain (Mai et al., 2015), and were taken from the left brain hemisphere.

Sections were stained with various histological (H&E and CV), and immunohistochemical markers for  $\alpha 2$ ,  $\alpha 7$  and  $\beta 2$  nicotinic acetylcholine receptor subunits counterstained with Hematoxylin using our standard laboratory protocol (Luijckx et al., 2020, Vivekanandarajah, 2017).

Initial visual inspection of sections was carried out to determine whether cell loss was evident using the 4 and 10x objective lens of a Leica Upright DM6000 Microscope (Leica Microsystems Ltd. Heerbrugg, Switzerland). Cell counts were then analysed manually using the Image J cell counter function in 3 regions (CA4, CA1 and subiculum) for each tissue section. 426x321µm representative snapshots were taken at random, non-overlapping locations in each region of interest (4 snapshots in CA4, 5 snapshots for both CA1 and subiculum) using the 40X objective lens. These regions were selected for analysis given that the ILAE specifies that CA4 and CA1 regions must be present within the tissue section when assessing for HS subtypes, (Blümcke et al., 2013). The subiculum was also analysed given its characteristic preservation in HS resulting from medial temporal lobe epilepsy (Hatanpaa et al., 2014). The number of neurons for which the nucleus was visible were counted in each snapshot, and scores were averaged to produce a single score per region for each section.

A sub non-statistical analysis considering factors that could affect neuronal counts of PCA, PMI and fixation time was undertaken to determine any possible trends (Selemon et al., 1998, Kreczmanski et al., 2007, Bandeira et al., 2009).

#### **4.2.3: RESULTS**

Initial visual inspection did not reveal notable neuron loss (Figure 4.1). For manual counting, neuronal density was on average highest in the CA1, followed by subiculum, and then CA4. There was however a large variation in neuronal counts between cases, as well as within the same case between different sections analysed (Table 4.1; Figure 4.2).

##### *Between cases*

The number of neurons per area varied between cases, and neuron morphology of the same region often also differed between cases (Figure 4.3). Despite this, cases with low counts for one brain region often had low scores across the 3 regions studied. Case 6 had the lowest average counts across the CA4, CA1 and subiculum, and conversely, case 1 had the highest average counts across all 3 regions (Table 4.1, Figure 4.2).

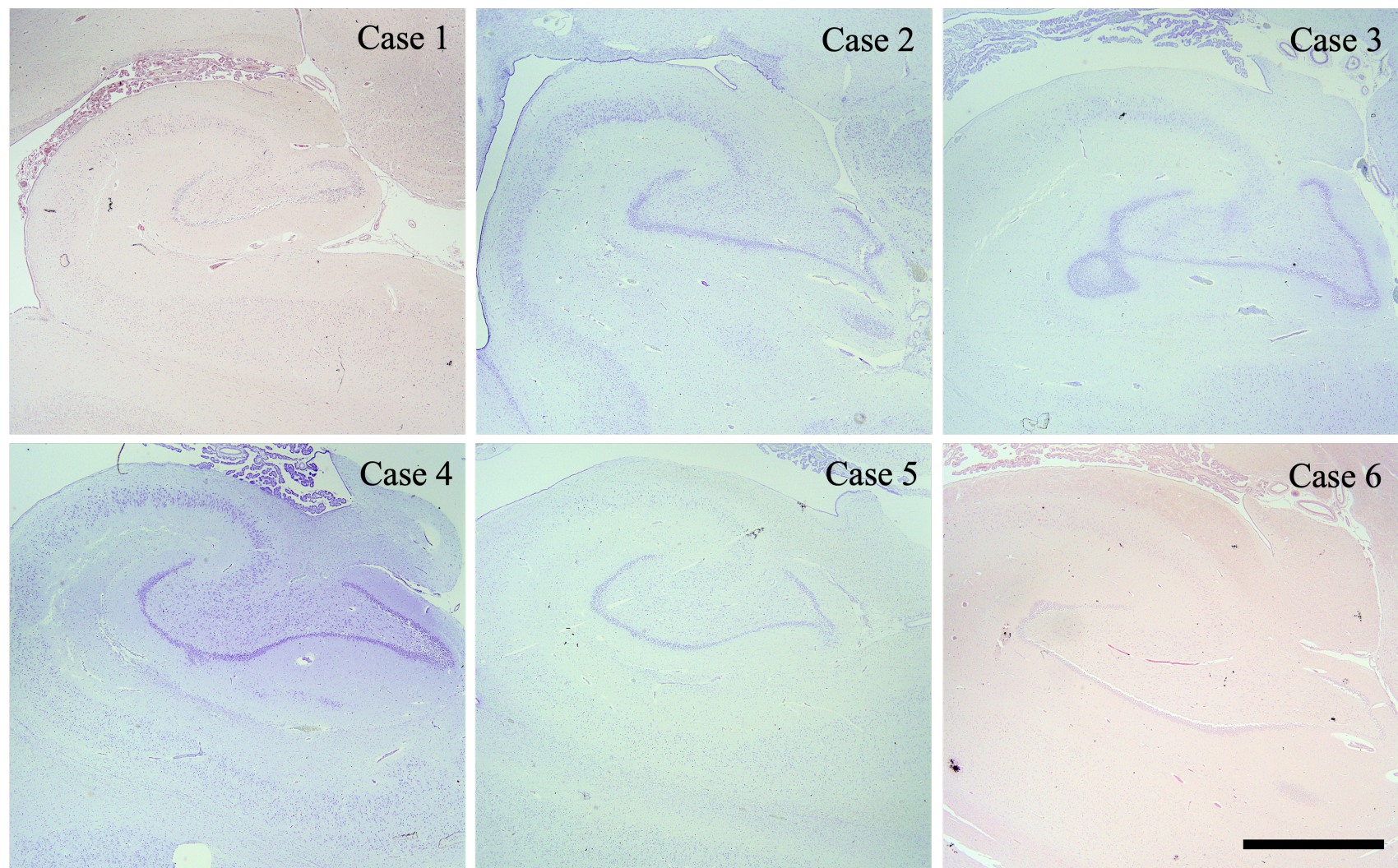
##### *Between sections*

There was a large variance in neuronal density between tissue sections of the same case. Some cases had counts that were more than double the lowest count (Table 4.1, indicated by SD).

### *Effect of PCA, PMI and Fixation time*

Although the number of cases was too low to run statistical analyses, visual analyses of the data in Table 4.1 indicated that there is no effect of fixation time, yet PCA and PMI possibly have a relationship. For example, there is approximately half of the number of neurons in all regions according to PCA of 80 vs 51 (average PCA of other cases), while for PMI, the case with a PMI of 80 had counts three-times higher than the PMI of 16.5 (Figure 4.2). Given that the case with the highest PCA had the lowest PMI, it is difficult to distinguish which of these factors contributed to this difference.





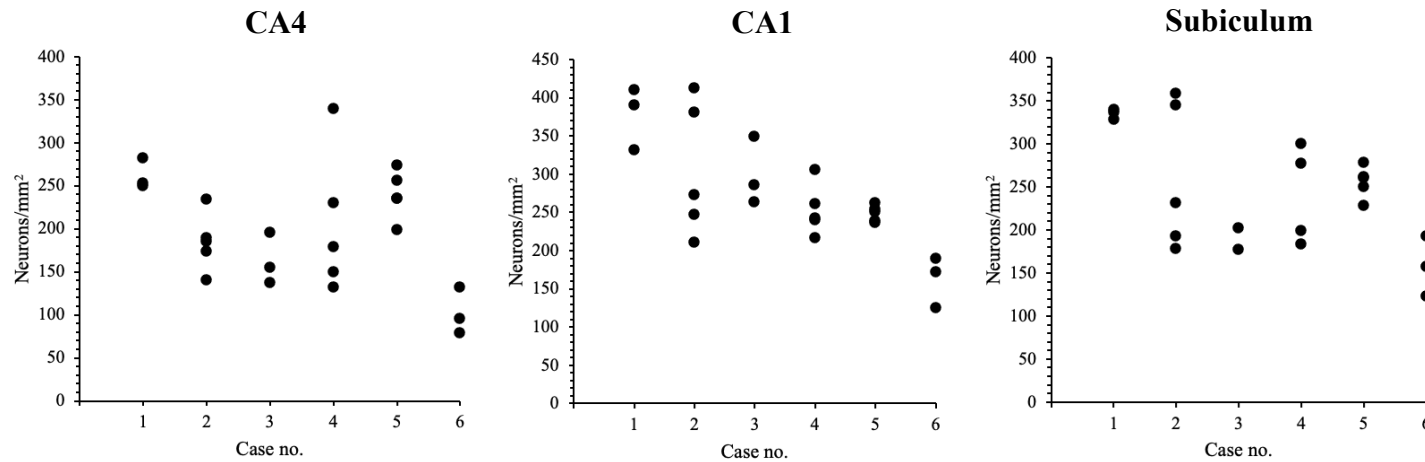
**Figure 4.1: Overview of hippocampal morphology visualised via H&E or Cresyl Violet histochemistry. Scale = 2mm.**

**Table 4.1: Neuronal density in the SUDI hippocampus.**

| Case (no. of sections) | PCA (weeks) | Fixation (days) | PMI (hours) | CA4             | CA1             | Subiculum       |
|------------------------|-------------|-----------------|-------------|-----------------|-----------------|-----------------|
| 1 (3)                  | 40          | 3               | 80          | 262 (18)        | 377 (41)        | 335 (6)         |
| 2 (5)                  | 41          | 17              | 25.5        | 184 (34)        | 305 (87)        | 261 (85)        |
| 3 (3)                  | 53          | 5               | 23.5        | 163 (31)        | 300 (44)        | 190 (18)        |
| 4 (5)                  | 48          | 8               | 24.5        | 206 (83)        | 253(33)         | 280 (57)        |
| 5 (5)                  | 71          | 13              | 44.25       | 240 (25)        | 244 (15)        | 255 (17)        |
| 6 (3)                  | 80          | 7               | 16.5        | 102 (27)        | 162(33)         | 158 (35)        |
| <b>Total average</b>   |             |                 |             | <b>193 (57)</b> | <b>273 (72)</b> | <b>240 (62)</b> |

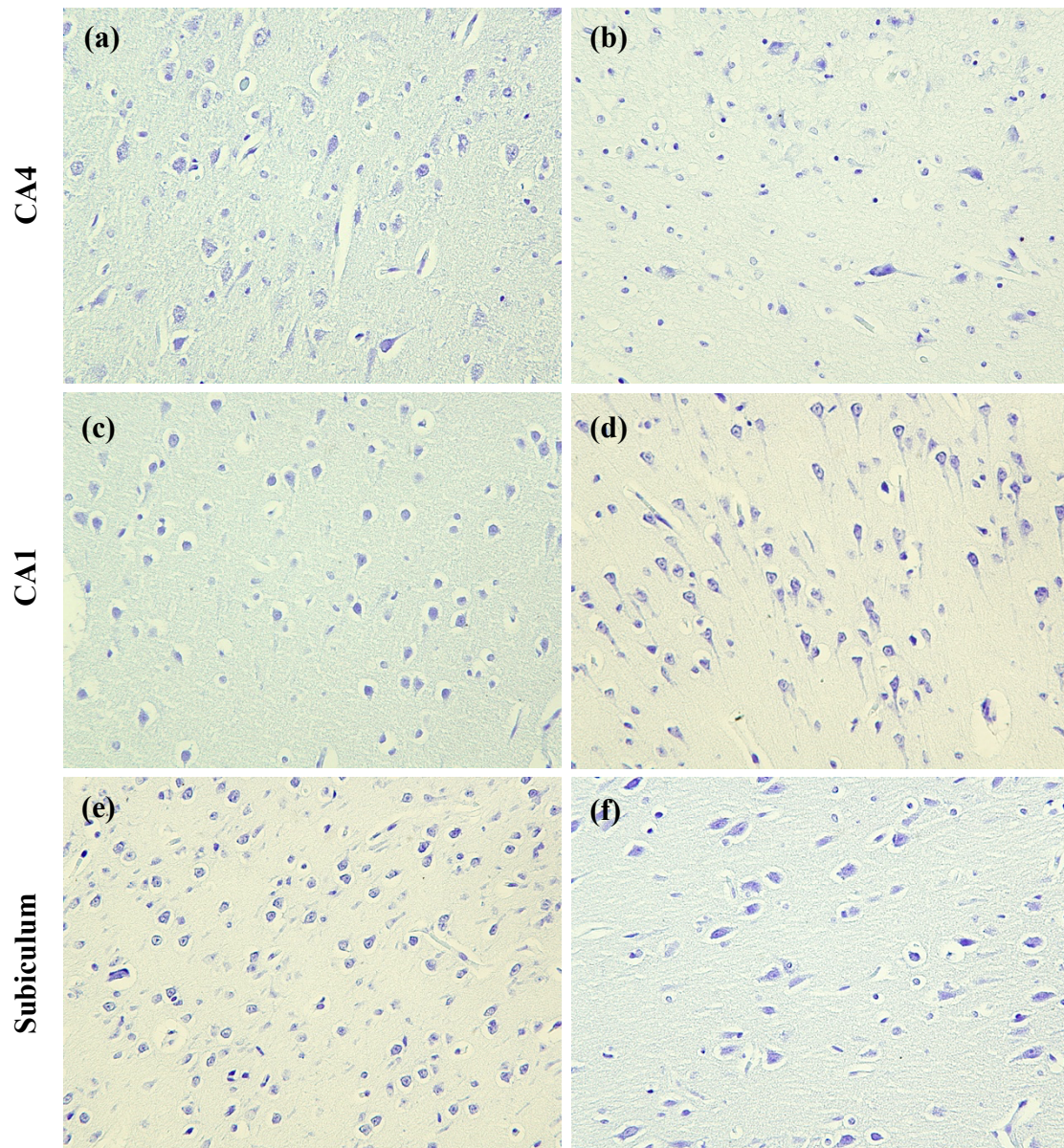
Values represented as mean neuronal density (neurons/mm<sup>2</sup>) across all sections per case (standard deviation).

*Abbreviations: PCA* post conception age, *PMI* post-mortem interval, *CA* Cornu Ammonis



**Figure 4.2: Scatterplots showing the spread of scores across different sections for each case in the CA4, CA1 and subiculum.**





**Figure 4.3: Micrograph illustrating different cell morphologies present amongst different cases in the same hippocampal regions. CA4 (a, b), CA1 (c, d) and subiculum (e, f) stained with Cresyl Violet. Scale bar = 200  $\mu$ m.**

#### 4.2.4: DISCUSSION

This study aimed to determine whether the average neuronal density could be reliably estimated in the post-mortem infant hippocampus, to subsequently investigate whether by comparison, a proportion of SIDS infants met the 2013 ILAE criteria for HS; or otherwise demonstrated more subtle, yet significant neuron loss. Cell density measures were initially carried out on multiple serial hippocampal tissue sections from a subset of the dataset. There was, however, large variation in neuronal density between cases, as well as across serial sections of the same case, with some cases having sections that produced counts two-times higher than another section taken from the same brain tissue block. This large variance was seen in all of the 3 regions studied, and is highly significant because a neuron loss of >50% in the CA4 alone meets the criteria for HS (Type 3 subtype) (Blümcke et al., 2013).

Producing unbiased estimates of neuronal cell numbers based on analysis of only one tissue section can be difficult given that there are marked variations along the longitudinal axis of each hippocampi (Dam, 1980, Dam, 1982, Thom et al., 2012), largely dependent on whether the pathology is focal versus diffuse (Thom et al., 2012), unilateral or bilateral (Thom et al., 2011) as well as due to the age of seizure onset (Thom et al., 2012). Given that HS pathology is unilateral in around half of post-mortem HS cases (Thom et al., 2011), HS may be overlooked by only investigating sections from the left hemisphere of the brain in each infant at only one level. Two-dimensional quantification methods are further biased due to variations arising from sampling biases, and also because they fail to take into account neuronal density with reference to the total volume of the hippocampus (West and Gundersen, 1990). Neuronal densities were not adjusted for in the current study with regards to the total area of the region representative counts were performed in, due to the known effects of shrinkage caused by fixation parameters (composition, time spent in fixative); as well as additional alterations that may occur as a result of variations in dehydration, clearing, paraffin embedding, tissue cutting, and mounting processes (Boonstra et al., 1983, Quester and Schröder, 1997, Dorph-Petersen et al., 2001). Systematic stereological principles and systematic sampling techniques based at multiple levels of the hippocampus are proposed in order to account for these limitations (West and Gundersen, 1990), however, this was not possible for our study.

Case-to-case variation resulting from variations in age, brain weight, head circumference, fixation, and PMI pose further challenges when attempting to detect subtle differences in cell number. Determining ‘normal’ neuronal densities in the infant brain is difficult giving that the

brain is undergoing processes of apoptosis, axonal outgrowth, synaptogenesis, pruning, and myelination that will greatly affect neuronal density counts at different developmental time-points (Mrzljak et al., 1991). The current study found a lower neuron density in our case of 80 weeks PCA (up to 50%), compared with other cases. Variations in neuron size, shape and orientation were also seen between cases in the current study, likely the result of pathological conditions at the time of death (i.e. hypoxia/ischemia) (Park et al., 1996). This poses additional challenges when distinguishing neuron from glia.

The results from this mini analysis suggest that producing reliable cell counts to detect neuron loss in the infant brain is a challenge, given the wide variation in counts between serial sections, and more importantly between different cases (and ages). This poses additional challenges when attempting to assess for subtle patterns of cell loss, which would require a large number of controls matched for PCA. Other markers such as MAP2 and synaptophysin can be useful when investigating for pathological features associated with HS (Thom, 2014, Prada Jardim et al., 2018), although are not formally recommended under the 2013 ILAE classification of HS. Even so, the use of these antibodies will first need to be validated and optimised for use in the developing brain.

## **CHAPTER 5: QUANTIFYING GFAP**

### **IMMUNOHISTOCHEMISTRY IN THE BRAIN-**

### **INTRODUCTION OF THE REACTIVITY SCORE (R-SCORE)**

### **AND HOW IT COMPARES TO OTHER METHODOLOGIES**

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#### Summary:

Although GFAP has become a prototypical marker for astrogliosis, standardised measures are lacking. This chapter introduces a novel semi-quantitative assessment of GFAP, which we term reactivity (R)-score. Reproducibility of the R-score was compared against other commonly used markers and findings demonstrated that this method was reproducible and sensitive to detect clinical astrogliosis. We therefore propose its validity in future research purposes and clinical application.

#### Statement of contribution:

Input to project design was by RM and LL. All tissue sectioning, experimentation, analysis and drafting of the manuscript was performed by LL, and RM and MR provided intellectual input into feedback while drafting. The manuscript has been submitted to 'Journal of Neuroscience Methods' and is currently under review. This chapter is presented as submitted, with tables and figures referred to as in submitted manuscript, although headings have been numbered in accordance with the thesis.

Piglet tissue used in this study was obtained from an experimental model in our laboratory. Experimentation and image analysis was performed at the SIDS and Sleep Apnea Laboratory at the University of Sydney, Sydney, Australia.

## ABSTRACT

**Background:** Astrogliosis occurs in response to homeostatic instability of the central nervous system (CNS), and involves a diverse number of morphological, molecular, and functional changes. Immunohistochemical upregulation of glial fibrillary acidic protein (GFAP) is most commonly used to detect astrogliosis in tissue sections and includes measurement of intensity and/or distribution of staining, cell counting, or graded scoring. There remains a lack of consensus, however, for standard objective measures when diagnosing astrogliosis and its severity, even among neuropathologists.

**New method:** The aim of this study was to test a novel semi-quantitative assessment of GFAP, which we term reactivity (R)-score, on its reproducibility and sensitivity to objectively measure astrogliosis. The R-score, which is based on the proportion of astrocytes seen at each level of reactivity, was compared to 3 other commonly employed quantification methods used in research: (1) thresholding, (2) point-counting, and (3) semi-quantitative assessment via subjective judgement by the observer about severity of pathology to produce a score. Sub-regions of the hippocampus, medulla, and cerebellum were studied in piglet and 4 human cases with clinically reported astrogliosis. Intra-assay coefficient of variation (CV) and percentage agreement cut-offs of  $\leq 20\%$  and  $\geq 75\%$  were used respectively to compare amongst the methods, with outcome measures being reproducibility across serial and non-sections, resilience to changes in experimental conditions, and inter- and intra-rater concordance.

**Results:** Averaged across 3 brain regions, the intra-assay coefficient of variation (CV) was 5% for R-score, with inter and intra-rater kappa scores being 0.99 and 0.95 respectively.

**Comparison with existing methods and conclusions:** Based on CV values, the R-score was superior to thresholding (CV of 51%) and point-counting (CV of 16%), with the semi-quantitative score being found to be on par (percentage agreement 95%). Given the ease, reproducibility and selectivity of the R-score, we propose its validity in future research purposes and clinical application.

**Keywords:** Astrogliosis, gliosis, astrocyte, glial fibrillary acidic protein, quantification

## 5.1: INTRODUCTION

Astrocytes are a subtype of glial cell that play an integral role in the development, structure, and function of the central nervous system (CNS). Astrogliosis is a highly characteristic but non-specific response to homeostatic instability of the CNS (such as in the case of injury) whereby astrocytes become ‘reactive’, undergoing a range of morphological, molecular and functional changes, dependent on the nature and severity of the insult (Sofroniew and Vinters, 2010).

Glial fibrillary acidic protein (GFAP) is the main component of astrocyte intermediate filaments, and has become a prototypical marker of reactive astrocytes (reviewed in (Preston et al., 2019)). Injury and/or insult to the CNS increases the production of GFAP so that previously immunonegative cells may become immunopositive (thus, an increase in the number of GFAP-positive cells), and astrocyte cell bodies and their processes may appear hypertrophied (thus, a change in morphology), both indicative of astrogliosis (Sun and Jakobs, 2012, Zhu et al., 2004). Acute and diffuse trauma is sufficient to evoke a transient upregulation of GFAP, even in the absence of tissue damage (Wilhelmsson et al., 2006), while longer and more severe injuries/insults lead to more significant GFAP upregulation, with more marked hypertrophy of processes and distal processes potentially extending into adjacent astrocyte domains (Myer et al., 2006). The degree of GFAP upregulation depends on the type of insult (Zamanian et al., 2012, Zhang et al., 2016), the brain region affected (Sun and Jakobs, 2012, Emsley and Macklis, 2006, Messing and Brenner, 2020), and the time between injury and assessment (Hausmann et al., 2000).

Different methods of quantification have been used to assess changes in GFAP expression in formalin-fixed, paraffin-embedded tissue using immunohistochemistry (Summarised in Supplementary Table 1), with outcome findings differing pending the method employed (Weber et al., 2013, McNeal et al., 2016). Animal studies often rely on thresholding or point-counting, or other more complex methods of assessment (Summarised in Supplementary Table 1), afforded by tissue availability, time and funding. In a clinical setting, time, money and tissue availability are restricted, and as such, the commonly employed method is the qualitative assessment of ‘mild’, ‘moderate’ or ‘severe’, which take into account both cell morphology and number, but with no set criteria for either, thus being subjective at best, and resulting in major disagreement between pathologist (Escartin et al., 2019, Leitner et al., 2022). Semiquantitative, graded scoring systems have been proposed to correspond to the qualitative



descriptions (Gouw et al., 2008, Sofroniew and Vinters, 2010), for application in a research setting to allow for comparison across groups, yet these are also subjective with no set criteria (Summarised in Supplementary Table 1).

In an attempt to address this issue, we introduce a novel method which we term the Reactivity score (R-score), which standardizes the semiquantitative, graded scoring method. This R-score, a derivative of the Histochemical score (H-score; Supplementary Section 2), quantitatively combines assessment of cell number and morphology, providing a weighted score indicative of astrogliosis severity. Utilising piglet brain tissue, we apply the R-score method and compare it to three other methods commonly used to assess GFAP expression by immunohistochemistry, including the graded scoring assessment purely based on cell morphology, and quantitative assessment based on the area of GFAP immunoreactivity or the number of GFAP positive cells, both of which do not take cell morphology into account. We therefore test the hypothesis that the R-score will be reproducible, and subsequently apply it to four clinical cases to validate its clinical applicability in diagnosing astrogliosis.

## **5.2: MATERIALS AND METHODS**

### **5.2.1: TISSUE COLLECTION**

Piglet brains were those collected previously in our laboratory (Machaalani and Waters, 2003b, Machaalani and Waters, 2003a, Peiris et al., 2004) following approval by the Animal Ethics Committee of the University of Sydney (K14/1-2000/3/3075 and K14/2-2003/3/3708). On extraction, the brain was fixed as a whole, in 10% neutral-buffered formalin for 2 weeks, sectioned into 4mm blocks, and returned to the fixative for a further 1 week before paraffin-embedding. The post-mortem interval never exceeded 30 minutes. 3 piglets aged 13-14 days were studied; 2 piglets were not exposed to any insult while the 3<sup>rd</sup> had been exposed to intermittent hypercapnic hypoxia (model and exposure detailed in (Waters and Tinworth, 2003)), known to stimulate astrogliosis (Li et al., 2010, Turlejski et al., 2016). The formalin fixed paraffin embedded (FFPE) blocks of the hippocampus, cerebellum and the medulla were serially sectioned at 7µm thickness using a rotary microtome, mounted onto silanized glass slides, dried overnight, and stored at room temperature in a dust-free environment for at least 1-week prior to immunostaining.

FFPE tissue sections of hippocampus and cerebellum from 4 paediatric cases of sudden unexpected deaths were also studied. These cases were derived from a cohort in our laboratory

with approval to utilise brain tissue for analyses by the Ethics Committee of the University of Sydney (X13-0038 & 2019/ETH06915) and the NSW state Coroner. Mild to moderate (2 cases) and moderate to severe (1 case) astrogliosis had been identified in the hippocampus and the cerebellum (1 case) at autopsy

### **5.2.2: GFAP IMMUNOHISTOCHEMISTRY**

Sections were immunostained for GFAP manually using a standard laboratory protocol (Luijckx et al., 2020) unless otherwise stated. Briefly, sections were deparaffinised in xylene and rehydrated through a graded series of ethanol washes. Microwave antigen retrieval was performed with 10% Tris-EDTA buffer (1mM EDTA, 1mM Na Citrate, 2mM Tris; pH 9.0) for 14 minutes. Sections were quenched for 20 minutes in a 3% H<sub>2</sub>O<sub>2</sub>, methanol and phosphate-buffered saline (PBS) solution and subsequently blocked with 10% normal horse serum (NHS) for 30 minutes. Overnight incubation was carried out using GFAP primary antibody (Z033401-2, Agilent, details and specificity described in our previous manuscript (Luijckx et al., 2021)) diluted to the relevant concentration using 1% normal horse serum (NHS). The following day, sections were incubated at room temperature for 1 hour in an affinity-purified anti-mouse/anti-rabbit secondary antibody (1:400; BA-400, Vector Laboratories Inc), followed by 1 hour incubation with avidin-biotin horseradish peroxidase complex (VEPH-4000, Vector Stain ABC kit; Vector Laboratories Inc). Colour labelling was achieved with 3,3'-diaminobenzidine (DAB) (K346811, DAKO). Slides were counterstained with haematoxylin before dehydration through a graded ethanol series and clearing in xylene prior to cover slipping.

Given clinically GFAP IHC is automated, tissue from the paediatric case identified as having moderate to severe astrogliosis was subjected to automated IHC using the Dako Autostainer plus (S3800, DAKO). The settings for this included incubation with GFAP primary antibody (Z033401-2, 1:3000 dilution) for 30 minutes.

### **5.2.3: MICROSCOPY AND REGIONS OF ANALYSIS**

Imaging, capturing, and quantification were performed by a single observer (LL). Stained sections were viewed using light microscopy (Olympus Upright BX51 Microscope, Olympus Optical Co., Ltd Japan) and imaged using image capture software (DP Controller, Olympus Optical Co, Ltd, Japan).

Four microscopically defined regions of interest (ROI) were analysed: the hypoglossal nucleus (XII) in the rostral medulla; the internal granular layer of the cerebellar cortex (IGL); and the CA1 and CA4 regions in the hippocampus (Figure 1). For each ROI, multiple, non-overlapping images were captured using a 40x objective lens, avoiding large blood vessels and artefacts. Where the ROI was smaller than the captured image, a grid overlay was applied using the inbuilt 'grid' plugin, and analysis was carried out within multiple 0.01mm<sup>2</sup> subregions. This was mostly applied to the CA1 of the hippocampus. The Red Green Blue images were then exported to Fiji imaging software (Schindelin et al., 2012) for GFAP quantification.

#### **5.2.4: GFAP QUANTIFICATION**

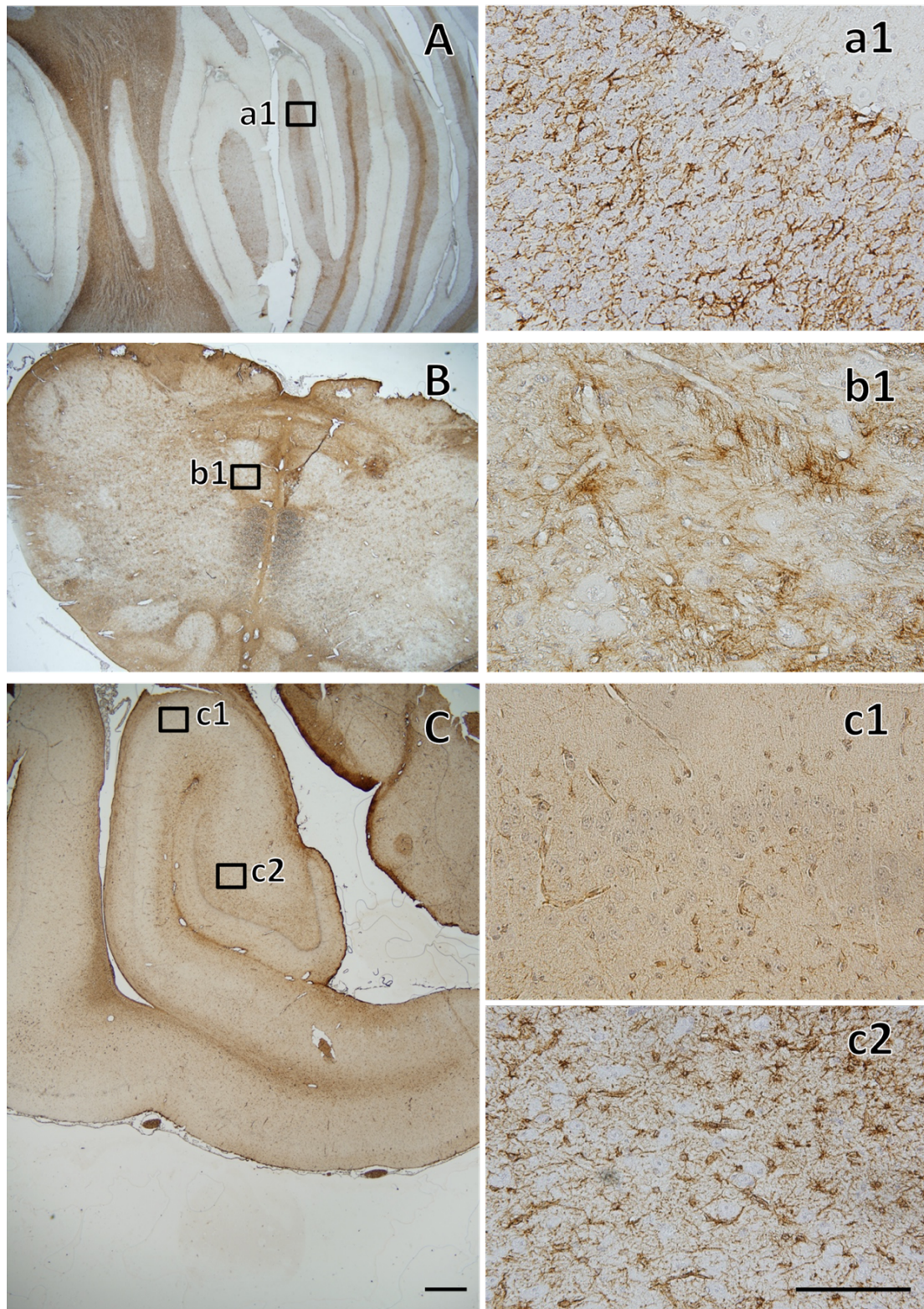
Four different methods were applied and included: thresholding to determine the area of GFAP staining, point-counting to determine the number of GFAP positive cells per mm<sup>2</sup>, semi-quantitative graded scoring based on cell morphology, and the R-score which is a combination of the graded score and point counting, utilising an equation. These particular methods were chosen given their common use in research settings, and also due to the fact that they are often used in combination with each other within the same study due to their ability to provide information about different types of changes astrocytes undergo in response to injury) (Summarised in Supplementary Table 1).

1- Mean area of GFAP expression (thresholding): For each image, colour deconvolution was performed on RGB images using the inbuilt 'H DAB' plug-in to isolate blue, brown and green colour layers. The brown layer (R:0.268, G:0.570, B:0.777) representing positive DAB-GFAP staining, was selected for analysis via manual thresholding using the histogram, which displays the distribution of pixel intensity values across the image (example provided in Supplementary Figure 3). A threshold value that effectively separates GFAP-immunostaining from background was selected. This method accounts for staining of astrocyte cell bodies, as well as staining from distal processes of other astrocytes. The area of the image above threshold was calculated as a percentage using the inbuilt 'measure' function. An average percentage was taken for ROI's with more than one image.

2- GFAP-positive astrocytes per mm<sup>2</sup>(point counting): The number of GFAP-positive astrocytes (for which the nucleus was visible) was counted manually in each image using the in-built 'multi point' function and the value was converted to counts per mm<sup>2</sup>.

Each image was captured at 400X magnification, measuring 0.135mm<sup>2</sup>. Total counts for a given region were divided by the total area to produce the average count per mm<sup>2</sup>.

- 3- A graded score based on the morphology of GFAP-positive cells adapted from (Gouw et al., 2008, Sofroniew and Vinters, 2010) and examples provided in Figure 2. For each image, the astrogliosis was assessed using a 4 tier scoring system: '0' = no astrogliosis (Visible astrocyte cell bodies and processes, with weak staining of processes); '1' = mild astrogliosis (Slight cellular hypertrophy, with enlarged cell body and increased staining of glial processes), '2' = moderate astrogliosis (significant cellular hypertrophy involving individual glial processes and nucleus, and/or individual processes no longer visible due to increased staining, although potentially few long, thick processes), '3' = severe gliosis (gemistocytic appearance of cell bodies and dense staining of glial processes). An average score was taken for ROI whereby a score of  $\geq 1$  was applied when at least 2 GFAP positive cells were of this feature.
- 4- Reactivity score (R- score); an adaptation of the H-score equation (Supplementary Section 2). Of the various formats and possibilities of applying the R-score equation (Discussed in Supplementary Section 2) the one we have chosen and adapted herein is  $R\text{-score} = \sum P(r + 1)$ , where P represents the percentage of cells, and "r" represents the reactivity/astrogliosis score as defined in method #3 above. Score range is from 0-400, with a score >100 indicating the presence of reactive astrocytes. Of note, a score of 0 is obtained when no cells within the ROI are stained positive for GFAP, and 100 when 100% of cells are a score of 0 (i.e. non-reactive). Thus, this method relies on all cells present within the image capture of the ROI to be provided with a score. Applying this equation allows for weighting the amount of astrogliosis given that a mixture of the scores could potentially be present (e.g., Figure 2).



**Figure 1: GFAP staining in the piglet** (A) cerebellum, (B) medulla and (C) hippocampus, with regions of interest quantified magnified on the right, including (a1) the granular layer, (b1) hypoglossal nucleus, and (c1) CA1 and (c2) CA4. Scale bar = 500 $\mu$ m (100 $\mu$ m for magnifications).



### 5.2.5: OUTCOME MEASURES AND STATISTICAL ANALYSIS

The outcome measures, assessed on both piglet and human brain tissue, were the reproducibility of the 4 quantification methods across:

1. Serial sections: to determine whether the quantification method yielded reproducible outcomes when minimising variation in the experimental method. Up to 4 serial tissue sections (per case) were stained for each ROI and quantified. Serial sections were stained with the same antibody concentration (1:3000) and within the same experimental run.
  - For continuous outcome values (mean area of positive GFAP expression, GFAP-positive astrocytes per mm<sup>2</sup> and R-score), the intra-assay coefficient of variation (CV) was calculated. The intra-assay CV refers to the variation obtained from replicates in the same experiment, and is calculated by (SD duplicates)/(mean duplicates) x 100% (van der Hoeven et al., 2022). For ligand-binding assays (van der Hoeven et al., 2022) and immunohistochemistry (Smith and Womack, 2014), CV ≤20% is accepted.
  - For semi-quantitative outputs with ordinal values, percentage agreements were calculated (Araujo and Born, 1985). Agreement ≥75% was considered acceptable (Graham et al., 2012).
2. Non-serial sections: to determine whether the quantification method yielded similar values for each ROI. This was determined by using the 2-way-mixed, consistency analysis intraclass correlation coefficients (ICC) for comparison.
3. Different antibody concentrations: the magnitude of change in scoring outcomes was assessed across different antibody concentrations (1:5000, 1:3000 and 1:1000) using correlation analysis: Pearson correlations for continuous data and Kendall Tau correlations ordinal data. This was only done in hippocampal tissue, thus CA1 and CA4.

For validation, additional outcome measures were undertaken on our human brain tissue samples, across:

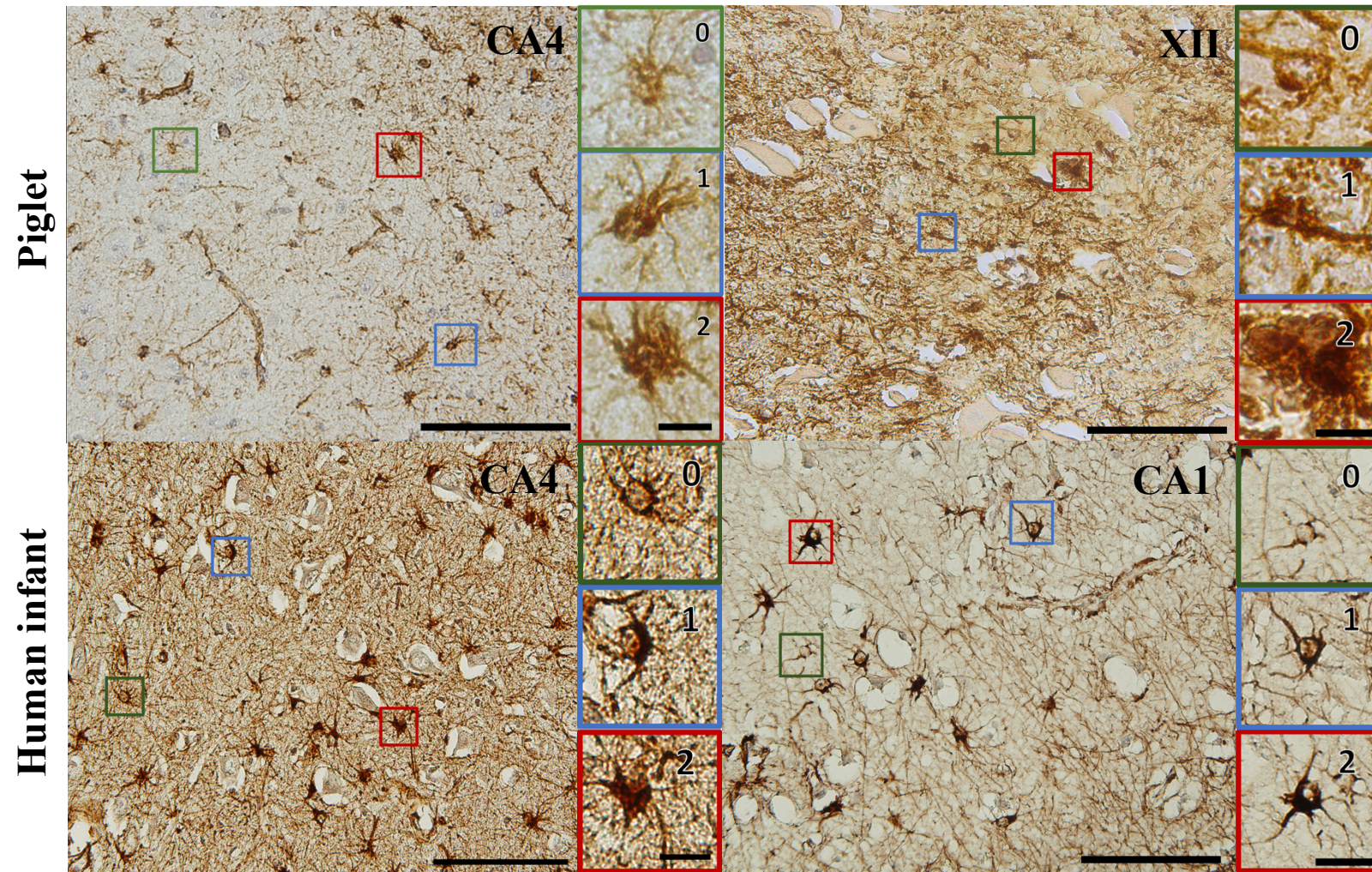
4. Different time-points (test-retest reliability): quantification was undertaken 2 weeks apart using ICC for comparison.

5. Different raters (inter-rater reliability): quantification was undertaken by 2 different raters (LL and RM) using ICC for comparison for continuous data, and the Kappa statistic for ordinal data.

Automated IHC is another common method applied by research and clinical groups, and thus was also trialled in human tissue for comparison (Supplementary Table 3).

All data obtained was entered into Microsoft Excel and exported to SPSS for Windows (version 25; SPSS (IBM) Inc., Illinois, USA) for statistical analysis. For all statistical analysis, Significance was taken at  $p \leq 0.05$ .





**Figure 2:** GFAP immunostaining in CA4 and XII of the piglet hippocampus and CA4 and CA1 of the human infant, denoting examples of astrocytes given a score of 0 (green), 1 (blue) and 2 (red) within the same tissue section. Regions selected on the basis of having all 3 astrocyte sub-types present. Scale bar = 100 $\mu$ m for main panel, and 10 $\mu$ m for the enlargements of 0-2.

## **5.3: RESULTS**

### **5.3.1: COMPARISON OF THE 4 METHODS IN PIGLET BRAIN**

#### **5.3.1.1: THRESHOLDING**

Threshold values ranged from 0.03 to 3.5% (Table 1, and Supplementary excel showing individual case values). There was marked variation in values across consecutive sections (CV=51%, Table 1), non-consecutive sections (ICC=0.16,  $p=0.43$ , Table 1), changes in primary antibody concentration (significant correlation  $r(36)=-0.38$ ,  $p<0.01$ ) (Figure 3a), and between manual IHC vs automated methods (CV=33%, Supplementary Table 3).

#### **5.3.1.2: GFAP-POSITIVE ASTROCYTES PER MM<sup>2</sup>**

Values ranged from 100 to 550 positive cells/mm<sup>2</sup> (Table 1). Values across consecutive sections were comparable (CV=16%, Table 1), but not across non-consecutive sections (ICC=0.69,  $p=0.11$ , Table 1) or due to primary antibody concentration ( $r(36)=-0.30$ ,  $p=0.02$ , Figure 3b). Values across manual IHC vs automated methods were comparable (CV=15%, Supplementary Table 3).

#### **5.3.1.3: GRADED- SCORE (0-3)**

Overall staining patterns for each image ranged between 0-1, with only a few cells scoring 2 and none receiving a score of 3. In several cases, all 3 patterns of 0, 1 and 2 were seen within the 1 tissue image capture (e.g., in Figure 2). Values across consecutive sections were comparable with percentage agreements for qualitative scores being 95% (Table 1), yet they were not for non-consecutive sections (ICC=0.40,  $p=0.29$ , Table 1). There was no statistically significant correlation between primary antibody dilution factor and the qualitative score (data not shown). Values across manual IHC vs automated methods were comparable with percentage agreements for qualitative scores being 100% (Supplementary Table 3).

#### **5.3.1.4: R-SCORE**

R-score values ranged between 0 and 167. There was very high homology in R-score values across consecutive sections (CV = 5%, Table 1), non-consecutive sections (ICC=0.85,  $p=0.03$ , Table 1), according to primary antibody dilution factor ( $r(36)=-0.02$ ,  $p=0.90$ , Figure 3c), and across manual IHC vs automated methods (CV=7%, Supplementary Table 3).

**Table 1:** Mean scores across serial sections for the 4 quantification methods of thresholding, counts per mm<sup>2</sup>, R-score and semi-quantitative score.

| Quantification method                           |                 |                        |              |             |
|-------------------------------------------------|-----------------|------------------------|--------------|-------------|
| Subject                                         | Thresholding    | Counts/mm <sup>2</sup> | R-score      | Score       |
| <b>CA4 hippocampus</b>                          |                 |                        |              |             |
| <b>1 (n=4)</b>                                  | 0.86±0.78 (90%) | 391±35 (9%)            | 114±9 (8%)   | 1 (75%)     |
| <b>2a (n=2)</b>                                 | 2.48±1.37 (55%) | 437±10 (2%)            | 123±5 (4%)   | 1 (100%)    |
| <b>2b (n=3)</b>                                 | 1.33±1.05 (79%) | 453±127 (28%)          | 120±20 (16%) | 1 (67%)     |
| <b>3a (n=2)</b>                                 | 0.70±0.52 (74%) | 423±100 (24%)          | 135±17 (12%) | 1 (100%)    |
| <b>3b (n=3)</b>                                 | 0.59±0.35 (58%) | 469±20 (4%)            | 138±7 (5%)   | 0 (100%)    |
| <b>CA1 hippocampus</b>                          |                 |                        |              |             |
| <b>1 (n=4)</b>                                  | 0.10±0.05 (54%) | 155±44 (28)            | 100±0 (0%)   | 0 (100%)    |
| <b>2a (n=2)</b>                                 | 1.50±0.57 (38%) | 378±43 (11%)           | 101±1 (1%)   | 0 (100%)    |
| <b>2b (n=3)</b>                                 | 0.76±0.34 (45%) | 243±72 (29%)           | 100±0 (0%)   | 0 (100%)    |
| <b>3a (n=2)</b>                                 | 0.05±0.05 (99%) | 148±67 (46%)           | 100±0 (0%)   | 0 (100%)    |
| <b>3b (n=3)</b>                                 | 0.44±0.21 (48%) | 165±42 (25%)           | 100±0 (0%)   | 0 (100%)    |
| <b>XII nucleus medulla</b>                      |                 |                        |              |             |
| <b>1a (n=3)</b>                                 | 1.30±0.32 (25%) | 254±18 (7%)            | 120±5 (4%)   | 0 (100%)    |
| <b>1b (n=2)</b>                                 | 7.21±6.41 (89%) | 457±60 (13%)           | 148±27 (18%) | 1 (100%)    |
| <b>2a (n=3)</b>                                 | 1.73±0.10 (6%)  | 342±21 (6%)            | 111±6 (5%)   | 0 (100%)    |
| <b>2b (n=2)</b>                                 | 1.66±0.63 (38%) | 230±10 (5%)            | 103±4 (3%)   | 0 (100%)    |
| <b>3 (n=4)</b>                                  | 1.44±0.65 (45%) | 243±33 (14%)           | 107±5 (4%)   | 0 (100%)    |
| <b>IGL cerebellum</b>                           |                 |                        |              |             |
| <b>1 (n=3)</b>                                  | 1.33±0.54 (41%) | 295±24 (8%)            | 102±2 (2%)   | 0 (100%)    |
| <b>2 (n=3)</b>                                  | 1.09±23 (21%)   | 344±69 (20%)           | 101±1 (0%)   | 0 (100%)    |
| <b>3 (n=3)</b>                                  | 1.48±0.22 (15%) | 381±22 (6%)            | 123±0 (0%)   | 1 (67%)     |
| <b>Intra-assay CV% (n=18<sup>^</sup>):</b>      | <b>51</b>       | <b>16</b>              | <b>5</b>     | <b>-</b>    |
| <b>Percentage agreement (n=18<sup>^</sup>):</b> | <b>-</b>        | <b>-</b>               | <b>-</b>     | <b>95</b>   |
| <b>ICC (non-consecutive blocks, n=6)</b>        | <b>0.16</b>     | <b>0.69</b>            | <b>0.85*</b> | <b>0.40</b> |

‘a’ & ‘b’ denote different blocks of the same brain region.

Case #1 & 2 are naïve piglets, case #3 is the hypoxia piglet.

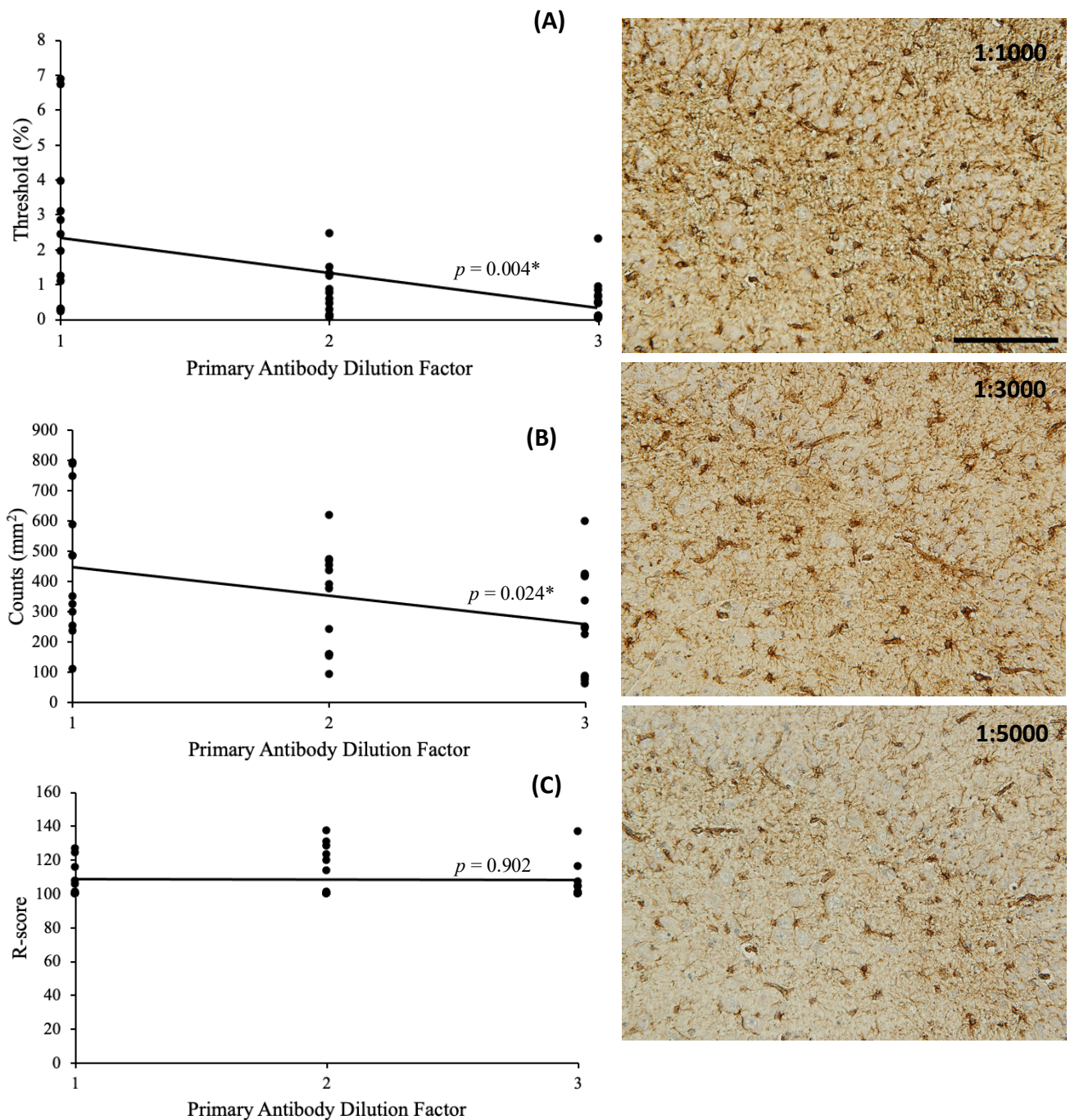
n= number of tissue sections, <sup>^</sup> n values represent the total of each case at each brain level.

Abbreviations: CA Cornu Ammonis, XII hypoglossal, ICC intraclass correlation coefficient, IGL internal granular layer of the cerebellar cortex, CV coefficient of variation.

Values expressed as mean±SD (individual CV%) for continuous data and mean (% agreement) for ordinal data.

\*Indicates statistical significance, p≤0.05.





**Figure 3:** Relationship between primary antibody dilution factor and: **(A)** mean area of positive GFAP expression (%), **(B)** number of GFAP-positive astrocytes per mm<sup>2</sup> and **(C)** R score, in the hippocampus (combining CA1 & CA4 (n=12 for each concentration.)). Primary antibody dilution factor of 1=1:1000, 2=1:3000 and 3=1:5000. Micrographs are examples taken from approximately the same region of the CA4 in the same case. Scale bar= 100µm. Significance  $p < 0.05^*$ .

### 5.3.2: CORRELATION AMONGST THE METHODS

Correlation analyses across all tissue sections quantified (n=54) showed that all methods were highly correlated with each other except for threshold value and score (p=0.10) (Table 2).

**Table 2:** Correlation between the methods.

|                                  | <b>Thresholding<br/>(n=54)</b> | <b>Counts per<br/>mm<sup>2</sup> (n=54)</b> | <b>R score (n=54)</b>    | <b>Score (n=54)</b>      |
|----------------------------------|--------------------------------|---------------------------------------------|--------------------------|--------------------------|
| <b>Thresholding</b>              | -                              | <b>0.35 (p&lt;0.01)</b>                     | <b>0.55 (p&lt;0.001)</b> | 0.19 (p=0.10)            |
| <b>Counts per mm<sup>2</sup></b> |                                | -                                           | <b>0.72 (p&lt;0.001)</b> | <b>0.52 (p&lt;0.001)</b> |
| <b>R score</b>                   |                                |                                             | -                        | <b>0.54 (p&lt;0.001)</b> |

Pearson correlation was used for continuous variables and Kendall's tau for ordinal values.

Values expressed as Pearson's r/Kendall's Tau B (p value).

Significance taken at  $p \leq 0.05$ .

### 5.3.3: COMPARISON AMONGST BRAIN REGIONS

Despite the above correlations amongst the methods, when analysing the individual regions to determine the patterns of expression, we found thresholding to differ greatly compared to the other 3 methods (Table 1). Considering the 2 control piglet cases, the pattern of expression for the thresholding method showed highest in the XII nucleus and lowest in the CA1, while counts and R-score showed CA4 to have highest and CA1 lowest, with score not differentiating between them (Table 3).

**Table 3:** Pattern of GFAP levels across the brain regions according to quantification method

|                                  |                  |
|----------------------------------|------------------|
| <b>Threshold</b>                 | XII>IGL>CA4>CA1  |
| <b>Counts per mm<sup>2</sup></b> | CA4>XII>IGL>CA1  |
| <b>R score</b>                   | CA4>XII>IGL= CA1 |
| <b>Score</b>                     | CA4>IGL=XII=CA1  |

### 5.3.4: VALIDATION VIA APPLICATION TO HUMAN CASES

For human cases, counts per mm<sup>2</sup> ranged between 22 in the CA1 and 498 in the cerebellum, R-score values ranged between 100 (seen in all regions analysed) and 150 in the CA4, while scores ranged between 0 and 2. Given the point-counting, R-score and semi-quantitative score were deemed to have given good CV and agreement values, these were subsequently applied to 4 human cases for validation. Across consecutive sections, values were comparable for the R-score (CV =5%) and score (percentage agreement of 100%), but not for the point counting

method, (CV = 24%), (Table 4), while across non-consecutive tissue sections, values were comparable for all 3 methods (ICC>0.97 and  $p<0.001$  for all, Table 4).

When assessing test-retest reliability, values were comparable for all 3 methods (ICC>0.99 and  $p<0.001$  for all) (Table 4). Inter-rater concordance was also high (ICC >0.80 and  $p<0.01$ ; kappa value 0.58,  $p>0.01$ ) (Table 4).

There was good correlation between all methods, including: point-counting and R-score ( $r(58)=0.53$ ,  $p<0.001$ ), point counting and score ( $r(58)=0.55$ ,  $p<0.001$ ) and R-score and count ( $r(58)=0.55$ ,  $p<0.001$ ).

The R-score values were also consistent with clinical data for the cases with reported mild to moderate astrogliosis whereby the case with mild had values averaging 130 while the moderate case averaged 170 (Table 4 and Figure 4).

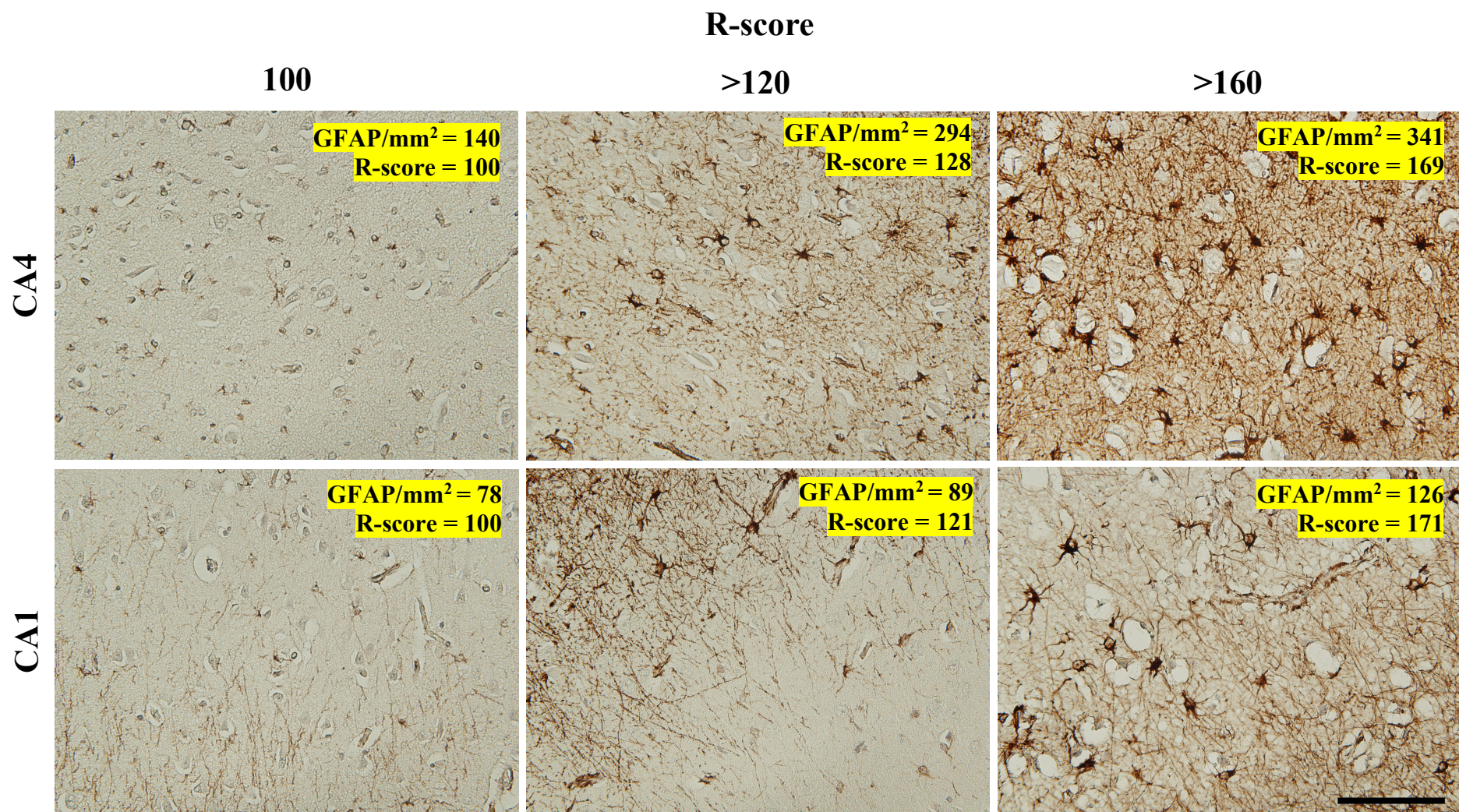
**Table 4:** GFAP-positive astrocytes per mm<sup>2</sup>, R-score and semi-quantitative scores applied to human cases.

|                                  |                            | Quantification method |          |
|----------------------------------|----------------------------|-----------------------|----------|
| Subject                          | Counts per mm <sup>2</sup> | R-score               | Score    |
| CA4 hippocampus                  |                            |                       |          |
| 1a (n=4)                         | 167±27 (16%)               | 103±5 (5%)            | 0 (100%) |
| 1b (n=3)                         | 107±30 (28%)               | 103±3 (3%)            | 0 (100%) |
| 2a (n=4)                         | 77±31 (40%)                | 104±5 (5%)            | 0 (100%) |
| 2b (n=3)                         | 70±28 (39%)                | 102±3 (2%)            | 0 (100%) |
| 3a (n=4)                         | 273±37 (14%)               | 137±10 (7%)           | 1 (100%) |
| 3b (n=4)                         | 211±14 (7%)                | 130±5 (4%)            | 1 (100%) |
| 4a (n=4)                         | 312±49 (16%)               | 169±6 (4%)            | 2 (100%) |
| 4b (n=2)                         | 318±31 (10%)               | 172±4 (3%)            | 2 (100%) |
| CA1 hippocampus                  |                            |                       |          |
| 1a (n=4)                         | 77±27 (35%)                | 102±3 (3%)            | 0 (100%) |
| 1b (n=3)                         | 40±11 (29%)                | 100±0 (0%)            | 0 (100%) |
| 2a (n=4)                         | 79±36 (45%)                | 103±3 (3%)            | 0 (100%) |
| 2b (n=3)                         | 101±9 (8%)                 | 101±2 (2%)            | 0 (100%) |
| 3a (n=4)                         | 41±18 (44%)                | 104±8 (8%)            | 0 (100%) |
| 3b (n=4)                         | 51±26 (50%)                | 105±10 (10%)          | 0 (100%) |
| 4a (n=4)                         | 114±8 (7%)                 | 178±18 (10%)          | 2 (100%) |
| 4b (n=2)                         | 111±21 (19%)               | 177±11 (6%)           | 2 (100%) |
| IGL cerebellum                   |                            |                       |          |
| 1 (n=2)                          | 457±0                      | 126±4                 | 1        |
| Intra-assay CV% (n=58):          | 24                         | 5                     | -        |
| Percentage agreement (n=58):     | -                          | -                     | 100      |
| ICC (non-consecutive blocks n=8) | 0.97*                      | 1.00*                 | 1.00*    |
| ICC (test-retest n=25)           | 0.99*                      | 0.99*                 | 0.99*    |
| ICC/Kappa (inter-rater n=10)     | 0.83*                      | 0.95*                 | 0.58*    |

Values expressed as mean ± SD or score.

\*Indicates statistical significance. Significance taken at  $p \leq 0.05$ .





**Figure 4:** Examples of sections that received an overall R-score =100, >120 and >160 in the CA4 and CA1 of human cases. As the R-score increases, the proportion of astrocytes stained with a higher reactivity score (>0) also increases. Scale = 100µm.

## **5.4: DISCUSSION**

The results from this study demonstrated that the R-score, which relied on assessing the severity of the pathology (based on morphology) for each individual GFAP-positive cell, was the most reproducible quantification method, with high inter and intra-rater concordance. This was initially demonstrated in brain tissue sourced from an experimental piglet model but was also consistent with clinical diagnosis when applied to 4 paediatric cases with varying levels of astrogliosis severity.

### **5.4.1: CURRENT METHODS FOR QUANTIFYING GFAP**

Many methods exist in the research setting for GFAP (summarised Supplementary Table 1). Stereological counting is considered the ‘gold standard’ for obtaining accurate estimates of a quantitative parameter, such as number of cells, length of processes, volume etc. (McNeal et al., 2016), however, it is highly time-consuming and expensive, and not feasible from a diagnostics standpoint. Quantification methods that are time/cost efficient, but also reproducible and accurate when applied on the minimum number of tissue sections, are warranted. Herein, we sought to determine whether the R-score method is suitable, and how it compared to other commonly used methods of assessment. To our knowledge, this is the first paper to compare different quantification methods simultaneously, and assess for measures of reproducibility, as well as sensitivity (assessed via ability to detect an upregulation in GFAP indicative of astrogliosis), and selectivity (assessed via ability to correspond with clinical diagnosis and severity) across serial and non-serial sections in an experimental model where methodological parameters can be tightly controlled for.

In a clinical setting, ‘astrogliosis’ is usually assessed qualitatively, which is highly cost and time-efficient, although subjective with poor inter-rater agreement (Escartin et al., 2019, Leitner et al., 2022), limited in ability to distinguish the range of phenotypic changes that can occur (McNeal et al., 2016), and biased, given that GFAP is differentially upregulated in different brain regions following injury (Boos et al., 2021). Similarly, in the research setting, “astrogliosis” is poorly defined and may refer to an increase in overall GFAP immunostaining, a change in astrocyte morphology, or increase in number of GFAP positive cells (or a combination of these). Quantitative methods offer certain advantages, including being (arguably) less subjective and more sensitive to detecting subtle changes (McNeal et al., 2016), while semi-quantitative assessment is only sensitive to detect large, observable differences (McNeal et al., 2016). Importantly, these different methods of assessment are likely to be



measuring different types of changes that can occur. For example, an upregulation in the amount of GFAP staining without morphological consideration may be reflective of early or more subtle changes in GFAP, given that astrocyte hypertrophy may not be maintained under certain conditions (Petito et al., 1990, Moeton et al., 2016).

We found the thresholding method to not be reliable, having consistently failed to satisfy our measures of reproducibility. Furthermore, it appeared to be highly inconsistent with the other methods when comparing amongst brain regions. Despite the advantage of thresholding to account for all stained particles, detecting and quantify astrocytic bodies and fibres (Muñoz-Castro et al., 2022, Healy et al., 2018, Johnson and Walker, 2015), differentiation between signal and background or ‘noise’, for example via background normalisation, is based on parameters set by the end user, thus making it subjective.

The point-counting method had some merit, showing consistency when assessing values across serial tissue sections, yet not in non-consecutive sections or according to antibody dilution factor. The lack of homology across non-consecutive sections is not surprising when considering that GFAP cells are at most 400µm in diameter (although often <100µm) (Oberheim et al., 2009). Given that this method is purely based on cell quantity, numbers would differ by a certain proportion across the rostral/caudal or ventral to dorsal aspect of a particular ROI. As such, stereological counting would be recommended to overcome this. Variation in counts according to changes in antibody dilution factor are likely due to increased binding of antibodies to their respective antigens with increased concentrations. This did not appear to affect the proportion of cells stained at each level of reactivity, given that the R-score values remained consistent across antibody concentrations.

The semi-quantitative score was deemed superior to the other 2 methods of comparison, showing consistency across serial tissue sections and different tissue blocks (human only), and having acceptable inter and intra-rater concordance. A baseline standard was set in the current study, whereby at least 2 GFAP positive cells had to be of a particular score to apply this to the whole tissue section. Parameters such as these are not clearly defined in the literature however (Lyck et al., 2008, McNeal et al., 2016).

#### **5.4.2: R-SCORE**

While semi-quantitative scoring was shown to meet our acceptable criteria and may be useful when detecting large, observable differences due to severe pathology, the R-score is likely to

offer several additional advantages. The first is that it standardises the semi-quantitative method by providing a numerical value representing the proportion of astrocytes at each severity level. Although this remains a somewhat subjective process (given the diverse morphological changes that can occur and that specific criteria, for example the number of processes present to differentiate a score of 2 or 3, are not set), we have provided several examples of each score within different brain regions (Figure 2), assessed in accordance with current scoring systems cited in the literature (Gouw et al., 2008, Sofroniew and Vinters, 2010), and showed that R-score was also consistent when assessed across different time-points, and between different raters, when applying the same criteria. The second is that the R-score will likely be able to detect even subtle group differences when undertaking statistical analysis on larger groups, and unlike other quantitative methods, the outcome value is directly meaningful. Validation of this will need to be tested in larger clinical datasets in future studies, however, an R score >100 indicates reactive processes occurring, and a higher score represents more significant pathology. Given that in our piglets, the range of astrocytes with a semi-quantitative score above 1 was low, we applied our methods on several human case of known astrogliosis and demonstrated R-scores >120 in cases diagnosed with mild astrogliosis (CA4 of the hippocampus and internal granular layer of the cerebellum), and >160 in a case with moderate astrogliosis (CA4 and CA1 of the hippocampus) (Figure 4).

## **5.5: CONCLUSIONS**

This study compared several commonly used methods for quantifying GFAP-immunohistochemistry to detect “astrogliosis” and validated that the R score, a derivative of the H-Score that takes GFAP positive cell morphology into consideration rather than staining intensity, is a highly advantageous method for evaluating astrogliosis via GFAP staining and may be a more systematic approach for analysis when compared with semi-quantitative methods. Its time and cost efficiency, as well as high reproducibility and accuracy, makes its clinical applicability eminent.

## **5.6: ACKNOWLEDGMENTS**

This research was supported by an Australian Government Research Training Program (RTP) Scholarship awarded to L. Luijckink.

## **5.7: APPENDIX**

This Appendix contains the supplementary material available. For ease of reference, the supplement tables retain their titles as referred to in the publication.

## Supplementary Section 1: comparison of methods for quantifying GFAP

**Supplementary Table 1:** Features of commonly used methods for quantifying GFAP immunohistochemistry in the context of astrogliosis.

|                                 | Method                                                                                                                                                                                                                                                                                                              | Outcome measure                                                                   | Advantages                                                                                                                                   | Disadvantages                                                                                                                                                                                                                                                                    | Example of studies that have utilised method | Presentation in the current study         |
|---------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------|-------------------------------------------|
| <b><i>Semi-quantitative</i></b> |                                                                                                                                                                                                                                                                                                                     |                                                                                   |                                                                                                                                              |                                                                                                                                                                                                                                                                                  |                                              |                                           |
| Incidence method                | Pre-defined phenotype or boundary of what is considered “normal”                                                                                                                                                                                                                                                    | Pathological feature is either present or not e.g., yes vs no astrogliosis        | <ul style="list-style-type: none"> <li>- Time and cost effective</li> </ul>                                                                  | <ul style="list-style-type: none"> <li>- Subjective judgement bias</li> <li>- Distinguishing ranges of phenotype can be limited</li> </ul>                                                                                                                                       | (Kinney et al., 2015) (Kon et al., 2020)     | NA                                        |
| Graded scoring system           | <p>Ordinal value indicating the level of severity of astrogliosis. This can be based on various criteria, such as intensity, distribution, morphology, and overall pattern of staining. Requires a trained observer utilising a self-standardised criterion.</p> <p>Ranks should reflect the range of phenotype</p> | Level of pathological severity, normally graded between no astrogliosis to severe | <ul style="list-style-type: none"> <li>- Takes overall staining pattern into account</li> <li>- Time-efficient and cost-effective</li> </ul> | <ul style="list-style-type: none"> <li>- Subjective judgement bias</li> <li>- Less sensitive to subtle changes in pathological state</li> <li>- GFAP expression differs between brain regions at baseline- may overlook changes in regions with lower GFAP expression</li> </ul> | (Lyck et al., 2008, McNeal et al., 2016)     | 4-tier grading system- scores ranging 0-3 |

|                            |                                                                                                                                                                         |                                                                                    |                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                                                 |                                                                |                   |
|----------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------|-------------------|
|                            | present in the data to effectively distinguish group differences, although standardisation of methods will allow for comparability between studies and research groups. |                                                                                    |                                                                                                                                                                                                                                               | <ul style="list-style-type: none"> <li>- No standard criteria for assigning score; e.g, how many cells need to be of the feature of '1' to assign a '1' for the whole ROI.</li> </ul>                                                                                                                           |                                                                |                   |
| H-score                    | Determined by addition or multiplication of the percentage of cells, with staining intensity; ordinal value.                                                            | Value that represents intensity and proportion of staining at each intensity level | <ul style="list-style-type: none"> <li>- Standardisation of the semi-quantification method allowing for comparability of findings between studies/laboratories</li> <li>- Gradations can be adapted to fit specific study outcomes</li> </ul> | <ul style="list-style-type: none"> <li>- More time-consuming than traditional semi-quantitative methods</li> <li>- Subjective judgement required</li> <li>- Variations in formulas across research groups (Supplementary Table 2).</li> <li>- Lack of standardised cut-offs when interpreting scores</li> </ul> | (Hazzaa et al., 2020) & please refer to Supplementary Table 2. | R-score;          |
| <b><u>Quantitative</u></b> |                                                                                                                                                                         |                                                                                    |                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                                                 |                                                                |                   |
| Counts                     | Number of immuno-positive astrocytes (or                                                                                                                                | Counts per field/area                                                              | <ul style="list-style-type: none"> <li>- May pick up subtle or early indications</li> </ul>                                                                                                                                                   | <ul style="list-style-type: none"> <li>- Time consuming and</li> </ul>                                                                                                                                                                                                                                          | (Buser et al., 2012)                                           | Number of immuno- |



|                                                                       |                                                                                                                                                                                                                                                               |                                                            |                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                                          |                                                                                                                       |                                         |
|-----------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|-----------------------------------------|
|                                                                       | feature of interest e.g., astroglial projections, astrocytes of reactive phenotype) in a given field/area                                                                                                                                                     |                                                            | <p>of reactive processes occurring as detecting upregulation of GFAP</p> <ul style="list-style-type: none"> <li>- A form of objective assessment and is therefore repeatable</li> <li>- Can be automated using imaging software</li> </ul> | <p>may require specialised software to automate the process – different algorithms can produce different results affecting the outcome of the analysis</p> <ul style="list-style-type: none"> <li>- Requires high quality images</li> </ul>                                                              | (Meadowcroft et al., 2015, Chaaya et al., 2023)                                                                       | positive astrocytes per mm <sup>2</sup> |
| Image segmentation- Binary image thresholding (pixel intensity level) | ‘Signal’ (immunolabelled material) to ‘noise’ (non-specific material attributed to immunolabelling process) ratio. These parameters are defined manually and remain constant between groups. The number of pixels within the signal range is then quantified. | Percentage of the image covered by positive immunostaining | <ul style="list-style-type: none"> <li>- Allows for a standardised comparison of the amount of staining</li> <li>- Accounts for changes in both the cell body and its processes</li> <li>- Can be automated</li> </ul>                     | <ul style="list-style-type: none"> <li>- Threshold parameters rely on subjective judgement</li> <li>- The assumption that the threshold range is the same across groups needs to be met (including staining parameters that may affect signal/noise)</li> <li>- Pixel intensity histogram may</li> </ul> | (Muñoz-Castro et al., 2022, Healy et al., 2018, Johnson and Walker, 2015, Tang et al., 2009, Machaalani et al., 2019) | % area covered by staining              |

|                                                      |                                                                                                                                                                                                            |                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                |                                                                  |    |
|------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------|----|
|                                                      |                                                                                                                                                                                                            |                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                  | complement standard thresholding                                                                                                                                                                                                                               |                                                                  |    |
| Image segmentation of individual cells or structures | Requires image to have staining separated from background via thresholding, edge detection or region growing. Individual structures can then be labelled or quantified i.e. length of astrocyte processes. | Area, perimeter or other properties of staining in each object                                    | <ul style="list-style-type: none"> <li>- Object based analysis rather than overall intensity of staining</li> <li>- Can be less affected by variations in stains and background, such as in the case of thresholding</li> <li>- Can provide spatial context to give context about staining distribution</li> <li>- Can be automated saving time and effort for researchers</li> <li>- Can provide multiple parameters</li> </ul> | <ul style="list-style-type: none"> <li>- Requires specialised software and hardware</li> <li>- Highly sensitive to image quality</li> <li>- Application of threshold introduces user bias and error</li> <li>- Over or under-segmentation can occur</li> </ul> | (Healy et al., 2018)                                             | NA |
| Mean optical density (MOD)                           | Average intensity of staining in the ROI (sum of intensity values divided by number of pixels). Can be done on a thresholded image (optional). MOD                                                         | Units of optical density (logarithmic scale representing the ratio of light transmitted through a | <ul style="list-style-type: none"> <li>- Reduced subjectivity methods</li> <li>- Can provide results that are reproducible, thus, can detect changes in staining over</li> </ul>                                                                                                                                                                                                                                                 | <ul style="list-style-type: none"> <li>- Subjectivity in defining the area of interest</li> <li>- Lack of sensitivity to spatial variations in staining</li> </ul>                                                                                             | (Eldomiaty et al., 2020, Petito et al., 1990, Tang et al., 2009) | NA |

|            |                                                                                                                                                                                                                         |                                                                                                                                                                            |                                                                                                                                                                                                                                                                                                                                                      |                                                                                                                                                                                                                                                                                                                                                                                     |                       |    |
|------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|----|
|            | values are normalised if necessary to correct for variations in image acquisition or staining protocol                                                                                                                  | sample to the light that would have passed through if the sample were clear)                                                                                               | time/between different samples                                                                                                                                                                                                                                                                                                                       | intensity in the tissue<br>- Lack of information on cell morphology                                                                                                                                                                                                                                                                                                                 |                       |    |
| Stereology | Involves mathematical methods to estimate 3-dimensional properties (such as in relation to density, distribution, and morphology of GFAP) in structures such as tissue sections, based off multiple (8-10) 2D sections. | Wide range of possible outcome measures such as estimation of the number of GFAP-positive cells, astrocyte volume, astrocyte density or spatial distribution of astrocytes | <ul style="list-style-type: none"> <li>- Considered unbiased and higher precision in measurement can be obtained given all parts of the tissue are sampled- thus minimising sampling errors</li> <li>- 3D reconstruction allows for better understanding of spatial relationships between GFAP positive cells and other tissue structures</li> </ul> | <ul style="list-style-type: none"> <li>- Laborious and complex approach often reserved for specialised labs.</li> <li>- Requires specialist training and software</li> <li>- Time consuming</li> <li>- Still based on 2D images or sections which may lead to errors</li> <li>- Accuracy can be affected by sample preparation and staining parameters</li> <li>- Costly</li> </ul> | (McNeal et al., 2016) | NA |

NA= Not applied. Yellow highlighted indicate studies from our own laboratory.

## Supplementary Section 2: The H-Score

The H-score is a method used in immunohistochemistry to quantify the expression of a protein in a tissue calculated by multiplying the **staining intensity** by the **percentage of cells stained at that specific intensity**. Different research groups may use different formulas and methods to calculate H-scores (Supplementary Table 2), and this will determine the range of values for final scores, and the comparability of outcome values between different research groups. Variations in methods and formulas for calculating the H-score can include whether negative cells are initially counted, whether negative cells or lowest scores are given weight in the H-score formula, and the range of intensity values (e.g., negative, weak, strong vs. negative, weak, moderate and strong, vs weak, moderate, strong). Of note, GFAP-negative astrocytes can be difficult to distinguish from other cells in the tissue, such as small neurons (García-Cabezas et al., 2016), particularly in the infant brain where neurons are not fully mature and have not yet reached their full size. Thus, this can lead to why some groups account for them while others do not (summarised in Table 2).

**Supplementary Table 2:** Examples of studies that have assessed GFAP immunohistochemistry via H-score between 2016-current.

| <b>Tissue type</b>                                      | <b>Formula stated</b>                                                                                                                                              | <b>Were negatives counted (according to methods)</b> | <b>Were negatives or lowest score included in the model (based on score range)</b> | <b>Score range</b> | <b>Reference</b>                           |
|---------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------|------------------------------------------------------------------------------------|--------------------|--------------------------------------------|
| Cerebral cortex, cerebellum (rat)                       | $\Sigma Pi(i + 1)$                                                                                                                                                 | Yes                                                  | Yes                                                                                | 100-300            | (Seker et al., 2016)                       |
| Temporal cortex (rat)                                   | $\Sigma Pi(i + 1)$                                                                                                                                                 | Yes                                                  | Yes                                                                                | 100-300            | (Yilmaz et al., 2018)                      |
| Cortex, cerebellum and brainstem (rat)                  | $\Sigma Pi(i + 1)$                                                                                                                                                 | Yes                                                  | Yes                                                                                | 100-300            | (Yorulmaz et al., 2015)                    |
| Cortex/ Hippocampus (rat)                               | $\Sigma Pi(i + 1)$                                                                                                                                                 | Yes                                                  | Yes                                                                                | 100-400            | (Delen et al., 2021)                       |
| Hippocampus and spinal cord (rat)                       | Initial grading 0-4.<br>$H = 1 \times (\% \text{ of } 1 + \text{cells}) + 2 \times (\% \text{ of } 2 + \text{cells}) + 3 \times (\% \text{ of } 3 + \text{cells})$ | Yes                                                  | No                                                                                 | 0-250              | (Hazzaa et al., 2020, Hazzaa et al., 2021) |
| Inner limiting membrane of the eye (human)              | Summing two values: (intensity 0-3) and (percentage (0 = 0; $1 \leq 10\%$ ; $2 = 10-25\%$ ; $3 \geq 25\%$ ))                                                       | Yes                                                  | Yes                                                                                | 1-4.6              | (Romano et al., 2018)                      |
| Several brain sub-regions including hippocampus (mouse) | Strong intensity; 3x%, moderate intensity; 2x%, mild intensity; 1x%, negative staining; 0x%                                                                        | Yes                                                  | No                                                                                 | 0-300              | (ATIA et al., 2021)                        |
| Brain (whole tissue sections) and hippocampus (mouse)   | $(\text{Percentage of weak staining}) \times 1 + (\text{percentage of moderate staining}) \times 2 + (\text{percentage of strong staining}) \times 3.$             | No                                                   | Yes                                                                                | 100-200            | (Fei et al., 2023)                         |

|                     |                                                                   |     |     |       |                      |
|---------------------|-------------------------------------------------------------------|-----|-----|-------|----------------------|
| Hippocampus (mouse) | multiplying the percentage score and its intensity category (0-3) | Yes | Yes | 0-300 | (Zhang et al., 2020) |
|---------------------|-------------------------------------------------------------------|-----|-----|-------|----------------------|

$\sum P_i(i + 1)$ - where “pi” represents the percentage of cells in each intensity category, and “i” represents the intensity value.

One of the major differences in interpretation of the H-score is whether all cells counted are included in the model, or not. When all cells that are scored are given weight in the H-score (i.e., by adding a value of 1 to each intensity value ( $\sum P_i(i + 1)$ )), the total proportion of cells accounted for in the model totals 100%, and thus, H-scores are always greater than or equal to 100 (Seker et al., 2016, Yilmaz et al., 2018, Yorulmaz et al., 2015, Delen et al., 2021). Alternatively, the lowest intensity value can be given no weight in the H-score formula, and the proportion of cells with this intensity value will be multiplied by 0 (i.e.,  $i = 0$ ). The total proportion of cells accounted for in the model will likely total <100% (unless all cells in the tissue receive a score > 0), thus H-score values can range from 0-400 (Hazzaa et al., 2020, Hazzaa et al., 2021, ATIA et al., 2021, Zhang et al., 2020).

The discrepancy in the application of the H-score between different studies stresses the importance of detailing the methods carefully to allow for valid comparisons in study outcomes. For example, using the scenario below, it is clear how the data can differ greatly depending on the H-score formula used.

### Example:

20% negative, 40% weak, 30% moderate, 10% strong

*Equation 1:*  $\sum P_i(i + 1)$  [ $i = 0$  for negative, 1 for weak, 2 for moderate, 3 for strong]

$$= 20(0 + 1) + 40(1 + 1) + 30(2 + 1) + 10(3 + 1)$$

$$= 230$$

*Equation 2:*  $\sum P_i(i + 1)$  [ $i = 1$  for weak, 2 for moderate, 3 for strong]

$$= 40(1 + 1) + 30(2 + 1) + 10(3 + 1)$$

$$= 210$$

*Equation 3:* (Percentage of weak staining)  $\times$  1 + (percentage of moderate staining)  $\times$  2 + (percentage of strong staining)  $\times$  3

$$= (40 \times 1) + (30 \times 2) + (10 \times 3)$$

$$= 130$$

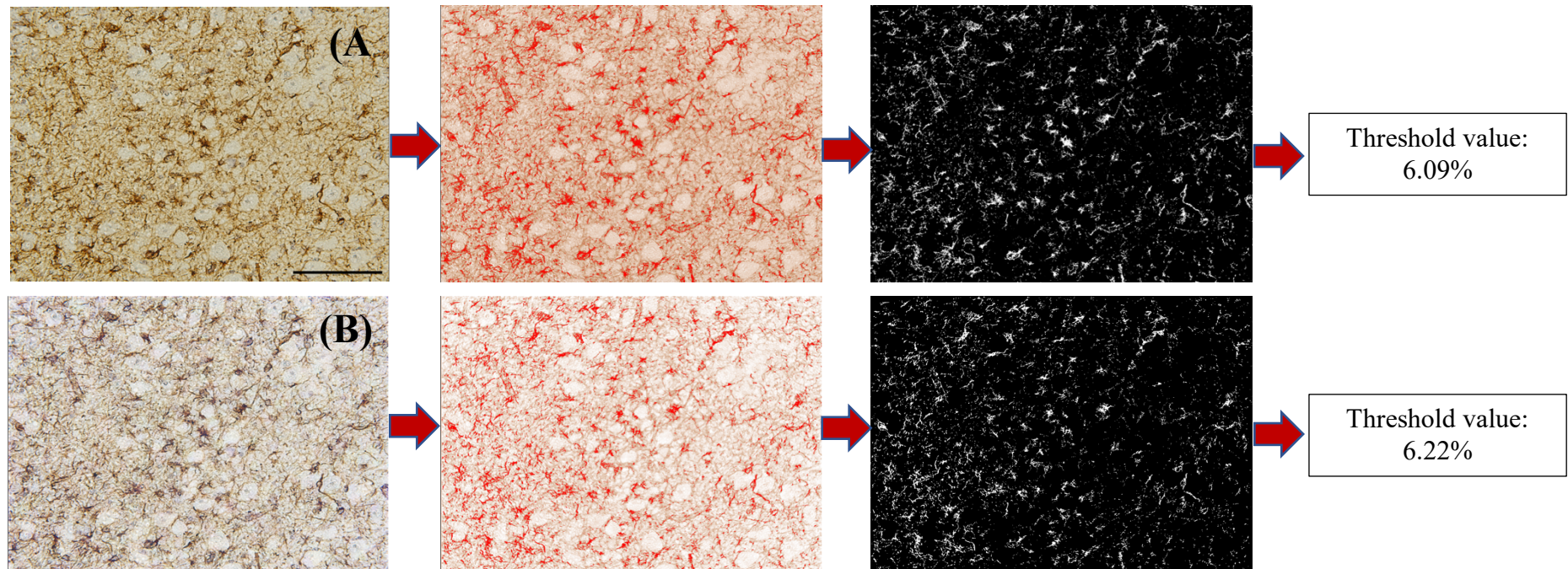
$$\begin{aligned} \text{Equation 4: } & (\text{Percentage of negative staining}) \times 1 + (\text{percentage of weak staining}) \times 2 + \\ & (\text{percentage of strong staining}) \times 3 \\ & = (20 \times 1) + (40 \times 2) + (10 \times 3) \\ & = 130 \end{aligned}$$

$$\begin{aligned} \text{Equation 5: } & (\text{Percentage of negative staining}) \times 1 + (\text{percentage of weak staining}) \times 2 + \\ & (\text{percentage of moderate staining}) \times 3 (\text{percentage of strong staining}) \times 4 \\ & = (20 \times 1) + (40 \times 2) + (30 \times 3) + (10 \times 4) \\ & = 230 \end{aligned}$$



### Supplementary Section 3: Background reduction for thresholding

Manual thresholding performed using the histogram, measuring the area of GFAP staining. In order to address uneven illumination and extract background in RGB images, we attempted the rolling ball algorithm for background subtraction, using the ‘subtract background’ plugin in Fiji (Supplementary Figure 1). This method involves independently applying the algorithm to each colour channel (red, green, and blue). For each channel, a ‘rolling ball’ is slid across the image, calculating the local background value, and subtracting this from the corresponding channel’s original pixel intensities.



**Supplementary Figure 1.** (A) Original image of GFAP staining in the CA4 of the piglet, and (B) the same image after undergoing background subtraction via the rolling ball algorithm. Scale = 100 $\mu$ m.

**Supplementary Table 3:** %CV for each method for serial sections that were stained using the manual method (n=4) and automated method (n=2).

|                                   | Quantification method |                        |         |          |
|-----------------------------------|-----------------------|------------------------|---------|----------|
|                                   | Threshold             | Counts/mm <sup>2</sup> | R-score | Score    |
| <b>Mean±SD</b>                    | 3.8±1.3               | 595±86                 | 167±12  | 0 (100%) |
| <b>Intra-assay CV% (n=6)</b>      | 33                    | 15                     | 7       | -        |
| <b>Percentage agreement (n=6)</b> | -                     | -                      | -       | 100      |

## Supplementary Excel File

Raw scores for quantification methods in the piglet brain are provided below.

|          | Hscore    | mm2       | threshold | score |
|----------|-----------|-----------|-----------|-------|
| 1a CA4   | 131.0757  | 619.75309 | 0.2905    | 1     |
| 1b CA4 1 | 123.76238 | 374.07407 | 0.371     | 1     |
| 1b CA4 2 | 106.47059 | 419.75309 | 0.0986667 | 1     |
| 1b CA4 3 | 119.71831 | 350.61728 | 1.8073333 | 1     |
| 1b CA4 4 | 105.29412 | 419.75309 | 1.1805    | 0     |
| 2a CA4 1 | 126.66667 | 444.44444 | 1.5063333 | 1     |
| 2a CA4 2 | 119.82759 | 429.62963 | 3.4456667 | 1     |
| 2b CA4 1 | 139.01345 | 550.61728 | 2.5406667 | 1     |
| 2b CA4 2 | 100       | 308.64198 | 0.6093333 | 0     |
| 2b CA4 3 | 120.79208 | 498.76543 | 0.8393333 | 1     |
| 3a CA4 1 | 146.5     | 493.82716 | 1.0603333 | 1     |
| 3a CA4 2 | 123.15789 | 351.85185 | 0.331     | 1     |
| 3b CA4 1 | 141.05263 | 469.1358  | 0.9813333 | 0     |
| 3b CA4 2 | 129.67033 | 449.38272 | 0.3193333 | 0     |
| 3b CA4 3 | 141.66667 | 488.88889 | 0.4756667 | 0     |
| 1a CA1   | 100       | 95        | 0.06      | 0     |
| 1b CA1 1 | 100       | 211.11111 | 0.1293333 | 0     |
| 1b CA1 2 | 100       | 104.54545 | 0.0753333 | 0     |
| 1b CA1 3 | 100       | 150       | 0.032     | 0     |
| 1b CA1 4 | 100       | 153.84615 | 0.1463333 | 0     |
| 2a CA1 1 | 100       | 347.05882 | 1.0966667 | 0     |
| 2a CA1 2 | 102.04082 | 408.33333 | 1.906     | 0     |
| 2b CA1 1 | 100       | 261.53846 | 1.069     | 0     |
| 2b CA1 2 | 100       | 164.28571 | 0.394     | 0     |
| 2b CA1 3 | 100       | 303.84615 | 0.8173333 | 0     |
| 3a CA1 1 | 100       | 195       | 0.0926667 | 0     |
| 3a CA1 2 | 100       | 100       | 0.0163333 | 0     |
| 3b CA1 1 | 100       | 206.66667 | 0.6753333 | 0     |
| 3b CA1 2 | 100       | 123.07692 | 0.2646667 | 0     |
| 3b CA1 3 | 100       | 165       | 0.3756667 | 0     |
| 3b CA1 4 | 100       | 144.44444 | 0.5026667 | 0     |
| 1a XII 1 | 122.38806 | 248.14815 | 1.331     | 0     |
| 1a XII 2 | 113.84615 | 240.74074 | 0.955     | 0     |
| 1a XII 3 | 122.97297 | 274.07407 | 1.5985    | 0     |
| 1b XII 1 | 128.57143 | 414.81481 | 2.672     | 1     |
| 1b XII 2 | 166.66667 | 500       | 11.7425   | 1     |
| 2a XII 1 | 112.37113 | 359.25926 | 1.8165    | 0     |
| 2a XII 2 | 116.27907 | 318.51852 | 1.745     | 0     |
| 2a XII 3 | 105.31915 | 348.14815 | 1.6135    | 0     |
| 2b XII 1 | 105       | 222.22222 | 2.1095    | 0     |
| 2b XII 2 | 100       | 237.03704 | 1.217     | 0     |
| 3 XII 1  | 114.08451 | 262.96296 | 1.3075    | 0     |
| 3 XII 2  | 106.66667 | 277.77778 | 0.745     | 0     |
| 3 XII 3  | 103.50877 | 211.11111 | 2.3165    | 0     |
| 3 XII 4  | 105.08475 | 218.51852 | 1.408     | 0     |
| 1 IGL 1  | 104       | 308.64198 | 1.6193333 | 0     |
| 1 IGL 2  | 100       | 308.64198 | 1.657     | 0     |
| 1 IGL 3  | 102.77778 | 266.66667 | 0.7023333 | 0     |
| 2 IGL 1  | 100.72464 | 340.74074 | 0.827     | 0     |
| 2 IGL 2  | 100.89286 | 276.54321 | 1.239     | 0     |
| 2 IGL 3  | 100       | 414.81481 | 1.2166667 | 0     |
| 3 IGL 1  | 123.60248 | 397.53086 | 1.5766667 | 1     |
| 3 IGL 2  | 122.78481 | 390.12346 | 1.6366667 | 1     |
| 3 IGL 3  | 122.91667 | 355.55556 | 1.221     | 0     |

## **CHAPTER 6: GFAP EXPRESSION AND ASTROGLIOSIS IN THE DEVELOPING HUMAN BRAIN AND CHANGES SEEN IN SIDS- UNDERTAKEN IN A UK COHORT**

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### Attribution statement

Input to project design was by RM, LL and MC. All tissue sectioning, experimentation, analysis and drafting of the manuscript was performed by LL and reviewed by RM and MC. This chapter is formatted according to criteria to be submitted for publication.

Human tissue used in this study was obtained from Sheffield Children's Hospital under the Sheffield Children's NHS Foundation Trust. Experimentation and image analysis was performed at the Sheffield Children's Hospital, Sheffield, UK.

## 6.1: INTRODUCTION

Astrocytes are a type of glial cell that play an integral role in the development, structure and function of the central nervous system (CNS) and are also known to become ‘reactive’ following almost any type of CNS injury or disease. This reactive process is termed ‘astrogliosis’, and involves diverse morphological, transcriptional and functional changes (Sofroniew and Vinters, 2010). Astrogliosis is commonly visualised immunohistochemically via an increase in the intermediate filament protein, GFAP (glial fibrillary acidic protein), such that: 1) there is an increase in the number of immunopositive astrocytes, and/or 2) astrocyte cell bodies and their processes become hypertrophied (Sun and Jakobs, 2012, Zhu et al., 2004).

Sudden infant death syndrome (SIDS) is the sudden and unexpected death of a seemingly healthy infant <1 year of age, occurring during a sleep period, that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history (Krous et al., 2004). The presence of astrogliosis has been studied previously in the SIDS brain (summarised in Table 2.5, Chapter 2) and identified in the brainstem (Bruce and Becker, 1991) (Storm et al., 1994, Obonai et al., 1996, Storm et al., 1999, Sawaguchi et al., 2002, Matturri et al., 2006, Spinelli et al., 2018), predominantly in nuclei of the medulla, and cerebellum (Lavezzi et al., 2018), while other studies reported no significant levels of astrogliosis among SIDS infants (Reske-Nielsen et al., 1987, Oehmichen et al., 1989a, Pamphlett and Treloar, 1996, Kinney et al., 2015). Contradictions in the literature may arise due to variations in experimental protocols and quantification methods, different definitions of SIDS and compositions of the control group, and differences in gestational age, which is an important factor of consideration given that changes in gliosis can be age-specific (Mickel and Gilles, 1970).

Interestingly, some studies correlated astrogliosis with known SIDS risk factors, such as pre-natal cigarette smoke exposure (Storm et al., 1999), and repeated apnoeic episodes/prone-sleeping (Sawaguchi et al., 2002). It is becoming increasingly apparent across SIDS research that analysis according to the presence of risk factors is imperative (Luijckx et al., 2020, Ambrose et al., 2020, Vivekanandarajah et al., 2021). SIDS is hypothesised to be the end-point of a heterogeneous group of pathophysiologies, rather than the result of a single underlying cause (Byard and Jensen, 2008) and thus, unlike diagnosis, a particular risk factor may represent a common mechanism that is identifiable at death.

The first aim of this study is to report on the immunohistochemical expression of GFAP in sub-regions of the medulla, cerebellum, and hippocampus of the developing human infant brain. These brain regions were chosen given that astrogliosis has been documented previously in the cerebellum and extensively in the medulla of SIDS (see above), and the hippocampus, due to the limbic seizure hypothesis thought to occur in a proportion of SIDS infants (Kinney et al., 2015, Kinney et al., 2018), given that limbic seizures are known to lead to significant hippocampal astrogliosis (Devinsky et al., 2013). The second aim was to determine whether there were any differences in expression between SIDS and non-SIDS (explained sudden unexpected deaths in infancy (eSUDI)). Thirdly, we sought to determine whether there were any differences in GFAP expression according to the presence of major SIDS risk factors, including cigarette smoke exposure, sleep position found, co-sleep/bed-sharing, the presence of a recent upper respiratory tract infection (URTI) and male gender.

Past studies investigating astrogliosis in SIDS using GFAP immunohistochemistry have predominantly based their assessment on either: quantification of the number of GFAP-positive astrocytes per unit area (Becker and Takashima, 1985, Obonai et al., 1996, Sawaguchi et al., 2002, Spinelli et al., 2018), or qualitative assessment (presence of astrogliosis and/or severity) based on astrocyte morphology (Reske-Nielsen et al., 1987, Kinney et al., 2015, Lavezzi et al., 2018) (summarised in Table 2.5, Chapter 2). Different methods of quantification consider different types of astrocyte changes that occur following CNS damage (Weber et al., 2013, McNeal et al., 2016). An upregulation in the number of GFAP-positive cells without significant morphological changes may be reflective of early or more subtle changes in GFAP (Petito et al., 1990), while an increase in astrocyte hypertrophy and/or proliferation may be indicative of severe astrogliosis (Sofroniew, 2015).

For our data to be comparable to past studies investigating astrogliosis in SIDS, we applied 2 quantification methods on GFAP immunohistochemically stained tissue: (1) point-counting of immunopositive cells, and (2) a qualitative assessment based on astrocyte morphology. In order to standardise the qualitative method, our laboratory recently characterised a novel quantification method termed ‘reactivity (R)-score’, which produces a single score based on the proportion of astrocytes scored at each level of reactivity (Chapter 5). Herein, we apply this R-score method for the first time in a clinical context and will assess how its data compares to the point-counting method.

## **6.2: METHODS**

### **6.2.1: DATA AND TISSUE COLLECTION**

A total of 34 cases were utilised in the current study from infants who died between 1-10 postnatal months. All infant brain tissue and information regarding infant characteristics and the presence of risk factors was collected by the Sheffield Children's Hospital under the Sheffield Children's NHS Foundation Trust in the UK between the years 2012-2021. Tissue and information were only accessed after a final diagnosis had been provided following ethical approval from the Health Research Authority (HRA) and Health and Care Research Wales (HCRW) (reference no: 22/HRA/2992), and all procedures were undertaken in accordance with the Human Tissue Act (2004). All cases were de-identified prior to experimentation and analysis using case codes. Reclassification of diagnosis was undertaken by the authors of the study in accordance with the San Diego criteria (Krous et al., 2004).

Brain tissue from the medulla, cerebellum, and hippocampus were collected at the time of death as part of the routine autopsy protocol for coronial investigation of the cause of death. Post-mortem interval (PMI) ranged between 22 and 180 hours. On extraction, whole brains were fixed in 20% neutral-buffered formalin for a maximum of 5 days, regions of interest dissected, placed in tissue cassettes, and fixed in 10% neutral buffered formalin (average 1-2 days) until paraffin-embedding; thus fixation time estimated to range from 3-10 days. Tissue was sectioned at 7µm thickness using a rotary microtome, mounted on FLEX IHC microscope slides, dried overnight at 37°C and stored overnight at room temperature prior to immunohistochemical staining.

### **6.2.2: GFAP IMMUNOHISTOCHEMISTRY**

Automated immunohistochemistry was performed using the Dako Autostainer Link 48 following the standard protocol. Sections were oven-baked at 60°C using the Leica Autostainer XL (ST5010), prior to undergoing deparaffinization, rehydration and heat-induced antigen retrieval via the DAKO PT Link (HIS072 with high pH DAKO Antigen Retrieval solutions, K8004), where the temperature for antigen retrieval reached a maximum of 90°C for 20 minutes. Sections were rinsed with buffer (EnVision FLEX wash buffer K8007), blocked for endogenous peroxidase for 5 minutes using FLEX peroxidase blocking agent (SM801, DAKO), rinsed, blocked with serum free protein block (X0909, DAKO) for 10 minutes, and incubated with prediluted primary-antibody GFAP (polyclonal rabbit, IR524, DAKO) for 30



minutes. Sections were rinsed and labelled with EnVision FLEX/HRP (SM802, DAKO) for 30 minutes, rinsed, and colour labelling was achieved using EnVision FLEX DAB+ Chromogen (DM827) in substrate buffer (SM803, DAKO) for 5 minutes, counterstained with EnVision FLEX Hematoxylin (K8008), and transferred to the Leica Autostainer XL (Leica CV5030, HIS096) to coverslip automatically using ExPert Mountant (SEA-1600-00A, CellPath). Duplicate staining was performed on at least 20% of sections for each brain region, and each case had more than one section/level of the medulla, with analysis undertaken on both left and right hemispheres

### **6.2.3: QUANTITATIVE ANALYSIS**

Stained sections were viewed using light microscopy (Nikon Eclipse, 80i) and imaged using Touptek image capture software (Toupcam industrial digital camera and Toupview version: x64, 3.7.13059.20181003). For each case, 21 microscopically defined regions were examined including 9 nuclei of the medulla (arcuate nucleus (AN), cuneate nucleus (Cun), dorsal motor nucleus of the vagus (DMNV), hypoglossal (XII), inferior olivary nucleus (ION), lateral reticular formation (LRF), nucleus of the spinal trigeminal tract (NSTT), nucleus of the solitary tract (NTS) and vestibular nucleus (Vest), from obex levels -2mm-8mm as defined by (Paxinos and Huang, 2013), 4 regions of the cerebellar cortex (dentate nucleus, internal granular layer (IGL), molecular layer (ML) and white matter, and 5 regions of the hippocampus (dentate gyrus (DG), cornu ammonis (CA)4, CA3, CA2, CA1, as well as the subiculum, entorhinal cortex (EC) and the fusiform gyrus of the temporal cortex., all taken at the level of the hippocampal body. For each nucleus/region, 3-5 non-overlapping images were taken using either the 40x objective lens (1 20x snapshot was taken per section for XII + 5 20x snapshots were taken for the temporal cortex). Images were exported to Fiji imaging software (Schindelin et al., 2012) for analysis of staining, applying 2 different quantification methods. Where possible, only the region of interest (ROI) was captured in the field of view, otherwise, the ROI was manually outlined in Fiji using the inbuilt 'polygon line tool' and 'ROI manager' plugin, such that subsequent analyses would only be within the defined region. Duplicates were averaged to produce a single score. All imaging and quantification were performed by a single observer, blind to the diagnostic group. Methods of quantification included:

1. Quantitative analysis of number of astrocytes

For each image, the number of GFAP-positive astrocytes (represented as brown staining in the cell bodies, Figure 6.1) was manually counted using the in-built ‘multi point’ function. For each brain region, the total number of GFAP-positive astrocytes was divided by the total area, to obtain a single value that represented the number of GFAP-positive cells per mm<sup>2</sup>. Only astrocytes for which the cell nucleus was visible, were quantified.

## 2. Semi-quantitative analysis of astrocyte morphology

Each GFAP-positive cell was scored a semi-quantitative score based on a qualitative judgement by the observer, using a 4-tier scoring system (‘0’ = no astrogliosis, ‘1’ = mild astrogliosis, ‘2’ = moderate astrogliosis, ‘3’ = severe gliosis), adapted from (Gouw et al., 2008, Sofroniew and Vinters, 2010) (and represented in Figure 6.1). An R-score was calculated according to the equation  $R = \sum Pr(r + 1)$ , where P represents the percentage of cells, and “r” represents the reactivity/astrogliosis score (Chapter 5).

### 6.2.4: STATISTICAL ANALYSIS

Data was exported to SPSS for Windows (version 25; SPSS (IBM) Inc., Illinois, USA) for statistical analysis, and outcome data was tested for normality. Independent samples t-tests were used to compare the mean number of GFAP-positive cells per mm<sup>2</sup> between diagnostic groups and for the presence of clinical risk factors. Mann-Whitney U tests were used to compare R-scores between diagnostic groups and for the presence of risk factors, as well as chi-square testing to compare the proportions of cases with R-scores >100, >120 and >160. The relationship between GFAP-positive cells per mm<sup>2</sup> and clinical data was determined using Pearson correlations, while Kendall’s Tau correlations were used to compare R-score and clinical data. GFAP-positive cells per mm<sup>2</sup> data is presented as mean  $\pm$  standard error of the mean (SEM), and R-score is presented as median (interquartile range). P-values  $\leq 0.05$  were taken as statistically significant.

## 6.3: RESULTS

### 6.3.1: INFANT CHARACTERISTICS

The 34 SUDI cases were classified into two groups of eSUDI (n=9) and SIDS (n=25). eSUDI included infants who died of myocarditis (n=3), infection (n=3), Waterhouse-Friderichsen Syndrome (n=1), tension pneumothorax (n=1) and asphyxiation (n=1). Diagnostic groups were matched for clinical and autopsy parameters (Table 6.1), with the exception of a higher PMI for the SIDS group ( $p=0.05$ ). Subsequent correlation analysis did not reveal a significant relationship between PMI and GFAP-positive cells per mm<sup>2</sup> or R-score for any of the brain regions studied (data not shown), thus no correction for PMI was undertaken. The prevalence of the risk factors of cigarette smoke exposure and a recent URTI were not statistically different between the groups. Sleep-related risk factors were only relevant to the SIDS group, with a prevalence of being found prone of 30%, and bed-sharing and/or co-sleeping arrangements of 44%. One infant was suspected to have had a seizure prior to death but this could not be confirmed, and another infant had a maternal sibling with epilepsy.

Due to the proposal that the mechanism of death between bed-shared and non-bed-shared infants may differ according to the uncertainty of the contribution of asphyxia (Byard, 2013) and subsequent brain findings of differences accordingly (Spinelli et al., 2018, Jensen et al., 2014, Machaalani and Waters, 2008, Luijckx et al., 2020), analysis was undertaken between these two groups. The growth-related parameters differed, with non-bed-sharing infants having higher body length ( $p=0.02$ ). Although not statistically significant, additional trends for lower parameters of birth weight ( $p=0.07$ ), body weight ( $p=0.08$ ), and head circumferences ( $p=0.07$ ) in those who were bed-sharing were noted (Table 6.1).

**Table 6.1: Clinical, autopsy and risk factor data for the current study dataset.**

| Data characteristic          | eSUDI (n = 9) | SIDS (n = 25) | <i>p</i> -value <sup>a</sup> | SIDS non-bed-shared (n=14) | SIDS bed-shared (n=11) | <i>p</i> -value |
|------------------------------|---------------|---------------|------------------------------|----------------------------|------------------------|-----------------|
| <i>Clinical data</i>         |               |               |                              |                            |                        |                 |
| Male: female                 | 7:2           | 19:6          | 1.00                         | 12:2                       | 7:4                    | 0.35            |
| Gestational age (wks)        | 40±1.5        | 39±1.6        | 0.92                         | 39±1.5                     | 38±1.7                 | 0.19            |
| Birth weight (kg)            | 3.5±0.5       | 3.0±0.7       | 0.10                         | 3.2±0.6                    | 2.5±0.4                | 0.07            |
| Vit D (nmol/L)               | 49±24.2       | 53±36.2       | 0.82                         | 45±25.6                    | 85±59.7                | 0.36            |
| <i>Autopsy data</i>          |               |               |                              |                            |                        |                 |
| Age at death (months)        | 5.0±3.0       | 3.2±2.7       | 0.10                         | 3.5±2.6                    | 2.8±2.0                | 0.54            |
| PCA (weeks)                  | 62±14         | 53±12         | 0.79                         | 55±11                      | 52±12                  | 0.52            |
| Body weight (kg)             | 6.9±2.4       | 6.0±2.0       | 0.33                         | 6.7±2.1                    | 5.3±1.6                | 0.08            |
| Brain weight (g)             | 757±208       | 703±201       | 0.50                         | 747±182                    | 648±219                | 0.23            |
| Body length (cm)             | 68±10.0       | 64±8.8        | 0.27                         | 67±7.0                     | 59±9.2                 | <b>0.02</b>     |
| Head circumference (cm)      | 41±3.6        | 40±3.6        | 0.61                         | 42±3.2                     | 39±3.7                 | 0.07            |
| Thymus weight (g)            | 33±21.3       | 36±12.6       | 0.54                         | 40±12.5                    | 31±11.6                | 0.10            |
| <i>Autopsy parameters</i>    |               |               |                              |                            |                        |                 |
| PMI (hours)                  | 61±37         | 95±44         | <b>0.05</b>                  | 99±43*                     | 91±47                  | 0.64            |
| <i>Risk factors</i>          |               |               |                              |                            |                        |                 |
| Cigarette smoke exposure (%) | 3/6 (50)      | 16/20 (80)    | 0.29                         | 11/12 (92)                 | 5/8 (63)               | 0.26            |
| Recent URTI <sup>b</sup> (%) | 5/9 (56)      | 10/25 (40)    | 0.46                         | 7/14 (50)                  | 3/11 (27)              | 0.41            |
| Found prone (%)              | NA            | 6/20 (30)     | NA                           | 3/12                       | 3/8 (38)               | 0.64            |
| Bed-share/co-sleeping (%)    | NA            | 11/25 (44)    | NA                           | NA                         | 11/11 (100)            | NA              |

Results presented as the mean ± SD or proportions (%)

Bold used to highlight statistical significance

\*indicates statistical significance compared to eSUDI

<sup>a</sup>Obtained using Fischer's exact test for nominal data and independent samples t-test for continuous data. Significance taken at  $p \leq 0.05$

<sup>b</sup>Parent reported presence of an URTI in the 2 weeks prior to death

*Abbreviations: eSUDI explained sudden unexpected death in infancy, Gest. gestational, PCA post conception age, PMI post-mortem interval, SIDS sudden infant death syndrome, URTI upper respiratory tract infection*

### **6.3.2: GFAP EXPRESSION**

#### **6.3.2.1: ACROSS BRAIN REGIONS (FOCUS ON ESUDI)**

Considering the eSUDI group for baseline values, the number of GFAP positive cells varied between 3/mm<sup>2</sup> in ML and 273/mm<sup>2</sup> in the IGL of the cerebellum (Table 6.2).

For the R score, values ranged between 0 and 185 (Table 6.2), with 0 indicating no cells were stained positive, 100 indicating positive cells but not of reactive morphology, and >100 indicating astrocytes of reactive morphology (Figure 6.1).

#### **6.3.2.2: CORRELATIONS WITH DEVELOPMENTAL PARAMETERS**

Significant associations between the number of GFAP-positive astrocytes/mm<sup>2</sup> and R-score values were run for the whole cohort, although were additionally run for the eSUDI and SIDS groups alone to distinguish whether these parameters were altered in SIDS.

Mild-to-moderate negative correlations (r value ranging from -0.35 to -0.72, p value from <0.001 to 0.05) were observed between the number of GFAP-positive astrocytes/mm<sup>2</sup> in the infant brain and developmental parameters, with regions that were consistently affected by the majority of the parameters being the IGL and white matter of the cerebellum (birth, body & brain weight, body length, PCA and head circumference), and subiculum, temporal cortex and NSTT (body & brain weight, body length, PCA and head circumference) with a few others only correlating with one or two parameters (Supplementary Table 1). Other negative correlations included thymus weight in the subiculum and ION, and vitamin D in the DMNV and XII (Supplementary Table 1). Apart from the subiculum, associations between developmental parameters in the hippocampus were predominantly seen amongst the SIDS cohort.

Mild-moderate correlations (r value ranging from -0.39 to 0.29, p value from 0.01 to 0.05) were observed between the R-score and developmental parameters, including: body weight in the EC and temporal cortex, body length in the temporal cortex, PCA in CA4, head circumference and PCA in the EC and temporal cortex, thymus weight in the CA1 and vitamin D in the LRF (Supplementary Table 2).

### **6.3.2.3: COMPARISON ACCORDING TO DIAGNOSIS**

Although age and growth parameters were associated with differences in expression (as detailed above), given these were not consistent for all brain regions, or diagnostic categories, we did not include them as covariates in our analysis. Further, covariate analysis was not warranted given the two groups did not differ statistically for these parameters (Table 6.1). Compared to eSUDI, SIDS infants had higher GFAP/mm<sup>2</sup> in the IGL (p=0.02), CA4 (p=0.05), temporal cortex (p<0.01) and DMNV (p=0.02) (Table 6.2). When removing infants who were bed-shared, higher GFAP expression was only maintained in the IGL (p=0.04) and temporal cortex (p<0.01) (Table 6.2).

For R-score, SIDS infants had a higher value in the IGL (p=0.04) (Table 6.2), whereby 36% of SIDS infants received an R-score over 120, compared with 0% of eSUDI infants (Table 6.3). This effect was maintained when removing bed-sharers (p=0.03) (Table 6.2).

Given that the IGL, CA4 and subiculum showed the most variation in R-score values (Table 6.3), with many cases receiving a score >120, which we deem to be mild astrogliosis (Chapter 5), further analyses were undertaken to determine whether cases with R-Scores ≥120 differed to those <120. No differences were present for the IGL or for CA4, but for the subiculum, those with scores >120 had lower thymus weights (g) (29±3 vs 41±3, p=0.01) (data not shown).

### **6.3.2.4: COMPARISONS ACCORDING TO RISK FACTORS**

In order to determine whether risk factors differentially affected GFAP amongst SIDS, risk factors were run on the whole cohort first, and subsequently only on the SIDS only cohort. Sleep related risk factors of sleeping prone and bed-sharing were only run on the SIDS group.

When analysis was carried out on the whole cohort for GFAP/mm<sup>2</sup>, infants exposed to cigarette smoke had higher expression than non-smoke-exposed infants in the XII (p=0.02) and LRF (p=0.04) (Table 6.4). When risk-factor analysis was carried out on only the SIDS cohort, male infants had higher GFAP expression than female infants in the dentate nucleus (p=0.02) and molecular layer (p=0.01), infants exposed to cigarette smoke had lower GFAP expression than non-smoke-exposed infants in the IGL (p=0.03) and infants with a reported UTRI had higher GFAP expression in the XII (p=0.02) (Table 6.5). For the sleep related risk factors, infants found prone had lower GFAP expression in the IGL (p=0.05), white matter (p=0.01) and higher expression in the DG (p=0.05) (Table 6.5).

When analysis was carried out on the whole cohort, there were no significant differences in R-score values for any of the risk factors studied (Table 6.6). When risk-factor analysis was carried out on only the SIDS cohort, infants who were exposed to cigarette smoke had higher scores in the CA4 ( $p=0.05$ ), subiculum ( $p=0.03$ ) and temporal cortex ( $p=0.05$ ) (Table 6.7).



**Table 6.2: Comparison of the number of GFAP-positive astrocytes/mm<sup>2</sup> and R-score values between diagnostic groups.**

| Brain Region             | Number of GFAP cells (point-counting) |                |                                        |                                   |                                                            | Morphology (R-score) |                 |                                     |                                      |                                                        |
|--------------------------|---------------------------------------|----------------|----------------------------------------|-----------------------------------|------------------------------------------------------------|----------------------|-----------------|-------------------------------------|--------------------------------------|--------------------------------------------------------|
|                          | eSUDI<br>(n=9)                        | SIDS<br>(n=25) | <i>p</i> -value<br>SIDS<br>vs<br>eSUDI | SIDS non-<br>bed-shared<br>(n=14) | <i>p</i> -value<br>SIDS non-<br>bed-<br>shared vs<br>eSUDI | eSUDI<br>(n=9)       | SIDS<br>(n=25)  | <i>p</i> -value<br>SIDS vs<br>eSUDI | SIDS<br>non-bed-<br>shared<br>(n=14) | <i>p</i> -value<br>SIDS non-<br>bed-shared<br>vs eSUDI |
| <i>Cerebellum</i>        |                                       |                |                                        |                                   |                                                            |                      |                 |                                     |                                      |                                                        |
| Dentate Nucleus          | 179±39                                | 172±13         | 0.86                                   | 180±19                            | 0.99                                                       | 100 (0)              | 100 (0)         | 0.80                                | 100 (0)                              | 0.75                                                   |
| IGL                      | <b>237±39</b>                         | <b>332±20</b>  | <b>0.02</b>                            | <b>322±20</b>                     | <b>0.04</b>                                                | <b>106 (12)</b>      | <b>114 (26)</b> | <b>0.03</b>                         | <b>115 (28)</b>                      | <b>0.04</b>                                            |
| ML                       | 3±2                                   | 3±1            | 0.84                                   | 4±2                               | 0.92                                                       | 0 (100)              | 0 (50)          | 0.70                                | 0 (100)                              | 0.70                                                   |
| White matter             | 118±29                                | 141±12         | 0.40                                   | 145±19                            | 0.43                                                       | 105 (8)              | 100 (5)         | 0.34                                | 100 (4)                              | 0.36                                                   |
| <i>Hippocampus</i>       |                                       |                |                                        |                                   |                                                            |                      |                 |                                     |                                      |                                                        |
| CA4                      | <b>187±23</b>                         | <b>258±17</b>  | <b>0.05</b>                            | 256±26                            | 0.10                                                       | 117 (41)             | 111 (32)        | 0.17                                | 110 (36)                             | 0.12                                                   |
| CA3                      | 115±26                                | 145±19         | 0.43                                   | 142±27                            | 0.53                                                       | 100 (0)              | 102 (0)         | 0.64                                | 100 (0)                              | 0.42                                                   |
| CA2                      | 76±24                                 | 113±18         | 0.32                                   | 110±26                            | 0.21                                                       | 100 (100)            | 100 (0)         | 0.64                                | 100 (0)                              | 0.50                                                   |
| CA1                      | 126±11                                | 147±14         | 0.43                                   | 141±21                            | 0.53                                                       | 100 (0)              | 100 (9)         | 0.44                                | 100 (7)                              | 0.31                                                   |
| DG                       | 216±34                                | 215±19         | 1.00                                   | 236±28                            | 0.67                                                       | 100 (13)             | 100 (8)         | 0.80                                | 100 (14)                             | 0.70                                                   |
| EC                       | 186±55                                | 162±15         | 0.69                                   | 168±18                            | 0.76                                                       | 100 (1)              | 102 (17)        | 0.54                                | 100 (8)                              | 0.27                                                   |
| Subiculum                | 159±11                                | 176±11         | 0.44                                   | 177±16                            | 0.48                                                       | 124 (41)             | 112 (37)        | 0.54                                | 112 (34)                             | 0.50                                                   |
| Temporal cortex          | <b>71±12</b>                          | <b>140±10</b>  | <b>&lt;0.01</b>                        | <b>137±13</b>                     | <b>&lt;0.01</b>                                            | 100 (3)              | 102 (13)        | 0.64                                | 101 (7)                              | 0.18                                                   |
| <i>Medulla (rostral)</i> |                                       |                |                                        |                                   |                                                            |                      |                 |                                     |                                      |                                                        |
| AN                       | 165±31                                | 197±28         | 0.52                                   | 221±49                            | 0.36                                                       | 100 (0)              | 100 (0)         | 0.67                                | 100 (2)                              | 0.70                                                   |
| Cun                      | 123±36                                | 143±23         | 0.63                                   | 111±33                            | 0.81                                                       | 100 (26)             | 100 (0)         | 0.72                                | 100 (0)                              | 0.41                                                   |
| DMNV                     | <b>46±13</b>                          | <b>102±19</b>  | <b>0.02</b>                            | 90±24                             | 0.13                                                       | 100 (50)             | 100 (0)         | 0.24                                | 100 (0)                              | 0.16                                                   |
| XII                      | 26±8                                  | 39±8           | 0.34                                   | 34±8                              | 0.46                                                       | 100 (0)              | 100 (0)         | 0.72                                | 100 (0)                              | 0.71                                                   |
| ION                      | 92±20                                 | 138±13         | 0.07                                   | 141±22                            | 0.13                                                       | 100 (0)              | 100 (0)         | 0.71                                | 100 (0)                              | 0.93                                                   |

|             |        |        |      |        |      |          |          |      |          |      |
|-------------|--------|--------|------|--------|------|----------|----------|------|----------|------|
| <b>LRF</b>  | 43±13  | 51±10  | 0.63 | 53±13  | 0.58 | 100 (50) | 100 (0)  | 0.82 | 100 (0)  | 0.72 |
| <b>NSTT</b> | 135±26 | 170±17 | 0.27 | 140±17 | 0.89 | 100 (14) | 106 (18) | 0.77 | 104 (16) | 0.48 |
| <b>NTS</b>  | 70±20  | 59±9   | 0.55 | 51±9   | 0.41 | 100 (0)  | 100 (2)  | 0.42 | 105 (10) | 0.29 |
| <b>Vest</b> | 60±22  | 80±10  | 0.34 | 79±15  | 0.47 | 100 (00) | 100 (0)  | 0.70 | 100 (0)  | 0.64 |

Bold used to highlight statistical significance. Obtained using independent samples t-test. Significance taken at  $p \leq 0.05$

*Abbreviations:* *AN* Arcuate nucleus, *CA* Cornu Ammonis, *Cun* Cuneate nucleus, *DG* dentate gyrus, *DMNV* dorsal motor nucleus of the vagus, *EC* entorhinal cortex, *eSUDI* explained sudden unexpected death in infancy, *XII* hypoglossal nucleus, *IGL* internal granular layer of the cerebellar cortex, *ION* inferior olivary nucleus, *LRF* lateral reticular formation, *ML* molecular layer of the cerebellar cortex, *NSTT* nucleus of the spinal trigeminal tract, *NTS* nucleus of the solitary tract, *PCA* post conception age, *SIDS* sudden infant death syndrome, *Vest* vestibular nucleus.

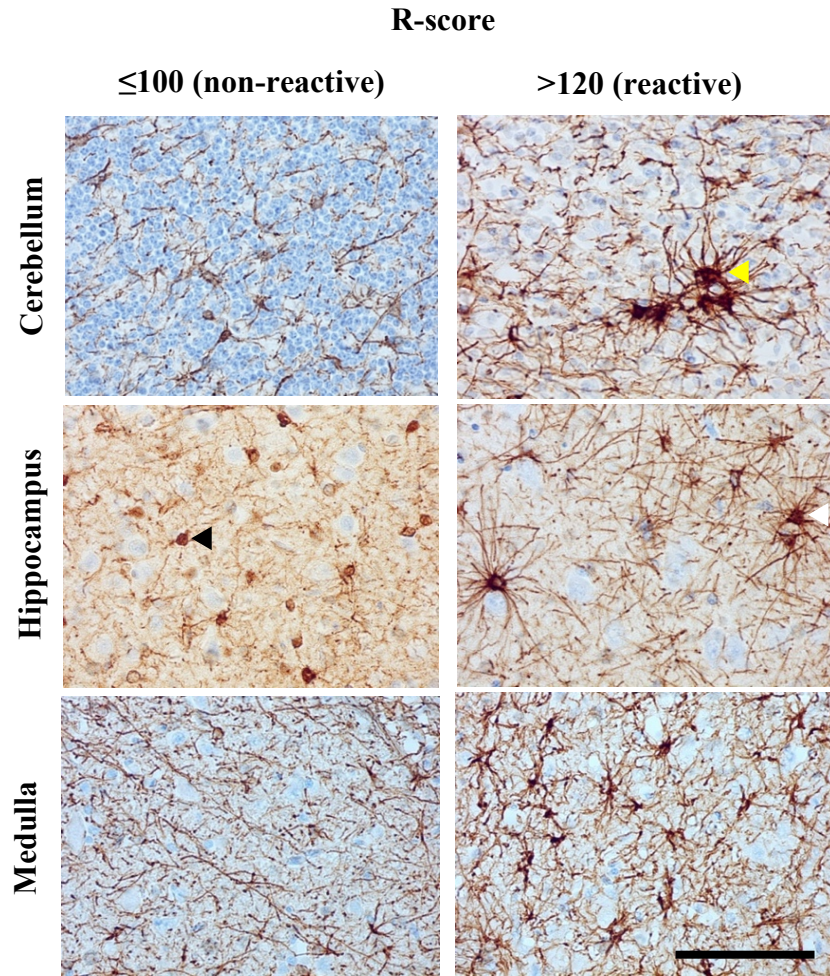
**Table 6.3: Comparison of diagnostic groups according to proportion of cases with R-score sub-categories of >100, >120 and >160.**

| Brain Region              | R-score |      |                 |                           |        |                 |
|---------------------------|---------|------|-----------------|---------------------------|--------|-----------------|
|                           | >100    |      |                 | >120 (>160 <sup>^</sup> ) |        |                 |
|                           | eSUDI   | SIDS | <i>p</i> -value | eSUDI                     | SIDS   | <i>p</i> -value |
| <b><i>Cerebellum</i></b>  |         |      |                 |                           |        |                 |
| <b>Dentate Nucleus</b>    | 0       | 8    | 1.00            | 0                         | 0      | -               |
| <b>IGL</b>                | 78      | 92   | 0.28            | 0                         | 36 (4) | 0.07            |
| <b>ML</b>                 | 0       | 0    | -               | 0                         | 0      | -               |
| <b>White matter</b>       | 44      | 36   | 0.70            | 0                         | 4      | 1.00            |
| <b><i>Hippocampus</i></b> |         |      |                 |                           |        |                 |
| <b>CA4</b>                | 100     | 79   | 0.56            | 43 (14)                   | 33     | 0.68            |
| <b>CA3</b>                | 0       | 8    | 0.64            | 0                         | 4      | 1.00            |
| <b>CA2</b>                | 0       | 0    | -               | 0                         | 0      | -               |
| <b>CA1</b>                | 12      | 33   | 0.39            | 12                        | 8      | 1.00            |
| <b>DG</b>                 | 43      | 25   | 0.38            | 0                         | 21     | 0.56            |
| <b>EC</b>                 | 29      | 50   | 0.41            | 0                         | 21     | 0.56            |
| <b>Subiculum</b>          | 100     | 92   | 1.00            | 57                        | 38 (4) | 0.41            |
| <b>Temporal cortex</b>    | 29      | 67   | 0.10            | 14                        | 17     | 1.00            |
| <b><i>Medulla</i></b>     |         |      |                 |                           |        |                 |
| <b>AN</b>                 | 13      | 21   | 1.00            | 0                         | 5      | 1.00            |
| <b>Cun</b>                | 22      | 20   | 1.00            | 0                         | 0      | -               |
| <b>DMNV</b>               | 0       | 11   | 0.54            | 0                         | 6      | 0.67            |
| <b>XII</b>                | 0       | 6    | 1.00            | 0                         | 6      | 1.00            |
| <b>ION</b>                | 11      | 10   | 1.00            | 0                         | 0      | -               |
| <b>LRF</b>                | 0       | 0    | -               | 0                         | 0      | -               |
| <b>NSTT</b>               | 44      | 62   | 0.44            | 11                        | 19     | 1.00            |
| <b>NTS</b>                | 0       | 27   | 0.26            | 0                         | 7      | 1.00            |
| <b>Vest</b>               | 0       | 0    | -               | 0                         | 0      | -               |

Values expressed as percentages rounded to nearest whole integer.

Bold highlights statistical significance using Chi square with Fisher's Exact test. Significance taken at  $p \leq 0.05$

<sup>^</sup>where applicable, >160 was applied based on previous chapter where this cut-off is proposed to be indicative of moderate astrogliosis.



**Figure 6.1: GFAP immunohistochemistry in the SUDI brain.** Examples of staining that received an R-score of  $\leq 100$  vs  $> 120$  in the IGL of the cerebellum, CA4 of the hippocampus and NSTT in the medulla. Scoring of each GFAP-positive astrocytes included 0' = no astrogliosis (black arrow), '1' = mild astrogliosis (white arrow), '2' = moderate astrogliosis (yellow arrow). Scale bar = 100 $\mu$ m.

**Table 6.4: Comparison of number of GFAP-positive cells per mm<sup>2</sup> according to major SIDS risk factors amongst the whole cohort.**

| SIDS risk factors (whole cohort) |             |              |                 |                          |              |                 |                                   |            |                 |
|----------------------------------|-------------|--------------|-----------------|--------------------------|--------------|-----------------|-----------------------------------|------------|-----------------|
|                                  | Sex         |              |                 | Cigarette smoke exposure |              |                 | Upper respiratory tract infection |            |                 |
| Brain Region                     | Male (n=26) | Female (n=8) | <i>p</i> -value | No (n=7)                 | Yes (n=19)   | <i>p</i> -value | No (n=19)                         | Yes (n=15) | <i>p</i> -value |
| <i>Cerebellum</i>                |             |              |                 |                          |              |                 |                                   |            |                 |
| Dentate Nucleus                  | 186±16      | 137±23       | 0.12            | 125±35                   | 193±16       | 0.06            | 168±18                            | 180±22     | 0.67            |
| IGL                              | 303±22      | 321±41       | 0.69            | 319±56                   | 312±23       | 0.89            | 317±27                            | 294±27     | 0.56            |
| ML                               | 3±1         | 3±2          | 0.91            | 6±2                      | 3±1          | 0.11            | 3±1                               | 3±1        | 0.87            |
| White matter                     | 135±14      | 135±21       | 0.99            | 145±29                   | 144±15       | 0.49            | 139±14                            | 130±20     | 0.69            |
| <i>Hippocampus</i>               |             |              |                 |                          |              |                 |                                   |            |                 |
| CA4                              | 251±17      | 208±30       | 0.23            | 222±35                   | 253±22       | 0.49            | 258±20                            | 218±21     | 0.19            |
| CA3                              | 142±18      | 126±34       | 0.67            | 141±27                   | 133±24       | 0.86            | 141±17                            | 134±30     | 0.83            |
| CA2                              | 103±18      | 112±31       | 0.81            | 146±35                   | 92±21        | 0.21            | 121±21                            | 83±22      | 0.22            |
| CA1                              | 145±12      | 133±25       | 0.66            | 149±17                   | 138±17       | 0.65            | 148±14                            | 134±19     | 0.53            |
| DG                               | 224±20      | 187±28       | 0.37            | 196±32                   | 207±22       | 0.80            | 236±19                            | 188±28     | 0.15            |
| EC                               | 162±19      | 187±33       | 0.54            | 194±40                   | 147±18       | 0.24            | 175±20                            | 159±29     | 0.64            |
| Subiculum                        | 174±10      | 167±19       | 0.75            | 169±18                   | 169±11       | 1.00            | 183±12                            | 158±11     | 0.14            |
| Temporal cortex                  | 116±10      | 153±23       | 0.38            | 142±23                   | 126±12       | 0.53            | 136±14                            | 110±13     | 0.18            |
| <i>Medulla (rostral)</i>         |             |              |                 |                          |              |                 |                                   |            |                 |
| AN                               | 191±26      | 178±40       | 0.81            | 131±36                   | 203±33       | 0.22            | 196±36                            | 177±22     | 0.68            |
| Cun                              | 131±22      | 152±37       | 0.64            | 104±39                   | 154±30       | 0.39            | 112±23                            | 162±29     | 0.19            |
| DMNV                             | 88±17       | 67±24        | 0.55            | 90±34                    | 96±20        | 0.88            | 85±20                             | 82±20      | 0.91            |
| XII                              | 35±7        | 31±14        | 0.79            | <b>17±4</b>              | <b>40±8</b>  | <b>0.02</b>     | 25±6                              | 45±10      | 0.11            |
| ION                              | 118±14      | 146±22       | 0.32            | 105±22                   | 139±19       | 0.34            | 119±16                            | 130±18     | 0.66            |
| LRF                              | 52±8        | 37±18        | 0.42            | <b>29±8</b>              | <b>59±11</b> | <b>0.04</b>     | 38±9                              | 60±12      | 0.17            |
| NSTT                             | 164±17      | 146±29       | 0.61            | 201±26                   | 167±23       | 0.40            | 182±12                            | 134±26     | 0.10            |

|             |       |       |      |       |       |      |       |       |      |
|-------------|-------|-------|------|-------|-------|------|-------|-------|------|
| <b>NTS</b>  | 57±8  | 86±31 | 0.20 | 92±38 | 60±9  | 0.47 | 69±13 | 57±12 | 0.51 |
| <b>Vest</b> | 74±11 | 75±19 | 0.95 | 72±23 | 84±15 | 0.66 | 71±13 | 78±15 | 0.74 |

Results presented as the mean ± SEM

Bold used to highlight statistical significance, obtained using independent samples t-test. Significance taken at  $p \leq 0.05$ .

Table 6.5: Comparison of number of GFAP-positive cells per mm<sup>2</sup> according to major risk factors amongst the SIDS cohort.

| Brain Region             | SIDS Risk Factors (SIDS only) |              |         |                          |            |         |                                   |            |         |                         |            |         |             |           |         |
|--------------------------|-------------------------------|--------------|---------|--------------------------|------------|---------|-----------------------------------|------------|---------|-------------------------|------------|---------|-------------|-----------|---------|
|                          | Sex                           |              |         | Cigarette smoke exposure |            |         | Upper respiratory tract infection |            |         | Co-sleeping/Bed-sharing |            |         | Found prone |           |         |
|                          | Male (n=19)                   | Female (n=6) | p-value | No (n=4)                 | Yes (n=16) | p-value | No (n=15)                         | Yes (n=10) | p-value | No (n=14)               | Yes (n=11) | p-value | No (n=14)   | Yes (n=6) | p-value |
| <i>Cerebellum</i>        |                               |              |         |                          |            |         |                                   |            |         |                         |            |         |             |           |         |
| Dentate Nucleus          | 184±16                        | 133±12       | 0.02    | 146±30                   | 181±17     | 0.35    | 161±15                            | 187±24     | 0.34    | 180±19                  | 162±18     | 0.52    | 177±18      | 151±29    | 0.51    |
| IGL                      | 322±23                        | 366±35       | 0.35    | 425±24                   | 316±22     | 0.03    | 344±28                            | 316±26     | 0.50    | 322±20                  | 345±38     | 0.58    | 368±23      | 283±32    | 0.05    |
| ML                       | 4±1                           | 0±0          | 0.01    | 5±6                      | 3±2        | 0.55    | 3±1                               | 4±2        | 0.66    | 4±2                     | 2±1        | 0.50    | 4±2         | 4±2       | 0.96    |
| White matter             | 143±15                        | 134±21       | 0.74    | 179±12                   | 138±15     | 0.21    | 146±14                            | 134±23     | 0.64    | 145±19                  | 136±15     | 0.74    | 166±13      | 93±13     | 0.01    |
| <i>Hippocampus</i>       |                               |              |         |                          |            |         |                                   |            |         |                         |            |         |             |           |         |
| CA4                      | 266±19                        | 227±34       | 0.37    | 268±50                   | 257±24     | 0.86    | 267±25                            | 244±22     | 0.50    | 256±26                  | 259±20     | 0.93    | 264±28      | 243±19    | 0.63    |
| CA3                      | 148±21                        | 134±45       | 0.76    | 145±39                   | 143±26     | 0.98    | 138±22                            | 155±35     | 0.67    | 142±27                  | 149±27     | 0.87    | 142±26      | 143±45    | 0.98    |
| CA2                      | 111±21                        | 124±44       | 0.78    | 185±64                   | 100±23     | 0.17    | 134±24                            | 84±27      | 0.19    | 110±26                  | 118±27     | 0.83    | 123±30      | 89±19     | 0.35    |
| CA1                      | 150±15                        | 137±36       | 0.71    | 162±32                   | 136±19     | 0.61    | 155±17                            | 136±24     | 0.50    | 141±21                  | 156±17     | 0.61    | 146±20      | 151±30    | 0.89    |
| DG                       | 222±23                        | 192±35       | 0.56    | 298±57                   | 213±24     | 0.92    | 237±22                            | 186±34     | 0.20    | 236±28                  | 186±23     | 0.21    | 193±24      | 280±30    | 0.05    |
| EC                       | 161±17                        | 168±31       | 0.85    | 198±51                   | 153±19     | 0.37    | 176±21                            | 143±19     | 0.29    | 168±18                  | 154±26     | 0.65    | 177±22      | 162±22    | 0.69    |
| Subiculum                | 178±11                        | 167±28       | 0.68    | 181±36                   | 168±11     | 0.67    | 192±15                            | 154±13     | 0.07    | 177±16                  | 175±14     | 0.94    | 181±14      | 157±12    | 0.30    |
| Temporal cortex          | 131±10                        | 165±23       | 0.15    | 166±27                   | 132±13     | 0.25    | 149±14                            | 128±14     | 0.31    | 137±13                  | 144±16     | 0.72    | 155±14      | 120±16    | 0.15    |
| <i>Medulla (rostral)</i> |                               |              |         |                          |            |         |                                   |            |         |                         |            |         |             |           |         |
| AN                       | 196±33                        | 199±59       | 0.97    | 145±77                   | 196±40     | 0.56    | 198±45                            | 196±29     | 0.97    | 221±49                  | 175±32     | 0.43    | 218±51      | 141±6     | 0.17    |
| Cun                      | 133±26                        | 171±50       | 0.48    |                          |            | NA^     | 127±28                            | 161±37     | 0.48    | 111±33                  | 179±28     | 0.14    | 128±35      | 175±46    | 0.42    |
| DMNV                     | 112±22                        | 67±32        | 0.33    |                          |            | NA      | 96±27                             | 110±27     | 0.71    | 90±24                   | 117±31     | 0.50    | 121±30      | 86±37     | 0.47    |
| XII                      | 42±19                         | 42±19        | 0.86    |                          |            | NA      | 22±7                              | 59±14      | 0.02    | 34±8                    | 44±17      | 0.58    | 41±9        | 51±21     | 0.70    |
| ION                      | 132±15                        | 159±29       | 0.41    | 132±18                   | 147±21     | 0.76    | 129±15                            | 151±24     | 0.43    | 141±22                  | 135±16     | 0.85    | 149±20      | 140±30    | 0.81    |



|             |        |        |      |        |        |      |        |        |      |        |        |      |        |        |      |
|-------------|--------|--------|------|--------|--------|------|--------|--------|------|--------|--------|------|--------|--------|------|
| <b>LRF</b>  | 56±10  | 33±26  | 0.30 | 19±11  | 58±12  | 0.16 | 40±11  | 65±16  | 0.20 | 53±13  | 48±14  | 0.79 | 55±15  | 42±19  | 0.60 |
| <b>NSTT</b> | 178±19 | 146±41 | 0.44 | 255±16 | 168±23 | 0.11 | 195±14 | 137±34 | 0.10 | 140±17 | 202±28 | 0.07 | 177±23 | 170±44 | 0.87 |
| <b>NTS</b>  | 59±11  | 59±8   | 0.99 |        |        | NA   | 53±4   | 67±21  | 0.54 | 51±9   | 68±15  | 0.34 | 61±5   | 64±25  | 0.92 |
| <b>Vest</b> | 85±12  | 65±14  | 0.43 | 77±24  | 85±14  | 0.78 | 72±41  | 93±45  | 0.32 | 79±15  | 82±14  | 0.91 | 79±12  | 75±23  | 0.87 |

Results presented as the mean ± SEM

Bold used to highlight statistical significance. Obtained using independent samples t-test. Significance taken at  $p \leq 0.05$ .

^NA due to low case numbers (n<3) due to tissue availability.

**Table 6.6: Comparison of R-score values according to major SIDS risk factors amongst the whole cohort.**

| Brain Region             | SIDS risk factors (whole cohort) |              |                 |                          |            |                 |                                   |            |                 |
|--------------------------|----------------------------------|--------------|-----------------|--------------------------|------------|-----------------|-----------------------------------|------------|-----------------|
|                          | Sex                              |              |                 | Cigarette smoke exposure |            |                 | Upper respiratory tract infection |            |                 |
|                          | Male (n=26)                      | Female (n=8) | <i>p</i> -value | No (n=7)                 | Yes (n=19) | <i>p</i> -value | No (n=19)                         | Yes (n=15) | <i>p</i> -value |
| <i>Cerebellum</i>        |                                  |              |                 |                          |            |                 |                                   |            |                 |
| <b>Dentate Nucleus</b>   | 100 (0)                          | 100 (0)      | 0.75            | 100 (0)                  | 100 (0)    | 0.70            | 100 (0)                           | 100 (0)    | 0.93            |
| <b>IGL</b>               | 114 (25)                         | 110 (14)     | 0.31            | 111 (15)                 | 114 (23)   | 0.19            | 110 (12)                          | 114 (26)   | 0.49            |
| <b>ML</b>                | 0 (100)                          | 0 (75)       | 0.95            | 100 (100)                | 0 (0)      | 0.17            | 0 (100)                           | 0 (100)    | 1.00            |
| <b>White matter</b>      | 100 (9)                          | 100 (2)      | 0.37            | 100 (0)                  | 102 (10)   | 0.17            | 100 (2)                           | 103 (8)    | 0.39            |
| <i>Hippocampus</i>       |                                  |              |                 |                          |            |                 |                                   |            |                 |
| <b>CA4</b>               | 114 (32)                         | 114 (40)     | 0.76            | 107 (34)                 | 118 (38)   | 0.34            | 110 (22)                          | 121 (36)   | 0.12            |
| <b>CA3</b>               | 100 (0)                          | 100 (0)      | 0.87            | 100 (0)                  | 100 (0)    | 1.00            | 100 (0)                           | 100 (0)    | 0.74            |
| <b>CA2</b>               | 100 (0)                          | 100 (0)      | 0.95            | 100 (0)                  | 100 (13)   | 0.45            | 100 (0)                           | 100 (0)    | 0.98            |
| <b>CA1</b>               | 100 (9)                          | 100 (0)      | 0.62            | 100 (0)                  | 100 (9)    | 0.25            | 100 (8)                           | 100 (7)    | 0.89            |
| <b>DG</b>                | 100 (18)                         | 100 (10)     | 0.76            | 100 (3)                  | 100 (15)   | 0.54            | 100 (5)                           | 100 (12)   | 0.57            |
| <b>EC</b>                | 100 (12)                         | 100 (7)      | 0.84            | 100 (27)                 | 100 (7)    | 0.92            | 106 (22)                          | 100 (3)    | 0.12            |
| <b>Subiculum</b>         | 112 (41)                         | 117 (19)     | 0.66            | 108 (12)                 | 117 (42)   | 0.09            | 108 (41)                          | 117 (33)   | 0.44            |
| <b>Temporal cortex</b>   | 102 (13)                         | 107 (11)     | 0.76            | 108 (28)                 | 101 (13)   | 0.42            | 107 (17)                          | 100 (3)    | 0.19            |
| <i>Medulla (rostral)</i> |                                  |              |                 |                          |            |                 |                                   |            |                 |
| <b>AN</b>                | 100 (0)                          | 100 (16)     | 0.51            | 100 (0)                  | 100 (0)    | 0.66            | 100 (0)                           | 100 (0)    | 1.00            |
| <b>Cun</b>               | 100 (0)                          | 100 (8)      | 0.50            | 100 (50)                 | 100 (8)    | 0.20            | 100 (0)                           | 100 (4)    | 0.33            |
| <b>DMNV</b>              | 100 (0)                          | 100 (63)     | 0.29            | 100 (25)                 | 100 (0)    | 0.50            | 100 (0)                           | 100 (0)    | 0.98            |
| <b>XII</b>               | 100 (0)                          | 100 (25)     | 0.55            | 100 (0)                  | 100 (0)    | 0.89            | 100 (0)                           | 100 (0)    | 0.79            |
| <b>ION</b>               | 100 (0)                          | 100 (0)      | 0.81            | 100 (4)                  | 100 (0)    | 0.86            | 100 (0)                           | 100 (0)    | 0.40            |
| <b>LRF</b>               | 100 (0)                          | 100 (50)     | 0.60            | 100 (25)                 | 100 (0)    | 0.86            | 100 (0)                           | 100 (0)    | 0.79            |
| <b>NSTT</b>              | 104 (19)                         | 104 (8)      | 0.50            | 103 (10)                 | 108 (21)   | 0.64            | 106 (18)                          | 100 (11)   | 0.33            |

|             |         |         |      |         |         |      |         |         |      |
|-------------|---------|---------|------|---------|---------|------|---------|---------|------|
| <b>NTS</b>  | 100 (0) | 100 (7) | 0.89 | 100 (0) | 100 (8) | 0.38 | 100 (1) | 100 (0) | 0.61 |
| <b>Vest</b> | 100 (0) | 100 (0) | 0.88 | 100 (0) | 100 (0) | 0.83 | 100 (0) | 100 (0) | 0.74 |

Results presented as the medians (interquartile range).

Bold used to highlight statistical significance. Obtained using Mann-Whitney U test. Significance taken at  $p \leq 0.05$ .

**Table 6.7: Comparison of R-score values according to major risk factors amongst the SIDS cohort.**

| Brain Region           | SIDS Risk Factors (SIDS only) |              |         |                          |                 |             |                                   |            |         |                         |            |         |             |           |         |
|------------------------|-------------------------------|--------------|---------|--------------------------|-----------------|-------------|-----------------------------------|------------|---------|-------------------------|------------|---------|-------------|-----------|---------|
|                        | Sex                           |              |         | Cigarette smoke exposure |                 |             | Upper respiratory tract infection |            |         | Co-sleeping/Bed-sharing |            |         | Found prone |           |         |
|                        | Male (n=19)                   | Female (n=6) | p-value | No (n=4)                 | Yes (n=16)      | p-value     | No (n=15)                         | Yes (n=10) | p-value | No (n=14)               | Yes (n=11) | p-value | No (n=14)   | Yes (n=6) | p-value |
| <i>Cerebellum</i>      |                               |              |         |                          |                 |             |                                   |            |         |                         |            |         |             |           |         |
| <b>Dentate Nucleus</b> | 100 (0)                       | 100 (0)      | 0.72    | 100 (0)                  | 100 (0)         | 0.74        | 100 (0)                           | 100 (0)    | 0.89    | 100 (0)                 | 100 (0)    | 1.00    | 100 (0)     | 100 (0)   | 0.83    |
| <b>IGL</b>             | 115 (29)                      | 111 (17)     | 0.44    | 113 (10)                 | 114 (26)        | 0.55        | 113 (13)                          | 124 (36)   | 0.34    | 115 (28)                | 111 (28)   | 0.61    | 114 (14)    | 124 (37)  | 0.54    |
| <b>ML</b>              | 0 (100)                       | 0 (0)        | 0.27    | 50 (100)                 | 0 (0)           | 0.39        | 0 (0)                             | 0 (100)    | 0.68    | 0 (100)                 | 0 (0)      | 0.69    | 0 (100)     | 0 (100)   | 0.90    |
| <b>White matter</b>    | 100 (8)                       | 100 (1)      | 0.24    | 100 (8)                  | 100 (0)         | 0.29        | 100 (2)                           | 100 (7)    | 0.98    | 100 (4)                 | 100 (7)    | 0.73    | 100 (4)     | 101 (32)  | 0.90    |
| <i>Hippocampus</i>     |                               |              |         |                          |                 |             |                                   |            |         |                         |            |         |             |           |         |
| <b>CA4</b>             | 110 (31)                      | 114 (37)     | 1.00    | <b>100 (0)</b>           | <b>118 (35)</b> | <b>0.05</b> | 106 (23)                          | 116 (28)   | 0.29    | 110 (36)                | 113 (23)   | 0.89    | 109 (35)    | 111 (21)  | 0.52    |
| <b>CA3</b>             | 100 (0)                       | 100 (0)      | 0.73    | 100 (0)                  | 100 (0)         | 0.88        | 100 (0)                           | 100 (5)    | 0.44    | 100 (0)                 | 100 (5)    | 0.44    | 100 (0)     | 100 (6)   | 0.58    |
| <b>CA2</b>             | 100 (0)                       | 100 (50)     | 0.73    | 100 (0)                  | 100 (0)         | 0.63        | 100 (0)                           | 100 (0)    | 0.89    | 100 (0)                 | 100 (0)    | 0.84    | 100 (0)     | 100 (0)   | 0.64    |
| <b>CA1</b>             | 100 (9)                       | 100 (8)      | 0.68    | 100 (0)                  | 100 (8)         | 0.42        | 100 (10)                          | 100 (7)    | 0.80    | 100 (7)                 | 100 (11)   | 0.59    | 100 (0)     | 104 (14)  | 0.21    |
| <b>DG</b>              | 100 (25)                      | 100 (5)      | 0.73    | 100 (0)                  | 100 (21)        | 0.42        | 100 (6)                           | 100 (14)   | 0.80    | 100 (14)                | 100 (6)    | 0.75    | 100 (0)     | 113 (32)  | 0.18    |
| <b>EC</b>              | 100 (19)                      | 105 (19)     | 0.89    | 121 (0)                  | 100 (8)         | 0.25        | 107 (27)                          | 100 (6)    | 0.10    | 100 (8)                 | 106 (24)   | 0.37    | 105 (26)    | 103 (10)  | 0.64    |

|                          |             |             |      |                     |                     |             |             |             |      |             |             |      |             |             |      |
|--------------------------|-------------|-------------|------|---------------------|---------------------|-------------|-------------|-------------|------|-------------|-------------|------|-------------|-------------|------|
| <b>Subiculum</b>         | 112<br>(39) | 117<br>(24) | 0.73 | <b>102 (0)</b>      | <b>117<br/>(40)</b> | <b>0.03</b> | 107<br>(42) | 115<br>(34) | 0.63 | 112<br>(34) | 112<br>(45) | 0.89 | 112<br>(23) | 138<br>(40) | 0.21 |
| <b>Temporal cortex</b>   | 102<br>(15) | 108<br>(15) | 0.72 | <b>101<br/>(10)</b> | <b>119<br/>(30)</b> | <b>0.05</b> | 108<br>(16) | 101 (8)     | 0.26 | 101 (7)     | 109<br>(20) | 0.06 | 108<br>(20) | 102<br>(26) | 0.31 |
| <b>Medulla (rostral)</b> |             |             |      |                     |                     |             |             |             |      |             |             |      |             |             |      |
| <b>AN</b>                | 100 (0)     | 101<br>(43) | 0.31 | 100 (0)             | 100 (0)             | 0.66        | 100 (0)     | 100 (9)     | 0.72 | 100 (3)     | 100 (1)     | 0.91 | 100 (0)     | 100 (5)     | 0.61 |
| <b>Cun</b>               | 100 (0)     | 100 (9)     | 0.44 |                     |                     | NA^         | 100 (2)     | 100 (4)     | 0.97 | 100 (0)     | 100 (8)     | 0.45 | 100 (0)     | 100 (8)     | 0.61 |
| <b>DMNV</b>              | 100 (0)     | 100<br>(75) | 0.33 |                     |                     | NA          | 100 (0)     | 100 (0)     | 0.76 | 100 (0)     | 100<br>(11) | 0.70 | 100 (0)     | 100<br>(29) | 1.00 |
| <b>XII</b>               | 100<br>(38) | 100<br>(75) | 0.38 |                     |                     | NA          | 100 (0)     | 100 (0)     | 1.00 | 100 (0)     | 100 (0)     | 1.00 | 100 (0)     | 100<br>(25) | 0.66 |
| <b>ION</b>               | 100 (0)     | 100 (0)     | 0.72 | 100 (0)             | 100 (0)             | 0.70        | 100 (0)     | 100 (0)     | 0.55 | 100 (0)     | 100 (0)     | 0.47 | 100 (0)     | 100 (0)     | 1.00 |
| <b>LRF</b>               | 100 (0)     | 100<br>(75) | 0.31 | 100 (0)             | 100 (0)             | 0.52        | 100 (0)     | 100 (0)     | 0.81 | 100 (0)     | 100 (0)     | 0.81 | 100 (0)     | 100<br>(25) | 1.00 |
| <b>NSTT</b>              | 105<br>(22) | 106 (8)     | 0.60 | 108 (0)             | 109<br>(30)         | 1.00        | 107<br>(16) | 100<br>(16) | 0.19 | 104<br>(16) | 107<br>(24) | 0.51 | 106<br>(16) | 102<br>(27) | 0.96 |
| <b>NTS</b>               | 100 (2)     | 100 (0)     | 0.84 |                     |                     | NA          | 100 (6)     | 100 (3)     | 0.69 | 100<br>(10) | 100 (2)     | 0.96 | 100<br>(17) | 100 (0)     | 0.43 |
| <b>Vest</b>              | 100 (0)     | 100 (0)     | 1.00 | 100 (0)             | 100 (0)             | 1.00        | 100 (0)     | 100 (0)     | 1.00 | 100 (0)     | 100 (0)     | 1.00 | 100 (0)     | 100 (0)     | 1.00 |

Results presented as the medians (interquartile range)

Bold used to highlight statistical significance. Obtained using Mann-Whitney U test. Significance taken at  $p \leq 0.05$ .

^Not provided due to low case numbers ( $n < 3$ ) due to tissue availability.

## **6.4: DISCUSSION**

This study is unique in that it quantitatively assessed both the number of GFAP-positive astrocytes, as well as their level of ‘reactivity’ based on their morphology in the developing human brain and reports on factors that affect GFAP expression, including developmental parameters, sub-categorisation of SIDS, and major SIDS risk factors (findings summarised in Table 6.8) The main findings were that negative correlations between GFAP and growth parameters of brain and body weight, length and head circumference, were evident, although were region-dependent, SIDS infants had higher GFAP expression in the IGL of the cerebellum, CA4 of the hippocampus, temporal cortex, and DMNV of the medulla, and further differences were seen according to the presence of major SIDS risk factors, with the most changes seen according to the presence of cigarette smoke exposure. The only consistent finding between the number of GFAP positive cells and astrocyte morphology (R-score) was the increase in the IGL of SIDS compared to eSUDI, with most alterations being specific to the number of GFAP positive cells.

**Table 6.8: Heat map of brain regions where GFAP was significantly associated with developmental and risk factor parameters.**

|                             | Dentate nucleus | IGL | ML | White matter | CA4 | CA3 | CA2 | CA1 | DG | Sub | EC | Temporal cortex | AN | Cun | DMNV | XII | ION | LRF | NSTT | NTS | Vest |
|-----------------------------|-----------------|-----|----|--------------|-----|-----|-----|-----|----|-----|----|-----------------|----|-----|------|-----|-----|-----|------|-----|------|
| <b>GFAP+/mm<sup>2</sup></b> |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Brain weight                |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Head circumference          |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| PCA                         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| SIDS                        |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Male Sex                    |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Cigarette smoke exposure    |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| URTI                        |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Bed-sharing                 |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Prone sleeping              |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| <b>R-score</b>              |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Brain weight                |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Head circumference          |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| PCA                         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| SIDS                        |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Male sex                    |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Cigarette smoke exposure    |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| URTI                        |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Bed-sharing                 |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Prone sleeping              |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |



Developmental parameters of brain weight, head circumference and PCA (whole cohort) and according to risk factors (whole cohort + SIDS only included).

p-values indicated as  $\leq 0.05$ ,  $\leq 0.01$  or  $\leq 0.001$  for parameters associated with higher expression, and  $\leq 0.05$ ,  $\leq 0.01$  or  $\leq 0.001$  for parameters associated with lower expression.

#### **6.4.1: CHANGES IN THE NUMBER OF GFAP-POSITIVE CELLS VS CHANGES IN MORPHOLOGY**

Findings of a change in one aspect of GFAP expression (either upregulation in the number of GFAP-positive cells, or changes in morphology) without corresponding changes in the other may provide valuable information about the specific nature of the cellular response or underlying pathology. There is marked regional heterogeneity in expression of GFAP both at baseline (Emsley and Macklis, 2006) and in response to injury (Boos et al., 2021), and astrocyte hypertrophy appears to be dependent on the upregulation of specific GFAP isoforms (Moeton et al., 2016). A change in the number of GFAP cells without morphological changes may be indicative of early or mild response to central nervous system damage or injury (Petito et al., 1990), while significant astrocyte hypertrophy may be indicative of more severe response to injury or disease (Sofroniew, 2015). This study found that most of the changes related to the number of GFAP-positive astrocytes, not reflected by corresponding changes in morphology based on the R-score, potentially indicating mild or early gliosis is occurring.

#### **6.4.2: COMPARATIVE ACCORDING TO DEVELOPMENTAL PARAMETERS**

Of the studies available to date, quantitative assessment of GFAP in the human brain in relation to PCA has been undertaken in the DMNV (Oehmichen et al., 1989a), ION (Storm et al., 1994), hippocampus (Kinney et al., 2015), and across the cerebellum and brainstem stratified by age groups of <4months, 4-8 months and >8 months (Ambler et al., 1981), indicating GFAP expression to decrease postnatally. However, this is contrasted with no change according to PCA in the DMNV (Bruce and Becker, 1991) and XII (Pamphlett and Treloar, 1996) in two other studies. Astrocytes proliferate in high numbers in the early postnatal period, becoming proliferation quiescent and morphologically and functionally mature thereafter, subsequently declining in density as shown in the rodent brain (dependent on brain region) (Ge et al., 2012, Shoneye et al., 2020, Schreiner et al., 2014). The current study found negative correlations between developmental parameters and the number of GFAP-positive cells, such that infants who were older, and with larger morphometric indices (body weight, brain weight, head circumference, body length) had lower GFAP expression. These associations were region-dependent and were consistently seen within the IGL and white matter of the cerebellum, subiculum, temporal cortex and NSTT, although this did differ between eSUDI and SIDS, with growth related parameters being specific to the SIDS group in the hippocampus. Some negative

correlations were also seen between developmental parameters and R-score (although positive for the CA4 and subiculum), although to a lesser degree, with only the temporal cortex being associated with all of the above parameters. This indicates that while the number of astrocytes may change as a factor of development, the reactivity status remains relatively constant.

#### **6.4.3: COMPARATIVE BETWEEN DIAGNOSTIC GROUPS**

The current study found that compared to eSUDI, SIDS infants had an increased number of GFAP positive cells in the IGL, CA4, temporal cortex and DMNV, as well as more severe reactive-astrocyte morphological profiles in the IGL. To our knowledge, only two studies have reported on astrogliosis in the SIDS hippocampus previously, with one reporting decreased astrogliosis (based on GFAP immunohistochemistry) in the CA3 and subiculum of SIDS (Kinney et al., 2015) and the other reporting increased prevalence (although not specific to sub-region) of astrogliosis (using basic histochemical stain), amongst SIDS (Kon et al., 2020). Both studies were based on qualitative/subjective judgements about the presence of astrogliosis (i.e., yes vs no astrogliosis). CA4 is a hypoxia sensitive region (Kirino, 1982), and GFAP has been shown to be significantly upregulated in this region following hypoxic injury in accordance with notable cell death/cell loss (Kitamura, 1994, Otellin et al., 2013). Importantly, GFAP-specific changes in the CA4 were only reported in cases with survival times longer than 4 hours (Kitamura, 1994), suggesting that astrogliosis in SIDS may follow hypoxic exposures that greatly precede death. Interestingly, SIDS infants also have significant cell death in CA4 (Luijckx et al., 2020, Waters et al., 1999). Astrogliosis has not been investigated in the temporal cortex of SIDS, however, this region is known to be prone to facilitating epileptic discharges (Nishio et al., 2000, Sarac et al., 2009) and is also hypoxia-sensitive (Julian et al., 2015).

The DMNV is known for its role in parasympathetic motor innervation of cardiorespiratory systems (Yuan and Silberstein, 2016), and a number of abnormalities have been reported in the DMNV of SIDS (reviewed (Bejjani et al., 2013)), including findings of astrogliosis (Takashima et al., 1978, Takashima and Becker, 1985, Becker and Takashima, 1985). In accordance with the current study, these studies involved counting the number of GFAP-positive cells (although (Takashima et al., 1978) utilised basic histochemical stain). Past studies have supported more extensive brainstem astrogliosis in SIDS (Bruce and Becker, 1991, Storm et al., 1994, Obonai et al., 1996, Storm et al., 1999, Sawaguchi et al., 2002, Matturri et al., 2006, Spinelli et al., 2018), however, we did not find this to be the case. It is often the case that only a proportion

of SIDS demonstrate significant glial pathology (Storm et al., 1994), and findings of astrogliosis are largely dependent on the composition of SIDS and controls. This is difficult to control for amongst the SIDS group given that the cause of death is unknown, although, the SIDS group in the current study were all of the SIDS II diagnostic subgroup. SIDS II category includes cases that have developmental or environmental parameters that may have contributed to the mechanism of death, and inclusion of different SIDS sub-categories may yield different results. Other reasons for discrepancies may be due to different definitions/categorisation of SIDS, variations in the composition and profile of the eSUDI (“control”) groups, and methods for quantifying stains as described in Chapter 5.

The cerebellum is involved in regulating a wide range of functions, such as motor control and balance (Manto et al., 2012, Thach and Bastian, 2004), and also plays a significant role in regulating breathing, heart rate and other critical homeostatic functions via connections to other brain structures such as the brainstem (Calton et al., 2016, Calton et al., 2014, Harper et al., 2000). The cerebellum is hypoxia-sensitive (Hausmann et al., 2007, Brierley, 1984) and highly vulnerable to hypoxia-mediated astrogliosis (Coimbra-Costa et al., 2021). Astrogliosis in the SIDS cerebellum has been reported in one study previously, with approximately 50% of their SIDS demonstrating astrogliosis in the IGL (Lavezzi et al., 2018). Our previous study investigating astrocyte morphology using the R-score suggested that mild astrogliosis was occurring in the human infant brain when R scores were >120, and moderate-severe in subjects with an R-score >160 (Chapter 5). In the current dataset, 36% of SIDS had R-scores > 120 in the IGL compared to 0% of eSUDI, suggesting at least mild astrogliosis in approximately 1/3<sup>rd</sup> of our SIDS cases, with only 1 case being moderate (R-score >160). The IGL is involved primarily in the integration of sensory-motor input and damage/injury may indicate issues with proprioception (Huang et al., 2013). Thus, an infant who is sleeping prone and faced with a hypoxic stress/suffocation challenge may be unable to turn their head to overcome this.

#### **6.4.4: REMOVAL OF BED-SHARERS FROM ANALYSIS BY DIAGNOSIS**

Past studies have found significant pathological differences between bed-shared and non-bed-shared SIDS infants (Spinelli et al., 2018, Jensen et al., 2014, Machaalani and Waters, 2008, Luijckx et al., 2020). A major hypothesis is that a proportion of bed-shared SIDS infants die from mechanical asphyxia or suffocation from overlay (Byard, 2015, Hayman et al., 2015), and this more rapid mechanism of death would subsequently not allow sufficient time for markers to upregulate at the protein level. This is particularly relevant in the context of GFAP

immunohistochemistry, given that it may take many hours, or even days following an injury to upregulate (O'Callaghan et al., 2014, Hausmann et al., 2000, Kirino, 1982, Trautz et al., 2019), and also given that GFAP has been shown to be upregulated in brainstem nuclei of non-bed-shared infants (Spinelli et al., 2018). Undertaking statistical analysis on a group of infants that have died of different underlying causes may mask trends in the data. Repeating the analysis after removing bed-sharers resulted in the loss of the finding of increased GFAP in the CA4 and DMNV, with no further additional findings revealed. Importantly, infants who did bed-share were on average smaller in body length than non-bed-shared infants, with other developmental parameters such as head circumference, birth weight and body weight also being lower amongst bed-sharers (Table 6.1, p-values ranging 0.6-0.9). Many of these parameters were negatively correlated with GFAP expression in many brain regions (Supplement Table 1), such that they were associated with higher GFAP expression. Additionally, we did not distinguish between infants who were routinely bed-shared and those who were only bed-shared on the night of death. Infants who are routinely bed-shared may be subject to repeated hypoxic exposures and have longer survival time, which is known to result in more significant GFAP upregulation (Trautz et al., 2019).

#### **6.4.5: COMPARATIVE ACCORDING TO PRESENCE OF OTHER SIDS RISK FACTORS**

When risk-factor analysis was carried out on the whole cohort, the most changes in GFAP-expression were seen amongst cigarette-smoke exposed infants, whereby increased numbers of GFAP-positive astrocytes were in the XII and LRF. Amongst the SIDS only cohort, differences were seen in relation to all of the risk factors, including increased expression in nuclei of the cerebellum of male infants, decreased expression in the IGL of smoke exposed infants, higher expression in the XII of infants with a recent URTI, and lower expression in nuclei of the cerebellum/higher expression in the DG of infants found prone. Based on morphology, stronger levels of astrocyte-reactivity were present in the CA4, subiculum and temporal cortex of cigarette smoke-exposed SIDS infants.

Past studies have supported that astrogliosis is increased in the SIDS cerebellum and brainstem as a consequence of maternal cigarette use (Storm et al., 1999, Lavezzi et al., 2007). In animal models, smoke exposure increased GFAP expression via immunoblot analysis in the adult rat hippocampus and cerebellum (Fuller et al., 2010). Tobacco smoke produces its harmful neurological effects through the release of nicotine and/or carbon monoxide, which can lead to

hypoxia (Avni et al., 2016, Chan et al., 2016), oxidative stress and mitochondrial dysfunction (Chan et al., 2016), inflammation (Chan et al., 2016, Vani et al., 2015) and in some cases, astrogliosis (Vani et al., 2015, Abdel-Rahman et al., 2003). When carrying out the analysis on the SIDS group, it is curious that we saw decreased expression in the IGL of smoke exposed infants. However, when considering the composition of the non-smoke exposed group, 3/4 non smoke exposed infants also bed shared (Table 1), with only 1 non CSE not bed shared. This did not appear to be the case for morphological changes, given that astrocytes appeared to be more reactive in the hippocampus of smoke-exposed infants, even amongst those who were bed-shared.

The proposed pathophysiological mechanism of death associated with sleeping prone is that it involves rebreathing of expired gasses and the development of an asphyxiating (hypoxic and hypercapnic) microenvironment (Campbell et al., 1997). Hypoxia may lead to significant astrogliosis in the hippocampus, particularly the dentate gyrus (Khuu et al., 2019), which we found to be the case in the current study. Infants slept prone had significantly higher head circumference (data not shown), which was associated with a lower number of GFAP-positive cells in the IGL and white matter, although was not correlated with expression in the DG. Given that we do not have prone-sleepers matched on these parameters available for comparison, we cannot conclude whether expressional alterations are directly due to prone-sleeping.

## **6.5: CONCLUSION**

Increased GFAP expression was predominant in the IGL of SIDS, indicative of brain damage or injury to this region, and as such, could correlate with the physiological changes seen in SIDS related to the function of the IGL in integrating sensory-motor information, and responding to external stimuli via proprioception.

## **6.6: APPENDIX**

This Appendix contains the supplementary material available.

**Supplement Table 1: An overview of the significant associations between the number of GFAP-positive astrocytes/mm<sup>2</sup> in the infant brain and developmental parameters.**

| Brain Region                  | Infant Parameter   | ALL<br>r-value <sup>a</sup> | ALL<br>p-value <sup>b</sup> | eSUDI<br>(n=9)<br>p-value | SIDS<br>(n=25)<br>p-value |
|-------------------------------|--------------------|-----------------------------|-----------------------------|---------------------------|---------------------------|
| <b><i>Cerebellum</i></b>      |                    |                             |                             |                           |                           |
| <b>Dentate nucleus</b>        | Body weight        | -0.37                       | 0.04                        | 0.03                      |                           |
|                               | Head circumference |                             |                             | 0.01                      |                           |
| <b>IGL</b>                    | Birth weight       | -0.52                       | 0.02                        |                           |                           |
|                               | Body weight        | -0.61                       | <0.001                      | 0.04                      | <0.01                     |
|                               | Brain weight       | -0.61                       | <0.001                      | 0.05                      | <0.01                     |
|                               | Body length        | -0.41                       | 0.02                        |                           |                           |
|                               | Head circumference | -0.60                       | <0.001                      | 0.04                      | <0.01                     |
|                               | PCA                | -0.72                       | <0.001                      | 0.01                      | <0.001                    |
| <b>Molecular layer</b>        | Body length        |                             |                             |                           | 0.04                      |
| <b>White matter</b>           | Birth weight       | -0.50                       | 0.02                        |                           |                           |
|                               | Body weight        | -0.42                       | 0.02                        |                           |                           |
|                               | Brain weight       | -0.38                       | 0.03                        |                           |                           |
|                               | Head circumference | -0.41                       | 0.02                        |                           |                           |
|                               | PCA                | -0.42                       | <0.001                      |                           | 0.03                      |
| <b><i>Hippocampus</i></b>     |                    |                             |                             |                           |                           |
| <b>CA4</b>                    | PCA                | -0.35                       | <0.01                       |                           | 0.02                      |
| <b>CA1</b>                    | PCA                | -0.46                       | <0.01                       |                           | 0.01                      |
| <b>Subiculum</b>              | Body weight        | -0.58                       | <0.001                      | 0.04                      | <0.01                     |
|                               | Brain weight       | -0.46                       | <0.01                       | 0.04                      | 0.04                      |
|                               | Body length        | -0.39                       | 0.03                        |                           |                           |
|                               | Head circumference | -0.53                       | <0.01                       |                           | 0.01                      |
|                               | PCA                | -0.58                       | <0.001                      | 0.03                      | <0.01                     |
|                               | Thymus weight      | -0.42                       | 0.02                        |                           |                           |
| <b>EC</b>                     | PCA                |                             |                             |                           | <0.01                     |
| <b>Temporal cortex</b>        | Body weight        | -0.38                       | 0.04                        |                           | 0.01                      |
|                               | Brain weight       | -0.39                       | 0.03                        |                           | <0.01                     |
|                               | Body length        | -0.39                       | 0.04                        |                           |                           |
|                               | Head circumference | -0.40                       | 0.03                        |                           | <0.01                     |
|                               | PCA                | -0.46                       | <0.01                       |                           | <0.01                     |
| <b><i>Rostral medulla</i></b> |                    |                             |                             |                           |                           |
| <b>DMNV</b>                   | Vitamin D          | -0.50                       | 0.03                        |                           |                           |
| <b>XII</b>                    | PCA                |                             |                             | 0.04                      |                           |
|                               | Vitamin D          | -0.59                       | 0.01                        |                           |                           |
| <b>LRF</b>                    | Birth weight       | -0.50                       | 0.03                        |                           |                           |
| <b>NSTT</b>                   | Body weight        | -0.37                       | 0.05                        |                           |                           |



|            |                    |       |        |      |
|------------|--------------------|-------|--------|------|
|            | Brain weight       | -0.47 | <0.001 | 0.02 |
|            | Body length        | -0.41 | 0.03   | 0.05 |
|            | Head circumference | -0.41 | 0.03   |      |
|            | PCA                | -0.51 | <0.01  | 0.01 |
| <b>ION</b> | Thymus weight      | -0.48 | <0.01  |      |
| <b>VN</b>  | Birth weight       | -0.53 | 0.03   |      |

Correlations run for whole cohort. *p*-values provided for correlations in eSUDI and SIDS groups alone for risk factors of PCA, body weight, brain weight, body length and head circumference.

Unless otherwise stated, significant correlations were negative associations thus indicating as the parameter increases, GFAP levels decrease.

<sup>a</sup> Pearson's correlation coefficient

<sup>b</sup> Significance taken at  $p \leq 0.05$

*Abbreviations:* CA cornu ammonis, *Cun* Cuneate nucleus, *DMNV* dorsal motor nucleus of the vagus, *XII* hypoglossal nucleus, *IGL* internal granular layer of the cerebellar cortex, *ION* inferior olivary nucleus, *IGL* internal granular layer, *LRF* lateral reticular formation, *NSTT* nucleus of the spinal trigeminal tract, PCA postconceptional age, *Vest* vestibular nucleus.

**Supplement Table 2: An overview of the significant associations between R-score values in the infant brain and in the infant brain and developmental parameters.**

| Brain Region                  | Infant Parameter   | ALL<br>r-value <sup>a</sup> | ALL<br>p-value <sup>b</sup> | eSUDI<br>(n=9)<br>p-value | SIDS<br>(n=25)<br>p-value |
|-------------------------------|--------------------|-----------------------------|-----------------------------|---------------------------|---------------------------|
| <b><i>Hippocampus</i></b>     |                    |                             |                             |                           |                           |
| <b>CA4</b>                    | Body weight        |                             |                             |                           | 0.05                      |
|                               | Head circumference |                             |                             |                           | 0.03                      |
|                               | PCA                | 0.29                        | 0.03                        |                           | <0.01                     |
| <b>CA1</b>                    | Thymus weight      | -0.28                       | 0.05                        |                           |                           |
| <b>EC</b>                     | Body weight        | -0.29                       | 0.04                        |                           | 0.04                      |
|                               | Head circumference | -0.29                       | 0.04                        |                           |                           |
|                               | PCA                | -0.31                       | 0.03                        |                           | 0.04                      |
| <b>Subiculum</b>              | Brain weight       |                             |                             | 0.01                      |                           |
|                               | PCA                |                             |                             | 0.02                      |                           |
| <b>Temporal cortex</b>        | Body weight        | -0.32                       | 0.02                        |                           | <0.01                     |
|                               | Brain weight       | -0.30                       | 0.02                        |                           | <0.01                     |
|                               | Body length        | -0.32                       | 0.02                        |                           | 0.02                      |
|                               | Head circumference | -0.35                       | 0.01                        |                           | <0.01                     |
|                               | PCA                | -0.32                       | 0.02                        |                           | <0.01                     |
| <b><i>Rostral medulla</i></b> |                    |                             |                             |                           |                           |
| <b>LRF</b>                    | Vitamin D          | -0.39                       | 0.05                        |                           |                           |

<sup>a</sup> Kendall's Tau correlation coefficient

<sup>b</sup> Significance taken at p≤0.05

## **CHAPTER 7: GFAP EXPRESSION AND ASTROGLIOSIS IN THE DEVELOPING HUMAN BRAIN AND CHANGES SEEN IN SIDS- UNDERTAKEN IN AN AUSTRALIAN COHORT**

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### Attribution statement

Input to project design was by RM and LL. Tissue sectioning was performed by NN. All experimentation, analysis and drafting of the manuscript was performed by LL. RM and KW provided intellectual input and feedback while drafting. This chapter has been written up to be submitted for publication.

Human tissue used in this study and the accompanying autopsy and infant data characteristic data were obtained from the NSW Forensic and Analytical Science Services (formerly known as the Department of Forensic Medicine). Experimentation and image analysis was performed at the SIDS and Sleep Apnea Laboratory at the University of Sydney, Sydney, Australia.

## 7.1: INTRODUCTION

Astrocytes play an integral role in the development, structure and function of the central nervous system (CNS) (Ullian et al., 2001, Simard and Nedergaard, 2004, Iadecola and Nedergaard, 2007, Pellerin et al., 2007, Zhang et al., 2016). As constant surveyors of the CNS, astrocytes respond to disruptions in metabolic processes such as hypoxia, hypercapnia, and reduced brain perfusion (Angelova et al., 2015, Marina et al., 2018). Disruptions in CNS homeostasis, such as in the case of injury, can trigger ‘astrogliosis’, a process involving diverse morphological and functional changes, including astrocyte hypertrophy and proliferation (Sofroniew and Vinters, 2010). Glial fibrillary acidic protein (GFAP) is the main component of astrocyte intermediate filaments and has become a prototypical marker of astrogliosis given that its expression is proportionate to the degree of astrocyte hypertrophy and proliferation. Astrogliosis can be visualised as an increase in the number of GFAP-positive cells, and/or hypertrophy of astrocyte cell bodies.

Astrogliosis has been investigated in the sudden infant death syndrome (SIDS) brain previously, focusing on nuclei of the medulla (summarised Table 2.5, Chapter 2) (Bruce and Becker, 1991, Storm et al., 1994, Obonai et al., 1996, Storm et al., 1999, Sawaguchi et al., 2002, Matturri et al., 2006, Spinelli et al., 2018, Reske-Nielsen et al., 1987, Shaw et al., 1989, Oehmichen et al., 1989a, Pamphlett and Treloar, 1996, Sawaguchi et al., 2003, Kinney et al., 2015), although there has been major disagreement as to whether or not it is a feature of SIDS, and if so, in which brain regions. Surmounting evidence suggests the involvement of additional brain regions in SIDS pathogenesis, including the hippocampus (Sparks and Hunsaker, 1991, Waters et al., 1999, Kinney et al., 2015, Machaalani et al., 2022, Luijckx et al., 2020) and cerebellum (Lavezzi et al., 2016a, Lavezzi et al., 2018). The amygdala, basal ganglia and cortex are now also being investigated in the context of SIDS with findings including altered apoptotic expression (Ambrose et al., 2019, Ambrose et al., 2020), and impaired catecholaminergic neurons (Ozawa et al., 1999). The presence of astrogliosis in these regions in SIDS is yet to be determined.

As reported in Chapter 6, based on GFAP immunohistochemistry in a UK SUDI cohort, the main findings were that: (1) GFAP expression decreased with development and growth-related parameters, including age, body and brain weight, body length and head circumference (although this was region-dependent), (2) SIDS infants had higher GFAP expression in the internal granular layer (IGL) of the cerebellum, cornu ammonis (CA)4 of the hippocampus,

temporal cortex, and dorsal motor nucleus of the vagus (DMNV) of the medulla, and (3) further differences were seen according to the presence of major SIDS risk factors, with the most changes seen according to the presence of cigarette smoke exposure. Expressional changes were predominantly related to the number of GFAP-positive cells, and not morphological changes, thus suggesting early or mild astrogliosis (Petito et al., 1990, Sofroniew, 2015).

Given the vast contradictions in past literature investigating astrogliosis in SIDS, the current study sought to determine whether findings amongst the UK cohort were reproducible when the same diagnostic criteria (San Diego Criteria (Krous et al., 2004)) and experimental and quantification methods were applied on an Australian SUDI cohort. We also investigated several additional brain regions, including nuclei of the amygdala and basal ganglia, regions that have not been explored in the context of astrogliosis in SIDS before. Rationale for the inclusion of these regions was to determine whether astrogliosis in SIDS, if present, involves more widespread damage occurring in other regions, and to provide a direct comparison against the apoptotic and microglial findings in this exact same cohort and amongst the same brain regions (Ambrose et al., 2019, Ambrose et al., 2020). Microglia are readily activated under a range of pathological conditions, and can activate astrocytes into a reactive state (Zhang et al., 2010, Goc et al., 2014) (Liddelow et al., 2017). Furthermore, cell death, which is upregulated in the SIDS cerebellum (Lavezzi et al., 2006), hippocampus (Luijckx et al., 2020, Waters et al., 1999), brainstem (Machaalani and Waters, 2008, Ambrose et al., 2018, Ambrose et al., 2019) amygdala (Ambrose et al., 2019) and basal ganglia (Ambrose et al., 2019), is known to occur in accordance with astrogliosis following CNS damage (Lee et al., 2022, Cullen et al., 2007, Guttenplan et al., 2021).

The aims of this study were therefore:

- 1- To report on the immunohistochemical expression of GFAP for 24 regions of the human infant brain, including regions of the medulla, cerebellum, basal ganglia, hippocampal formation, temporal cortex, and amygdala, and determine the relationship with growth and developmental parameters.
- 2- To determine whether there were any differences in expression according to SUDI subgroup; herein, we had 3 groups of eSUDI, SIDS I and SIDS II.
- 3- To determine whether there were any differences in GFAP expression according to the presence of major SIDS risk factors, including cigarette smoke exposure, sleep position

found, co-sleep/bed-sharing, the presence of a recent upper respiratory tract infection (URTI), and male gender.

- 4- To compare findings to cell death (Ambrose et al., 2019) and microglial expression (Ambrose et al., 2020), which were undertaken in the exact same dataset.

## **7.2: METHODS**

### **7.2.1: DATA AND TISSUE COLLECTION**

All infant brain tissue and information regarding infant characteristics and the presence of risk factors was collected by the NSW Forensic and Analytical Science Services (FASS; formerly, the Department of Forensic Medicine) between 2008-2012 as part of their routine autopsy protocol for coronial investigation of the cause of death. Previous students from our laboratory (Ambrose, 2021, Vivekanandarajah, 2017) accessed this tissue and information after a final diagnosis had been provided in accordance with ethical approval from the NSW Health RPAH Zone (X13-0038 and HREC/13/RPAH/54) and the Human Ethics Committees of The University of Sydney (Approval No. 3013/235), and the tissue and the information obtained was utilised herein.

The cases included in this study were the exact same ones studied previously for microglial expression (Ambrose et al., 2020) and for which re-classification was undertaken (Ambrose et al., 2018) following the San Diego criteria (Krous et al., 2004). The San Diego criteria further sub-categorises SIDS into SIDS I and SIDS II, with the main distinction being that SIDS I have had all necessary investigations performed, with results showing the absence of unsafe sleep practices (i.e. bed-sharing) and no further insight into the mechanism of death, while SIDS II includes cases that may have not undergone all the necessary investigations, have pre-existing vulnerabilities such as prematurity, or where resolved developmental delays, unsafe sleeping conditions or acute illness are present but are insufficient to explain the cause of death. It is this final classification that has been employed herein.

Brain tissue was fixed in 20% neutral buffered formalin, and paraffin embedded. Post-mortem interval (PMI) ranged between 8 and 85 hours, and fixation time ranged between 2 and 23 days. Tissue was sectioned at 7µm thickness using a rotary microtome and mounted on 2% 3-aminopropyltriethoxysilane treated slides, which were dried overnight and stored in a dust-free environment prior to staining.

### **7.2.2: GFAP IMMUNOSTAINING**

Manual immunohistochemistry for GFAP was performed as per our standard laboratory protocol, described in detail previously ((Luijckx et al., 2020), Chapter 3) unless otherwise stated. Briefly, sections were deparaffinised in xylene and rehydrated through a graded series of ethanol prior to microwave antigen retrieval in 10% Tris-EDTA buffer (1mM EDTA, 1mM Na Citrate, 2mM Tris; pH 9.0) for 14 minutes. Sections were allowed to cool and were quenched for 20 minutes in a 3% H<sub>2</sub>O<sub>2</sub>, methanol and phosphate-buffered saline (PBS) solution. Sections were blocked with 10% normal horse serum (NHS) for 30 minutes, followed by overnight incubation with GFAP primary antibody (Z033401-2, Agilent, 1:3000 dilution). Sections were washed with PBS and incubated at room temperature for 1 hour in an affinity-purified anti-mouse/anti-rabbit secondary antibody (1:400; BA-400, Vector Laboratories) and subsequently were washed in PBS and incubated for 1 hour with avidin-biotin horseradish peroxidase complex (VEPH-4000, Vector Stain ABC kit; Vector Laboratories Inc). Colour labelling was achieved with 3,3'-diaminobenzidine (DAB) (K346811, DAKO). Following counterstaining with Harris haematoxylin, sections were dehydrated through a graded ethanol series, cleared in xylene and cover slipped. Duplicate staining was performed on 15- 20% of sections for each brain region. The specificity of the GFAP antibody has been demonstrated in ((Luijckx et al., 2021), Chapter 4).

### **7.2.3: QUANTITATIVE ANALYSIS**

All tissue sections stained for GFAP were viewed using light microscopy (Olympus Upright BX51 Microscope, Olympus Optical Co., Ltd Japan) and imaged using image capture software (DP Controller, Olympus Optical Co, Ltd, Japan). For each case, 24 microscopically defined brain regions of interest (ROI) were examined (Figure 7.1), including 9 nuclei of the medulla (arcuate nucleus (AN), cuneate nucleus (Cun), DMNV, hypoglossal (XII), inferior olivary nucleus (ION), lateral reticular formation (LRF), nucleus of the spinal trigeminal tract (NSTT), nucleus of the solitary tract (NTS) and vestibular nucleus (Vest)), from obex levels -2mm-8mm as defined by (Paxinos and Huang, 2013), 4 regions of the cerebellar cortex (dentate nucleus, IGL, molecular layer (ML) and white matter) (Naidich, 2007) and 5 regions of the hippocampus taken at or near the lateral geniculate nucleus (dentate gyrus (DG), CA4, CA3, CA2, CA1, as well as the subiculum, entorhinal cortex (EC) and fusiform gyrus of the temporal cortex, all taken at the level of the hippocampal body as defined by (Duvernoy, 2013), the

basolateral region of the amygdala, and the caudate nucleus and putamen of the anterior basal ganglia (striatum) (Figure 7.1).

For each brain region, multiple, random, non-overlapping images were taken using either the 20x or 40x objective lens (number of images in each region of interest (ROI) and magnification utilised, are indicated in Figure 7.1). Large blood vessels and other artefacts were avoided in the field of view. Red Green Blue images were exported to Fiji imaging software (Schindelin et al., 2012) for analysis of staining, applying 2 different quantification methods; the number of GFAP-positive astrocytes per mm<sup>2</sup>, and the R-score based on astrocyte morphology. Where possible, only the ROI was captured in the field of view, otherwise, the ROI was manually outlined in Fiji using the inbuilt 'polygon line tool' and 'ROI manager' plugin, such that subsequent analyses would only be within the defined region. All imaging and quantification were performed by a single observer, blind to the diagnostic group.

### 3. GFAP-positive astrocytes per mm<sup>2</sup>

For each image, the number of GFAP-positive astrocytes was manually counted using the in-built 'multi point' function. For each brain region, the total number of GFAP-positive astrocytes was divided by the total area, to obtain a single value that represented the number of GFAP-positive cells per mm<sup>2</sup>. Only astrocytes for which the cell body was visible, were counted as positive.

### 4. R-score (morphology)

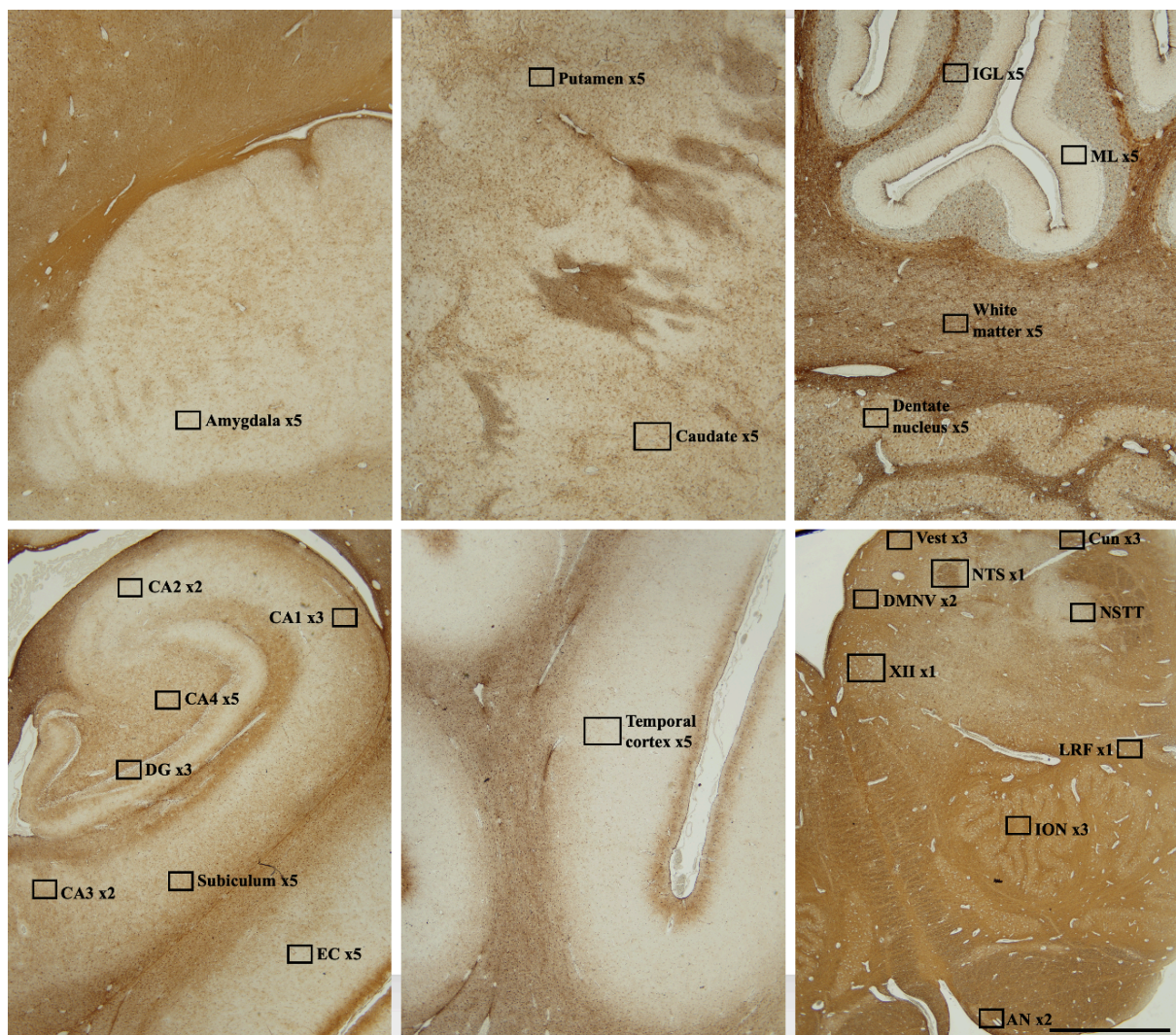
Each GFAP-positive cell was scored a semi-quantitative score based on a qualitative judgement by the observer, using a 4-tier scoring system ('0' = no astrogliosis, '1' = mild astrogliosis, '2' = moderate astrogliosis, '3' = severe gliosis), adapted from (Gouw et al., 2008, Sofroniew and Vinters, 2010) (and represented in Figure 7.1). An R-score was calculated according to the equation  $R = \sum Pr(r + 1)$ , where P represents the percentage of cells, and "r" represents the reactivity/astrogliosis score (as defined in Chapter 5).

## 7.2.1: STATISTICAL ANALYSIS

Data was exported to SPSS for Windows (version 25; SPSS (IBM) Inc., Illinois, USA) for statistical analysis, and outcome data was tested for normality. ANOVA with Bonferroni post hoc analysis was used to compare the mean number of GFAP-positive astrocytes per mm<sup>2</sup>



between diagnostic groups, and independent samples t-tests were used for risk factors. Kruskal-Wallis tests were used to compare R-scores between diagnostic groups, with Mann-Whitney U tests for pairwise comparisons and for the presence of risk factors, as well as chi-square testing to compare the proportions of cases with R-scores >100 and >120. The relationship between GFAP-positive cells per mm<sup>2</sup> and continuous clinical data was determined using Pearson correlations, while Kendall's Tau correlations were used to compare R-score and continuous clinical data. GFAP-positive cells per mm<sup>2</sup> data is presented as mean  $\pm$  standard error of the mean (SEM), and R-score is presented as median (interquartile range). P-values  $\leq$  0.05 were taken as statistically significant.



**Figure 7.1: Representative GFAP staining per brain section stained in the Australian dataset.** Indication of the location of the specific ROI analysed is included, with the number of snapshots analysed per ROI in brackets. Box sizes represent captured at 20x (larger boxes) and 40x (smaller boxes). Scale bar = 2mm.

## **7.3: RESULTS**

### **7.3.1: INFANT CHARACTERISTICS**

As characterised previously (Ambrose et al., 2019), the dataset included 28 SUDI cases (17 males, 11 females), categorised as eSUDI (n=7), SIDS I (n=8) and 13 SIDS II (n=13), with cause of death in the eSUDI group including acute pneumonia (n = 1), thrombotic occlusion of Blalock Taussig Shunt (n=1), complications of congenital heart disease (n=2), myocarditis (n=2) and meningoencephalitis of uncertain aetiology (n=1). The diagnostic groups were matched for clinical and autopsy data, as well as for the prevalence of major SIDS risk factors (Table 7.1), with the exception of bed-sharing being exclusive to the SIDS II group, consistent with diagnostic criteria (Krous et al., 2004).

**Table 7.1: Clinical, autopsy and risk factor data for the study dataset.**

|                               | Data Characteristic |                |                  | <i>p</i> -value <sup>a*</sup> |
|-------------------------------|---------------------|----------------|------------------|-------------------------------|
|                               | eSUDI (n = 7)       | SIDS I (n = 8) | SIDS II (n = 13) |                               |
| <i>Clinical data</i>          |                     |                |                  |                               |
| Male: female                  | 3:4                 | 6:2            | 8:5              | 0.44                          |
| Gest. Age (wks)               | 38.7±2.2            | 39.3±1.7       | 38.7±1.8         | 0.77                          |
| Birth weight (kg)             | 2.8 ± 0.9           | 3.0 ± 0.5      | 3.1 ± 0.7        | 0.22                          |
| <i>Autopsy data</i>           |                     |                |                  |                               |
| Age at death (months)         | 4.14 ± 3.3          | 4.5 ± 2.0      | 3.7 ± 2.5        | 0.77                          |
| PCA (weeks)                   | 55 ± 15             | 57 ± 8         | 53 ± 11          | 0.73                          |
| Body weight (kg)              | 5.7 ± 2.9           | 6.9 ± 1.6      | 6.0 ± 2.0        | 0.51                          |
| Brain weight (g)              | 650 ± 230           | 765 ± 147      | 717 ± 172        | 0.48                          |
| Body length (cm)              | 57 ± 12.7           | 64 ± 5.3       | 61 ± 7.3         | 0.29                          |
| Head circumference (cm)       | 39 ± 5.1            | 42 ± 2.4       | 40 ± 4.6         | 0.49                          |
| <i>Tissue parameters</i>      |                     |                |                  |                               |
| PMI (hours)                   | 25 ± 25             | 39.0 ± 12      | 30.0 ± 18        | 0.35                          |
| Fixation (days)               | 5.0 ± 1.4           | 8.6 ± 5.5      | 8.4 ± 6.1        | 0.38                          |
| <i>Risk factor prevalence</i> |                     |                |                  |                               |
| Cigarette smoke exposure (%)  | 1/5 (20)            | 3/8 (38)       | 4/10 (40)        | 0.73                          |
| Recent URTI <sup>b</sup> (%)  | 1/7 (14)            | 2/8 (25)       | 6/13 (46)        | 0.30                          |
| Found prone (%)               | -                   | 5/8 (63)       | 7/11 (64)        | 1.00                          |
| Bed-share/co-sleeping (%)     | -                   | -              | 9/13 (69)        | NA                            |

Results presented as the mean ± SD or percentage with characteristic. Fractions are provided where data was not complete for the diagnostic group's total n-value

<sup>a</sup>Obtained using Fischer's exact test for nominal data and one-way ANOVA with Bonferroni adjustment for continuous data.

<sup>b</sup>Reported presence of an URTI in the 2 weeks prior to death

\* Significance taken at  $p \leq 0.05$

*Abbreviations: eSUDI explained sudden unexpected death in infancy, Gest. gestational, PCA post conception age, PMI post-mortem interval, SIDS sudden infant death syndrome, URTI upper respiratory tract infection*

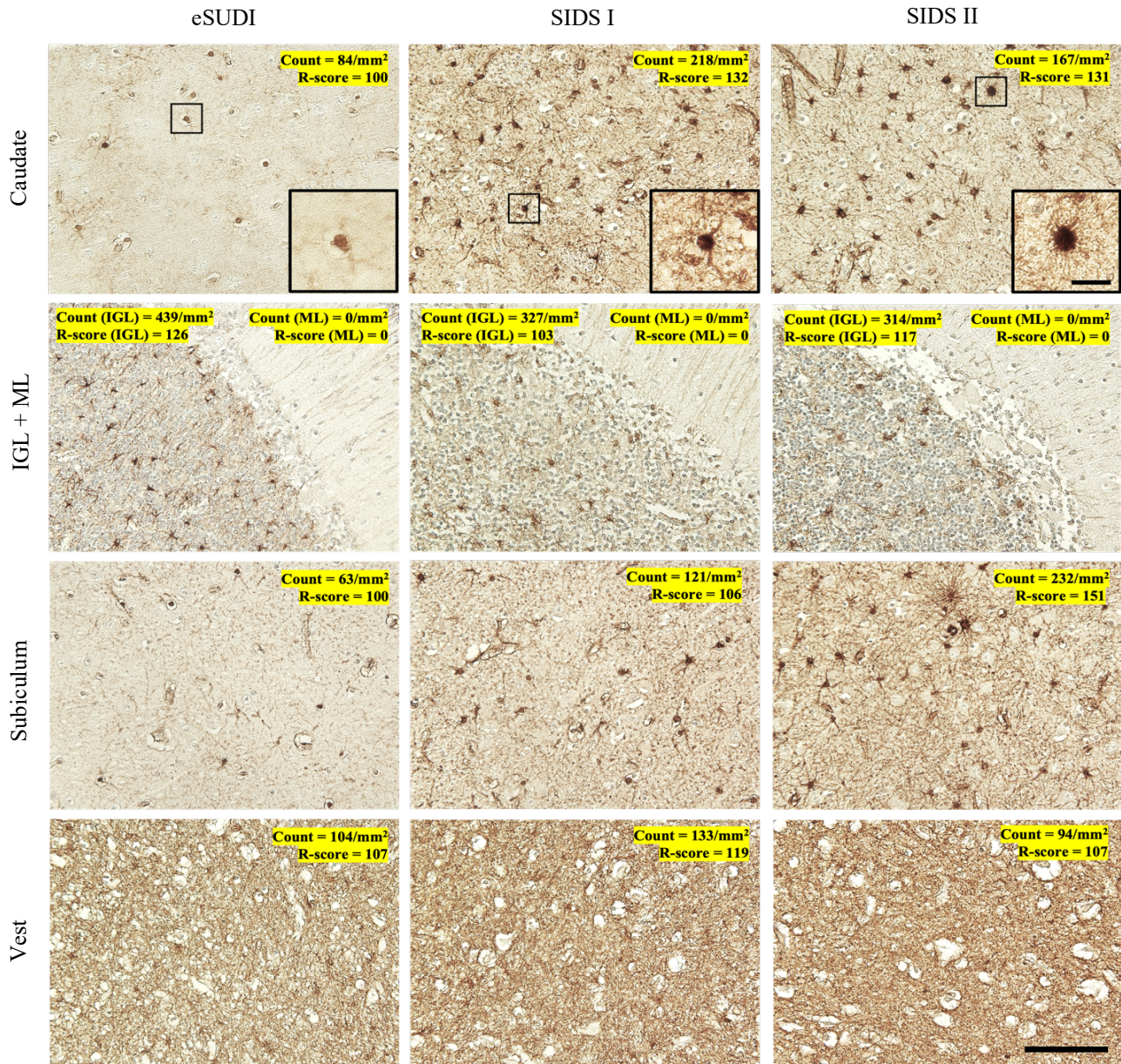
### **7.3.2: GFAP EXPRESSION**

#### **7.3.2.1: ACROSS BRAIN REGIONS (FOCUS ON ESUDI)**

Considering the eSUDI group for baseline values, the number of GFAP positive cells varied between 1/mm<sup>2</sup> in ML and 401/mm<sup>2</sup> in the IGL of the cerebellum (Table 7.2).

For the R score, values ranged between 0 and 185 (Table 7.2) (examples of R-score values and corresponding counts shown in Figure 7.2), with 0 indicating no cells were stained positive, 100 indicating positive cells but not of reactive morphology, and >100 indicating astrocytes of reactive morphology.





**Figure 7.2: Examples of GFAP expression in the Caudate nucleus, IGL and ML, subiculum, and Vestibular nucleus, across 3 SUDI sub-groups.** Images represent one field of microscope view for each case with final GFAP+/mm<sup>2</sup> and R-score values annotated in yellow. Magnified inserts within the Caudate panels represent examples of astrocytes scored as 0, 1 and 2, respectively in panel left to right. Scale bar= 100µm for main panels, and 20µm for magnified panels of the caudate.

### **7.3.2.2: CORRELATIONS WITH DEVELOPMENTAL PARAMETERS (WHOLE COHORT)**

Associations between GFAP staining and infant developmental parameters were explored in all cases, regardless of diagnosis. Weak to strong negative correlations ( $r = -0.38$  to  $-0.76$ ,  $p = <0.001$  to  $0.05$ ) were observed between the number of GFAP-positive cells per  $\text{mm}^2$  developmental parameters, with regions where GFAP+/ $\text{mm}^2$  correlated with the majority of the parameters being the dentate nucleus, IGL, white matter, EC, temporal cortex, ION, LRF, NTS (body weight, brain weight, body length and head circumference), subiculum (body weight, body length, head circumference and PCA), Cun, XII (body weight, body length and PCA), and DMNV (body weight, brain weight, body length, PCA), with others only correlating with one or two parameters (Supplementary Table 1). When analysing within the diagnostic categories, the majority of these correlations seem to be due to the SIDS group (Supplementary Table 1).

For the R-score, weak-moderate negative correlations ( $r = -0.29$  to  $-0.56$ ,  $p = <0.001$  to  $0.05$ ) were observed with the majority being in the white matter, EC, temporal cortex, Cun, ION (body weight, brain weight, body length, head circumference, PCA), caudate (body weight, brain weight, head circumference, PCA), and DMNV (body weight, brain weight and body length, PCA) (Supplementary Table 2). When analysing within the diagnostic categories, the correlations in the cerebellum and hippocampus were specific to the SIDS group (Supplementary Table 2).

### **7.3.2.3: COMPARISON ACCORDING TO DIAGNOSIS**

Although age and growth parameters were associated with differences in expression (as detailed above), given these were not consistent for all brain regions and tended to be swayed by the SIDS group, we did not include them as covariates in our analysis. Further, covariate analysis was not warranted given the three SUDI subgroups did not differ statistically for these parameters (Table 7.1).

There were no differences in GFAP/ $\text{mm}^2$  or R-score values between any of the diagnostic subcategories for any of the brain regions studied (Table 7.2). Based on proportion, eSUDI included a higher proportion of cases with scores  $>120$  in the white matter of the cerebellum (Table 7.3).

In order for data to be comparable with our previous cohort (Chapter 6) which only included SIDS II infants, comparisons were also made between eSUDI and SIDS II (Table 7.2 & 7.3). No differences were seen between eSUDI and SIDS II for counts per mm<sup>2</sup> or R-score values, although SIDS had a higher proportion of cases with scores >100 in the caudate nucleus (97% vs 57%, p=0.05).

#### **7.3.2.4: COMPARISONS ACCORDING TO RISK FACTORS**

In order to determine whether risk factors differentially affected GFAP in SIDS, analyses were run on the whole cohort first, and then the SIDS only cohort. Sleep related risk factors of sleeping prone and bed-sharing were only run on the SIDS group.

When analysis was run on the whole cohort for GFAP/mm<sup>2</sup>, infants exposed to cigarette smoke had higher expression than non-smoke-exposed infants in the temporal cortex (p=0.02) and infants with a recent URTI had lower expression in the DG (p=0.04) (Table 7.4). When risk-factor analysis was carried out only on the SIDS cohort, the effect was lost for smoke exposure, but maintained for URTI (p=0.03) (Table 7.5). Infants who bed-shared had higher expression in the Vest (p=0.04), and infants who were found prone had lower expression in the subiculum (p=0.02) (Table 7.5).

When analysis was run on the whole cohort for R-score, female infants had higher values in the CA1 (p=0.03) and Cun (p<0.01), and infants with a recent URTI had higher values in the caudate nucleus (p<0.01) (Table 7.6). When analysis was run only on the SIDS cohort, the finding of female infants having higher values was maintained in the Cun (p=0.03) but not the CA1, infants exposed to cigarette smoke had lower values in the CA2 (p=0.04), and infants with a recent URTI had lower values in the XII (p=0.01) and LRF (p=0.05), although the effect in caudate nucleus was lost (Table 7.7). Infants found prone had lower values in the AN (p<0.01) (Table 7.7).

**Table 7.2: Comparison of the number of GFAP-positive astrocytes/mm<sup>2</sup> and R-score values between diagnostic groups.**

| Brain Region             | Number of GFAP cells<br>(point-counting) |                 |                   |                             |                                                      | Morphology (R-score) |               |                   |                                |                                                      |
|--------------------------|------------------------------------------|-----------------|-------------------|-----------------------------|------------------------------------------------------|----------------------|---------------|-------------------|--------------------------------|------------------------------------------------------|
|                          | eSUDI<br>(n=7)                           | SIDS I<br>(n=8) | SIDS II<br>(n=13) | <i>p</i> -value<br>3 groups | <i>p</i> -value 2<br>groups<br>(SIDS II vs<br>eSUDI) | eSUDI<br>(n=7)       | SIDS<br>(n=8) | SIDS II<br>(n=13) | <i>p</i> -value<br>3<br>groups | <i>p</i> -value 2<br>groups<br>(SIDS II vs<br>eSUDI) |
| <i>Amygdala</i>          | 132±22                                   | 162±19          | 112±21            | 0.29                        | 0.94                                                 | 103 (5)              | 107 (10)      | 104 (3)           | 0.28                           | 0.32                                                 |
| <i>Basal Ganglia</i>     |                                          |                 |                   |                             |                                                      |                      |               |                   |                                |                                                      |
| <b>Caudate nucleus</b>   | 109±18                                   | 129±25          | 127±10            | 0.66                        | 0.36                                                 | 102 (13)             | 102 (21)      | 103 (13)          | 0.79                           | 0.53                                                 |
| <b>Putamen</b>           | 163±27                                   | 144±25          | 163±9             | 0.74                        | 0.99                                                 | 114 (54)             | 127 (55)      | 109 (23)          | 0.60                           | 0.39                                                 |
| <i>Cerebellum</i>        |                                          |                 |                   |                             |                                                      |                      |               |                   |                                |                                                      |
| <b>Dentate Nucleus</b>   | 399±57                                   | 322±44          | 390±34            | 0.42                        | 0.56                                                 | 102 (4)              | 103 (5)       | 105 (11)          | 0.22                           | 0.26                                                 |
| <b>IGL</b>               | 401±56                                   | 362±24          | 392±34            | 0.76                        | 0.68                                                 | 124 (43)             | 123 (16)      | 117 (30)          | 0.83                           | 0.60                                                 |
| <b>ML</b>                | 1±1                                      | 1±1             | 5±4               | 0.63                        | 0.55                                                 | 0 (100)              | 0 (0)         | 0 (0)             | 0.63                           | 0.53                                                 |
| <b>White matter</b>      | 176±42                                   | 121±31          | 146±21            | 0.50                        | 0.32                                                 | 129 (38)             | 107 (22)      | 110 (11)          | 0.70                           | 0.44                                                 |
| <i>Hippocampus</i>       |                                          |                 |                   |                             |                                                      |                      |               |                   |                                |                                                      |
| <b>CA4</b>               | 269±32                                   | 218±24          | 280±27            | 0.28                        | 0.76                                                 | 108 (15)             | 108 (21)      | 106 (21)          | 0.92                           | 0.80                                                 |
| <b>CA3</b>               | 93±14                                    | 98±24           | 101±19            | 0.97                        | 0.80                                                 | 100 (0)              | 100 (0)       | 100 (0)           | 0.40                           | 0.72                                                 |
| <b>CA2</b>               | 51±21                                    | 77±19           | 57±11             | 0.53                        | 0.52                                                 | 100 (100)            | 100 (0)       | 100 (25)          | 0.90                           | 1.00                                                 |
| <b>CA1</b>               | 135±18                                   | 114±20          | 116±11            | 0.61                        | 0.32                                                 | 100 (8)              | 103 (12)      | 103 (10)          | 0.82                           | 0.57                                                 |
| <b>DG</b>                | 184±40                                   | 171±24          | 176±24            | 0.96                        | 0.79                                                 | 100 (15)             | 100 (1)       | 100 (0)           | 0.49                           | 0.57                                                 |
| <b>EC</b>                | 71±11                                    | 100±21          | 130±18            | 0.09                        | 0.06                                                 | 100 (0)              | 100 (2)       | 100 (6)           | 0.68                           | 0.53                                                 |
| <b>Subiculum</b>         | 150±33                                   | 145±18          | 147±16            | 0.99                        | 0.89                                                 | 100 (1)              | 104 (31)      | 102 (8)           | 0.18                           | 0.08                                                 |
| <b>Temporal cortex</b>   | 59±18                                    | 87±26           | 93±13             | 0.38                        | 0.16                                                 | 100 (1)              | 100 (9)       | 100 (2)           | 0.90                           | 0.96                                                 |
| <i>Medulla (rostral)</i> |                                          |                 |                   |                             |                                                      |                      |               |                   |                                |                                                      |



|             |        |        |                     |      |      |          |          |          |      |      |
|-------------|--------|--------|---------------------|------|------|----------|----------|----------|------|------|
| <b>AN</b>   | 180±35 | 186±29 | 245±18              | 0.14 | 0.29 | 100 (26) | 100 (11) | 102 (24) | 0.60 | 0.89 |
| <b>Cun</b>  | 260±92 | 183±38 | 192±27              | 0.54 | 0.49 | 105 (36) | 107 (19) | 107 (14) | 0.98 | 0.88 |
| <b>DMNV</b> | 141±25 | 86±15  | 105±18              | 0.22 | 0.11 | 109 (24) | 108 (22) | 106 (16) | 0.93 | 0.84 |
| <b>XII</b>  | 43±11  | 35±6   | 45±8                | 0.70 | 0.91 | 105 (13) | 110 (14) | 107 (11) | 0.88 | 0.67 |
| <b>ION</b>  | 191±74 | 107±15 | 133±17              | 0.29 | 0.40 | 122 (58) | 105 (20) | 117 (23) | 0.38 | 0.36 |
| <b>LRF</b>  | 69±23  | 43±10  | 55±7                | 0.46 | 0.28 | 108 (47) | 100 (25) | 100 (12) | 0.24 | 0.20 |
| <b>NSTT</b> | 188±47 | 157±20 | 194±21              | 0.59 | 0.81 | 110 (26) | 108 (8)  | 108 (21) | 0.49 | 0.53 |
| <b>NTS</b>  | 113±38 | 51±13  | 59±12               | 0.11 | 0.20 | 110 (12) | 105 (29) | 111 (26) | 0.98 | 0.87 |
| <b>Vest</b> | 61±12  | 49±20  | 118±21 <sup>^</sup> | 0.06 | 0.33 | 103 (14) | 100 (6)  | 107 (8)  | 0.35 | 1.00 |

Results presented as the mean ± SEM for point-counting and medians (interquartile range) for R-score.

P values were obtained using one-way ANOVA with Bonferroni pairwise comparisons for point counting, and Kruskal-Wallis with Mann-Whitney U test for pairwise comparisons for R-score. Significance taken at  $p \leq 0.05$

<sup>^</sup>  $p=0.08$  bonferroni compared to SIDS I.

*Abbreviations:* *AN* Arcuate nucleus, *CA* Cornu Ammonis, *Cun* Cuneate nucleus, *DG* dentate gyrus, *DMNV* dorsal motor nucleus of the vagus, *EC* entorhinal cortex, *eSUDI* explained sudden unexpected death in infancy, *XII* hypoglossal nucleus, *IGL* internal granular layer of the cerebellar cortex, *ION* inferior olivary nucleus, *LRF* lateral reticular formation, *ML* molecular layer of the cerebellar cortex, *NSTT* nucleus of the spinal trigeminal tract, *NTS* nucleus of the solitary tract, *PCA* post conception age, *SIDS* sudden infant death syndrome, *Vest* vestibular nucleus.

**Table 7.3: Comparison of diagnostic groups according to percentage of cases with R-score sub-categories of >100, >120 and >160.**

| Brain region             | R-score |        |         |                  |                                     |              |           |          |                  |                                     |
|--------------------------|---------|--------|---------|------------------|-------------------------------------|--------------|-----------|----------|------------------|-------------------------------------|
|                          | >100    |        |         |                  |                                     | >120 (>160^) |           |          |                  |                                     |
|                          | eSUDI   | SIDS I | SIDS II | p-value 3 groups | p-value 2 groups (SIDS II vs eSUDI) | eSUDI        | SIDS I    | SIDS II  | p-value 3 groups | p-value 2 groups (SIDS II vs eSUDI) |
| <i>Amygdala</i>          | 80      | 100    | 82      | 0.52             | 1.00                                | 0            | 17        | 9        | 0.63             | 1.00                                |
| <i>Basal ganglia</i>     |         |        |         |                  |                                     |              |           |          |                  |                                     |
| <b>Caudate nucleus</b>   | 57      | 100    | 92      | 0.07             | <b>0.05</b>                         | 14           | 17        | 17       | 0.52             | 0.5                                 |
| <b>Putamen</b>           | 86      | 83     | 100     | 0.36             | 0.49                                | 43           | 50 (29)   | 42 (17)  | 0.94             | 1.00                                |
| <i>Cerebellum</i>        |         |        |         |                  |                                     |              |           |          |                  |                                     |
| <b>Dentate Nucleus</b>   | 67      | 62     | 85      | 0.48             | 0.63                                | 0            | 0         | 0        | -                | -                                   |
| <b>IGL</b>               | 100     | 100    | 100     | -                | -                                   | 71 (14)      | 75        | 46       | 0.34             | 0.67                                |
| <b>ML</b>                | 14      | 0      | 0       | 0.21             | 0.25                                | 14 (14)      | 0         | 0        | 0.21             | 0.25                                |
| <b>White matter</b>      | 57      | 62     | 92      | 0.14             | 0.32                                | <b>57</b>    | <b>38</b> | <b>8</b> | <b>0.05</b>      | 0.14                                |
| <i>Hippocampus</i>       |         |        |         |                  |                                     |              |           |          |                  |                                     |
| <b>CA4</b>               | 86      | 87     | 92      | 0.89             | 1.00                                | 14           | 25        | 31       | 0.72             | 0.64                                |
| <b>CA3</b>               | 14      | 12     | 0       | 0.39             | 0.44                                | 0            | 0         | 0        | -                | -                                   |
| <b>CA2</b>               | 14      | 0      | 0       | 0.21             | 0.25                                | 0            | 0         | 0        | -                | -                                   |
| <b>CA1</b>               | 43      | 75     | 69      | 0.39             | 0.21                                | 14           | 0         | 8        | 0.56             | 0.44                                |
| <b>DG</b>                | 29      | 25     | 8       | 0.42             | 0.57                                | 0            | 0         | 8        | 0.55             | 1.00                                |
| <b>EC</b>                | 14      | 29     | 31      | 0.71             | 0.63                                | 0            | 14        | 16 (8)   | 0.55             | 0.55                                |
| <b>Subiculum</b>         | 29      | 75     | 77      | 0.08             | 0.06                                | 14           | 25        | 8        | 0.55             | 1.00                                |
| <b>Temporal cortex</b>   | 29      | 17     | 31      | 0.81             | 1.00                                | 14           | 17        | 8        | 0.82             | 1.00                                |
| <i>Medulla (rostral)</i> |         |        |         |                  |                                     |              |           |          |                  |                                     |

|             |     |    |     |      |      |         |    |    |      |      |
|-------------|-----|----|-----|------|------|---------|----|----|------|------|
| <b>AN</b>   | 40  | 37 | 64  | 0.47 | 1.00 | 40      | 13 | 36 | 0.44 | 0.61 |
| <b>Cun</b>  | 60  | 71 | 80  | 0.71 | 0.59 | 40      | 14 | 20 | 0.56 | 0.55 |
| <b>DMNV</b> | 67  | 75 | 67  | 0.91 | 1.00 | 33      | 25 | 17 | 0.72 | 0.60 |
| <b>XII</b>  | 67  | 81 | 73  | 0.97 | 1.00 | 17      | 0  | 18 | 0.49 | 1.00 |
| <b>ION</b>  | 100 | 75 | 100 | 0.09 | 1.00 | 50 (17) | 38 | 33 | 0.79 | 0.64 |
| <b>LRF</b>  | 67  | 43 | 25  | 0.23 | 0.18 | 33 (17) | 29 | 0  | 0.11 | 0.23 |
| <b>NSTT</b> | 100 | 75 | 100 | 0.09 | 1.00 | 33      | 0  | 33 | 0.18 | 0.60 |
| <b>NTS</b>  | 100 | 71 | 73  | 0.35 | 0.28 | 17      | 43 | 27 | 0.58 | 0.63 |
| <b>Vest</b> | 60  | 33 | 73  | 0.29 | 1.00 | 20      | 0  | 9  | 0.52 | 0.41 |

Values expressed as percentages rounded to nearest whole integer.

Bold highlights statistical significance using Chi square test with Fisher's Exact test. Significance taken at  $p \leq 0.05$

^where applicable, >160 was applied based on previous chapter where this cut-off is proposed to be indicative of moderate astrogliosis.

Table 7.4: Comparison of number of GFAP-positive cells per mm<sup>2</sup> according to major SIDS risk factors amongst the whole cohort.

| Brain Region             | SIDS risk factors (whole cohort) |               |         |                          |               |             |                                   |               |             |
|--------------------------|----------------------------------|---------------|---------|--------------------------|---------------|-------------|-----------------------------------|---------------|-------------|
|                          | Sex                              |               |         | Cigarette smoke exposure |               |             | Upper respiratory tract infection |               |             |
|                          | Male (n=17)                      | Female (n=11) | p-value | No (n=15)                | Yes (n=8)     | p-value     | No (n=19)                         | Yes (n=9)     | p-value     |
| <i>Amygdala</i>          | 127±19                           | 136±17        | 0.74    | 134±16                   | 113±37        | 0.54        | 128±15                            | 138±28        | 0.72        |
| <i>Basal ganglia</i>     |                                  |               |         |                          |               |             |                                   |               |             |
| <b>Caudate nucleus</b>   | 126±10                           | 116±18        | 0.58    | 114±12                   | 131±22        | 0.47        | 150±12                            | 114±10        | 0.08        |
| <b>Putamen</b>           | 152±10                           | 169±23        | 0.43    | 147±12                   | 190±26        | 0.09        | 151±12                            | 181±14        | 0.21        |
| <i>Cerebellum</i>        |                                  |               |         |                          |               |             |                                   |               |             |
| <b>Dentate Nucleus</b>   | 341±31                           | 425±34        | 0.10    | 330±28                   | 381±49        | 0.34        | 349±31                            | 418±34        | 0.19        |
| <b>IGL</b>               | 356±18                           | 433±45        | 0.14    | 378±23                   | 391±42        | 0.77        | 372±23                            | 417±48        | 0.34        |
| <b>ML</b>                | 4±3                              | 1±1           | 0.57    | 5±3                      | 1±1           | 0.39        | 3±3                               | 1±1           | 0.56        |
| <b>White matter</b>      | 134±19                           | 165±30        | 0.38    | 124±23                   | 192±35        | 0.11        | 132±20                            | 177±28        | 0.21        |
| <i>Hippocampus</i>       |                                  |               |         |                          |               |             |                                   |               |             |
| <b>CA4</b>               | 251±22                           | 273±25        | 0.52    | 262±27                   | 260±26        | 0.95        | 263±20                            | 251±31        | 0.74        |
| <b>CA3</b>               | 103±14                           | 91±18         | 0.61    | 110±14                   | 75±22         | 0.17        | 101±14                            | 92±21         | 0.71        |
| <b>CA2</b>               | 62±11                            | 59±15         | 0.87    | 69±11                    | 51±20         | 0.40        | 70±11                             | 42±13         | 0.14        |
| <b>CA1</b>               | 122±9                            | 118±18        | 0.81    | 119±7                    | 124±24        | 0.86        | 124±10                            | 112±16        | 0.54        |
| <b>DG</b>                | 185±19                           | 163±29        | 0.50    | 191±25                   | 151±22        | 0.30        | <b>198±19</b>                     | <b>130±22</b> | <b>0.04</b> |
| <b>EC</b>                | 99±11                            | 120±24        | 0.45    | 89±10                    | 117±22        | 0.20        | 102±12                            | 118±26        | 0.54        |
| <b>Subiculum</b>         | 132±12                           | 171±22        | 0.10    | 144±13                   | 149±29        | 0.87        | 145±15                            | 153±19        | 0.73        |
| <b>Temporal cortex</b>   | 82±13                            | 82±16         | 1.00    | <b>63±9</b>              | <b>112±23</b> | <b>0.02</b> | 83±12                             | 81±17         | 0.95        |
| <i>Medulla (rostral)</i> |                                  |               |         |                          |               |             |                                   |               |             |
| <b>AN</b>                | 215±23                           | 208±21        | 0.81    | 187±24                   | 251±20        | 0.09        | 190±19                            | 248±21        | 0.07        |
| <b>Cun</b>               | 173±26                           | 250±49        | 0.15    | 174±32                   | 259±52        | 0.16        | 222±34                            | 176±41        | 0.41        |
| <b>DMNV</b>              | 97±10                            | 121±23        | 0.37    | 106±16                   | 110±25        | 0.89        | 101±12                            | 120±25        | 0.42        |

|             |        |        |      |        |        |      |        |        |      |
|-------------|--------|--------|------|--------|--------|------|--------|--------|------|
| <b>XII</b>  | 41±7   | 43±7   | 0.86 | 39±7   | 52±8   | 0.28 | 39±5   | 46±10  | 0.54 |
| <b>ION</b>  | 120±14 | 164±41 | 0.27 | 124±19 | 174±58 | 0.30 | 135±27 | 145±25 | 0.80 |
| <b>LRF</b>  | 44±8   | 69±13  | 0.09 | 57±12  | 49±9   | 0.70 | 46±6   | 70±17  | 0.21 |
| <b>NSTT</b> | 172±21 | 193±23 | 0.50 | 167±20 | 233±30 | 0.07 | 181±22 | 181±16 | 0.97 |
| <b>NTS</b>  | 56±10  | 91±24  | 0.15 | 65±13  | 91±32  | 0.36 | 67±16  | 76±19  | 0.74 |
| <b>Vest</b> | 65±11  | 117±27 | 0.06 | 66±16  | 88±15  | 0.40 | 70±11  | 115±32 | 0.22 |

Results presented as the mean ± SEM

Bold used to highlight statistical significance, obtained using independent samples t-test. Significance taken at  $p \leq 0.05$ .

**Table 7.5: Comparison of number of GFAP-positive cells per mm<sup>2</sup> according to major risk factors amongst the SIDS cohort.**

| Brain Region             | SIDS Risk Factors (SIDS only) |              |         |                          |           |         |                                   |               |             |                         |           |         |               |               |             |
|--------------------------|-------------------------------|--------------|---------|--------------------------|-----------|---------|-----------------------------------|---------------|-------------|-------------------------|-----------|---------|---------------|---------------|-------------|
|                          | Sex                           |              |         | Cigarette smoke exposure |           |         | Upper respiratory tract infection |               |             | Co-sleeping/Bed-sharing |           |         | Found prone   |               |             |
|                          | Male (n=14)                   | Female (n=7) | p-value | No (n=11)                | Yes (n=7) | p-value | No (n=13)                         | Yes (n=8)     | p-value     | No (n=12)               | Yes (n=9) | p-value | No (n=7)      | Yes (n=12)    | p-value     |
| <i>Amygdala</i>          | 123±22                        | 146±17       | 0.54    | 135±20                   | 113±37    | 0.57    | 130±19                            | 130±33        | 0.99        | 130±20                  | 130±28    | 0.99    | 112±28        | 134±21        | 0.55        |
| <i>Basal Ganglia</i>     |                               |              |         |                          |           |         |                                   |               |             |                         |           |         |               |               |             |
| <b>Caudate nucleus</b>   | 130±11                        | 123±24       | 0.77    | 118±12                   | 137±26    | 0.45    | 121±13                            | 145±13        | 0.30        | 123±17                  | 134±11    | 0.62    | 129±20        | 124±14        | 0.82        |
| <b>Putamen</b>           | 159±11                        | 150±24       | 0.72    | 145±13                   | 170±19    | 0.30    | 151±13                            | 171±12        | 0.38        | 149±15                  | 167±13    | 0.39    | 154±21        | 150±11        | 0.86        |
| <i>Cerebellum</i>        |                               |              |         |                          |           |         |                                   |               |             |                         |           |         |               |               |             |
| <b>Dentate Nucleus</b>   | 341±33                        | 426±56       | 0.24    | 329±32                   | 360±51    | 0.59    | 328±34                            | 422±38        | 0.09        | 355±35                  | 376±45    | 0.71    | 430±31        | 317±38        | 0.06        |
| <b>IGL</b>               | 358±19                        | 426±56       | 0.17    | 364±22                   | 363±36    | 0.97    | 366±19                            | 405±52        | 0.42        | 358±19                  | 412±47    | 0.25    | 404±54        | 354±23        | 0.33        |
| <b>ML</b>                | 4±4                           | 1±1          | 0.59    | 6±5                      | 0±0       | 0.28    | 5±4                               | 1±1           | 0.51        | 1±1                     | 7±6       | 0.24    | 1±1           | 1±1           | 0.91        |
| <b>White matter</b>      | 142±22                        | 125±30       | 0.65    | 113±24                   | 172±33    | 0.16    | 121±22                            | 162±27        | 0.25        | 137±26                  | 136±26    | 0.98    | 142±18        | 129±28        | 0.71        |
| <i>Hippocampus</i>       |                               |              |         |                          |           |         |                                   |               |             |                         |           |         |               |               |             |
| <b>CA4</b>               | 264±25                        | 242±32       | 0.62    | 256±34                   | 247±27    | 0.86    | 270±26                            | 235±29        | 0.40        | 230±18                  | 292±37    | 0.12    | 268±24        | 223±17        | 0.14        |
| <b>CA3</b>               | 100±17                        | 99±27        | 0.96    | 108±19                   | 73±25     | 0.28    | 104±19                            | 93±24         | 0.72        | 83±18                   | 122±23    | 0.20    | 89±27         | 90±15         | 0.97        |
| <b>CA2</b>               | 61±12                         | 72±17        | 0.59    | 76±10                    | 41±21     | 0.12    | 75±13                             | 47±13         | 0.17        | 60±15                   | 70±11     | 0.62    | 56±12         | 64±15         | 0.73        |
| <b>CA1</b>               | 122±10                        | 102±20       | 0.36    | 116±10                   | 108±22    | 0.72    | 118±12                            | 111±18        | 0.74        | 106±14                  | 127±13    | 0.30    | 117±22        | 108±11        | 0.35        |
| <b>DG</b>                | 191±22                        | 139±24       | 0.16    | 175±26                   | 148±25    | 0.50    | <b>203±20</b>                     | <b>127±25</b> | <b>0.03</b> | 167±20                  | 183±31    | 0.67    | 168±31        | 163±19        | 0.44        |
| <b>EC</b>                | 106±13                        | 151±34       | 0.15    | 97±13                    | 117±26    | 0.44    | 118±15                            | 123±30        | 0.95        | 97±16                   | 147±22    | 0.07    | 140±30        | 98±12         | 0.12        |
| <b>Subiculum</b>         | 141±13                        | 157±25       | 0.55    | 154±14                   | 125±20    | 0.24    | 146±15                            | 147±20        | 0.86        | 136±14                  | 160±19    | 0.31    | <b>172±21</b> | <b>121±12</b> | <b>0.02</b> |
| <b>Temporal cortex</b>   | 92±14                         | 88±23        | 0.89    | 64±9                     | 110±27    | 0.07    | 96±15                             | 80±15         | 0.53        | 87±17                   | 95±17     | 0.75    | 107±25        | 82±12         | 0.33        |
| <i>Medulla (rostral)</i> |                               |              |         |                          |           |         |                                   |               |             |                         |           |         |               |               |             |
| <b>AN</b>                | 221±24                        | 220±23       | 0.99    | 195±27                   | 258±23    | 0.14    | 202±23                            | 246±34        | 0.21        | 214±29                  | 228±17    | 0.68    | 241±31        | 205±24        | 0.37        |

|             |        |        |      |        |        |      |        |        |      |              |               |             |        |        |      |
|-------------|--------|--------|------|--------|--------|------|--------|--------|------|--------------|---------------|-------------|--------|--------|------|
| <b>Cun</b>  | 189±29 | 188±35 | 0.98 | 167±30 | 213±31 | 0.33 | 222±28 | 141±25 | 0.06 | 200±30       | 173±32        | 0.56        | 179±25 | 181±34 | 0.96 |
| <b>DMNV</b> | 94±11  | 104±29 | 0.71 | 99±20  | 91±18  | 0.79 | 89±12  | 110±25 | 0.41 | 94±12        | 101±24        | 0.77        | 131±20 | 83±16  | 0.08 |
| <b>XII</b>  | 44±7   | 38±9   | 0.64 | 36±8   | 52±10  | 0.23 | 41±6   | 42±10  | 0.93 | 38±7         | 46±9          | 0.48        | 35±9   | 46±9   | 0.42 |
| <b>ION</b>  | 126±14 | 117±23 | 0.72 | 117±19 | 118±18 | 0.96 | 119±15 | 128±21 | 0.71 | 113±16       | 134±18        | 0.41        | 123±15 | 117±19 | 0.82 |
| <b>LRF</b>  | 46±9   | 58±10  | 0.38 | 48±11  | 48±10  | 0.99 | 45±7   | 57±12  | 0.38 | 48±8         | 53±11         | 0.75        | 60±9   | 46±11  | 0.47 |
| <b>NSTT</b> | 183±21 | 171±19 | 0.70 | 177±23 | 208±18 | 0.38 | 181±23 | 175±18 | 0.84 | 183±21       | 174±23        | 0.78        | 170±21 | 179±24 | 0.79 |
| <b>NTS</b>  | 56±11  | 57±15  | 0.97 | 56±13  | 61±11  | 0.82 | 51±9   | 64±18  | 0.46 | 54±9         | 59±16         | 0.77        | 49±17  | 59±12  | 0.63 |
| <b>Vest</b> | 66±13  | 144±35 | 0.08 | 71±22  | 97±15  | 0.42 | 75±15  | 115±32 | 0.26 | <b>58±16</b> | <b>126±25</b> | <b>0.04</b> | 135±40 | 64±13  | 0.14 |

Results presented as the mean ± SEM

Bold used to highlight statistical significance. Obtained using independent samples t-test. Significance taken at  $p \leq 0.05$ .

Table 7.6: Comparison of R-score values according to major SIDS risk factors amongst the whole cohort.

| Brain Region             | SIDS risk factors (whole cohort) |                 |                 |                          |           |         |                                   |                 |                 |
|--------------------------|----------------------------------|-----------------|-----------------|--------------------------|-----------|---------|-----------------------------------|-----------------|-----------------|
|                          | Sex                              |                 |                 | Cigarette smoke exposure |           |         | Upper respiratory tract infection |                 |                 |
|                          | Male (n=17)                      | Female (n=11)   | P value         | No (n=15)                | Yes (n=8) | P value | No (n=19)                         | Yes (n=9)       | P value         |
| <i>Amygdala</i>          | 142 (139)                        | 132 (66)        | 0.92            | 138 (56)                 | 97 (160)  | 0.50    | 136 (119)                         | 150 (92)        | 1.00            |
| <i>Basal ganglia</i>     |                                  |                 |                 |                          |           |         |                                   |                 |                 |
| <b>Caudate nucleus</b>   | 122 (59)                         | 128 (94)        | 0.30            | 108 (59)                 | 122 (88)  | 0.15    | <b>112 (30)</b>                   | <b>159 (37)</b> | <b>&lt;0.01</b> |
| <b>Putamen</b>           | 149 (50)                         | 148 (117)       | 0.45            | 148 (50)                 | 177 (98)  | 0.27    | 145 (60)                          | 157 (81)        | 0.16            |
| <i>Cerebellum</i>        |                                  |                 |                 |                          |           |         |                                   |                 |                 |
| <b>Dentate Nucleus</b>   | 104 (6)                          | 105 (8)         | 0.90            | 103 (8)                  | 104 (6)   | 0.98    | 102 (6)                           | 105 (7)         | 0.25            |
| <b>IGL</b>               | 124 (25)                         | 123 (30)        | 0.78            | 123 (18)                 | 131 (47)  | 0.98    | 123 (23)                          | 126 (29)        | 0.74            |
| <b>ML</b>                | 0 (0)                            | 0 (100)         | 0.49            | 0 (0)                    | 0 (0)     | 0.78    | 0 (0)                             | 0 (0)           | 0.66            |
| <b>White matter</b>      | 107 (20)                         | 113 (33)        | 0.43            | 107 (21)                 | 113 (24)  | 0.36    | 104 (20)                          | 116 (13)        | 0.16            |
| <i>Hippocampus</i>       |                                  |                 |                 |                          |           |         |                                   |                 |                 |
| <b>CA4</b>               | 106 (17)                         | 114 (22)        | 0.46            | 108 (26)                 | 112 (14)  | 0.73    | 106 (14)                          | 121 (34)        | 0.36            |
| <b>CA3</b>               | 100 (0)                          | 100 (0)         | 0.89            | 100 (0)                  | 100 (0)   | 0.83    | 100 (0)                           | 100 (0)         | 0.85            |
| <b>CA2</b>               | 100 (0)                          | 100 (100)       | 0.89            | 100 (0)                  | 75 (100)  | 0.36    | 100 (0)                           | 100 (50)        | 0.81            |
| <b>CA1</b>               | <b>101 (3)</b>                   | <b>106 (10)</b> | <b>0.03</b>     | 103 (6)                  | 107 (24)  | 0.44    | 102 (7)                           | 106 (12)        | 0.40            |
| <b>DG</b>                | 100 (0)                          | 100 (15)        | 0.43            | 100 (0)                  | 100 (14)  | 0.33    | 100 (0)                           | 100 (8)         | 0.77            |
| <b>EC</b>                | 100 (0)                          | 100 (14)        | 0.33            | 100 (0)                  | 100 (7)   | 0.21    | 100 (0)                           | 100 (4)         | 0.59            |
| <b>Subiculum</b>         | 102 (7)                          | 101 (13)        | 1.00            | 102 (9)                  | 105 (12)  | 0.88    | 102 (8)                           | 101 (11)        | 0.96            |
| <b>Temporal cortex</b>   | 100 (0)                          | 100 (11)        | 0.36            | 100 (0)                  | 100 (34)  | 0.22    | 100 (0)                           | 100 (1)         | 0.60            |
| <i>Medulla (rostral)</i> |                                  |                 |                 |                          |           |         |                                   |                 |                 |
| <b>AN</b>                | 100 (16)                         | 105 (24)        | 0.73            | 101 (17)                 | 100 (26)  | 0.64    | 100 (26)                          | 104 (22)        | 0.52            |
| <b>Cun</b>               | <b>105 (8)</b>                   | <b>124 (28)</b> | <b>&lt;0.01</b> | 105 (14)                 | 115 (20)  | 0.07    | 105 (18)                          | 111 (22)        | 0.97            |
| <b>DMNV</b>              | 106 (14)                         | 112 (23)        | 0.80            | 104 (18)                 | 111 (21)  | 0.41    | 111 (18)                          | 103 (19)        | 0.53            |



|             |          |          |      |          |          |      |          |          |      |
|-------------|----------|----------|------|----------|----------|------|----------|----------|------|
| <b>XII</b>  | 107 (12) | 108 (10) | 0.69 | 107 (11) | 108 (5)  | 0.69 | 110 (18) | 104 (9)  | 0.06 |
| <b>ION</b>  | 115 (19) | 117 (41) | 0.76 | 113 (28) | 120 (31) | 0.89 | 115 (30) | 117 (16) | 1.00 |
| <b>LRF</b>  | 100 (15) | 100 (29) | 0.50 | 100 (20) | 100 (4)  | 0.64 | 103 (17) | 100 (0)  | 0.11 |
| <b>NSTT</b> | 108 (13) | 109 (20) | 0.80 | 108 (20) | 109 (9)  | 0.32 | 109 (16) | 108 (16) | 0.50 |
| <b>NTS</b>  | 105 (22) | 113 (18) | 0.17 | 106 (21) | 114 (15) | 0.49 | 111 (23) | 108 (13) | 0.53 |
| <b>Vest</b> | 100 (9)  | 103 (6)  | 0.85 | 101 (7)  | 101 (11) | 1.00 | 105 (12) | 101 (6)  | 0.40 |

Results presented as the medians (interquartile range)

Bold used to highlight statistical significance. Obtained using Mann-Whitney U test. Significance taken at  $p \leq 0.05$ .

**Table 7.7: Comparison of R-score values according to major risk factors amongst the SIDS cohort.**

| Brain Region           | SIDS Risk Factors (SIDS only) |              |         |                          |           |         |                                   |           |         |                         |           |         |             |            |         |
|------------------------|-------------------------------|--------------|---------|--------------------------|-----------|---------|-----------------------------------|-----------|---------|-------------------------|-----------|---------|-------------|------------|---------|
|                        | Sex                           |              |         | Cigarette smoke exposure |           |         | Upper respiratory tract infection |           |         | Co-sleeping/Bed-sharing |           |         | Found prone |            |         |
|                        | Male (n=14)                   | Female (n=7) | p-value | No (n=11)                | Yes (n=7) | p-value | No (n=13)                         | Yes (n=8) | p-value | No (n=12)               | Yes (n=9) | p-value | No (n=7)    | Yes (n=12) | p-value |
| <i>Amygdala</i>        | 105 (7)                       | 104 (15)     | 0.67    | 105 (7)                  | 104 (13)  | 0.70    | 105 (7)                           | 104 (16)  | 0.57    | 105 (8)                 | 104 (3)   | 0.74    | 103 (25)    | 105 (5)    | 0.31    |
| <i>Basal Ganglia</i>   |                               |              |         |                          |           |         |                                   |           |         |                         |           |         |             |            |         |
| <b>Caudate nucleus</b> | 102 (16)                      | 103 (21)     | 0.44    | 102 (11)                 | 103 (23)  | 0.31    | 102 (3)                           | 115 (26)  | 0.06    | 102 (20)                | 103 (13)  | 0.46    | 109 (31)    | 102 (11)   | 0.37    |
| <b>Putamen</b>         | 113 (32)                      | 102 (41)     | 0.34    | 115 (28)                 | 113 (49)  | 0.44    | 105 (34)                          | 127 (29)  | 0.34    | 110 (45)                | 116 (31)  | 0.83    | 114 (49)    | 110 (34)   | 0.79    |
| <i>Cerebellum</i>      |                               |              |         |                          |           |         |                                   |           |         |                         |           |         |             |            |         |
| <b>Dentate Nucleus</b> | 104 (7)                       | 105 (11)     | 0.80    | 104 (6)                  | 105 (9)   | 1.00    | 101 (20)                          | 106 (5)   | 0.21    | 105 (6)                 | 104 (11)  | 0.86    | 104 (5)     | 105 (10)   | 0.43    |
| <b>IGL</b>             | 122 (23)                      | 123 (32)     | 0.74    | 124 (15)                 | 126 (35)  | 0.86    | 121 (23)                          | 129 (31)  | 0.37    | 123 (17)                | 107 (31)  | 0.55    | 108 (23)    | 125 (22)   | 0.14    |
| <b>ML</b>              | 0 (0)                         | 0 (0)        | 1.00    | 0 (100)                  | 0 (100)   | 0.38    | 0 (0)                             | 0 (0)     | 0.92    | 0 (0)                   | 0 (50)    | 0.60    | 0 (0)       | 0 (0)      | 0.84    |
| <b>White matter</b>    | 108 (19)                      | 110 (16)     | 0.87    | 107 (16)                 | 113 (16)  | 0.38    | 104 (12)                          | 115 (13)  | 0.12    | 108 (18)                | 110 (15)  | 0.81    | 116 (19)    | 107 (13)   | 0.43    |
| <i>Hippocampus</i>     |                               |              |         |                          |           |         |                                   |           |         |                         |           |         |             |            |         |
| <b>CA4</b>             | 108 (21)                      | 108 (22)     | 0.56    | 109 (30)                 | 109 (15)  | 0.93    | 106 (16)                          | 114 (38)  | 0.65    | 107 (14)                | 118 (27)  | 0.51    | 105 (19)    | 108 (21)   | 0.71    |
| <b>CA3</b>             | 100 (0)                       | 100 (0)      | 0.80    | 100 (0)                  | 100 (0)   | 0.79    | 100 (0)                           | 100 (0)   | 0.65    | 100 (0)                 | 100 (0)   | 0.75    | 100 (0)     | 100 (0)    | 0.65    |

|                          |                |                     |             |                |                     |             |                     |                |             |             |             |      |                     |                |                 |
|--------------------------|----------------|---------------------|-------------|----------------|---------------------|-------------|---------------------|----------------|-------------|-------------|-------------|------|---------------------|----------------|-----------------|
| <b>CA2</b>               | 100<br>(13)    | 100 (0)             | 0.86        | <b>100 (0)</b> | <b>50<br/>(100)</b> | <b>0.04</b> | 100<br>(25)         | 100 (0)        | 0.75        | 100<br>(75) | 100 (0)     | 0.55 | 100 (0)             | 100<br>(88)    | 0.26            |
| <b>CA1</b>               | 102 (7)        | 106<br>(10)         | 0.12        | 103 (5)        | 103<br>(14)         | 0.89        | 103<br>(10)         | 105<br>(14)    | 0.68        | 102 (5)     | 106<br>(13) | 0.26 | 105<br>(12)         | 102 (6)        | 0.54            |
| <b>DG</b>                | 100 (0)        | 100 (0)             | 0.97        | 100 (0)        | 100 (2)             | 0.54        | 100 (0)             | 100<br>(11)    | 0.50        | 100 (0)     | 100 (0)     | 0.92 | 100 (2)             | 100 (0)        | 0.54            |
| <b>EC</b>                | 100 (0)        | 102<br>(46)         | 0.27        | 100 (0)        | 100<br>(16)         | 0.53        | 100 (4)             | 100 (4)        | 0.97        | 100 (0)     | 100<br>(22) | 0.30 | 102<br>(43)         | 100 (0)        | 0.10            |
| <b>Subiculum</b>         | 102 (8)        | 102<br>(12)         | 0.97        | 102<br>(38)    | 102 (8)             | 0.33        | 102 (7)             | 102<br>(12)    | 0.59        | 102 (7)     | 102<br>(10) | 0.51 | 108<br>(40)         | 102 (5)        | 0.43            |
| <b>Temporal cortex</b>   | 100 (1)        | 100<br>(11)         | 0.70        | 100 (0)        | 100<br>(10)         | 0.43        | 100 (1)             | 100 (2)        | 0.90        | 100 (1)     | 100 (4)     | 0.72 | 100<br>(13)         | 100 (0)        | 0.53            |
| <b>Medulla (rostral)</b> |                |                     |             |                |                     |             |                     |                |             |             |             |      |                     |                |                 |
| <b>AN</b>                | 101<br>(22)    | 105<br>(20)         | 0.84        | 103<br>(21)    | 100 (7)             | 0.15        | 100<br>(13)         | 104<br>(22)    | 0.27        | 100<br>(17) | 102<br>(22) | 0.50 | <b>120<br/>(29)</b> | <b>100 (0)</b> | <b>&lt;0.01</b> |
| <b>Cun</b>               | <b>105 (8)</b> | <b>120<br/>(22)</b> | <b>0.03</b> | 105<br>(14)    | 111<br>(15)         | 0.18        | 106<br>(16)         | 108<br>(15)    | 0.60        | 105<br>(16) | 113<br>(20) | 0.23 | 114<br>(23)         | 105 (7)        | 0.68            |
| <b>DMNV</b>              | 107<br>(14)    | 102<br>(21)         | 0.64        | 104<br>(18)    | 109<br>(14)         | 0.73        | 112<br>(21)         | 103<br>(14)    | 0.24        | 105<br>(14) | 111<br>(19) | 0.77 | 112<br>(33)         | 102<br>(14)    | 0.15            |
| <b>XII</b>               | 110<br>(11)    | 106<br>(10)         | 0.33        | 108<br>(11)    | 108 (8)             | 0.71        | <b>110<br/>(15)</b> | <b>102 (7)</b> | <b>0.01</b> | 108<br>(15) | 108 (9)     | 0.97 | 105<br>(12)         | 108<br>(12)    | 0.56            |
| <b>ION</b>               | 120<br>(24)    | 108<br>(16)         | 0.54        | 113<br>(20)    | 114<br>(22)         | 1.00        | 118<br>(30)         | 115<br>(17)    | 0.73        | 108<br>(19) | 118<br>(22) | 0.30 | 117 (8)             | 103<br>(21)    | 0.38            |
| <b>LRF</b>               | 100<br>(13)    | 100<br>(16)         | 1.00        | 100 (4)        | 100 (9)             | 0.79        | <b>104<br/>(17)</b> | <b>100 (0)</b> | <b>0.05</b> | 100 (9)     | 100<br>(16) | 1.00 | 100<br>(15)         | 100 (0)        | 0.32            |
| <b>NSTT</b>              | 108<br>(13)    | 108<br>(16)         | 0.49        | 108<br>(23)    | 109 (4)             | 0.73        | 108<br>(19)         | 107<br>(11)    | 0.31        | 108 (6)     | 109<br>(28) | 0.33 | 105<br>(14)         | 108 (6)        | 0.25            |

|             |             |             |      |             |             |      |             |             |      |             |             |      |             |             |      |
|-------------|-------------|-------------|------|-------------|-------------|------|-------------|-------------|------|-------------|-------------|------|-------------|-------------|------|
| <b>NTS</b>  | 106<br>(25) | 111<br>(34) | 0.39 | 106<br>(26) | 113<br>(19) | 0.49 | 114<br>(29) | 105<br>(12) | 0.43 | 105<br>(23) | 113<br>(24) | 0.41 | 107<br>(30) | 106<br>(16) | 0.79 |
| <b>Vest</b> | 100 (8)     | 103<br>(11) | 0.66 | 100 (7)     | 101<br>(14) | 0.61 | 108<br>(14) | 101 (6)     | 0.37 | 100 (6)     | 107 (8)     | 0.17 | 107<br>(10) | 100 (3)     | 0.07 |

Results presented as median (interquartile range)

Bold used to highlight statistical significance. Obtained using Mann-Whitney U test. Significance taken at  $p \leq 0.05$ .

## **7.4: DISCUSSION**

Using the number of GFAP-positive cells and their reactivity-status as assessed by morphology, we document GFAP expression in the developing human brain, and the region-specific association with developmental parameters and SIDS risk factors (findings summarised in Table 7.8). No differences were seen in GFAP expression as a factor of SIDS diagnosis, while analysis by the risk factors showed region-specific differences, including higher counts in the temporal cortex associated with cigarette smoke exposure, lower counts in the DG associated with URTI, and females having higher reactivity than males in the Cun. Sleep related risk factors were specific to the SIDS group and showed an association between prone sleeping and lower GFAP expression in the subiculum and AN, while bed-sharers had higher GFAP in the Vest. Findings will be assessed in accordance with findings of cell death and microglia, which were studied amongst the same cohort.

Comparisons between the findings of this Chapter to those from the UK cohort (Chapter 6), will be discussed in detail in the next Chapter.

**Table 7.8: Heat map of brain regions where GFAP was significantly associated with developmental and risk factor parameters.**

|                             | Amygdala | Caudate nucleus | Putamen | Dentate nucleus | IGL | ML | White matter | CA4 | CA3 | CA2 | CA1 | DG | Sub | EC | Temporal cortex | AN | Cun | DMNV | XII | ION | LRF | NSTT | NTS | Vest |
|-----------------------------|----------|-----------------|---------|-----------------|-----|----|--------------|-----|-----|-----|-----|----|-----|----|-----------------|----|-----|------|-----|-----|-----|------|-----|------|
| <b>GFAP+/mm<sup>2</sup></b> |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Brain weight                |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Head circumference          |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| PCA                         |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| SIDS                        |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Male sex                    |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Cigarette smoke exposure    |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| URTI                        |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Bed-sharing                 |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Prone sleeping              |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| <b>R-score</b>              |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Brain weight                |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Head circumference          |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| PCA                         |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| SIDS                        |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Male sex                    |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Cigarette smoke exposure    |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| URTI                        |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Bed-sharing                 |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Prone sleeping              |          |                 |         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |

Developmental parameters of brain weight, head circumference and PCA (whole cohort) and according to risk factors (whole cohort + SIDS only included).

*p*-values indicated as  $\leq 0.05$ ,  $\leq 0.01$  or  $\leq 0.001$  for parameters associated with higher expression, and  $\leq 0.05$ ,  $\leq 0.01$  or  $\leq 0.001$  for parameters associated with lower expression.

#### **7.4.1: COMPARATIVE ACCORDING TO DEVELOPMENTAL PARAMETERS**

Negative associations were seen between developmental parameters and GFAP such that increased age, body weight, brain weight, body length and head circumference were associated with a lower number of GFAP-positive cells. These associations were consistently (counting and R-score) seen in the regions of the cerebellum, EC, subiculum, temporal cortex and Cun, DMNV and ION, however separation by sub-categories of eSUDI and SIDS (I+II combined) showed that most appeared to be specific to the SIDS group. As discussed in Chapter 6, negative correlations of growth and astrocyte expression during early postnatal development are not unusual and have been reported in animal models; for example a decrease in the number of astrocytes per area measurement in accordance with increased brain volumes (Ge et al., 2012, Shoneye et al., 2020, Schreiner et al., 2014). Thus, our data appear to be consistent with this.

#### **7.4.2: COMPARATIVE BETWEEN DIAGNOSTIC GROUPS**

Despite previous reports of increased astrogliosis in the SIDS cerebellum (Lavezzi et al., 2018) and brainstem (Bruce and Becker, 1991, Storm et al., 1994, Obonai et al., 1996, Storm et al., 1999, Sawaguchi et al., 2002, Matturri et al., 2006, Spinelli et al., 2018), the findings from the current study were consistent with the other studies who report the incidence of astrogliosis and/or its severity is not altered in SIDS brainstem compared to controls (Reske-Nielsen et al., 1987, Oehmichen et al., 1989a, Pamphlett and Treloar, 1996, Kinney et al., 2015). The findings of this study extend this literature to indicate differences according to the SIDS diagnostic category did not translate to other brain regions of the EC, temporal cortex, hippocampus and basal ganglia.

Although we have selected controls that died suddenly of known causes, and who were matched on clinical and autopsy parameters, astrogliosis may still be present under certain conditions, with some evidence of abnormal brain development in cases of congenital heart disease (Kinney et al., 2005, Miller et al., 2007). Repeated hypoxic-ischemic episodes due to congenital heart conditions has been shown to result in significant astrogliosis (Kinney et al., 2005). These hypoxic ischemic episodes are thought to greatly precede death, with long survival times post-injury, allowing for more significant GFAP upregulation (Trautz et al., 2019, Goc et al., 2014). 5/7 eSUDI cases in the current study dataset were cardiac deaths,



potentially skewing baseline GFAP levels for comparison. Infant deaths due to trauma (such as drowning or motor vehicle accidents with no neurological damage or head injury) may represent the most “normal” postnatal brain, however, we did not have any such cases in our study.

Integration of epidemiological, physiological, and neuropathological data has emphasised the multifactorial nature of SIDS, with its diagnosis representing the common end point for a range of pathophysiologies, rather than a single underlying cause (Byard and Jensen, 2008). As such, analysing a group of infants who died of different causes may mask patterns and trends in the data, further accounting for why some studies report astrogliosis in SIDS, while others do not. Given that the underlying mechanism of death remains unknown, this is something that we are unable to control for. Investigation of astrogliosis according to the presence of known SIDS risk factors may be more appropriate than correlating outcomes against diagnosis, given that unlike diagnosis, a particular risk factor may represent a common mechanism that is identifiable at death.

#### **7.4.3: COMPARISONS ACCORDING TO SIDS RISK FACTORS**

Repeated disruptions in CNS homeostasis, such as in the case of hypoxia or infection can trigger astrocyte reactivity (Kang and Hébert, 2011, Burda et al., 2016, Burda and Sofroniew, 2014) with activation and signalling pathways differing depending on the type of insult. Broadly, astrocytes can either become neurotoxic (A1 subtype) or neuroprotective (A2 subtype), triggered by inflammation and hypoxia/ischemia respectively (Zamanian et al., 2012, Chai et al., 2017, Yu et al., 2021). Exposure to SIDS risk factors could potentially lead to significant astrogliosis, such as in the case of intermittent hypercapnic hypoxia hypothesised to occur as a result of sleeping prone (Campbell et al., 1997), although human studies exploring the relationship between astrogliosis and SIDS risk factors are limited. The current study found that infants exposed to cigarette smoke had an almost two-fold increase in the number of GFAP-positive cells in the temporal cortex, however, for many of the risk factors including male sex, URTI and prone sleeping, GFAP expression was decreased, either in number, or ‘reactivity’ status. Interestingly, expression of microglia markers were also decreased amongst infants with reported risk factors in the same cohort (discussed below) (Ambrose et al., 2020).

The presence of an URTI showed the most changes in GFAP, with decreased expression seen in the DG, decreased reactivity seen in the XII and LRF, and increased reactivity seen in the

caudate nucleus. One hypothesis is that infection may have a causative association with SIDS. Viral and bacterial URTI have been noted in many SIDS cases (McKendrick et al., 1992, Morris, 1999) and downstream inflammatory responses have been suggested to trigger the events leading to SIDS (Lundemose et al., 1993, Vege et al., 1995, Raza and Blackwell, 1999, Ferrante et al., 2008, Moscovis et al., 2015). Epidemiological data supports that the risk of sleeping prone is enhanced by thermal stress and excessive thermal insulation which can occur when an infant is unwell and has excessive bedding/bundling (Mitchell, 2009, Bach and Libert, 2022). In this case, URTI could lead to downstream effects of inflammation and subsequent astrogliosis. Whether the relationship between URTI and SIDS is causal, however, remains contested (Weber et al., 2010). An alternative mechanism by which URTI may increase the risk of SIDS is due to heightened risk of febrile seizures during illness/fever (Tang et al., 2014, Gourabi et al., 2012, Sharawat et al., 2016), potentially leading to sudden death, particularly if occurring during a sleep period and/or lying prone (Hesdorffer et al., 2015). This more rapid mechanism of death may not allow sufficient time for GFAP to upregulate, and thus, may account for why at least a proportion of infants with URTI show reduced GFAP expression.

Increased astrocyte number and reactivity was also seen in the Cun and CA1 of female infants. GFAP may be differentially upregulated in males compared to females following injury, as seen following exposure to cigarette smoke in animal models (Peixoto et al., 2019, Arias et al., 2009), potentially due to complex interactions with hormones. It is not known whether there are sex-based vulnerabilities to GFAP upregulation in the infant brain, or following exposure to other risk factors, although these findings confirm that GFAP may be differentially upregulated in response to injury between males and females.

Infants who bed-shared had a higher number of GFAP-positive cells in the Vest, which is in the opposite direction found in the same region by (Spinelli et al., 2018), whereby they compared GFAP expression according to bedsharing status in their SIDS cohort. Interestingly, baseline values were similar with (Spinelli et al., 2018) reporting 66 (vs 58 in the current study) GFAP-positive cells per mm<sup>2</sup>. It is unclear why there would be a difference in direction of change, but possibilities could include different characteristics of the datasets, and the fact that we did not distinguish between inferior and superior portions of the Vest. For our analysis of bed-sharing, we included the SIDS I as non-bed-sharers to improve the statistical power of the analysis.

One possible explanation for other findings of decreased expression is that expressional alterations are the result of developmental differences between comparison groups, and thus, decreased expression is independent of the risk factor itself. Infants found prone had higher body weight, brain weight, body length and head circumference on average, all factors which were associated with reduced GFAP expression. Given that SIDS is multifactorial in nature, baseline values are likely further skewed by the presence of other risk-factors, such that the ‘no risk factor’ group (i.e., no recent URTI) in each comparison likely contains infants with who have a number of other risk factors present. Multifactorial analysis may be more appropriate in this case, although we were limited by our small sample size and incomplete clinical and risk factor data for some infants.

#### **7.4.4: COMPARISON OF FINDINGS AGAINST CELL DEATH AND MICROGLIA**

For the SIDS II cohort, we found no evidence of a relationship between the numbers, or reactivity of astrocytes compared to the other diagnostic subgroups which precluded correlations with differences seen in the cell death and microglial studies. However, outcomes of the different markers could be correlated with growth parameters of the infants, and with exposure to SIDS risk factors.

Differences in cell death and microglial markers were found for the SIDS II cohort compared to the other two diagnostic sub-categories (eSUDI and SIDS I). Markers of cell death (Caspase-3 and TUNEL) were increased in SIDS II infants throughout regions studied compared to both eSUDI and SIDS I diagnostic groups (Ambrose et al., 2019). The microglial markers evaluated resting (ionized calcium-binding adaptor molecule 1 (Iba1)) and activated (cluster of differentiation 68 (CD68) and human leukocyte antigens (HLA)) microglial states (Ambrose et al., 2020). The SIDS II infants had reduced microglia (identified as the area of Iba1) while the SIDS I group displayed fewer activated microglia in the hippocampus compared to SIDS II and eSUDI. There were no correlations between the findings for microglial changes and cell death amongst the SIDS infants, thus it was not possible to conclude any relationship between these pathways.

Although microglial and astrocyte changes were not seen within the same ROI, the same direction of change i.e., reduced markers, was also seen according to growth parameters and for the presence of SIDS risk factors. In the Cun and DMNV in the medulla, negative

correlations were seen for the mean area of Iba1, the number of GFAP-positive cells and the R-score in the Cun (brain weight, head circumference) and DMNV (brain weight, body weight). There were also negative correlations for the mean area of Iba1 and the number of GFAP-positive cells in the XII (PCA) and NTS (brain weight, head circumference).

Although astrocyte activation can be triggered by microglia under certain conditions such as CNS infection (Zhang et al., 2010, Goc et al., 2014, Liddelow et al., 2017), the timing of their activation may differ. Microglia are early responders to damage in the CNS, and can become activated within minutes of insult (Lier et al., 2020), while at the protein level, GFAP upregulation may not be present until at least 12 hours after injury (O'Callaghan et al., 2014). It is likely the case that underlying brain pathology in SIDS, and as a result of exposure to risk factors is highly complex, involving many other cell types such as inflammatory cells, chemokines and neuron-glia interactions expressed at different time-points (Ransohoff and El Khoury, 2016). The next step would be to study markers, such as those associated with inflammation to further reveal the underlying processes that are occurring.

## **7.5: CONCLUSION**

In this study we showed regional heterogeneity in both the number of GFAP-positive cells and their level of reactivity in 24 different brain regions of the developing human brain, as well as their association with developmental parameters. GFAP levels did not differ amongst the diagnostic groups, but they did for the risk factors of male sex, exposure to cigarette smoke, URTI, bed-sharing and sleeping prone, albeit being region specific.

## **7.6: APPENDIX**

This Appendix contains the supplementary material available.

**Supplement Table 1. An overview of the significant associations between the number of GFAP-positive astrocytes/mm<sup>2</sup> in the infant brain and developmental parameters.**

| Brain Region             | Infant Parameter   | ALL<br>r-value <sup>a</sup> | ALL<br>p-<br>value <sup>b</sup> | eSUDI<br>(n=7)<br>p-value | SIDS<br>(n=21)<br>p-value |
|--------------------------|--------------------|-----------------------------|---------------------------------|---------------------------|---------------------------|
| <i>Basal ganglia</i>     |                    |                             |                                 |                           |                           |
| <b>Caudate</b>           | PCA                | -0.40                       | 0.05                            |                           | 0.05                      |
| <i>Cerebellum</i>        |                    |                             |                                 |                           |                           |
| <b>Dentate nucleus</b>   | Body weight        | -0.75                       | <0.001                          | <0.01                     | <0.001                    |
|                          | Brain weight       | -0.76                       | <0.001                          | <0.01                     | <0.001                    |
|                          | Body length        | -0.70                       | <0.001                          |                           | <0.001                    |
|                          | Head circumference | -0.74                       | <0.001                          | <0.01                     | <0.001                    |
|                          | PCA                | -0.74                       | <0.001                          | 0.01                      | <0.001                    |
| <b>IGL</b>               | Body weight        | -0.46                       | 0.01                            |                           |                           |
|                          | Brain weight       | -0.44                       | 0.02                            |                           |                           |
|                          | Body length        | -0.53                       | <0.01                           |                           | 0.04                      |
|                          | Head circumference | -0.46                       | <0.01                           |                           | 0.05                      |
|                          | PCA                | -0.56                       | <0.01                           |                           | <0.01                     |
| <b>White matter</b>      | Body weight        | -0.65                       | <0.001                          | 0.04                      | <0.01                     |
|                          | Brain weight       | -0.65                       | <0.001                          |                           | <0.01                     |
|                          | Body length        | -0.74                       | <0.001                          | <0.01                     | <0.01                     |
|                          | Head circumference | -0.59                       | <0.01                           |                           | 0.03                      |
|                          | PCA                | -0.68                       | <0.001                          | 0.02                      | <0.01                     |
| <i>Hippocampus</i>       |                    |                             |                                 |                           |                           |
| <b>CA2</b>               | Body length        | 0.41                        | 0.03                            |                           |                           |
| <b>EC</b>                | Body weight        | -0.50                       | <0.01                           |                           | <0.01                     |
|                          | Brain weight       | -0.47                       | 0.01                            |                           | <0.001                    |
|                          | Body length        | -0.41                       | 0.04                            |                           | <0.001                    |
|                          | Head circumference | -0.50                       | <0.01                           |                           | <0.001                    |
|                          | PCA                | -0.58                       | <0.01                           |                           | <0.001                    |
| <b>Subiculum</b>         | Body weight        | -0.44                       | 0.02                            |                           |                           |
|                          | Body length        | -0.38                       | 0.05                            |                           |                           |
|                          | Head circumference | -0.39                       | 0.04                            |                           |                           |
|                          | PCA                | -0.43                       | 0.02                            |                           |                           |
| <b>Temporal cortex</b>   | Body weight        | -0.51                       | <0.01                           |                           | <0.01                     |
|                          | Brain weight       | -0.40                       | 0.04                            |                           | <0.001                    |
|                          | Body length        | -0.40                       | 0.04                            |                           | <0.001                    |
|                          | Head circumference | -0.41                       | 0.04                            |                           | <0.01                     |
|                          | PCA                | -0.55                       | <0.01                           |                           | <0.001                    |
| <i>Medulla (rostral)</i> |                    |                             |                                 |                           |                           |
| <b>Cun</b>               | Body weight        | -0.49                       | 0.02                            | 0.05                      |                           |
|                          | Brain weight       |                             |                                 | 0.01                      |                           |

|             |                    |       |        |       |
|-------------|--------------------|-------|--------|-------|
|             | Body length        | -0.66 | <0.01  | 0.04  |
|             | Head circumference | -0.44 | 0.04   | 0.02  |
|             | PCA                |       |        | 0.02  |
| <b>DMNV</b> | Body weight        | -0.46 | 0.02   | 0.02  |
|             | Brain weight       | -0.39 | 0.05   |       |
|             | Body length        | -0.49 | 0.01   |       |
|             | PCA                | -0.40 | 0.05   |       |
| <b>XII</b>  | Body weight        | -0.50 | 0.01   | 0.04  |
|             | Body length        | -0.55 | <0.01  | 0.03  |
|             | PCA                | -0.41 | 0.05   |       |
| <b>ION</b>  | Body weight        | -0.58 | <0.01  | 0.01  |
|             | Brain weight       | -0.49 | 0.01   |       |
|             | Body length        | -0.62 | <0.001 | 0.03  |
|             | Head circumference | -0.50 | <0.01  | 0.05  |
|             | PCA                | -0.49 | 0.01   |       |
| <b>LRF</b>  | Body weight        | -0.51 | <0.01  | <0.01 |
|             | Brain weight       | -0.48 | 0.02   | 0.04  |
|             | Body length        | -0.69 | <0.001 | 0.01  |
|             | Head circumference | -0.47 | 0.02   | 0.03  |
|             | PCA                | -0.40 | 0.05   |       |
| <b>NSTT</b> | Body weight        | -0.50 | 0.01   |       |
|             | Body length        | -0.40 | 0.04   |       |
|             | PCA                |       |        | 0.03  |
| <b>NTS</b>  | Body weight        | -0.56 | <0.01  |       |
|             | Brain weight       | -0.45 | 0.03   |       |
|             | Body length        | -0.59 | <0.01  |       |
|             | Head circumference | -0.46 | 0.02   |       |
|             | PCA                | -0.44 | 0.03   |       |

<sup>a</sup> Pearson's correlation coefficient.

<sup>b</sup> Significance taken at  $p \leq 0.05$

*Abbreviations:* CA cornu ammonis, *Cun* Cuneate nucleus, *DG* dentate gyrus, *DMNV* dorsal motor nucleus of the vagus, *EC* entorhinal cortex, *XII* hypoglossal nucleus, *IGL* internal granular layer of the cerebellar cortex, *ION* inferior olivary nucleus, *IGL* internal granular layer, *LRF* lateral reticular formation, *NSTT* nucleus of the spinal trigeminal tract, *NTS* nucleus of the solitary tract, *PCA* postconceptional age, *PMI* post-mortem interval.

**Supplement Table 2. An overview of the significant associations between R-score values in the infant brain and developmental parameters.**

| Brain Region                    | Infant Parameter   | ALL<br>r-value <sup>a</sup> | ALL<br>p-value <sup>b</sup> | eSUDI<br>(n=7)<br>p-value | SIDS<br>(n=21) p-<br>value |
|---------------------------------|--------------------|-----------------------------|-----------------------------|---------------------------|----------------------------|
| <b><i>Basal ganglia</i></b>     |                    |                             |                             |                           |                            |
| <b>Caudate</b>                  | Brain weight       | -0.34                       | 0.02                        | 0.03                      | 0.03                       |
|                                 | Body length        | -0.37                       | 0.01                        |                           |                            |
|                                 | Head circumference | -0.32                       | 0.03                        |                           |                            |
|                                 | PCA                | -0.52                       | <0.001                      |                           |                            |
| <b><i>Cerebellum</i></b>        |                    |                             |                             |                           |                            |
| <b>Dentate nucleus</b>          | PCA                |                             |                             |                           | 0.01                       |
| <b>White matter</b>             | Body weight        | -0.43                       | <0.01                       |                           | 0.03                       |
|                                 | Brain weight       | -0.50                       | <0.01                       |                           |                            |
|                                 | Body length        | -0.50                       | <0.001                      |                           | 0.05                       |
|                                 | Head circumference | -0.45                       | <0.01                       |                           | 0.05                       |
|                                 | PCA                | -0.45                       | <0.01                       |                           |                            |
| <b><i>Hippocampus</i></b>       |                    |                             |                             |                           |                            |
| <b>CA4</b>                      | PCA                |                             |                             |                           | <0.01*                     |
| <b>EC</b>                       | Body weight        | -0.40                       | 0.01                        |                           | <0.01                      |
|                                 | Brain weight       | -0.37                       | 0.02                        |                           | 0.01                       |
|                                 | Body length        | -0.32                       | 0.04                        |                           | 0.02                       |
|                                 | Head circumference | -0.38                       | 0.02                        |                           | 0.02                       |
|                                 | PCA                | -0.36                       | 0.02                        |                           | 0.03                       |
| <b>Subiculum</b>                | PCA                |                             |                             |                           | 0.05                       |
| <b>Temporal cortex</b>          | Body weight        | -0.55                       | <0.001                      |                           | <0.01                      |
|                                 | Brain weight       | -0.54                       | <0.001                      |                           | <0.001                     |
|                                 | Body length        | -0.50                       | <0.01                       |                           | <0.01                      |
|                                 | Head circumference | -0.56                       | <0.001                      |                           | <0.001                     |
|                                 | PCA                | -0.53                       | <0.01                       |                           | <0.01                      |
| <b><i>Medulla (rostral)</i></b> |                    |                             |                             |                           |                            |
| <b>Cun</b>                      | Body weight        | -0.38                       | 0.02                        | 0.02                      |                            |
|                                 | Brain weight       | -0.36                       | 0.02                        | 0.02                      |                            |
|                                 | Body length        | -0.36                       | 0.02                        |                           |                            |
|                                 | Head circumference | -0.43                       | <0.01                       | 0.02                      |                            |
|                                 | PCA                | -0.33                       | 0.04                        | 0.02                      |                            |
| <b>DMNV</b>                     | Body weight        | -0.33                       | 0.02                        |                           |                            |
|                                 | Brain weight       | -0.29                       | 0.05                        |                           |                            |
|                                 | Body length        | -0.30                       | 0.04                        |                           |                            |
|                                 | PCA                | -0.32                       | 0.03                        |                           |                            |
| <b>XII</b>                      | Birth weight       | -0.39                       | 0.03                        |                           |                            |
| <b>ION</b>                      | Body weight        | -0.35                       | 0.01                        |                           |                            |



|             |                    |       |       |      |      |
|-------------|--------------------|-------|-------|------|------|
|             | Brain weight       | -0.31 | 0.03  |      |      |
|             | Body length        | -0.37 | <0.01 | 0.04 |      |
|             | Head circumference | -0.34 | 0.02  |      |      |
|             | PCA                | -0.32 | 0.03  |      |      |
| <b>Vest</b> | PCA                |       |       | 0.04 | 0.04 |

<sup>a</sup> Kendall's Tau correlation coefficient

<sup>b</sup> Significance taken at  $p \leq 0.05$ . \*indicates positive correlation

## **CHAPTER 8: GFAP EXPRESSION AND ASTROGLIOSIS IN THE DEVELOPING HUMAN BRAIN AND CHANGES SEEN IN IN SIDS- A COMPARISON ACROSS TWO SEPARATE RESEARCH COHORTS**

### **8.1: INTRODUCTION**

Astrogliosis was investigated via GFAP immunohistochemistry in two separate research cohorts, the first in the UK (Chapter 6), and the second in Australia (Chapter 7). Both cohorts were reclassified for diagnosis using the San Diego criteria (Krous et al., 2004) and common brain tissue blocks for analysis included those taken at the level of the cerebellum, hippocampal body and rostral medulla. Two methods of quantification were applied, one representing the number of GFAP-positive cells and the other representing their level of reactivity via assessment of morphology and corresponding R-score. Across both cohorts, negative correlations between GFAP and growth parameters of brain and body weight, length and head circumference were evident, thus showing that as the parameter increases postnatally, GFAP expression decreases. These associations were region-dependent and differed between diagnostic sub-categories. When analysing GFAP expression according to sub-categorisation of SIDS and major SIDS risk factors, findings were not consistent across cohorts. These inconsistencies include: findings of increased expression/reactivity in SIDS in the IGL, CA4, temporal cortex and DMNV, with no changes seen according to diagnosis in Australia, various alterations in expression/reactivity (predominantly increased) for risk factors of male sex, cigarette smoke exposure, URTI and sleeping prone in the UK, while the Australian cohort showed differential changes in expression according to risk factors (both increases and decreases), in different brain regions to the UK (summarised in Table 8.1).

**Table 8.1: Comparative data between the UK and Australian cohorts when analysed according to developmental parameters, diagnosis, and risk factors.**

|                                  | Dentate nucleus | IGL | ML | White matter | CA4 | CA3 | CA2 | CA1 | DG | Sub | EC | Temporal cortex | AN | Cun | DMNV | XII | ION | LRF | NSTT | NTS | Vest |
|----------------------------------|-----------------|-----|----|--------------|-----|-----|-----|-----|----|-----|----|-----------------|----|-----|------|-----|-----|-----|------|-----|------|
| <b>GFAP+/mm<sup>2</sup> (UK)</b> |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Brain weight                     |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Head circumference               |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| PCA                              |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| SIDS                             |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Male Sex                         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Cigarette smoke exposure         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| URTI                             |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Bed-sharing                      |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Prone sleeping                   |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| <b>R-score (UK)</b>              |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Brain weight                     |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Head circumference               |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| PCA                              |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| SIDS                             |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Male sex                         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Cigarette smoke exposure         |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| URTI                             |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Bed-sharing                      |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |
| Prone sleeping                   |                 |     |    |              |     |     |     |     |    |     |    |                 |    |     |      |     |     |     |      |     |      |

| GFAP+/ $\text{mm}^2$ (Aus) |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|----------------------------|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|
| Brain weight               |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Head circumference         |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| PCA                        |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| SIDS                       |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Male Sex                   |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Cigarette smoke exposure   |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| URTI                       |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Bed-sharing                |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Prone sleeping             |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| R-score (Aus)              |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Brain weight               |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Head circumference         |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| PCA                        |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| SIDS                       |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Male sex                   |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Cigarette smoke exposure   |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| URTI                       |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Bed-sharing                |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Prone sleeping             |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

Developmental parameters of brain weight, head circumference and PCA (whole cohort) and according to risk factors (whole cohort + SIDS only included).

$p$ -values indicated as  $\leq 0.05$ ,  $\leq 0.01$  or  $\leq 0.001$  for parameters associated with higher expression, and  $\leq 0.05$ ,  $\leq 0.01$  or  $\leq 0.001$  for parameters associated with lower expression

Similar inconsistencies in findings according to analysis by diagnosis have been reported in the brainstem previously (summarised Table 2.5, Chapter 2), likely due to variations in compositions of both control and SIDS groups, as well as variations in experimental methods. A major strength of the 2 studies undertaken herein is that we were able to standardise parameters, including application of diagnostic criteria, brain regions analysed, methods of analysis (i.e., location and number of images captured for each region) and method of quantification. Despite this, other parameters could not be controlled for resulting in different limitations within each research cohort, including the small sample size of eSUDI groups, homogeneity of eSUDI in the Australian cohort, given that 5/7 deaths were cardiac related (compared to 3/9 in the UK), and limited cases for risk factor analysis (i.e., only 4/20 SIDS in the UK were not exposed to cigarette smoke, with only 1 of these infants not bed-shared).

Combining datasets to form a ‘multi-centre’ study is particularly useful for less common disorders, such as in the case of SIDS, offering advantages such as increased sample size and statistical power, reduced bias and improved generalizability of findings (Youssef et al., 2008, Das, 2022). The purpose of this chapter is two-fold: 1) to combine the data from the 2-studies and determine which effects are maintained, and whether any new trends are highlighted, and 2) to compare the eSUDI and SIDS groups from the 2 cohorts to account for inconsistencies in findings.

## **8.2: METHODS**

### **8.2.1: DATA**

Characteristic and GFAP quantitative data from the eSUDI and SIDS groups across the two research cohorts, as detailed in Chapters 6 and 7, were pooled for analysis, with 21 microscopically defined regions being common across the two cohorts, including 9 nuclei of the medulla (AN, cun, DMNV, XII, ION, LRF, NSTT, NTS and vest, 4 regions of the cerebellum (dentate nucleus, IGL, ML and white matter), and 5 regions of the hippocampus (DG, CA1-4), as well as the subiculum, EC and temporal cortex.

### **8.2.2: STATISTICAL ANALYSIS**

Data was exported to SPSS for Windows (version 25; SPSS (IBM) Inc., Illinois, USA) for statistical analysis. 3 comparative tests were applied:

- 1- Pooling the eSUDI across the two cohorts and the SIDS across 2 cohorts and comparing between these diagnostic pooled groups,
- 2- Comparing between the eSUDI UK and eSUDI Australia
- 3- Comparing between the SIDS UK and SIDS Australia

Independent samples t-tests were used to compare the mean number of GFAP-positive cells per mm<sup>2</sup> between diagnostic groups, and for the presence of clinical risk factors. Mann-Whitney U tests were used to compare R-scores between diagnostic groups and for the presence of risk factors, as well as chi-square testing to compare the proportions of cases with R-scores >120 and >160. The relationship between GFAP-positive cells per mm<sup>2</sup> and clinical data was determined using Pearson correlations, while Kendall Tau correlations were used to compare R-score and clinical data. GFAP-positive cells per mm<sup>2</sup> data is presented as mean  $\pm$  standard error of the mean (SEM), and R-score is presented as median (interquartile range). P-values  $\leq$  0.05 were taken as statistically significant.

## **8.3: RESULTS**

### **8.3.1: INFANT CHARACTERISTICS**

The pooled dataset consisted of 62 SUDI cases, classified into two groups of eSUDI (n=16) and SIDS (SIDS I+II combined) (n=46). When pooled, diagnostic groups of eSUDI and SIDS were comparable across clinical, autopsy and risk factor data (Table 8.2). When comparing eSUDI groups between the UK and Australia, PMI was significantly higher in the UK cohort (p=0.05) (Table 8.2). When comparing SIDS groups between the UK and Australia, PMI was also significantly higher in the UK cohort (p<0.001), and the prevalence of cigarette smoke exposure was higher in the UK (p=0.01). GFAP immunohistochemistry is not reported to be sensitive to increased PMI (Chandana et al., 2009), and we did not find it to affect expression significantly for either cohort.

**Table 8.2: Clinical, autopsy and risk factor data for when combined for the UK and Australia.**

|                                 | Data Characteristic |                  |                          |                                |                               |
|---------------------------------|---------------------|------------------|--------------------------|--------------------------------|-------------------------------|
|                                 | eSUDI<br>(n = 16)   | SIDS (n =<br>46) | p-<br>value <sup>a</sup> | p-value<br>eSUDI UK<br>vs Aus* | p-value<br>SIDS UK<br>vs Aus* |
| <i>Clinical data</i>            |                     |                  |                          |                                |                               |
| Male: female                    | 10:6                | 33:13            | 0.54                     | 0.30                           | 0.48                          |
| Gest. Age (wks)                 | 39±1.8              | 39±1.7           | 0.29                     | 0.31                           | 0.53                          |
| Birth weight (kg)               | 3.3±0.7             | 3.0±0.6          | 0.30                     | 0.10                           | 0.93                          |
| <i>Autopsy data</i>             |                     |                  |                          |                                |                               |
| Age at death (months)           | 4.6±3.1             | 3.6±2.5          |                          | 0.60                           | 0.30                          |
| PCA (weeks)                     | 59±14               | 54±11            | 0.14                     | 0.39                           | 0.60                          |
| Body weight (kg)                | 6.3±2.6             | 6.2±1.9          | 0.79                     | 0.39                           | 0.60                          |
| Brain weight (g)                | 711±218             | 718±182          | 0.90                     | 0.35                           | 0.56                          |
| Body length (cm)                | 63±12               | 63±8             | 0.99                     | 0.08                           | 0.46                          |
| Head circumference<br>(cm)      | 40±4.3              | 41±3.7           | 0.73                     | 0.31                           | 0.83                          |
| <i>Tissue parameters</i>        |                     |                  |                          |                                |                               |
| PMI (hours)                     | 45±36               | 67±46            | 0.10                     | <b>0.05</b>                    | <b>&lt;0.001</b>              |
| <i>Risk factor prevalence</i>   |                     |                  |                          |                                |                               |
| Cigarette smoke<br>exposure (%) | 4/11 (36)           | 23/38 (61)       | 0.19                     | 0.56                           | <b>0.01</b>                   |
| Recent URTI <sup>b</sup> (%)    | 6/16 (38)           | 18/46 (39)       | 1.00                     | 0.16                           | 0.89                          |
| Found prone (%)                 | -                   | 18/39 (46)       | NA                       | NA                             | 0.06                          |
| Bed-share/co-sleeping<br>(%)    | -                   | 20/46 (44)       | NA                       | NA                             | 0.94                          |

Results presented as the mean ± SD or proportions (%)

<sup>a</sup>Obtained using Fischer's exact test for nominal data and independent samples t-test for continuous data. Actual values can be found in Tables 6.1 & 7.1.

Significance taken at  $p \leq 0.05$

Bold used to highlight statistical significance

### 8.3.2: GFAP EXPRESSION

#### 8.3.2.1: CORRELATIONS WITH DEVELOPMENTAL PARAMETERS

Significant associations between the number of GFAP-positive astrocytes/mm<sup>2</sup> and R-score values were observed for the whole cohort, and according to diagnosis.

*GFAP-positive astrocytes/mm<sup>2</sup>*: For the whole cohort, mild-moderate negative correlations (r value ranging from -0.26 to -0.69, p value from <0.001 to 0.05) were observed with developmental parameters, with regions that were consistently affected by the majority of parameters being the dentate nucleus, IGL and white matter of the cerebellum, EC subiculum, temporal cortex, cuneate nucleus, ION and NSTT (body weight, brain weight, body length, head circumference and PCA) (Table 8.3) with other regions correlating with 1-3 parameters. When comparing associations by diagnosis, correlations in the hippocampus were specific to the SIDS group (Table 8.3).

*R-score*: For the whole cohort, mild correlations (r value ranging from -0.19 to -0.36, p value from <0.001 to 0.04) were observed with developmental parameters, with regions that were consistently affected by the majority of parameters being the white matter of the cerebellum, CA4, CA1, EC, temporal cortex and cuneate nucleus (body weight, brain weight, body length and head circumference), with other regions only affected by 1-3 parameters (Table 8.4).

By diagnosis, correlations in the cerebellum and medulla were specific to the eSUDI group, with new correlations resulting in the IGL and Dentate nucleus, as well as CA1 for eSUDI, while the correlations in the hippocampus were specific for the SIDS group (Table 8.4).



**Table 8.3: An overview of the significant associations between the number of GFAP-positive astrocytes/mm<sup>2</sup> in the infant brain and developmental parameters.**

| Brain Region              | Infant Parameter   | ALL<br>r-value <sup>a</sup> | ALL<br>p-value <sup>b</sup> | eSUDI<br>(n=16)<br>p-value | SIDS<br>(n=46)<br>p-value |
|---------------------------|--------------------|-----------------------------|-----------------------------|----------------------------|---------------------------|
| <b><i>Cerebellum</i></b>  |                    |                             |                             |                            |                           |
| <b>Dentate nucleus</b>    | Body weight        | -0.42                       | <0.01                       | <0.01                      |                           |
|                           | Brain weight       | -0.39                       | <0.01                       | <0.01                      |                           |
|                           | Body length        | -0.45                       | <0.001                      | 0.01                       | 0.03                      |
|                           | Head circumference | -0.43                       | <0.001                      | <0.001                     |                           |
|                           | PCA                | -0.37                       | <0.01                       | 0.02                       |                           |
| <b>IGL</b>                | Body weight        | -0.52                       | <0.001                      | <0.01                      | <0.01                     |
|                           | Brain weight       | -0.51                       | <0.001                      | 0.01                       | <0.001                    |
|                           | Body length        | -0.50                       | <0.001                      | <0.01                      | <0.01                     |
|                           | Head circumference | -0.51                       | <0.001                      | 0.01                       | <0.001                    |
|                           | PCA                | -0.62                       | <0.001                      | <0.01                      | <0.001                    |
| <b>White matter</b>       | Body weight        | -0.54                       | <0.001                      | <0.01                      | <0.01                     |
|                           | Brain weight       | -0.50                       | <0.001                      | 0.01                       | 0.02                      |
|                           | Body length        | -0.53                       | <0.001                      | <0.01                      | <0.01                     |
|                           | Head circumference | -0.51                       | <0.001                      | <0.01                      | <0.01                     |
|                           | PCA                | -0.53                       | <0.001                      | 0.02                       | <0.001                    |
| <b><i>Hippocampus</i></b> |                    |                             |                             |                            |                           |
| <b>CA4</b>                | Body weight        | -0.27                       | 0.04                        |                            |                           |
|                           | Body length        | -0.29                       | 0.03                        |                            |                           |
|                           | PCA                | -0.31                       | 0.02                        |                            | 0.02                      |
| <b>CA1</b>                | Brain weight       | -0.26                       | 0.05                        |                            |                           |
|                           | Head circumference | -0.28                       | 0.04                        |                            |                           |
|                           | PCA                | -0.37                       | <0.01                       |                            | <0.01                     |
| <b>DG</b>                 | Birth weight       | 0.34                        | 0.04                        |                            |                           |
| <b>EC</b>                 | Body weight        | -0.27                       | 0.05                        |                            | <0.01                     |
|                           | Brain weight       | -0.28                       | 0.03                        |                            | <0.001                    |
|                           | Body length        |                             |                             |                            | 0.03                      |
|                           | Head circumference | -0.26                       | 0.05                        |                            | <0.001                    |
|                           | PCA                | -0.30                       | 0.02                        |                            | <0.001                    |
| <b>Subiculum</b>          | Body weight        | -0.47                       | <0.001                      |                            | <0.01                     |
|                           | Brain weight       | -0.35                       | <0.01                       |                            | 0.02                      |
|                           | Body length        | -0.31                       | 0.02                        |                            |                           |
|                           | Head circumference | -0.42                       | <0.01                       |                            | <0.01                     |
|                           | PCA                | -0.48                       | <0.001                      |                            | <0.01                     |
| <b>Temporal cortex</b>    | Body weight        | -0.38                       | <0.01                       |                            | <0.001                    |
|                           | Brain weight       | -0.35                       | <0.01                       |                            | <0.001                    |
|                           | Head circumference | -0.34                       | 0.01                        |                            | <0.001                    |

|                          |                    |       |        |       |        |
|--------------------------|--------------------|-------|--------|-------|--------|
|                          | PCA                | -0.44 | <0.001 |       | <0.001 |
| <b>Medulla (rostral)</b> |                    |       |        |       |        |
| <b>Cun</b>               | Body weight        |       |        | 0.02  |        |
|                          | Brain weight       |       |        | 0.04  |        |
|                          | Body length        | -0.35 | 0.01   | <0.01 |        |
|                          | Head circumference |       |        | 0.01  |        |
|                          | PCA                |       |        | 0.04  |        |
| <b>ION</b>               | Birth weight       | -0.35 | 0.04   |       |        |
|                          | Body weight        | -0.37 | <0.01  |       | 0.04   |
|                          | Body length        | -0.35 | <0.01  | 0.02  |        |
|                          | Head circumference | -0.29 | 0.03   |       |        |
| <b>LRF</b>               | Body weight        | -0.34 | 0.01   |       | 0.02   |
|                          | Body length        | -0.69 | <0.001 | <0.01 |        |
|                          | Head circumference | -0.30 | 0.03   |       |        |
| <b>NSTT</b>              | Birth weight       |       |        | <0.01 |        |
|                          | Body weight        | -0.42 | <0.01  | 0.02  | 0.05   |
|                          | Brain weight       | -0.38 | <0.01  | 0.04  | 0.05   |
|                          | Body length        | -0.42 | <0.01  | 0.04  | 0.03   |
|                          | Head circumference | -0.36 | <0.01  | 0.02  |        |
|                          | PCA                | -0.45 | <0.001 | 0.02  | 0.03   |

<sup>a</sup> Pearson's correlation coefficient

<sup>b</sup> Significance taken at  $p \leq 0.05$

*Abbreviations:* CA cornu ammonis, *Cun* Cuneate nucleus, *DG* dentate gyrus, *DMNV* dorsal motor nucleus of the vagus, *EC* entorhinal cortex, *XII* hypoglossal nucleus, *IGL* internal granular layer of the cerebellar cortex, *ION* inferior olivary nucleus, *IGL* internal granular layer, *LRF* lateral reticular formation, *NSTT* nucleus of the spinal trigeminal tract, *NTS* nucleus of the solitary tract, *PCA* postconceptional age, *PMI* post-mortem interval.

**Table 8.4: An overview of the significant associations between R-score values in the infant brain and developmental parameters.**

| Brain Region             | Infant Parameter   | ALL<br>r-value <sup>a</sup> | ALL<br>p-value <sup>b</sup> | eSUDI<br>(n=16)<br>p-value | SIDS<br>(n=46) p-<br>value |
|--------------------------|--------------------|-----------------------------|-----------------------------|----------------------------|----------------------------|
| <i>Cerebellum</i>        |                    |                             |                             |                            |                            |
| <b>Dentate nucleus</b>   | Birth weight       |                             |                             | 0.03                       |                            |
|                          | PCA                |                             |                             | 0.04                       |                            |
| <b>IGL</b>               | Body weight        |                             |                             | 0.03                       |                            |
|                          | Body length        |                             |                             | 0.03                       |                            |
| <b>Molecular layer</b>   | Body length        |                             |                             |                            | 0.03                       |
| <b>White matter</b>      | Body weight        |                             |                             | 0.03                       |                            |
|                          | Brain weight       | -0.20                       | 0.03                        | 0.03                       |                            |
|                          | Body length        | -0.24                       | 0.01                        | <0.01                      |                            |
|                          | Head circumference | -0.19                       | 0.04                        | 0.01                       |                            |
| <i>Hippocampus</i>       |                    |                             |                             |                            |                            |
| <b>CA4</b>               | Birth weight       | 0.25                        | 0.03                        |                            |                            |
|                          | Body weight        | 0.21                        | 0.02                        |                            | <0.01                      |
|                          | Brain weight       | 0.21                        | 0.02                        |                            | 0.01                       |
|                          | Body length        | 0.22                        | 0.02                        |                            | 0.01                       |
|                          | Head circumference | 0.23                        | 0.01                        |                            | <0.01                      |
|                          | PCA                | 0.26                        | <0.01                       |                            | <0.001                     |
| <b>CA1</b>               | Body weight        |                             |                             | <0.01                      |                            |
|                          | Brain weight       |                             |                             | <0.01                      |                            |
|                          | Body length        |                             |                             | 0.02                       |                            |
|                          | Head circumference |                             |                             | <0.01                      |                            |
|                          | PCA                |                             |                             | 0.01                       |                            |
| <b>EC</b>                | Body weight        | -0.32                       | <0.01                       |                            | <0.001                     |
|                          | Brain weight       | -0.31                       | <0.01                       |                            | <0.01                      |
|                          | Body length        | -0.21                       | 0.04                        |                            | 0.01                       |
|                          | Head circumference | -0.32                       | <0.01                       |                            | <0.01                      |
|                          | PCA                | -0.32                       | <0.01                       |                            | <0.01                      |
| <b>Subiculum</b>         | PCA                |                             |                             |                            | 0.03                       |
| <b>Temporal cortex</b>   | Body weight        | -0.35                       | <0.001                      |                            | <0.001                     |
|                          | Brain weight       | -0.36                       | <0.001                      |                            | <0.001                     |
|                          | Body length        | -0.27                       | <0.01                       |                            | <0.001                     |
|                          | Head circumference | -0.38                       | <0.001                      |                            | <0.001                     |
|                          | PCA                | -0.35                       | <0.001                      |                            | <0.001                     |
| <i>Medulla (rostral)</i> |                    |                             |                             |                            |                            |
| <b>Cun</b>               | Body weight        |                             |                             | 0.01                       |                            |
|                          | Brain weight       |                             |                             | 0.02                       |                            |
|                          | Body length        | -0.30                       | <0.01                       | <0.001                     |                            |

|             |                    |       |      |       |
|-------------|--------------------|-------|------|-------|
|             | Head circumference |       |      | 0.01  |
|             | PCA                |       |      | 0.03  |
| <b>DMNV</b> | Body length        | -0.25 | 0.02 |       |
| <b>XII</b>  | Birth weight       | -0.29 | 0.03 |       |
| <b>ION</b>  | Body length        | -0.25 | 0.01 | 0.04  |
| <b>NSTT</b> | Birth weight       |       |      | 0.03  |
|             | PCA                |       |      | 0.04  |
| <b>NTS</b>  | Birth weight       | -0.31 | 0.02 | <0.01 |

<sup>a</sup> Kendall's Tau correlation coefficient

<sup>b</sup> Significance taken at  $p \leq 0.05$ .

### 8.3.2.2: COMPARISON ACCORDING TO DIAGNOSIS (POOLED)

Compared to eSUDI, SIDS infants had higher GFAP/mm<sup>2</sup> in the temporal cortex ( $p < 0.01$ ) (Table 8.5).

For R-score, values did not differ significantly between groups (Table 8.5).

### 8.3.2.3: COMPARISON BETWEEN UK VS AUSTRALIA DIAGNOSTIC GROUPS

*GFAP/mm<sup>2</sup>*: Comparing between eSUDI groups, expression was higher in the Australian cohort in the dentate nucleus ( $399 \pm 57$  vs  $179 \pm 39$ ,  $p < 0.01$ ), IGL ( $402 \pm 56$  vs  $237 \pm 39$ ,  $p = 0.03$ ) and DMNV ( $141 \pm 25$  vs  $46 \pm 13$ ,  $p < 0.01$ ), while between SIDS groups, expression was higher in the Australian cohort in the dentate nucleus ( $364 \pm 27$  vs  $172 \pm 13$ ,  $p < 0.001$ ), but lower in the CA2 ( $64 \pm 10$  vs  $113 \pm 18$ ,  $p = 0.03$ ) and temporal cortex ( $91 \pm 12$  vs  $140 \pm 10$ ,  $p < 0.01$ ) (Table 8.5).

*R-score*: Comparing scores between eSUDI groups, they were higher in the Australian cohort in the dentate nucleus (102 (4) vs 100 (0),  $p = 0.04$ ), IGL (124 (43) vs 106 (12),  $p = 0.01$ ), and majority of the medulla nuclei including DMNV (109 (24) vs 100 (0),  $p = 0.01$ ), XII (105 (13) vs 100 (0),  $p = 0.03$ ), ION (122 (58) vs 100 (0),  $p < 0.01$ ), LRF (108 (47) vs 100 (50),  $p = 0.02$ ) and NTS (110 (12) vs 100 (0),  $p < 0.001$ ), but lower in the subiculum (100 (1) vs 124 (41),  $p = 0.02$ ) (Table 8.5). Between SIDS groups, values were higher in the Australian cohort in the dentate nucleus (105 (8) vs 100 (0),  $p < 0.001$ ) and white matter of the cerebellum (110 (16) vs 100 (5),  $p < 0.01$ ), and all nuclei of the medulla with the exception of the NSTT, and lower in the EC (102 (8) vs 112 (37),  $p = 0.02$ ) and temporal cortex (100 (1) vs 102 (13),  $p = 0.03$ ) (Table 8.5). An example IHC stained GFAP medulla section of a case from each cohort is provided in Figure 8.1 to show the staining pattern and distribution.

**Table 8.5: Comparison of the number of GFAP-positive astrocytes/mm<sup>2</sup> and R score between diagnostic groups.**

| Brain Region             | Number of GFAP cells<br>(Point-counting) |                |                                     |                                                       |                                                   | Morphology (R-score) |                |                                     |                                                       |                                                   |
|--------------------------|------------------------------------------|----------------|-------------------------------------|-------------------------------------------------------|---------------------------------------------------|----------------------|----------------|-------------------------------------|-------------------------------------------------------|---------------------------------------------------|
|                          | eSUDI<br>(n=16)                          | SIDS<br>(n=46) | <i>p</i> -value<br>SIDS vs<br>eSUDI | <i>p</i> -value <sup>^</sup><br>eSUDI<br>UK vs<br>Aus | <i>p</i> -value <sup>^</sup><br>SIDS UK<br>vs Aus | eSUDI<br>(n=16)      | SIDS<br>(n=46) | <i>p</i> -value<br>SIDS vs<br>eSUDI | <i>p</i> -value <sup>^</sup><br>eSUDI<br>UK vs<br>Aus | <i>p</i> -value <sup>^</sup><br>SIDS UK<br>vs Aus |
| <i>Cerebellum</i>        |                                          |                |                                     |                                                       |                                                   |                      |                |                                     |                                                       |                                                   |
| Dentate Nucleus          | 274±44                                   | 261±20         | 0.78                                | <0.01                                                 | <0.001                                            | 100 (1)              | 100 (5)        | 0.32                                | <b>0.04</b>                                           | <0.001                                            |
| IGL                      | 309±38                                   | 354±15         | 0.28                                | <b>0.03</b>                                           | 0.11                                              | 111 (20)             | 116 (26)       | 0.23                                | <b>0.01</b>                                           | 0.33                                              |
| ML                       | 2±1                                      | 3±1            | 0.69                                | 0.21                                                  | 0.89                                              | 0 (100)              | 0 (0)          | 0.29                                | 1.00                                                  | 0.41                                              |
| White matter             | 143±25                                   | 139±10         | 0.88                                | 0.27                                                  | 0.82                                              | 105 (25)             | 102 (12)       | 0.47                                | 0.30                                                  | <0.01                                             |
| <i>Hippocampus</i>       |                                          |                |                                     |                                                       |                                                   |                      |                |                                     |                                                       |                                                   |
| CA4                      | 228±22                                   | 257±13         | 0.27                                | 0.06                                                  | 0.97                                              | 115 (25)             | 109 (26)       | 0.37                                | 0.07                                                  | 0.73                                              |
| CA3                      | 104±15                                   | 124±12         | 0.41                                | 0.47                                                  | 0.07                                              | 100 (0)              | 100 (0)        | 0.46                                | 0.46                                                  | 0.60                                              |
| CA2                      | 63±16                                    | 91±11          | 0.23                                | 0.45                                                  | <b>0.03</b>                                       | 100 (100)            | 100 (0)        | 0.51                                | 0.81                                                  | 0.54                                              |
| CA1                      | 131±10                                   | 132±9          | 0.90                                | 0.67                                                  | 0.07                                              | 100 (4)              | 101 (9)        | 0.17                                | 0.28                                                  | 0.13                                              |
| DG                       | 200±26                                   | 196±13         | 0.90                                | 0.56                                                  | 0.12                                              | 100 (13)             | 100 (0)        | 0.34                                | 0.81                                                  | 0.28                                              |
| EC                       | 129±32                                   | 143±11         | 0.58                                | 0.08                                                  | 0.04                                              | 100 (0)              | 100 (8)        | 0.12                                | 0.71                                                  | <b>0.02</b>                                       |
| Subiculum                | 155±17                                   | 162±8          | 0.68                                | 0.79                                                  | 0.07                                              | 106 (34)             | 106 (32)       | 0.67                                | <b>0.02</b>                                           | 0.22                                              |
| Temporal cortex          | <b>65±11</b>                             | <b>118±8</b>   | <0.01                               | 0.60                                                  | <0.01                                             | 103 (2)              | 100 (9)        | 0.26                                | 1.00                                                  | <b>0.03</b>                                       |
| <i>Medulla (rostral)</i> |                                          |                |                                     |                                                       |                                                   |                      |                |                                     |                                                       |                                                   |
| AN                       | 171±22                                   | 209±16         | 0.23                                | 0.77                                                  | 0.48                                              | 100 (2)              | 100 (4)        | 0.43                                | 0.35                                                  | <b>0.05</b>                                       |
| Cun                      | 172±42                                   | 165±16         | 0.84                                | 0.22                                                  | 0.16                                              | 100 (6)              | 100 (9)        | 0.23                                | 0.11                                                  | <0.01                                             |

|             |        |        |      |                 |      |          |          |      |                  |                  |
|-------------|--------|--------|------|-----------------|------|----------|----------|------|------------------|------------------|
| <b>DMNV</b> | 84±17  | 100±11 | 0.45 | <b>&lt;0.01</b> | 0.83 | 100 (6)  | 100 (12) | 0.16 | <b>0.01</b>      | <b>&lt;0.001</b> |
| <b>XII</b>  | 33±6   | 40±5   | 0.41 | 0.22            | 0.78 | 100 (1)  | 100 (9)  | 0.30 | <b>0.03</b>      | <b>&lt;0.001</b> |
| <b>ION</b>  | 131±33 | 131±9  | 0.98 | 0.25            | 0.39 | 100 (14) | 100 (16) | 0.30 | <b>&lt;0.01</b>  | <b>&lt;0.001</b> |
| <b>LRF</b>  | 53±12  | 51±6   | 0.82 | 0.29            | 0.97 | 100 (3)  | 100 (0)  | 0.67 | <b>0.02</b>      | <b>&lt;0.01</b>  |
| <b>NSTT</b> | 156±25 | 174±11 | 0.45 | 0.31            | 0.71 | 105 (18) | 108 (15) | 0.80 | 0.11             | 0.32             |
| <b>NTS</b>  | 87±19  | 57±6   | 0.16 | 0.29            | 0.82 | 100 (7)  | 102 (14) | 0.33 | <b>&lt;0.001</b> | <b>0.01</b>      |
| <b>Vest</b> | 60±14  | 87±10  | 0.15 | 0.98            | 0.50 | 100 (1)  | 100 (1)  | 0.47 | 0.07             | <b>&lt;0.001</b> |

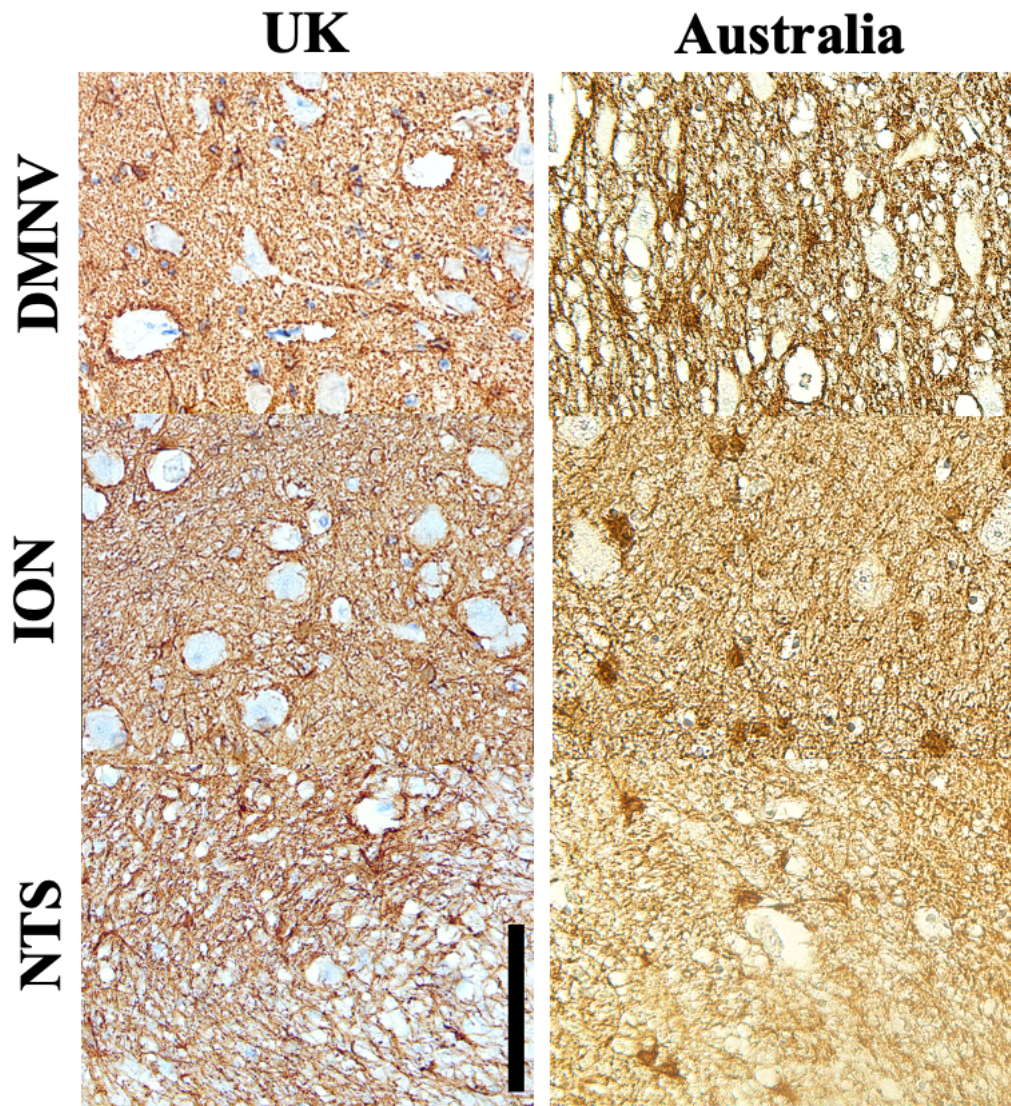
*p*-values were obtained using independent t-test for point counting, and Mann Whitney U test for R-score. Significance taken at  $p \leq 0.05$

Bold used to highlight statistical significance. Significance taken at  $p \leq 0.05$

^Actual values can be found in Tables 6.2&7.2.

*Abbreviations:* *AN* Arcuate nucleus, *CA* Cornu Ammonis, *Cun* Cuneate nucleus, *DG* dentate gyrus, *DMNV* dorsal motor nucleus of the vagus, *EC* entorhinal cortex, *eSUDI* explained sudden unexpected death in infancy, *XII* hypoglossal nucleus, *IGL* internal granular layer of the cerebellar cortex, *ION* inferior olivary nucleus, *LRF* lateral reticular formation, *ML* molecular layer of the cerebellar cortex, *NSTT* nucleus of the spinal trigeminal tract, *NTS* nucleus of the solitary tract, *PCA* post conception age, *SIDS* sudden infant death syndrome, *Vest* vestibular nucleus.





**Figure 8.1: Comparison of staining profiles in the medulla of the Australian and UK cohort.** Cases selected both scored ‘high’ for their cohort in the ION and NTS and low in the DMNV. Zoomed in panels show DMNV, ION and NTS. Scale bar = 100 $\mu$ m.

#### **8.3.2.4: PERCENTAGE OF CASES WITH ‘MILD’ AND ‘MODERATE’ ASTROGLIOSIS**

Considering the pooled dataset, the percentage of cases with R-scores >120 and >160 did not differ between eSUDI and SIDS (Table 8.6). The percentage of eSUDI cases with R-scores >120 was lower in IGL ( $p<0.01$ ) and higher in the white matter of the cerebellum ( $p=0.01$ ) and ION ( $p<0.01$ ) in the Australian cohort (Table 8.6), and higher in the ION of SIDS of the Australian cohort (Table 8.6).



**Table 8.6: Comparison of diagnostic groups according to percentage of cases with R-score sub-categories of >120 and >160.**

| Brain Region       | >120 (>160) |        |                 |                                       |                                      |
|--------------------|-------------|--------|-----------------|---------------------------------------|--------------------------------------|
|                    | eSUDI       | SIDS   | <i>p</i> -value | <i>p</i> -value<br>eSUDI UK<br>vs Aus | <i>p</i> -value<br>SIDS UK<br>vs Aus |
| <i>Cerebellum</i>  |             |        |                 |                                       |                                      |
| Dentate Nucleus    | 0           | 0      | -               | -                                     | -                                    |
| IGL                | 31 (6)      | 46 (2) | 0.32            | <b>&lt;0.01</b>                       | 0.15                                 |
| ML                 | 6 (6)       | 0      | 0.26            | 0.44                                  | -                                    |
| White matter       | 25          | 11     | 0.22            | <b>0.02</b>                           | 0.16                                 |
| <i>Hippocampus</i> |             |        |                 |                                       |                                      |
| CA4                | 29 (7)      | 31     | 1.00            | 0.56                                  | 0.73                                 |
| CA3                | 0           | 2 (2)  | 1.00            | -                                     | 1.00                                 |
| CA2                | 0           | 0      | -               | -                                     | -                                    |
| CA1                | 13          | 7      | 0.59            | 1.00                                  | 1.00                                 |
| DG                 | 0           | 13     | 0.32            | -                                     | 0.19                                 |
| EC                 | 0           | 18 (2) | 0.18            | -                                     | 0.71                                 |
| Subiculum          | 36          | 27 (2) | 0.52            | 0.27                                  | 0.08                                 |
| Temporal cortex    | 14          | 14     | 1.00            | 1.00                                  | 0.68                                 |
| <i>Medulla</i>     |             |        |                 |                                       |                                      |
| AN                 | 15          | 16 (3) | 1.00            | 0.13                                  | 0.18                                 |
| Cun                | 14          | 8      | 0.61            | 0.11                                  | 0.10                                 |
| DMNV               | 13          | 13     | 1.00            | 0.14                                  | 0.34                                 |
| XII                | 7           | 8      | 1.00            | 0.40                                  | 1.00                                 |
| ION                | 20 (7)      | 17     | 1.00            | <b>0.04</b>                           | <b>&lt;0.01</b>                      |
| LRF                | 13 (7)      | 5      | 0.30            | 0.14                                  | 0.22                                 |
| NSTT               | 20          | 20     | 1.00            | 0.53                                  | 1.00                                 |
| NTS                | 7           | 21     | 0.41            | 0.40                                  | 0.10                                 |
| Vest               | 8           | 3      | 0.47            | 0.39                                  | 0.49                                 |

Values expressed as percentages rounded to nearest whole integer.

Bold highlights statistical significance using Chi square test with Fisher's Exact. Significance taken at  $p \leq 0.05$ .

#### 8.3.2.5: COMPARISONS ACCORDING TO RISK FACTORS

Risk factors were run on the whole cohort first, and subsequently on the SIDS only cohort. Sleep related risk factors of sleeping prone and bed-sharing were only run on the SIDS group.

*GFAP/mm<sup>2</sup>*: For the whole cohort, infants exposed to cigarette smoke had higher expression than non-smoke-exposed infants in the temporal cortex ( $p=0.03$ ), and infants with a recent URTI had lower expression in the DG ( $p=0.03$ ) (Table 8.7). Amongst SIDS, infants with a recent URTI had lower expression in the DG ( $p=0.02$ ) and infants found prone had lower expression in the subiculum ( $p<0.01$ ) and temporal cortex ( $p=0.01$ ) (Table 8.8).

*R-score*: For the whole cohort, female infants had higher values in the cuneate nucleus ( $p<0.01$ ) and NTS ( $p=0.04$ ), infants exposed to cigarette smoke had higher values in the subiculum ( $p=0.03$ ), and infants with a recent URTI had higher values in the CA4 ( $p=0.03$ ) (Table 8.9). Amongst SIDS, female infants had higher values in the cuneate nucleus ( $p=0.03$ ) and those found prone had lower values in the EC ( $p=0.02$ ) and temporal cortex ( $p=0.02$ ) (Table 8.10).

**Table 8.7: Comparison of number of GFAP-positive cells per mm<sup>2</sup> according to major SIDS risk factors amongst the whole cohort.**

| Brain Region             | SIDS risk factors (whole cohort) |               |             |                          |               |             |                                   |               |             |
|--------------------------|----------------------------------|---------------|-------------|--------------------------|---------------|-------------|-----------------------------------|---------------|-------------|
|                          | Sex                              |               |             | Cigarette smoke exposure |               |             | Upper respiratory tract infection |               |             |
|                          | Male (n=43)                      | Female (n=19) | p-value     | No (n=22)                | Yes (n=27)    | p-value     | No (n=38)                         | Yes (n=24)    | p-value     |
| <i>Cerebellum</i>        |                                  |               |             |                          |               |             |                                   |               |             |
| Dentate Nucleus          | 250±20                           | 297±41        | 0.31        | 265±30                   | 250±25        | 0.70        | 259±23                            | 273±31        | 0.71        |
| IGL                      | <b>324±15</b>                    | <b>386±33</b> | <b>0.05</b> | 359±24                   | 335±21        | 0.46        | 344±18                            | 340±27        | 0.89        |
| ML                       | 3±1                              | 2±1           | 0.54        | 5±2                      | 2±1           | 0.21        | 3±1                               | 2±1           | 0.58        |
| White matter             | 135±11                           | 153±19        | 0.41        | 130±18                   | 158±15        | 0.24        | 136±12                            | 148±17        | 0.56        |
| <i>Hippocampus</i>       |                                  |               |             |                          |               |             |                                   |               |             |
| CA4                      | 251±13                           | 248±20        | 0.76        | 251±21                   | 255±17        | 0.87        | 260±14                            | 232±18        | 0.21        |
| CA3                      | 126±12                           | 104±17        | 0.33        | 119±13                   | 115±19        | 0.88        | 121±11                            | 117±20        | 0.86        |
| CA2                      | 86±12                            | 80±16         | 0.76        | 91±14                    | 79±16         | 0.60        | 95±12                             | 66±14         | 0.14        |
| CA1                      | 135±8                            | 124±14        | 0.45        | 128±7                    | 134±14        | 0.71        | 136±9                             | 125±13        | 0.47        |
| DG                       | 208±14                           | 172±20        | 0.17        | 192±19                   | 190±17        | 0.93        | <b>216±14</b>                     | <b>164±20</b> | <b>0.03</b> |
| EC                       | 136±13                           | 148±21        | 0.64        | 119±17                   | 139±14        | 0.36        | 138±13                            | 143±21        | 0.81        |
| Subiculum                | 157±8                            | 170±15        | 0.41        | 151±11                   | 162±11        | 0.47        | 163±10                            | 156±10        | 0.62        |
| Temporal cortex          | 102±8                            | 112±15        | 0.58        | <b>87±12</b>             | <b>122±11</b> | <b>0.03</b> | 108±10                            | 100±10        | 0.63        |
| <i>Medulla (rostral)</i> |                                  |               |             |                          |               |             |                                   |               |             |
| AN                       | 200±18                           | 197±19        | 0.92        | 167±21                   | 219±23        | 0.12        | 193±20                            | 208±17        | 0.60        |
| Cun                      | 147±17                           | 208±33        | 0.08        | 154±26                   | 187±28        | 0.40        | 167±23                            | 167±23        | 1.00        |
| DMNV                     | 92±11                            | 102±18        | 0.61        | 102±14                   | 101±15        | 0.96        | 93±11                             | 98±16         | 0.83        |
| XII                      | 38±5                             | 38±7          | 0.94        | 34±6                     | 44±6          | 0.27        | 32±4                              | 46±7          | 0.12        |
| ION                      | 119±10                           | 157±26        | 0.10        | 119±15                   | 150±22        | 0.25        | 127±15                            | 136±14        | 0.70        |
| LRF                      | 49±6                             | 57±11         | 0.49        | 48±9                     | 56±8          | 0.51        | 42±5                              | 64±10         | 0.06        |
| NSTT                     | 167±13                           | 175±18        | 0.72        | 177±16                   | 187±19        | 0.69        | 182±13                            | 152±18        | 0.17        |

|             |      |        |      |       |       |      |       |       |      |
|-------------|------|--------|------|-------|-------|------|-------|-------|------|
| <b>NTS</b>  | 57±6 | 89±19  | 0.11 | 71±13 | 72±13 | 0.97 | 68±10 | 65±11 | 0.84 |
| <b>Vest</b> | 70±8 | 101±18 | 0.08 | 68±13 | 85±11 | 0.31 | 71±8  | 92±16 | 0.19 |

Results presented as the mean ± SEM

Bold used to highlight statistical significance, obtained using independent samples t-test. Significance taken at  $p \leq 0.05$ .

Table 8.8: Comparison of number of GFAP-positive cells per mm<sup>2</sup> according to major risk factors amongst the SIDS cohort.

| SIDS Risk Factors (SIDS only) |             |               |         |                          |            |         |                                   |            |         |                         |            |         |             |            |         |
|-------------------------------|-------------|---------------|---------|--------------------------|------------|---------|-----------------------------------|------------|---------|-------------------------|------------|---------|-------------|------------|---------|
|                               | Sex         |               |         | Cigarette smoke exposure |            |         | Upper respiratory tract infection |            |         | Co-sleeping/Bed-sharing |            |         | Found prone |            |         |
| Brain Region                  | Male (n=33) | Female (n=13) | p-value | No (n=15)                | Yes (n=23) | p-value | No (n=28)                         | Yes (n=18) | p-value | No (n=26)               | Yes (n=20) | p-value | No (n=21)   | Yes (n=18) | p-value |
| Cerebellum                    |             |               |         |                          |            |         |                                   |            |         |                         |            |         |             |            |         |
| Dentate Nucleus               | 253±22      | 282±47        | 0.57    | 280±33                   | 238±27     | 0.32    | 241±24                            | 292±35     | 0.23    | 264±26                  | 258±33     | 0.55    | 266±32      | 262±33     | 0.94    |
| IGL                           | 337±16      | 398±34        | 0.07    | 380±19                   | 330±19     | 0.08    | 354±17                            | 355±28     | 0.97    | 339±14                  | 375±30     | 0.28    | 380±23      | 331±20     | 0.12    |
| ML                            | 4±2         | 1±1           | 0.24    | 6±3                      | 2±1        | 0.18    | 4±2                               | 3±1        | 0.71    | 2±1                     | 4±3        | 0.48    | 3±1         | 2±1        | 0.65    |
| White matter                  | 143±12      | 129±18        | 0.55    | 131±19                   | 148±15     | 0.46    | 134±13                            | 147±17     | 0.56    | 141±15                  | 136±12     | 0.81    | 158±10      | 117±20     | 0.09    |
| Hippocampus                   |             |               |         |                          |            |         |                                   |            |         |                         |            |         |             |            |         |
| CA4                           | 265±15      | 236±22        | 0.32    | 259±28                   | 254±19     | 0.89    | 269±18                            | 240±17     | 0.27    | 244±16                  | 275±20     | 0.24    | 266±20      | 230±13     | 0.15    |
| CA3                           | 128±15      | 113±24        | 0.61    | 116±17                   | 122±21     | 0.83    | 122±14                            | 127±23     | 0.83    | 115±17                  | 136±18     | 0.41    | 123±20      | 108±18     | 0.57    |
| CA2                           | 89±14       | 94±21         | 0.87    | 99±19                    | 82±18      | 0.53    | 106±15                            | 68±16      | 0.10    | 87±16                   | 96±16      | 0.71    | 100±21      | 72±12      | 0.26    |
| CA1                           | 138±10      | 117±19        | 0.29    | 126±11                   | 129±15     | 0.87    | 137±11                            | 125±15     | 0.49    | 125±13                  | 142±11     | 0.37    | 136±15      | 123±12     | 0.50    |
| DG                            | 209±16      | 161±21        | 0.12    | 184±24                   | 193±19     | 0.78    | 220±15                            | 160±22     | 0.02    | 204±19                  | 185±18     | 0.47    | 184±19      | 202±21     | 0.54    |
| EC                            | 138±12      | 159±23        | 0.40    | 119±18                   | 143±16     | 0.31    | 148±14                            | 135±16     | 0.56    | 137±14                  | 151±17     | 0.53    | 164±18      | 121±13     | 0.06    |
| Subiculum                     | 163±9       | 161±18        | 0.93    | 159±13                   | 155±11     | 0.78    | 170±11                            | 151±11     | 0.25    | 158±11                  | 168±11     | 0.54    | 178±11      | 133±9      | <0.01   |
| Temporal cortex               | 115±9       | 127±19        | 0.54    | 93±16                    | 126±12     | 0.10    | 123±11                            | 110±13     | 0.44    | 115±11                  | 122±13     | 0.70    | 141±13      | 95±10      | 0.01    |
| Medulla (rostral)             |             |               |         |                          |            |         |                                   |            |         |                         |            |         |             |            |         |
| AN                            | 207±21      | 212±24        | 0.89    | 183±26                   | 218±27     | 0.39    | 200±25                            | 221±19     | 0.53    | 217±27                  | 200±19     | 0.61    | 228±31      | 181±17     | 0.19    |
| Cun                           | 158±20      | 180±28        | 0.53    | 165±27                   | 169±24     | 0.91    | 175±22                            | 152±23     | 0.49    | 155±24                  | 176±20     | 0.52    | 146±24      | 179±27     | 0.38    |
| DMNV                          | 103±12      | 91±22         | 0.60    | 104±19                   | 105±16     | 0.95    | 92±13                             | 110±18     | 0.41    | 92±12                   | 109±19     | 0.46    | 125±18      | 84±16      | 0.09    |
| XII                           | 40±6        | 39±9          | 0.92    | 35±7                     | 47±7       | 0.26    | 32±5                              | 51±9       | 0.07    | 36±5                    | 45±9       | 0.40    | 39±6        | 47±9       | 0.43    |
| ION                           | 129±10      | 134±18        | 0.80    | 120±15                   | 138±15     | 0.43    | 124±11                            | 140±16     | 0.38    | 127±14                  | 134±12     | 0.68    | 139±13      | 125±16     | 0.51    |

|             |        |        |      |        |        |      |        |        |      |        |        |      |        |        |      |
|-------------|--------|--------|------|--------|--------|------|--------|--------|------|--------|--------|------|--------|--------|------|
| <b>LRF</b>  | 52±7   | 48±12  | 0.74 | 41±9   | 55±9   | 0.30 | 43±7   | 62±10  | 0.11 | 51±8   | 50±9   | 0.95 | 56±10  | 44±9   | 0.40 |
| <b>NSTT</b> | 180±14 | 160±19 | 0.43 | 194±20 | 180±17 | 0.61 | 188±13 | 155±20 | 0.15 | 162±14 | 189±18 | 0.24 | 175±16 | 176±21 | 0.96 |
| <b>NTS</b>  | 57±7   | 58±10  | 0.99 | 56±12  | 59±6   | 0.81 | 52±5   | 66±13  | 0.34 | 53±6   | 63±11  | 0.39 | 55±9   | 61±11  | 0.71 |
| <b>Vest</b> | 77±9   | 112±25 | 0.10 | 73±17  | 89±10  | 0.39 | 73±10  | 104±18 | 0.11 | 70±11  | 105±15 | 0.07 | 100±17 | 69±12  | 0.15 |

Results presented as the mean ± SEM

Bold used to highlight statistical significance. Obtained using independent samples t-test. Significance taken at  $p \leq 0.05$ .

**Table 8.9: Comparison of R-score values according to major SIDS risk factors amongst the whole cohort.**

| Brain Region             | SIDS risk factors (whole cohort) |                 |                 |                          |                 |             |                                   |                 |             |
|--------------------------|----------------------------------|-----------------|-----------------|--------------------------|-----------------|-------------|-----------------------------------|-----------------|-------------|
|                          | Sex                              |                 |                 | Cigarette smoke exposure |                 |             | Upper respiratory tract infection |                 |             |
|                          | Male (n=43)                      | Female (n=19)   | p-value         | No (n=22)                | Yes (n=27)      | p-value     | No (n=38)                         | Yes (n=24)      | p-value     |
| <i>Cerebellum</i>        |                                  |                 |                 |                          |                 |             |                                   |                 |             |
| Dentate Nucleus          | 100 (3)                          | 100 (5)         | 0.68            | 100 (5)                  | 100 (1)         | 0.12        | 100 (3)                           | 100 (5)         | 0.96        |
| IGL                      | 115 (25)                         | 114 (26)        | 0.87            | 116 (17)                 | 114 (29)        | 0.92        | 115 (20)                          | 116 (30)        | 0.74        |
| ML                       | 0 (0)                            | 0 (100)         | 0.58            | 0 (100)                  | 0 (0)           | 0.29        | 0 (0)                             | 0 (25)          | 0.76        |
| White matter             | 103 (12)                         | 102 (16)        | 0.70            | 101 (13)                 | 105 (14)        | 0.60        | 101 (12)                          | 107 (14)        | 0.26        |
| <i>Hippocampus</i>       |                                  |                 |                 |                          |                 |             |                                   |                 |             |
| CA4                      | 109 (23)                         | 114 (26)        | 0.58            | 108 (25)                 | 116 (32)        | 0.16        | <b>108 (15)</b>                   | <b>121 (35)</b> | <b>0.03</b> |
| CA3                      | 100 (0)                          | 100 (0)         | 0.96            | 100 (0)                  | 100 (0)         | 0.97        | 100 (0)                           | 100 (0)         | 0.38        |
| CA2                      | 100 (0)                          | 100 (25)        | 0.89            | 100 (0)                  | 100 (63)        | 0.18        | 100 (0)                           | 100 (0)         | 0.82        |
| CA1                      | 100 (7)                          | 103 (9)         | 0.22            | 101 (5)                  | 100 (13)        | 0.61        | 100 (8)                           | 100 (8)         | 0.92        |
| DG                       | 100 (0)                          | 100 (11)        | 0.77            | 100 (0)                  | 100 (13)        | 0.09        | 100 (0)                           | 100 (11)        | 0.31        |
| EC                       | 100 (7)                          | 100 (7)         | 0.63            | 100 (0)                  | 100 (7)         | 0.12        | 100 (8)                           | 100 (3)         | 0.46        |
| Subiculum                | 106 (36)                         | 107 (19)        | 0.45            | <b>102 (9)</b>           | <b>112 (40)</b> | <b>0.03</b> | 105 (29)                          | 111 (31)        | 0.33        |
| Temporal cortex          | 100 (3)                          | 100 (10)        | 0.53            | 100 (1)                  | 101 (15)        | 0.13        | 100 (87)                          | 100 (3)         | 0.71        |
| <i>Medulla (rostral)</i> |                                  |                 |                 |                          |                 |             |                                   |                 |             |
| AN                       | 100 (1)                          | 100 (22)        | 0.10            | 100 (4)                  | 100 (0)         | 0.34        | 100 (3)                           | 100 (9)         | 0.56        |
| Cun                      | <b>100 (5)</b>                   | <b>109 (25)</b> | <b>&lt;0.01</b> | 100 (9)                  | 102 (11)        | 0.38        | 100 (8)                           | 100 (9)         | 0.99        |
| DMNV                     | 100 (7)                          | 100 (17)        | 0.74            | 100 (12)                 | 100 (12)        | 0.73        | 100 (12)                          | 100 (4)         | 0.35        |
| XII                      | 100 (6)                          | 100 (10)        | 0.68            | 102 (10)                 | 100 (7)         | 0.19        | 100 (10)                          | 100 (1)         | 0.06        |
| ION                      | 100 (13)                         | 102 (21)        | 0.09            | 105 (21)                 | 100 (8)         | 0.06        | 101 (18)                          | 100 (13)        | 0.25        |
| LRF                      | 100 (0)                          | 100 (6)         | 0.34            | 100 (11)                 | 100 (0)         | 0.14        | 100 (4)                           | 100 (0)         | 0.12        |
| NSTT                     | 107 (18)                         | 107 (12)        | 0.96            | 106 (15)                 | 109 (18)        | 0.55        | 108 (15)                          | 104 (14)        | 0.18        |

|             |                |                 |             |          |          |      |          |          |      |
|-------------|----------------|-----------------|-------------|----------|----------|------|----------|----------|------|
| <b>NTS</b>  | <b>100 (6)</b> | <b>111 (15)</b> | <b>0.05</b> | 103 (16) | 102 (14) | 0.76 | 102 (18) | 100 (11) | 0.20 |
| <b>Vest</b> | 100 (0)        | 100 (5)         | 0.10        | 100 (5)  | 100 (0)  | 0.26 | 100 (6)  | 100 (1)  | 0.35 |

Results presented as the medians (interquartile range)

Bold used to highlight statistical significance. Obtained using Mann-Whitney U test. Significance taken at  $p \leq 0.05$ .



**Table 8.10: Comparison of R-score values according to major risk factors amongst the SIDS cohort.**

| Brain Region           | SIDS Risk Factors (SIDS only) |                  |             |                          |               |             |                                   |               |             |                         |               |             |                     |                |             |
|------------------------|-------------------------------|------------------|-------------|--------------------------|---------------|-------------|-----------------------------------|---------------|-------------|-------------------------|---------------|-------------|---------------------|----------------|-------------|
|                        | Sex                           |                  |             | Cigarette smoke exposure |               |             | Upper respiratory tract infection |               |             | Co-sleeping/Bed-sharing |               |             | Found prone         |                |             |
|                        | Male<br>(n=33)                | Female<br>(n=13) | p-<br>value | No<br>(n=15)             | Yes<br>(n=23) | p-<br>value | No<br>(n=28)                      | Yes<br>(n=18) | p-<br>value | No<br>(n=26)            | Yes<br>(n=20) | p-<br>value | No<br>(n=21)        | Yes<br>(n=18)  | p-<br>value |
| <i>Cerebellum</i>      |                               |                  |             |                          |               |             |                                   |               |             |                         |               |             |                     |                |             |
| <b>Dentate Nucleus</b> | 100 (3)                       | 100 (6)          | 0.82        | 101 (5)                  | 100 (2)       | 0.10        | 100 (2)                           | 100 (6)       | 0.45        | 100 (5)                 | 100 (4)       | 0.98        | 100 (3)             | 100 (7)        | 0.12        |
| <b>IGL</b>             | 119<br>(24)                   | 114<br>(28)      | 0.56        | 121<br>(15)              | 115<br>(31)   | 0.62        | 115<br>(20)                       | 127<br>(32)   | 0.26        | 120<br>(18)             | 111<br>(28)   | 0.31        | 114<br>(20)         | 125<br>(24)    | 0.07        |
| <b>ML</b>              | 0 (50)                        | 0 (0)            | 0.21        | 0 (100)                  | 0 (0)         | 0.14        | 0 (0)                             | 0 (25)        | 0.72        | 0 (0)                   | 0 (0)         | 0.95        | 0 (50)              | 0 (0)          | 0.59        |
| <b>White matter</b>    | 103<br>(12)                   | 100<br>(12)      | 0.62        | 102<br>(12)              | 103<br>(13)   | 0.88        | 101<br>(11)                       | 107<br>(14)   | 0.52        | 102<br>(13)             | 103<br>(11)   | 0.73        | 101<br>(16)         | 104<br>(11)    | 0.75        |
| <i>Hippocampus</i>     |                               |                  |             |                          |               |             |                                   |               |             |                         |               |             |                     |                |             |
| <b>CA4</b>             | 109<br>(24)                   | 111<br>(29)      | 0.70        | 106<br>(26)              | 117<br>(32)   | 0.13        | 106<br>(18)                       | 116<br>(34)   | 0.16        | 109<br>(30)             | 114<br>(23)   | 0.68        | 107<br>(30)         | 110<br>(19)    | 0.47        |
| <b>CA3</b>             | 100 (0)                       | 100 (0)          | 0.29        | 100 (0)                  | 100 (0)       | 0.75        | 100 (0)                           | 100 (0)       | 0.30        | 100 (0)                 | 100 (0)       | 0.35        | 100 (0)             | 100 (0)        | 0.91        |
| <b>CA2</b>             | 100 (0)                       | 100 (0)          | 0.84        | 100 (0)                  | 100<br>(50)   | 0.13        | 100 (0)                           | 100 (0)       | 0.56        | 100 (0)                 | 100 (0)       | 0.33        | 100 (0)             | 100<br>(13)    | 0.33        |
| <b>CA1</b>             | 100 (9)                       | 104<br>(12)      | 0.46        | 102 (6)                  | 100<br>(12)   | 0.76        | 102<br>(10)                       | 100 (7)       | 0.86        | 100 (6)                 | 103<br>(13)   | 0.21        | 100 (6)             | 102 (9)        | 0.21        |
| <b>DG</b>              | 100 (0)                       | 100 (0)          | 0.66        | 100 (0)                  | 100<br>(10)   | 0.10        | 100 (0)                           | 100<br>(11)   | 0.31        | 100 (0)                 | 100 (0)       | 0.62        | 100 (0)             | 100 (6)        | 0.65        |
| <b>EC</b>              | 100 (8)                       | 104<br>(32)      | 0.30        | 100 (2)                  | 100 (7)       | 0.41        | 100<br>(21)                       | 100 (5)       | 0.35        | 100 (6)                 | 104<br>(21)   | 0.75        | <b>103<br/>(28)</b> | <b>100 (3)</b> | <b>0.04</b> |
| <b>Subiculum</b>       | 106<br>(36)                   | 107<br>(16)      | 0.59        | 102<br>(15)              | 112<br>(33)   | 0.13        | 105<br>(33)                       | 110<br>(31)   | 0.56        | 105<br>(34)             | 106<br>(30)   | 0.13        | 108<br>(29)         | 104<br>(16)    | 0.48        |

|                          |                |                 |             |          |          |      |          |          |      |          |          |      |                 |                |             |
|--------------------------|----------------|-----------------|-------------|----------|----------|------|----------|----------|------|----------|----------|------|-----------------|----------------|-------------|
| <b>Temporal cortex</b>   | 100 (4)        | 103 (11)        | 0.60        | 100 (5)  | 100 (9)  | 0.49 | 100 (11) | 100 (4)  | 0.56 | 100 (2)  | 103 (13) | 0.10 | <b>104 (18)</b> | <b>100 (2)</b> | <b>0.02</b> |
| <i>Medulla (rostral)</i> |                |                 |             |          |          |      |          |          |      |          |          |      |                 |                |             |
| <b>AN</b>                | 100 (2)        | 102 (20)        | 0.13        | 101 (17) | 100 (0)  | 0.85 | 100 (2)  | 101 (18) | 0.15 | 100 (4)  | 100 (5)  | 0.71 | 102 (26)        | 100 (1)        | 0.07        |
| <b>Cun</b>               | <b>100 (6)</b> | <b>109 (26)</b> | <b>0.03</b> | 102 (12) | 102 (11) | 0.89 | 105 (12) | 100 (9)  | 0.46 | 100 (7)  | 107 (14) | 0.20 | 100 (11)        | 105 (8)        | 0.30        |
| <b>DMNV</b>              | 100 (12)       | 100 (13)        | 0.54        | 103 (16) | 100 (12) | 0.54 | 100 (13) | 100 (4)  | 0.52 | 100 (6)  | 100 (16) | 0.51 | 100 (12)        | 100 (12)       | 0.77        |
| <b>XII</b>               | 100 (10)       | 100 (8)         | 0.55        | 106 (11) | 100 (7)  | 0.18 | 103 (13) | 100 (3)  | 0.09 | 100 (9)  | 100 (10) | 0.78 | 100 (4)         | 100 (10)       | 0.43        |
| <b>ION</b>               | 100 (18)       | 101 (15)        | 0.81        | 102 (21) | 100 (8)  | 0.14 | 101 (19) | 100 (15) | 0.63 | 100 (9)  | 101 (18) | 0.27 | 100 (16)        | 100 (14)       | 0.52        |
| <b>LRF</b>               | 100 (0)        | 100 (0)         | 0.61        | 100 (0)  | 100 (0)  | 0.84 | 100 (4)  | 100 (0)  | 0.12 | 100 (0)  | 100 (0)  | 0.77 | 100 (0)         | 100 (0)        | 0.71        |
| <b>NSTT</b>              | 108 (20)       | 107 (9)         | 0.44        | 108 (18) | 109 (16) | 0.83 | 108 (15) | 103 (12) | 0.10 | 106 (11) | 108 (23) | 0.25 | 106 (15)        | 108 (14)       | 0.35        |
| <b>NTS</b>               | 100 (13)       | 111 (23)        | 0.21        | 104 (26) | 105 (14) | 0.94 | 102 (25) | 100 (11) | 0.42 | 101 (15) | 102 (14) | 0.74 | 102 (23)        | 100 (12)       | 0.60        |
| <b>Vest</b>              | 100 (0)        | 101 (5)         | 0.17        | 100 (6)  | 100 (0)  | 0.43 | 100 (6)  | 100 (1)  | 1.00 | 100 (0)  | 100 (7)  | 0.12 | 100 (6)         | 100 (0)        | 0.41        |

Results presented as the medians (interquartile range)

Bold used to highlight statistical significance. Obtained using Mann-Whitney U test. Significance taken at  $p \leq 0.05$ .

## 8.4: DISCUSSION

Pooling the data for GFAP immunohistochemistry from chapters 6 and 7 with the aim of identifying commonalities and differences between the 2 cohorts, this chapter's main findings were that: 1) in accordance with findings of the previous two chapters, GFAP expression decreased with parameters of growth and development (including PCA, body weight, body length, brain weight and head circumference), although these associations were region-dependent and also differed according to diagnosis, 2) SIDS infants had an increased number of GFAP-positive cells in the temporal cortex, 3) expressional and/or morphological alterations were seen according to the presence of SIDS risk factors, including increased expression in the NTS and Cun of female infants, increased expression in the temporal cortex and subiculum for infants exposed to cigarette, decreased expression in the DG/increased expression in the CA4 of infants with a recent URTI, and decreased expression in the white matter, subiculum and temporal cortex of infants found prone, and 4) there were major differences in GFAP expression between the individual diagnostic groups comparing Australian to UK eSUDI and SIDS, particularly in the medulla.

A summary of the comparative data of each individual cohort alongside the pooled data is presented in Table 8.11 showing that when pooling samples for analyses, some findings are lost/retained.

**Table 8.11: Comparative data between the UK and Australian cohorts when analysed according to diagnosis and risk factors.**

|                                          | <b>UK</b>                                 |                                        | <b>Australian</b> |                                                               | <b>Combined</b>                |                         |
|------------------------------------------|-------------------------------------------|----------------------------------------|-------------------|---------------------------------------------------------------|--------------------------------|-------------------------|
| <i>Parameter:</i>                        | <b>Counts/mm<sup>2</sup></b>              | <b>R-score</b>                         | <b>Counts/mm</b>  | <b>R-score</b>                                                | <b>Counts/mm</b>               | <b>R-score</b>          |
| <b>SIDS</b>                              | ↑IGL<br>↑CA4<br>↑temporal cortex<br>↑DMNV | ↑IGL                                   | No difference     | <b>No difference</b>                                          | ↑temporal cortex               | <b>No difference</b>    |
| <b>R score &lt;120</b>                   | NA                                        | No difference                          | NA                | eSUDI > SIDS<br>white matter<br>SIDS>eSUDI<br>cuneate nucleus | No difference                  | No difference           |
| <b>Male sex</b>                          | ↑dentate nucleus<br>↑ML                   | No difference                          | No difference     | ↓CA1<br>↓Cun                                                  | ↓IGL                           | ↓Cun<br>↓NTS            |
| <b>Cigarette smoke exposure</b>          | ↓IGL<br>↑XII<br>↑LRF                      | ↑CA4<br>↑subiculum<br>↑temporal cortex | ↑temporal cortex  | ↓CA2                                                          | ↑temporal cortex               | ↑subiculum              |
| <b>Upper respiratory tract infection</b> | ↑XII                                      | No difference                          | ↓DG               | ↓XII<br>↓LRF                                                  | ↓DG                            | ↑CA4                    |
| <b>Bed-sharing</b>                       | No difference                             | <b>No difference</b>                   | ↑Vest             | <b>No difference</b>                                          | No difference                  | <b>No difference</b>    |
| <b>Found Prone</b>                       | ↓IGL<br>↓white matter<br>↑DG              | No difference                          | ↓Subiculum        | ↓AN                                                           | ↓subiculum<br>↓temporal cortex | ↓EC<br>↓temporal cortex |

Bold indicates common findings between cohorts.

#### **8.4.1: COMPARATIVE ACCORDING TO DEVELOPMENTAL PARAMETERS**

The findings from this comparison study confirmed that as developmental and growth-related parameters increase postnatally, there are region-dependent decreases in GFAP. Increasing the sample size by combining data allows for improvement on statistical power, and as such, many of the discrepancies seen between eSUDI and SIDS in the cerebellum and medulla in the past two chapters were resolved, with growth related associations now common to both diagnostic groups. GFAP expression in the hippocampus and temporal cortex, however, continued to only correlate with developmental parameters for the SIDS group. When considering astrocyte reactivity based on R-score, negative correlations between values and developmental parameters were predominantly seen in the cerebellum and medulla for eSUDI, and hippocampus and temporal cortex for SIDS. Associations for one group but not the other may indicate heterogeneity in GFAP independent of ‘normal’ growth and development. As such, a proportion of eSUDI infants may have altered GFAP expression in the hippocampus. For reactivity status, reactive morphologies decreased with age only amongst eSUDI, indicating that normal developmental processes may be impaired in a subset of SIDS.

#### **8.4.2: COMPARATIVE BETWEEN DIAGNOSTIC GROUPS**

When analysing GFAP data (pooled) according to diagnostic sub-groups, there was an increased number of GFAP-positive cells in the temporal cortex of SIDS. No differences were seen in reactivity status, assessed by astrocyte morphology. The lack of findings in the CA4, IGL and DMNV reported previously in the UK cohort indicates that increased GFAP is only present in a subset of SIDS and not present in many cases in the Australian dataset, thus cancelling out patterns in the data when undertaking statistical analysis, or alternatively, that GFAP expression was abnormal in the UK or Australian baseline group. Australian eSUDI infants had a significantly higher number of GFAP-positive cells in the IGL and DMNV compared to UK eSUDI infants, although this was not the case when comparing the two SIDS cohorts. Furthermore, the proportion of infants with R-scores >120, indicating mild astrogliosis, was also significantly higher in the Australian eSUDI group compared to the UK eSUDI group. Although we found GFAP expression was altered as a factor of postnatal development, we hypothesise that scores >120 would not be present under ‘normal’ conditions, as was the case in the UK. Given that astrogliosis may be increased under certain conditions, such as in the case of hypoxic-episodes related to cardiac-abnormalities (Kinney et al., 2005),

and that the IGL is a hypoxia-sensitive region (Hausmann et al., 2007, Brierley, 1984), this study indicates that astrogliosis may in fact occur in a proportion of eSUDI infants. By contrast, the UK SIDS infants had a significantly higher number of GFAP-positive astrocytes in the temporal cortex compared to Australian SIDS infants, with no differences for eSUDI. Baseline GFAP may have been swayed for certain brain regions in our Australian dataset, although this did not appear to be the case for the temporal cortex.

Vast differences were seen for GFAP staining between the UK and Australian cohorts in the medulla, with the number of GFAP-positive cells and reactivity-status being consistently higher in Australia. This could either be a result of variations in laboratory protocols or could be due to true biological variation. Differences in experimental method between our cohorts included manual (Australia) vs automated (UK) immunohistochemistry, higher PMI in the UK and higher tissue storage times in Australia prior to staining. Biological differences seem to lie within the SIDS group given that many correlations between GFAP and growth parameters were specific to eSUDI group, thus indicating ‘normal’ development. This could possibly explain the inconsistency of the literature related to the SIDS medulla and astrogliosis (Summarised Table 2.5, Chapter 2).

#### **8.4.3: COMPARATIVE ACCORDING TO RISK FACTORS**

Various changes were seen according to risk factors, with additional findings for decreased expression/reactivity in the IGL and NTS of male infants, increased reactivity in the CA4 of infants with a recent URTI and decreased expression/reactivity in the subiculum, EC and temporal cortex of infants found prone. Additional changes seen in the IGL and NTS of female infants is supportive of previous literature that suggests differential sensitivities to astrogliosis in response to disruptions in CNS homeostasis and injury (Peixoto et al., 2019, Arias et al., 2009). Furthermore, astrogliosis was upregulated in the CA4 of infants with a recent URTI, and cell death has been shown to be upregulated in the CA4 as a factor of URTI previously (Luijckx et al., 2020). Downstream inflammatory responses have been suggested to trigger the events leading to SIDS (Lundemose et al., 1993, Vege et al., 1995, Raza and Blackwell, 1999, Ferrante et al., 2008, Moscovis et al., 2015) and astrocytes activated under inflammatory conditions can subsequently induce death of neurons (Liddelow et al., 2017). Our findings therefore increase support for this mechanism. In accordance with the findings in chapter 6&7, infants found prone had significantly higher age and growth-parameters of PCA, body weight,

brain weight and head circumference, thus, no conclusions can be made about the effects of sleeping prone.

Most notably, infants exposed to cigarette smoke showed increased GFAP expression in the temporal cortex, as well as increased reactivity in the subiculum. Interestingly, infants did not show significantly increased expression in the temporal cortex as a factor of diagnosis in our Australian cohort yet showed upregulation as a factor of cigarette smoke exposure (Chapter 7, Table 8.1). In the UK, the opposite was true, with increased expression seen as a factor of SIDS diagnosis, but not cigarette smoke-exposure (Table 8.1). In the UK cohort, the non-smoke-exposed group was of a small sample size, and there may have not been enough statistical power to detect an effect despite its likely contribution, given that a high proportion of SIDS infants had reported exposure. This finding in the SIDS group may well be an effect of cigarette smoke exposure.

Despite various alterations seen, vast inconsistencies within and between cohorts show that the presence of astrogliosis is highly heterogeneous, and increased GFAP following exposure to risk factors may only be present in a proportion of infants, such as those with survival times >12 hours following insult (O'Callaghan et al., 2014). A major limitation in our studies is that we did not distinguish the strength of the risk-factor exposure (e.g., routinely slept prone vs. only slept prone on the night of death). Future studies will need to focus on stratifying infants according to specific aetiology that indicate a causal pathway using specific markers to identify astrogliosis caused by hypoxia vs inflammation (or both), given that increasing evidence suggests that these two pathways differ (Zamanian et al., 2012, Chai et al., 2017, Yu et al., 2021). In theory, this would be advantageous given that it would: 1) indicate the nature of the risk factors that led to damage, and 2) the level of damage would reflect the severity of the insult.

## **8.5: CONCLUSION**

The data from the two cohorts (UK and Australia) when pooled, confirm that GFAP expression decreases in a region-specific manner postnatally, and maintain that astrogliosis does occur in the SIDS in the temporal cortex. However, changes related to the presence of major SIDS risk factors varied, as did the data within the eSUDI group, thus suggesting changes in GFAP are highly heterogeneous in nature and may not be of diagnostic potential in relation to SUDI deaths.

## CHAPTER 9: CONCLUDING REMARKS

### 9.1: OVERALL SUMMARY

This thesis investigated a mechanism for cell death via nAChRs in the hippocampus, and astrogliosis throughout a range of regions in the developing human brain. It also reports on changes in SIDS and its major risk factors.

Chapter 3 investigated IHC markers of cell death (caspase-3, and TUNEL) and nAChR subunits ( $\alpha 7$  and  $\beta 2$ ) in 5 sub-regions of the hippocampus. Considering the eSUDI group as our control group, representing ‘normal’ postnatal development, we found up to 10% of neurons stained for markers of cell death, while SIDS infants showed up to 39%, with significant increases in markers of cell death seen throughout almost all regions studied, although appearing only after excluding bed-sharers. The  $\alpha 7$  nAChR subunit was also upregulated in the CA2 of SIDS. Markers of cell death and the nAChR subunits correlated overall, and further expressional changes in all markers were found for the risk factors. These findings support a likely mechanism for cell death in the SIDS hippocampus.

Chapter 4 sought to determine whether HS, based on NeuN histochemistry, was present in a subset of SIDS infants. In the absence of a finding of HS in SIDS, more subtle anatomical changes in neuron density and GFAP expression were hypothesised be present, indicative of a precursor to the development of HS. Preliminary studies using NeuN IHC on FFPE human brain tissue revealed staining to be low or absent, and alterations in our experimental protocol could not improve signal to a level suitable for assessment. Undertaking a follow-up pilot study to determine whether assessment on basic histochemical stain would provide reliable cell counts to detect subtle neuron loss, showed it to be challenging resulting in a wide variation in counts.

Chapter 5 introduced a novel method to quantify GFAP IHC, termed R-score, which attempts to standardise typical qualitative judgments about astrogliosis and its associated severity. The reproducibility of this method was tested against other commonly used methods and its sensitivity was compared against clinical diagnosis. The R-score was shown to be reproducible across tissue sections, different time points, and different raters, and was also sensitive to detect astrogliosis. Thus, we propose its validity in clinical diagnosis and future research.



Chapter 6, utilising tissue derived from a UK cohort of SUDI infants, investigated astrogliosis via GFAP IHC, in 21 microscopically defined regions in the developing human cerebellum, hippocampus and brainstem medulla. The R-score was applied for the first time in a research setting, in combination with the traditional cell-counting method. GFAP was found to be associated with region-specific postnatal development, and SIDS infants showed higher GFAP expression in the IGL of the cerebellum, CA4 of the hippocampus, temporal cortex, and DMNV of the medulla, with further differences seen according to the presence of major SIDS risk factors.

Chapter 7, utilising brain tissue derived from an Australian cohort, aimed to determine whether findings in chapter 6 were reproducible, with analyses of additional 3 regions of the amygdala, caudate and putamen. GFAP was again shown to be associated with region-specific postnatal development, although no changes in expression were seen according to SIDS classification. Various alterations were seen in accordance with SIDS risk factors, although these were different brain regions to those seen in Chapter 6, and were often decreased, which was in the opposite direction to what was originally hypothesised.

Chapter 8 sought to combine and analyse data from Chapter 6 and Chapter 7 and compare differences between the eSUDI and SIDS groups to account for inconsistencies seen. This chapter confirmed that GFAP expression decreases in a region-specific manner postnatally and maintained that astrogliosis does occur in the SIDS temporal cortex, however, changes related to the presence of major SIDS risk factors varied.

## **9.2: CONTRIBUTION OF THIS THESIS TO SUDI RESEARCH**

Cell death, beyond what is expected during normal postnatal development, has been documented previously in the SIDS brainstem and hippocampus, however, it is not known by which mechanism this occurs. nAChRs are known to have a role in regulating cell death, with  $\alpha 7$  being either pro or anti-apoptotic, and  $\beta 2$  thought to be neuroprotective. This study confirms that cell death is increased in the SIDS hippocampus, but also investigates nAChRs in the SUDI hippocampus for the first time (summarised Table 9.1), and as such, identifies their potential role in regulating cell death. Increased cell death and dysregulated expression of nAChR subunits may be further accounted for by SIDS risk factors, particularly URTI.

GFAP expression has not been assessed quantitatively in as many regions of the developing human brain previously, nor in the context of SIDS and associated risk factors. The studies

included in this thesis were also unique in that they quantitatively assessed changes in both the number of GFAP-positive cells, as well as their pathological severity based on assessment of morphology. GFAP expression was shown to decrease postnatally in a region-dependent manner, and also differ between eSUDI and SIDS groups. Various expressional alterations were seen as a factor of diagnosis and risk factors, and differences were often in the number of GFAP-positive cells, or in the level of reactivity, but not both. These findings highlight that discrepancies in past studies may be dependent on the types of changes assessed as well as due to the heterogeneity within the profile of the study groups.

**Table 9.1: Summary of common findings across this thesis.**

| <i>Parameter:</i>               | <b>Subregion</b>                                          |              |                         |                  |                      |
|---------------------------------|-----------------------------------------------------------|--------------|-------------------------|------------------|----------------------|
|                                 | <b>CA4</b>                                                | <b>CA3</b>   | <b>CA2</b>              | <b>CA1</b>       | <b>Sub</b>           |
| <b>SIDS</b>                     | ↑TUNEL<br>↑GFAP                                           | ↑TUNEL       | ↑ $\alpha$ 7<br>↑Casp-3 | ↑ TUNEL          | ↑TUNEL               |
| <b>Male sex</b>                 | ↑ $\beta$ 2                                               | ↑ $\beta$ 2  |                         | ↓Casp-3<br>↓GFAP |                      |
| <b>Cigarette smoke exposure</b> | ↓ $\beta$ 2<br>↑GFAP                                      |              | ↓GFAP                   | ↓TUNEL           | ↓TUNEL<br>↑GFAP      |
| <b>URTI</b>                     | ↑ $\alpha$ 7<br>↓ $\beta$ 2<br>↑Casp-3<br>↑TUNEL<br>↑GFAP | ↑Casp-3      | ↓ $\alpha$ 7            | ↓Casp-3          |                      |
| <b>Bed-sharing</b>              |                                                           |              |                         |                  | ↓ $\beta$ 2          |
| <b>Found Prone</b>              | ↓ $\alpha$ 7                                              | ↑ $\alpha$ 7 |                         |                  | ↓ $\beta$ 2<br>↓GFAP |

Pale red filled boxes highlight common findings between cell death and astrogliosis. Note, all the changes in TUNEL relate to findings when analysed after removal of bed-sharers.

Summarising the findings across the thesis for the hippocampus regions of CA4, CA3, CA2, CA1 and subiculum (Table 9.1), the major overlap according to diagnosis was that SIDS infants have both increased GFAP, indicative of astrogliosis, and increased TUNEL, indicative of late-stage cell death in the CA4. However, these exact changes were also seen when analysed according to the presence of an URTI, with additional changes of increased Casp-3 and  $\alpha$ 7, and decreased  $\beta$ 2. Given evidence of  $\alpha$ 7 being either pro or anti-apoptotic (Orr-Urtreger et al., 2000, Laudenbach et al., 2002, Hejmadi et al., 2003, Gergalova et al., 2012, Lykhmus et al., 2014)

and  $\beta 2$  neuroprotective (Zoli et al., 1999, Laudenbach et al., 2002, Parikh et al., 2010), the differential expression of  $\alpha 7$  and  $\beta 2$ , in addition to cell death and astrogliosis is interesting, and suggests a relationship between nAChRs and brain injury in light of an inflammatory process (underlying the presence of URTI). The finding that this was specific to the CA4 region is interesting given that compared to other CA regions, the CA4 is highly vulnerable to damage from hypoxia (Kirino, 1982) (in combination with CA1) and (Jono et al., 2002).

Considering findings of expressional alterations in GFAP amongst other surrounding brain regions, those that seemed to relate to SIDS are the IGL, temporal cortex, and DMNV (Table 9.2). Given that the IGL and temporal cortex were also associated with exposure to cigarette smoke and being found prone, this may indicate that the change is not specific to SIDS. The increase in the DMNV was not associated with any of the studied risk factors, suggesting its specific role in SIDS pathogenesis, in keeping with our laboratory proposing the DMNV to play a prominent role in SIDS (Bejjani et al., 2013).

**Table 9.2: Summary of GFAP findings (indicative of astrogliosis) for SIDS diagnosis and associated risk factors in UK, Australia and combined.**

|                    | Dentate nucleus | IGL | ML | White matter | DG | EC | Temporal cortex | AN | Cun | DMNV | XII | ION | LRF | NSTT | NTS | Vest |
|--------------------|-----------------|-----|----|--------------|----|----|-----------------|----|-----|------|-----|-----|-----|------|-----|------|
| <b>SIDS</b>        |                 | ↑   |    |              |    |    | ↑               |    |     | ↑    |     |     |     |      |     |      |
| <b>Male sex</b>    | ↑               |     | ↑  |              |    |    |                 |    | ↓   |      |     |     |     |      | ↓   |      |
| <b>CSE</b>         |                 | ↓   |    |              |    |    | ↑               |    |     |      | ↑   |     | ↑   |      |     |      |
| <b>URTI</b>        |                 |     |    |              | ↓  |    |                 |    |     |      | ↑/↓ |     | ↓   |      |     |      |
| <b>Bed-sharing</b> |                 |     |    |              |    |    |                 |    |     |      |     |     |     |      |     | ↑    |
| <b>Found Prone</b> | ↓               | ↓   |    | ↓            | ↑  |    | ↓               | ↓  |     |      |     |     |     |      |     |      |

↑/↓ occurs when expression/reactivity is differentially higher/lower in a brain region for a given parameter.

### 9.3: PROPOSED MECHANISMS INVOLVING A1 AND A2 ASTROCYTES

Repeated disruptions in CNS homeostasis can trigger astrocyte reactivity (Kang and Hébert, 2011, Burda et al., 2016, Burda and Sofroniew, 2014), and in line with the most current model that suggests that astrocytes either become neurotoxic (A1) or neuroprotective (A2), under inflammation and hypoxia/ischemia respectively (Zamanian et al., 2012, Chai et al., 2017, Yu et al., 2021), we propose that exposure to SIDS risk factors can trigger these pathways. Differential expression may occur under different intrinsic conditions, one being sex, and in our study, we found that male and female infants had differences in GFAP for some brain regions (Tables 9.1 and 9.2) thus suggesting they may have different responses to injury.

Although investigation of A1/A2 astrocytes was not a focus of this thesis, given the vast brain regions affected between SIDS cohorts, subsequent investigation is warranted given that it will help to further subtype SIDS according to mechanistic pathways, giving further insight into the different causes of death in this diagnosis, and aiding at post-mortem examination. A schematic depicting the possible pathways SIDS and related risk factors may trigger astrogliosis is presented in Figure 9.1 and discussed in detail below.

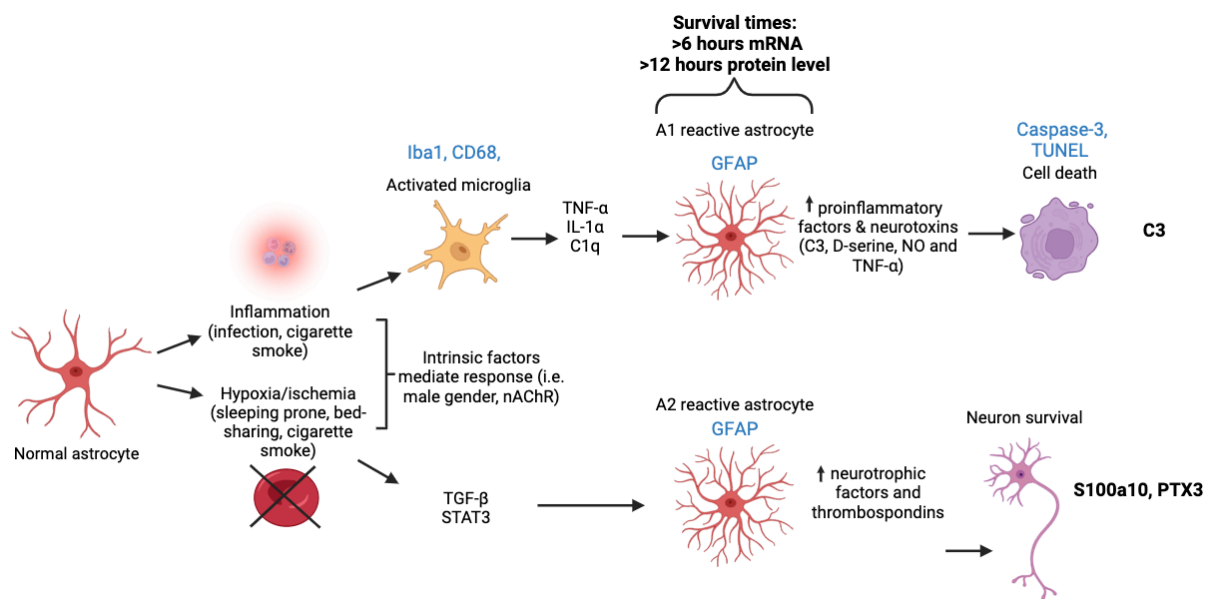
A1/A2 astrocytes have not been studied in the context of cigarette smoke before, although cigarette smoke can lead to hypoxia (Avni et al., 2016, Chan et al., 2016) and/or inflammation (Chan et al., 2016, Vani et al., 2015). A1 and A2 astrocytes may upregulated together, and loss of balance of these two sub-types and over-expression of A1 can subsequently lead to microglia activation and inflammation (Lun et al., 2022). Cigarette smoke exposure was shown to significantly affect GFAP expression in a number of brain regions in this thesis. Our Australian dataset did not reveal GFAP upregulation to be in accordance with cell death and microglia changes, which may either be due to the fact that these markers may be differentially affected at different survival times, or due to the fact that cigarette smoke exposure in fact lead to activation of the A2 sub-type. This is interesting given that we found cigarette smoke to be neuroprotective against cell death in Chapter 3.

Although nAChRs are activated by exogenous nicotine, they are also activated by endogenous ACh. Given that nAChR were shown to be differentially expressed in SIDS independent of cigarette smoke exposure, and the fact that recent data from our group showed altered BChE in the blood of SIDS infants (Harrington et al., 2022), cholinergic dysregulation is currently being further investigated as a biomarker for SIDS. Furthermore, activation of  $\alpha 7$  nAChR may promote activation of A2 astrocytes, and silence activation of A1 (Lun et al., 2022). Thus,

nAChR expression may also be involved in regulating the level of astrocyte damage following exposure to risk factors.

The mechanism by which URTI increases the risk of SIDS is not known, although may be in relation to downstream inflammatory responses (Lundemose et al., 1993, Vege et al., 1995, Raza and Blackwell, 1999, Ferrante et al., 2008, Moscovis et al., 2015), in theory leading to activation of A1 astrocytes. Alternatively, infants with an infection may be at heightened risk of cardiorespiratory and arousal dysfunction, particularly if other risk factors, such as prone sleeping, are present (Ponsonby et al., 1993), suggesting a hypoxic pathway. It is not known which pathway URTI would activate, although potentially both could occur depending on the timing and brain region affected.

Sleep-related risk factors would likely trigger A2 given that the mechanism of death is likely related to hypoxia (Campbell et al., 1997). Cell death in relation to hypoxia may occur, although not as a consequence of astrocyte activation as is the case in the A1 pathway, given that A2 astrocytes are neuroprotective. Our findings did not support that risk factors related to hypoxia lead to astrocyte activation. This may suggest a more rapid mechanism of death following exposure to these risk factors, not allowing sufficient time for GFAP to upregulate.



**Figure 9.1: Proposed mechanism by which exposure to SIDS risk factors may lead to changes in brain pathology of SIDS**, with the inflammatory pathway involving activated microglia signalling with TNF-  $\alpha$ , IL-1  $\alpha$  and C1q, inducing a neurotoxic astrocyte phenotype ‘A1’, which subsequently begin to secrete neurotoxic factors such as C3. By contrast, the A2 pathway, activated following hypoxia/ischemia, is less well understood, although is neuroprotective via release of factors such as TGF- $\beta$  and STAT3. IHC markers in bold represent specific markers for A1/A2 astrocytes, and blue indicate markers studied in our laboratory that we hypothesise to be altered in accordance. Figure created using biorender.com.

## **9.4: LIMITATIONS AND FUTURE DIRECTIONS**

### **9.4.1: VARIATIONS IN AUTOPSY AND DIAGNOSIS**

A number of different pathologists were involved in undertaking post-mortem investigations and assigning diagnosis to cases in this study. Variations exist between examiners, although this is particularly relevant in the instance where cases were from different countries, given that different countries have different protocols and standards in place. We were able to control for this to a large degree by having each case undergo reclassification by the research teams involved in the study cohorts according to the San Diego criteria (Krous et al., 2004), including a forensic pathologist for each panel. As such, it is expected that classification was standardised accordingly, thus allowing valid comparisons.

### **9.4.2: COMPOSITION OF DIAGNOSTIC GROUPS**

It remains unclear whether the data obtained from the eSUDI groups can be truly considered the “normal” baseline expression of the markers, given the heterogeneous composition of this group. This is a common limitation of SIDS pathology research where true representative controls would include deaths due to trauma, such as drowning or motor vehicle accidents with no neurological damage or head injury, which are fortunately very rare these days. Based on the cell death data, it seems that the SIDS I group had lower values overall and less variability, suggesting that this group may in fact represent ‘normal’ pathology, having died suddenly and with most having absence of many risk factors simultaneously. This was also suggested in our laboratory when microglia were analysed (Ambrose et al., 2020) and thus supports application of the San Diego criteria in future SIDS pathology research, classifying and separating the groups for analysis accordingly.

### **9.4.3: SAMPLING RATE**

Tissue availability is a further limitation of this study. Many infants only had one brain block available per brain region, thus, IHC markers investigated could not be investigated throughout the length of each region, with data provided representing a ‘snap-shot’ of each region. Despite this, for each brain region investigated, roughly 20% of cases had duplicates and reproducibility was acceptable for the analysis performed.



#### **9.4.4: FUTURE DIRECTIONS**

The findings in this thesis suggest a role for the nAChR in regulating cell death. The brainstem is the major site responsible for regulating cardio-respiratory functions and has been the main region of investigation in SIDS. Although cell death and nAChR have been examined in the SIDS brainstem separately, their role in combination has not been investigated. This research is therefore warranted, and is highly practical given that antibodies, as used in this thesis, already exist.

The findings also highlighted the heterogeneity of baseline protein expression in the SIDS group, resulting in differential expression between cohorts. Future studies should stratify infants according to common biological pathways, for example via differentiation of A1 and A2 astrocytes to help to elucidate underlying mechanisms of death and resolve for inconsistencies between research groups. This is feasible given that specific antibodies for A1 and A2 astrocytes exist, with C3 for A1 astrocytes, and S100a10 and PTX3 for A2 astrocytes, however, their use in formalin-fixed and paraffin-embedded infant brain tissue would need to be tested and optimised given that no literature to date has utilised them on such tissue.

Given the perceived relationship between cell death, microglia and astrocytes, running correlation analyses between these markers in the same cases, and identifying any patterns, would be advantageous. Regression analysis to adjust for clinical variables such as PCA and risk factors could also be undertaken on a larger sample. This analysis is feasible given data in our laboratory has been collected and can be undertaken in accordance with a statistician.

Recent scientific progress has shown that GFAP can be measured in cerebrospinal fluid (CSF)/serum for adult neurological disorders of multiple sclerosis (Abdelhak et al., 2018, Abdelhak et al., 2019), Alzheimer's disease (Pereira et al., 2021, Benedet et al., 2021) and Alexander's disease (Jany et al., 2015). Given our GFAP findings were inconsistent, and in light of the fact that GFAP is very much time dependent in activation after insult, measurement in CSF may be resolve whether GFAP is upregulated overall. Furthermore, a difference in GFAP as demonstrated in CSF/serum levels could serve as a prognostic biomarker to identify infants who are at high risk of SIDS This is feasible as CSF is collected as part of routine post-mortem examination.

## **9.5: OVERALL CONCLUSION**

The findings of this study confirm that cell death, regulated by nAChRs, is a feature of the SIDS hippocampus, although the presence of an URTI may underlie this. Astrogliosis is present in SIDS, although this is highly variable by cohort, and as such, remains unclear its true role in the pathophysiology of SIDS. However, its presence and regions affected, associated with the presence of risk factors, support that hypoxia and inflammation may lead to abnormal brain pathology in SIDS.

## CHAPTER 10: REFERENCES

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