# Exploring social fear and avoidance through biological and behavioural models of autism spectrum disorder and social anxiety

**Mia Louise Langguth**

Bachelor of Science (Hons I)

THE UNIVERSITY OF SYDNEY

A thesis submitted in fulfilment

of the requirements for the degree of

*Doctor of Philosophy*

School of Psychology

Faculty of Science

The University of Sydney

Australia

31st January 2024

## Statement of Authorship

I, Mia Louise Langguth, hereby declare that I was the principal researcher of all the work included in this thesis. This thesis has not been submitted for any other degree or purpose. 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.

All procedures were performed with approval from The University of Sydney Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia. The research reported in this thesis was supported by the award of an International Research Training Program scholarship and by the Dr Anna Donald Gift granted to the PhD candidate.

Mia L Langguth

31st January 2024

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# List of Abbreviations

| | |
|---|---|
| AD | Anxiety Disorders |
| AHA | Anterior Hypothalamus |
| AP | Anterior/Posterior |
| ASD | Autism Spectrum Disorders |
| BLA | Basal Lateral Amygdala |
| BNST | Bed Nucleus of the Stria Terminalis |
| CA | *Cornu Ammonis (regions of the hippocampus)* |
| $Ca^{2+}$ | Calcium |
| CBT | Cognitive Behavioural Therapy |
| CCG | Cytosine-Guanine-Guanine |
| CeA | Central Amygdala |
| CRF | Corticotrophin-releasing factor |
| CRH | Corticotrophin-Releasing Hormone |
| Crhr2 | Corticotrophin-Releasing Hormone Receptor Type 2 |
| CS | Conditioned Stimulus |
| CSDS | Chronic Social Defeat Stress |
| CSP | Cavum Septum Pellucidum |
| DCZ | Deschloroclozapine |
| DG | Dentate Gyrus |
| dLS | Dorsal Lateral Septum |
| DREADDs | Designer Receptors Exclusively Activated by Designer Drugs |
| DV | Dorsal/Ventral |
| EPM | Elevated Plus Maze |
| fMRI | Functional Magnetic Resonance Imaging |
| *FMR1* | Fragile-Mental-Retardation-1 (*human gene*) |
| *Fmr1* KO | Fragile-X gene knockout (*mouse*) |
| *fmrp* | Fragile-Mental-Retardation Protein |
| FXS | Fragile-X Syndrome |
| GWAS | Genome Wide Association Study |
| HPC | Hippocampus |
| IL | Infralimbic Cortices (*of the mPFC*) |
| i.c.v | Intracerebroventricular (*injection*) |
| i.p. | Intraperitoneal (*injection*) |

| | |
|---|---|
| ITI | Intertrial Interval |
| LH | Lateral Hypothalamus |
| LS | Lateral Septum |
| ML | Medial/Lateral |
| mPFC | Medial Prefrontal Cortex |
| MRI | Magnetic Resonance Imaging |
| MS | Medial Septum |
| NaC | Nucleus Accumbens |
| nS1-nS6 | Non-Social Stimulus 1 to 6 |
| nSFC | Non-Social Fear Conditioning |
| NT | Neurotensin |
| OFT | Open Field Test |
| OXT | Oxytocin |
| OXTR | Oxytocin Receptor |
| PAG | Periaqueductal Gray |
| PET | Positron Emission Tomography |
| PFC | Prefrontal Cortex |
| PND | Post Natal Day |
| PrL | Prelimbic Cortices (*of the mPFC*) |
| PV+ | Parvalbumin-expressing |
| PVN | Paraventricular Nucleus |
| S1-S10 | Stimulus 1 to 10 |
| SAD | Social Anxiety Disorder |
| SFC | Social Fear Conditioning |
| SON | Supraoptic Nucleus |
| sS1-sS6 | Social Stimulus 1 to 6 |
| UBE3A | Ubiquitin protein ligase E3A |
| UR | Unconditioned Response |
| US | Unconditioned Stimulus |
| VMH | Ventromedial Hypothalamus |

## Conference Presentations

**Langguth, M.L.**, Everett, N.E., Lynch, E.L., Turner, K.M. and Bowen, M.T. (2023). The lateral septum is associated with fleeing social contact in mice – but is this effect social-specific? Poster to be presented at the International Brain Research Organisation (IBRO) World Congress, Granada, Spain, September 2023

**Langguth, M.L.**, Everett, N.E., Lynch, E.L., Turner, K.M. and Bowen, M.T. (2023). The lateral septum is associated with fleeing social contact in mice – but is this effect social-specific? Data Blitz Travel Awardee and poster presented at the European Behavioural Pharmacology Society (EBPS), Mannheim-Heidelberg, Germany, August 2023

**Langguth, M.L.**, Everett, N.E., Lynch, E.L., Turner, K.M. and Bowen, M.T. (2022). In vivo fibre photometry reveals increased neural activity in the lateral septum is associated with fleeing social contact in mice. Invited to present at the Biological Psychiatry Australia Conference, Newcastle, NSW, Australia, November 2022

**Langguth, M.L.**, Everett, N.E., Lynch, E.L., Turner, K.M. and Bowen, M.T. (2022). In vivo fibre photometry reveals increased neural activity in the lateral septum is associated with fleeing social contact in mice. Poster presented at Federation of European Neuroscience Societies (FENS), Paris, France, July 2022

**Langguth, M.L.**, & Bowen, M.T. (2021). Exploring social avoidance in the Fragile X Mouse model of Autism Spectrum Disorder using a social fear conditioning paradigm. Poster presented at the Society for Social Neuroscience (S4SN) Conference, Online

**Langguth, M.L.**, & Bowen, M.T. (2020). Modelling social anxiety symptoms in Fragile X Syndrome using *Fmr1* KO mice. Poster presented at the International Behavioural Neuroscience Society (IBNS) Conference, Online

**Langguth, M.L.**, & Bowen, M.T. (2020). Modelling social anxiety symptoms in Fragile X Syndrome using *Fmr1* KO mice. Poster presented at the Brain and Mind Centre Symposium, Sydney, NSW, Australia

**Langguth, M.L.**, & Bowen, M.T. (2020). Modelling social anxiety symptoms in Fragile X Syndrome using *Fmr1* KO mice. Poster presented at Emerging Management Therapies for Childhood Neurodevelopmental Disorders, Brisbane, QLD, AU. Awarded Highly Commended.

## Abstract

Social anxiety disorder (SAD) is a common and debilitating disorder marked by excessive and persistent fear and avoidance of social situations in which individuals fear risk of negative evaluation, embarrassment, and judgement. More prevalent in females, SAD commonly emerges during childhood and adolescence, a critical period for social development. Further, those with neurodevelopmental disorders, such as Fragile-X Syndrome or autism spectrum disorder experience SAD at greater rates compared to the general population.

Social anxiety-like behaviour can be modelled in mice using behavioural tasks which produce behaviours homologous to some symptoms of the human disorder. One such task is the social fear conditioning (SFC) paradigm which pairs mild foot shock with interaction with novel, same-sex caged conspecifics. During fear extinction, mice are presented a series of novel social stimuli. Mice which spend increasingly more time investigating the social stimulus demonstrate social fear extinction, however others are resistant to fear extinction and persistently avoid social interaction.

The first experimental chapter of this thesis used the SFC task to explore genetic, developmental, and biological correlates of social anxiety-like behaviours in mice. As those with Fragile-X have substantially increased risk of SAD, we used a genetic animal model of the disorder, *Fmr1* KO mice, to explore potential correlates of SAD. Further, as the literature indicates age and sex are important risk factors in developing SAD, we examined adolescent and adult as well as male and female mice of each genotype using the SFC task. We found there was no significant difference between conditioned wildtype and *Fmr1* KO mice, nor males and females during social fear extinction. We did however find, that irrespective of genotype and sex, fear conditioned adolescent mice showed increased

social approach over extinction, whereas adult mice showed no signs of extinction of conditioned social fear.

In the second experimental chapter, we used the SFC paradigm to examine neural correlates for social fear and avoidance behaviours. Prior use of the SFC task has uncovered a role for the dorsal lateral septum (dLS) in social anxiety-like avoidance of social stimuli. However, the methods previously used lacked temporal specificity to identify specific behaviours i.e. approach, investigation or flee, that the dLS is involved in. Further, we wanted to examine if activity in the dLS was social-specific. Thus, the second experimental chapter used fibre photometry, a temporally precise calcium imaging technique which allows for recording of neural activity in freely moving mice, together with the SFC task to examine which specific social approach and avoidance behaviours the dLS is associated with during social fear extinction. In Experiment 1a, we first examined social behaviour under non-fearful conditions to establish dLS activity in response to novel same-and opposite-sex social and non-social stimuli. We found both male and female mice spent more time investigating social compared to non-social stimuli. However, photometry revealed dLS activity was similar across social and non-social stimulus investigation suggesting the dLS signal did not appear to be social specific. Using the same cohort of mice, we conducted a preliminary SFC pilot study. We found dLS activity was increased in social fear conditioned (SFC+) mice during social stimulus investigation and preceding proximal flee, a rapid avoidance behaviour initiated within the quadrant containing the stimulus, relative to unconditioned mice (SFC-). Further, we found dLS activity diminished alongside the extinction of social fear. In Experiment 2, we aimed to replicate these findings in a larger cohort of mice and examine if the dLS signal, preceding proximal flee, was social specific. Mice underwent SFC and non-social fear conditioning (nSFC), a modified SFC task which closely mimicked social fear conditioning but used scented

tennis balls as novel non-social stimuli. We observed peaks in dLS activity preceding proximal flee in social *and* non-social fear conditioned (nSFC+) mice. However, contrary to SFC+ mice, we did not observe a reduction of dLS activity in nSFC+ mice nor did they extinguish fear of the novel stimuli. Together the findings of this chapter demonstrated a novel role of the dLS in specific approach and avoidance behaviours that was not social-specific.

In the final experimental chapter, we used a chemogenetic approach to silence the dLS prior to social fear extinction to investigate if activity in the dLS played a *causal* role in behaviours associated with elevated dLS activity, specifically in proximal flee. It was hypothesised that inhibiting the LS would result in reduced fleeing behaviour and this would in turn lead to an increase in social investigation. Our results indicated there was a greater decrease in fleeing over repeated stimulus exposures when the dLS was inhibited and this was only apparent with proximal flees. However, this reduction in fleeing behaviour did not result in increased social investigation in socially fear conditioned mice.

This thesis has used the SFC paradigm to explore biological and behavioural correlates of social fear and avoidance. Our use of this task has revealed age as an important factor in likelihood to extinguish social fear, that *Fmr1* KO mice did not show greater social avoidance compared to wildtypes and that there was no effect of sex on conditioned social avoidance. Further, in combination with fibre photometry and chemogenetic techniques, we used SFC and extinction to uncover a nuanced role of the LS in distinct social approach and avoidance behaviour. Together these findings not only advance our understanding of social anxiety-like behaviours but also hold promise for more targeted interventions and treatment strategies for individuals with SAD in the future.

# Chapter 1. General Introduction

## Drivers of social engagement, avoidance, and fear.

## 1. 1. Background

Social behaviour is at the core of what makes us human. From birth, we begin to engage in non-verbal and verbal communication to connect with our parents and families. Growing up, we learn to interact with strangers, to distinguish friend from foe, and to build meaningful interactions which can be transformative to our lived experience and help to protect us from emotional harm. During childhood, adolescence, and into adulthood we form lifelong bonds, and our social ability begins to shape our future interpersonal experiences and occupational outcomes (Feldman, 2020; Wood et al., 2018). On the flipside, difficulties engaging and navigating social interaction can lead to social anxiety, avoidance, isolation, and subsequent development of secondary co-morbid conditions such as depression, substance-use disorders, or self-harm (Barzeva et al., 2020; Brook & Schmidt, 2008; Lemyre et al., 2019). With the advent of technology, we have become increasingly isolated (Smith et al., 2021), with loneliness now considered to be as harmful to long term health as poor diet and smoking (Paul et al., 2021).

Importantly, however, state anxiety and acute social avoidance are innate adaptive functions which serve to prime the body for potential harm in response to adverse environmental situations (Saviola et al., 2020). It is only when this anxiety and avoidance is excessive to the relative risk and for a sustained period of time (> 3 months) that the behaviour comes to be considered pathological (Kenwood et al., 2022). Social anxiety disorder (SAD) is thus characterised by persistent, intense fear of specific social situations coupled with a fear of being negatively evaluated, embarrassed or judged (American Psychiatric Association, 2013). Irrespective of country of birth, most of the approximately 8 to 15% of the population likely to experience social anxiety in their lifetime (Koyuncu et al., 2019) will develop symptoms in early adolescence (Jefferies & Ungar, 2020; D. J. Stein et al., 2017). Within the global population, individuals who identify as sexual or

gender minorities, or those with developmental or neuropsychiatric disorders, are at a markedly greater risk of developing SAD (Ezell et al., 2019; Mahon et al., 2022; McEnery et al., 2019). Once social anxiety-like behaviours are identified, those who seek treatment will most likely be referred for cognitive behavioural therapy (CBT), take prescription anti-anxiety medication, or a combination of the two (Pelissolo et al., 2019). However, the increasing rate of diagnoses of SAD has not been matched with the development of new, more effective treatments (Garakani, Freire, et al., 2021; T. Williams et al., 2020). Thus, there remains an urgent need to develop more effective model systems with improved internal and external construct validity to further our foundational knowledge about the development of social avoidance (Pound & Ritskes-Hoitinga, 2018), and provide critical information to guide development of new therapeutics.

## 1. 2. Behavioural models of social behaviour

To better understand and treat SAD, we need to use models which conservatively recapitulate social anxiety-like behaviours, with translational validity being paramount. Whilst computational modelling is increasingly being used as an adjunct in clinical settings (Abend et al., 2022), animal models are still considered the most effective tool for exploring potential mechanisms, underlying neural circuits and investigating novel therapeutic targets for psychiatric disorders (Monteggia et al., 2018; Uliana et al., 2022). Fortunately, a variety of tests have been developed to examine different components of social behaviour in animals. The most commonly used behavioural paradigms to study *general social behaviour* are the three-chamber test (Crawley, 2004) and the direct social interaction test (Silverman et al., 2010). The three-chamber test measures preference for interacting with a novel conspecific compared to an inanimate object (social preference) or a previously encountered conspecific (social novelty preference). The direct social

interaction test has many variations but common across these is the measurement of social behaviours during free interaction between two conspecifics, usually in a neutral context, which provides a more naturalistic assessment of social behaviour. In contrast, to measure *social stress*, subjects are introduced into the homecage of an unfamiliar, often larger, aggressor animal either acutely (resident-intruder task) or repeatedly (chronic social defeat stress (CSDS) task), resulting in marked social avoidance (Patel et al., 2019). Finally, in the examination of *social avoidance* and *social anxiety-like behaviour*, the social fear conditioning (SFC) task has emerged as a translationally relevant behavioural paradigm (Reus et al., 2014). SFC pairs mild foot shocks with investigation of caged, weight- and sex-matched conspecifics, such that social interaction comes to be associated with a negative outcome. Animals then undergo behavioural fear extinction in their homecage (Toth et al., 2012) or familiar open field arenas (Raymond et al., 2019); which involves exposing the animals to a series of novel conspecifics (social stimuli) over the course of the extinction session and measuring the time spent interacting with each successive social stimulus. Critically, it is both the initial expression of social fear and the rate at which subjects extinguish this social fear that provides a readout for the degree of social anxiety-like behaviour. The use of these behavioural tasks enables the controlled examination of the effect of genetic, environmental, neuronal, or pharmacological manipulations on social avoidance and social fear behaviours.

## 1. 3. The aetiology of social anxiety disorder

### *Genetic correlates for social anxiety*

Within the field of psychiatry, the emergence of new quantitative tools and techniques provides the opportunity to gain insight into the genetic, epigenetic, and

environmental factors contributing to social anxiety. The first of its kind SAD-specific genome wide association study (GWAS) found two risk loci for SAD, *rs78924501* on chromosome 1 in an African American population sample, and *rs708012* on chromosome 6 in a European American sample (M. B. Stein et al., 2017). These findings confirmed results identified from prior twin studies (Torvik et al., 2016), suggesting a mild heritable basis for social anxiety. More recently, a shift towards "precision medicine" has led to the incorporation of multi-omics, brain imaging (Bas-Hoogendam & Westenberg, 2020), and wearable devices (Kazanskiy et al., 2022). These varying techniques allow for the collection of multiple potential biomarkers for SAD, which may help to reveal specific biological mechanisms and lead to better patient stratification. Omics-based approaches have led to the identification of striatal-expressing genes co-occurring across all anxiety disorder (AD) sub-types (Karunakaran et al., 2021). Several of the genes identified are implicated in the pre-clinical literature as associated with perturbations in social behaviour across species (*see* H. Hu et al. (2023); Locci et al. (2021); Page et al. (2009); Palle et al. (2020) and Vithayathil et al. (2018)). Indeed, a notable study which adopted a multi-omics approach, unearthed mitochondrial pathways associated with anxiety-like behaviours in both mice and humans. Using the chronic social defeat stress (CSDS) model and focusing on the bed nucleus of the stria terminalis (BNST) as a key brain region in stress and anxiety, Misiewicz et al. (2019) found differential expression of mRNA, miRNA and proteins within the BNST and blood cells following CSDS in mice. Translating these findings across species, the authors found patients with panic disorder had similar mitochondrial-related alterations, before and after exposure-induced panic attacks, indicating an evolutionarily conserved stress response. However, even with the advent of omics-based approaches allowing us to better harness information from insufficiently powered GWAS (Otowa et al., 2016), we are scarcely closer to understanding the genetic

variants responsible for susceptibility to SAD nor the mechanisms behind the behavioural phenotypes (Korologou-Linden et al., 2021). Acknowledging the limitations of a "one-size fits all" approach, there are significant advantages that can be derived from studies which focus on discrete sub-groups within the wider population (Govender et al., 2020; Kambeitz-Ilankovic et al., 2022), recognising there are likely multiple genetic phenotypes that can result in increased likelihood of SAD. Thus, given the heritable nature of SAD, studies of models of genetic disorders in which social dysfunction and social anxiety are at an increased prevalence, may improve the mechanistic understanding of the causes of social impairments in at least some individuals, and guide the development of targeted therapeutics.

In the pursuit of models of social anxiety and avoidance, much of the literature has focused on the use of mouse models of autism spectrum disorders (ASD), which demonstrate reasonable face validity for social anxiety-like behaviours. Behaviourally and diagnostically distinct from SAD, those on the autism spectrum develop moderate to severe social dysfunction with verbal and non-verbal communication impairments in early childhood, in addition to stereotyped and repetitive sensory–motor behaviours (Lord et al., 2018). Neurodiverse populations, including those with ASD, are up to twice as likely to experience social anxiety (~30%, (Ezell et al., 2019; Grebe et al., 2022; Kerns et al., 2014) compared to neurotypical populations (8 to 15%, (Koyuncu et al., 2019)). Despite genetic studies indicating between 74 and 93% heritability (Tick et al., 2016), the pathophysiology of ASD is unknown in the majority of cases. Akin to SAD, ASD is highly heterogenous and whilst GWAS have uncovered several common genetic risk variants (Grove et al., 2019), the majority of cases are likely polygenic (Cirnigliaro et al., 2023; Weiner et al., 2017).

Amongst the heterogeneity, there are a number of individual monogenetic mutations with high penetrance of significance (Monteggia et al., 2018). Approximately 5 to 10% (Wisniowiecka-Kowalnik & Nowakowska, 2019) of those with autism have syndromic ASD – where individuals have a primary diagnosis of a monogenetic disorder with a secondary diagnosis of ASD (Ziats et al., 2021). The most prevalent monogenetic cause of ASD is Fragile-X Syndrome (FXS), closely followed by Angelman Syndrome (AS) (Sztainberg & Zoghbi, 2016). AS is caused by loss of function mutation of the *ubiquitin protein ligase E3A* (*UBE3A)*, which is a maternally imprinted gene that encodes proteins important in neuronal morphological maturation and cortical development (Khatri & Man, 2019). However, unlike in ASD, one of the defining characteristics of AS is hypersociability, with high emotional co-dependence and an excessive need for social contact (Roy et al., 2015). In mice, loss of function mutations in the *UBE3A* gene typically recapitulate these *prosocial* behaviours making them a poor model of social anxiety-like behaviour. Further, given there is limited clinical evidence of SAD in AS populations (Grebe et al., 2022; Hong et al., 2017), this mutation does not represent a good candidate for the study of social anxiety-like behaviours in mice.

Whilst not identified as a specific genetic risk factor in SAD, individuals with mutations in the *FMR1* gene have a two to three times higher likelihood of experiencing social anxiety (~13 to 35%, (Cordeiro et al., 2011; Ezell et al., 2019) compared to the general population (Koyuncu et al., 2019). Mutations in the *FMR1* gene exceeding 200 Cytosine-Guanine-Guanine (CGG) repeats, leads to FXS, which is the leading cause of intellectual disability (Duy & Budimirovic, 2017). The developmental disorder has significant overlap and co-morbidity with ASD where those with FXS experience similar communication deficits and social dysfunction (Kaufmann et al., 2017). Indeed, mutations in the *FMR1* gene are the most prevalent monogenetic cause of ASD, occurring in

approximately 3% of those with the disorder (Fyke & Velinov, 2021). Importantly, more evidence exists on the association between FXS and SAD compared to other less common monogenetic developmental disorders. In infants as young as 12-months old, those with FXS are found to display behavioural inhibition and physiological symptoms during a stranger approach task indicative of social anxiety-like changes in behaviour (Black et al., 2021). Adults with FXS demonstrate varying degrees of emotional recognition deficits in recognising happy (Williams et al., 2014) and angry faces (Shaw & Porter, 2013) with evidence suggesting these deficits centre on social anxiety rather than socioemotional processing, as more commonly seen in ASD. Further, adults with FXS display a willingness to interact with familiar and unfamiliar people while concurrently experiencing elevated social anxiety, indicating that social anxiety *interrupts* motivated desire to engage in social settings (Cornish et al., 2008). This finding is reinforced by a more recent eye-tracking study that found both neurotypical controls and those with FXS demonstrated heightened social preference where those with ASD did not (Hong et al., 2019). This suggests that FXS social deficits centre on social anxiety, rather than decreased social interest, as is more commonly seen in ASD (Cregenzan-Royo et al., 2022).

Like humans with *FMR1* loss of function mutations, some studies have found *Fmr1* KO mice have deficits in social interaction as well as cognitive impairments, repetitive behaviours, and hyperactivity. *Fmr1* KO mice demonstrate reduced social preference and novelty in the three-chamber test (Pietropaolo et al., 2011) and during direct interactions (Dahlhaus & El-Husseini, 2010). Yet they do not typically exhibit generalised anxiety-like behaviours (Liu & Smith, 2009), indicating social-specific avoidance behaviour. The FXS model is thus a genetic model that offers potential for investigating social-avoidance and social anxiety-like behaviours in mice.

***Sex and age are important risk factors in SAD understudied in the human and animal literature.***

Since social phobia (DSM-III, American Psychiatric Association, 1981), now more commonly referred to as SAD (DSM-IV, American Psychiatric Association, 1996), was first defined, it has been consistently more prevalent in females compared to males (*see reviews* Brook and Schmidt (2008), Rapee and Spence (2004) and Weinstock (1999)). A recent systematic review supports historical evidence of a gender disparity within SAD (Asher et al., 2017), with females, especially adolescent women, almost twice as likely (odds ratio: 1.5 -1.6) to experience SAD than men (Crome et al., 2015; MacKenzie & Fowler, 2013; Ohayon & Schatzberg, 2010) coupled with reporting greater symptom severity (Xu et al., 2012).

There are two prevailing psychosocial theories as to why these sex differences in SAD might occur, the self-construal and self-discrepancy theories (Asher et al., 2017). In short, women are more likely than men to create interdependent self-constructs, which tends to include others as part of the self (*self-construal theory*, Cross et al. (2011)). Second, whilst men report lower symptoms severity, they may experience heightened emotional distress as they are less resilient than females to discrepancies between their actual-, ideal- and ought-selves (*self-contextual theory,* Higgins (1987); Roberts et al. (2011)), which in part explains why men seek treatment more often than women (Asher et al., 2017).

While illuminating, neither psychosocial theory speculates which biological differences, if any, might be driving these disparities. Further, whilst brain imaging studies exist which probe potential sex differences in brain regions which modulate emotional regulation (i.e. limbic system activation), they are predominantly in healthy controls and

so do not assess pathological social anxiety (Kaczkurkin et al., 2016; Wang et al., 2007; Yang et al., 2017). To investigate biological differences which may be driving these gender disparities, animal models of SAD offer the opportunity to examine potential physiological variables while controlling for psychosocial factors.

Animal models allow specific variables to be manipulated and enable the examination of biological factors which may be at the core of the consistent sex differences seen in SAD. However, there is a significant shortage of studies which examine sex differences in social avoidance behaviours. In contrast to the human literature, female rats demonstrate reduced anxiety-like behaviours across several behavioural tasks including the elevated plus maze (EPM), open field test (OFT) (Scholl et al., 2019) and following two-hour restraint stress (Gupta & Chattarji, 2021) in comparison to males. However, in BALB/c mice, an inbred murine model of social avoidance, there are conflicting findings within the three-chambered social and adapted resident-intruder tasks as to whether sex differences exist (An et al., 2011; Boivin et al., 2017; Langguth et al., 2018). In social stress studies, a common justification for the lack of inclusion of females is they tend not to be as naturally aggressive or territorial, invalidating social stress tasks (*see comprehensive sex differences review* Bangasser and Cuarenta (2021). However, in recent years the use of strains and species pre-disposed to these behaviours has overcome this particular limitation (Kim et al., 2022; Newman et al., 2019; Silva et al., 2010). Unfortunately, the persistent exclusion of females in the pre-clinical literature has led to the majority of existing studies providing few insights into sex differences in social anxiety (Borchers et al., 2022). To better examine risk factors and correlates driving social anxiety, our animal models must include important variables such as sex to allow us to better recapitulate the human disorder.

Another key factor when calculating an individual's risk of developing SAD, together with genetic background and sex, is age. Whilst SAD has a lifetime prevalence of up to 15% (Koyuncu et al., 2019), 80-90% of those who develop social anxiety will do so before the age of 24 with a median onset of 13 years (Kessler et al., 2007; Solmi et al., 2022). This constitutes a peri-adolescence time period spanning late childhood through to adolescence (Larsen & Luna, 2018). Adolescence represents a critical window of development during which individuals tend to spend increased time with peers, place significant emphasis on social acceptance, and develop a sense of self (Crone & Fuligni, 2020; Sawyer et al., 2018). Compared to childhood, adolescents are influenced by and rely on others within their age group for social enjoyment, support, and self-esteem (Sentse et al., 2010). Those who are more socially anxious tend to have smaller friend groups, find it harder to make new friends, are typically less likeable and unfortunately more likely to be ignored - feeding the narrative of negative evaluation (Luchetti & Rapee, 2014; Oh et al., 2008).

Neuroimaging studies can facilitate our understanding of the neural basis and pathophysiology of SAD through non-invasive techniques sensitive to neural changes across the lifespan (Bethlehem et al., 2022; Xie et al., 2021). A number of sub-cortical brain regions are implicated in SAD; in particular the amygdala and striatum, which were historically considered the "accelerator" and "break" in emotionally salient processing (Ernst et al., 2006). In more recent years, together with the prefrontal cortex (PFC) and intermediate relay centres, evidence suggests these subcortical regions are integral in co-ordinating external motivational cues with internal emotional states important in navigating diverse social situations (Lago et al., 2017; Qi et al., 2018; Stanley et al., 2021). Compared to adults, adolescence have increased activation in the striatum when processing

reward stimuli and in processing emotionally salient stimuli together with the amygdala (Crone & Dahl, 2012; Lago et al., 2017).

Crucially, adolescence coincides with puberty, an endocrine-sensitive life stage which centres on sexual maturation - a sexually dimorphic process which may underlie gender differences seen in SAD in adolescence and adulthood (Moisan, 2022; van Honk et al., 2015). Under the influence of sex hormones during adolescence, the brain – in particular the frontal lobe and limbic system - undergoes dramatic morphological and functional changes (Arain et al., 2013). Indeed, a maturational gap between prefrontal, cognitive control and affective, limbic processing has been hypothesised for increased proclivity to engage in impulsive, risky and less rational behaviours (Crone & Dahl, 2012). Clinical and pre-clinical evidence provides support for sex differences in the rate of frontal cortex maturation where the PFC in females maturates earlier than in males (Cullity et al., 2019; Gennatas et al., 2017). In parallel, adolescent men have greater amygdala and hippocampal volumes compared to women – where earlier maturation of limbic structures may prime females for increased likelihood of emotional dysregulation in early adulthood (Frere et al., 2020). To examine the role of sex hormones on the development of anxiety-like behaviours, Delevich et al. (2020) performed gonadectomies on C57BL/6 mice prior to puberty in early adolescence. Removal of the gonads *induced* in males and *reduced* in females anxiety-like behaviours in the OFT and EPM compared to intact mice which showed no sex differences (Delevich et al., 2020). These results support findings of the differential role of sex and stress hormones on limbic and cortical development (*see reviews* Heck and Handa (2019); Premachandran et al. (2020) and Perry et al. (2021)). Together, current evidence in the literature highlights the importance of examining sex and age and the need to further interrogate the interaction between them in the development and maintenance of social anxiety-like behaviours.

### *Neural correlates of social anxiety*

With the advent of novel techniques and technologies developed for use within humans and rodents comes the opportunity to explore neural correlates that underlie social anxiety behaviours. Using magnetic resonance imaging (MRI), functional MRI (fMRI) and positron emission tomography (PET) to image cortical and sub-cortical brain regions, it is possible to study normative social behaviours and how brain activity diverges in people with social anxiety. From these studies, there is mounting data in favour of hyperactive fear circuitry, in excess to the relative harm, in SAD. Individuals with SAD typically have larger grey matter volume in the putamen of the right dorsal striatum (Bas-Hoogendam et al., 2017) and higher co-expression of dopamine and serotonin within the amygdala, putamen, and dorsomedial thalamus (Hjorth et al., 2021). Processing of negative and neutral emotional faces (Gentili et al., 2016; Schulz et al., 2013; Stein et al., 2002), threatening voices and language (Schmidt et al., 2010; Simon et al., 2017), and unintentional social transgressions (Blair et al., 2010) results in greater activation of the amygdala and prefrontal cortices in those with SAD.

Across species, the amygdala is associated with the rapid response to innate or acquired threat stimuli (Ren & Tao, 2020), whilst the PFC is typically associated with the maintenance of excessive and long-lasting fear in those with SAD. Of note, human imaging studies demonstrate increased activation in the PFC in response to social stress or threat stimuli in those with SAD compared to healthy controls (Buff et al., 2016; Kawashima et al., 2016). This is supported by the pre-clinical literature, where using a modified SFC paradigm, Xu et al. (2019) demonstrated increased expression of c-Fos positive cells, an indicator of neuronal activity, in the dorsomedial PFC and other prelimbic cortices (PrL) following conditioning. Subsequent pharmacological inactivation

of the PrL increased social investigation in the three-chambered social interaction task in socially fear conditioned mice *and* in the approach-avoidance task following social defeat. Finally, via implanted tetrodes for *in vivo* electrophysiology, the authors identified an elevated firing rate of pyramidal neurons coupled with a diminished firing rate of fast-spiking parvalbumin-expressing (PV+) interneurons during a social task following SFC; the inverse of cell-type specific activity seen in unconditioned mice. These results indicate that PFC pyramidal neurons may be important in modulating approach/avoidance behaviours during initial social investigation following conditioning, but that inhibitory PV+ interneurons may be facilitating social fear extinction via downstream targets.

As seen in humans, amygdala hyperactivity in response to social fear stimuli is conserved in rodents. Following acute social defeat stress, mice had heightened expression of immediate early genes (IEGs) markers, zif268, arc and c-Fos, in the amygdala compared to the ventral hippocampus (vHPC) and medial PFC (mPFC). However, one and two weeks post social defeat, mice spent more time investigating novel social stimuli which subsequently corresponded with increased IEGs expression in the vHPC and mPFC coupled with diminished expression in the amygdala (Qi et al., 2018). Another study using rats, demonstrated how intra-amygdala circuits facilitate observational fear learning, highlighting the role of the amygdala in the transmission and integration of relevant external cues in emotionally salient decision-making (Twining et al., 2017). However, mounting evidence suggests that the PFC and amygdala do not work in isolation to co-ordinate the most appropriate response to threatening stimuli (*see reviews* Aupperle and Paulus (2010); Grimm et al. (2021); (Grogans et al., 2023)). Adoption of a systems neuroscience approach has revealed several other subcortical brain regions related to social anxiety-like behaviours (Carlton et al., 2020; Steinman et al., 2019; Wang et al., 2021). Amongst them, is the lateral septum (LS) – a brain region previously associated with

vicious social aggressive and defensive behaviour, which now emerges as a novel candidate brain region in the co-ordination of social fear and avoidance.

In humans, the role of the septum in social behaviour is understudied (Menon et al., 2022). One of the first characterisations of the septum in social behaviour came from septal forebrain tumour studies, which reported that lesions and damage to the septum increased aggressiveness and irritability. Patients were described as having "spontaneous outbursts of anger" and "overreactions", leading to the coining of the phrase "septal rage" (Albert & Chew, 1980; Zeman & King, 1958). Indeed, these findings were corroborated with lesion studies in rats, which found removal of the whole or part of the septum led to uncontrollable, excessive fear and terror (Brady & Nauta, 1953; Slotnick et al., 1973). Another indication of the septum in social behaviour is the finding that presence of cavum septum pellucidum (CSP) i.e. agenesis of the septum pellucidum, in adults is associated with increased incidence of psychosis, bipolar disorder and depression (Galarza et al., 2004; Nopoulos et al., 1998; Shioiri et al., 1996). Whilst not necessarily a predictor of psychosis (Dremmen et al., 2019), recent evidence suggests that CSP is more common than previously expected and that CSP of any size are more prevalent in individuals with psychiatric disorders than the general population (Wang et al., 2020). However, it is the *septum verum* which contains the septal nuclei (Kamali et al., 2023), rather than the *septum pellucidum* which contains white matter fibre tracts (Cho et al., 2015; Griffiths et al., 2009), which are predominantly studied in relation to the limbic system and social behaviour. While it is still not common for the septum to be considered part of the "social brain" (Menon et al., 2022), it is increasingly gaining attention in studies examining social memory (Diaconescu et al., 2017) and trust (Krueger et al., 2007).

The septum is optimally located to relay information from the prefrontal cortices to the limbic system and motor afferents (Besnard & Leroy, 2022). While the septum has not

yet been found to be involved in SAD, those with ASD have abnormal hippocampal, amygdala and septal structures compared to neurotypical controls (Bauman & Kemper, 2005). Further, those with FXS display greater white matter volume, commonly associated with cognitive disability in psychiatric disorders including ASD (Blackmon et al., 2016), in the body of the fornix adjacent to septal nuclei and superior to the amygdala (Sandoval et al., 2018). The septum is an evolutionarily well-conserved part of the limbic system between mammals (Lanuza & Martínez-García, 2009), and in mice is divided into the lateral (LS) and medial septum (MS). The MS is associated with spatial and object recognition and, in contrast to the LS, has been minimally studied in relation to social behaviours (Menon et al., 2022). The LS, on the other hand, is functionally connected to several cortical and sub-cortical regions related to social behaviour not limited to the amygdala, HPC, lateral- (LH) and ventromedial hypothalamus (VMH) and PFC. The structural location of the LS ideally positions this brain region as a relay and co-ordination hub for emotionally salient information, including aggression (Leroy et al., 2018), pair bonding (Sailer et al., 2022), social memory (Horiai et al., 2020), social investigation (de Leon Reyes et al., 2023) and more recently, evidence has emerged for a role in social fear and avoidance (Menon et al., 2018; Zoicas et al., 2014).

Using the previously described SFC paradigm, which pairs mild foot shock with interaction with a caged same-sex conspecific, Zoicas et al. (2014) found that infusion of oxytocin (OXT) into the LS increased social interaction during fear extinction. Building on this work, Menon et al. (2018) demonstrated that pharmacological inhibition of LS OXT signalling re-established social avoidance and silencing of LS oxytocin receptor (OXTR) expressing neurons abolished social interaction. A system-level computational model further supports a role of the LS in social fear extinction, via inhibition of the VMH (Alfieri et al., 2022); corroborating Krzywkowski et al. (2020) findings in an adapted

CSDS model. Following social trauma (via CSDS), susceptible mice differentially perceive social rewards as threatening via activation of a sub-population of LS neurotensin-expressing GABAergic neurons ($LS^{NT}$), which promotes sustained social avoidance to non-threatening social situations (Li et al., 2023). Collectively these findings support the involvement of the LS in mediating and coordinating social behaviour during fearful contexts. Notably absent from the existing body of literature is the integration of the SFC task with cutting-edge neuronal recording and manipulation methodologies, which holds the promise of affording specific characterisation of the role of the LS in distinct approach and avoidance behaviours.

New technologies provide the opportunity to gain a deeper understanding of the neural processes supporting social interactions. Two such techniques are fibre photometry and chemogenetics. Fibre photometry is a calcium imaging technique which takes advantage of rapid changes in intracellular calcium ions ($Ca^{2+}$), a close proxy of neuronal activity, allowing for the disentanglement of specific behaviours (e.g., fight, flight, and freeze), in freely moving animals (Mejaes et al., 2022; Zhang et al., 2023). Previous use of this technique has uncovered the role of several neuronal pathways in the co-ordination of specific social behaviours e.g. the role of corticotrophin-releasing hormone (CRH) transients in the disinhibition of the LS in social investigation of familiar stimuli (de Leon Reyes et al., 2023); and the role of $LS^{NT+}$ GABAergic neurons ($LS^{NT+}$) in social aggression and defensive behaviours following social defeat (Li et al., 2023). Opto- and chemogenetic techniques on the other hand are used to infer a *causal* role between particular brain regions and specific behaviours (Atasoy & Sternson, 2018; Luchkina & Bolshakov, 2018; Siuda et al., 2016). Both allow for the remote manipulation of a defined collection of neurons, with optogenetics affording high temporal precision delivered via light pulses. On the other hand, chemogenetic techniques allow for more long-term manipulation of neural

activity via infusion of designer ligands which exclusively act upon designer receptors expressed in the target region using viral gene delivery. While we know the LS is involved in social fear, we do not know how or when it is critical, and the importance of this brain region has only recently been examined in this specific paradigm (Menon et al., 2018; Zoicas et al., 2014). These innovative tools in rodents will allow us to investigate how these circuits might participate in the moment-to-moment decisions to engage or flee social interaction. Knowing this information will allow us to link together biological factors, such as genetics, age, and sex, with changes in the neural circuits that control behaviour. Answering these fundamental questions will help to advance our understanding and treatment of SAD.

## 1. 4. Thesis aims and hypotheses.

The overarching goal of this research is to examine potential genetic, developmental, biological, and neural correlates of social anxiety-like behaviours in mice (*Figure 1.1*).

**Figure 1.1: Exploring genetic, development, biological and neural correlates of social anxiety-like behaviours in mice.**

Based on the literature reviewed above, mutations in the *FMR1* gene lead to the development of FXS, are high penetrance for ASD, associated with an increased prevalence of social anxiety in humans, and result in social behaviour deficits in mice.

However, it can be argued that standard social behavioural assays used in mouse models, such as the three-chambered and direct social interaction tasks, do not specifically measure social avoidance, but rather only quantify an interest or disinterest in social engagement; this ambiguity means these previous findings cannot be clearly interpreted as social avoidance. The chronic social defeat stress (CSDS) and resident-intruder tasks in part overcome these limitations. Social avoidance in the face of social aggression is an important and ecologically valid behavioural phenotype (Lopez-Moraga et al., 2022; Lyons et al., 2023). Yet it may not hold good translational validity where social fear and avoidance in humans is not typically maintained through chronic social aggression. In this regard the SFC task holds greater construct validity to SAD behaviours through exposure to an initial negative experience (~3 to 4 mild foot shocks during conditioning) followed by the opportunity to engage and extinguish social avoidance in a neutral context with a constrained, non-aggressor, sex- and weight-matched social stimulus (*Figure 1.2*).

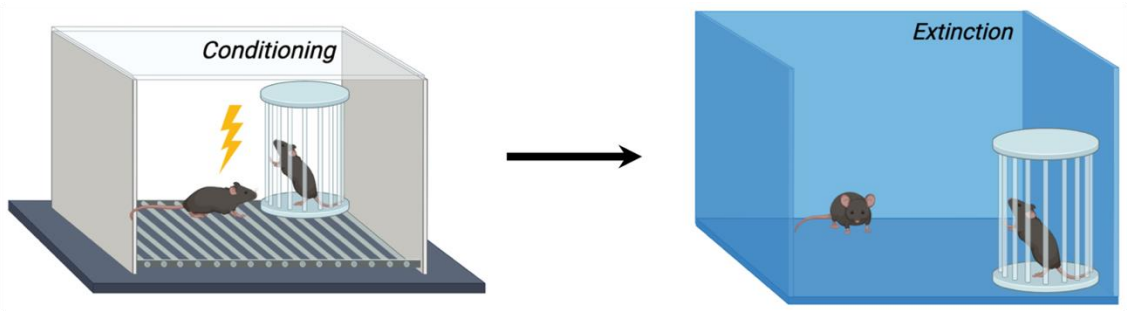



**Figure 1.2: The social fear conditioning task**
Mice are conditioned to associate social interaction with an aversive, mild foot shock. Later in extinction with the absence of foot shocks, mice are presented a series of novel social stimuli. Over stimulus exposures, mice extinguish avoidance of the social stimuli where persevering avoidance of novel social stimuli is indicative of persistent social fear.

For these reasons, the aim of the work presented in the first experimental chapter was to examine social anxiety-like behaviours in *Fmr1* KO mice using the SFC task, which until now has only been performed in C57BL/6 and CD-1 mice. It was hypothesised that

socially fear conditioned *Fmr1* KO mice would spend less time investigating novel social stimuli and would be slower to extinguish social fear across repeated exposures to novel same-sex mice. Given the outlined importance of sex and age, the first experimental study included males and females, as well as adolescent (PND35) and adult (PND70) wildtype C57BL/6 and *Fmr1* KO mice. Based on the literature demonstrating adolescence is a critical period for the development of SAD, one would predict that adolescent mice might have the most pronounced deficits, or that an interaction may exist between *Fmr1* genotype and age. In contrast, the literature presented on increased risk taking in adolescents lends more to a prediction of reduced social anxiety, at least in the WT mice. An exploration of these competing hypotheses was a focus of the study. Finally, whilst women experience higher rates of SAD compared to men, this was hypothesised to be driven primarily by psychosocial factors, despite some evidence of biological drivers, and therefore we did not predict any major sex differences in the mouse model.

Previous use of the SFC task, along with CSDS studies, have identified the LS as integral to the social fear and avoidance response. However, what previous studies have lacked is the temporal specificity to identify the potential role of the LS in specific behaviours (e.g., stimulus approach, stimulus investigation, or fleeing the stimulus). Thus, the objective of the second experimental chapter was to record, using fibre photometry, LS calcium activity during a social interaction task and later during SFC extinction in conditioned mice. Given the evidence of the role of OXTR-expressing GABAergic neurons in the extinction of social fear (Menon et al., 2018; Zoicas et al., 2014), it was hypothesised that we would see *inhibition* of dLS activity during initial social investigation during extinction in conditioned mice. Second, that dLS activity would increase across extinction and return to pre-conditioning baseline as social avoidance extinguished. The second objective was to determine if changes in the observed LS activity patterns were

social-specific. With the exception of a study from our own lab (Raymond et al., 2019), the SFC task has not been adapted to analyse whether avoidance of the conditioned stimulus is social-specific. Operant fear conditioning of non-social stimuli may also recruit the LS; thus, fibre photometry was used in experiments that tightly methodologically mirror the SFC task but used non-social stimuli to examine the response during non-social fear conditioning and extinction. Based on evidence in the contextual fear conditioning literature reviewed in Chapter 3, we hypothesised there would be a comparable pattern of dLS activity during extinction following non-social fear conditioning (nSFC) and SFC.

The final experimental chapter examined if global chemogenetic inhibition of the dLS alters social fear behaviours. Based on our findings of increased dLS activity during social fear extinction in Chapter 3, we hypothesised that inhibiting the dLS via designer receptors exclusively activated by designer drugs (DREADDs) would promote social investigation and rescue social avoidance behaviour.

# Chapter 2. No effect of genotype or sex but significant impact of age on social fear extinction in wildtype and *Fmr1* knockout mice

## Overview

Social anxiety and avoidance are the most prevalent social deficits in patients with Fragile X Syndrome (FXS), present in as many as 75% of males. Until recently, however, phenotyping of social behaviour has been limited in its ability to isolate aspects of social interaction from social fear. Like humans with the *FMR1* loss of function mutation, *Fmr1* KO mice have deficits in social interaction and motivation as well as locomotion, repetitive grooming, and anxiety-like behaviours. Using a novel murine social fear conditioning (SFC) task, we paired a mild foot shock with conspecific social interaction and examined social avoidance and fear extinction in wildtype and *Fmr1* KO mice to isolate social fear and better understand the underlying aetiology. We examined how age (adolescent vs adult), sex (male vs female), genotype (wildtype vs *Fmr1* KO*)* and the interaction between these variables impacts conditioned social avoidance behaviour. Our results showed high levels of sociability in unconditioned mice and clear social avoidance in conditioned mice during social fear extinction. However, no significant difference was found between conditioned wildtype and *Fmr1* KO mice nor male and female mice during social fear extinction. Interestingly, irrespective of genotype or sex, fear conditioned adolescent mice showed increasing social approach over extinction, whereas adult mice showed no signs of extinguishing conditioned social fear. This was the first study to specifically examine social anxiety-like behaviour in *Fmr1* KO mice and our findings indicate the genetic model does not recapitulate the social anxiety aspects of the clinical phenotype, at least in the social fear conditioning task.

## 2. 1. Introduction

Fragile X Syndrome (FXS) is a neurodevelopmental disorder and the most common form of intellectual disability (Duy & Budimirovic, 2017). An X-linked disorder, FXS is caused by Cytosine-Guanine-Guanine (CGG) repeat expansions in the promoter region of the *fragile-X-mental-retardation-1* (*FMR1*) gene (Bagni & Oostra, 2013). Occurring in 1 in 7000 males and 1 in 11,000 females (Crawford et al., 2001; Hunter et al., 2014; Peprah, 2012), expansion of more than 200 CGG repeats leads to silencing of the *FMR1* gene and absence of the *fragile-X-mental-retardation-protein* (*fmrp*). As the leading cause of hereditary developmental delay and difficulties in children, cardinal symptoms of FXS include moderate to severe intellectual disability (Bagni et al., 2012; Bagni & Zukin, 2019), impaired social behaviour including social anxiety (Cregenzan-Royo et al., 2022; Garrett et al., 2004; Holsen et al., 2008), difficulties with attention and hyperactivity (Schmitt et al., 2019), and generalised anxiety (Bartholomay et al., 2019; Roberts et al., 2018).

FXS is the single most common monogenetic cause of ASD, identified in approximately 3% of those diagnosed with ASD (Fyke & Velinov, 2021), with as many as 50% of males and 20% of females with FXS having a secondary ASD diagnosis (Kaufmann et al., 2004; Kaufmann et al., 2017; Marlborough et al., 2021). Given both ASD and FXS are marked by social impairments, it is unsurprising that both disorders are highly co-morbid with social anxiety disorder (SAD). SAD is characterised by fear of social interactions and being negatively evaluated, and consequent avoidance of social situations (DSM-V, American Psychiatric Association, 2013). SAD occurs in 13-35% and 30% of those with FXS and ASD respectively (Cordeiro et al., 2011; Ezell et al., 2019; Simonoff et al., 2008), compared to 8 to 15% in the general population (Koyuncu et al., 2019). Individuals with FXS or ASD and co-morbid SAD experience exacerbated

functional impairment, reduced capacity for independent living (Mason et al., 2018; Spain et al., 2020), and report lower quality of life (Lau et al., 2021).

Animal models are a useful tool for better understanding the aetiology and pathophysiology of social anxiety-like behaviours in FXS and ASD. Like humans with *FMR1* loss of function mutations, some studies have reported cognitive impairments, social deficits, repetitive behaviours, and hyperactivity in *Fmr1* KO mice. Dahlhaus and El-Husseini (2010) found *Fmr1* KO mice lacked a preference for social stimuli and engaged in significantly less active and passive social interactions (e.g. sniffing or allogrooming), compared to wildtype mice. Similarly, Pietropaolo et al. (2011) reported a lack of preference for novel over familiar social stimuli in *Fmr1* KO mice compared to controls. To examine if social deficits seen in *Fmr1* KO mice were due to elevated generalised anxiety-like behaviour or were social specific, Liu and Smith (2009) used the EPM and three chamber social interaction test. The authors found *Fmr1* KO mice had reduced social approach in the three-chambered social interaction test yet spent more time in the open arms on the EPM, indicating social avoidance in *Fmr1* KO mice was not associated with generalised anxiety-like behaviour. Beyond genetic risk factors for SAD, clinical studies have identified other important risks, including sex and age. Utilising laboratory-based animals and employing appropriate behavioural assays, these factors can be effectively modelled to recapitulate social anxiety-like behaviours and in turn aid our understanding of their contribution in the pathophysiology of SAD.

Age is an important developmental risk factor in the aetiology of SAD in the general population and in those with comorbid FXS and ASD. Behavioural and physiological markers for social avoidance are detectable as early as 2 to 6 months in infants later diagnosed with ASD (Jones & Klin, 2013) and at 12 months in infants with FXS (Black et al., 2021). In a study on the relevance of age in the onset of social phobias

in the general population, Rosellini et al. (2013) found that 57% developed social anxiety in adolescence, compared to 31% in childhood and 11% in adulthood. As highlighted in Chapter 1, adolescence (10 to 24 years; (Sawyer et al., 2018), is a critical window of development and represents a period of biological, psychological, and social transformation (Blakemore & Mills, 2014). Adolescents start to spend more time with friends than family, with peer relationships and gaining social approval becoming increasingly important (Orben et al., 2020). It is therefore not surprising that childhood and adolescence is a period when ASD and SAD symptoms emerge in FXS. A study investigating the developmental trajectory of social avoidance in males with FXS, found social withdrawal during infancy in 81% of participants (Roberts et al., 2019). In another study of females, girls with FXS aged 10.5 to 16 years old scored higher in social avoidance and experienced greater difficulty in navigating reciprocal social interaction compared to their younger counterparts (Lightbody et al., 2022).

Given the considerable changes in brain function across childhood and adolescence, this critical period of socioemotional development represents an opportunity to investigate divergence from typical development. The prefrontal cortex (PFC) controls higher order cognitive functions and co-ordination of social behaviours (Bicks et al., 2015; Kolb et al., 2012). Using structural MRI, Bray et al. (2011) found prefrontal gyri showed significant aberrant maturation in adolescents with FXS compared to age-matched controls, indicating differential maturation trajectories may contribute towards persistent intellectual deficits present in FXS. Another study using fMRI, found decreased activation of prefrontal regions associated with social cognition in gender discrimination and social recognition memory tasks in adolescents with FXS compared to neurotypical controls (Holsen et al., 2008). Together, these studies suggest that social anxiety in FXS may be

linked to delayed neuronal maturation during adolescence leading to difficulties in recruiting higher-order social cognitive processes.

The impact of age on brain function and social behaviour can be modelled in rodents. Adolescence in mice occurs between postnatal day 23 (PND23) and PND60 (Brust et al., 2015). Without environmental or genetic perturbations, adolescent and adult C57BL/6 mice show similar social behaviours (Langguth et al., 2018; Shoji et al., 2016). However, a two-week social isolation period in adolescent mice is enough to perturb the establishment of appropriate adult social behaviours, where the same isolation period in adult mice showed no impact on these same social behaviours (Bicks et al., 2020). When juvenile and adolescent (PND21 to PND35) mice are socially isolated for an extended period of time (≥ 1 month up to 12 months), they no longer show preference for novel social stimuli over an empty stimulus cage (Y. Y. Hu et al., 2023; Medendorp et al., 2018). Furthermore, wildtype and *Fmr1* KO mice subjected to isolation rearing from early adolescence and tested as adults (between 5 to 7 months old), show similar preference for novel social stimuli over an empty stimulus cage, with subtle social novelty differences indicating long-term social isolation in adolescence may impact social behaviour to a greater extent than genetic background (Heitzer et al., 2013). Medendorp et al. (2018) demonstrated that a lack of social enrichment during adolescence removed necessary stimulation of the PFC, resulting in altered spine morphology, hypoactivation and impaired plasticity in the medial PFC (mPFC). Extending on these findings, Bicks et al. (2020) used *in vivo* calcium imaging in freely moving mice to explore the role of parvalbumin-expressing (PV+) interneurons in the mPFC during social behaviour. In adult group housed mice, PV+ interneurons were found to be activated immediately prior to active social behaviour. However, mice socially isolated during adolescence demonstrated blunted event-related activity in the dorsal region of the mPFC and engaged in significantly less

active social behaviour. Thus, the impact of a brief social isolation period during adolescence disrupting the establishment of adult social behaviours, underscores the vulnerability of this developmental phase. Given adolescence, which coincides with the onset of puberty, is a period with increased risk and diagnoses of SAD (Rosellini et al., 2013), using animal models to understand the role of critical periods of development on risk of developing social anxiety-like behaviour is of paramount importance.

The examination of sex differences, amid the sexually dimorphic changes during puberty, emerges as an essential factor in comprehending the intricate dynamics of genetics, age, and sex on the development of social fear (Alloy et al., 2016; Tannenbaum et al., 2017). Being X-linked, fewer females are diagnosed with FXS (having >200 CCG repeats); however carrying a premutation (with 55-200 CCG repeats in the *FMR1* gene) is much more common in females (1 in 130-250) than males (1 in 250-810) (Fernandez-Carvajal et al., 2009; Tassone et al., 2012). While women with FXS tend to experience less severe symptoms (Hunter et al., 2014; Schneider et al., 2020) than males due to X-chromosome inactivation (Wang et al., 2014), studies have shown that irrespective of sex the likelihood of experiencing cognitive deficits and social difficulties increases relative to CGG repeat length (Cordeiro et al., 2015; Del Hoyo Soriano et al., 2018). SAD is present in as many as 38% of FXS females (Cordeiro et al., 2011) where women and girls who carry a *FMR1* premutation have an elevated likelihood of social disorders, attenuated cognitive disability (Bartholomay et al., 2019) and altered socio-emotional and language processing (Winston et al., 2020) compared to neurotypical controls. While only a few studies of SAD in people with autism disaggregate based on sex (Spain et al., 2018), Maddox and White (2015) showed autistic women were more than twice as likely as men to meet criteria for SAD, which is in line with trends in the general population (Bandelow & Michaelis, 2015).

Given the increased prevalence of FXS and ASD in the male population and the bias towards males in animal research, it is unsurprising that most pre-clinical *Fmr1* studies use male mice (Nolan et al., 2017; Reynolds et al., 2016; Z. Wang et al., 2023). Indeed, in a recent review on mouse models of fragile X-related disorders, while females were acknowledged for their increased prevalence of *Fmr1* premutations, sex differences in FXS mouse models were not discussed (Willemsen & Kooy, 2023). Using the three-chamber social preference test, Nolan et al. (2017) found no sex differences between male and female *Fmr1* KO mice but later demonstrated subtle variations in vocal repertoire and call frequency between sexes (Nolan et al., 2020). Given the gender disparity in FXS and ASD prevalence, a bias towards use of male mice in FXS research is to be expected. However, the development of effective treatments for SAD and for social symptoms of FXS and ASD may be accelerated if the field can incorporate/explore interactions with other factors when designing mouse models to improve translation of pre-clinical findings to the clinical population (Karp & Reavey, 2019; Kat et al., 2022).

As outlined in Chapter 1, to better understand and treat SAD, we need to use models which conservatively recapitulate social anxiety-like behaviours and are translationally relevant. In the treatment of SAD, those who seek treatment will most likely be offered cognitive behavioural therapy (CBT) either in isolation or together with prescription anti-anxiety medication namely selective serotonin-reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) (Pelissolo et al., 2019). However, up to 60% of people with SAD are treatment-resistant to CBT and/or pharmacotherapy leading to poorer quality of life (Bystritsky, 2006). In addition, there are currently no pharmacological interventions shown to be effective in the management or improvement of the social and communication deficits found in those with FXS and ASD (Parellada et al., 2021). The reality is that CBT is inaccessible and expensive with a high

proportion of patients who are treatment resistant (Bystritsky, 2006; Garakani, Murrough, et al., 2021). With a dearth of new pharmacological treatment discoveries, there remains an urgent need to improve animal models which accurately represent important risk factors and use optimally designed behavioural tasks (Kat et al., 2022; Lyons et al., 2023; Uliana et al., 2022).

Most studies in mouse models of FXS and ASD rely on the three-chamber and/or the direct social interaction test (Kat et al., 2022; Moy et al., 2004; Nadler et al., 2004). However, these assays were not designed to specifically measure social anxiety-like or social fear behaviours in mice, rather they examine *general social behaviour*. As outlined in Chapter 1, the three-chambered and direct social interaction tasks, do not specifically measure social avoidance, but rather only quantify an interest or disinterest in social engagement. Some studies have attempted to overcome these issues by examining social vigilance (A. V. Williams et al., 2020) or using multimodal approaches to quantify "affective" states (Jabarin et al., 2022); however these tasks are still not optimally designed to examine social-specific fear and avoidance.

Given social anxiety and avoidance are hallmark features experienced by those with FXS and ASD, it is imperative to identify assays that allow more precise examination of social anxiety-like behaviours in murine models of these disorders. The social fear conditioning (SFC) task is one assay which may address this issue. The SFC assay uses instrumental fear conditioning to induce social-specific fear and avoidance, pairing a mild foot shock with investigation of a novel same-sex social stimuli (Toth et al., 2012; Zoicas et al., 2014) and subsequently assessing social approach and avoidance of novel conspecifics in a different context. A review by Reus et al. (2014) found that unlike resident-intruder or other social stress tasks, the SFC task has good face and construct validity for SAD. The model also has good predictive validity as both acute

benzodiazepine exposure and chronic antidepressants treatment, which are approved pharmacotherapies for SAD (Laurito et al., 2018; Pelissolo et al., 2019), were found to reduce social avoidance in socially fear conditioned animals (Toth et al., 2012). However, it remains to be determined whether the SFC task can be used to detect differences in social anxiety-like behaviour in genetic mouse models related to social dysfunction.

To address this gap, we used the SFC paradigm to examine social-specific anxiety-related behaviours for the first time in an animal model of FXS - *Fmr1* KO mice. Based on the literature, we hypothesised that *Fmr1* KO mice would be more resistant to social fear extinction. Given the apparent influence of both age and sex on the emergence of social anxiety-like symptoms and FXS and ASD symptoms more broadly, we examined both adolescent and adult, male and female mice.

## 2. 2. Methods

### *2. 2. 1. Subjects*

Adolescent *Fmr1 wildtype (WT)* (n = 40, 21 female) and *Fmr1* KO mice (n = 47, 21 female) aged between PND35 and 42 and adult *Fmr1 WT* (n = 57, 18 female) and *Fmr1* KO mice (n = 43, 19 female) aged between PND70 and 77 were bred by pairing 6-week-old heterozygous *Fmr1* females with either wildtype *C57BL/6* or hemizygous *Fmr1 KO* (Stock #003025, The Jackson Laboratory, Maine, USA) male mice on *C57BL/6* background (Animal Resource Centre, WA) for a maximum of six breeding rounds (Bosch Lab Animal Services, NSW and Animal BioResources (ABR), NSW). Offspring were weaned between 3 and 4 weeks-old, ear notched, genotyped (samples sent to Transnetyx, Tennessee, USA) and housed with mixed genotype littermates according to sex. Mice were transferred to the test facility at either 4 weeks-old (adolescents) or 9 weeks-old (adults) and given one week to acclimatise to the new facility before commencement of testing. Mice were group housed (3 to 5 per cage) under standard laboratory conditions (12/12h light/dark cycle, light phase 0700 to 1900, 22 ± 2ºC, 50-70% humidity, food, and water *ad libitum*) in transparent IVC cages and weighed twice per week. Males were always tested before females and all experiments were performed in the light phase by a female experimenter. All procedures were performed with approval from The University of Sydney Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia.

### *2. 2. 2. Materials*

Fear conditioning was performed in Med Associates chambers (32 × 26 × 21 cm; East Fairfield, VT, USA, *see Figure 2.1A*). Chamber design consists of floor made of

evenly spaced metal rods, which administered manual foot shocks, with clear Perspex walls, one which opened to allow access to the inner chamber, and ceiling. Chambers were housed within sound- and light-attenuating cabinets. Prior to conditioning, chambers and wire-frame stimulus cages (5 x 5 x 5cm wire-frame cage with rubber base, made in-house) were cleaned with ethanol (80% v/v). Habituations and extinction took place in blue, opaque Perspex test arenas (40 x 40 x 40cm, Plastix, NSW, Australia, *see Figure 2.1B*) which were cleaned between subjects with diluted 1:25 F10. For habituation and extinction, a stimulus cage was placed in the top right-hand corner of the test arena for each stimulus exposure where it was either empty or contained a social stimulus. Age-matched, same-sex C57BL/6 mice (Animal BioResources (ABR), NSW) were used as social stimuli. Social stimuli were acclimatised to stimulus cages for two 25-minute sessions prior to the commencement of testing and used up to five rounds of testing subject to age in line with the Three R's of Animal Research. Extinction was recorded using an overhead camera and TopScan (Version 3.00, Cleversys Inc., VA, USA), behavioural tracking software, was used to detect and automatically score behaviour. Experimenter was blind to genotype but not age or sex during testing, data processing and analysis.

### *2. 2. 3. Procedure*

#### ***Behavioural Testing***

*Social Fear Conditioning Paradigm*

This task is used to model social fear and social anxiety-like avoidance behaviours in mice and was adapted from Toth et al. (2013) to involve testing in a neutral context instead of the home-cage as previously reported (Raymond et al., 2019). Group-housed

mice were socially isolated seven days prior to social fear conditioning (Day 0*, see Figure 2.2*) and remained individually housed for the remainder of the experiment.

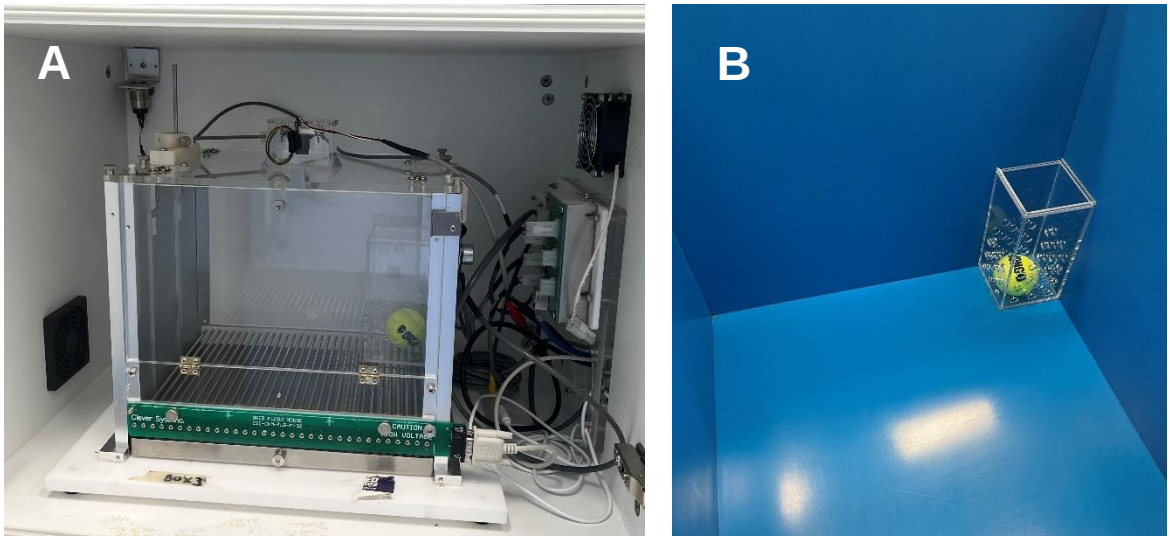



**Figure 2.1: Social and non-social fear conditioning chamber and extinction arena experimental setup.**
**A** Fear conditioning was performed in Med Associates chambers (32 × 26 × 21 cm; East Fairfield, VT, USA). Chamber design consists of floor made of evenly spaced metal rods, which administered manual foot shocks, with clear Perspex walls, one which opened to allow access to the inner chamber, and ceiling. Chambers were housed within sound- and light-attenuating cabinets. Chambers were cleaned with ethanol (80% v/v) prior to each conditioning. **B** Habituations and extinction took place in smooth, blue, opaque Perspex test arenas (40 x 40 x 40cm, Plastix, NSW, Australia) which were cleaned between subjects with diluted 1:25 F10. For all behavioural tests, a stimulus cage was placed in the top right-hand corner of the test arena for each stimulus exposure where it was either empty or contained a social (same- or opposite sex conspecific) or non-social (scented tennis ball) stimulus. Across all experiments, stimuli were located within stimulus cages; Chapter 2 and Chapter 3 Experiment 1 utilised a 5H x 5W x 5L cm wire-frame cage (built in-house) and in Chapter 3 Experiment 2 and Chapter 4 this was changed to 14H x 7W x 7L cm clear Perspex cage with 1cm round holes on all sides on lower half of cage (Plastix, Arncliffe, NSW, Australia), due to issues with the patch cord during photometry recordings and test mice mounting the wire frame cage.

*Habituation*

Mice were acclimated to the behavioural room for at least 30 minutes before testing. Prior to habituations, mice were handled for 2 minutes to acclimate to the experimenter (Day 4, 5 and 6) before being placed into the test arena for 10 minutes, which

was empty except for an empty stimulus cage. Two further habituations occurred after conditioning; the first, a minimum of 1-hour post-conditioning (Day 7), and the second, 1-hour prior to extinction (Day 8, *see Figure 2.2*).

*Social fear conditioning*

Social fear conditioning involved the administration of mild foot shocks upon interaction with a same-sex social stimulus. Conditioning began with a 30 second acclimatisation period where experimental mice were placed into the empty operant chamber. Subsequently, an empty stimulus cage was positioned in the top-right corner of the chamber and experimental mice were given 3 minutes to freely explore. Next, the empty stimulus cage was replaced with a caged same-sex social stimulus in the same location. Mice in the conditioned treatment group (SFC+), were manually administered a short electric foot shock (1 s, 0.7 mA, constant current) upon initial social stimulus investigation, defined as direct contact with the stimulus cage of at least 3 seconds duration. After this first bout of investigation, a foot shock was applied immediately upon each instance of direct investigation of the stimulus cage. Conditioning was terminated once no further investigation had occurred within a 2-minute period following the last foot shock. In line with Raymond et al. (2019), we determined *a priori* mice would be excluded if they received *less than two or more than five* foot shocks during the procedure, however no mice met this exclusion criteria. Mice in the unconditioned (SFC-) treatment group were allowed to freely investigate the social stimulus for 5 minutes and no foot shocks were administered. After conditioning, both SFC- and SFC+ mice had an empty stimulus cage, cleaned with diluted 1:25 F10, placed into their individual homecage overnight, to extinguish any conditioned fear to the stimulus cage.

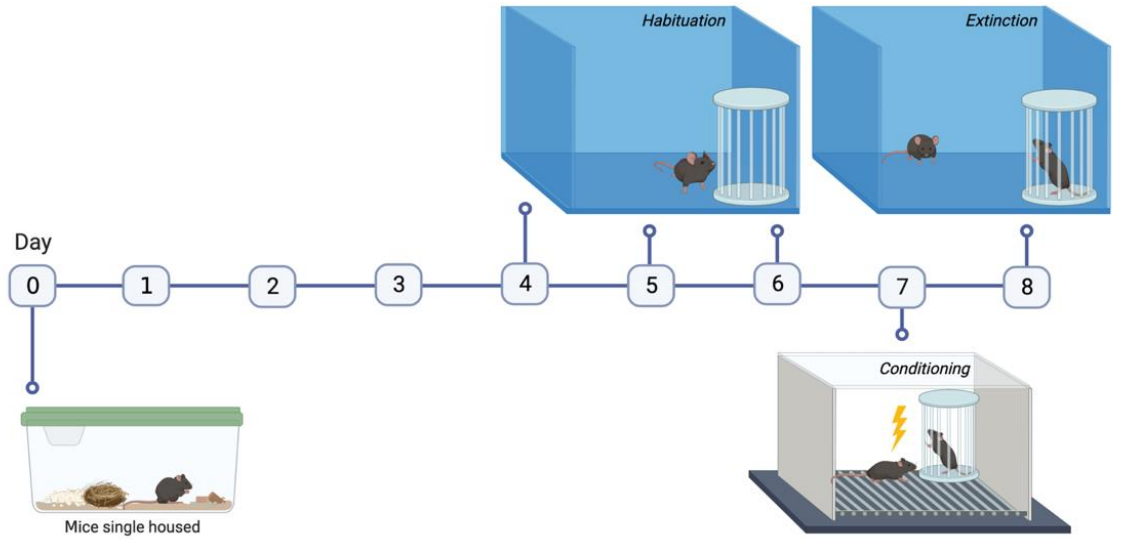



**Figure 2.2: Experimental timeline for wildtype and *Fmr1* KO mice undergoing social fear conditioning and extinction.**
Adolescent and adult wildtype and *Fmr1* KO mice were social isolated. On days 4, 5 and 6 mice were habituated to experimenter handling and the test arena for 10 minutes. On day 7, mice underwent social fear conditioning which involved the administration of a mild foot shock (~ 3 to 4) upon investigation of a caged, same-sex social stimulus; after 2 minutes without investigation of the social stimulus, mice were removed from the conditioning chamber and returned to their homecage. On day 8, mice underwent social fear extinction; this involved presenting mice with three empty stimulus cages followed by ten novel, same-sex social stimuli – each trial lasting 3 minutes with a 3-minute intertrial interval. The outcome measure was the percent of each trial spent investigating the stimulus.

*Social fear extinction*

Social fear extinction involves sequentially presenting conditioned mice with novel same-sex social stimuli to assess if they avoid investigation of caged social stimuli and if the avoidance persists over repeated exposure. To begin, mice were placed into the testing arena and presented an empty stimulus cage three times, for 3 minutes each with a 3-minute intertrial interval. Following the third non-social stimulus exposure (NS3), mice were presented ten novel, same-sex social stimuli for 3 minutes with a 3-minute intertrial interval. Within the same experimental room, a maximum of eight test mice were tested in individual test arenas and the ten novel social stimuli were rotated clockwise through the

test mice. At the end of extinction, the test arenas and stimulus cages were cleaned with 1:25 F10.

### *Statistical analysis*

*A priori* power analysis was conducted using G*Power version 3.1.9.2 (Faul et al., 2007) to determine the approximate sample size based on previous data (Raymond et al., 2019; Zoicas et al., 2014). Results indicated the required sample size to achieve 80% power for detecting a large effect, at a significance criterion of $\alpha = .05$ was N = 10 to 12 for mixed model ANOVA, considering the inclusion of *Fmr1* KO mice which have not previously been tested in the SFC paradigm. Final sample size per condition were as follows: n = 40 adolescent wildtype (n = 21 SFC-, 11 female and n = 19 SFC+, 10 female), n = 47 adolescent *Fmr1* KO (n = 23 SFC-, 11 female and n = 24 SFC+, 10 female), n = 57 adult wildtype (n = 31 SFC-, 10 female and n = 26 SFC+, 8 female) and n = 43 adult *Fmr1* KO mice (n = 23 SFC-, 10 female and n = 20 SFC+, 9 female). Thus, the obtained sample size of approximately n = 10 per group was adequate to test the study hypothesis. Variable group sizes can be attributed to i) viability of offspring from HET females x hemizygous *Fmr1* KO males, ii) female heterozygous mice made up most females in any given litter and iii) surplus adults due to delay in receiving genotype results.

Data analysis was performed in R Studio (V4.3.1) using packages *tidyr*, *dplyr*, *stringr*, *ggplot2* (Wickham et al., 2019) and *Rmisc* (Hope, 2022) for data visualisation. For statistical analysis *Tidyverse* (Wickham et al., 2019), *ggpubr* (Kassambara, 2023a), *rstatix* (Kassambara, 2023b), *afex* (Singmann et al., 2023) and *performance* (Lüdecke et al., 2021) were used for mixed ANOVA analyses using the Greenhouse-Geisser correction for lack of sphericity. Separate models were run for the 3 non-social stimulus exposures (NS1 to

NS3) and 10 social stimulus exposures (S1 to S10). The dependent variable was time spent investigating the stimulus cage (% of total trial duration) during each 3-minute stimulus cage exploration. Investigation of the empty stimulus cage or social stimulus was defined as when the nose of the test mouse entered the 20 mm space perimeter of the stimulus cage. Independent variables were SFC condition, genotype, age, sex, and stimulus exposure. Interactions involving SFC condition and stimulus exposures with $p < 0.1$ were followed up with polynomial trend analysis examining linear and quadratic trends. For all comparisons, significance was set at $p < 0.05$ and all data are presented as Mean ± SEM.

## 2. 3. Results

SFC+ mice engaged in less investigation of social stimuli compared to SFC- mice, averaged over stimulus exposures [*F(1, 171) = 228.41, p < 0.001, Figure 2.3*], indicating the fear conditioning was successful. There was an interaction between SFC condition and stimulus exposure [*F(5.75, 982.70) = 21.39, p < 0.001*]. SFC- mice showed a linear decrease in sociability over the ten stimulus exposures [*t(171) = -4.56, p <0.0001*], whereas SFC+ mice displayed an initial increase in social stimulus investigation between S1 and S5 (extinction), which then plateaued [linear trend: *t(171) = 6.32, p < 0.0001;* quadratic trend: *t(171) = -5.15, p < 0.0001*], and the trends differed significantly between SFC+ and SFC- mice [linear: *t(171) = 7.72, p < 0.0001*; quadratic: *t(171) = -3.18, p = 0.0017*].

The interaction between SFC condition, age and stimulus exposure approached significance [*F(5.75, 982.70) = 1.80, p = 0.099*]. Examination of the data presented in Figure 2.3 suggested a possible difference in extinction between adolescent and adult mice. This was confirmed by trend analysis, which revealed social fear extinguished in adolescent SFC+ mice, and this was most pronounced over S1 – S6 [linear trend: *t(81) = 5.83, p < 0.0001*; quadratic trend: *t(81) = -5.20, p < 0.0001, Figure 2.4*]. In contrast, there was no significant linear (*p = 0.263*) or quadratic (*p = 0.353*) trend in adult SFC+ mice, and the trends differed significantly between the adolescent and adult SFC+ mice [linear trend: *t(81)= -3.33, p = 0.0013*; quadratic trend: *t(81)= 3.01, p = 0.004*]. There was also a significant main effect of age [*F(1, 171) = 44.85, p < 0.001*], main effect of stimulus [*F(5.75, 982.70) = 4.05, p < 0.001*] and interaction between age and stimulus [*F(5.75, 982.70) = 7.68, p < 0.001*], but these are not interpreted due to the aforementioned three-way interaction between SFC condition, age and stimulus exposure.

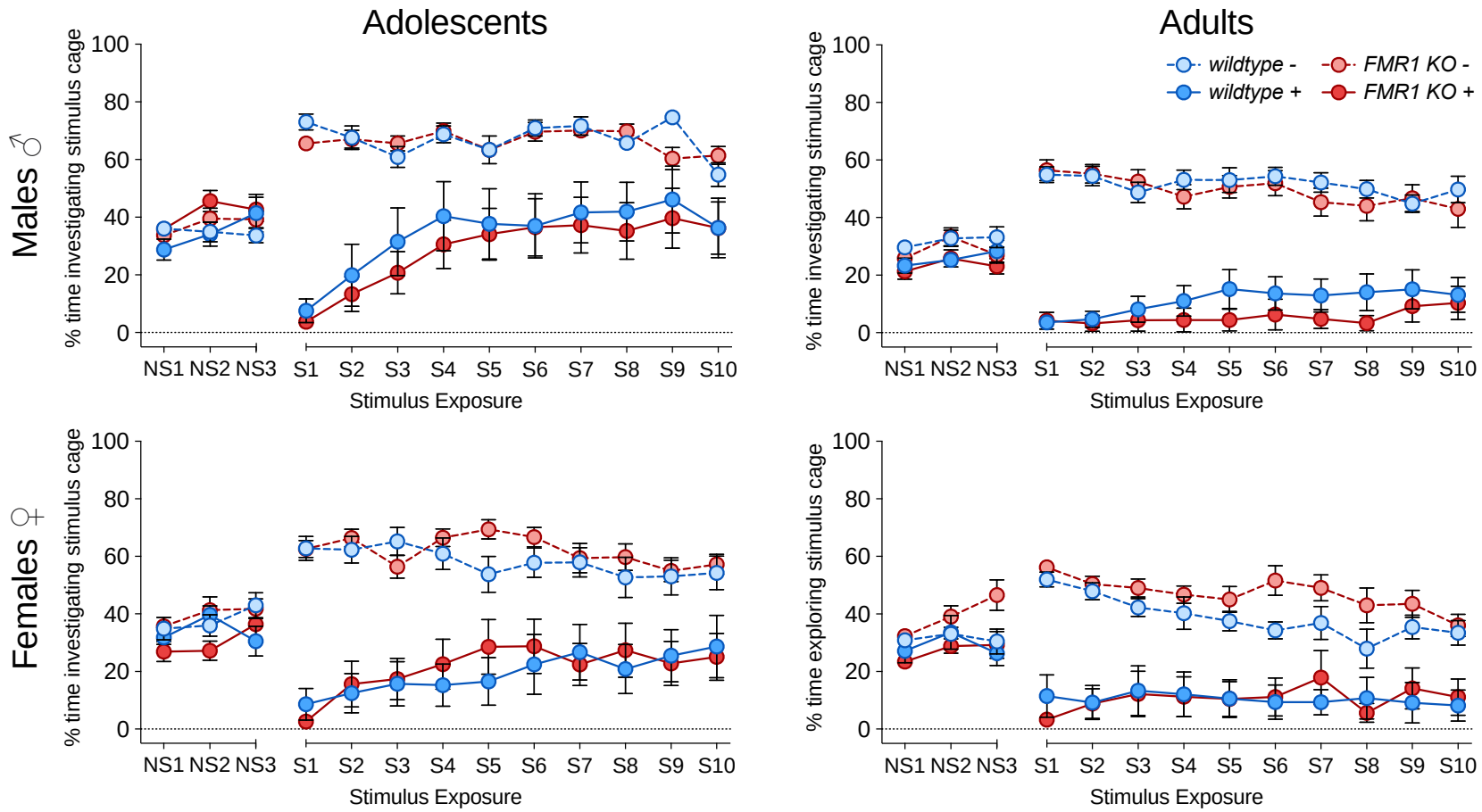



**Figure 2.3: Social investigation during fear extinction in adolescent and adult, wildtype and *Fmr1* KO male and female mice.**
Mice were presented three empty, non-social stimuli (NS1 to NS3) followed by ten novel, same-sex social stimuli (S1 to S10). SFC+ mice engaged in less investigation of social stimuli compared to SFC- mice indicating the fear conditioning was successful. Genotype and sex had no significant impact on social fear, with no significant two-way or three-way interactions including SFC condition (all $p \geq 0.157$). However, adolescent mice appeared to extinguish social fear more than adult mice and so further analysis was performed. Data presented are Mean ± SEM. n = 40 adolescent wildtype (n = 21 SFC-, 11 female and n = 19 SFC+, 10 female), n = 47 adolescent *Fmr1* KO (n = 23 SFC-, 11 female and n = 24 SFC+, 10 female), n = 57 adult wildtype (n = 31 SFC-, 10 female and n = 26 SFC+, 8 female) and n = 43 adult *Fmr1* KO mice (n = 23 SFC-, 10 female and n = 20 SFC+, 9 female).

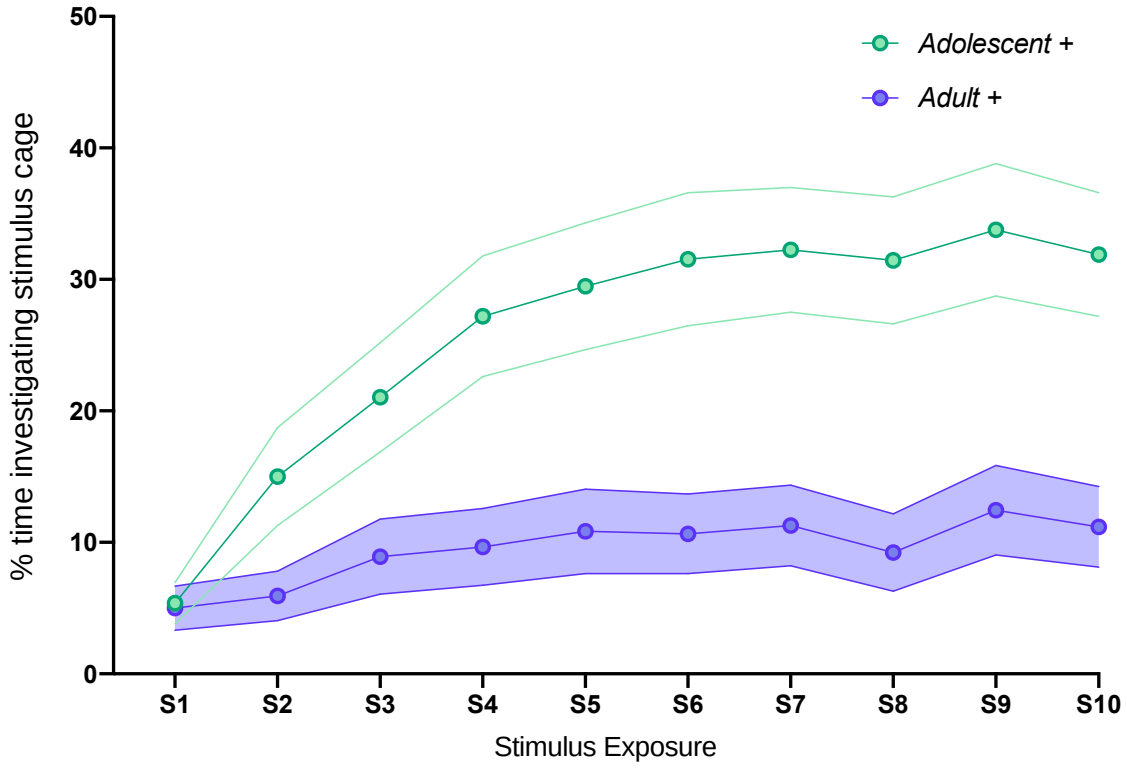



**Figure 2.4: SFC+ adolescent mice extinguish social fear across stimulus exposures compared to adult mice.**
Trend analysis revealed social fear extinction in adolescent SFC+ mice but not adult SFC+ mice. In adolescent mice only, there was a significant linear trend found across S1 to S6 and overall, there was a quadratic change in stimulus investigation. Data presented are Mean ± SEM values for conditioned adolescent (n = 43) and adult (n = 46) mice (pooled across sex and genotype).

Genotype and sex had no significant impact on social fear, with no significant two-way or three-way interactions including SFC condition (all $p \geq 0.157$, *Figure 2.3*). However, averaged across stimulus exposures, male mice spent more time investigating social stimuli compared to females [$F(1, 171) = 5.46, p = 0.021$], and an interaction was found between sex and stimulus exposure [$F(5.75, 982.70) = 2.78, p = 0.012$]. At S1, social stimulus investigation was similar between males and females, but male mice showed an increase then plateau in social stimulus investigation over the remaining social stimulus exposures [linear: $t(171) = 3.0, p = 0.0029$; quadratic: $t(171) = -4.4, p < 0.0001$], whereas females showed no change in social stimulus investigation (linear $p = 0.461$,

quadratic $p = 0.075$), and the linear trends differed significantly between sexes [$t(171) = -2.55, p = 0.0116$].

In terms of investigation of the empty stimulus cage (NS1 to NS3), SFC+ mice engaged in less investigation of the empty stimulus cage compared to SFC- mice, when averaged over stimulus exposures [$F(1, 171) = 10.32, p = 0.002$, *Figure 2.3*], indicating some residual fear of the stimulus cage. However, there was no interaction between SFC condition and stimulus exposure [$F(1.82, 311.39) = 0.24, p = 0.769$]. Unlike in S1 to S10, there was no interaction between age, SFC and stimulus exposure [$F(1.82, 311.39) = 0.66, p = 0.505$] nor was there an interaction between sex, SFC condition and stimulus exposure [$F(1.82, 311.39) = 2.83, p = 0.066$]. Follow-up trend analysis revealed male and female SFC- mice spent similar amount of time investigating the empty cage during NS1 and NS2 however during NS3, female mice continue to increase their time investigating the empty stimulus cage [linear: $t(90) = 3.55, p = 0.0006$] whereas there was no significant linear trend ($p = 0.275$) in male SFC- mice; the linear trends between males and females mice did not reach statistical significance [$t(90) = 1.91, p = 0.059$]. There was also a significant main effect of age [$F(1, 171) = 23.37, p < 0.001$] and stimulus exposure [$F(1.82, 311.39) = 14.92, p < 0.001$] but no interaction found between age and stimulus exposure [$F(1.82, 311.39) = 0.75, p = 0.462$], unlike when interacting with social stimuli, and so was not followed up.

## 2. 4. Discussion

Fragile-X is a monogenetic neurodevelopmental disorder associated with high rates of social anxiety. The aim of this study was to use the SFC task to model social anxiety-like behaviour in *Fmr1* KO mice as well as study important biological factors associated with social fear and avoidance, specifically age and sex. *Fmr1* KO mice did not express greater levels of social avoidance compared to WT mice following social fear conditioning. In contrast, age was identified as an important moderator of social avoidance behaviour, with adolescent mice showing more pronounced extinction of conditioned social fear than adult mice. There was no effect of sex on conditioned social avoidance.

There are several possible explanations, which range from subtleties in background strain, the utility of single gene knockouts in highly heterogenous disorders to the temperamental replicability in *Fmr1* KO mouse models, as to why we were unable to recapitulate social avoidance in a mouse model of FXS using the SFC paradigm. First, the majority of studies which examine social fear use CD1 mice (Menon et al., 2018; Zoicas et al., 2014), an outbred strain which is typically considered to be more aggressive (Hsieh et al., 2017) yet also more socially motivated compared to C57BL/6 mice (Ramsey et al., 2022). The background strain of the mice used in this study was C57BL/6. A study examining the impact of background strain, found *Fmr1* x C57BL/6 mice showed increased active social behaviour compared to wildtype littermates but that wildtype and *Fmr1* KO mice on a CD1 background were significantly more social than *Fmr1* x C57BL/6 mice (Spencer et al., 2011). In contrast, a recent study used both CD1 and C57BL6 mice and found limited strain difference across multiple behavioural tasks including the SFC paradigm (Zoicas et al., 2023). Further, a recent meta-analysis determining the reproducibility of *Fmr1* mice on a range of background strains found no significance differences across strains (Kat et al., 2022). Thus, these results indicate background strain

would likely have, at most, only a minimal impact on behavioural phenotype within the SFC task.

FXS and autism are both highly heterogeneous disorders (Masi et al., 2017; Verdura et al., 2021) influenced by gene-gene and gene-environment interactions (Bagni & Zukin, 2019; Tordjman et al., 2014). This suggests that single gene knockdown of *FMR1* may not be able to capture the complexity of the genetic manifestation of impaired social behaviour. Epidemiological data show that exposure to environmental risk factors, such as bacterial or viral infections during pregnancy and subsequent maternal immune activation, increases the likelihood of offspring developing ASD (Massrali et al., 2022). In *Fmr1* KO mice, gene-environmental interactions find that gestational exposure to environmental insults, such as lipopolysaccharide bacteria or *H1N1 virus*, alters microglia function and disrupts *fmrp* signalling, altering downstream translation targets (Fatemi et al., 2017; Parrott et al., 2021). Models which examine gene-gene interaction models in *Fmr1* KO mice (e.g. *Fmr1* x *Tsc2*$^{+/-}$(Auerbach et al., 2011) or *Fmr1* x *Cpeb*$^{-/-}$ (Udagawa et al., 2013) mice), found these particular double mutants demonstrated rescue of FXS-related behavioural phenotypes. In contrast, eIF4E transgenic mice, an ASD mouse model which exhibits excessive levels of translation, crossed with *Fmr1* mice exhibited pronounced social deficits and cognitive impairments which far exceed those displayed in single eIF4E or *Fmr1* mutants (Huynh et al., 2015). By increasing levels of eIF4E and deleting *fmrp*, which work in tandem to disrupt translation (Santini et al., 2017), the authors generated a model which more closely recapitulates autism-related behaviours. Indeed, a common theme in the impact of gene-gene and gene-environmental interactions in FXS- and ASD-related behavioural phenotypes is the loss of production of *fmrp*. *Fmrp* is important in translation, mRNA stability and transcription through chromatin modulation (Richter & Zhao, 2021). Several studies which have focused on examining the role of *fmrp* in the

brain have made encouraging progress towards understanding the impact loss of function in *FMR1* has on neuronal development and pathways associated with social behaviour (Hoeft et al., 2010; Lightbody & Reiss, 2009; Parrott et al., 2021). Together these studies indicate, the genetic and epigenetic diversity among those with FXS may limit the utility of highly inbred mouse models like *Fmr1* KO mice (Richter & Zhao, 2021). Single gene mutations may not be able to recapitulate the diverse ASD and FXS phenotypes in mice and better understanding of *fmrp* and associated epigenetic mechanisms may hold the key to developing better animal models of the disorder.

Within the literature, the *Fmr1* mouse behavioural phenotype is highly variable, with low replication of reported results across several behavioural domains (Kat et al., 2022); Richter and Zhao (2021). In a recent meta-analysis and systematic review, Kat et al. (2022) attributed discrepancies between findings in *Fmr1* KO mice to diverse methodological approaches, lack of transparency in reporting of methods and results, inappropriate statistical analysis, and insufficient sample sizes. The authors recommended large sample sizes of approximately 100 mice per genotype for studies examining social and anxiety-related behavioural tasks to reach statistical power (0.8). In the present study, when pooled across sex and age, this study included 97 wildtype and 90 *Fmr1* KO mice. Given the multiple group comparisons (age, sex, genotype and SFC condition) we were able to reach statistical power in our overall sample. We are thus in a position to confidently conclude that the lack of effect of genotype in our study is a robust finding – contrary to many previously published studies. Whilst some social and communication deficits seen in FXS have been reproduced in *Fmr1* KO mice (Cregenzan-Royo et al., 2022), these behaviours are not core to the FXS behavioural phenotype. The meta-analysis found that in attention, aggression and anxiety, symptoms more strongly associated with FXS, there was a phenotypic mismatch between *Fmr1* KO mice and the clinical population

(Kat et al., 2022). The poor translation of pre-clinical models of FXS is hindering the drug discovery process where drug candidates of interest which prove promising in rodents are not translating to viable treatment options in clinical trials (*see reviews* Berry-Kravis et al. (2018) and Gross et al. (2015)). Together with our results, these findings cast doubt on the face validity of the *Fmr1* mouse model, their utility in understanding drivers of social avoidance in mice and the translatability of pre-clinical *Fmr1* behavioural studies to the clinical population.

While we did not observe genetic differences, our study was successful in supporting the face and construct validity of the SFC task (Reus et al., 2014), and confirmed it provides a solid platform to probe the effects of age and sex on social fear and avoidance. A key finding of this study was that adolescent mice showed more rapid extinction of social fear than adult mice. During adolescence, there is a greater willingness to take risks if there is a potential reward (Pfeifer & Berkman, 2018; Schreuders et al., 2018) and, in evolutionary terms, this may afford survival advantages (Kopetz et al., 2019; Salas-Rodriguez et al., 2021). Importantly, increased risk-taking during adolescence is observed across human and non-human species, with, for example, adolescent but not juvenile or adult mice, engaging in more risk-taking and novelty-seeking behaviours (Macri et al., 2002; Rosati et al., 2023). Consistent with the present findings, following chronic social defeat, adolescent mice were found to be more resilient to social stress. A possible mechanism for this is hippocampal neurogenesis, which is the generation and integration of new neurons in the hippocampus. Prior to CSDS, adult mice subjected to one week transitory neural ablation (PND28 to PND42) did not have altered social behaviour compared to controls (Kirshenbaum et al., 2014). Following CSDS however, adolescence mice had a significant reduction in hippocampal neurogenesis which promoted resilience to social defeat but in mice subject to adult transitory ablation, it did not. This is significant

as it implies adolescence is a period of resilience to social stress which suggests the behavioural phenotype can be "rescued" prior to adulthood. Neurogenesis occurs across the lifespan but is 300% greater during adolescence compared to adulthood (He & Crews, 2007). It is therefore possible that increased risk appetite during adolescence in conjunction with increased hippocampal plasticity, is driving the more rapid extinction of social avoidance behaviours in the fear conditioned mice. The peak in social drive and capacity to overcome social stress during adolescence likely contributes to social anxiety and avoidance first being identified during this critical period.

Another possibility is that it is not age *per se,* but instead an interaction between age and social isolation that is driving the differences in social fear extinction observed between adults and adolescents. Bicks et al. (2020) found two weeks social isolation altered social behaviour in adolescent but not adult mice. However, most studies looking at the effect of social isolation include an isolation period much longer (6 to 12 weeks) than the one used in our study (7 days). The consensus among these studies is that social isolation impacts social behaviour in adolescents more than adult mice (Rivera-Irizarry et al., 2020). A possible mechanism for driving this interaction may be the role of inhibitory neurons, specifically those expressing parvalbumin (PV+), which play a critical role in excitatory and inhibitory balance in the central nervous system (CNS) (Ferguson & Gao, 2018). Environmental perturbations during this critical window can have differential effects on adolescent versus adult mice. Mice socially isolated during peri-adolescence (PND21 to PND56), have significantly less PV+ interneurons in the dentate gyrus (DG) and CA1 region of the hippocampus, brain region important in learning and memory, compared to controls (Ueno et al., 2017). Bicks et al. (2020) identified early adolescence (PND21 to PND25) as a unique developmental window in PV+ interneuron maturation, with perturbations during this period responsible for decreased excitability and subsequent

reduction in social motivation. Thus, future studies could vary the duration of social isolation to assess whether this impacts social fear extinction in adolescent mice.

Our study explored potential sex differences which might arise during social fear extinction. We found overall sex difference in social behaviour in terms of time spent investigating social stimuli, however we did not observe any sex difference in social fear and avoidance behaviours. The inclusion of sex as a biological variable is important in better understanding where gender differences arise in the clinical population. Thus far, systematic sex bias in pre-clinical research has resulted in a knowledge gap and has been identified as a contributing factor in the poor translatability of pre-clinical findings into successful pharmacotherapies (Karp & Reavey, 2019; Kokras et al., 2019). If animal models serve to recapitulate characteristics of human mental disorders to inform pharmacotherapies, it is of the utmost importance to include females and examine sex as a biological variable. Thus, whilst we did not observe sex difference in fear and avoidance behaviours our findings contribute towards greater understanding of sex differences, or lack thereof, in social behaviours.

A limitation of the present study is that we did not examine non-social fear and thus cannot comment on whether the findings generalise to conditioned avoidance outside of a social context. In tasks designed to measure hippocampal and amygdala-based fear learning, Nolan et al. (2017) found that *Fmr1* KO mice have impairments in trace and delayed fear conditioning. These conditioning tasks employ the use of audiogenic stimuli and measure freezing response. In contrast, the SFC paradigm uses instrumental fear conditioning, so comparison between the two must be made with considerable caution. Our lab has previously designed and used a modified version of the SFC, pairing mild foot shocks with interaction with a scented tennis ball (Raymond et al., 2019); this paradigm would be useful in future studies comparing social and non-social fear.

There are two clear strengths of the current study: the inclusion of important biological factors leading to the finding that age has a greater impact on behavioural phenotype than genetic background, and our finding that *Fmr1* KO mice do not recapitulate the social anxiety phenotype observed in the clinical population. The design of our study was comprehensive and rigorous. We determined *a priori* the necessary effect size; we examined age and sex in the hopes that had there been a main effect of genotype we would be sufficiently powered to detect and interpret possible interactions. With the finding in a recent meta-analysis that *Fmr1* KO mice have reduced anxiety-like behaviours (Kat et al., 2022), a direct contrast to the clinical population who experience higher levels of anxiety (Cordeiro et al., 2011), the use of the *Fmr1* KO mouse as a behavioural model for FXS is called into question. We hope that our thorough use of a task designed to examine social-specific fear and avoidance, holds conclusive evidence of the poor translatability of the *Fmr1* mouse model in recapitulating social anxiety-like behaviour as seen in SAD. Thus, future studies which use *Fmr1* KO mice should focus on better understanding the role of *fmrp* in brain development and maturation (Berry-Kravis et al., 2018; Gross et al., 2015) if we are to make strides in developing successful pharmacotherapies for symptoms of FXS (Richter & Zhao, 2021). Finally, given the heterogeneity in the general population and in those with FXS and ASD, studies like our own further support the growing consensus that the age of intervention is crucial in social disorders (Larsen & Luna, 2018; Moisan, 2022).

Consistent with previous studies (Menon et al., 2018; Toth et al., 2012), our study effectively used the SFC paradigm to accurately demonstrate the acquisition of social fear; all SFC+ groups spent significantly less time exploring the social stimulus cage during S1 compared to empty cage stimulus exposures (NS1-NS3) and SFC- mice. Thus, our study has provided further evidence of the efficacy of the SFC paradigm in measuring the

acquisition and extinction of social fear. As investigation of genetic correlates for social anxiety-like behaviour has been unsuccessful, priority should be place on employing the robust and translatable SFC paradigm (Reus et al., 2014) to reveal the underlying neural mechanisms driving social fear behaviours. The SFC task, along with studies on social avoidance, have identified the lateral septum (LS) (Menon et al., 2018; Zoicas et al., 2014) and PFC (Xu et al., 2019) as key brain regions of interest in the acquisition and maintenance of social fear.

# Chapter 3. Exploring the association between lateral septum signalling and specific social fear and avoidance behaviours

## Overview

The lateral septum (LS) modulates social avoidance in mice, yet the specific behaviours affected by the LS in this context are not well characterised. Our aim was to examine the relationship between LS dynamics and specific social approach and avoidance behaviours with high temporal precision using fibre photometry and social fear conditioning (SFC). C57BL/6 mice underwent surgery to infuse a fluorescent calcium sensor (jGCaMP8f) and implant a fibre optic cannula within the dLS. Three weeks post-surgery, mice were socially isolated and remained so throughout the study. For Experiment 1, we examined social behaviour under non-fearful and fearful conditions. First, we examined dLS activity during investigation of novel non-social, and same- and opposite-sex social stimuli under non-fearful conditions (Experiment 1a). Using the same cohort of mice, we then ran a preliminary SFC study (Experiment 1b); to capture changes in dLS activity as a result of social fear conditioning, we adapted the SFC task to create a within-subject baseline of dLS activity during extinction in unconditioned animals. This meant mice underwent an initial session, whereby they were placed in the conditioning chamber with a caged same-sex conspecific, but not administered foot shocks; this was analogous to the procedure used for the SFC- mice in Chapter 2, but allowed the mice to act as their own within-subject control. To record basal dLS activity, unconditioned mice were then presented a series of six novel social stimuli for 3-min each with a 3-min intertrial interval (ITI). One week later, mice underwent social fear conditioning (SFC+), and were again presented with a series of six social stimuli during fear extinction. Using fibre photometry and frame-locked video recordings, we found dLS activity was increased in SFC+ mice during social stimulus investigation and preceding proximal fleeing from the stimulus relative to SFC-, and this response reduced alongside the extinction of social fear. Further, under non-fearful conditions, the results from Experiment 1a demonstrated both male and female mice spent

more time investigating social compared to non-social stimuli. However, photometry revealed dLS activity was similar across social and non-social stimulus investigation. Thus, the aim of Experiment 2 was to examine if peaks in dLS activity were social specific. In a naïve cohort of mice, we infused jGCaMP8f and implanted an optic cannula within the dLS. Mice were assigned to social or non-social fear conditioning and extinction. For non-social fear conditioning (nSFC), we modified the SFC task used in Experiment 1b to instead pair investigation of novel non-social stimuli with mild foot shocks (nSFC+). We observed peaks in dLS activity preceding proximal stimulus flee in SFC+ *and* nSFC+ mice. However, contrary to SFC+ mice, we did not observe diminution of dLS activity in nSFC+ mice nor did they extinguish fear of the novel stimuli. Thus, using fibre photometry we have uncovered a novel dLS signal associated with specific stimulus avoidance behaviours following instrumental fear conditioning.

## 3. 1. Introduction

In a special issue on the *"Neurobiology of Human Fear and Anxiety",* international experts across disciplines reached a consensus that there is likely no singular fear circuitry. Instead, they agreed multiple brain circuits work in tandem to actively suppress or initiate neural processes based on moment-to-moment assessment of environmental risks (Grogans et al., 2023). This is to say that whilst much of the human literature focuses on the amygdala and prefrontal cortex as important hubs in processing fearful situations (*see reviews* Aupperle and Paulus (2010); Gangopadhyay et al. (2021); Ren and Tao (2020)), they cannot work in isolation - fear and anxiety pathways encompass the entire brain. The discussants concluded that common and dissociable circuits are broadly engaged by a range of threats, confirmed within humans, non-human primates, and other mammals, in the co-ordination of adaptive and appropriate fear behaviours (Grogans et al., 2023). Thus, examination of brain regions within identified fear and anxiety-related pathways will allow us to better understand the genesis of excessive fear and how imbalance within one or more regions can lead to the manifestation of anxiety disorders (AD) (Grogans et al., 2023).

In the study of social-specific fear and avoidance, the use of the translationally relevant social fear conditioning (SFC) task has highlighted a role of the lateral septum in social anxiety-like behaviour. First established to better model key aspects of social anxiety and improve the face validity of animal models of SAD (Toth et al., 2012); the SFC task was later used to establish the role of oxytocin (OXT) in social fear and extinction (Zoicas et al., 2014). When administered via intracerebroventricular (i.c.v) injection immediately before social fear extinction, OXT increased investigation of novel social stimuli compared to vehicle. Further, pharmacological blockade of OXT receptors (OXTR) followed by administration of OXT demonstrated that OXT abolished social fear

via OXTR (Zoicas et al., 2014). Using autoradiography, the authors examined OXT receptor binding and found social fear conditioned (SFC+) mice had significantly greater OXT receptor binding, compared to unconditioned (SFC-) mice in the dorsal LS (dLS), right dentate gyrus (DG), right *cornu ammonis* (CA) 1, medial preoptic area (MPOA) and right central amygdala (CeA). However, after extinction OXT receptor binding no longer differed between SFC+ and SFC- mice. Using microdialysis, Zoicas et al. (2014) demonstrated SFC *limited* OXT release in the dLS and subsequent OXT administration directly into the dLS abolished social fear during extinction. In a separate investigation by the same research team, they explored social fear in lactating female mice as they have naturally occurring high OXT levels and do not express social fear (Menon et al., 2018). Using c-Fos immunohistochemistry, they found SFC+ lactating mice had lower c-Fos expression in the LS compared to SFC+ virgin mice who displayed typically elevated c-Fos expression. This suggested lactating females showed suppressed neuronal activation of the LS due to elevated OXT expression (Menon et al., 2018). Strengthening these findings, pharmacologically blocking OXT signalling in SFC+ lactating females increased social fear expression where enhancement of OXT signalling in virgin females decreased social avoidance. These results are consistent with those previously reported in male mice (Zoicas et al., 2014), providing strong support for a role of OXT signalling and moreover the LS in social fear extinction.

The evolution of novel tools and technologies, with ever- improving temporal resolution and spatial accuracy, have significantly shaped our characterisation of the role of the LS, and associated pathways, in various social behavioural processes. Prior to the identification of the LS as important in social fear and avoidance in mice, the septum was long considered as a hub for aggression. In pioneering lesioning studies of the lateral and medial septum (MS), rats exhibited marked fear together with defensive and aggressive

behaviours leading to the coining of the phrase "septal rage" (Albert & Chew, 1980; Albert & Wong, 1978; Brady & Nauta, 1953). In rats, other rodent species and birds, subsequent pharmacological inhibition and chemical lesioning studies corroborated this behavioural phenotype solidifying a role of the LS in aggressive behaviours (Lee & Gammie, 2009; McDonald et al., 2012; Potegal et al., 1981; Ramirez et al., 1988; Slotnick et al., 1973). Now with the use of more advanced techniques, evidence suggests the LS is anxiolytic i.e. dampens fear and anxiety, which explains why lesioning of this brain region disinhibits aggression. Where previous studies had focused on inhibition, Wong et al. (2016) sought to examine the effect of optogenetic activation, i.e. light stimulation of distinct neuronal populations via an implanted optic probe to promote activity, of the LS on social aggression. The authors demonstrated that acute activation of the LS immediately after the onset of an attack, was highly effective in terminating continued attack behaviour (Wong et al., 2016). Histological analysis revealed the LS was functionally connected to the ventrolateral region of the ventromedial hypothalamus (VMH), a downstream brain region previously identified as important in social aggression (Lin et al., 2011). To investigate the role of this pathway in motivated, aggressive behaviours, Wong et al. (2016) subsequently activated the LS-VMH pathway and demonstrated suppressed aggression. Building on this body of work, Leroy et al. (2018) sought to understand the upstream, cognitive processes involved in the decision to engage in aggressive behaviour. Given the LS is densely innervated by all CA regions of the hippocampus (Sheehan et al., 2004), Leroy et al. (2018) explored the role of CA1 and CA2 hippocampal projections to the dLS using fibre photometry. A calcium ($Ca^{2+}$) imaging technique, fibre photometry takes advantage of rapid changes in intracellular calcium dynamics as a proxy of neural activity (Akerboom et al., 2012). Together with the resident intruder task, Leroy et al. (2018) used photometry to observed peaks in brain activity in pyramidal CA2 neurons or CA2 inputs in the LS paired

with specific behaviours (e.g. in social exploration, dominance, and aggression). Further, the authors showed recording activity in the CA2 was associated with a broad range of social behaviours, however recording specifically from CA2 projections in the dLS was found to be highly selective for specific aggressive behaviours, such as biting. Finally, the authors demonstrated that optogenetic activation of the dorsal CA2, excited the dLS and promoted aggression via downstream disinhibition of the dLS to ventral LS (vLS) leading to activation of the VMH thus revealing a novel circuit in social aggression. Together the findings from Wong et al. (2016) and Leroy et al. (2018) highlight the utility of employing novel techniques to advance and extend our current understanding of facets of social behaviours within the wider fear network.

The use of the SFC task has provided strong evidence for the involvement of the LS in co-ordinating social fear acquisition and subsequent extinction. However, alongside the development of the theory of the LS-OXT pathway in social fear, the LS is more broadly implicated in the co-ordination of fear and threat behaviours via alternative neuronal pathways. For example, using a hippocampal-dependent contextual fear learning task, which pairs auditory cues with aversive foot shocks, Besnard et al. (2019) found activity in CA3 hippocampal neurons was highly correlated with activity in the dLS, where increased dLS activity preceded reductions in the freezing response. Further, the authors identified LS somatostatin-expressing GABAergic neurons receive monosynaptic input from CA3 neurons suggestive of a role for this circuit in contextual fear learning (Besnard et al., 2019) and discrimination (Besnard et al., 2020). Further, use of contextual fear learning together with the utilisation of a vast array of assays which examined (social) stress, defeat and predatory fear behaviours, has uncovered a role for LS expressing neurotensin ($LS^{NT}$) (Azevedo et al., 2020) and type 2 corticotrophin-releasing hormone ($LS^{Crhr2}$) (Hashimoto et al., 2023) receptors as specifically activated in the LS during fear

acquisition and extinction. Together, the evidence in the non-social fear conditioning literature suggests that multiple neuronal pathways are recruited in the fear response and, moreover, that the LS is involved in both social and non-social fear acquisition and extinction behaviours.

Whilst immunohistochemistry, microdialysis and electrophysiology have been pivotal in determining a role of the LS in social fear, little is known about the discrete behaviours during social fear extinction which the LS governs, and determination of whether the LS is also involved in non-social stimulus avoidance following instrumental fear conditioning. The use of fibre photometry has so far proven invaluable in understanding the role and distinct pathways in aggression (Leroy et al., 2018) and threat learning (Azevedo et al., 2020; Hashimoto et al., 2023). Using fibre photometry, we will be able to capture millisecond to millisecond fluctuations in neuronal activity within the dLS to be precisely paired with specific approach and avoidance behaviours (e.g. approach, interaction, and escape), enabling disentanglement of broad behavioural phenotypes into defined, distinct actions. Thus, this experimental chapter aims to use fibre photometry time-locked to video recordings to characterise behaviour-specific calcium signalling in the dLS under non-fearful and fearful conditions, using both social and non-social stimuli. Based on the literature, we hypothesised that during social fear extinction dLS activity will be suppressed during stimulus approach, increase as avoidance extinguishes, and not be social specific.

## 3. 2. Methods

### *3. 2. 1. Subjects*

Six- to seven-week-old *C57BL/6J* mice (n = 50 test mice, 24 female, n = 48 stimulus mice, 24 female) were obtained from Australian BioResources (ABR, NSW, Australia). Mice were group housed (3 to 4 per cage) under standard laboratory conditions (12/12h light/dark cycle, light phase 0700 to 1900, 22 ± 2ºC, 50-70% humidity, food, and water *ad libitum*) in transparent IVC cages and weighed twice per week. Subjects were distributed into two experiments examining the association between dLS dynamics and specific stimulus approach, interaction, and avoidance behaviours: Experiment 1a (n = 18 test mice, 9 female; n = 8 stimulus mice, 4 female), which examined dLS activity in mice presented with social and non-social stimuli in non-fearful contexts; Experiment 1b, which used the same test mice from Experiment 1a to conduct a pilot examining dLS activity during extinction following SFC (n = 20 stimulus mice, 10 female); and Experiment 2 (n = 32 mice, 15 female; n = 20 stimulus mice, 10 female), which examined dLS activity during extinction following SFC versus non-social fear conditioning (nSFC). Males were always tested before females and all experiments were performed in the light phase by a female experimenter. Upon completion of behavioural testing, mice were perfused, and brains collected for histology to confirm anatomical placement. All procedures were performed with approval from The University of Sydney Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia.

### *3. 2. 2. Materials*

Fear conditioning took place in Med Associates conditioning chambers (East Fairfield, VT, USA) housed within sound- and light-attenuating boxes which were cleaned

with 70% ethanol between subjects. The social investigation test, social and non-social fear extinction all took place in blue, opaque Perspex test arenas (40 x 40 x 40cm, Plastix, Arncliffe, NSW, Australia) which were cleaned between subjects with diluted 1:25 F10 *(see Figure 2. 1 for experimental setup).* All behavioural tasks were captured with a webcam (Logitech C920e HD Pro Webcam, Logitech, The Netherlands) mounted 60 cm above the test arena floor which recorded at 30Hz and outputted a MP4 and timestamp (CSV) file for video playback and time-matching to the photometry signal, respectively.

For all behavioural tests, a stimulus cage was placed in the top right-hand corner of the test arena for each stimulus exposure where it was either empty or contained a social (same- or opposite sex conspecific) or non-social (scented tennis ball) stimulus. In Experiment 1a, age-matched male and female C57BL/6 mice were used as social stimuli in the social interaction test. Age- *and* sex-matched conspecifics were used in for both conditioning and extinction for Experiment 1b and 2. For Experiment 2 only, the SFC paradigm was adapted to allow examination of non-social stimuli, whereby a separate cohort of subject mice were presented novel objects to examine nSFC and extinction. For this, we used miniature, yellow tennis balls (~45mm in diameter, Kong, Colorado, USA) each individually rubbed in food essences (Coconut, Lemon, Salted Caramel, Strawberries and Cream, Rosewater, Vanilla, or Baked Cheesecake; Queen Fine Foods, Alderley, QLD, Australia). Across all experiments, stimuli were located within stimulus cages; Experiment 1 utilised a 5H x 5W x 5L cm wire-frame cage (built in-house) and in Experiment 2 this was changed to 14H x 7W x 7L cm clear Perspex cage with 1cm round holes on all sides on lower half of cage (Plastix, Arncliffe, NSW, Australia). The adjustment in cage design was made for two reasons; the patch cord would occasionally catch on the wire frame cage and subjects would mount, bite or pull at the wire frame cage interrupting behavioural scoring. The adapted cage design, based on Figure 5B in Ueno et al. (2019), allowed for

olfactory and visual investigation yet removed the aforementioned issues through smooth edges and a taller design. Social stimuli were acclimatised to the cage for two 25-minute sessions prior to the commencement of testing and used up to five rounds of testing subject to age in line with the Three R's of Animal Research.

Fibre photometry was used to record bulk calcium activity in dLS neurons during arena habituations, social interaction, and fear extinction tests. To perform longitudinal fibre photometry recordings, we simultaneously recorded jGCaMP8f and control fluorescence in the dLS using a commercial fibre photometry system (FP3002, Neurophotometrics Ltd., San Diego, CA). Two LEDs (470nm: $Ca^{2+}$-dependent GCaMP fluorescence; 415nm: autofluorescence, isosbestic control, $Ca^{2+}$-independent GCaMP fluorescence) were reflected off dichroic mirrors and coupled via a suspended 3m patch cord (Neurophotometrics) and white ceramic sleeve to the implanted optical ferrule. The excitation light was decreased to 4% and interleaved between 415nm and 470nm excitations channels with 2.5ms period at 40Hz. Photometry data was acquired using a custom Bonsai (Lopes et al., 2015) workflow with integrated timestamp data for the initiation of baseline (B), entry of non-social (N), tennis ball (T) or social (S) stimulus or completion of stimulus exposure (E) using the "KeyDown" function. Data was saved using Bonsai and exported to jupyter notebook (Kluyver et al., 2016) for analysis using python.

### *3. 2. 3. Procedures*

*Surgery*

Mice were anesthetised with isoflurane (3% induction, 0.7-1.5% maintenance) and non-steroidal anti-inflammatory and antibiotic agents were administered subcutaneously based on body weight. Mice were unilaterally infused with adeno-associated virus (AAV)

expressing the genetically encoded calcium indicator jGCaMP8f under the control of the ultrafast synapsin (Syn) promoter (pGP-AAV-syn-jGCaMP8f-WPRE, Addgene, Watertown, MA, USA) to drive expression preferentially in projection neurons. Virus (300-350 nl) was infused at a rate of 50 nl/min into the dLS (AP: -0.35; ML +0.5; DV -2.7 mm from bregma) using a glass pipette. The pipette was left in place for ~ 6 to 7 minutes and drawn up at a rate of 0.5 mm/minute to ensure sufficient diffusion and minimize off-target spread. The optical fibre (200 µm diameter, 4.5 mm in length with 0.37 numerical aperture (NA), Neurophotometrics, San Diego, CA) was implanted (AP: -0.35; ML +0.5; DV -2.65 mm from bregma) and held in place with the insertion of stainless steel screws (5/64 slotted 82° flathead, Antrin Miniature Specialties, Inc., Fallbrook, CA, USA) into the skull and dental cement (Vertex, The Netherlands) covering up to halfway of the black ceramic ferrule. Saline was administered post-operatively to mitigate dehydration and mice were maintained in group housing for recovery and full viral expression (~3 to 4 weeks). One week prior to behavioural testing, mice were individually housed and remained isolated throughout.

*Habituation*

Mice were acclimated to the behaviour room for at least 30 minutes before testing. A rubber reinforced glove was used to restrain mice during the attachment of the patch cord. Mice were habituated to the restraint hold repeatedly for up to 30s to acclimatise the animal to the handling, allow time to clean the optic ferrule (70% ethanol) and attach the subject to the patch cord cable. To ensure proper acclimatisation to the restraint, the test arena and the head mounted patch cord cable mice were habituated over three 10-minute sessions to the test arena prior to the commencement of social tasks (*Day 25 to 27, Figure 3.1*).

*Experiment 1a: Stimulus Interaction Test*

Mice were restrained, attached to the patch cord, and placed in the test arena which contained an empty stimulus cage. Using a custom Bonsai workflow, video capture and fibre photometry recordings were initiated simultaneously. To establish a baseline signal, mice were habituated to the arena for 10 minutes. Next, a series of novel stimuli (non-social, same- or opposite-sex) were presented for three minutes followed by a three-minute inter-exposure interval (*Day 28, Figure 3.1*). The order of presentation of stimuli was randomised between each subject.

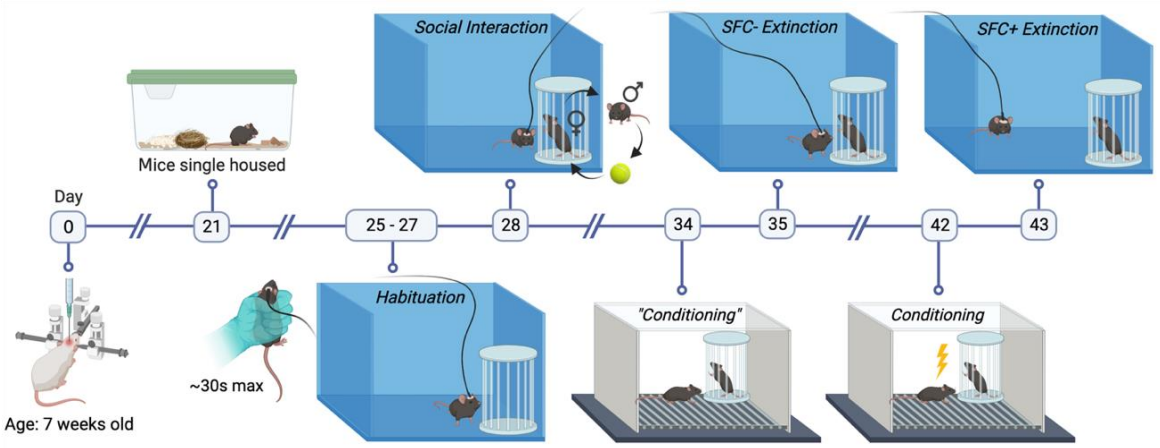



**Figure 3.1: Experiment 1 timeline.**
Male and female mice underwent intracranial surgery for the infusion of jGCaMP8f into the dLS and the implantation of an optic probe. After 21 days, mice were socially isolated and remained so throughout the experiment. Mice were habituated to handling and the test arena (10 mins/day over three days). On Day 28, mice were placed into the test arena for the stimulus interaction test (Experiment 1a). This involved presenting mice with either a novel non-social or same or opposite sex social stimulus for 3 minutes with a 3-minute intertrial interval whilst recording dLS activity. To examine the change in dLS signal before and after social fear conditioning, we used a within-subject control design (Experiment 1b). On Day 34, mice underwent "conditioning" (SFC-) whereby they were placed in the conditioning chamber with an empty stimulus cage for three minutes followed by a novel social stimulus but did not receive foot shock. On Day 35, mice underwent standard "extinction" establishing baseline dLS activity under non-fearful conditions. On Day 42, mice underwent SFC+ and received foot shock upon investigation of novel social stimulus (~ 3 to 5 shocks which ceased after 2 minutes without re-engaging the stimulus). On Day 43, mice were attached to the patch cord and presented six novel social stimuli to examine social fear extinction.

*Fear Conditioning paradigm*

The standard SFC paradigm, involves splitting cohorts of mice into unconditioned (SFC-) and conditioned groups (SFC+). For photometry, it was important to establish the relationship between photometry signal and behaviour in each, individual mouse to understand LS activity in non-fearful contexts and then observe changes in dLS dynamics following fear conditioning. Thus, subjects were allocated to social or non-social stimuli exposure conditions which remained constant throughout the experiment.

To create within-subject controls of behaviour within the SFC and nSFC tasks, subject mice first underwent "conditioning" (henceforth referred to as SFC-) whereby mice were placed in the conditioning chamber for ~ 8 minutes (30 seconds acclimatising to chamber, 3 minutes with empty stimulus cage and 5 minutes with a caged same-sex (Experiment 1b and 2) or scented tennis ball stimulus (*Experiment 2 only, Figure 3.2*). The following day, mice underwent SFC- extinction. Similar to standard SFC, SFC- extinction involves the presentation of three empty stimulus cages for 3 minutes with a 3-minute intertrial interval and then the presentation of 6 novel same-sex or differently scented tennis ball stimuli for 3 minutes each. One-week later mice underwent standard fear conditioning, as in Chapter 2, where mice were acclimatised to the empty chamber (30 seconds), then the empty stimulus cage (3 minutes) and presented with a not previously encountered social or non-social stimulus. Upon stimulus investigation, mice were manually administered a mild foot sock (~3 to 4) until investigation was ceased/not initiated for a minimum of 2 minutes. Mice were removed from the conditioning chamber and returned to their homecage where an empty stimulus cage was placed (without the lid) to allow mice to habituate to the stimulus cage to ensure stimulus specific avoidance. As in SFC- extinction, one day following fear conditioning (SFC+ and nSFC+) mice underwent fear extinction (*Experiment 1b: Day 43, Figure 3.1, and Experiment 2: Day 36, Figure*

*3.2*). Each stimulus exposure session lasted three minutes with a three-minute inter-exposure interval. The order of stimulus exposures was three successive empty stimulus cages, followed by six social or non-social stimuli exposures each containing a stimulus mouse (sS1+ to sS6+) or scented tennis ball (nS1+ to nS6+) respectively. dLS activity was recorded using calcium imaging during SFC- and SFC+ extinction and the baseline activity was defined by a two-minute window within the inter-exposure interval between the third empty stimulus cage and the first stimulus exposure (S1+).

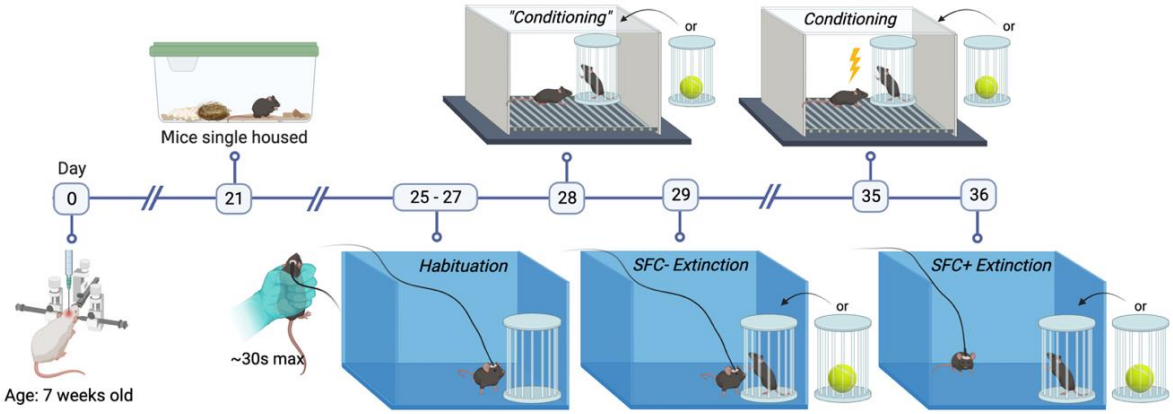



**Figure 3.2: Experiment 2 timeline – Social and non-social fear conditioning.** Mice underwent intracranial surgery for the infusion of jGCaMP8f into the dLS and the implantation of an optic probe. After 21 days, mice were socially isolated and remained so throughout the experiment. Mice were habituated to handling and the test arena. On Day 28, mice underwent SFC- to either a novel social or non-social stimulus followed by extinction the next day, which served as a baseline for normative social behaviour. One week later, mice were conditioned whereby investigation of a novel non-social or same-sex social stimulus was paired with a mild foot shock. On Day 36, dLS activity was recorded during social or non-social fear extinction.

*Histology*

To confirm viral and probe placement in the dLS, mice were deeply anesthetized first with 3% isoflurane induction and then administered 30% pentobarbital. Mice were euthanised by transcardial perfusion of phosphate buffered solution (PBS) followed by 10% neutral buffered formalin. Brains were extracted, post-fixed with formalin at 4$^{o}$C for

24 to 48 hours, washed with PBS and embedded in PBS with 3% agarose. Brains were sectioned using a vibratome (Leica VT1000S) into 50-60 μm coronal slices and stored in PBS. Samples were mounted onto glass slides (Corning, NY, USA) and coverslipped using Fluoroshield™ with DAPI (Sigma-Aldrich, North Ryde, NSW Australia). Sections were imaged on a VS-120 Virtual Slide Microscope (Olympus, Notting Hill. VIC, Australia). Representative image of fluorescents expression from Experiment 2 can be found in *Figure 3.8A.*

### *3. 2. 4. Data Analysis*

*Behavioural analysis*

To ensure precise, time-locked assessment of individual bouts of behaviour, the custom-designed event-tracking software MorLog was used to characterise four distinct behaviours for Experiment 1: proximal (stimulus) approach, proximal (stimulus) flee, stimulus investigation and stimulus disengage and re-engagement (*see Figure 3.3A, C, E and F respectively*). When scoring the social interaction and SFC- and SFC+ tasks, proximal approach was defined as orienting toward the stimulus followed by immediate locomotor activity towards the stimulus which ended in Q4 (*Figure 3.3A*). Proximal flee was defined as the rapid escape from the quadrants containing the stimulus (Q4) in which the subject did not re-engage in any kind of stimulus investigation for more than 5 seconds post initiation of the flee behaviour (*Figure 3.3C).* Stimulus investigation was scored as any instance in which the subject's nose was within the stimulus cage area (SC, *Figure 3.3E*), as scoring of social interaction cannot strictly be assumed when interacting with a caged stimulus where behaviour is often times not mutually directed. Finally, stimulus disengage/reengage was defined as a brief pulling away and immediate re-engagement of

investigation with the stimulus (*Q4 in Figure 3.3F*) within a 3 second period. To examine the duration and frequency to investigate the stimulus cage, we used TopScan (Version 3.0, CleverSys, Reston, VA, USA) software to automatically detect and quantify behavioural activity. Data from MorLog was processed in conjunction with the fibre photometry output and data from TopScan (Cleversys) was processed with Microsoft Excel (Microsoft, Redmond, WA, USA).

In Experiment 2, to improve scoring of stimulus directed behaviour, an additional four behaviours were characterised on top of those used previously in Experiment 1: distal approach, distal flee, non-stimulus behaviours and immobile in the corner. Distal approach was defined as an approach which started and ended in Q1-Q3 (*Figure 3.3B*). Counter to approach behaviours which were defined by the quadrant in which they *terminated*, flee behaviour are defined by the quadrant they *commenced*. Thus, while proximal flee was defined by a stimulus flee which commenced in Q4 (*Figure 3.3C*), distal flee was defined by an escape which was initiated within Q1-Q3 (*Figure 3.3D*). To label the behaviour of the subject at any given moment, Experiment 2 also examine two non-stimulus behaviours: non-stimulus behaviour which included arena exploration, grooming and rearing (*Figure 3.3G*) and corner immobile when a subject was immobile and vigilant towards the SC in the furthermost corner from the SC (*Figure 3.3H*). Under this scoring system all behaviours were mutually exclusive and simultaneously recorded, meaning the total duration of behaviours during each three-minute stimulus exposure could be calculated as a percentage of time spent engaged in a behaviour.

In Experiment 2, in addition to exploring dLS activity during social and non-social fear extinction we observed that within the social fear conditioned group there was a subset of mice which spent less than 1% time investigating the social stimulus at each stimulus exposure across extinction. Thus, these mice were referred to as *non-extinguishers* and

mice who showed an increase in social investigation across stimulus exposures during extinction were referred to as *extinguishers* .

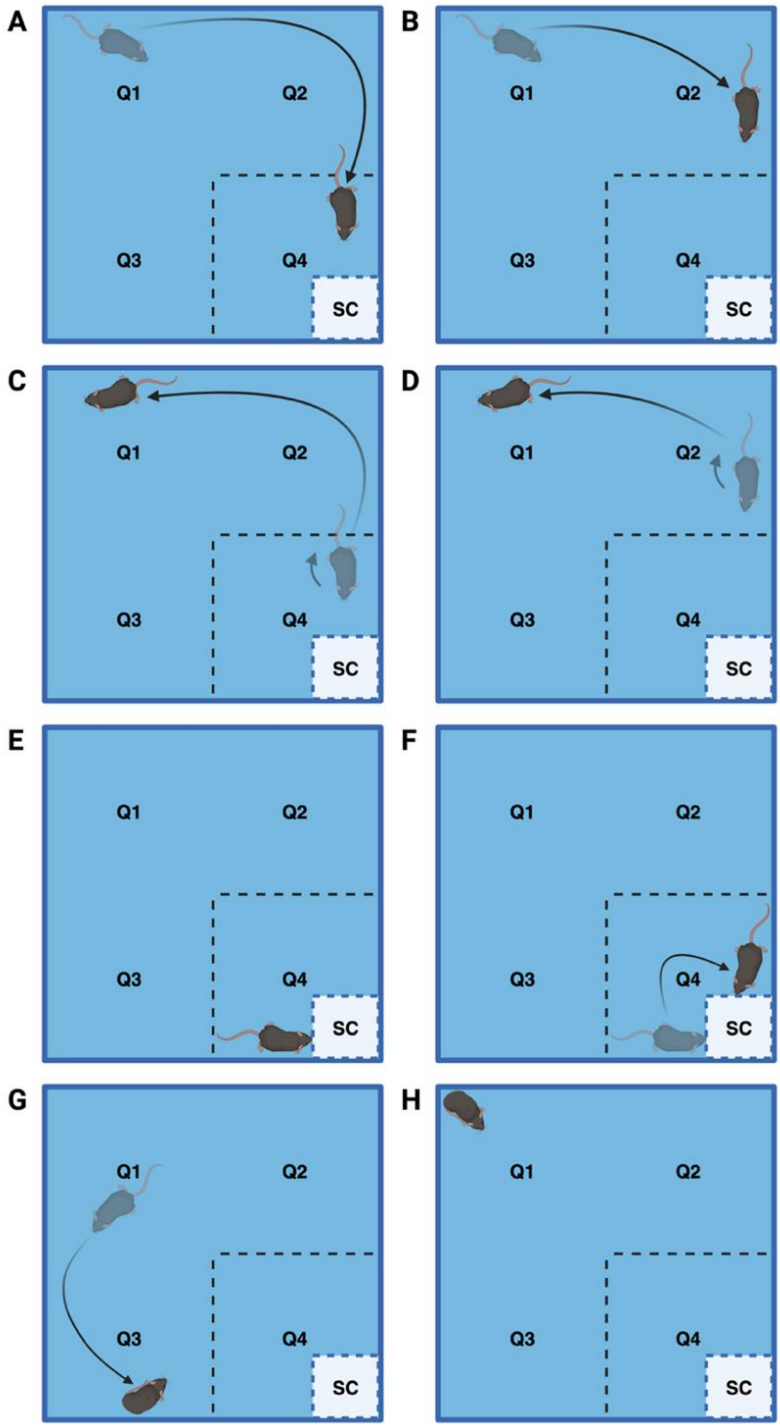



**Figure 3.3: The eight distinct behaviours used to characterise social behaviour during social and non-social tasks.**
The test arena was divided into four equal sized quadrants (Q1 - Q4 of the 40 x 40 cm test arena. The stimulus cage (SC) was always placed in Q4. Approach behaviours were defined

by the quadrant in which the behaviour terminated; thus **A** Proximal approach began in any quadrant but must have ended within Q4 whereas **B** Distal Approach was an approach which terminated in Q1 to Q3. For escape/flee behaviours, the inverse was true, flee behaviours were defined by the location in which they commenced. **C** Proximal flee was defined by a flee which started in Q4 where **D** Distal flee was defined as a flee which started in Q1 to Q3. **E** A visual representation of stimulus investigation which was any moment the nose of the subject mouse was within the SC area. A more complex behaviour can be found in **F** which was termed stimulus disengage/re-engage. This behaviour was defined by the set of movements where a subject investigates the SC and then moved to another section/side of the SC within 3 seconds *and* did not leave Q4. **G wa**s an example of non-stimulus directed behaviour which can include grooming, rearing and arena exploration but did not involve any approach or interaction with the stimulus and **H** if during non-stimulus directed behaviour the subject was immobile in the corner of Q1 (i.e., not grooming or exploring) and vigilant towards the SC this was defined as immobile in corner. The onset of scoring each behaviour started at the initiation of the event e.g. flee started when a subject turned to rapidly escape.

*Fibre photometry analysis*

Data was pre-processed and peri-event analysis performed using a custom-written pipeline in python. Data from the calcium-independent 415 nm isosbestic control channel was used to correct for motion artifacts and photobleaching. Using least-squares linear regression, the 415 nm signal was fit to the 470nm signal. Change in fluorescence (ΔF/F) at each time point was calculated by subtracting the fitted 415 signal from the 470 signal and normalizing to the fitted 415 data [(470-fitted 415)/(fitted 415)]. For Experiment 1a, the baseline was calculated based on the mean signal during an 8-minute window within the 10-minute habituation to the arena. For fear conditioning (Experiment 1b and 2), the baseline was calculated based on the mean signal during a two-minute window between the removal of the third empty stimulus cage and before the entry of the first stimulus (S1) exposure. Using the calculated baseline, the z-score was then calculated at each individual time point to produce a signal z-score.

$$\text{z-score} = \frac{\Delta F/_F - (\textit{mean of values in baseline period})}{\textit{Standard deviation of values in baseline period}}$$

To examine calcium activity for each individual bout of behaviour in each subject, peri-event analysis was performed whereby z-scored traces were aligned and frame-matched with the manually scored behaviour and video (30Hz) files, as outlined above. A 5s pre- and post-window (15s for Experiment 2) for each behavioural event was then exported to Excel and trials were grouped and analysed according to within-subject variable stimulus type. Following peri-event analysis, peak analysis was conducted for conditioned (S1+) versus unconditioned (S1-) proximal flee events using Guided Photometry Analysis in Python (GuPPy) (Sherathiya et al. (2021) to examine the proportion of manually scored behavioural events which correlated with automatically detected peaks in dLS activity and the corresponding mean amplitude of these peaks.

*Exclusions*

Mice were excluded and no longer tested if during the last habituation there was no significant difference in transient calcium fluctuations between the calcium-dependent and isosbestic control signals when analysed using python (Experiment 1 n = 6 (2 female), Experiment 2 n= 7, (5 female)), likely due to inaccurate viral or probe placement. Mice were excluded if they lost their headcap during the experiment (No exclusions in Experiment 1, Experiment 2 n = 7, (5 female)). In Experiment 1b, in post-hoc analysis we excluded mice which spent the same or more time investigating the social stimulus during S1+ compared to S1- (n = 7, 4 female) as this indicated unsuccessful fear conditioning. In Experiment 2, we determined *a priori* not to include subjects which had the same or higher rates of social behaviour between S1- and S1+ as they would not meet the threshold for fear conditioning (n = 2 males in social. condition). However, in examination of the dLS activity in extinguishers and non-extinguishers, these mice were included in the analysis.

No mice were excluded following histological confirmation of anatomical viral and probe placement.

*Statistical analysis*

Statistical analysis was performed in R Studio version 4.3.1. Time spent exploring the stimulus cage was analysed using mixed measures ANOVA using packages *dplyr, tidyr, stringr, ggplot2* (Wickham et al., 2019) and *Rmisc* (Hope, 2022). For peri-event analysis of photometry data, to account for the fact that events are not independent, we used a hierarchical model, which nested events within subjects; to do this we used *lme4* (Bates et al., 2015), *lmerTest* (Kuznetsova et al., 2017), *pracma* (Borchers, 2022), *emmeans* (Lenth, 2023) and *car* (Fox & Weisberg, 2019). For behaviours where, across all conditions, dLS activity showed no discernible peak in signal or clear elevation of baseline activity in the 3 seconds preceding and proceeding the onset of the behaviour (defined as the 95% CI for AUC containing 0), we did not proceed with area under the curve (AUC) analysis; no behaviours were excluded from Experiment 1a and 1b, and in Experiment 2, AUC analysis was not performed for distal approach (*Figure 3.10C-D*) or distal flee (*Figure 3.11C-D*). For stimulus investigation (*Figure 3.12A-B*), to ensure capture of stimulus investigation and not proximal flee as the two events are often paired, we removed all photometry events less than 1s. This left very few events and no discernible peaks in dLS activity thus we did not perform AUC analysis for stimulus investigation. A similar trend was seen for disengage and re-engage of the stimulus where no mice in the non-social fear conditioning group engaged in this behaviour (*Figure 3.12C-D*). Further, AUC analysis was not performed for other non-stimulus directed behaviours (*Figure 3.12E-F*) and corner immobility (*Figure 3.12G-H*) as these behaviours had no discernible peaks in signal or clear elevation from baseline. A generalized linear mixed model was

used to assess the effect of fear conditioning and stimulus type, and their interaction, on the probability of automatically detecting a peak prior to proximal flee events in Experiment 2, with random intercepts included for individual mice to account for events being nested within mice. The model was fit using a binomial distribution with a logit link function.

Behavioural data were analysed using a two-way ANOVA in GraphPad Prism. The dependent variable were time spent exploring the stimulus cage or social stimulus (% of total trial duration) during each 3-min trial and the frequency to engage in proximal approach (Experiment 1a, 1b and 2), distal approach (Experiment 2), stimulus investigation (Experiment 1a, 1b and 2), disengage and re-engage with the stimulus (Experiments 1a and 1b), proximal flee (Experiments 1a, 1b and 2), distal flee (Experiments 2). Investigation with the cage or social stimulus was defined as when the nose of the test mouse was in contact with the stimulus cage. In Experiment 1a, the independent variables were sex and stimulus type (same-sex social stimulus, opposite-sex social stimulus, and non-social stimulus). For Experiment 1b and 2, which examined fear conditioning, following exclusions, the sample was underpowered to assess sex differences. As such, analyses were pooled across sex. In Experiment 1b, the independent variable was stimulus exposure (S1-, S1+, S2+ and S3+). For Experiment 2, the independent variables were stimulus exposure (S1-, S1+, S2+, S3+ and S6+) and stimulus type (social *vs* non-social). In Experiment 2, in post-hoc analysis we examined the dLS activity preceding proximal flee in socially fear conditioned mice divided into the two subgroups, extinguishers and non-extinguishers. The independent variable was stimulus exposure (S1-, S1+, S2+, S3+ and S6+) and the extinction condition (extinguishers *vs* non-extinguishers). For all comparisons, significance was set at $p < 0.05$ and all behavioural data are presented as Mean ± SEM.

## 3. 3. Results

### *3. 3. 1. Experiment 1a: Stimulus Interaction Test*

To assess whether dLS neurons were activated by salient stimuli in real time, we used in vivo fibre photometry to record $Ca^{2+}$ signals following exposure to ethologically relevant novel non-social, and same- and opposite-sex social stimuli. Mice were first placed in the open field test arena with an empty stimulus cage placed in one corner for 10 minutes (baseline). They were then presented with stimuli in a pseudorandom order for three minutes with three minutes between each stimulus presentation (*see Figure 3.4A*). Behaviours were hand scored and 3s windows before and after the time from start of each behaviour were analysed separately.

#### *Stimulus investigation*

There was a main effect of stimulus type on the time spent investigating the stimulus cage [$F(1.49, 14.86) = 21.05$, $p < 0.001$, *Figure 3.4B*]. Mice spent significantly more time investigating opposite-sex [$t(11) = 5.94$, $p < 0.001$] and same-sex social stimuli [$t(11) = 3.74$, $p = 0.003$] compared to non-social stimuli. Additionally, there was a main effect of stimulus type on frequency to investigate the stimulus [$F(1.41, 14.07) = 11.71$, $p = 0.002$, *Figure 3. 4C*] where mice made fewer attempts to engage with a non-social stimulus compared with an opposite- [$t(11) = 3.82$, $p = 0.003$] or same-sex [$t(11) = 7.23$, $p < 0.001$] stimulus.

However, there was no main effect of sex [$F(1, 10) = 1.54$, $p = 0.244$], and no interaction between sex and stimulus type [$F(2, 20) = 1.83$, $p = 0.186$] on time spent investigating the stimulus cage nor was there a main effect of sex [$F(1, 10) = 0.20$, $p = 0.660$] and no interaction between sex and stimulus type [$F(2, 20) = 1.16$, $p = 0.334$] on

frequency to investigate the stimulus. Further, when presented with a non-social, same-sex or opposite-sex stimulus, there was no main effect of sex [$F(1, 10) = 1.50, p = 0.249$], stimulus type [$F(1.88, 18.77) = 2.42, p = 0.119$] or interaction [$F(2, 20) = 0.62, p = 0.549$] on the frequency to approach the stimulus (*see Figure 3. 4D*).

There was a main effect of stimulus type on the frequency to disengage and re-engage a social or non-social stimulus, a pro-investigatory behaviour [$F(1.89, 18.85) = 11.25, p < 0.001$, *Figure 3. 4E*]. Subjects disengage/re-engaged with a non-social stimulus less frequently than a same-sex [$t(11) = 5.06, p < 0.001$] or opposite-sex [$t(11) = 3.62, p = 0.004$] stimulus. Additionally, there was a main effect of stimulus type on the frequency to engage in proximal flee, the rapid escape from the immediate vicinity of the caged stimulus [$F(1.97, 19.68) = 3.96, p = 0.037$, *Figure 3. 4F*] where planned contrasts revealed subjects more frequently fled an opposite-sex than non-social [$t(11) = 2.66, p = 0.022$] or same-sex [$t(11) = 2.26, p = 0.045$] stimulus. Finally, there was no main effect of sex [$F(1, 10) = 0.05, p = 0.836$] or interaction between sex and stimulus type [$F(2, 20) = 1.05, p = 0.367$] in the frequency to disengage and re-engage a social or non-social stimulus nor was there a main effect of sex [$F(1, 10) = 0.75, p = 0.406$] or interaction between sex and stimulus type [$F(2, 20) = 1.79, p = 0.192$] in the frequency to proximally flee the stimulus.

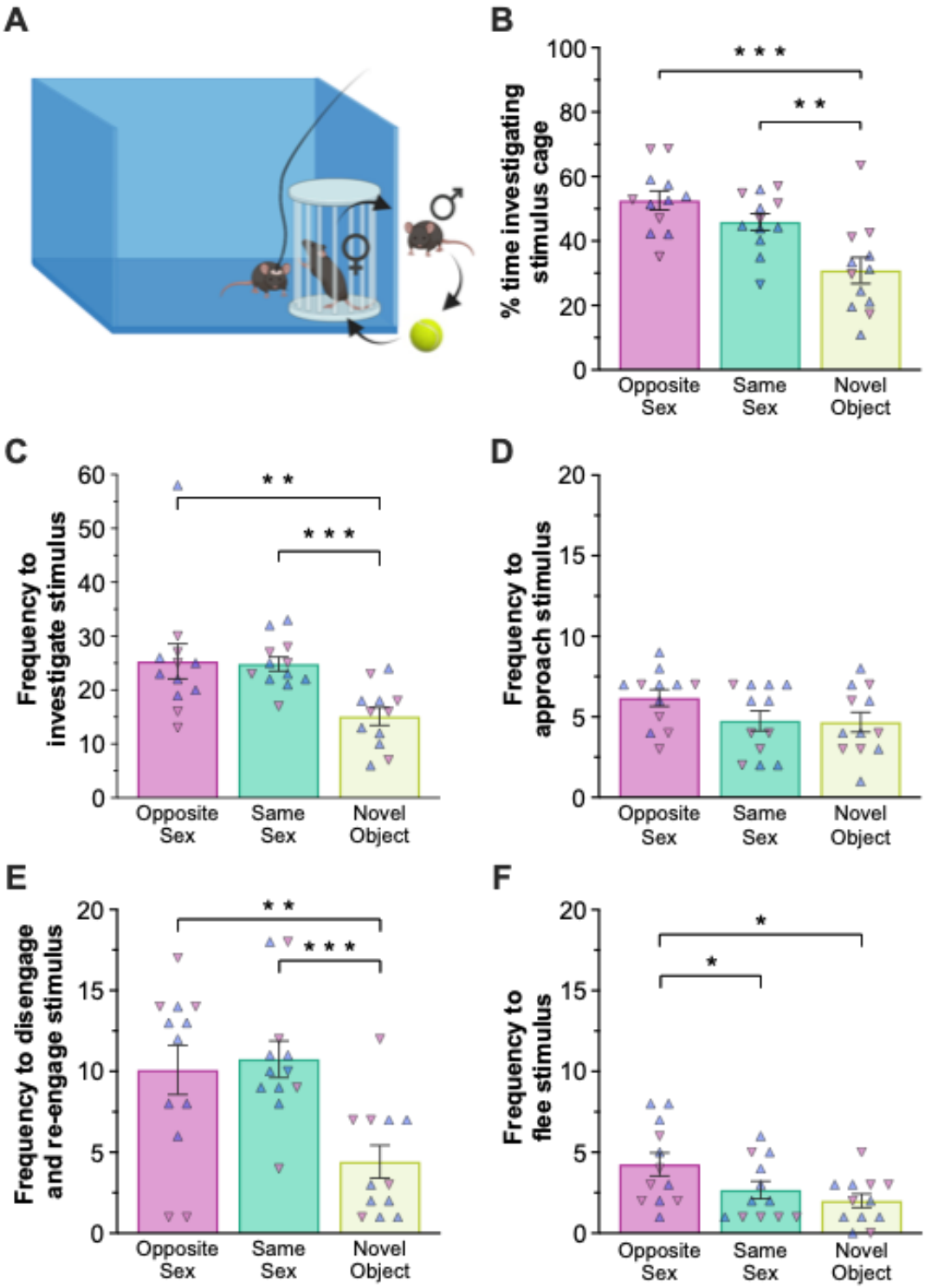



**Figure 3.4: Investigation of the stimulus cage during the social interaction task.** There was no effect of sex or interaction between sex and stimulus type, as such bars are averaged across sex, but sex is indicated for individual data points (see below). **A** The task involved placing subjects in a test arena with only an empty stimulus cage for 10 minutes to habituate to the optic fibre and establish a baseline for photometry. For the test, mice (n = 12, 5 females) were randomly presented with a same- or opposite-sex social or non-social

stimulus for three minutes with a three-minute inter exposure interval. **B** Mice spent significantly less time investigating a novel object compared to an opposite-sex or same-sex social stimulus. **C** Mice engaged in more bouts of stimulus investigation with an opposite-sex and same-sex stimuli compared to non-social stimuli, **D** but displayed no preference to approach any stimulus type more than any another. **E** Similar to stimulus investigation, mice initiated disengagement and re-engagement with opposite- and same-sex social stimuli more than non-social stimuli. **F** Subjects engaged in stimulus flee more frequently when interacting with an opposite-sex social stimulus compared to same-sex and non-social stimuli. Data are Mean ± SEM, triangles represent individual datapoints (upward point = male, downward point = female). N = 12 (5 female). $\alpha$ is 0.05, ****p < 0.001, **p < 0.01, *p < 0.05.*

***Fibre photometry***

*Stimulus investigation and approach behaviours*

In the 3s preceding and proceeding the onset of investigation of same-sex (*Figure 3.5A-B*), opposite-sex or non-social stimuli, no stimulus type demonstrated dLS activity greater than baseline (*see Appendix B: Table 3. 1),* therefore no further analyses were examined. However, in the 3s prior to the onset of stimulus approach behaviour, female mice approaching novel non-social, same-sex, or opposite sex social stimuli all had an AUC above baseline, whereas male mice did not (*see Appendix B: Table 3. 2*). Consistent with this, there was a main effect of sex, with AUC photometry signal greater for females than males [*F(1, 9.37) = 5.95, p = 0.036, Figure 3. 5C-D*]. There was no main effect of stimulus type [*F(2, 160.38) = 0.30, p = 0.742*] or interaction between stimulus type and sex [*F(2, 160.38) = 0.37, p = 0.693*].

In the 3s following stimulus approach, AUC was greater than baseline only for females following approach to an opposite-sex or same-sex social stimulus (*see Appendix B: Table 3. 2*). There was no main effect of sex [*F(1, 9.05) = 3.82, p = 0.082*] or stimulus type [*F(2, 158.84) = 0.88, p = 0.417*], but there was an interaction between sex and

stimulus type [*F(2, 158.84) = 3.10, p = 0.047, Figure 3. 5C-D*]. AUC was greater following opposite-sex stimulus approach [*t(14.1) = 3.00, p = 0.01*] or non-social stimulus approach [*t(16.2) = 2.54, p = 0.022*] for females compared to males.

*Stimulus avoidance behaviours*

When engaging in disengage/re-engage behaviour, there was no change in fluorescence and (*see Figure 3.5E-F*) no condition had an AUC greater than baseline in the 3s prior to or following the onset of the behaviour (*see Appendix B: Table 3. 3*), as such no further analyses were examined. However, when examining proximal stimulus flee behaviour, defined by a stimulus flee that occurs in the quadrant containing the stimulus cage only females interacting with an opposite sex social stimulus had an AUC in the 3s preceding proximal stimulus flee that was greater than baseline (*see Appendix B: Table 3. 4*). There was no main effect of sex [*F(1, 8.31) < 0.01, p = 0.995*] or stimulus type [*F(2, 89.02) = 0.49, p = 0.617*] on AUC. An interaction between sex and stimulus type for AUC [*F(2, 89.02) = 10.05, p < 0.001, Figure 3.5G-H*] was found where when interacting with the opposite sex, the different in AUC preceding proximal stimulus flee between females and males was more pronounced than when interacting with a same sex stimulus [*t(87.4) = 3.42, p = 0.001*] or non-social stimulus [*t(89.8) = 3.87, p < 0.001*]. In the 3s following proximal flee behaviour, none of the conditions had an AUC that was greater than baseline (*see Appendix B: Table 3. 4*).

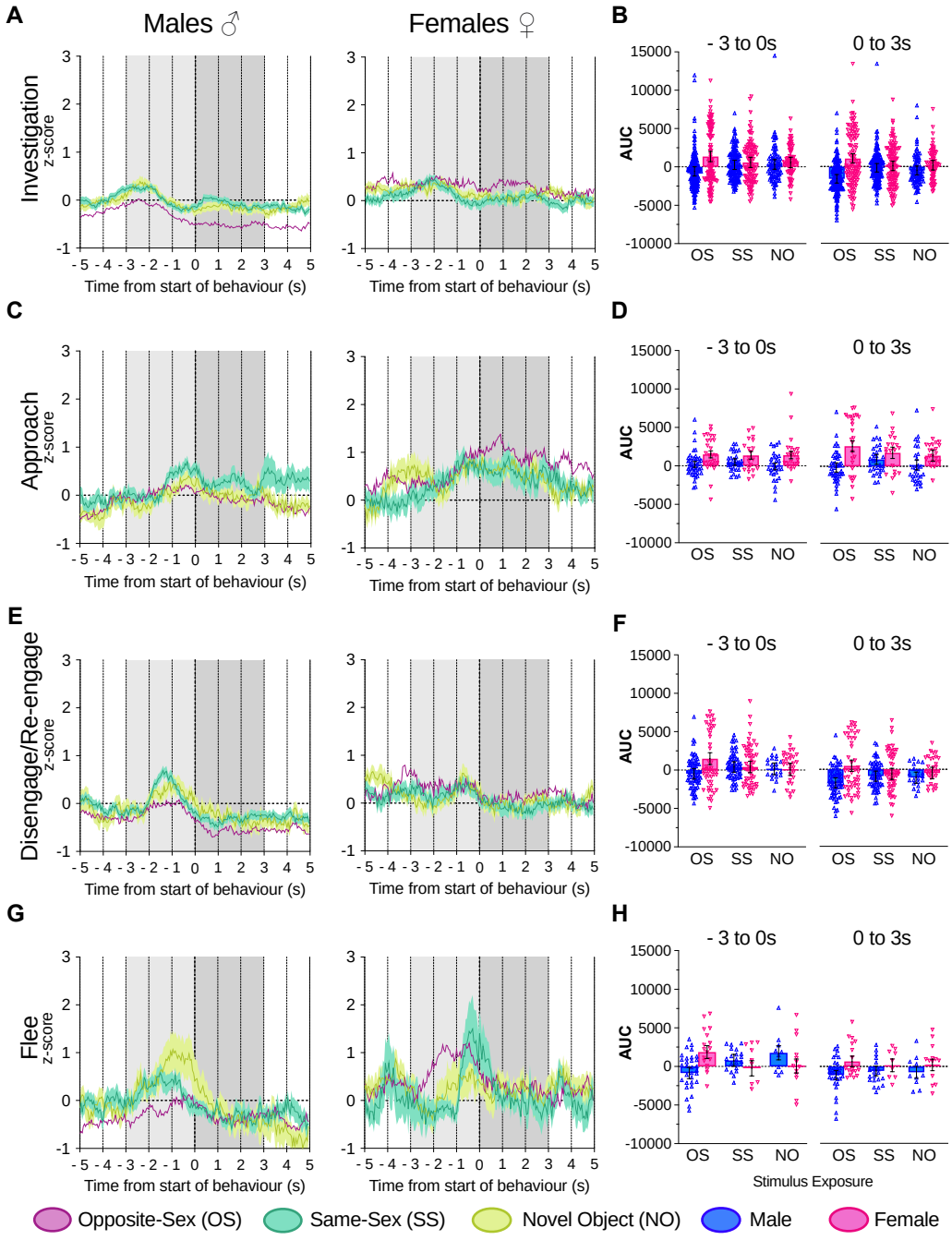



**Figure 3.5: dLS activity in male and female mice during the stimulus interaction test.**

Mice were randomly presented a series of novel same- (SS, green), opposite-sex (OS, purple) or non-social (NO, yellow) stimuli for three minutes with a three-minute inter-exposure interval. Data represents the 3s before and after the initiation of each behaviour. **A-B** Immediately before and after stimulus investigation, no condition had elevated dLS activity compared to baseline. **C-D** In the 3s preceding stimulus approach, the area under the curve (AUC) photometry signal was greater for females than males. In the 3s following stimulus approach, the AUC was greater than baseline only for females following approach of opposite-sex or same-sex social stimulus. AUC was greater, compared to baseline, following opposite-sex and non-social stimulus approach for females compared to males. **E-F** No

condition had elevated dLS activity compared to baseline before or after the onset of dis-engagement and re-engagement behaviour. **G-H** Photometry revealed only females interacting with an opposite sex social stimulus had an AUC in the 3s preceding stimulus flee that was greater than baseline, however following stimulus flee no condition had an AUC greater than baseline. Data are Mean ± SEM. N = 12 (5 female).

### *3. 3. 2. Experiment 1b: Social Fear Conditioning Pilot*

*Stimulus investigation*

One-way ANOVA revealed a main effect of stimulus exposure condition on percentage of time spent investigating the social stimulus [*F(1.60, 6.40) = 12.97, p = 0.007, Figure 3. 6B*]. When mice were conditioned, they spent less time exploring the social stimulus compared to unconditioned (SFC-) mice during S1+ [*t(4) = 8.46, p = 0.001]*, S2+ [*t(4) = 4.10, p = 0.015*], and S3+ [*t(4) = 4.42, p = 0.012*]. Between S1+ and S2+ and S3+, the difference between time spent exploring the stimulus approached significance [*S2+, t(4) = 2.45, p = 0.071* and S3+, *t(4) = 2.69, p = 0.055*]. There was a main effect of stimulus exposure on frequency to investigate the social stimulus [*F(1.74, 6.94) = 13.12, p = 0.005, Figure 3.6C*]. When mice were unconditioned (S1-), they engaged in social stimulus investigation more frequently than during S1+ [*t(4) = 6.61, p = 0.003*] and S3+ [*t(4) = 3.60, p = 0.023*], and frequency to engage in social stimulus investigation during S2+ approached significance [*t(4) = 2.54, p = 0.064*]. Mice engaged in social stimulus investigation less frequently during S1+ compared to S2+ [*t(4) = 2.95, p = 0.042*] and S3+ [*t(4) = 5.69, p = 0.005*]. One-way ANOVA revealed no significant difference in frequency to approach the social stimulus between stimulus exposure conditions [*F(1.10, 4.38) = 0.32, p = 0.618, Figure 3. 6D*].

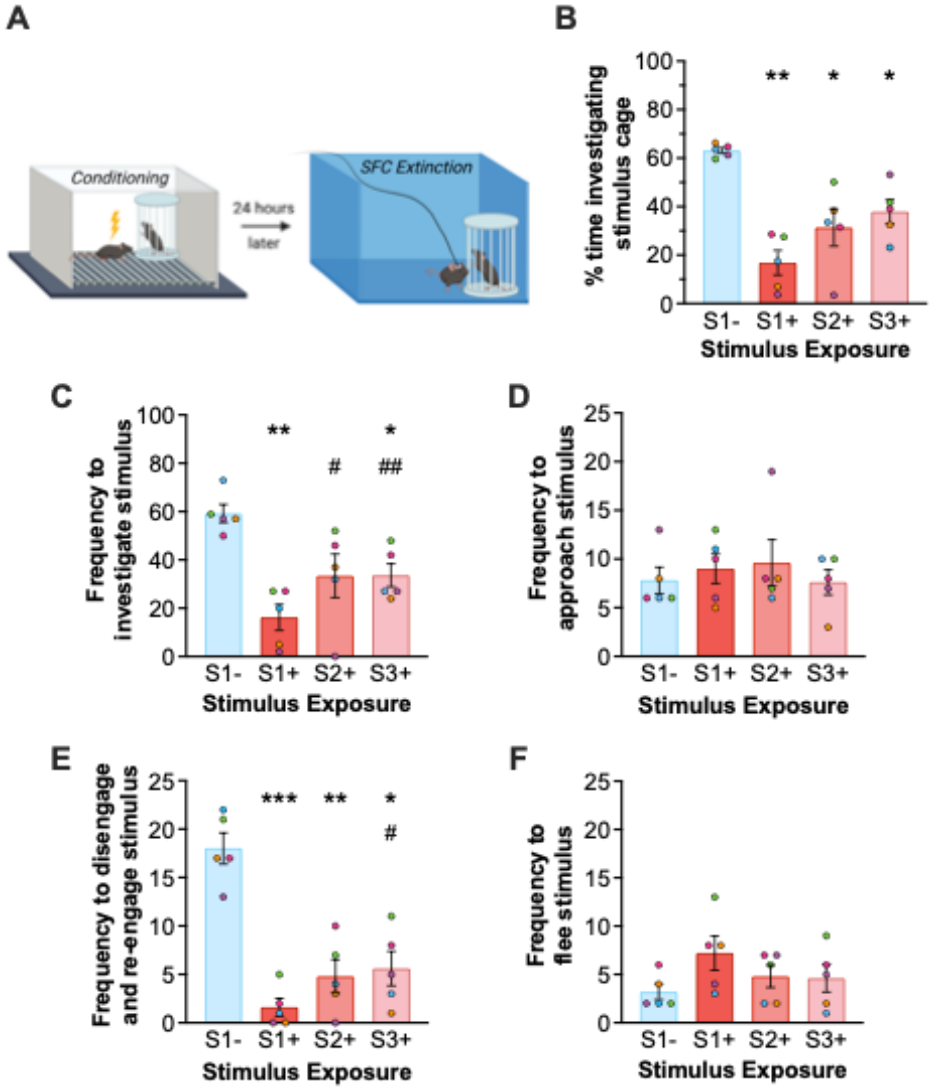



**Figure 3.6: Investigation of the social stimuli in SFC- and SFC+ mice.**
**A** To create within-subject controls of behaviour within the social fear conditioning (SFC) task, mice first underwent "conditioning" (SFC-) then extinction and one week later underwent standard SFC+ followed by extinction. Activity in the dLS was recorded during both SFC- and SFC+ extinction using fibre photometry. **B** Mice spent significantly less time investigating the social stimulus at S1+, S2+ and S3+ compared to S1-, where **C** mice also made fewer attempts to investigate at S1+ compared to S1-. By S3+, mice engaged in stimulus investigating more frequently compared to S1+. **D** Across stimulus exposures there was no difference between frequency to engage in stimulus approach, but **E** mice engaged in much less disengagement and re-engagement of the social stimulus at S1+, S2+ and S3+ compared to S1-. **F** There was no difference in frequency to flee the social stimulus across extinction. N = 5 mice. Data are Mean ± SEM and α is 0.05. S1- *vs* S1+, S2+ or S3+, ****p < 0.001, **p < 0.01, *p < 0.05*. S1+ vs S2+ or S3+, *## p < 0.01, #p < 0.05.*

There was a main effect of stimulus exposure condition on the frequency to disengage and swiftly re-engage with the social stimulus [*F(1.94, 7.78) = 42.32, p < 0.001, Figure 3. 6E*]. Frequency to disengage and re-engage in S1- was significantly higher compared to S1+ [*t(4) = 12.36, p < 0.001*], S2+ [*t(4) = 7.45, p = 0.002*], S3+ [*t(4) = 5.74, p = .005*]. The frequency to disengage and re-engage with the stimulus was higher during S3+ compared to S1+ [*t(4) = 3.81, p = 0.019*]. There was no main effect of stimulus exposure on the frequency to flee the novel stimulus [*F(1.90, 7.61) = 2.53, p = 0.145, Figure 3.6F*].

***Fibre photometry***

*Stimulus investigation and approach behaviours*

In the 3s preceding stimulus investigation, AUC was greater than baseline under all stimulus exposure conditions (*see Appendix B: Table 3. 5*). There was a main effect of stimulus exposure condition [*F(3, 706.56) = 21.26, p < 0.001, Figure 3.7A-B*]. AUC was greater during S1+ than S1- [*t(707) = 7.76, p < 0.001*], S2+ [*t(705) = 6.08, p < 0.001*], and S3+ [*t(706) = 6.96, p < 0.001*]; there was no difference between S1- and S2+ [*t(708) = 1.69, p = 0.092*] or S3+ [*t(705) = 0.45, p = 0.650*]. In the 3s following the initiation of stimulus investigation, AUC for activity in dLS was greater than baseline under all stimulus exposure conditions except for S3+ (*see Appendix B: Table 3. 5*). There was a main effect of stimulus exposure [*F(3, 707.07) = 24.05, p < 0.001, Figure 3.7A-B*]. As seen prior to investigation, AUC was greater during S1+ than S1- [*t(708) = 7.62, p < 0.001*], S2+ [*t(705) = 6.68, p < 0.001*], and S3+ [*t(707) = 8.10, p < 0.001*]; there was no difference between S1- and S2+ [*t(708) = 0.69, p = 0.493*] or S3+ [*t(706) = 1.34, p = 0.182*]. However, there appears to be an elevated baseline over the entire 10s trace (*Figure*

*3. 7A*) indicating that perhaps mice have elevated tonic activity during S1+.In the 3s preceding stimulus approach, AUC was greater than baseline under all exposure conditions (*see Table 3. 6*) and there was no main effect of stimulus exposure [*F(3, 165.57) = 0.76, p = 0.516, Figure 3. 7C-D*]. In the 3s following stimulus approach, the AUC was greater across all exposures compared to baseline and there was a main effect of stimulus exposure [*F(3, 165.9) = 6.03, p < 0.001*]. AUC for the dLS signal was greater during S1+ than S1- [*t(166) = 2.01, p = 0.046*], S2+ [*t(164) = 2.45, p = 0.016*], and S3+ [*t(163) = 4.23, p < 0.001*].

*Stimulus avoidance behaviours*

In the 3s preceding each disengagement and re-engagement with the stimulus, all conditions had an AUC greater than baseline (*see Appendix B: Table 3. 7*). There was a trend toward a main effect of stimulus exposure [*F(3, 144.67) = 2.52, p = 0.060, Figure 3. 7E-F*]. Pairwise comparisons revealed a greater AUC for S1+ compared to S1- [*t(145) = 2.74, p = 0.007*], S2+ [*t(143) = 2.11, p = 0.0362*] and S3+ [*t(143) = 2.22, p = 0.028*]. In the 3s proceeding the onset of disengagement and re-engagement, only S1+ and S3+ had an AUC greater than baseline (*see Appendix B: Table 3. 7*). There was a main effect of stimulus exposure [*F(3, 144.44) = 4.61, 0.004*]. AUC was greater for S1+ compared to S1- [*t(143) = 2.69, p = 0.008*] and S2+ [*t(146) = 2.61, p = 0.010*].

In the 3s prior to each proximal stimulus flee (flee) event, all conditions had an AUC greater than baseline except for S3+ (*see Appendix B: Table 3. 8*). There was a main effect of SFC exposure [*F(3, 93.81) = 13.01, p < 0.001*]. AUC preceding flees was greater during S1+ than S1- [*t(92.9) = 2.64, p = 0.010*], S2+ [*t(93.8) = 2.71, p = 0.008*] and S3+ [*t(93.8) = 2.63, p = 0.010, Figure 3.7G-H*]. There was no difference in AUC preceding flee between S1- and S2+ [*t(93.5) = 0.23, p = 0.819*], and AUC during S3+ was lower than

S1- [*t(93.8) = 2.63, p = 0.010*]. In the 3s proceeding the onset of stimulus flee, only S1+ had an AUC greater than baseline (*see Appendix B: Table 3. 8*). There was a main effect of SFC exposure for AUC [*F(3, 92.45) = 5.88, p = 0.001*], where S1+ had significantly greater AUC compared to S2+ [*t(93.0) = 2.07, p = 0.041*] and S3+ [*t(92.3) = 4.18, p = 0.041*]. Further, the AUC for S1- was greater than S3+ [*t(93.0) = 2.14, p = 0.035*].

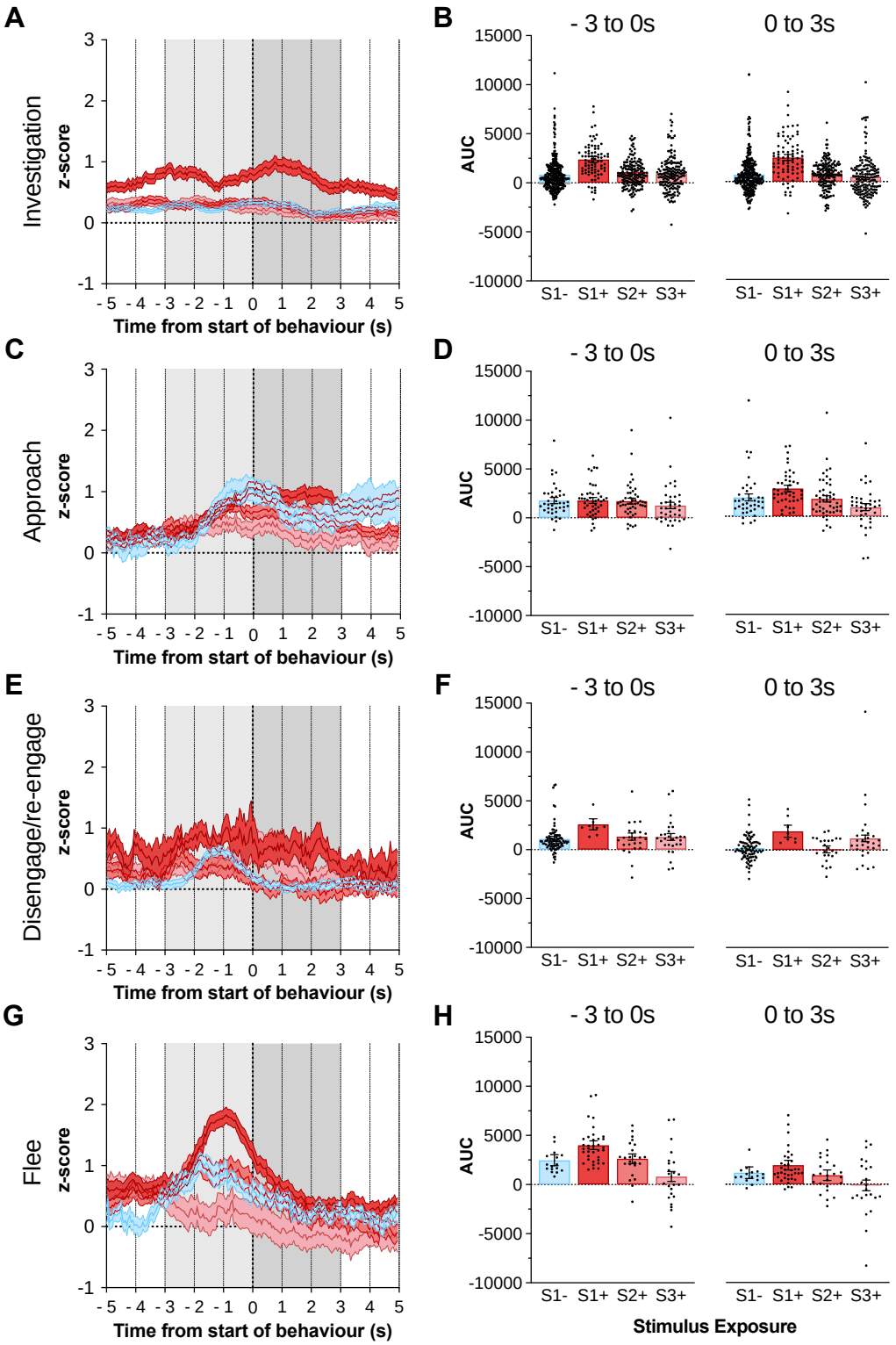



**Figure 3.7: dLS activity for specific approach and avoidance behaviours during SFC extinction.**

Mice underwent social fear conditioning (SFC) and received foot shock upon investigation of novel social stimulus. The next day, mice were attached to the patch cord and presented six novel social stimuli to examine social fear extinction. Data presented here are pre-conditioning baseline of social behaviour (S1-) and the first three stimulus exposures post-

conditioning (S1+ to S3+). **A-B** In the 3s preceding and proceeding stimulus investigation, area under the curve (AUC) for dLS activity was greater than baseline under all stimulus exposure conditions except for S3+ post-onset of the behaviour. Further, the AUC was greatest for S1+ compared to S1-, S2+ and S3+ before and after the onset of stimulus investigation. **C-D** For stimulus approach of unconditioned and preconditioned social stimuli, all conditions had AUC of dLS activity greater than baseline however there was no effect of stimulus exposure. **E-F** In the 3s preceding each disengagement and re-engagement with the stimulus, all conditions had an AUC greater than baseline. In the 3s proceeding the behaviour, only S1+ and S3+ had an AUC greater than baseline. The AUC for dLS activity was greater for S1+ compared to S1- and S2+. **G-H** For stimulus flee, all conditions had an AUC greater than baseline except for S3+. AUC preceding flees was greater during S1+ than S1-, S2+ and S3+. The dLS activity prior to flee was no different between S1- and S2+, and AUC for dLS activity during S3+ was lower than S1-. Data are Mean ± SEM. N = 5.

### *3. 3. 3. Experiment 2: Social and Non-Social Fear Conditioning and Extinction*

#### *Social versus Non-Social Fear Extinction*

To determine the reproducibility of our SFC results in behaviourally naïve mice, we replicated the SFC photometry study piloted in the cohort from Experiment 1 in a new, larger cohort of mice who did not undergo social interaction testing prior to SFC (*see Figure 3. 2*). Experiment 2 extended upon Experiment 1b by assessing if the elevated signal in the dLS preceding stimulus flee was social-specific using a separate group of mice who underwent non-social fear conditioning (nSFC); this involved mice being conditioned with a mild foot shock upon investigation of novel non-social stimulus (scented tennis balls; *see Figure 3.8B*). Further, based on observations from the pilot, and to better understand the behavioural dynamics within the fear conditioning tasks, we expanded upon the four behaviours examined in Experiment 1, to divide approach and flee into proximal and distal, and include corner immobility and a general category of behaviour to capture other non-stimulus related behaviours (*see Figure 3. 3*).

***Stimulus investigation***

There was a main effect of stimulus exposure [*F(1, 30) = 41.61, p < 0.001*] but no main effect of stimulus type [*F(1, 30) = 1.02, p = 0.321*] nor interaction between SFC and stimulus type [*F(1, 30) = 0.26, p = 0.617*]. When examining conditioned mice, contrast analysis revealed mice spent significantly more time investigating novel social stimuli at sS6+ compared to sS1+ [*t(15) = 3.31, p = 0.005*] with an upward linear trend [*t(15) = 3.40, p = 0.004, Figure 3. 8C*]. Mice in the non-social stimuli demonstrated no fear extinction at nS6+ [*t(15) = 0.22, p = 0.829, Figure 3.8C-D*]. Finally, subjects during pre-conditioning extinction, demonstrated a negative quadratic trend where they had higher sociability at the beginning of fear conditioning, which decreased over time [*t(30) = 2.13, p = 0.041*].

There was a main effect of stimulus type [*F(1, 13) = 5.77, p = 0.*032] and stimulus exposure [*F(1.60, 20.80) = 31.22 , p < 0.001*] and an interaction between stimulus type and exposure [*F(4, 52) = 2.86, p = 0.032*]. SFC+ mice made significantly more attempts to engage with stimuli than nSFC+ mice at S6+ [*t(5.24) = 2.59, p = 0.47, Figure 3.8E*] but there was no difference between stimulus types at S1- [*t(5.89) = 1.22, p = 0.268*], S1+ [*t(10.07) = 0.70, p = 0.500*], S2+ [*t( 5.91) = 1.18, p = 0.283*], S3+ [*t(5.31) = 1.50, p = 1.90*]. Conditioned mice during S1+ made significantly fewer stimulus investigations than unconditioned mice [SFC- mice [*t(5) = 3.54, p = 0.015*] and nSFC mice [*t(8) = 10.54, p < 0.001*]. Finally, SFC+ mice engaged in stimulus investigation more during S6+ compared to S1+ [*t(5) = 2.80, p = 0.38*] however nSFC+ mice did not [*t(8) = 2.23, p = 0.56*].

In terms of proximal approach, there was no main effect of stimulus type [*F(1, 13) = 2.68, p = 0.125*], stimulus exposure [*F(2.64, 34.31) = 2.48, p = 0.084*] or an interaction between stimulus type and stimulus exposure [*F(4, 52) = 0.74, p = 0.567, Figure 3.9A*].

For distal approach of the stimulus, there was a main effect of stimulus exposure [*F(2.56, 33.34) = 3.63, p = 0.028, Figure 3.9B*] but no main effect of stimulus type [*F(1, 13) = 2.66, p = 0.127*] or an interaction between stimulus type and stimulus exposure [*F(4, 52) = 0.97, p = 0.433*].

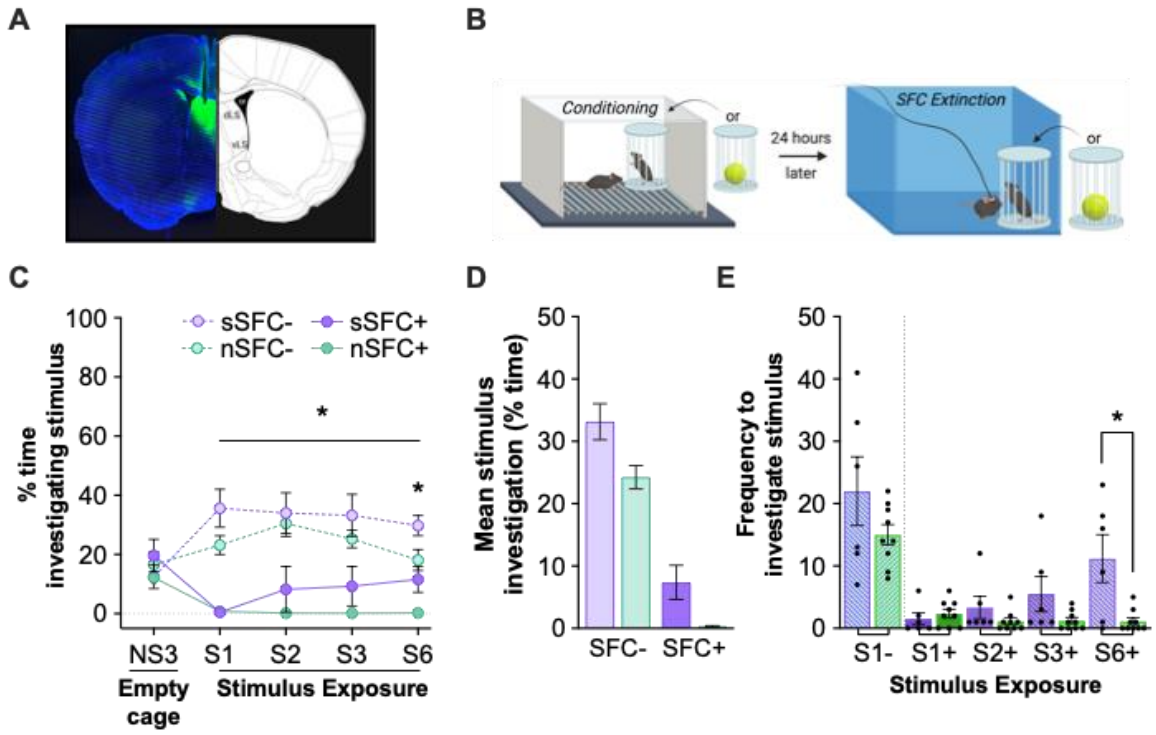



**Figure 3.8: Investigation of social and non-social stimuli during fear extinction.**
**A** Representative example of AAV-jGCaMP8f expression in the dorsal lateral septum (dLS) in an experimental mouse. **B** To create within-subject controls of behaviour within the social fear conditioning (SFC) and non-social fear conditioning (nSFC) tasks, mice first underwent "conditioning" (SFC-) then extinction and one week later underwent standard SFC+ followed by extinction. Activity in the dLS was recorded during extinction using fibre photometry. **C** SFC+ and nSFC+ mice spent significantly less time investigation the stimulus across extinction (S1 to S6). Mice conditioned to social stimuli demonstrated fear extinction however mice conditioned to non-social stimuli did not. **D** Mean stimulus investigation across stimulus exposures showed overall greater social investigation in SFC+ than nSFC+ mice. **E** Mice frequently engaged with stimuli during S1-, however following fear conditioning there was a significant reduction in frequency to engage stimuli. SFC+ mice made significantly more attempts to engage in stimulus investigation than nSFC+ during the final stimulus exposure (S6). Data are Mean ± SEM. N = 15 (6 SFC and 9 nSFC mice). $\alpha$ is 0.05, *$p < 0.05$.

In terms of proximal stimulus flee, there was no main effect of stimulus type [*F(1, 13) = 2.35, p = 0.150, Figure 3.9C*], stimulus exposure [*F(2.76, 35.93) = 0.73, p = 0.528*] or an interaction between stimulus type and stimulus exposure [*F(4, 52) = 0.63, p = 0.640*]. For distal flee, there was a main effect of stimulus exposure [*F(2.47, 32.20) = 3.21, p = 0.044, Figure 3.9D*] but no main effect of stimulus type [*F(1, 13) = 2.96, p = 0.109*] or an interaction between stimulus exposure and stimulus type [*F(4, 52) = 1.10, p = 0.365*].

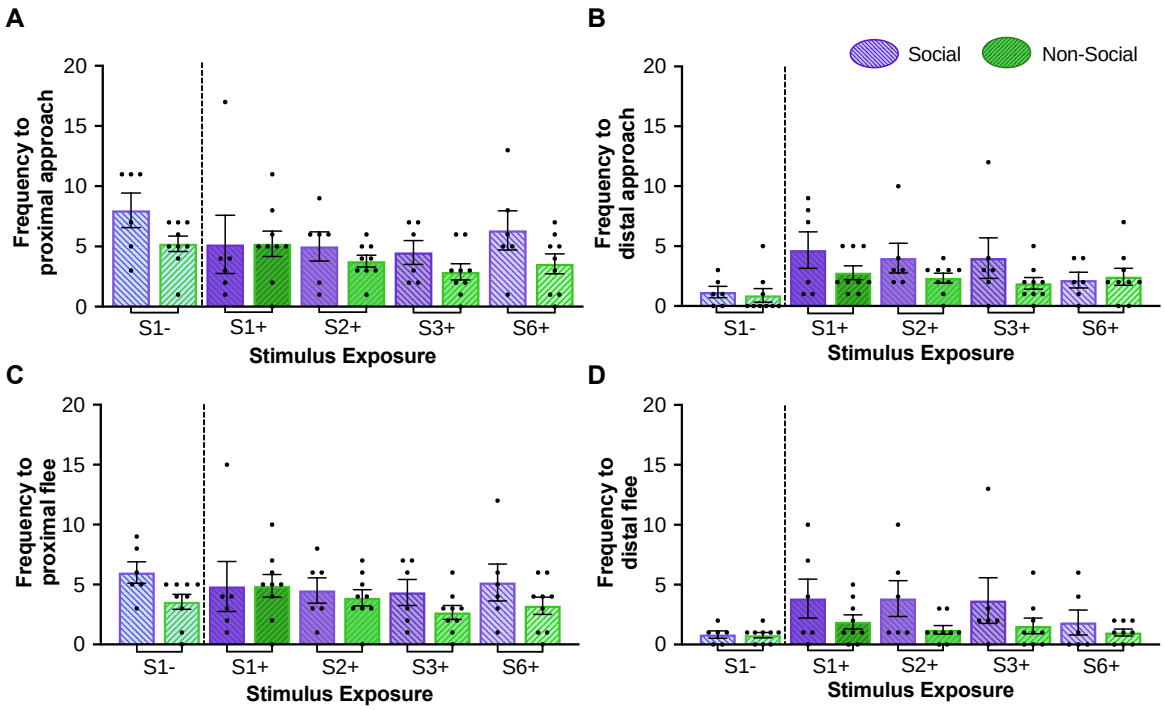



**Figure 3.9: Frequency to approach and flee stimulus during fear extinction.**
Across fear extinction, there was no significant difference between mice conditioned to social or non-social fear in frequency to engage in **A** proximal approach, however for **B** distal approach there was a main effect of stimulus exposure but not for stimulus type. There was no difference across stimulus types or exposure for **C** proximal flee but **D** there was a main effect of stimulus exposure on distal flee. Grey dots represent individual data points for behavioural events. Data are Mean ± SEM.

***Fibre Photometry***

*Approach behaviours*

In the 3s preceding proximal approach of the stimulus, all stimulus types and stimulus exposures had an AUC greater than baseline (*see Appendix B: Table 3. 9*). There was a main effect of stimulus exposure [*F(4, 79.99) = 3.09, p = 0.020*], where interaction contrasts revealed a greater AUC for S6+ compared to S2+ [*t(330.3) = 2.98, p = 0.0031*] and S3+ [*t(329.4) = 2.95, p = 0.0034, Figure 3.10A, B and E*]. There was no main effect of stimulus type [*F(1, 22.67) = 0.16, p = 0.697*] or interaction between stimulus exposure and type [*F(4, 79.99) = 0.32, p = 0.862*].

In the 3s following proximal approach, all conditions continued to have an AUC greater than baseline (*see Appendix B: Table 3. 9*). There was a main effect of stimulus exposure [*F(4, 74.55) = 7.23, p < 0.0001*] and an interaction between stimulus type and stimulus exposure [*F(4, 74.55) = 2.75, p = 0.034, Figure 3.10A, B and E*]; there was no main effect of stimulus type [*F(1, 19.38) = 0.38, p = 0.546*]. AUC of activity in the dLS increased between pre-conditioning (S1-) and S1+ under the non-social condition [*t(33.6) = 2.62, p = 0.013*] and there was a trend toward an increase under the social condition [*t(32.1) = 1.90, p = 0.067*]; the magnitude of increase between S1- and S1+ did not differ significantly between social and non-social conditions [*t(32.7) = 0.23, p = 0.817*]. The AUC of dLS activity following proximal approach of social stimuli was greater during S1+ than S2+ [*t(332.9) = 3.44, p = 0.0007*], S3+ [*t(331.5) = 4.53, p < 0.0001*] and S6+ [*t335.8) = 4.91, p < 0.0001*]. However, in mice conditioned to non-social stimuli there was no difference between S1+ and S2+ [*t(330.1) = 1.37, p = 0.172*], S3+ [*t(330.9) = 1.20, p = 0.231*] and S6+ [*t(333.5) = 1.56, p = 0.121*]. Pairwise comparisons reveal, an increased AUC in dLS activity in social and non-social stimuli at S1+ compared to S3+ [*t(331.2) =*

*2.53, p = 0.012*] and S6+ *t(334.8) = 2.57, p = 0.011*), however not for S2+ [*t(334.8) = 2.57, p = 0.084*]. Finally, we did not analyse the AUC for the dLS signal for distal approach (*Figure 3.10C-D)* as outlined in the Methods (*see Section 3.2.4*).

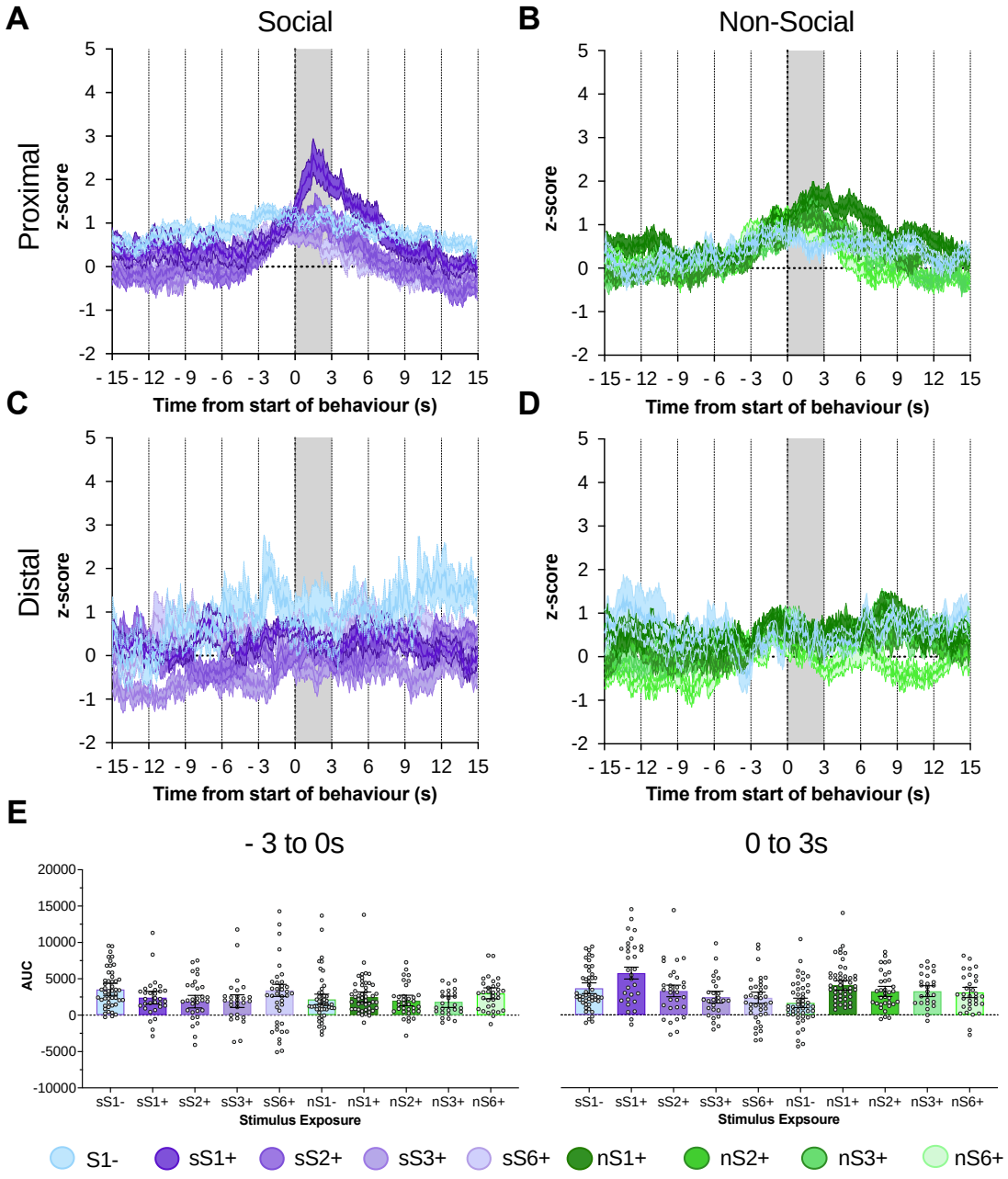



**Figure 3.10: Proximal and distal approach during extinction of social and non-social conditioned fear.**

Mice underwent social- (SFC) and non-social fear conditioning (nSFC) and received foot shock upon investigation of a novel social or non-social stimulus. The next day, mice were attached to the patch cord and presented six novel social or non-social stimuli to examine

fear extinction. Data presented here are pre-conditioning baseline of social and non-social behaviour (S1-) and the first three stimulus exposures post-conditioning (S1+ to S3+). **A-B** In the 3s preceding and proceeding proximal approach of the stimulus, all conditions and stimulus exposures had an area under the curve (AUC) greater than baseline. In the 3s prior to proximal approach, the AUC was greater during S6+ compared to S2+ and S3+. In the 3s after the onset of proximal approach, the AUC at S1+ was significantly greater than S1-. Across extinction (S1+ to S6+), mice demonstrated a reduction in AUC following onset of proximal approach to the social stimulus, a trend not seen in after in mice after approaching the non-social stimulus. **C-D** There were no discernible peaks in dLS activity immediately before or after distal approach of stimuli and thus we did not perform AUC analysis. **E** The AUC for the 3s preceding and proceeding proximal stimulus approach by stimulus exposure (S1-, S1+ to S6+) and stimulus type (social, purple and non-social, green). Grey dots represent individual proximal approach events.

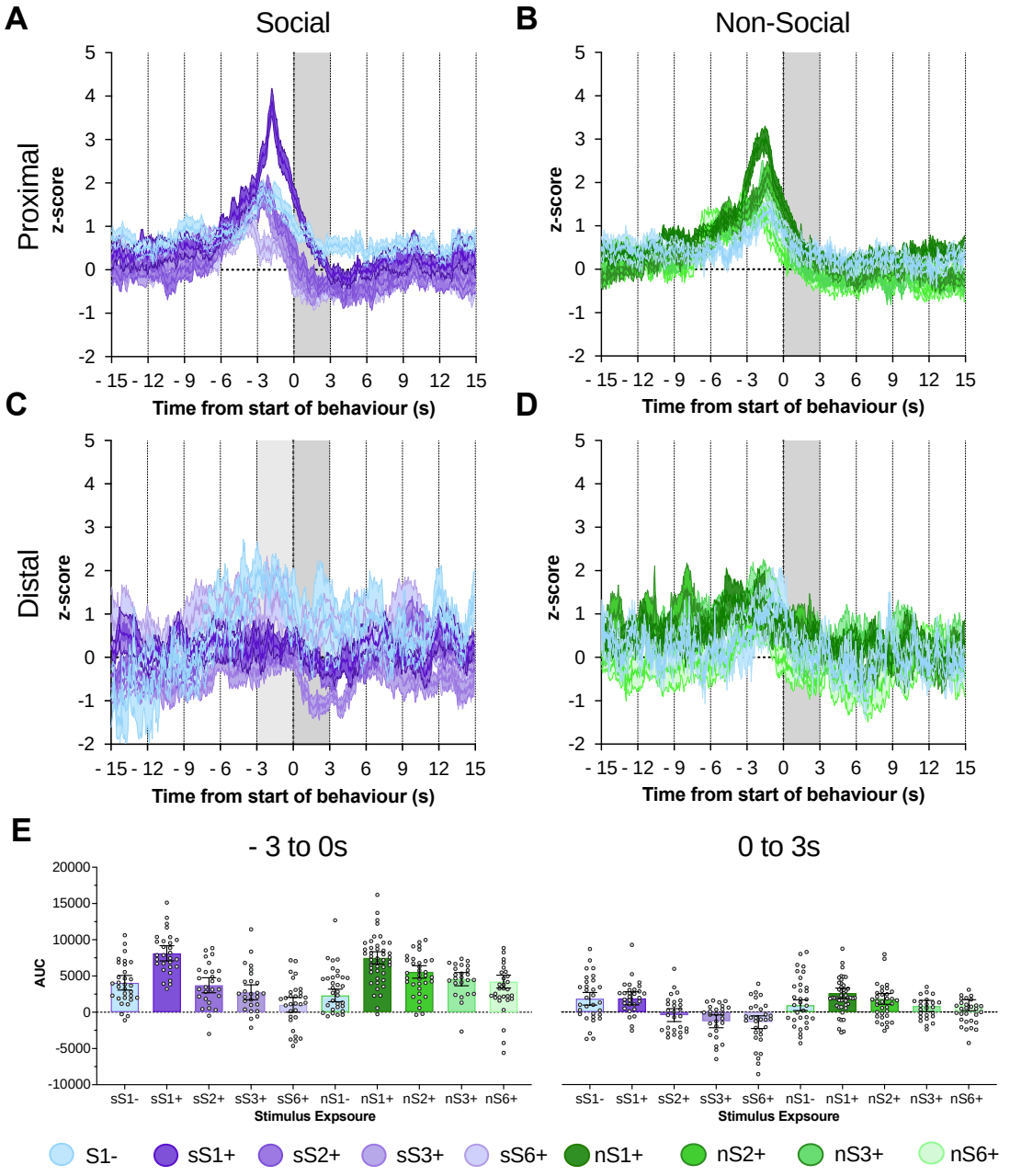
*Flee behaviours*

In the 3s preceding proximal flee from a novel stimulus, all stimulus exposures had an AUC greater than baseline except for S6+ in the social condition (*see Appendix B:Table 3. 10*). There was a main effect of stimulus exposure [$F(4, 290.42) = 39.74, p < 0.001$] and an interaction between stimulus type and stimulus exposure [$F(4, 290.42) = 9.49, p < 0.001$]; there was no main effect of stimulus type [$F(1, 12.58) = 0.09, p = 0.772$, *Figure 3.11A, B and E*]. AUC was greater for first stimulus exposure post-conditioning (S1+) compared to when unconditioned (S1-) in both social [$t(295.3) = 5.77, p < 0.001$] and non-social [$t(295.7) = 8.28, p < 0.001$] stimulus type, and the increase was of a similar magnitude [$t(295) = 9.85, p < 0.0001$]. Social stimulus exposures sS2+ had an AUC similar to pre-fear conditioning (sS1-; $t(294.3) = 0.68, p = 0.498$), whereas the AUC at sS3+ [$t(296.1) = 2.06, p = 0.040$] and sS6+ [$t(295.3) = 4.9, p < 0.0001$] was lower than at sS1-. In contrast, the AUC during non-social stimulus exposures nS2+ [$t(292.5) = 5.03, p < 0.0001$], nS3+ [$t(292.7) = 3.18, p = 0.002$], and nS6+ [$t(295.1) = 2.77, p = 0.006$] remained greater than pre-fear conditioning (nS1-). The magnitude of the differences in AUC of the signal preceding a proximal flee between S1- and S2+ [$t(293) = 3.97, p = 0.0001$], S3+ [$t(294) = 3.71, p = 0.0002$] and S6+ [$t(295) = 5.31, p < 0.0001$] differed

significantly between the social and non-social conditions. During fear extinction (S1+ to S6+), whilst the AUC decreased from S1+ to S2+ [*t(288) = 2.70, p = 0.007*], S3+ [*t(288) = 2.55, p = 0.011*] and S6+ [*t(289) = 4.11, p < 0.001*] for both social and non-social stimulus types, the magnitude of the decrease was greater for social compared to non-social stimulus type (S2+; *t(288) = 6.96, p < 0.*0001, S3+; *t(288) = 8.64, p < 0.*0001, and S6+; *t(289) = 11.16, p < 0.0001*).

In the 3s following proximal flee, AUC was greater than baseline during social stimulus exposures sS1- and sS1+, and during non-social stimulus exposures nS1+ and nS2+ (*see Appendix B: Table 3. 10*). There was a main effect of stimulus exposure [*F(4, 290.30) = 14.27, p < 0.001*] and an interaction between stimulus exposure and stimulus type [*F(4, 290.30) = 6.12, p < 0.001*]; there was no main effect of stimulus type [*F(1, 12) = 1.28, p = 0.280*]. There was no difference in AUC between sS1- and sS1+ [*t(295.9) = 0.01, p = 0.995*], whereas AUC for nS1+ was greater than nS1- [*t(296.3) = 3.10, p = 0.002*], and these differences differed significantly [*t(296) = 2.09, p = 0.*038]. AUC was significantly lower than sS1- during sS2+ [*t(294.7) = 4.03, p = 0.0001*], sS3+ [*t(296.7) = 5.30, p < 0.0001*], and sS6+ [*t(295.8) = 5.86, p < 0.0001*], whereas there was no significant difference between nS1- and nS2+ [*t(292.7) = 1.57, p = 0.117*], nS3+ [*t(293.0) = 0.19, p = 0.848*] or nS6+ [*t(295.6) = 0.004, p = 0.996*] and these differences differed significantly (all *p < 0.001*).

Peak analysis was conducted for conditioned (S1+) versus unconditioned (S1-) for proximal stimulus flee events, given the clear increase in AUC preceding proximal fleeing in conditioned mice, and that this increase was most pronounced during S1+. A peak in calcium signalling was 20 times more likely to be automatically detected prior to a proximal flee event in conditioned mice compared to unconditioned mice (*OR = 20.41, p = 0.006, Figure 3.11F*) and this did not differ as a function of stimulus type (*p = 0.203*).




**F**

| Stimulus | SFC Condition | |
|---|---|---|
| | *Unconditioned (S1-)* | *Conditioned (S1+)* |
| ***Social (s)*** | 61.1% | 96.9% |
| ***Non-Social (n)*** | 65.6% | 88.6% |

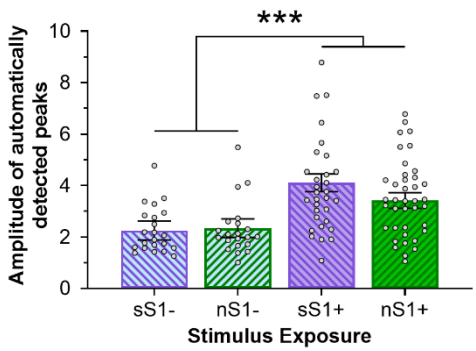


**Figure 3.11: Proximal and distal flee during extinction of social and non-social conditioned fear and proximal flee peak analysis.**
Mice underwent social- (SFC) and non-social fear conditioning (nSFC) and received foot shock upon investigation of a novel social or non-social stimulus. The next day, mice were attached to the patch cord and presented six novel social or non-social stimuli to examine fear extinction. Data presented here are pre-conditioning baseline of social and non-social behaviour (S1-) and the first three stimulus exposures post-conditioning (S1+ to S3+). **A-B** AUC was greater for first stimulus exposure post-conditioning (S1+) compared to when unconditioned (S1-) in both social and non-social conditions. Social stimulus exposures sS2+ had an AUC similar to pre-fear, whereas the AUC at sS3and sS6+ were lower than at sS1-. In contrast, the AUC during non-social stimulus exposures nS2+ to nS6+ remained greater than pre-fear conditioning (nS1-). During fear extinction (S1+ to S6+), whilst the AUC decreased from S1+ to S6+ for both social and non-social stimulus types, the magnitude of the decrease was greater for social compared to non-social stimulus type. There was no difference in AUC between sS1- and sS1+, whereas AUC for nS1+ was greater than nS1-. **C-D** There were no discernible peaks in dLS activity immediately before or after distal flee of stimuli and thus we did not perform AUC analysis. **E** The AUC for the 3s preceding and proceeding proximal stimulus approach by stimulus exposure (S1-, S1+ to S6+) and stimulus type (social, purple and non-social, green). In the 3s preceding proximal flee of the stimulus, all stimulus types and stimulus exposures had an area under the curve (AUC) greater than baseline except for S6+, however in the 3s proceeding proximal flee only social S1- and S1+ stimulus types and non-social S1+ and S2+ had AUC greater than baseline. **F** Peak analysis was conducted for conditioned (S1+) versus unconditioned (S1-) for proximal stimulus flee events. Results showed a peak in calcium signalling was 20 times more likely to be automatically detected prior to a proximal flee event in conditioned mice compared to unconditioned mice regardless of stimulus type. **G** The amplitude of automatically detected peaks in dLS activity preceding proximal stimulus flee was significantly greater for conditioned compared to unconditioned mice, irrespective of stimulus type. Data are Mean ± SEM and $\alpha$ is 0.05, grey dots represent individual proximal flee events and ***$p < 0.001$.

Furthermore, the amplitude of automatically detected peaks preceding proximal stimulus flees was significantly greater in conditioned animals [*$F(1, 24.12) = 17.90$, $p = 0.0003$, see Figure 3.11G*] irrespective of stimulus type [*$F(1, 24.12) = 0.73$, $p = 0.400$*] and no interaction between stimulus type and stimulus condition [*$F(1, 24.12) = 1.26$, $p = 0.273$*] was found. Finally, we did not analyse the AUC for the dLS signal for distal flee (*Figure 3.11C-D*) as outlined in the Methods (*see Section 3.2.4*).

*Other stimulus and non-stimulus behaviours*

We did not run statistical analysis on AUC for the dLS signal for the disengagement and re-engagement of the stimulus, as only pre-conditioned mice in the social exposure demonstrated this behaviour. Further, the photometry signal for mice engaged in non-stimulus directed behaviour e.g. grooming and exploring of test arena, did not show any significant peaks in 3s window before or after the onset of the behaviour. There was a peak in dLS activity preceding the 3s measurement window however, this is likely due to flee behaviour which typically occurs immediately preceding corner immobility and vigilance. Thus, only the 3s after window is relevant, however no significant peaks in activity were observed and so AUC analysis was not performed.

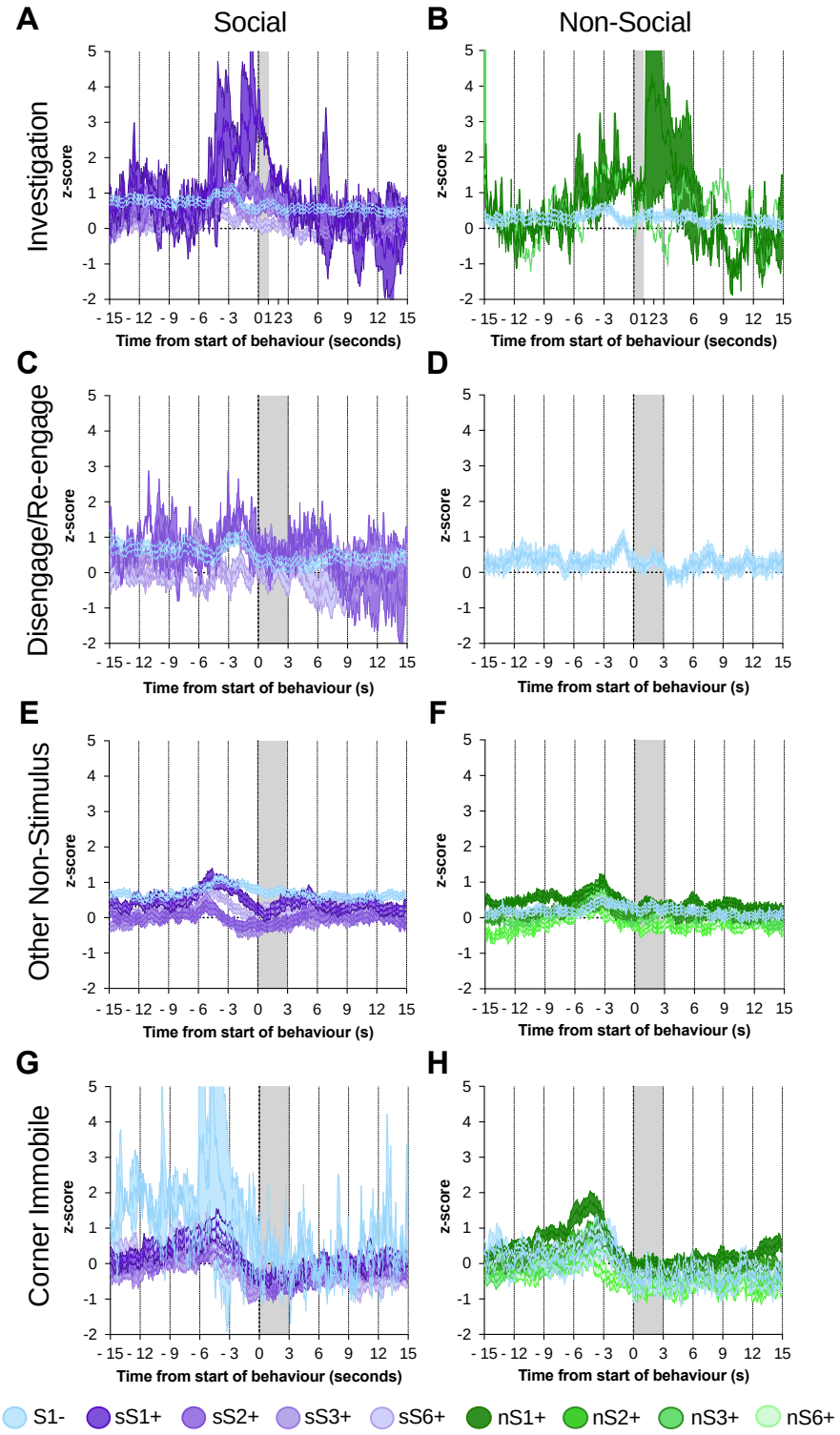



**Figure 3.12: dLS activity for stimulus investigation and other stimulus and non-stimulus directed behaviours.**

To examine which specific approach and avoidance behaviours were associated with changes in dLS activity compared to baseline we manually scored a total of eight behaviour. For **A-B** stimulus investigation, we removed all data that occurred in less than 1 second to remove events where a peak in dLS signal related to proximal stimulus flee and not investigation of the stimulus. This left very few events for analysis in all conditions except

for S1-, thus we did not perform area under the curve (AUC) analysis for the dLS signal. **C** We did not analyse the AUC for the dLS activity for disengagement and re-engagement of the stimulus as there were very few instances of the behaviour in conditioned mice and **D** no mice conditioned to non-social stimuli engaged in this behaviour after conditioning. **E-F** For non-stimulus directed behaviours e.g. grooming, rearing and **G-H** time spent immobile in the corner, no behaviour had discernible peaks in dLS activity in the 3s preceding or proceeding the onset of the behaviour and so additional analysis was not pursued.

### *Extinguishers and non-extinguishers following social fear conditioning.*

Within social fear conditioned animals, we observed variance between stimulus exposures S1+ to S6+ which indicated that not all mice conditioned to social stimuli had similar extinction profiles (*see Figure 3.13A-C*). In Experiment 2, we observed the most prominent dLS signal preceding proximal stimulus flee. Thus, for follow up analysis of extinguishers versus non-extinguishers, we analysed the dLS signal for the 3s preceding and proceeding proximal stimulus flee only.

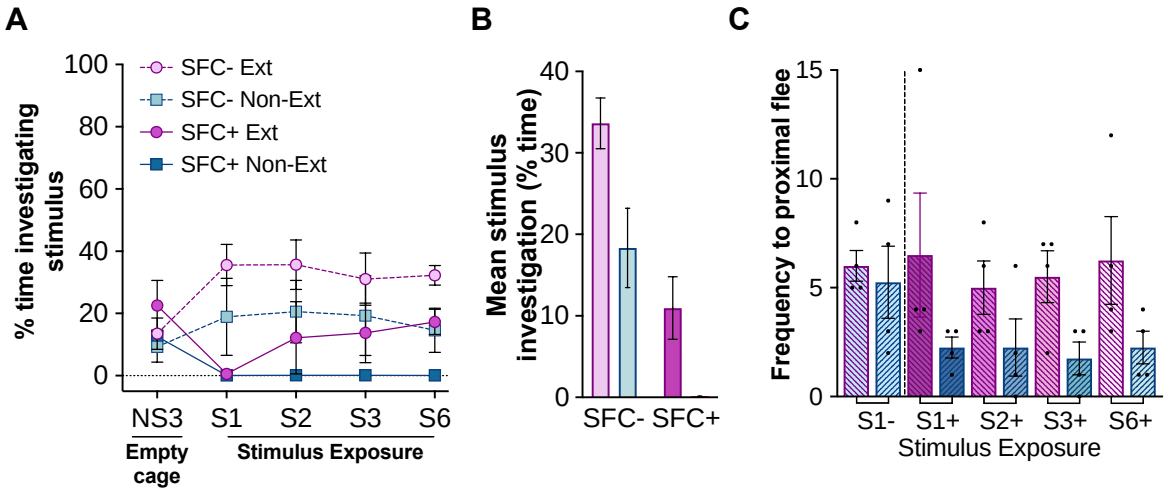



**Figure 3.13: Stimulus investigation and frequency to proximal flee the stimulus in social fear conditioned extinguishers versus non-extinguishers.**
Mice who underwent SFC and extinction were divided into two subgroups: extinguishers (Ext) and non-extinguishers (Non-Ext). **A-B** Highlights that extinguishers spent more time at S6+ investigating the social stimulus than non-extinguishers and also spent less time interacting when tested without having undergone SFC (SFC-). **C** Non-extinguishers also made fewer proximal flees, grey dots represent individual data, (n =8, 4 extinguishers).

In the 3s preceding proximal stimulus flee all conditions and stimulus exposures had an AUC of dLS activity greater than baseline except for extinguishers at S6+ (*see Appendix B: Table 3.11*). There was a main effect of stimulus exposure [*F(4, 34.37) = 10.64, p < 0.001*] and trend toward an interaction between stimulus exposure and extinction phenotype *[F(4, 34.37) = 2.59, p = 0.054, Figure 3.14A-B*]. Follow-up comparisons revealed there was no difference in photometry signal at S1+ between the extinguishers and non-extinguishers [*t(16.0) = 0.564, p = 0.581*]. However, the reduction in signal from S1+ to S6+ was significantly greater for the extinguishers compared to the non-extinguishers [*t(155.4) = 3.15, p = 0.002, Figure 3.14C*].

In the 3s seconds proceeding proximal stimulus flee from the social stimulus, no condition had AUC of dLS activity greater than baseline except for S1+ (*see Appendix B: Table 3.11*). There remained a main effect of stimulus exposure [*F(4, 35.33) = 4.24, p = 0.007*] but there was no main effect of extinction condition [*F(1, 10.30) = 0.53, p = 0.48*] nor an interaction between stimulus exposure and extinction condition [*F(4, 35.33) = 2.28, p = 0.080*].

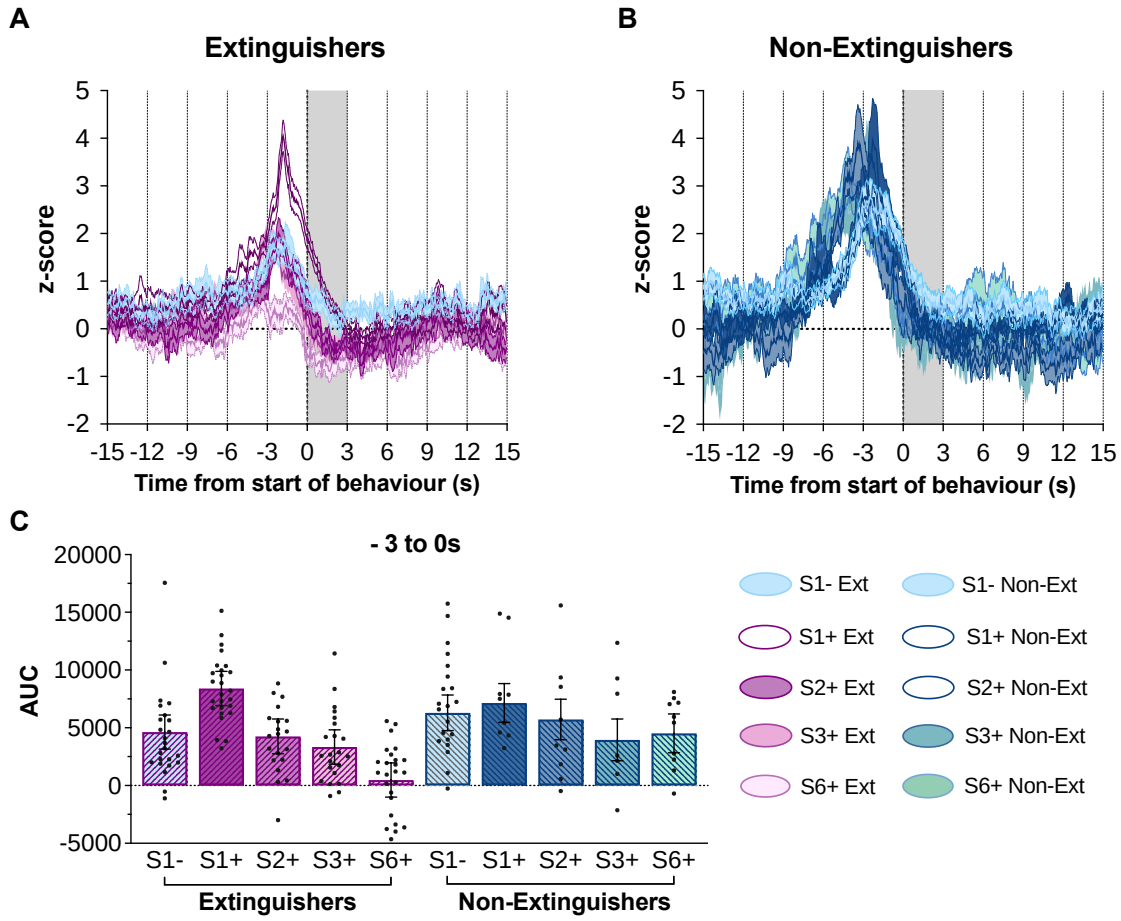



**Figure 3.14: dLS activity preceding proximal flee in extinguishers versus non-extinguishers during social fear extinction.**

Following the completion of behavioural testing, we observed that within the social fear conditioned group there was a subset of mice which spent less than 1% time investigating the social stimulus at each stimulus exposure across extinction. In post-hoc analysis, we examined the dLS activity in those sensitive to social fear extinction, referred to as extinguishers (Ext), and those which demonstrated persistent avoidance of social stimuli, referred to as non-extinguishers (Non-Ext). **A-B** there was no difference in photometry signal at S1+ between the extinguishers and non-extinguishers however, the reduction in signal from S1+ to S6+ was significantly greater for the extinguishers compared to the non-extinguishers. **C** In the 3s preceding flee, all extinction phenotypes and stimulus exposures had an AUC for dLS activity greater than baseline (defined as the 95% CI not containing 0) except for S6+ extinguishers. From S1+ to S6+, there was a significantly greater reduction in dLS signal in extinguishers compared to non-extinguishers whose dLS signal remain elevated across stimulus exposures. Data are Mean ± SEM, grey dots represent individual data values for each stimulus exposure within each extinction phenotype. N = 8 (4 extinguishers).

## 3. 4. Discussion

The LS is increasingly implicated in the co-ordination of social anxiety-like and fear related behaviours. Its role as a relay centre in the integration of external stimuli coupled with regulation of internal emotional states ideally positions the LS to play an integral role in social fear and avoidance behaviours (Menon et al., 2022). This study aimed to investigate the role of the dLS, using fibre photometry, in social anxiety-like behaviours using the SFC behavioural paradigm. To establish dLS activity under non-fearful conditions, we demonstrated in Experiment 1a that significant activity in the dLS was only observed preceding fleeing of an opposite-sex social stimulus. Using fibre photometry together with the SFC task within the same cohort of mice, we observed increased activity in the dLS preceded proximal stimulus flee behaviour and this signal reduced alongside the extinction of social fear, i.e. mice had the greatest increase in dLS activity, compared to baseline, during the first stimulus exposure post-conditioning (S1+), which normalised to pre-conditioning levels over extinction. Following up our findings, the aim of Experiment 2 was to reliably reproduce the results in a separate cohort of mice and determine if the signal in the dLS was social specific. Consistent with Experiment 1, we demonstrated that increased dLS activity preceded proximal stimulus flee in conditioned mice. Interestingly, increased activation of the dLS preceding stimulus flee was present during extinction in mice that underwent instrumental fear conditioning to a social or non-social stimuli, with no difference in the magnitude of the signal at S1+, indicating the dLS signal detected is associated with both social and non-social stimulus avoidance. However, whilst socially fear conditioned mice demonstrate an extinction of social fear, mice conditioned with scented tennis balls did not. Further, upon deeper inspection of mice within the SFC group in Experiment 2, we identified a subset of mice that showed evidence of at least some fear extinction, referred to as *extinguishers*, and a

subset which showed no social fear extinction, referred to as *non-extinguishers*. Extinguishers showed a significant reduction in dLS activity over extinction whereas non-extinguishers did not, consistent with the reduction in dLS activity alongside extinction of conditioned social fear observed in Experiment 1b. Together these studies demonstrate a novel role of the dLS in specific fear and avoidance behaviours that is not social-specific.

The most prominent findings of the present study were 1) the identification of elevated activity in the dLS preceding proximal stimulus flee only, 2) that this behaviour occurred irrespective of whether the stimulus was social or non-social and 3) that sustained activation of the dLS correlated with continued stimulus avoidance in those conditioned to non-social stimuli and those which were social fear non-extinguishers. Using fibre photometry, we achieved one of our primary aims and established which specific approach and avoidance behaviours were associated with changes in dLS activity during fear extinction, identifying proximal stimulus flee. In Experiment 2, with the added inclusion of distal flee, defined as stimulus flees which were initiated in quadrants Q1 to Q3, we were able to demonstrate that elevated dLS activity only occurred prior to stimulus flees initiated in the quadrant containing the stimulus (Q4, *see Figure 3.3*). This indicates that proximity is an important determining factor in initiating the fear response. Evidence in support of this finding comes from the clinical and pre-clinical literature. For example, people with SAD tend to feel more comfortable maintaining greater distances from unfamiliar people and perceiving them as closer than they are - which is associated with the increased avoidance behaviour seen in SAD (Givon-Benjio et al., 2020). Examination of sympathetic activation via skin conductance during *anticipation* of certain versus uncertain threats, found people with SAD had indiscriminate sympathetic activation of threat and safety cues (Evans et al., 2019). Further, those with SAD had greater sympathetic activation of proximal and certain threats, as opposed to distal and uncertain,

in line with mobilisation of reactive avoidance behaviours (Low et al., 2008; Low et al., 2015). In healthy participants, Faul et al. (2020) found proximal versus distal threats differentially engaged distinct neural circuits during the acquisition of fear whereby *near* threats activated reactive circuits e.g. anterior midcingulate and thalamus, greater than *far* threats which activated cognitive circuits e.g. amygdala, hippocampus, and ventromedial PFC. Finally, a study in rats explored the role of socially signalled imminent versus remote threat in differentially co-ordinating defence behaviours (Andraka et al., 2021). When an observer directly observed a cage mate receive foot shocks i.e. imminent danger, observer rats engaged in more freezing and less exploration behaviour, as opposed to when cage mates where administered foot shocks out of sight, which did not elicit the same response. Similar to Faul et al. (2020), the authors showed that these two types of threat responses recruited distinct neural circuits, in this instance different populations of CeA neurons (Andraka et al., 2021). These findings together with our own in Experiment 1b and 2, indicate that across species, proximity is likely a determining factor of which defensive behaviours are engaged (e.g. freeze or flee), and may differentially recruit different brain regions depending on the assessment of level of immediate threat.

Another key finding of our study is that the elevated dLS activity which precedes proximal flee from the stimulus is not social-specific. When closely replicating our SFC and extinction protocol in Experiment 2 with the added inclusion of a nSFC and extinction group to examine non-social fear, we demonstrated that in the absence of prior social testing in the extinction arena (*as seen in Experiment 1a*), there was indeed an even greater magnitude of activity in the dLS preceding proximal stimulus flee. Moreover, the elevated dLS signal was present when mice were conditioned to fear interacting with social or non-social stimuli. Additional peak analysis further supported this finding by demonstrating peaks in calcium signalling were substantially more likely to be detected prior to proximal

flee events in conditioned mice compared to unconditioned mice, and the amplitude of these automatically detected peaks was considerably greater irrespective of stimulus type. In light of these findings, it raises the question as to whether proximal stimulus flee in this context is, in fact, at least two distinct behaviours. One behaviour, associated with a preceding peak in calcium and substantially more likely in fear conditioned animals, a genuine fleeing of the stimulus; and the other, associated with no peaks in dLS activity and more likely in unconditioned animals, a disengagement from the stimulus unrelated to fear. Based on the prior literature using the SFC task, there was strong evidence in favour of OXTR-expressing GABAergic neurons playing an important role in driving extinction of social fear (Menon et al., 2018; Zoicas et al., 2014). Increased levels of oxytocin, via i.c.v local infusion (Zoicas et al., 2014) or when naturally occurring in lactating females (Menon et al., 2018), reduced social fear during extinction. Given this, we hypothesised that following SFC, mice would exhibit inhibition of the dLS during the first stimulus exposure post-conditioning (S1+), with activity increasing over the course of extinction (S2+ to S6+). In contrast to our hypothesis, here we demonstrated activation of the dLS was strongest at S1+ and reduced over subsequent stimulus exposures, with pronounced reduction of the signal only apparent when there was extinction of avoidance behaviour.

There are several possible explanations for the observed pattern of dLS activity during fear extinction. One possibility is that a non-oxytocin pathway may be dominant during S1+ before later inhibition of this pathway, perhaps by the previously identified oxytocin circuit (Menon et al., 2018; Zoicas et al., 2014), during active social fear extinction. It is well established that LS neurons are predominantly GABAergic (Besnard et al., 2019; Besnard & Leroy, 2022; Zhao et al., 2013), with only a subset of glutamatergic neurons in the more ventral LS (Vega-Quiroga et al., 2018). Thus, increased activation of the dLS preceding stimulus flee behaviour may indicate silencing of

GABAergic neurons through either upstream subcortical modulatory inputs or laterally within the LS.

In beginning to unravel potential circuits involved in the co-ordination of fleeing an instrumentally conditioned stimulus, a number of specific pathways emerge as candidates; most notably cell-type specific involvement of LS expressing-neurotensin ($LS^{NT}$) neurons, corticotrophin-related pathways, and hippocampal projections to the LS. Following acute restraint stress, Azevedo et al. (2020) observed increased activity in the LS, in particular in $LS^{NT}$ neurons. Subjecting mice to different forms of environmental stress, the authors identified a role of $LS^{NT}$ neurons in food intake suppression, tail suspension, experimenter mediated immobilisation, contextual fear learning and predator escape. In doing so, they established a role of $LS^{NT}$ in escape behaviours in response to "predator" stress and chemogenetic activation of $LS^{NT}$ neurons. The presence and activity of this cell population represents a strong candidate in the initial fear response we observed in S1+ in social and non-social fear conditioned animals. Another study, which employed CSDS, found susceptible mice i.e. those more avoidant during social interaction tasks, had higher activation in $LS^{NT}$ compared to resilient mice (Li et al., 2023). This might explain why in Experiment 2 some mice demonstrated reduced proclivity towards fear extinction and remain avoidant across stimulus exposures as seen in social fear conditioned non-extinguishers and those conditioned to non-social stimuli.

Another candidate cell-type specific modulator of the LS activity during social fear extinction is corticotrophin-releasing factor (CRF). In contextual fear learning study, Hashimoto et al. (2022) showed that mice presented with an unconditioned auditory tone (i.e. tone not paired with a foot shock) will at first demonstrate elevated dLS activity. However, as the auditory tone is successively not paired with a negative outcome, the dLS response habituates with repeated unreinforced presentations (Hashimoto et al., 2023). In

contrast, when mice were presented, a conditioned tone from the start, dLS activity increased across trials. This is relevant to our study, as whilst mice have previously been conditioned to pair stimulus investigation with a mild foot shock, when they repeatedly attempt to engage and the stimulus investigation is no longer negatively reinforced, the dLS signal decreases. Moreover, in this same study, authors recorded exclusively from type 2 corticotropin-releasing hormone releasing hormone expressing LS ($LS^{Crhr2}$) neurons though the use of Cre-dependent GCaMP6s, supporting a role for Crhr2 in mediating social fear behaviours via the dLS (Hashimoto et al., 2023). However, these circuits seem to apply more to the acquisition and initial fear response suggesting another pathway might be involved in the active extinction of social fear. Together with the results from Chapter 4, cortical and sub-cortical pathways associated with fear extinction, including whether previously described oxytocin circuits may be involved, will be explored in Chapter 5.

In terms of stimulus investigation there are two notable findings which need to be discussed: our observation of elevated tonic activity in the dLS during S1+ in Experiment 1b and evidence of a limited role of the dLS in stimulus investigation in the stimulus interaction task (Experiment 1a) and in SFC- mice (Experiment 2). In Experiment 1b, we observed elevated dLS activity during stimulus investigation. It is unlikely this tonic activity is an artefact of signal drift. Our defined baseline window was between NS3 and S1+, therefore if we had observed signal drift, it would have more likely occurred during late stimulus exposures S3+ and S6+ rather than during S1+ stimulus exposure. Thus, a potential explanation for the elevated tonic activity observed during S1+ might be that the dLS is primed, perhaps to facilitate a stimulus flee where the notable peak in activity was observed. To put it differently, the elevated tonic activity may facilitate the dLS signal reaching a threshold, which, when exceeded, may result in subsequent fleeing of the stimulus. However, in contrast to this theory is the important consideration that we are

recording a number of behaviours which follow on from each other in close succession. As an example, a subject attempting to engage in social investigation of a conditioned stimulus will likely first approach the stimulus, initiate investigation and immediately proximal flee – all behaviours occurring in rapid succession. Thus, the elevated baseline we observed, and likely elevated dLS activity in behaviours such as disengage/re-engage, a behaviour which is flanked by stimulus investigation, likely involved the capture of other stimulus directed behaviours during the 3s window either side of the onset of the recorded behaviour.

Evidence in the literature suggests the dLS is involved in stimulus investigation and novel versus familiar social preference, a finding not replicated in the present study. Recording the activity of dLS neurons using GCaMP6f in a control experiment, albeit more rostrally than our own study, a recent study showed increased activity in the dLS following social interaction in mice (de Leon Reyes et al., 2023). In addition, the authors demonstrated that while mice spent more time with novel stimuli, they demonstrated greater peak amplitude in the dLS during familiar rather than novel same-sex interaction (de Leon Reyes et al., 2023). On top of the role of CRH in threat learning and perception (Chudoba & Dabrowska, 2023; Hashimoto et al., 2023), de Leon Reyes et al. (2023) demonstrated that activity in the dLS in response to novelty was modulated by CRH-expressing infralimbic (IL) neurons projecting to the dLS. A potential explanation for why we did not see greater dLS activation during stimulus investigation in Experiment 1a, may lie in our use of novel rather than familiar social stimuli. This could be easily examined in future studies through the inclusion of a non-surgical cage mate prior to social isolation or through examination of the dLS signal upon repeated presentation of the same social stimulus.

A key objective of the present study was to explore if activity in the dLS was social specific. We observed that, following instrumental fear conditioning, mice conditioned to social and non-social stimuli had similar, significantly elevated activity in the dLS. However, whilst in SFC+ mice this signal reduced across extinction (Experiment 1b and 2), in mice conditioning to non-social stimuli we did observe a reduction of dLS activity or an extinction of conditioned fear. An important consideration in interpreting these findings is the role of motivation in extinguishing fear of non-social stimuli. Overcoming social fear towards conditioned social stimuli is arguably more important that overcoming fear of a non-social stimulus that offers little survival advantage as learning to disassociate negative valence towards non-harmful social stimuli is important in maintaining social systems and survival (Krishnan, 2014; Mobbs & Kim, 2015). However, extinguishing fear to an inedible, inanimate object may not hold high motivational value. Beyond its established role in social behaviour, the dLS is involved in diverse systems of reward from drug seeking, promoting feeding and foraging (Azevedo et al., 2020; Gabriella et al., 2022) via projections which support memory retrieval for food and water rewards (Decarie-Spain et al., 2022). This overlap of ethologically diverse reward systems ideally positions the dLS to evaluate value-weighted context-cues to integrate and assess the cost and benefit of specific behaviours (Wirtshafter & Wilson, 2021). To this end, extinguishing fear towards a previously encountered, non-rewarding object does not hold high value; whilst scented, the tennis ball cannot be consumed nor actively interacted with, extinction of avoidance of the tennis ball thus holds lower value than extinction of social fear. This corresponds with findings in our study, which showed that nSFC+ mice do not increase frequency to engage in investigation or approach of novel stimuli across extinction (Experiment 2). Thus, the limited fear extinction observed following non-social conditioning may represent an ethologically relevant cost-saving mechanism demonstrated by subject mice.

While exploring sex differences was not a main objective of the present study, in line with promoting more inclusion of females in pre-clinical research to aid in improved translational outcomes, we included equal number of sexes across experiments. In Experiment 1A, we identified sex differences in specific approach and avoidance behaviours under non-fearful conditions where females demonstrated greater activation in the dLS prior to and following fleeing from opposite-sex stimuli compared to males. On the other hand, males did not demonstrate AUC greater than baseline when interacting with social or non-social stimuli suggesting in males the dLS may not be involved in stimulus directed behaviours under non-fearful conditions. Given evidence of the role of the LS in pair bonding and mating behaviour (Ophir, 2017; Walum & Young, 2018), we might have expected to see increased activation of the dLS following investigation of opposite-sex stimuli, especially in males. However, many studies which examine mating and aggression use different strains and species and our study was not designed to identify mating-specific behaviours (Khotskina et al., 2023; Sailer et al., 2022). An alternative explanation as to the elevated dLS signal preceding flee behaviour in females not males, may be that males (opposite-sex) are larger and represent a greater potential threat of aggression than female (same-sex) conspecifics. This is consistent with findings from Experiment 1b and 2 where the signal preceding flee was most elevated when the fear was greatest (i.e. first stimulus exposure post-conditioning, S1+). This also indicates that the dLS is involved in fleeing conditioned and unconditioned stimuli, which warrants exploration in future studies.

A limitation of the study is that in Experiments 1b and 2, we were underpowered to continue exploration of potential sex differences in fear and avoidance behaviours. Whilst surgeries were completed on equal number of male and female mice, females were a) more susceptible to losing their head mounts prior to behavioural testing, b) had higher rates of

jGCaMP8f/optic probe misalignment and c) poorer performance in the SFC task resulting in a higher rate of attrition. Given there are established sex differences in fear learning (Day & Stevenson, 2020), future studies which examine cell-type specific pathways in the promotion and suppression of fear using the adapted SFC task would benefit from the inclusion of both sexes with sufficient power to examine sex-specific differences in the acquisition, extinction and maintenance of fear.

Another limitation of the present study is the ability to identify cell types of interest, following fear extinction in unconditioned and conditioned mice. As seen in Zoicas et al. (2014), examination of c-Fos expression post conditioning and extinction in SFC- and SFC+ mice led to the identification of the dLS as a target region of interest. Further, the co-labelling of c-Fos positive cells within the dLS and OXT using OXTR reporter mice, was pivotal in the association of OXT activity in the dLS positively contributing towards fear extinction. Whilst photometry overcomes the poor temporal resolution of immunohistochemistry studies which provide a single snapshot of activity, had mice been sacrificed after behavioural testing, this would have enabled the co-labelling of c-Fos positive cells and candidate neurotransmitters and peptides of interest e.g. NT (Woodworth et al., 2018), Crhr2 (de Leon Reyes et al., 2023; Hashimoto et al., 2023) and GAD65/67 (Zhao et al., 2013). In addition, it would have allowed examination of whether dLS OXT-mediated activity was involved in the extinction of social *and* non-social fear following fear conditioning. Instead, we decided to prioritise clear results from the photometry experiments and create within-subject controls of dLS activity during SFC- and SFC+ extinction, thus our rationale for not examining c-Fos expression and immunolabelling was the lack of a control group. In addition to immunolabelling of target cell-types of interest, to further supplement the finding of this study, additional mono- or transsynaptic antero- and retrograde tracing studies could identify upstream and

downstream targets as seen in studies by Leroy et al. (2018) and Hashimoto et al. (2023). Together, this would support predictions in the literature of either a role of excitatory cortical or subcortical mono and/or polysynaptic inputs into the dLS mediating social behaviour or implicate potential intraseptal pathways to explore. An alternative method would be to employ brain clearing methods coupled with whole brain imaging techniques which would provide an unbiased approach to selection of downstream targets of interest (Molbay et al., 2021; Renier et al., 2016). The inclusion of these tracing and imaging studies would help to guide the direction of future studies in further elucidating the role of the dLS in social fear.

Upon the identification of functional brain regions and circuits of interest, other common methods used to identify pathways involved in fear learning and social behaviours are optogenetics and chemogenetics. These genetic manipulation techniques allow for the reversible and minimally invasive activation or inhibition of specific brain regions of interest with remarkable temporal and spatial specificity (Aldrin-Kirk & Bjorklund, 2019; Kim et al., 2017). Elegant use of these techniques has contributed towards our increased understanding of the pathways involved in a wide range of social behaviours and fear learning (Besnard et al., 2019; Luchkina & Bolshakov, 2018; Sailer et al., 2022). To build on the findings of this study, neuromodulation techniques were used to expand on the results reported in this chapter and are reported in Chapter 4.

First identified as important in aggression and later as anxiolytic, the use of modern techniques has evolved our understanding of the LS in social behaviour. Here, using fibre photometry we revealed increased dLS activity precedes proximal stimulus flee, the signal reduces alongside fear extinction, and the signal is not social-specific. These findings suggest a potential *causal* role of dLS activity in the extinction of social and non-social fear, and specifically in the flee behaviour that is critical to social avoidance. The findings

presented here illuminate not only the role of a specific neurotransmitter, but rather the dynamics involved in the modulation of social fear behaviour. To further elucidate the role of the dLS, inhibition of this identified pathway would reveal the functional characteristics of this subcortical modulator of social behaviour.

# Chapter 4. Investigating the causal role of LS activity in social fear using chemogenetic inhibition

## Overview

In Chapter 3, we established dLS activity preceded proximal flee from social and non-social stimuli indicating a potential *causal* role in the extinction of fear. To probe the relationship between the dLS and fleeing from conditioned social stimuli, we used chemogenetic inhibition to silence the dLS prior to social fear extinction. The aim of the experiment presented was to use designer receptors exclusively activated by designer drugs (DREADDs) to establish a causal relationship between increased activity in the dLS and stimulus flee behaviour. It was hypothesised that dLS inhibition would reduce stimulus flee behaviour and subsequently increase stimulus investigation indicative of social fear extinction. To achieve this, mice underwent intracranial surgery to bilaterally infuse inhibitory DREADDs into the dLS. Following viral expression, mice were fear conditioned and administered the DREADD actuator deschloroclozapine (DCZ) via intraperitoneal (i.p.) injection prior to social fear extinction. Results indicate that dLS inhibition did not increase social investigation in fear conditioned mice; however, we did observe DCZ-mediated modulation of proximal but not distal stimulus flee behaviour. In sum, we find ubiquitous inhibition of the dLS did not significantly increase social investigation but did selective modify stimulus flee behaviour, suggesting multiple circuits may be involved in modulating the range of behaviours that are expressed during social fear and that cell-type specific or more temporally specific modulation of the dLS might be required to establish the causal role of the dLS in social fear.

## 4. 1. Introduction

Learning to adapt and make effective judgements, especially in social situations, is fundamental to everyday decision making and survival. Misinterpretation of potential risks or perceived stressors may be important in the development of anxiety-related disorders (Ramos-Cejudo & Salguero, 2017). Social anxiety alters the decision-making processes involved in weighing up the relative risk versus reward of engaging in or avoiding social situations (Hengen & Alpers, 2021; Richards et al., 2015). Within the pre-clinical literature, the LS is recognised as an important neural relay centre for social behaviour. It integrates incoming cognitive and experience-based information and transmits this information to subsequent downstream executive and motor function regions (Menon 2022). Utilisation of the SFC paradigm, which pairs mild foot shock with investigation of a caged conspecific resulting in social avoidance, has led to the identification of a role for the LS in social fear (Menon et al., 2018; Zoicas et al., 2014). In Chapter 3, we built upon these findings by demonstrating that specific avoidance behaviours are associated with changes in calcium dynamics in the dLS during social fear extinction. Specifically, there was a significant increase in calcium signalling in the dLS preceding instances of mice fleeing the social stimulus, and the magnitude of this dLS signal was more pronounced when the conditioned fear was greatest (i.e. earlier in extinction). Further, it was shown that a reduction in LS activity only occurred in individuals that extinguished social fear, whereas the heightened LS activity persisted in those that did not extinguish. This clear association between LS activity and stimulus avoidance behaviour warrants causal investigation.

Chemogenetic technologies are a valuable tool for selectively manipulating neuronal populations, allowing for better understanding of their functional and behavioural significance (Atasoy & Sternson, 2018; Roth, 2016). This knowledge can then be utilized

in the development of targeted pharmacotherapies for disorders associated with dysfunction in these neuronal populations and brain circuits. Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) enable reversible, remote control of neuronal populations and neural circuits through the insertion of bio-engineered, fluorescently tagged receptors via adeno-associated viruses (AAVs) vectors and subsequent systemic delivery of biologically inert, designer ligands (Aldrin-Kirk & Bjorklund, 2019). Thus, chemogenetic viruses can be used to selectively "switch on" or "switch off" specific brain regions following systemic delivery of a ligand which activates or silences the brain regions for approximately 2 hours, allowing for the examination of behavioural processes which are altered as a result of the neuronal manipulation.

Chemogenetics has been successfully utilised in various behavioural studies to uncover brain regions involved in sociability. In terms of general social behaviour, Horiai et al. (2020) found that excitation of OXTR-expressing GABAergic neurons in the LS was associated with increased sociability in both environmental and genetic mouse models of ASD. In a model of chronic social stress, Li et al. (2023) found chemogenetic inhibition of LS neurons expressing neurotensin ($LS^{NT}$), a neuropeptide associated with threat and fear response, increased social interaction where activation of $LS^{NT}$ neurons reduced social investigation following CSDS. Finally, silencing of hippocampal CA2-LS neurons was found to reduce social aggression in a resident-intruder task (Leroy et al., 2018).

Beyond social behaviour, genetic manipulation tools have also been employed to manipulate hippocampal LS pathways implicated in fear learning and memory (Rizzi-Wise & Wang, 2021; Wirtshafter & Wilson, 2021). Another genetic tool which has been used to identify causal associations between behaviour and neuronal pathways is optogenetics. Similar to chemogenetics, optogenetics involves the introduction of viral vectors into targeted brain regions. However, in the case of optogenetics, these vectors encode light-

sensitive proteins or "opsins". Activation or inhibition of neural activity is achieved via an implanted optic cannula, offering high temporal specificity during behavioural tasks (Kim et al., 2017). For instance, optogenetic inhibition of CA1-LS neurons during non-social fear extinction is found to reduce freezing (Opalka & Wang, 2020). Further, optogenetic silencing of dLS somatostatin neurons, which receive direct monosynaptic inputs from hippocampal CA3 neurons, increased, whereas activation attenuated, contextual fear responses in mice (Besnard et al., 2019). Another study using chemogenetics, found inhibition of ventral CA3-LS caudodorsal (cd), which corresponds to the dLS in mice, potentiated approach towards a learned conflict-eliciting stimulus in a Y-maze task (Yeates et al., 2022), implicating a reduction in the acquired fear and avoidance response. The combined use of these genetic manipulation tools has identified an important role for the LS in social behaviours but also general fear, anxiety, and avoidance. Thus, utilisation of these tools may unveil what role the LS performs in social fear.

If the LS does play a causal role in social avoidance and conditioned social fear does indeed alter social decision-making, then manipulating an essential part of the fear circuitry should influence avoidance behaviour. DREADDs is a tool that can be deployed to improve our causal understanding of the neurobiology of specific social approach and avoidance behaviours. Thus, this experimental chapter aims to investigate the functional characteristics of the LS in mediating social fear and avoidance using inhibitory DREADDs. It was hypothesised that inhibition of the LS would result in reduced flee behaviour and that this would be most pronounced in proximal stimulus flees. Subsequently, it was hypothesised that the reduction in flee behaviour would be associated with an increase in stimulus investigation.

## 4. 2. Methods

### *4. 2. 1. Subjects*

7-week-old *C57BL/6* mice (n=72, 36 female) were obtained from Animal Resource Centre (ARC, WA, Australia). Mice were group housed (3 to 4 per cage) under standard laboratory conditions (12/12h light/dark cycle, light phase 0700 to 1900, 22 ± 2ºC, 50-70% humidity, food, and water *ad libitum*) in transparent IVC cages and weighed twice per week. Subjects were allocated to either Experiment 1 or Experiment 2, with all mice tested between 10 and 13 weeks old (PND 70 to 91). Males were always tested before females and all experiments were performed in the light phase by female experimenters. All procedures were approved by The University of Sydney Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia.

### *4. 2. 2. Materials*

As in Chapter 2 and 3, fear conditioning took place in Med Associates conditioning chambers and extinction in blue, opaque test arenas (*for details see Chapter 2 and Figure 2.1 for experimental setup*). Deschloroclozapine (DCZ, #HB8555, Hello Bio, Bristol, United Kingdom), a potent, selective, and metabolically stable hM4Di muscarinic DREADD actuator. DCZ was dissolved in 0.9% saline and administered via intraperitoneal (i.p.) injection prior to extinction. Based on the pharmacokinetic profile (Nagai et al., 2020), DCZ was administered 15 minutes prior to commencement of extinction to ensure sufficient but more stable exposure by the first social stimulus exposure (S1, ~30 minutes post-injection). Mice received 0.9% saline (vehicle control), 50 µg/kg or 100 µg/kg DCZ.

Experimenters were not blinded to treatment condition during drug administration in the interest of observing potential adverse side effects.

Each extinction trial was recorded using an overhead Swann camera and DeepLabCut was trained to detect and automatically score nose in contact with the stimulus cage behaviour. For approach, flee and other stimulus and non-stimulus behaviours, data were manually scored by an experimenter blind to treatment (Cohen's $\kappa$ = - 0.21) and SFC condition (Cohen's $\kappa$ = 0.92) in line with the behavioural analysis criteria detailed in Chapter 3 (*Section 3. 2. 4, Figure 3.3*).

For behavioural tests, age- and sex-matched *C57BL/6* mice were used as social stimuli. Social stimuli were acclimatised to stimulus cages (14H x 7W x 7L cm clear cage with 1cm round holes on lower half of cage, as in Chapter 3 Experiment 2) for two 25-minute sessions prior to the commencement of testing and used for up to five rounds of testing subject to age in line with the Three R's of Animal Research.

### *4. 2. 3. Procedures*

#### *Surgery*

To examine DCZ-mediated inhibition of the dLS, mice (n = 18, 9 female) were anesthetised with isoflurane (3% induction, 0.7-1.5% maintenance). Mice were given subcutaneous injections of a non-steroidal anti-inflammatory and antibiotic pre-operatively to minimize pain, discomfort, and infection. Mice were bilaterally infused with adeno-associated virus (AAV) expressing the Gi-coupled hM4D DREADD fused with mCherry under the control of the human synapsin (hSyn) promoter (pAAV-hSyn-hM4D(Gi)-mCherry, 50475-AAV8, Addgene, Watertown, MA, USA). Virus (300-350 nl) was infused at rate of 50 nl/min into the dLS (AP: -0.35; ML +0.5; DV -2.7 mm from bregma) using a

glass pipette. The pipette was left in place for ~ 6 to 7 minutes and drawn up at a rate of 0.5 mm/minute to ensure sufficient diffusion and minimize off-target spread. Bone wax was used to fill cranial injection sites and mice were closed using dissolvable sutures. Saline was administered post-operatively and mice were monitored for 1.5 hours before returning to original group-housed home cages. Behavioural testing began at least 4 weeks post-surgery to allow for adequate viral-mediated protein expression.

### *Social Fear Conditioning Paradigm*

A similar SFC protocol to Chapter 2 was used with minor alterations (*see Figure 4.1*). Following social isolation, mice were habituated to scruffing and ventral i.p. injection of 0.9% saline 15 minutes prior to arena habituation (Day 25, 26, 27 and Day 28 during habituation post-conditioning). No changes were made to the social fear conditioning procedure (Day 28). For extinction, control mice in Experiment 1 were administered with saline, 50 µg/kg or 100 µg/kg DCZ (Day 29), In Experiment 2, pAAV-hSyn-hM4D(Gi)-mCherry DREADDs mice were administered saline or 100 µg/kg DCZ across two days (Day 29 and 30).

### *Histology*

To confirm viral placement in the dLS, mice were deeply anesthetized with 30% pentobarbital and transcardially perfused with phosphate buffered solution (PBS) followed by 10% neutral buffered formalin. Brains were extracted, post-fixed with formalin at 4ºC for 24 to 48 hours, washed with PBS and embedded in PBS with 3% agarose. Brains were sectioned using a vibratome (Leica VT1000S) into 50-60µm coronal slices and stored in PBS. Samples were mounted onto glass slides (Corning, NY, USA) and coverslipped using Fluoroshield™ with DAPI (Sigma-Aldrich, North Ryde, NSW Australia). Sections were

imaged on a VS-120 Virtual Slide Microscope (Olympus, Notting Hill. VIC, Australia) and analysed for bilateral expression of immunoreactive signals of hM4Di-mCherry (*see Figure 4.3A*). Mice with unilateral expression were excluded (n=10, 4 female).

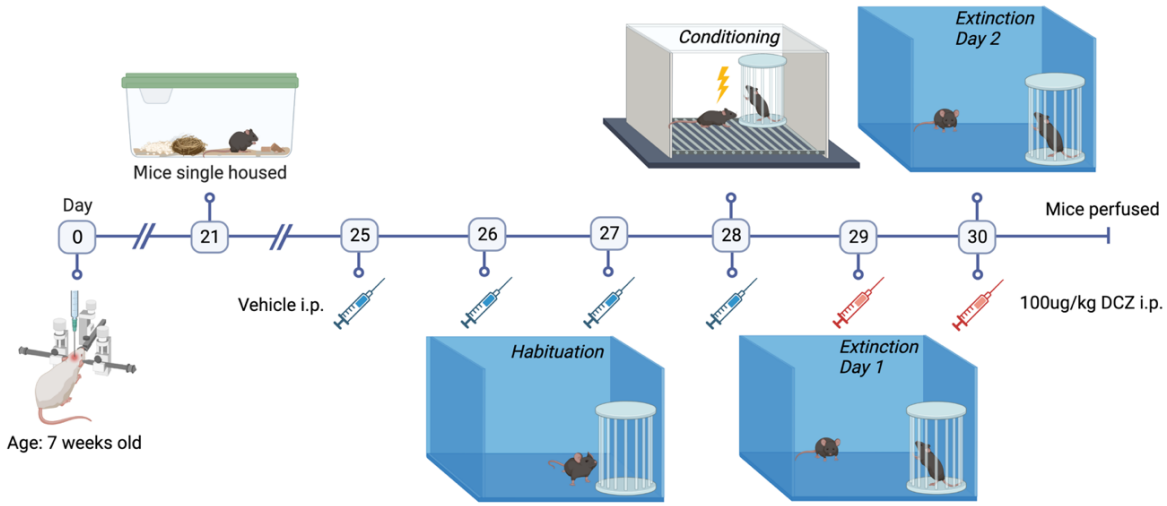



**Figure 4.1: Experimental timeline for DREADDs-Gi mice.**
7-week-old *C57BL/6* mice were anesthetised and bilaterally infused with adeno-associated virus (AAV) expressing the Gi-coupled hM4D DREADD fused with pAAV-hSyn-hM4D(Gi)-mCherry. 4 weeks post-surgery, mice were habituated to scruffing and i.p. injection of 0.9% saline 15 minutes prior to arena habituation (day 25, 26, 27 and day 28 during habituation post-conditioning). On day 28 mice underwent social fear conditioning, receiving between 3 to 4 shocks following investigation of a same-sex conspecific. On day 29 and 30, 15 minutes prior to extinction, mice received vehicle (blue) or 100 µg/kg DCZ (red) via i.p. injection. As a control, mice not expressing inhibitory DREADDs mice followed the same experimental timeline however did not undergo intracranial surgery and only underwent one day of extinction (day 29 only) and were administered vehicle (blue), 50 µg/kg or 100 µg/kg DCZ (red).

### *Statistical Analysis*

Statistical analysis was performed in R version 4.3.1 using packages *tidyr*, *dplyr*, *stringr*, *ggplot2* (Wickham et al., 2019) and *Rmisc* (Hope, 2022) for data visualisation, and *tidyverse* (Wickham et al., 2019), *ggpubr* (Kassambara, 2023a), *rstatix* (Kassambara, 2023b), *afex* (Singmann et al., 2023) and *performance* (Lüdecke et al., 2021) for mixed ANOVA analyses. The dependent variables were time spent exploring the stimulus cage or

social stimulus (% of total trial duration) during each 3-min trial, and the frequency to engage in proximal and distal approach and proximal and distal flees from the social stimulus as defined in Chapter 3 (*see Figure 3.3*). Investigation with the cage or social stimulus was defined as when the nose of the test mouse entered the 20 mm space perimeter of the stimulus cage. While we acknowledge sex as an important biological variable, following histological exclusions the sample was underpowered to assess sex differences. As such, analyses were pooled across sex. Thus, independent variables were DCZ treatment, SFC condition and stimulus exposure. For all comparisons, significance was set at $p < 0.05$ and all data are presented as Mean ± SEM.

## 4. 3. Results

### *4. 3. 1. DCZ does not alter social fear conditioning behaviour in mice not expressing inhibitory DREADDs.*

Prior to beginning behavioural experiments using the DREADD virus, we assessed whether low doses of DCZ administered to control subjects would impact social anxiety-like behaviour during fear extinction. Thus, the aim of the first experiment was to establish a safe and effective dose of DCZ in mice without adversely impacting behaviour.

There was no main effect of DCZ treatment in control subjects not expressing DREADDs [*F(2, 22) = 0.26, p = 0.776,*] and there was no interaction between treatment and stimulus exposure [*F(6.75, 74.28) = 0.50, p = 0.827*], or between treatment, SFC and stimulus exposure [*F(6.75, 74.28) = 0.63, p = 0.725*, *Figure 4.2A*]. Regardless of treatment, SFC+ mice spent significantly less time interacting with the social stimuli compared to SFC- mice *[F(1, 22 ) = 29.65, p < 0.001, Figure 4.2B].* There was no main effect of stimulus exposure *[F(3.38, 74.28) = 0.37, p = 0.797],* but there was an interaction between SFC and stimulus exposure *[F(3.38, 74.28) = 3.01, p = 0.030].* Trend analysis revealed SFC- mice showed a linear decrease in sociability over the six stimulus exposures *[t(22) = 2.23, p = 0.037]*, SFC+ mice showed no sign of extinction of social avoidance between S1 and S6 *[t(22) = 1.60, p = 0.123]*, and these difference across S1 to S6 for SFC+ and SFC- differed significantly [*t(22) = 2.72, p = 0.012*]. Finally, there was no main effect of treatment [*F(2, 21) = 0.008, p = 0.920*], SFC condition [*F(1, 21) = 1.75, p 0.200*] or an interaction between treatment and SFC condition [*F(2, 21) = 0.37, p = 0.697*] during investigation of an empty stimulus cage (NS1-3) at the start of extinction. There was however an effect of stimulus exposure [*F(1.79, 37.69) = 14.82, p<0.001*], where trend analysis revealed a quadratic relationship where subjects investigated the empty stimulus

cage more during NS2 than during NS1 and NS3 [*SFC-, t(2.81), p = 0.011 and SFC+, t(4.88), p < 0.0001*].

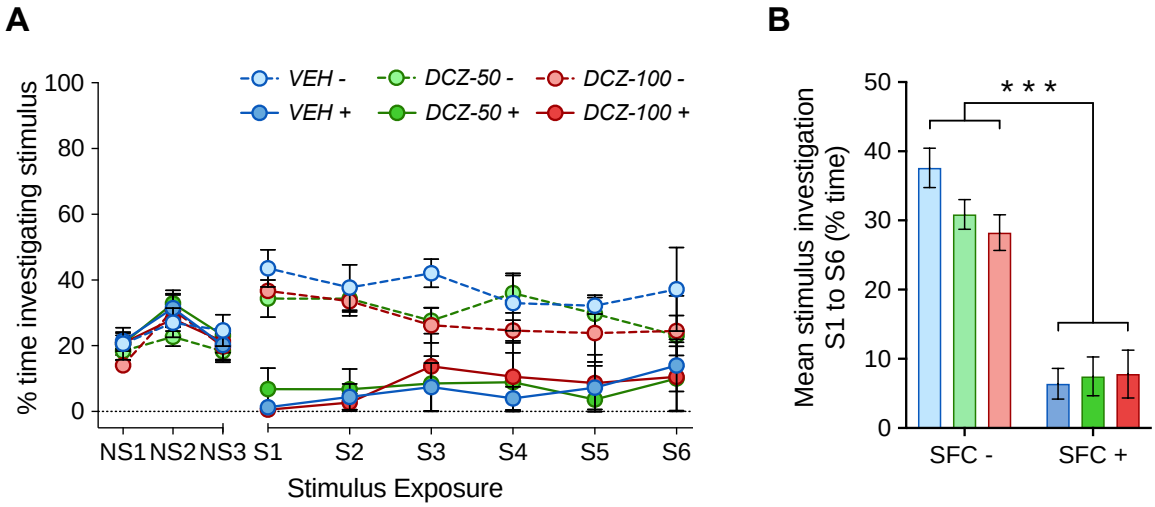



**Figure 4.2: DCZ treatment during SFC extinction in mice not expressing inhibitory DREADDs.**
In mice not expressing inhibitory DREADDs, we performed social fear conditioning (SFC) and extinction to establish a safe dose of deschloroclozapine (DCZ) which did not adversely impact behaviour. **A** During stimulus exposure to an empty stimulus cage (NS1-NS3), there was no difference in % time investigating the cage. From S1 to S6, SFC+ mice spent significantly less time interacting with the social stimuli compared to SFC- mice. **B** Across stimulus exposures, these results show that in control mice not expressing hM4Di-DREADDs administration of vehicle, 50 µg/kg or 100 µg/kg DCZ prior to social fear extinction does not alter social fear extinction behaviour in conditioned (SFC+) or unconditioned (SFC-) mice. Data presented are Mean ± SEM. n = 9 vehicle (4 SFC+), 10 DCZ-50 µg/kg (5 SFC+) and 9 DCZ-100 µg/kg (5 SFC+). *** *p < 0.001.*

### *4. 3. 2. Moderate effects of LS inhibition on frequency to flee stimulus and no effect on stimulus investigation time.*

Having established a safe dose of DCZ in control subjects that did not alter behaviour in the absence of DREADDs, we examined social fear and avoidance behaviours in subjects which expressed pAAV-hSyn-hM4D(Gi)-mCherry DREADDs *(see Figure 4.3A*) in the dLS following social fear conditioning.

On the first day of extinction (Day 29, *Figure 4.1*), SFC+ mice spent significantly less time interacting with the social stimuli compared to SFC- mice [*F(1, 20 ) = 97.56, p <*

*0.001*]. There was a main effect of stimulus exposure [*F(2.31, 46.28) = 3.91, p = 0.022*], and an interaction between SFC and stimulus exposure [*F(2.31, 46.28) = 4.34, p = 0.015*]. Trend analysis revealed SFC- mice showed a linear decrease in sociability over the six stimulus exposures [*t(20) = 4.14, p <0.001*], SFC+ mice showed no sign of extinction of social avoidance between S1 and S6 [*t(20) < 0.01, p >0.999*], and these difference across S1 to S6 for SFC+ and SFC- differed significantly [*t(20) = 3.19, p = 0.005*]. There was no main effect of DCZ treatment [*F(1, 20) = 0.73, p = 0.402, Figure 4.3B-C*]. There was no interaction between treatment and SFC [*F(1, 20) = 2.80, p = 0.110*], treatment and stimulus exposure [*F(2.31, 46.28) = 1.32, p = 0.280*], or between treatment, SFC and stimulus [*F(2.31, 46.28) = 1.53, p 0.224*].

To assess whether further stimulus exposures would result in extinction of social avoidance and emergence of any differences between treatment groups, mice underwent a second day of extinction following the same injection schedule (Day 30, *Figure 4.1)*. There was no main effect of treatment [*F(1, 20) = 1.91, p = 0.182*] or stimulus exposure [F*(2.94, 58.70) = 1.38, p = 0.258*], and no interaction between treatment and stimulus [*F(2.94, 58.70) = 0.45, p = 0.712*], SFC and stimulus [F*(2.94, 58.70) = 1.88, p = 0.144*], or treatment, SFC and stimulus [*F(2.94, 58.70) = 0.29, p = 0.827*]. SFC remained significant on day 2, with conditioned mice spending significantly less time interacting with the social stimuli [*F(1, 20) = 30.10, p<0.001, Figure 4.3D-E*].

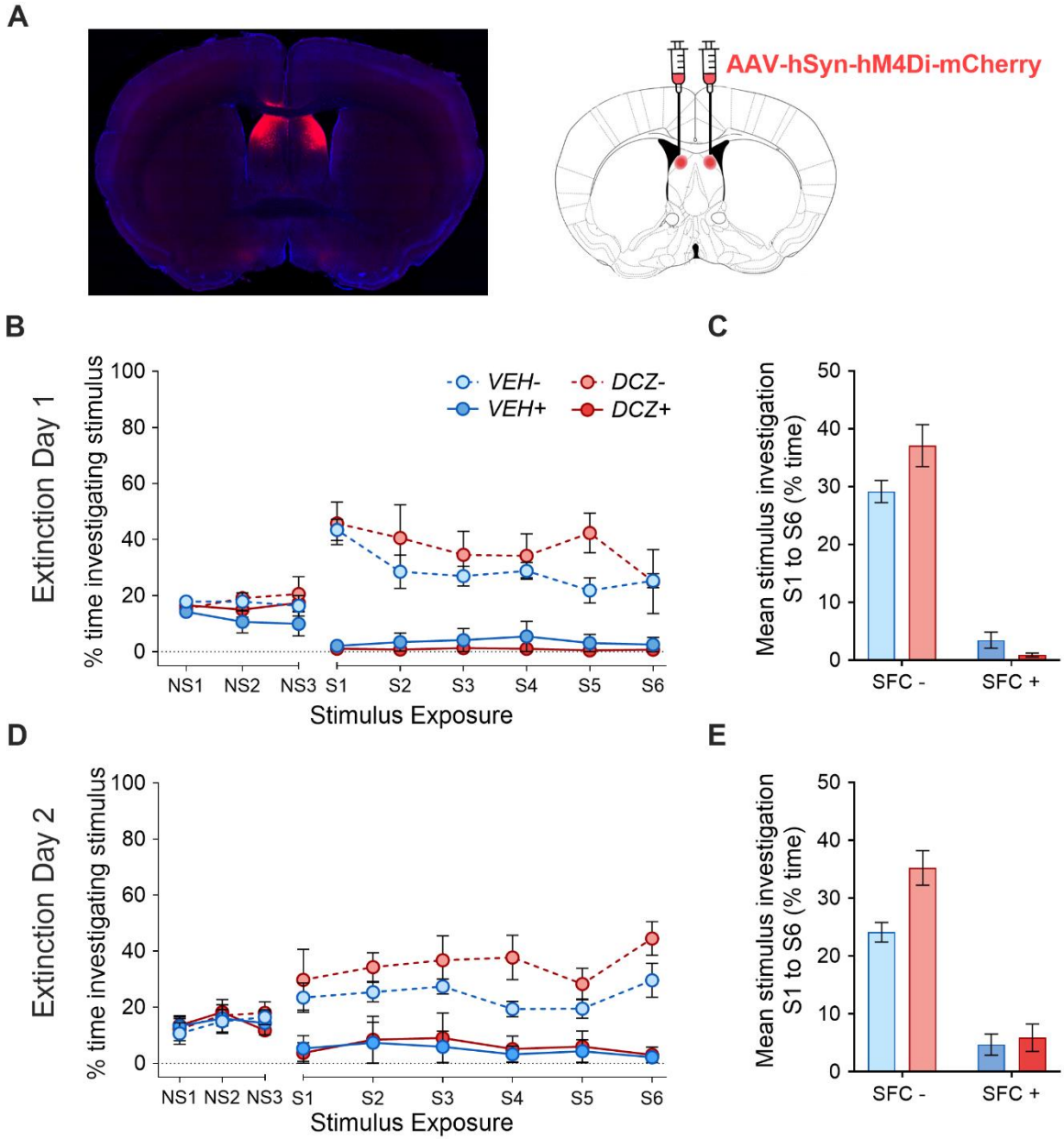



**Figure 4.3: Silencing the dLS does not extinguish social fear.**
Mice underwent surgery to infuse hM4Di-DREADDs into the dorsal lateral septum (dLS). After four weeks viral expression, mice were divided into two groups: unconditioned (SFC-) and conditioned (SFC+). Following conditioning, mice were administered 100 µg/kg DCZ prior to social fear extinction. **A** Representative example of AAV-hSyn-hM4Di-mCherry expression in the dLS in an experimental mouse. **B** On extinction day 1, SFC+ mice spent significantly less time interacting with the social stimuli compared to SFC- mice, demonstrating acquisition of social fear however there was no effect of treatment on social investigation in either condition. Further, SFC- mice reduced their time spent investigating the social stimulus across stimulus exposures. **C** SFC+ mice administered vehicle or DCZ did not mice show signs of extinction across S1 and S6. **D** On extinction day 2, SFC- mice continued to spend more time investigating social stimuli compared to SFC+. **E** SFC+ mice did not show signs of extinction in either treatment condition. Data presented are Mean ± SEM. n = 13 vehicle (7 SFC+) and 11 DCZ-100ug/kg (7 SFC+).

The results presented in Chapter 3 identified an LS signal associated with stimulus approach and fleeing, and Experiment 2 of Chapter 3 found the LS signal was clearest when the behaviour was initiated when the test mouse was proximal to the social stimulus; as such, in the present experiment we examined whether these specific stimulus approach and avoidance behaviours were altered following chemogenetic inhibition of the dLS. There was a main effect of conditioning where unconditioned (SFC-) mice engaged in proximal approach (those that reach the quadrant of the arena in which the stimulus is located) more frequently than SFC+ mice [$F(1, 21) = 6.47, p < 0.001$], but no main effect of treatment on frequency to approach the social stimulus [$F(1, 21) = 1.77, p = 0.198$] nor an interaction between SFC condition and treatment [$F(1, 21) = 0.05, p = 0.826$, *Figure 4.4A*]. There was a main effect of stimulus exposure, where mice more frequently approach social stimuli at the start compared to the end of extinction [$F(1.84, 38.62) = 12.77, p < 0.001$] and an interaction between stimulus exposure and condition [$F(1.84, 38.62) = 5.13, p = 0.012$]. There was no interaction between treatment and stimulus exposure [$F(1.84, 38.62) = 2.77, p = 0.079$] nor between SFC condition, treatment and stimulus exposure [$F(1.84, 38.62) = 0.74, p = 0.474$]. For frequency to distally approach social stimuli, there was a main effect of condition [$F(1, 21) = 20.75, p < 0.001$] but no main effect of treatment [$F(1, 21) = 0.12, p = 0.738$] nor interaction between treatment and condition [$F(1, 21) = 1.03, p = 0.322$, *Figure 4.4B*]. There was a main effect of stimulus exposure [$F(2.68, 56.34) = 2.93, p = 0.47$] but no interactions between stimulus exposure and any other variables.

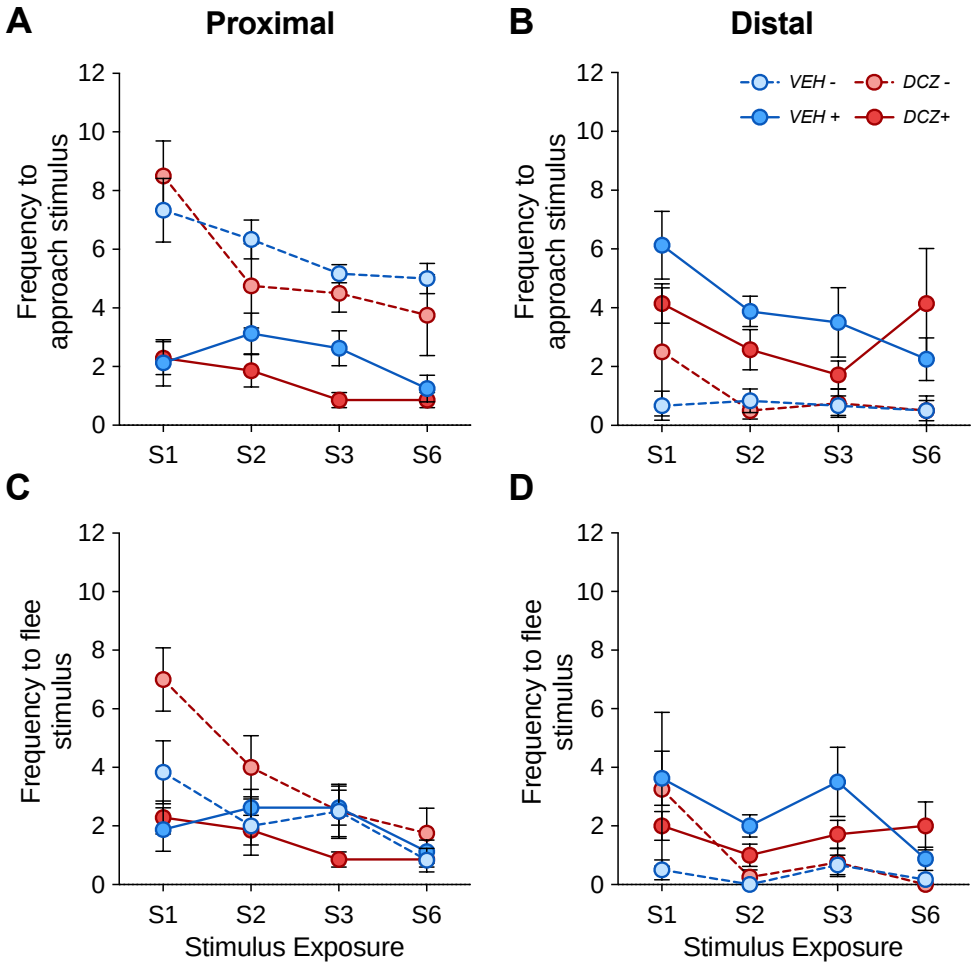



**Figure 4.4: dLS inhibition alters flee but not approach behaviour.**
We examined if stimulus approach and flee behaviour was altered by dLS inhibition for Extinction Day 1 **A** SFC- mice engaged in more proximal approach of the stimulus, B and **B** SFC+ mice made more distal approaches, those that do not reach the quadrant containing the stimulus cage, than SFC- mice. For both proximal and distal approach, dLS inhibition did not increase the frequency to approach novel social stimuli compared to vehicle. **C** SFC- mice made more proximal flees than SFC+ mice. dLS inhibition did decrease the frequency to engage in proximal flee from the social stimuli however more flees were made at S1 in both treatment groups. Those administered DCZ appeared to reduce the number of proximal flees across stimulus exposures so further analysis was performed. **D** SFC+ mice engaged in more distal flees than SFC- mice. Across conditions, dLS inhibition did conservatively reduce the frequency to flee between S1 and S3. Data are present Mean ± SEM. n = 13 vehicle (7 SFC+) and 11 DCZ-100ug/kg (7 SFC+).

SFC- mice engaged in proximal flees more frequently than SFC+ mice [*F(1, 21) = 5.06, p = 0.035*]. There was no main effect of treatment on frequency to engage in proximal flee from the stimulus [*F(1, 21) = 0.65, p = 0.430*], nor interaction between treatment and condition [*F(1, 21) = 3.42, p = 0.078, Figure 4.4C*]. There was a main effect

of stimulus exposure [*F(2.55, 53.47) = 18.95, p < 0.001*], where more proximal flees were made at S1 compared to S6 across groups. There were interactions between treatment and stimulus exposure [*F(2.55, 53.47) = 4.85, p = 0.007*] and condition and stimulus exposure [*F(2.55, 53.47) = 7.69, p < 0.001*]. Polynomial contrast analysis of the treatment by stimulus interaction revealed there was a significant linear decrease in proximal fleeing from S1 to S3 in the DCZ treated mice [*t(23) = 4.31, p < 0.001, Figure 4.5*], but not in the vehicle treated mice [*t(23) = 0.27, p = 0.788*], and the difference in these slopes was significant [*t(23) = 3.04, p = 0.006*].

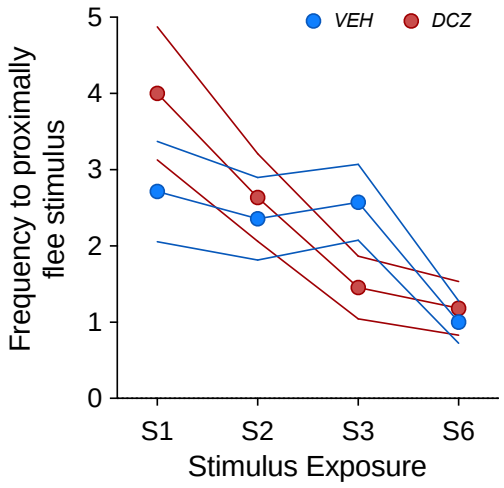



**Figure 4.5: Mice treated with DCZ engage in fewer proximal flees across extinction compared to mice treatment with vehicle.**
Trend analysis revealed there was a significant linear decrease in proximal fleeing from S1 to S3 in the DCZ treated mice, but vehicle treated mice. Further, the difference between the two slopes were significantly different. Data are present Mean ± SEM. n = 13 vehicle and 11 DCZ-100ug/kg.

For distal flee from the stimulus, there was a main effect of condition [*F(1, 21) = 9.74, p = 0.005*], with SFC+ mice engaging in more distal flees than SFC- mice. There was no main effect of treatment [*F(1, 21) = 0.00, p = 0.963*] nor an interaction between SFC condition and treatment [*F(1, 21) = 2.67, p = 0.117, Figure 4.4D*]. Similar to proximal flee, there was a main effect of stimulus exposure on distal flee [*F(1.83, 38.53) = 6.47,*

*0.005*] and an interaction between condition, treatment and stimulus [*F(1.83, 38.53) = 3.54, p = 0.042*]. Polynomial trend analysis of this three-way interaction revealed a significant linear decrease in distal flees in DCZ treated SFC- mice [*t(21) = 2.30, p = 0.032*], but no other conditions; however, this should be interpreted with caution as the three-way interaction for the slopes was not significant [*t(21) = 1.5, p = 0.148*]. There were no interactions between stimulus and treatment [*F(1.83, 38.53) = 0.90, p = 0.409*] or stimulus and condition [*F(1.83, 38.53) = 0.11, p = 0.883*].

## 4. 4. Discussion

The aim of this study was to chemogenetically silence the LS prior to fear extinction to determine if it results in any effects on social investigation, social approach, or social flee behaviours. In Chapter 3, we demonstrated LS activity was associated with stimulus flee behaviour, and specifically with proximal flees, which involve the subject mouse fleeing when in the same quadrant of the arena as the stimulus mouse. Thus, it was hypothesised that inhibiting the LS would result in reduced fleeing behaviour, and that this would be most pronounced with proximal flees. It was hypothesised that this would result in a subsequent increase in the time spent investigating the caged social stimuli over extinction. In line with our hypothesis, there was a greater decrease in fleeing over repeated stimulus exposures when the dLS was inhibited, and this was only apparent with proximal flees. In contrast to our hypothesis, silencing the dLS did not significantly impact the proportion of time spent investigating the social stimulus. Finally, we confirmed in a control study that the chemogenetic actuator DCZ does not affect social fear extinction in mice not expressing inhibitory DREADDs.

A key finding of the present study was that chemogenetic inhibition of the dLS resulted in a more pronounced decrease in proximal fleeing over the first three stimulus exposures. This was in line with our hypothesis and suggests the increased dLS signal identified as preceding proximal stimulus flee (reported in Chapter 3) may indeed play a causal role in driving this behaviour. Further supporting this, there was little evidence of major impact on distal fleeing, which was not associated with increased dLS activity. Interestingly, the effect of dLS inhibition on proximal fleeing appeared to be independent of whether or not the mice were fear conditioned. This is perhaps not surprising, as in the studies reported in Chapter 3, an increase in dLS calcium signalling was identified as preceding fleeing behaviour irrespective of whether the mice were conditioned or not,

suggesting the dLS may promote fleeing in the context of both conditioned and unconditioned responses.

An explanation as to why dLS inhibition did not significantly alter the total time spent engaged in social investigation may lie in our ubiquitous, rather than cell-type specific, silencing of dLS pathways. The LS is composed of predominantly GABAergic neurons, between 85% and 100% (Besnard et al., 2019; Wong et al., 2016; Zhao et al., 2013). As discussed in Chapter 3, within the LS there are a number of neuropeptide receptors including, but not limited to, OXTR (Menon et al., 2018), corticotropin-releasing factor (CRF) (Anthony et al., 2014; de Leon Reyes et al., 2023; Hashimoto et al., 2023) and neurotensin (NT) receptors (Azevedo et al., 2020; Li et al., 2023). In silencing the dLS via AAV8-hSyn-hM4D(Gi)-mCherry DREADDs, we inhibited all neuronal activity. Thus, if there are opposing roles of the dLS in the acquisition and/or extinction of social fear, then we silenced both *fear promoting* and *fear suppressing* pathways (Rizzi-Wise & Wang, 2021). Together with the results in Chapter 3, this will be discussed in more depth in Chapter 5.

Another possibility is that through silencing the dLS, we interrupted intraseptal GABAergic modulation of neuronal activity and subsequently behaviour. In a review on the top-down modulation of motivated behaviours through LS sub-circuits, Besnard and Leroy (2022) highlighted the importance of lateral inhibition occurring between sub-regions of the LS which promote fear suppression. For instance, glutamatergic projections from dorsal CA2 neurons promote aggression through the excitation of the dLS, which in turn inhibits ventral LS (vLS) neurons leading to disinhibition of the ventrolateral VMH (VHMvl) (Leroy et al., 2018). Through whole-cell recordings in the vLS, the authors demonstrated photoactivation of dCA2 projections produced hyperpolarisation of vLS cells, a direct contrast to the large depolarisation which occurred following photoactivation

of the dLS, thus solidifying a trisynaptic disinhibitory circuit to promote aggression. Beyond aggression, excitation of the dLS is implicated in increased anxiogenic and fear-related behaviours (Anthony et al., 2014; Li et al., 2023; Zoicas et al., 2014). Chemogenetic activation of vLS-projecting vCA1/vCA3 neurons suppresses anxiety-like behaviour in the EPM (Parfitt et al., 2017) and optogenetic activation of vCA3 terminals in the vLS suppress freezing during exposure to fearful contexts (Besnard et al., 2020). Thus, in inhibiting the dLS, we may have silenced fear suppression pathways within the LS.

Our results reflect those by Yeates et al. (2022), which elegantly transfected inhibitory DREADDs into the vCA1 or vCA3 of rats and placed guide cannula in the caudodorsal (cd) or rostrovenrtral (rv) LS to differentially manipulate the projections between these two distinct regions. Chemogenetic inhibition of the vCA3-LScd pathway, via local administration of CNO, potentiated approach towards a learned conflict-eliciting stimulus. However, inhibition of the vCA1-LSrv pathway potentiated approach non-specifically to both neutral and conflict stimuli. These findings further support the differential roles for anatomical distinct regions within the LS in the co-ordination of approach versus avoidance behaviours towards learned conflict-eliciting stimulus. Whilst Yeates et al. (2022) examined non-social contexts, inhibition of this glutamatergic circuit encouraged interaction with conflict-associative stimuli which may in part explain why conditioned mice in our study engage in less stimulus flees when presented with social stimuli. What is more, given our initial hypothesis in Chapter 3 that conditioned mice would demonstrate inhibition in the dLS during the SFC given the role of LS OXTR-expressing GABAergic neurons which are abundant in the dLS (Menon et al., 2018), identification of a glutamatergic pathway involved in potentiation towards learned conflict stimuli is meaningful.

Unconditioned mice, usually a control for normative social behaviour, showed lower sociability during extinction than expected in both the DCZ pharmacology and dLS inhibition study when compared to the literature (Zoicas et al., 2023). As the low sociability occurs in conditioned and unconditioned mice, it suggests that there may be external factors potentiating decreased engagement with social stimuli. The major deviation here from the SFC protocol used in Chapter 2, where unconditioned mice showed high sociability, is the chronic four-day i.p. administrations during habituation and testing (*see Figure 4.1*). Habituating mice to repeated scruffing and i.p. administration may lead to increased mild stress prior to behavioural testing, as measured through higher corticosterone levels, when compared to a single injection (Du Preez et al., 2020). This may have resulted in overall lower motivation to interact with novel social stimuli as subjects may have experience a heightened basal level of stress due to their environment. However, as we did not control for this within our study, we cannot be certain that injections adversely impacted social fear extinction. To overcome this potential issue and reduce stress in mice already exposed to multiple social stressors (social isolation and conditioning), future studies could administer DCZ via voluntary oral administration (Ferrari et al., 2022; Schalbetter et al., 2021). While this suggestion would further alter the standard SFC protocol, a reduction in aversive experimenter interaction may reduce confounding variables allowing for more accurate and comparable analysis of social fear behaviour. Replication of this study using a less aversive drug delivery regime, may promote social interaction in both SFC- and SFC+ allowing for clearer examination of the impact of dLS inhibition of social investigation and approach and avoidance behaviours.

Another explanation as to why dLS inhibition, via DREADDs, did not reduce stimulus flee behaviour and subsequently increased social investigation might be our use of chemogenetic rather than optogenetic silencing of the dLS. Using fibre photometry, we

revealed elevated activity in the dLS preceding proximal stimulus flee. As a technique, fibre photometry affords high temporal precision in the order of milliseconds, yet administration of DREADDs silenced the region in the order of hours. Our rationale for using DREADDs compared to optogenetics was the technological hurdle of timing dLS inhibition with stimulus flee events. Unlike stimulus approach which could technically be attenuated once the behaviour was initiated to halt the progression of the behaviour, given the dLS signal preceded the onset of the flee behaviour, this meant that once the behaviour had begun, and the animal had fled, it would be too late to silence the dLS activity which occurs prior to the behaviour. Thus, our aim was to time silencing of the dLS with the first stimulus exposure post-conditioning (S1) and observe gradual changes to social avoidance behaviour over stimulus exposures compared to controls.

A limitation of the study is the manual, rather than automated scoring of behaviour, which is subjective and time-consuming. Given the practical and time constraints of training a machine learning model on eight different behaviours, an experimenter not involved in the collection of behavioural data, was trained based on operationalised criteria and later assessed through interrater reliability scoring. Whilst employing this method helped to remove experimenter bias, manual scoring remains subjective and therefore will always be prone to inaccuracies and biases. Beyond improved objectivity and standardisation, another benefit of using a machine learning model would be the capacity to analyse velocity and in turn dissect freeze and flee behaviours. For example, in a study assessing the effect of looming stimulus location, which initiates instinctive approach and avoidance behaviours in mice, on the flight or freeze response, Solomon et al. (2023) used machine learning to track animal behaviour and operationally defined parameters for escape (i.e., flee as speed exceeding 40cm $s^{-1}$ before returning to a “nest” within 2 seconds) and freeze (an epochs when speed was less than 2cm $s^{-1}$ for more than 0.5 seconds).

Utilisation of velocity would allow future studies to investigate the behavioural shift from avoidance to approach behaviours, as studied in Yeates et al. (2022), and potential transition from "freeze and flee" to "freeze and approach", in mice actively extinguishing learned fear. Further, given much of the literature on fear conditioning includes freezing behaviour as a primary behavioural output, it would enable translation of our findings to the contextual fear literature given the increasing crossover and our own findings that LS activation during flee behaviour is not social specific.

Whilst DCZ is increasingly being used in chemogenetic studies, it was important to establish a safe and effective dose within our model and confirm the presence of no secondary behavioural phenotypes, such as changes in locomotion during social fear extinction. DCZ is a highly potent clozapine analogue which overcomes the limitations of clozapine-N-chloride (CNO) which can readily reverse-metabolises to the antipsychotic drug clozapine and subsequently have off-target effects (Manvich et al., 2018). When 50 or 100ug/kg DCZ was administered, there was no significant difference in social investigation compared to vehicle. Given the main objective of the control study was to establish gross changes in social investigation time, individual behaviours including approach and flee behaviours were not characterised during fear extinction. Whilst these behaviours were captured in the analysis of LS inhibition, there was no behavioural indication within the pharmacology study that supported the need for more in-depth investigation. This is consistent with *in vitro* studies which demonstrate DCZ superior selectivity for DREADDs (lower affinity for endogenous receptors) and *in vivo* findings which demonstrate a 20-fold and 60-fold lower dose of DCZ is necessary to achieve similar receptor occupancy compared to CNO and C21 respectively (Nagai et al., 2020). In the absence of DREADDs, a study in macaques examined the effects of actuator ligands in surgically-naïve animals and demonstrated that low dose CNO altered resting-state

functional connectivity and produced off-target effects where low dose DCZ (100ug/kg) did not (Cushnie et al., 2023). Further, Nentwig et al. (2022) showed 100ug/kg DCZ did not alter gross locomotor activity nor did it induce a place preference in surgically-naïve rats, in line with our own findings. Together with the results from our own study, we can conclude clozapine analog DCZ was an optimal actuator ligand for DREADDs with no detectable side effects.

Given our modest results, a limitation of the present study is the absence of additional pharmacological and behavioural positive controls to accurately assess the efficacy of dLS inhibition in altering social behaviour. It is possible that the receptor expression or the ligand dose was insufficient to observe a more pronounced behavioural effect. A review by Campbell and Marchant (2018), suggested the type of DREADD (i.e., inhibitory or excitatory) as well as the size of the target brain region can impact the potential efficacy of the ligand dose. Whilst not quantified, histology confirmed good viral placement and expression throughout the dLS. Further, the appropriate controls would have been performed had the results of the study been significant.

On the other hand, if we assume the dose was correct given it correlates with similar DCZ studies in mice (Nagai et al., 2020) and we have post-mortem histological confirmation of anatomical placement, an alternate positive control would be the inclusion of an assessment of behaviour in a task already established as perturbed by silencing of the LS. Given the historical association of the LS with social aggression, silencing of the dLS prior to presentation of an aggressor mouse would enable examination of alterations of social aggression and defensive behaviours following dLS inhibition as seen in lesioning and optogenetic studies (Albert & Chew, 1980; Albert & Wong, 1978; Leroy et al., 2018). Whilst C57BL/6 mice are generally considered to be less aggressive than other outbred mouse strains (Hsieh et al., 2017), Leroy et al. (2018) effectively demonstrated social

aggression in transgenic mice on a C57BL/6J background, thus it would be possible to examine aggression under the current experimental parameters. Future studies examining the role of the LS in social fear and avoidance could create positive within-subject controls by maintaining single housing in unconditioned SFC mice for six to eight weeks post-extinction. These mice could then undergo resident-intruder or open field dyadic social interaction testing to assess if administration of DCZ 15-mins prior to the behavioural task altered aggressive behaviour.

Finally, our study did not include an inert viral vector control (pAAV-hSyn-mCherry) which would have functioned to confirm DCZ, in the absence of hM4Di receptors, did not impact behaviour. However, it was decided the inclusion of this control group would only be necessary if there was a significant effect on social investigation. Thus, to ensure sufficient power, a decision was made to only follow up with additional control studies rather than split mice over more groups and risk being underpowered to detect a significant effect.

In conclusion, we found that chemogenetically silencing the LS did not increase the time spent with a social stimulus but did seem to accelerate the decline in stimulus flees over repeated stimulus exposures. This study focused on the analysis of inhibition of the LS in social contexts given the thesis aims to examine neural correlates of social anxiety-like behaviours. In future studies, silencing of cell-type specific pathways and using an optogenetic approach would enable dissection of the neural process driving flee behaviour in social fear and avoidance. In manipulating the LS and altering the flee response, we are closer to understanding the role this dynamic brain region holds in the interpretation and co-ordination of social behaviour.

# Chapter 5. General Discussion

This thesis used the social fear conditioning task to examine social anxiety-like behaviours in mice. Using this task, we explored several broad research questions: 1) what, amongst several factors of interest identified, influences social anxiety-like behaviour, 2) what specific social approach and avoidance behaviours is the dLS associated with; and 3) does the dLS play a causal role in these behaviours, and in extinction of social fear more generally?

As outlined in Chapter 1, genetic studies have thus far presented few viable candidate genes related to SAD. Instead, a logical alternative was to examine the aetiology of SAD in genetic disorders in which rates of social anxiety are substantially higher than in the general population. FXS, caused by a genetic mutation in the *FMR1* gene, is a neurodevelopmental disorder where individuals can be expected to experience SAD at two to three times the global average. Loss of function mutations in the *FMR1* gene are reproducible in mice. Thus, in work reported in Chapter 2, *Fmr1* KO mice underwent social fear conditioning and we hypothesised that, in comparison to wildtype controls, *Fmr1* KO mice would have impaired social fear extinction. Given the literature emphasises developmental age and sex as important risk factors for SAD, a secondary aim was to examine age (adolescent versus adult), sex (male versus female), and the interaction between these variables and genotype. We found that knockout of the *Fmr1* gene did not increase social anxiety-like avoidance of novel conspecifics during extinction. There was also no effect of sex on social fear. However, adolescent mice were more resilient to social fear conditioning than adults, demonstrating marked extinction of social fear in contrast to the extinction resistance observed in adult mice. Given clinical evidence that adolescents are more likely than adults to develop SAD (Kessler et al., 2007; Solmi et al., 2022), our finding that age is an important predictor of likelihood to extinguish learned social fear is meaningful and will be discussed further.

The work detailed in Chapter 3 explored the neural correlates of social anxiety using fibre photometry with social behavioural assays in pursuit of identifying associations between discrete behaviours and fluctuations in dLS activity. Experiment 1a assessed social behaviour towards novel *non-social* and *social* stimuli under non-fearful conditions. In line with the literature (Kopachev et al., 2022; Netser et al., 2017; Yang et al., 2011), we observed preference for same- and opposite-sex conspecifics over a novel object. During stimulus presentations, females had greater activation of the dLS preceding proximal stimulus approach and flee from an opposite-sex social stimuli, whilst males did not show increased dLS activity during any behaviour when compared to baseline. Utilising these same experimental mice to conduct an SFC photometry pilot study, in Experiment 1b we found fear conditioned mice showed significantly elevated dLS activity preceding proximal stimulus flee of novel social stimuli, which reduced alongside the extinction of social fear. Experiment 2 sought to reproduce these findings in a larger cohort of mice and assess whether any signal was social-specific by inclusion of a non-social fear conditioned (nSFC) control group. This study replicated the results from our pilot experiment with significantly elevated dLS signal preceding proximal flee *and* a similar extinction of social fear. Intriguingly, in contrast to our hypothesis that dLS activity would be social-specific, we found that mice conditioned to non-social stimuli also demonstrated activity in the dLS preceding proximal flee. In contrast to SFC mice, where extinction of photometry signal was coupled with an extinction of conditioned fear, the elevated dLS signal in nSFC mice persisted across stimulus exposures as did avoidance of the non-social stimuli, with mice showing no signs of fear extinction over the six stimulus exposures. In addition, during fear extinction of social and non-social fear, the elevated dLS activity observed preceded proximal flee, where the test mouse enters the quadrant containing the stimulus then flees, rather than distal flee, where the flee is initiated outside the quadrant containing the

stimulus. This finding indicates that proximity to the stimulus is an important factor in the fear response associated with the identified dLS signal. Finally, when the SFC group was divided into two subgroups based on time spent investigating the social stimulus across S2 to S6, mice sensitive to fear extinction demonstrated diminished dLS activity which correlated with increased investigation of social stimuli however, in mice resistant to social fear extinction the elevated signal in the dLS persisted across extinction. Thus, these results indicate that the dLS may indiscriminately co-ordinate active avoidance of aversive stimuli by facilitating fleeing behaviour and that extinction of dLS activity preceding stimulus fleeing is associated with extinction of learned fear.

In the work reported in the final experimental chapter (Chapter 4), we sought to examine if silencing the dLS could alter stimulus flee behaviour and subsequently increase social investigation in conditioned mice. Our results revealed that inhibition of the dLS did not increase the time spent investigating novel social stimuli. However, it did increase the rate at which proximal fleeing behaviour decreased over repeated stimulus exposures, and this appeared to be independent of whether the mice were conditioned. These results provide support for a causal role of dLS activity in stimulus flee behaviour, but that this does not necessarily translate into an increase in other social behaviours such as approach and investigation.

**The utility of the SFC task, motivation to extinguish learned fear and factors influencing fear extinction resistance.**

Pre-clinical biological psychiatry relies on animal models to experimentally interrogate the effect of alterations to genetic, environmental, biological, and neurological factors on behaviour. Given the inherent limitation that animals are unable to articulate their feelings, researchers must infer their affective or emotional states from their

*observable* behaviour (Dolensek et al., 2020; Krakenberg et al., 2020), recognising that no model can fully recapitulate the complexities of psychological disorders but, instead, strive to model important aspects of the disorders of interest (Monteggia et al., 2018). Throughout this thesis, the SFC paradigm has consistently emerged as a reliable and valuable tool for scrutinising fear and avoidance behaviours in mice, offering insights with potential relevance to understanding analogous human behaviours, which are relevant to many psychiatric disorders beyond SAD including depression and substance use disorders (Brook & Schmidt, 2008; Lemyre et al., 2019). Using the SFC task, mice reliably learnt the association between foot shock (unconditioned stimulus, US) and social interaction (conditioned stimulus, CS). First, across all experimental chapters, SFC+ mice spent significantly less time investigating the social stimuli compared to SFC- mice, which was highly consistent with previous studies using the SFC paradigm (Menon et al., 2018; Xu et al., 2019). Second, SFC- mice demonstrated greater levels of engagement with the stimulus during S1 to S6 than when investigating an empty stimulus cage (NS1 to NS3), which emphasises the specificity of the fear to the social stimulus, rather than the cage, a finding replicated in the literature (Menon et al., 2018; Zoicas et al., 2014). Finally, using a modified design which utilised scented tennis balls in place of social stimuli, we found the instrumental fear conditioning assay used is as robust for non-social fear conditioning (nSFC) as SFC as first demonstrated in Raymond et al. (2019). Specifically, in Experiment 2 mice showed stimulus specific avoidance of caged scented tennis balls compared to an empty stimulus cage, and the magnitude was similar to that observed in SFC. What distinguishes the SFC task, in comparison to the three chamber or direct social interaction tasks, is that it captures *active avoidance* rather than passive avoidance due to low sociability. Further, unlike behavioural tasks e.g. resident-intruder and CSDS, which rely on dominance and continual social trauma, the SFC task centres on a small number of

aversive events experienced during a very short time window (the single conditioned session), and then provides the opportunity to assess a subject's ability to overcome the conditioned avoidance by assessing fear in neutral environment where social interactions go unpunished. These distinctions underscore the SFC paradigm as a more translationally relevant tool for understanding conditioned social fear and active avoidance of social interactions, which more closely mirror elements of SAD in humans.

In this thesis, the combined use of instrumental fear conditioning with in-depth analysis of distinct behaviours (e.g. proximal approach, proximal flee, investigation, disengage and re-engagement etc.), has elevated the utility of the SFC task to one which captures *motivated* behaviours. Through expert manual scoring of individually defined, mutually exclusive behaviours, we have uncovered a role of the LS in the active avoidance or *fleeing* from aversive stimuli. Our findings support those in the literature in highlighting the role of the LS as an integral component of the "social behaviour network" (Menon et al., 2022) and more broadly in pathways involved in affect and motivation (Besnard & Leroy, 2022; Wirtshafter & Wilson, 2021). These results highlight the importance of employing tasks which promote the expression of behaviours relevant to the symptoms of psychological disorders being examined.

The SFC paradigm relies on the assumption that mice are socially motivated, intelligent animals that should choose to overcome fear of novel social stimuli to gain the opportunity to engage in social interaction. The SFC paradigm increases motivation to interact with social stimuli through social isolation, as discussed in Chapter 2, and the presentation of social stimuli in cages, is designed to maximise olfactory, auditory, visual, and tactile interactions between subject and conspecific in a controlled manner. It has previously been shown that differing emotional and physical states, in either conspecifics or subjects, altered the likelihood of subjects to engage in social interaction (Ferretti et al.,

2019; Scheggia et al., 2020; Ueno et al., 2018). For example, mice show diminished social recognition, novelty preference and impaired social stimulus discrimination following SFC when the social stimulus is anaesthetised as opposed to awake (de la Zerda et al., 2022). Further, the authors demonstrated preference for a novel versus familiar cage mate stimulus was disrupted if subject mice had loss of somatosensation (via whisker removal), hearing or sense of smell indicating these sensory modalities are important for social interaction. The apparent importance of multimodal sensory cues is why, in Chapter 3 Experiment 2, we tried to mimic the multisensory modalities of a social stimulus for the non-social fear conditioning task by using a colourful, furry objects with distinct scents (e.g. rosewater, coconut, vanilla etc.), to enhance novelty of each non-social stimulus exposure. However, given social stimulus cues may be an important factor driving motivation to interact and, consequently, contribute towards fear extinction, it could provide an explanation as to why mice conditioned to non-social stimuli were more resistant to fear extinction. Further, whilst these cues may positively contribute to fear extinction, if there are more salient environmental cues (e.g. increased stress due to injections prior to extinction as seen in Chapter 4), these may override social cues from conspecifics and thereby diminish motivation to interact. Understanding the interplay or prioritization of social and non-social cues in driving motivated behaviour is an important area for future research, particularly in the context of understanding social anxiety disorders.

In Chapter 2 we observed adolescent mice more readily extinguished social fear compared to adult mice, which might suggest a similar effect might be seen for non-social fear extinction. A study examining cued fear extinction following foot shocks (US) paired with an auditory tone (CS) found that extinction and recall of fear behaviour differed between adults and adolescents (Gerhard & Meyer, 2021). Interestingly, the authors found

that spacing of extinction trials across multiple days positively contributed towards extinction learning in adults but led to spontaneous re-emergence of fear behaviour in adolescents. This was followed by a rapid reduction in freezing similar to controls that underwent non-spaced extinction. Gerhard and Meyer (2021) speculated that adult and adolescent mice differ in their fear extinction profiles. They hypothesised that adults extinguish fear through inhibition of CS-US associations whereas adolescents use comparative trial by trial learning to rapidly update their behaviour based on their experience in the immediately preceding trial. These findings are supported by the clinical fear conditioning literature, which shows adolescents have greater skin conductance and reduced dorsolateral PFC activation during short-term extinction recall compared to adults, indicating differential behavioural and neural responses post-conditioning between adolescents and adults (Ganella et al., 2017). If so, it could provide an explanation as to why adolescent mice more readily extinguished social fear in Chapter 2, if the classical fear conditioning findings translate to instrumental fear learning. If in Chapter 2 adolescent mice used trial by trial learning as indicated in Gerhard and Meyer (2021), then as they engaged in investigation of the social stimulus across trials, they would have more rapidly updated their behaviour i.e. increased social interaction, based on learning that social investigation no longer resulted in a foot shock. Moreover, this hypothesis is consistent with evidence of enhanced neurogenesis in adolescent mice which could support greater neural plasticity as compared to adults (He & Crews, 2007). In Experiment 2 in Chapter 3, we identified a subset of mice conditioned to non-social stimuli that were resistant to fear extinction and a subset which did extinguish. If fear extinction in adults is related to disassociation of the CS-US pairing, then the persistent lack of interaction, as indicated by very low levels of stimulus investigation across extinction in mice conditioned to non-social stimuli (*see Figure 3.8C-E*) and non-extinguishers (*see Figure 3.13A-C*), may have

impeded inhibition of the CS-US pairing; in contrast, the perhaps greater drive to interact with the social stimuli may have facilitated extinction. Examination of social and non-social fear conditioning and extinction in adolescent versus adults with the added inclusion of spontaneous recovery and renewal effects, would help answer if age determines the neural mechanism recruited during fear extinction. Finally, based on findings in the literature and our own results in Chapter 2, had more precocious adolescent mice been used in non-social fear extinction in Chapter 3 rather than adult mice, we may have more readily observed fear extinction following nSFC. In turn, if we had observed non-social fear extinction, we may have also observed the dLS signal diminished alongside the extinction of fear as seen in SFC mice thus consolidating whether the pathway was social-specific or not. In any case, this evidence demonstrates the importance of considering age in the design and interpretation of fear conditioning studies.

Whilst adolescent mice more readily extinguished social fear in the study reported in Chapter 2, the decision to use adult mice, which had shown greater extinction resistance in the *FMR1* study, in Chapters 3 and 4 were threefold. Firstly, we wanted to closely replicate the SFC task originally used to identify the LS in social fear extinction and minimise any confounding variables (Menon et al., 2018; Zoicas et al., 2014). Secondly, using mice more resistant to fear extinction held a greater opportunity to observe gradual changes in dLS activity across extinction. In saying this, most of the literature uses adult mice to examine SFC and in Experiment 1b in Chapter 3, we did indeed observe social fear extinction in adult mice in the photometry pilot experiment. The more pronounced extinction in the adult mice in Experiment 1b, could have been due to the considerably greater handling the mice underwent and their greater familiarity with the testing arena and specifically with social interaction in that testing arena due to undergoing testing in Experiment 1a prior to being used for Experiment 1b. It was, however, somewhat

unexpected that we would see low levels of fear extinction in adult mice as other SFC studies in C57BL/6J mice have reported extinction in adults (Raymond et al., 2019; Zoicas et al., 2023). Possible explanations could be strain (Chapter 2 used the WT mice from the *Fmr1* KO breeding colony as opposed to C57BL/6 mice from a commercial supplier, although this does not explain the extinction resistance observed in Experiment 2 Chapter 3) or individual differences as seen in socially fear conditioned mice (Experiment 2 Chapter 3), of which half did not show any signs of extinguishing social fear. This observation of there being extinguishers and non-extinguishers amongst adult cohorts is consistent with recent studies which have found similar results and have identified an underlying genetic basis (Royer et al., 2022). Finally, there were practical considerations in terms of performing fibre photometry and DREADDs in adolescent mice. Specifically, in Chapter 2, adolescent mice were tested between PND35 and PND42 with the peri-adolescent time period spanning from PND23 to PND60 (Brust et al., 2015). However, the experimental timeline used for fibre photometry experiments in Experiment 2 in Chapter 3 was 36 days (*see Figure 3.2*); thus, to examine dLS activity in adolescent mice undergoing fear conditioning and extinction, intracranial surgery would need to be performed by PND24 immediately post-weaning. Alternatively, a shorter two week viral expression period would need to be implemented, as seen in de Leon Reyes et al. (2023), allowing for surgery at PND30 and behavioural testing to be complete by PND52. Given the innovative nature of the experiments in Chapter 3 and 4, it was deemed more appropriate and prudent to start exploring these questions in adults and future studies may now expand on these results using adolescent mice.

Differences in cost-benefit evaluation may provide another possible explanation for extinction resistance seen in adults and when using non-social stimuli (Schneider et al., 2021). The cost of engaging with either the novel non-social or social stimuli (e.g.

*potential for foot shock)* may initially outweigh the benefit (e.g. *opportunity to interact*); however, as the expectation of shock decreases with each exposure the cost reduces, leading to extinction of the fear response and increased investigation of social stimuli. However, as discussed in Chapter 3, there is a more limited need to extinguish fear of an inanimate, inedible object, relative to a novel conspecific, and this thus represents a *high cost, low benefit* scenario. Cost-benefit factors may also explain the observation from the experiment presented in Chapter 2 that adolescent mice were more likely to extinguish social fear than adults. Across species, there exists a greater evolutionary need to inhibit fear responses following stressful social interactions during adolescences, due to adolescent being a critical period for mating and establishment of social hierarchy in many species (*see review* MacLeod et al. (2023)). These evolutionary social pressures are somewhat reduced in adult mice, which may explain their greater extinction resistant relative to adolescents. This may explain why adults remain sensitive to value-based decision making, for example in our finding that there was increased investigation driven by individual differences in Chapter 3, or when we observed reduced investigation when the cost was higher due to a potentially aversive environments such as the increased experimenter handling and injections (Chapter 4), which while not controlled for might have contributed towards lower levels of investigation.

This thesis has identified a number of controllable and uncontrollable factors which influence sensitivity and resistance to fear extinction. Our findings suggest that age and stimulus type are important factors in determining the likelihood of fear extinction while social stimuli-driven engagement and individual differences cannot be controlled but efforts e.g. experimenter handling and reducing aversive environments, can be made towards minimising them where possible to influence sensitivity to social fear extinction. The behavioural results presented in this thesis underscore the robustness and utility of the

behavioural phenotype in the SFC paradigm. Yet, the paradigm has also proven sensitive enough to reveal subtle variations in social anxiety behaviour, offering valuable insights for targeted interventions and further exploration of neural correlates.

### *Neuronal pathways of the LS and cell-type specific neurons of interest implicated in social fear and anxiety.*

As outlined in Chapter 3, it was the development of the SFC paradigm that led to the discovery of a central role for the LS in social fear expression and extinction (Menon et al., 2018; Zoicas et al., 2014). Building on this framework, we used fibre photometry and inhibitory DREADDs to record and later inhibit dLS activity during social fear extinction. Our goal was to uncover which specific approach, avoidance and explorative behaviours involved dLS activity. Using jGCaMP8f, we recorded fluctuations in intracellular $Ca^{2+}$, which showed ramping activity in the dLS *preceding* flee behaviour, regardless of whether the stimulus was social or non-social (Chapter 3). Based on our findings, we predicted that chemogenetic silencing of the dLS during fear extinction would suppress flee behaviour leading to a reduction in the social fear response and increase the time spent exploring the social stimulus. However, only part of our prediction was supported by the experiment: dLS inhibition reduced flee behaviour over repeated stimulus exposures, but this did not result in a subsequent increase in social investigation. This suggests that flee behaviour and social investigation are driven by separate processes with different neural substrates. Evidence in support of this theory, comes from the presence of multiple neuronal cell-type expressed throughout the LS which have opposing roles in prosocial and antisocial behaviour (Leroy et al., 2018; Menon et al., 2018) as well as circuits which both promote and those which silence anxiety-related behaviours (Anthony et al., 2014; Chen et al.,

2021; Parfitt et al., 2017). Through our ubiquitous silencing of the dLS, we inhibited all neuronal activity within the region. Thus, our results from these two studies suggest there may be competing *fear promoting* and *fear suppressing* circuits within the LS which work in tandem to modulate the switch from approach – avoid to approach – engage, which likely depend on salient environmental cues.

In exploring the role of the LS in social fear and why the dominant signal recorded from in the LS in Chapter 3, and chemogenetically silenced in Chapter 4, is specifically involved in proximal stimulus flee but no other socially motivated behaviours related to social fear, we need to examine the function of the LS within the wider anxiety and fear response network (Grogans et al., 2023; Menon et al., 2022). Calhoon and Tye (2015) proposed an anxiety response framework consisting of four key phases. Briefly, in the initial "detection phase", sensory information (i.e. visual, auditory, olfactory, somatosensory) is processed through the thalamus and sensory cortex to the basolateral amygdala (BLA). This marks the commencement of the "interpretation" phase", where the LS together with several other key brain regions previously highlighted within this thesis (e.g. BLA, central amygdala (CeA), vHPC and BNST), integrates and relays salient information. From here, the "evaluation phase" ensues where excitatory projections to cortical and limbic brain regions (e.g. VTA, LH, AHA, NaC, prelimbic (PrL) and inframlibic (IL) subregions of the mPFC), contribute to an internal assessment of emotional states. This is paired with an external examination of events to determine if they align with expectations. Subsequently, the "response initiation phase" unfolds, whereby projections from the "interpretation" and "evaluation" phase initiate physiological (e.g. increased heart rate etc.) and behavioural responses (e.g. defence, attack, risk avoidance or freezing) via the motor cortex and brainstem nuclei including the periaqueductal gray (PAG). Crucially, it is postulated that anxiety occurs when there is *mis-interpretation* of

ambiguous environmental stimuli as threatening during the "interpretation phase" (Calhoon & Tye, 2015). Therefore, it is reasonable to conclude that persistent activity in the LS in response to fear-conditioned social cues could promote continued avoidance and resistance to fear extinction. Our results in Chapter 3 support this suggestion, yet how different circuits within the LS promote different behaviours remains to be fully elucidated. Thus, examination of cortical and sub-cortical inputs into the LS will provide support for common and dissociable fear promoting and fear suppressing pathways in social fear learning and extinction.

One of the key findings of this thesis is the ramping of dLS activity prior to the onset of proximal, and not distal, stimulus flee. This indicates a role of the dLS in the response to anticipation of an imminent threat. Ramping activity is indicative of an anticipatory behaviour, which ramps up arousal and sensory attentional processes to enable subjects to be more acutely sensitive to conspecific and environmental cues together with memory of the prior aversive event (Garfinkel & Critchley, 2014). In humans, there is evidence of a paradoxical relationship between fear and pain whereby anticipation or threat of pain and discomfort may increase pain-related fear (van Vliet et al., 2018). Evidence of ramping activity in anticipation of foot shock comes from studies examining the role of the ventrolateral PAG in cued fear discrimination. The PAG is associated with innate and conditioned fear-evoked freezing (Tovote et al., 2016), is functionally connected with the hippocampus via the hypothalamus and LS and the circuit is putatively associated with risk assessment response i.e. motor output (Motta et al., 2017; Sheehan et al., 2004). Recording from ventrolateral PAG cells, Wright and McDannald (2019) identified two subset of neurons which had opposing roles during cued fear discrimination. "Onset" cells switched on upon administration of the light cue and activity in these cells subsided across the trial, returning and remaining at baseline even after the administration of foot shock 12s later.

"Ramping" cells on the other hand, began a steady increase upon the light cue and sustained ramping activity until the foot shock was administered (Wright & McDannald, 2019). What this indicates is that within the ventrolateral PAG, there are distinct neurons which modulate ramping activity in anticipation of an aversive stimulus and some which do not, but alternatively signal impending threat probability. Applying these findings to our own study, it could be speculated that a subset of dLS neurons may be attuned to threat probability, perhaps even firing for the first-time during fear conditioning and initiating fear acquisition. These cells may then remain responsive to the CS i.e. the social or non-social stimulus, upon stimulus approach. In parallel, a second subset of "ramping" neurons may be responsive to threat probability and perhaps in the absence of foot shock, the ramping activity subsides, resulting in a subsequent decrease in fleeing and increased time spent investigating the stimulus. Unfortunately, Wright and McDannald (2019) did not examine fear extinction and although the ventrolateral PAG is associated with freezing (Tovote et al., 2016), the authors did not examine if freezing behaviour corresponded with "onset" or "ramping" cells. If a similar set of neurons were to exist within the dLS *or* if cell-type specific neuronal populations e.g. CRH or NT, were to respond to acquisition of aversive situations similarly, then it would support the theory that within the LS there are fear promoting and fear suppressing pathways. These may work synchronously to dampen the fear response or in the case of anxiety disorders, asynchronously potentially perpetuating fear behaviours. Alternatively, given the dLS is connected with the PAG via the fear response pathway (Motta et al., 2017), it is possible that the dLS serves to "integrate" the incoming salient information related to proximity to the aversive stimulus to downstream targets which include the PAG which co-ordinates the "response initiation phase".

Whilst this thesis was not focussed on dissecting the neural circuits within the LS, a principle aim was to use fibre photometry to identify the specific SFC and extinction-related behaviours modulated by the LS. An assessment of eight behaviours, distal and proximal approach, stimulus investigation, disengagement and re-engagement, proximal and distal flee together with corner immobility and non-stimulus directed behaviour (e.g. grooming and rearing), found only proximal flee was consistently associated with elevated dLS activity. The distinction between proximal and distal flee from a technical perspective is that proximal flees occur within the quadrant containing the stimulus cage whereas distal flees start within one of the other three quadrants (*see Figure 3.3*). Therefore, the key difference was that proximal flees were within close vicinity of the stimulus, whereas distal flees were not. As discussed in Chapter 3, across species proximity to threat is an important determining factor in initiation of specific fear related behaviours and recruitment of reactive over cognitive circuits (Andraka et al., 2021; Faul et al., 2020). When a proximal flee occurs, the perceived threat is more imminent however, when approaching and fleeing from a more distal position, the threat is more remote and less threatening, consistent with the clinical literature (Givon-Benjio et al., 2020; Low et al., 2015). This is consistent with previous studies which have shown the effect of particular circuits on behaviour are dependent on the imminence of threat, in some cases even having the opposite effect when threat is imminent vs distal (see review Olivera-Pasilio and Dabrowska (2020).

In Chapter 3, during fear acquisition via SFC and nSFC, mice received a foot shock upon investigation of the stimulus and so form an aversive association with the stimulus. The elevated dLS activity preceding proximal but not distal stimulus flee observed in Chapter 3 suggests that the closer the subject gets to the stimulus before fleeing is associated with the increased amplitude of the dLS activity, thus explaining why

fleeing from a greater distance from the stimulus does not elevate dLS activity. Further, in most cases, but not all, a distal flee is paired with a distal approach and a proximal flee with a proximal approach. Within approach behaviours, mice are likely engaging in risk assessment behaviours (e.g. stretch attend postures and sniffing) and so a distal flee may indicate a decision not to approach or investigate whereas a proximal flee is more closely related to fleeing contact with the stimulus. Finally, it might be assumed that mice engage in more distal approach/flees earlier in extinction when more fearful and transition to engage in more proximal approach/flees as they gain confidence to get closer to the stimulus. However, in Chapters 3 and 4, we assessed the frequency to engage in distal and proximal approach and flee behaviours and did not observe this trend. Thus, while dLS activity is elevated preceding proximal stimulus flee, the cessation of this signal is not necessarily associated with a reduction in the frequency to engage in this behaviour.

In Chapter 3, we observed dLS activation preceding proximal flee during preconditioning (S1-) and post-conditioning (S1+ to S6+) indicating the dLS is involved in this escape behaviour under fearful and non-fearful contexts; it further indicates the dLS is involved in conditioned and unconditioned fear responses. Typically, proximal flee will be preceded by one of two behaviours, proximal approach, or stimulus investigation. This indicates that proximal flee immediately succeeds an assessment of threat and risk which results in the decision to engage in a defensive behaviour and escape. It is intriguing that mice which are unconditioned engage in this behaviour and that it elicits a similar dLS signal to situations involving threat. For example, in Experiment 1a in Chapter 3, female mice demonstrated elevated dLS activity preceding fleeing for opposite-sex stimuli and in Experiment 1b and 2 using the social and non-social fear conditioning tasks we observed peaks in dLS activity preceding proximal stimulus flee in unconditioned and conditioned social and non-social fear conditioned mice. In support of our findings, Hashimoto et al.

(2023) found that LS neurons expressing type 2 corticotrophin-releasing hormone receptors ($LS^{Crhr2}$) respond to sensory stimuli associated with environmental threat and are necessary for stimulus-triggered defensive behaviours that prime the animal to engage in the appropriate threat response i.e. flight, fight or freeze. Another possibility is dLS activity, via the BLA (Rodriguez et al., 2023) or OXTR-expressing LS neurons (Horiai et al., 2020), is due to novelty rather than threat, where activity in either circuit is associated with improved social novelty recognition. However, if this were the case, then the dLS signal preceding proximal flee during S1- would be predicted to be of the same magnitude to the signal preceding S1+, which is not the case. Moreover, the reduction in dLS signal preceding flee over extinction reduced alongside the fear response, if it were simply reducing due to a reduction in novelty, it would be predicted to reduce equally in extinguishers and non-extinguishers. Additional analysis could examine dLS activity preceding proximal flee across S2- to S6- to observe if there was a continuation of dLS activity associated with proximal flee similar to S1-.

Given the identification of a key role of the LS and oxytocin in social fear acquisition and extinction (Menon et al., 2018; Zoicas et al., 2014), we initially hypothesised we would observe inhibition of the dLS during S1+ followed by increased dLS activity to baseline during extinction. Further, we might have predicted activity in the dLS would be associated with prosocial behaviours such as stimulus approach and investigation rather than fleeing due to oxytocin activity. In contrast, we found increased dLS activity preceding stimulus flee and activity in the dLS diminished across extinction. Further, the results of our study examining dLS activity during social and non-social fear extinction indicate that activity in the dLS is not social specific. Beyond social behaviour, the LS is involved in a variety of motivated behaviours including drug-seeking, feeding, foraging and spatial memory (*see* reviews Rizzi-Wise and Wang (2021) and Wirtshafter

and Wilson (2021)). Further, whilst the SFC has highlighted a key role of OXT in social fear extinction, OXT is not only associated with social behaviour, but also novelty preference (Haskal de la Zerda et al., 2020; Ribeiro et al., 2020) and accurate fear discrimination (Olivera-Pasilio & Dabrowska, 2020). Thus, given the evidence suggests dampening of LS activity via oxytocin is associated with social fear extinction, it is likely that OXT via OXTR-expressing GABAergic neurons is contributing towards the extinction of fear. This fear suppressing circuit, together with others implicated in social and non-social fear extinction, will be discussed.

***Upstream cortical and sub-cortical pathways of interest associated with extinction of social and non-social fear.***

Increased OXT signalling, via oxytocin neurons projecting from the supraoptic nucleus (SON) to the LS, is strongly implicated in social fear extinction (Menon et al., 2018; Zoicas et al., 2014). In Chapter 3, central to our hypothesis was that we would observe inhibition of the dLS during the first stimulus exposure post-conditioning (S1+) followed by a recovery of activity in the dLS to baseline. Specifically, previous studies have shown that there is reduced oxytocin release in the LS following social fear conditioning, that infusion of OXT or overexpression of OXTRs in the LS diminishes social fear during extinction, and that knockout of OXTRs impairs social fear extinction (Menon et al., 2018; Zoicas et al., 2014). Thus, this implied the OXT system, with its well established role in facilitating social behaviour (Menon & Neumann, 2023), is impaired by social fear conditioning and that extinction of social fear involves the oxytocin system becoming active again. However, in Chapter 3 we demonstrated the inverse – we observed significantly elevated activity in the dLS during S1+ which reduced across extinction alongside the extinction of social fear. Initially the results from our study suggest that we

are recording *fear promoting* pathways that are different to the *fear supressing* oxytocin pathway previously identified and described, and that this *fear promoting pathway* is the dominant pathway in the LS during early extinction and in mice that do not extinguish. We observed significantly elevated dLS activity following proximal stimulus flee, a behaviour which enables the rapid avoidance of the conditioned stimuli. As mice engaged in more social investigation (Experiment 1b and 2), the dLS signal subsided, suggesting a dampening of the fear promoting circuit, which may have been due to the previously described SON-LS OXT circuit becoming active and inhibiting the fear promoting circuit as extinction progressed. However, it is still possible that we are recording a signal from the known OXT LS system, and that it is perhaps activated in early extinction but the impact on social approach and avoidance is not immediate. Further, previous studies examining the OXT system in the context of social fear conditioning have used techniques that lack the temporal dynamics to characterise the kinetics of OXT system over the course of extinction and our studies represents the first to use a high temporal resolution technique in social fear conditioning to record the LS. Taking into consideration our findings that 1) dLS activity is associated with active avoidance behaviours, 2) the signal diminished alongside extinction of social fear, and 3) that mice which do not extinguish social fear fail to demonstrate a reduction in the dLS signal we were recording preceding flee, the most parsimonious explanation is that the signal we have identified that precedes stimulus flee is a separate, avoidance promoting circuit, that competes with the previously identified OXT circuit. It is also of interest to note that mice conditioned to non-social stimuli demonstrated similar activation of the dLS prior to proximal stimulus flee; it would be of interest to examine the previously identified OXT circuit using our modified SFC paradigm to examine if OXT activity is also involved in non-social fear extinction following instrumental fear conditioning. Evidence from the non-social, contextual fear

conditioning literature (Besnard et al., 2019) and studies examining aggression (Leroy et al., 2018) and threat responsivity (Azevedo et al., 2020; Hashimoto et al., 2023), suggest there are other LS fear promoting and fear suppressing circuits of interest to explore in light of our findings.

In the present study, each stimulus exposure presented a novel stimulus, suggesting that there is active learning through the transference of previous stimulus experience across social and non-social fear extinction. During social fear extinction, we also observed dLS activity reduced alongside the extinction of social fear. This indicates that the LS may be interfacing with social memory circuits to alter behaviour and encourage fear extinction. These circuits may provide constantly updated incoming information that investigation of stimuli is no longer being punished by foot shock administration. In recent years, several studies have identified CA2 pyramidal neurons in the hippocampus play an important role in social memory (Hitti & Siegelbaum, 2014; Leroy et al., 2018). The LS receives more inputs from the hippocampus than any other region (Sheehan et al., 2004) with different sub-regions of the hippocampus projecting to distinct regions of the LS (Rizzi-Wise & Wang, 2021). Like the LS, the hippocampus is functionally divided into distinct sub-regions and several tracing studies have identified hippocampal dorsal CA2 and CA3 regions project to the dLS (Besnard et al., 2020; Oh et al., 2014). In support for the role of dorsal CA2/3-LS projections in fear learning, silencing of CA2-LS projection neurons reduces aggression to novel conspecifics, indicating disrupted social novelty learning (Leroy et al., 2018). Further, dLS and CA3 neurons exhibit highly correlated activation during fear conditioning. Using miniature endoscopes, another form of *in vivo* calcium imaging, Besnard et al. (2019) demonstrated increased dLS activity preceding freezing. This mirrors the findings in Chapter 3, where we observed an increase in dLS activity immediately preceded proximal stimulus flee. A key finding from our study was the

increased activation of the dLS during the first stimulus exposure post-conditioning (S1+) followed by subsequent reduction in dLS activity compared to baseline across stimulus exposures, particularly in social fear conditioned mice. In a later study, Besnard et al. (2020) found optogenetic activation of ventral CA3 terminals in the dLS suppressed freezing upon exposure to fearful stimuli during contextual fear extinction (Besnard et al., 2020). These data suggest that inputs from CA2/CA3 might contribute towards the reduction of the conditioned response demonstrated by decreased activation of the dLS across extinction following proximal stimulus flee.

On top of hippocampal control of fear learning and extinction, regions of the mPFC are implicated in fear learning and might offer alternative circuits which are interacting with the LS to facilitate fear extinction. Of note, glutamatergic projections from the infralimbic cortex (IL) to the LS and the CeA have been found to have opposing roles in anxiety and fear related behaviours (Chen et al., 2021). Through pharmacological inactivation of glutamate receptors in the LS and CeA and photostimuluating of the IL, Chen et al. (2021) found IL-LS projections induced *anxiogenic* effects on behaviour whereas IL-CeA projections induced *anxiolytic* effects on behaviour in the EPM and open field test (OFT). Optogenetic and chemogenetic inhibition of the IL-LS circuit increased time spent in the open arms and centre of the EPM, thus indicating LS inhibition promoted an anxiolytic response. Subsequent activation of IL-LS projections during cued fear conditioning, demonstrated higher rates of freezing across extinction whereas IL-CeA activation decreased freezing compared to controls. Similar to inhibition during EPM, IL-LS inhibition following cued fear conditioning led to a significant reduction in freezing response across extinction (Chen et al., 2021). These results indicate that, at least under non-social fear conditioning, dampening of IL-LS activity is needed to extinguish fear.

In parallel, there is also a role for the prelimbic (PrL) subregion of the mPFC in *social* fear extinction. Using a modified SFC task, Xu et al. (2019) found elevated c-Fos expression in the PrL compared to the IL following extinction. Further, they demonstrated that GABAergic inactivation of the PrL silenced social fear expression, but inactivation of the IL had minimal effect on social fear extinction (Xu et al., 2019). What this might mean in the context of our study is unclear, as recording and silencing of IL-LS projection neurons clearly demonstrated a reduction in fear and anxiety (Chen et al., 2021). However, these tasks did not assess social specific fear. Whilst our findings in Chapter 3 suggest a similar fear pathway is likely involved in social and non-social fear, the mPFC still represents a brain region of interest in the extinction of social fear behaviours. Future studies recording from IL-LS and CA3-LS projections would reveal their respective roles in social fear extinction. Based on current evidence, inhibition of these projection-specific pathways would be expected to inhibit the expression of social fear. Together with septohippocampal evidence of modulation of fear behaviours, both the IL-LS and vCA3-LS represent good candidate circuits in the extinction, but not acquisition of social fear.

***Downstream brain regions implicated in the extinction of social fear to explore.***

A key finding in Chapter 3, was the discovery that dLS activity is greatest during the first stimulus exposure post-conditioning (S1+) and that heightened dLS activity persists in those that are resistant to fear extinction. Further, results from Chapter 3 and 4 indicate that proximity to the stimulus is a key predictor of dLS activity where mice engaging in proximal but not distal flee had elevated dLS activity. One common feature of both these situations is that these are likely to be times of peak stress. A key neuropeptide implicated in stress and threat response is corticotrophin (CRH) and activation of type 2 corticotropin-releasing factor receptors (CRFR2) may promote and sustain active

avoidance of threatening stimuli. Discussed previously was the top-down modulation of LS activity in social fear behaviour. Alongside this activity, the LS may indirectly modulate cortical states – supporting extinction or maintaining fear – through modulation of subcortical brain regions (e.g. hypothalamus and paraventricular nucleus (PVN)), which innervate neocortical targets. In a seminal study investigating the role of CRH in the LS, Anthony et al. (2014) found activation of CRFR2 promoted anxiety via projections to the medial hypothalamus. Subsequent inhibition of this pathway was anxiolytic, suggesting that *if* this pathway were recruited and chemogenetically silenced prior to social fear extinction, that you might expect to see abolition of social fear to novel social stimuli, similar to what has been observed in SFC with infusion of OXT into the LS (Menon et al., 2018). To identify indirect cortical projections and thus determine the possibility of LS-mediated neocortical modulation, Hashimoto et al. (2022) infused a transneuronal tracer into the LS and observed viral expression in the orbitofrontal cortex, PrL and IL five days post-injection. This suggests that the LS is embedded in a complex network with both direct and indirect connections to regions with key roles in the regulation of social behaviour and fear extinction. This is further complicated by the diversity of cell types and neurotransmitters within the LS.

What completes the loop is the discovery that CRH signalling from the IL-LS suppresses interaction with familiar mice, in turn promoting interaction with novel mice (de Leon Reyes et al., 2023). We previously described how silencing of IL-LS projection neurons induced an anxiolytic response, where Chen et al. (2021) observed increased exploration in the OFT and EPM. In this more recent study, de Leon Reyes et al. (2023) found no impact on anxiety-related behaviours. This suggests that in the presence of anxiety-inducing environments, IL-LS dampens fear, however in the absence of an aversive environment, these same projections *promote* exploration of novel stimuli.

Finally, given the dense input from the hippocampus, especially dorsal and ventral CA3 to dLS, it is likely that uncoupling of the conditioned stimulus from the unconditioned stimulus is positively contributing towards fear extinction. Together, these studies support top-down control of the LS and how silencing of these neurons, possibly through feed-forward hypothalamic control, leads to social fear extinction.

One important subpopulation within the LS implicated in control of extinction of anxiety-like behaviours is GABAergic-expressing neurons. D. Wang et al. (2023) found that following chronic restraint stress, a task used to increase physiological stress, mice had increased activation in LS GABAergic-expressing ($LS^{GABAergic}$) neurons during OFT and when exploring the open arms of the EPM. Anterograde tracing of axonal projection of $LS^{GABAergic}$ neurons found the NaC, LH, VMH, VTA and hippocampus were all downstream targets – incidentally regions implicated in the "evaluation phase" (Calhoon & Tye, 2015). The $LS^{GABAergic}$-LH circuit emerged as of particular importance, where through the use of a GABA sensor in the LH, large increases in GABA release were found to occur during open field and open-arm exploration demonstrating that this circuit functionally mediates anxiety-like behaviour under certain conditions (D. Wang et al., 2023). Given the established role of the LH in detection of threat cues (Giardino et al., 2018), silencing of this pathway would contribute towards social fear extinction via bottom-up silencing of the cortical inputs into the LS.

Finally, mice who were extinction resistant in Chapters 3 and 4, may have perceived persistent environmental threat due to conspecific, sensory cues, experienced heightened stress following injections or other uncontrollable factors (e.g. experimenter handling) and subsequently remained hypervigilant. This defensive behaviour may have resulted in persevering hyperactivation of the LS upon proximal stimulus flee via Crhr2 (Anthony et al., 2014). These extinction resistant mice continued to actively approach

social or non-social stimuli, as seen in the frequency to approach and flee during S6+, however these mice did not successfully "unlearn" the association between investigation of the social or non-social stimulus (CS) and foot shock (US). This is evident in the finding that extinction resistant mice maintain elevated dLS activity at S6+ compared to pre-conditioning baseline (S1-). To test if hyperactivation of the dLS is contributing to sustained social avoidance, in future studies we could quantify CRH release using microdialysis (de Leon Reyes et al., 2023) or through use of the recently developed CRH-biosensor CRF1.0 (H. Wang et al., 2023) together with fibre photometry. With this method, we could divide mice *extinguishers* and *non-extinguishers* as in Chapter 3 and as commonly seen in the CSDS literature (Li et al., 2023). We could then examine dLS activity during social fear extinction and determine if there is a difference between those who extinguish social fear (extinguishers) and those who do not (non-extinguishers). In addition, selectively activating or silencing IL-LS, or alternatively LS-hypothalamic projections, we could observe *increased* social avoidance in unconditioned mice and *reduced* social avoidance in conditioned mice, respectively.

Whilst there are other circuits and cell-type of interest to explore (e.g. LS neurotensin-expressing (Azevedo et al., 2020) and LS somatostatin-expressing neurons (Xiao et al., 2017)) as discussed in Chapter 3 and intraseptal inhibition as described in Chapter 4, in the absence of characterisation of the cell-type activity within the dLS, we are unable to reach more definitive conclusions. Future studies will need to use cell-type specific approaches in recording and chemogenetic manipulation of dLS neurons during social fear extinction to delineate these. The IL-LS-Hyp-IL pathway proposed here is a plausible suggestion. Referring back to the four-stage anxiety framework proposed by Calhoon and Tye (2015), what may be occurring with sustained social avoidance as seen in social anxiety could be the *misinterpretation* of the relative risk within social situations,

leading to the hyperactivation of the LS via IL-LS projections subsequently perpetuating socially avoidant behaviour.

### *Clinical implications of research findings*

In Australia and in many countries, if you are experiencing mild forms of social anxiety or depression, you do not typically need a formal diagnosis from a psychiatrist to access behavioural therapy or be prescribed anti-anxiety medication (Charlotte et al., 2022; Forslund et al., 2020; Harrison et al., 2012). This offers an advantage in terms of early intervention and low-cost solutions (Eek et al., 2021; Strawn et al., 2018). Research shows that early intervention is important in reducing the long-term impact of psychological disorders and in reducing the long-term financial impact of chronic mental health (Colizzi et al., 2020; Fisak et al., 2023; Seabury et al., 2019). In Chapter 2, we demonstrated adolescent mice were more likely to extinguish social fear compared to adult mice. Given 80-90% of those diagnosed with social anxiety will be 24 years old or younger (Kessler et al., 2007; Solmi et al., 2022), our findings further emphasise adolescence as a critical period for social development. Solutions which can positively impact adolescents are thus important. Modern behavioural therapies targeted towards younger persons include CBT delivered via video-call and smartphone assisted apps (Biagianti et al., 2023; Leigh et al., 2023; Nordh et al., 2021), which have shown promise owing to their accessibility. Thus, identification within this thesis of the increased resilience towards social anxiety further supports the importance of early intervention in children and young adults.

Unfortunately, a high proportion of those who seek treatment for social anxiety report poor response rates and remain symptomatic post-treatment (~35-45%, Blanco et al. (2013); Roy-Byrne (2015). This indicates CBT and currently available pharmacotherapies

do not sufficiently manage and alleviate symptoms in many patients (*see reviews* Pelissolo et al. (2019) and Lee and Lee (2019)). As highlighted in Calhoon and Tye (2015), the rate of new medications for anxiety disorders compared to other major public health burdens such as hypertension has not grown to meet demand. To further compound the issue, patients with increased symptom severity and co-morbid disorders are more likely to be treatment resistant, meaning those who have the greatest need for clinical intervention are unable to adequately manage their symptoms leading to poorer quality of life (Koyuncu et al., 2019; Wilmer et al., 2021). This thesis has uncovered a novel role of the LS within the "social brain network" (Menon et al., 2022). In Chapter 3 in Experiment 2, we demonstrated that sustained hyperactivation of dLS neurons preceding proximal stimulus flee was associated with persistent social avoidance behaviour and that a reduction in dLS calcium signalling is associated with extinction of social fear. As discussed, there a numerous ascending and descending cell-type specific pathways of interest to explore relative to our findings. Elucidation of the dominant cell-type specific circuit involved in the dLS following social fear conditioning in mice susceptible to fear extinction (extinguishers) versus those resistant (non-extinguishers) would enable single-cell analysis of the excitatory receptors present on inhibitory GABAergic LS interneurons. In this way, akin to Clark et al. (2021), we could examine cell to cell interaction together with a reconstructed view of the neuronal connectivity within the dLS via single cell RNA sequencing. This process would, in theory, enable the identification of drug targets which could guide a medicinal chemistry program aimed at identification of ligands which modulate dLS activity in the desired direction. Applying an omics-based approach to identifying candidate therapeutic molecules in the modulation of social fear behaviour would allow for fine-tuning of treatment strategies in subsets of the SAD population. In this way, pharmacological treatment could be tailored to individuals to develop

pharmacological solutions which accounts for divergent developmental pathways seen in those with co-morbid neurodevelopmental disorders (e.g., FXS and ASD) and acknowledge the heterogeneity within psychiatric disorders.

In Chapter 4 we demonstrated the utility of silencing a specific subset of neurons implicated in social fear in modifying social anxiety-related behaviours. Beyond pharmacological therapies, advances are being made in the use of chemogenetics to modify anxiety-like behaviours in non-human primates (Raper et al., 2019; Roseboom et al., 2021). These studies together with the nascent evolution of DREADDs receptor delivery solutions via systemic rather than intracranial routes (Chen et al., 2022), open the door to less invasive DREADDs administration in the future. Whilst the use of DREADDs as a therapeutic solution in SAD is unlikely due to its current receptor delivery mode, it's application in rodents and non-human primates is crucial in informing our understanding of the causal drivers of social behaviour. Together with the use of calcium imaging techniques, the translation of findings from pre-clinical research is a crucial step in drug discovery for social disorders (Seshadri et al., 2020).

This thesis has explored social fear through biological and behavioural models of ASD and SAD. The use of the SFC task has been fundamental to our gained understanding of the drivers of social engagement, avoidance, and fear. It has allowed the examination of active social approach and avoidance behaviour, and expanded our understanding of the genetic, developmental, biological, and neural correlates of social anxiety-like behaviour in mice. This thesis has demonstrated that age is an important factor in the likelihood to extinguish social fear. Further, in a task specifically designed to assess social-specific fear, we have found that *Fmr1* KO mice do not exhibit greater social avoidance compared to controls. Finally, using fibre photometry and DREADDs we built upon the existing literature and characterised the specific approach and avoidance behaviours mediated by

the dLS during social fear extinction. Together, this work represents a thorough examination of correlates of social anxiety-like behaviours with the goal that, in the future, improved understanding of drivers of social engagement and avoidance will lead to the development of better treatment for SAD.

# Acknowledgements

There are a number of people who I have met along my academic journey so far that I would like to thank – without you I would not be the scientist nor the person I am today.

First and foremost, I would like to thank my PhD supervisors Professor Michael Bowen and Dr Karly Turner. To Michael, I will forever be proud to be one of your first PhD students. When we first discussed me joining the lab, I knew there was a certain risk in joining a new lab that was just getting started but after those two weeks in March 2018 getting to know you and the lab, I knew I was making the right decision. It has been a real pleasure to come along for what has been a meteoric rise for your scientific career these last few years. I especially appreciate the early years of brainstorming ideas, the support during tough times in COVID and your assistance and resilience with me gaining confidence in my research and statistics. You champion your students and post-docs in whatever passions and careers they choose to pursue, and I am so lucky and immensely grateful to have had you as my supervisor. Thank you.

To Karly, am I glad I met you when I did! Your mentorship and support throughout my Honours, providing me with once-in-a-life time experiences interning with you at Cambridge and now my PhD has been invaluable. You have been a constant form of support during my PhD, especially during the tough days of learning intracranial surgeries in a pandemic lockdown. You are an excellent friend and an even better scientist. Your dedication to your day-to-day goals and research is truly inspiring. I can only hope to one day be half the scientist you are.

To Bianca, Gabi, Joel, Rhianne, Erin, Stela, Connie, Oliver, Nick and Michael and the rest of the Bowen Lab, I have absolutely loved spending these last few years with you - lunches in the sun on the benches, lab drinks at the end of a long week and heading off to conferences together have all made my PhD an unforgettable experience. In particular, I want to say thank you to Bianca for showing me the ropes when I first started, Harry and Tim for technical support with coding, Erin for teaching me surgeries and much more and Nick for his help with photometry. I am also grateful to Charlotte for helping me score my mountain of behavioural videos and Joel for being a fantastic support as a fellow PhD student and making me feel welcome right from the get-go. I would also like to extend my gratitude to all those who help support research and PhD students like me, in particular those in animal care, Janelle and Jen and those in microscopy, Pam and Michael.

Outside of the lab and research, I would like to first acknowledge and thank Maria and Chris. Together you inspired my journey into autism research. Spending time with you Chris, has forever shaped the direction of my life. You have both taught me so much, and I am so thankful to you Maria for taking a chance on me all those years ago. What I love most of all is that we are all still in touch and I still get to spend time with you together with Jack and Geena.

To Batoul, I am so grateful for you. I am so grateful you moved to Sydney to do your PhD, that we reconnected and that you became not only the most wonderful friend,

but also an important person to talk to outside of the lab about the stress of a PhD, testing and navigating it all. You made it all more fun and I love you immensely. To Georgina, you always went out of your way to check in with me daily, catch-up with me weekly and provide me with so much love and support. Since the day you moved to Sydney, my time here has been immeasurably more fun and fuller. Becoming best friends with you and Batoul in Sydney has been one of the most profoundly impactful experiences in such a transformative time in my life.

To Caelyn, my forever lab partner. Thank you for being my rock at the beginning of my PhD and for always making the best effort to come see me when you visited Sydney. To Alice, Chris and Winston for all the wine, food and Catan parties. To Kate for trips to the beach before or after a long day of testing and to being such an important person during my time in Australia and welcoming to Christmas's with you and your family. To all my wonderful housemates in particular Lucy, Jess, Rahul, George, and Gina, I've loved living at Harrow Road with you and love even more than we're all still in touch after moving out. And to Tully's mum Renee and partner Leigh for providing comfort and a sense of family in Sydney with fortnightly Monday night dinners, making my mum feel welcome during her visit and cups of tea over cuddles with Raj after many hours in the lab.

And of course, to my Tully, you have nourished my body and my heart, keeping me alive and healthy and full of love and laughter. Thank you for always creating a calm and cosy space to relax with you in and unwind ready to do it all again the next day. I am so grateful to have met you and very much looking forward to us both getting to enjoy what life after my PhD looks like. I don't think, I would be here in as full of a piece and as happy as I am without your love and support.

Finally, to my mother Tina and my sister and brother Emma and Nikolai. It has not been easy living so far away, but we've made it work through videocalls and making the effort to catch-up in wonderful places all over the world. Mummy, you have been the most wonderful parent to me, Nik and Emma. I will always remember and appreciate our almost daily phone calls during my PhD in COVID, you kept me going on some of the hardest of days. Thank you for coming to live with me in Sydney, it was the best thing ever, I loved getting to show you my Australia and to have you visit long term meant we got to catch-up on so much time apart. I am very grateful for you and love you very much. Finally, to my father Paul, who I would have loved nothing more than to share in this experience and my successes with. You always nurtured my ceaselessly curious mind and raised me, together with Mummy, to constantly question the world around me - providing me every opportunity in life. I know that, with you around, learning to understand Python and R would have been a much more enjoyable experience. I often think how cool you would have thought fibre photometry, chemogenetics and all the microscopy work I've done is and I wish I could have shared it with you. Instead, I dedicate this thesis to you and your memory.

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# Appendix A

*Python Photometry Scripts*

## Appendix A. 1 Pre-processing

**This notebook achieves several things.**

1. It combines the Bonsai output of 470nm and 415nm traces into one side-by-side sheet
2. It crops to a start and end time (primarily to remove start/end artifacts due to plug/unplug)
3. It fits the 470 to the 415 curve, plots these
4. It subtracts fitted 470 from fitted 415 for the deltaF
5. It has the user define a baseline period (start and end in seconds)
6. It calculates the median of this baseline and subtracts this from the entire trace
7. It calculates a % change in dF/F0
8. It calculates a Z-score
9. it combines the behaviour sheet and the camera sheet into one
10. Together this produces 3 output files (ID _SignalZscore; ID_SignalPercentDelta; ID_BehaviourTimeStamped), which are used as inputs for DLC heatmapping, or peri-event analysis

In [ ]:

```
# Import dependencies
import pandas as pd
import glob
import os
import numpy as np
import matplotlib.pyplot as plt
import array as arr
import scipy
from scipy.stats import zscore
from scipy import signal as ss
from matplotlib.pyplot import figure
from sklearn.linear_model import HuberRegressor
```

**All inputs for the entire sheet can be put in here, and then you should be able to run the whole notebook**

In [ ]:

```
# Input start time and duration of TOTAL recording in seconds, for cropping
#UserInputtedStartFrame = 3000 # From first S, frame number
UserInputtedStartSecs = 270 ## SECONDS. if using this for peri-event, keep at 0. it will cut the file to this point.
UserInputtedEndFrame = 136045 ### FRAME NUMBER - NOT ROW NUMBER:  based on E6 (or final 'end' timestamp to ensure we're not capturing data once the cable is removed)
# DEFINE your baseline period, start + duration
BaselineStartFrame = 49603 # FRAME NUMBER - NOT ROW NUMBER: eventually i will get this to pull keydown information from bonsai.
```

```
BaselineDur = 120 ## input duration in seconds
#####################
Hz = 20 ## Capture rate on NPM system for the 470nm channel
RowFromUserInputtedStartSecs = UserInputtedStartSecs*Hz
RowFromFrame_EndFrame = int(UserInputtedEndFrame/2)
#DUR = 3100 ## in seconds. make it at least your session, can be longer
FrameDUR = RowFromFrame_EndFrame-RowFromUserInputtedStartSecs
DUR = FrameDUR/Hz # in seconds
print("Duration in seconds between user inputted frames is: ", DUR)
print("Duration in minutes: ", DUR/60)
#INPUT YOUR FOLDER HERE with the 5 files (470, 410, cameracsv, DLCtracking, behaviour)
path = r"Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned" # use your path
Duration in seconds between user inputted frames is:  3131.1
Duration in minutes:  52.184999999999995

In [ ]:
animal_id = os.path.split(path)[1]

df470path = glob.glob(os.path.join(path, f"{animal_id}*_470*"))[0]
df415path = glob.glob(os.path.join(path, f"{animal_id}*_415*"))[0]
print("470 file is: ", df470path)
```

```
print("415 file is: ", df415path)

#Makes a new folder to save files in to
savepath = os.path.join(path,"Pre-processing")
if not os.path.exists(savepath):
    os.mkdir(savepath)
```

470 file is:  Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\5.1.1s-m-conditioned_470_2022-10-22T16_52_39.csv

415 file is:  Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\5.1.1s-m-conditioned_415_2022-10-22T16_52_39.csv

**1) Combine the Bonsai output of 470nm and 415nm traces into one side-by-side sheet**

In [ ]:

```
## Gets info from 470nm sheet
df470 = pd.read_csv(df470path, index_col=0)
df470.columns = ['Timestamp', 'Flags', '470nm']
## Gets only the 415nm trace
df415 = pd.read_csv(df415path, index_col=0)
df415.columns = ['Timestamp', 'Flags', '415nm']

# If there are a different number of rows between the two csv files, we need to check the data,
print(f"df470 rows: {df470.shape[0]}")
print(f"df415 rows: {df415.shape[0]}")
```

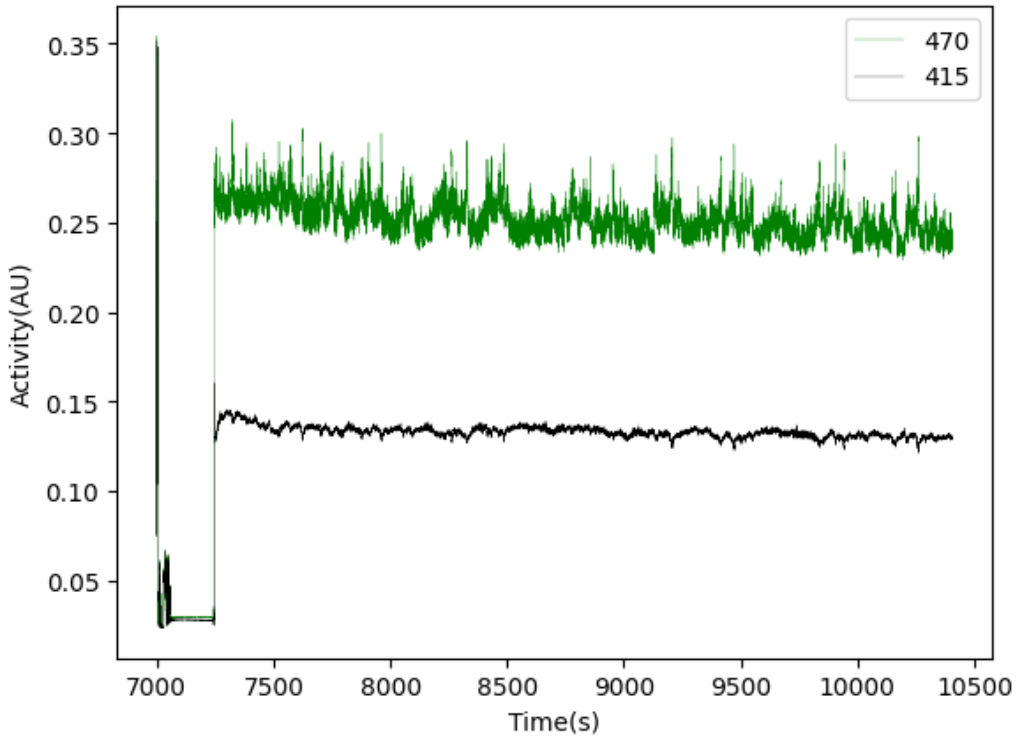
```
num_rows = np.min([df470.shape[0], df415.shape[0]])
df470 = df470.iloc[:num_rows]
df415 = df415.iloc[:num_rows]
## Combines the two dataframes so 470/415 are side-by-side
dfcombined = df470.copy()
dfcombined["415nm"] = df415["415nm"].values

# Plot and save the trace you've segmented for delta, 470 and 415. For Delta only, use below function
x = dfcombined['Timestamp']
y1 = dfcombined['470nm']
y2 = dfcombined['415nm']
plt.plot(x, y1, color='green', linewidth=0.2, label = "470")
plt.plot(x, y2, color='black', linewidth=0.2, label = "415")
plt.legend(['470', '415'], loc='best')
plt.xlabel('Time(s)')
plt.ylabel('Activity(AU)')
plt.savefig(os.path.join(savepath, f"{animal_id}_RawTraces.jpg"), dpi=600, orientation='landscape')

# Save combined df to savepath folder
dfcombined.to_csv(os.path.join(savepath, f"{animal_id}_RawTraces.csv"))
```

df470 rows: 68137

df415 rows: 68136




**Cropping combined Bonsai output (called dfcombined) to start/end, and plot the traces with a raw delta**

In [ ]:

```
UserInputtedStartFrame = dfcombined[dfcombined["Timestamp"] >= UserInputtedStartSecs + dfcombined.iloc[0]["Timestamp"]].index[0]
dfcombined_trimmed = dfcombined.loc[UserInputtedStartFrame:UserInputtedEndFrame]
dfcombined_trimmed = dfcombined_trimmed.iloc[:-1]
```

dfcombined_trimmed

Out[ ]:

| | Timestamp | Flags | 470nm | 415nm |
|---|---|---|---|---|
| **FrameCounter** | | | | |
| **10801** | 7268.959456 | 18 | 0.264708 | 0.139103 |
| **10803** | 7269.009504 | 18 | 0.264442 | 0.139208 |
| **10805** | 7269.059488 | 18 | 0.261728 | 0.138779 |
| **10807** | 7269.109504 | 18 | 0.261360 | 0.139103 |
| **10809** | 7269.159488 | 18 | 0.260737 | 0.139168 |
| **...** | ... | ... | ... | ... |
| **136035** | 10399.864768 | 18 | 0.238212 | 0.130255 |
| **136037** | 10399.914752 | 18 | 0.239415 | 0.130007 |
| **136039** | 10399.964768 | 18 | 0.241580 | 0.130462 |
| **136041** | 10400.014784 | 18 | 0.240151 | 0.129860 |
| **136043** | 10400.064800 | 18 | 0.242001 | 0.129516 |

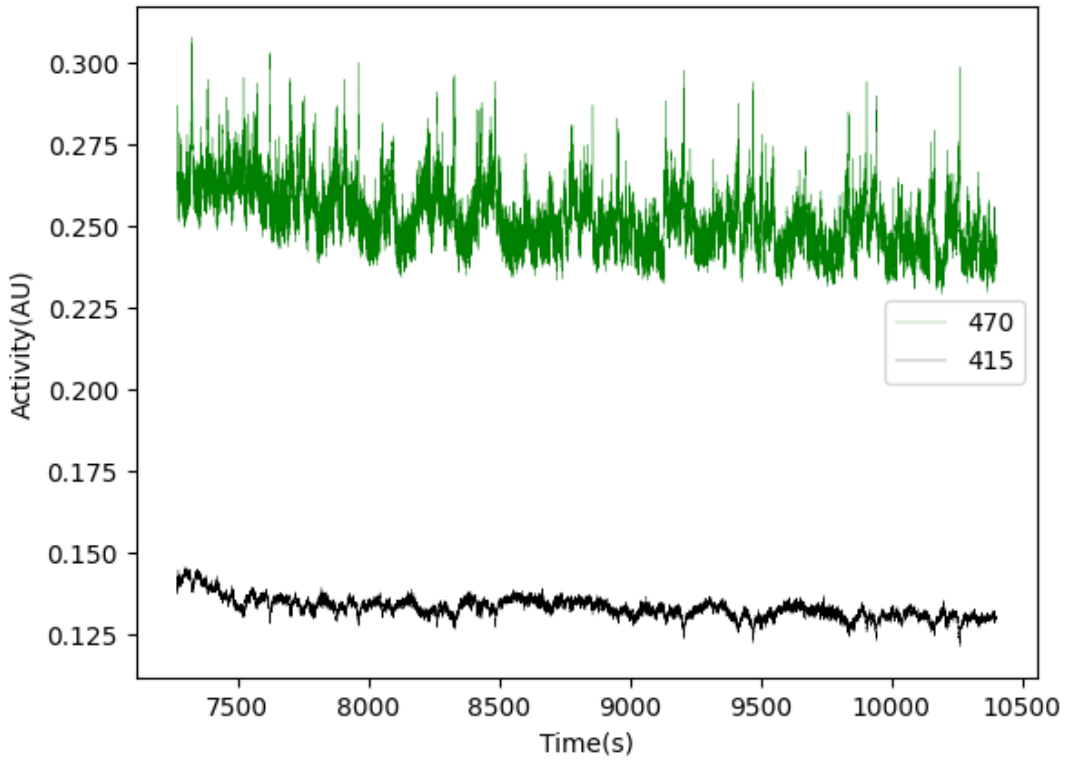
62622 rows × 4 columns

In [ ]:

```
x = dfcombined_trimmed['Timestamp']
y1 = dfcombined_trimmed['470nm']
y2 = dfcombined_trimmed['415nm']
plt.plot(x, y1, color='green', linewidth=0.2, label = "470")
plt.plot(x, y2, color='black', linewidth=0.2, label = "415")
plt.legend(['470', '415'], loc='best')
plt.xlabel('Time(s)')
plt.ylabel('Activity(AU)')
```

Out[ ]:

```
Text(0, 0.5, 'Activity(AU)')
```




**2) Fit 470 to 415 curve**

In [ ]:

signals_df = dfcombined_trimmed.rename(columns={"470nm": "raw_signal", "415nm": "raw_reference"})

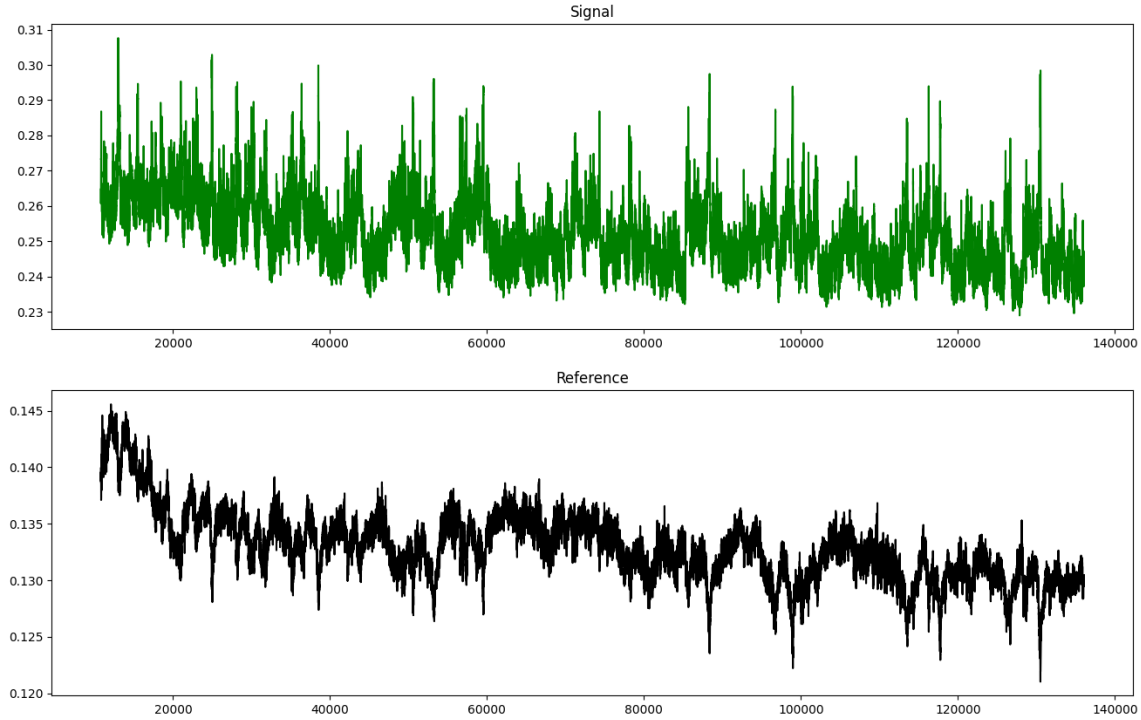
In [ ]:

*##### For now, if they are uneven # of frames, you'll need to manually remove the last frame from the one with the highest*

*#### Adjust this to take these values from the newly created m19_rawtraces.csv*

```
print("Signal, number of frames: ",signals_df["raw_signal"].shape)
print("Control, number of frames: ",signals_df["raw_reference"].shape)
Signal, number of frames:  (62622,)
Control, number of frames:  (62622,)
In [ ]:
##Plotting and comparing signal and reference, no manipulations occur here, just plots the same data from the above figure, but on different scales
fig = plt.figure(figsize=(16, 10))
ax1 = fig.add_subplot(211)
ax1.plot(signals_df["raw_signal"],'green',linewidth=1.5)
ax2 = fig.add_subplot(212)
ax2.plot(signals_df["raw_reference"],'black',linewidth=1.5)
ax1.set_title("Signal")
ax2.set_title("Reference")

plt.show()
```




In [ ]:

*##a linear regression of reference and signal*

*##we use the huberregressor as it is robust to large and infrequent outliers, and often not different to Linear Regression.*

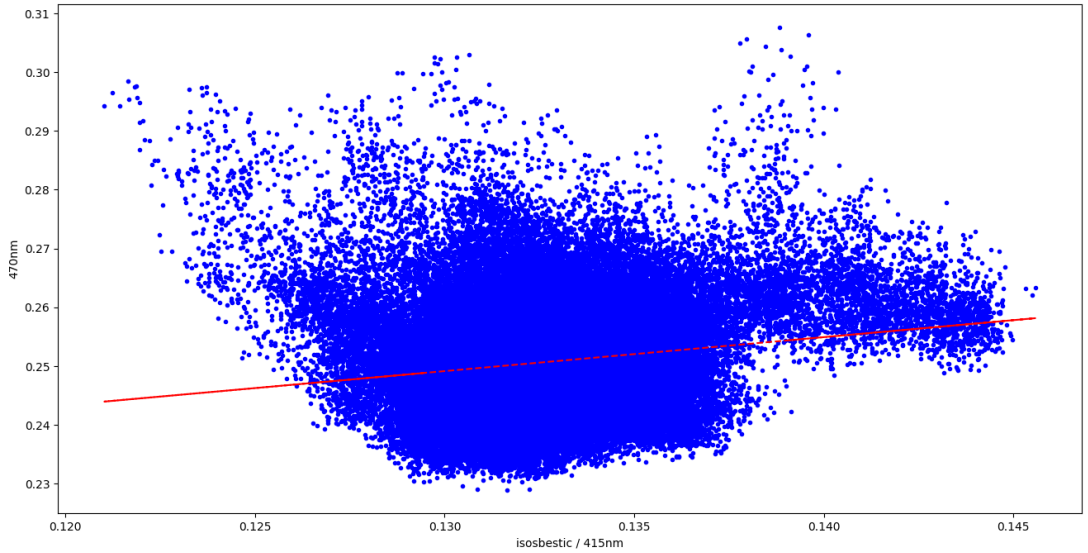
```
model = HuberRegressor(epsilon=1)
n=len(signals_df["raw_reference"])
model.fit(signals_df["raw_reference"].values.reshape(n, 1), signals_df["raw_signal"].values.reshape(n,1))
```

```
c:\Users\nicka\.conda\envs\DEEPLABCUT\lib\site-packages\sklearn\utils\validation.py:1184: DataConversionWarning: A column-vector y
was passed when a 1d array was expected. Please change the shape of y to (n_samples, ), for example using ravel().
  y = column_or_1d(y, warn=True)


Out[ ]:
HuberRegressor
HuberRegressor(epsilon=1)
In [ ]:
##we chart the relationship between reference and signal and show the linear fit. This is because any highly correlated activity is likely noise due to fibre bending, etc.


fig = plt.figure(figsize=(16, 8))
ax1 = fig.add_subplot(111)
ax1.plot(signals_df["raw_reference"],signals_df["raw_signal"], 'b.')

plt.xlabel("isosbestic / 415nm")
plt.ylabel("470nm")
signals_df["arr"] = model.predict(signals_df["raw_reference"].values.reshape(n, 1))
ax1.plot(signals_df["raw_reference"], signals_df["arr"], 'r--',linewidth=1.5)
Out[ ]:
[<matplotlib.lines.Line2D at 0x239c55d1460>]
```



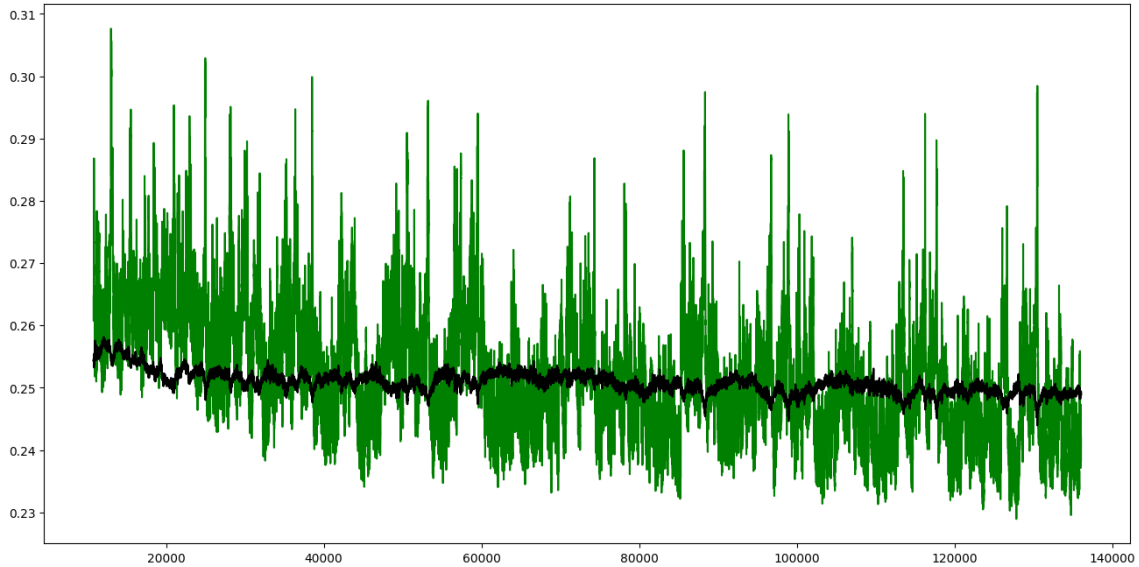

In [ ]:

*##the aligned control (arr) is the (control + y_intercept) * gradient of the control regressed to the signal*

```
fig = plt.figure(figsize=(16, 8))
ax1 = fig.add_subplot(111)
ax1.plot(signals_df["raw_signal"], 'green')
##yellow is signal aligned noise
ax1.plot(signals_df["arr"],'black')
```

Out[ ]:

[<matplotlib.lines.Line2D at 0x239c564dee0>]




In [ ]:

*##Now that we have the aligned control, we subtract it from the signal*

```
res = np.subtract(signals_df["raw_signal"], signals_df["arr"])
```

*##and then divide the signal by the control*

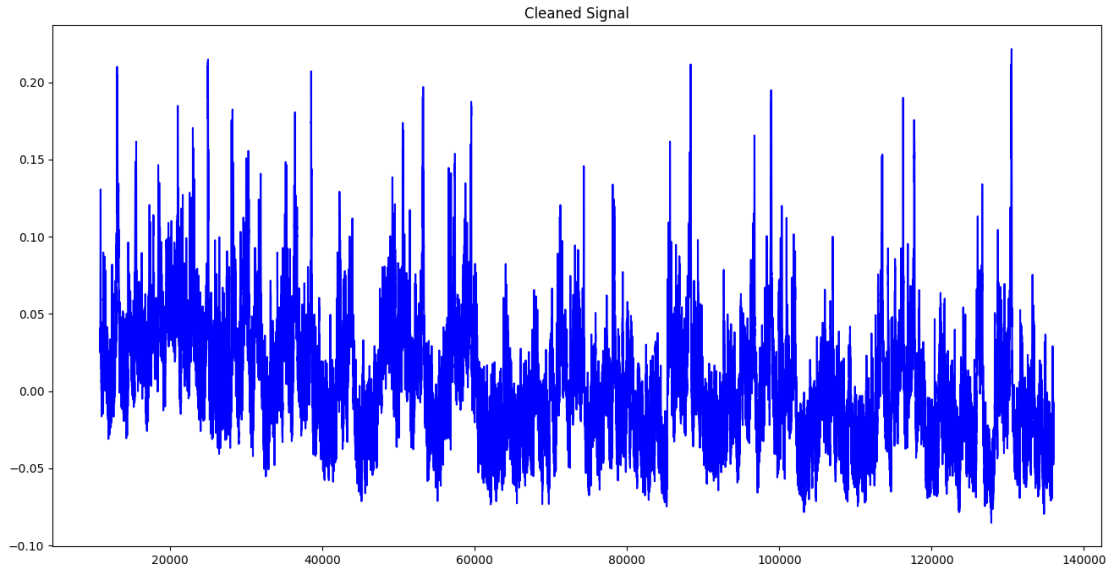
```
signals_df["norm"] = np.divide(res, signals_df["arr"])
```

In [ ]:

```
fig = plt.figure(figsize=(16, 8))
ax1 = fig.add_subplot(111)
ax1.set_title("Cleaned Signal")
ax1.plot(signals_df["norm"], color='blue')
```

Out[ ]:

[<matplotlib.lines.Line2D at 0x239c56e63d0>]




In [ ]:

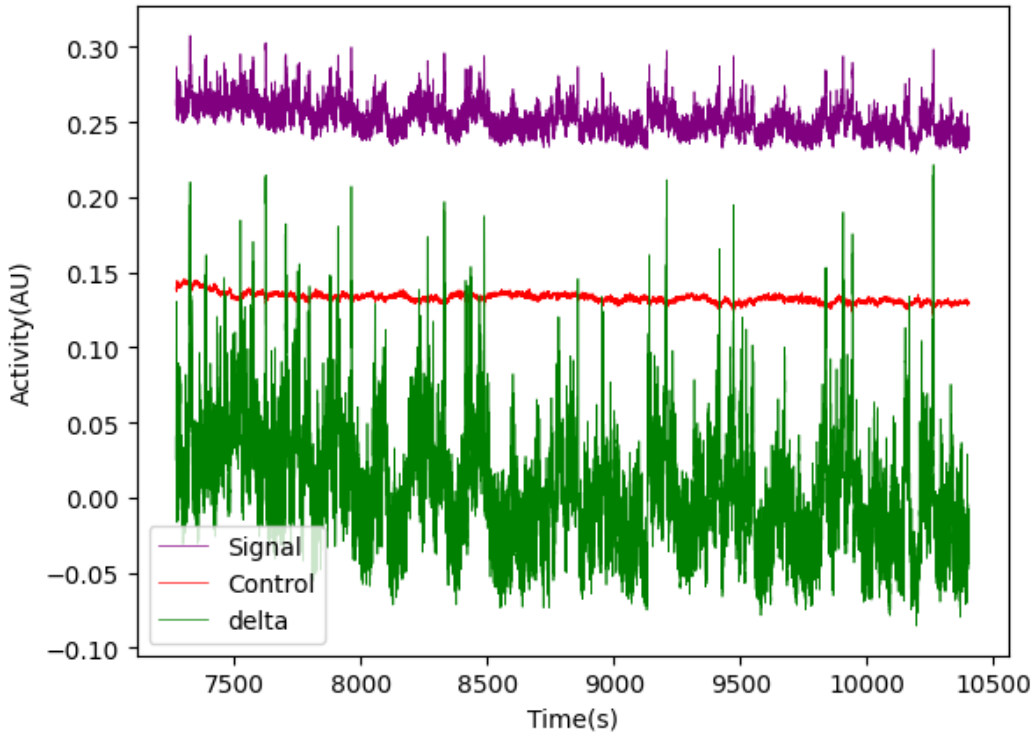
*# Plot and save the trace you've segmented for delta, 470 and 415. For Delta only, use below function*

```
x = signals_df['Timestamp']
y1 = signals_df["raw_signal"]
y2 = signals_df["raw_reference"]
y3 = signals_df["norm"]
```

```
plt.plot(x, y1, color='purple', linewidth=0.5, label = "Signal")
plt.plot(x, y2, color='red', linewidth=0.5, label = "Control")
plt.plot(x, y3, color='green', linewidth=0.5, label = "delta")
plt.legend(['Signal', 'Control', 'delta'], loc='best')
plt.xlabel('Time(s)')
plt.ylabel('Activity(AU)')

## Save the above plot
plt.savefig(os.path.join(savepath, f"{animal_id}_NormalisedTrace.jpg"), dpi=600, orientation='landscape')
print('Success! Saved as {}'.format(os.path.join(savepath, f"{animal_id}_NormalisedTrace.jpg")))
plt.show()

# Write combinedtwo df to csv, using animal ID
signals_df.to_csv(os.path.join(savepath, f"{animal_id}_NormalisedTraces.csv"), encoding='utf-8')
print('Success! Saved as {}'.format(os.path.join(savepath, f"{animal_id}_NormalisedTraces.csv")))
Success! Saved as Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Pre-processing\5.1.1s-m-conditioned_NormalisedTrace.jpg
```




Success! Saved as Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Pre-processing\5.1.1s-m-conditioned_NormalisedTraces.csv

In [ ]:

signals_df

Out[ ]:

| | Timestamp | Flags | raw_signal | raw_reference | arr | norm |
|---|---|---|---|---|---|---|
| **FrameCounter** | | | | | | |
| **10801** | 7268.959456 | 18 | 0.264708 | 0.139103 | 0.254392 | 0.040553 |
| **10803** | 7269.009504 | 18 | 0.264442 | 0.139208 | 0.254453 | 0.039258 |
| **10805** | 7269.059488 | 18 | 0.261728 | 0.138779 | 0.254205 | 0.029598 |
| **10807** | 7269.109504 | 18 | 0.261360 | 0.139103 | 0.254392 | 0.027393 |
| **10809** | 7269.159488 | 18 | 0.260737 | 0.139168 | 0.254430 | 0.024790 |
| **...** | ... | ... | ... | ... | ... | ... |
| **136035** | 10399.864768 | 18 | 0.238212 | 0.130255 | 0.249280 | -0.044398 |
| **136037** | 10399.914752 | 18 | 0.239415 | 0.130007 | 0.249137 | -0.039019 |
| **136039** | 10399.964768 | 18 | 0.241580 | 0.130462 | 0.249399 | -0.031355 |
| **136041** | 10400.014784 | 18 | 0.240151 | 0.129860 | 0.249051 | -0.035735 |

| FrameCounter | Timestamp | Flags | raw_signal | raw_reference | arr | norm |
|---|---|---|---|---|---|---|
| **136043** | 10400.064800 | 18 | 0.242001 | 0.129516 | 0.248853 | -0.027534 |

62622 rows × 6 columns

**3) Define Baseline period, removes it from dF to get dF-f0, to be used for %dF-F0/F0**

In [ ]:

```
BaselineStartTimestamp = signals_df.loc[BaselineStartFrame, "Timestamp"]
BaselineFinalTimestamp = signals_df.loc[BaselineStartFrame, "Timestamp"] + BaselineDur
BaselineFinalFrame = signals_df[signals_df["Timestamp"] >= BaselineFinalTimestamp].index[0]
print("Start timestamp: ", BaselineStartTimestamp)
print("BaselineStartFrame: ", BaselineStartFrame)
print("Final timestamp:", BaselineFinalTimestamp)
print("BaselineFinalFrame:", BaselineFinalFrame)

## Define Fzero
baseline_df = signals_df.loc[BaselineStartFrame:BaselineFinalFrame]
#Fzero or F0 is the median of the defined baseline period. this is for subtracting from each dF value
Fzero = baseline_df["norm"].median()
print("Baseline Fluorescence [Fzero] is: ", Fzero)
```

*# Calculates dF-Fzero*

signals_df["dfminusbaseline"] = signals_df["norm"] - Fzero

dfminusbaseline=pd.DataFrame(signals_df["dfminusbaseline"].values, columns = ["dF-Fzero"])

Start timestamp:  8239.02608

BaselineStartFrame:  49603

Final timestamp: 8359.02608

BaselineFinalFrame: 54403

Baseline Fluorescence [Fzero] is:  0.018619843374062743

In [ ]:

baseline_df

Out[ ]:

| | Timestamp | Flags | raw_signal | raw_reference | arr | norm |
|---|---|---|---|---|---|---|
| **FrameCounter** | | | | | | |
| **49603** | 8239.026080 | 18 | 0.257583 | 0.131130 | 0.249785 | 0.031216 |
| **49605** | 8239.076064 | 18 | 0.258523 | 0.131264 | 0.249862 | 0.034663 |
| **49607** | 8239.126080 | 18 | 0.255412 | 0.131250 | 0.249855 | 0.022240 |

| | Timestamp | Flags | raw_signal | raw_reference | arr | norm |
|---|---|---|---|---|---|---|
| **FrameCounter** | | | | | | |
| **49609** | 8239.176064 | 18 | 0.252554 | 0.131546 | 0.250025 | 0.010113 |
| **49611** | 8239.226080 | 18 | 0.254017 | 0.131214 | 0.249834 | 0.016745 |
| **...** | ... | ... | ... | ... | ... | ... |
| **54395** | 8358.828096 | 18 | 0.250495 | 0.132789 | 0.250744 | -0.000991 |
| **54397** | 8358.878080 | 18 | 0.248263 | 0.132407 | 0.250523 | -0.009022 |
| **54399** | 8358.928096 | 18 | 0.247745 | 0.133631 | 0.251230 | -0.013873 |
| **54401** | 8358.978080 | 18 | 0.247108 | 0.133440 | 0.251120 | -0.015974 |
| **54403** | 8359.028096 | 18 | 0.250689 | 0.133209 | 0.250986 | -0.001186 |

2401 rows × 6 columns

**4a) Uses dF-Fzero to calculate %dF as (dF-F0/F0)**

In [ ]:

*#Fzero is defined above, as is dfminusebaseline*

*#pctdFF = np.divide(dfminusbaseline, Fzero)*

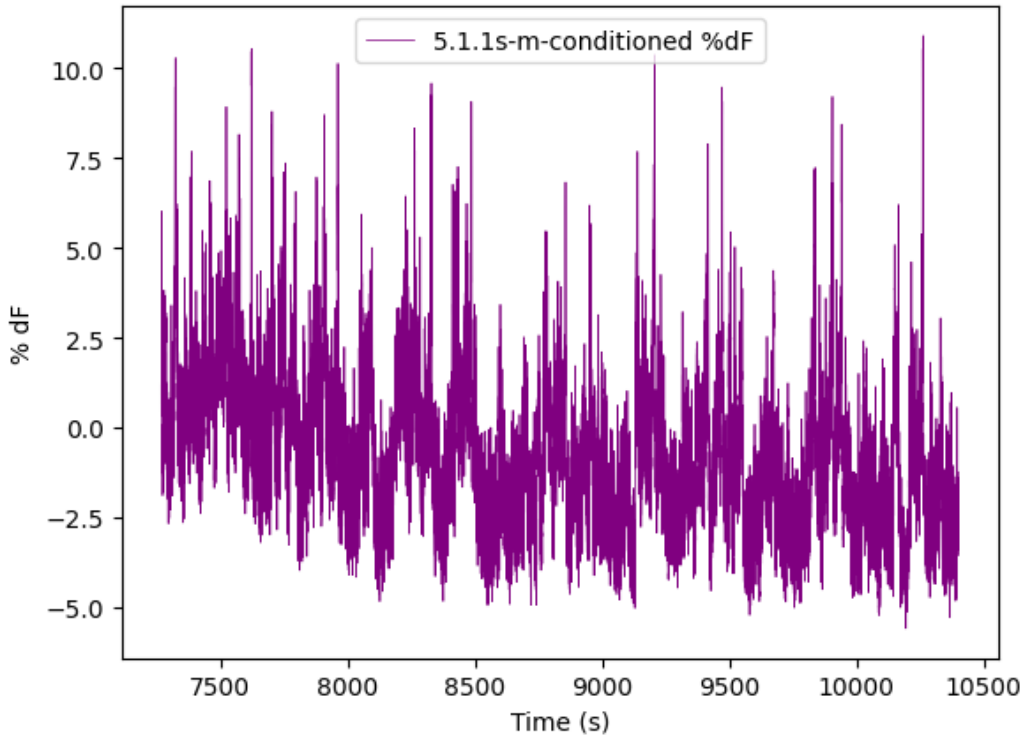
```
signals_df["pctdFF"] = signals_df["dfminusbaseline"]/Fzero

# Plot and save the trace you've segmented for delta, 470 and 415. For Delta only, use below function
x = signals_df['Timestamp']
y1 = signals_df["pctdFF"]

fig, ax = plt.subplots()
# Plot the 'pctdFF' column of the DataFrame against the x-axis
ax.plot(x, y1, color='purple', linewidth=0.5, label=f"{animal_id} %dF")

# Set the axis labels and legend
ax.set_xlabel('Time (s)')
ax.set_ylabel('% dF')
ax.legend()

## Save the above plot
plt.savefig(os.path.join(savepath, f"{animal_id}_pctdF.jpg"), dpi=600, orientation='landscape')
print('Percent dFF image saved')
plt.show()
Percent dFF image saved
```




**4b) Uses already-normalsied dF-Fzero (dfminusbaseline) to calculate ZdF1 (normalised to entire dataset) or uses norm_data to calculate ZdF2 (normalised to baseline period)**

In [ ]:

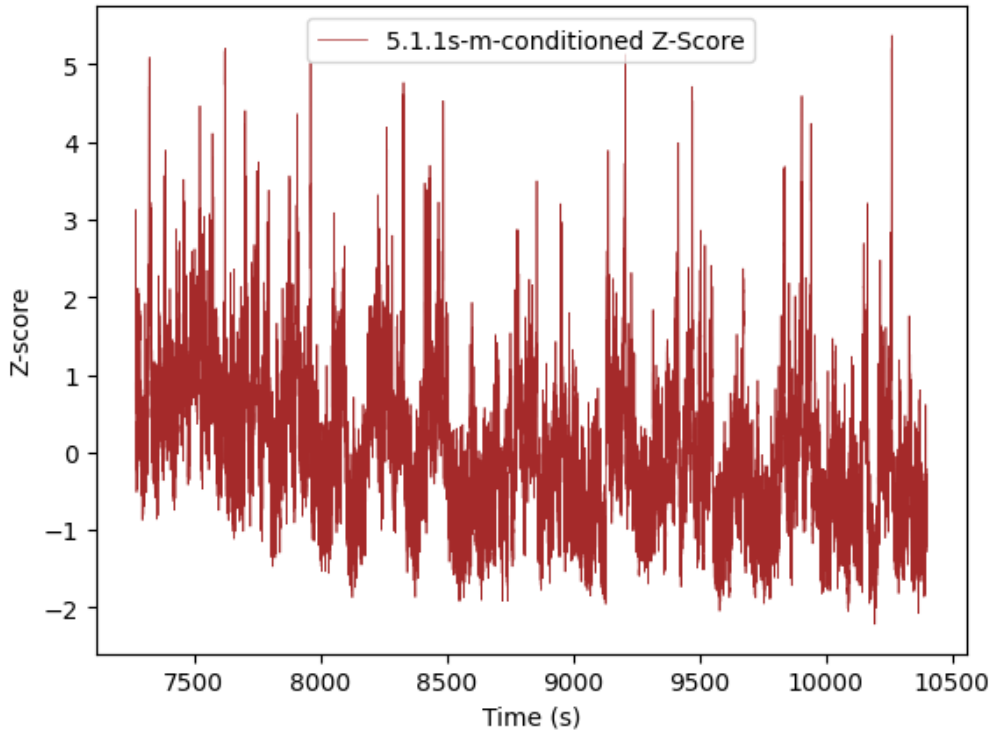
*#### ZdF uses dfminusbaseline, which is already normalised to the basleine period, and cals Z-score based on the entire data.*

*# e.g. How many SDs is each frame from the mean of the entire data?*

```
### calculates z-score
signals_df["ZdF"] = scipy.stats.zscore(signals_df["dfminusbaseline"], axis=0, ddof=0, nan_policy='propagate')
signals_df

# Plot and save the trace you've segmented for delta, 470 and 415. For Delta only, use below function
x = signals_df['Timestamp']
y1 = signals_df["ZdF"]

fig, ax = plt.subplots()
# Plot the 'pctdFF' column of the DataFrame against the x-axis
ax.plot(x, y1, color='brown', linewidth=0.5, label=f"{animal_id} Z-Score")
ax.set_xlabel('Time (s)')
ax.set_ylabel('Z-score')
ax.legend()
## Save the above plot
plt.savefig(os.path.join(savepath, f"{animal_id}_Z-score.jpg"), dpi=600, orientation='landscape')
print('Z-score image saved')
plt.show()
Z-score image saved
```




In [ ]:

*#### ZdF2 uses norm_data, which is not normalised to a baseline period*

*#### and then calculates a Z-score of all data, based on the mean/SD of the baseline period.*

*#### e.g. # e.g. How many SDs is each frame from the baseline data?*

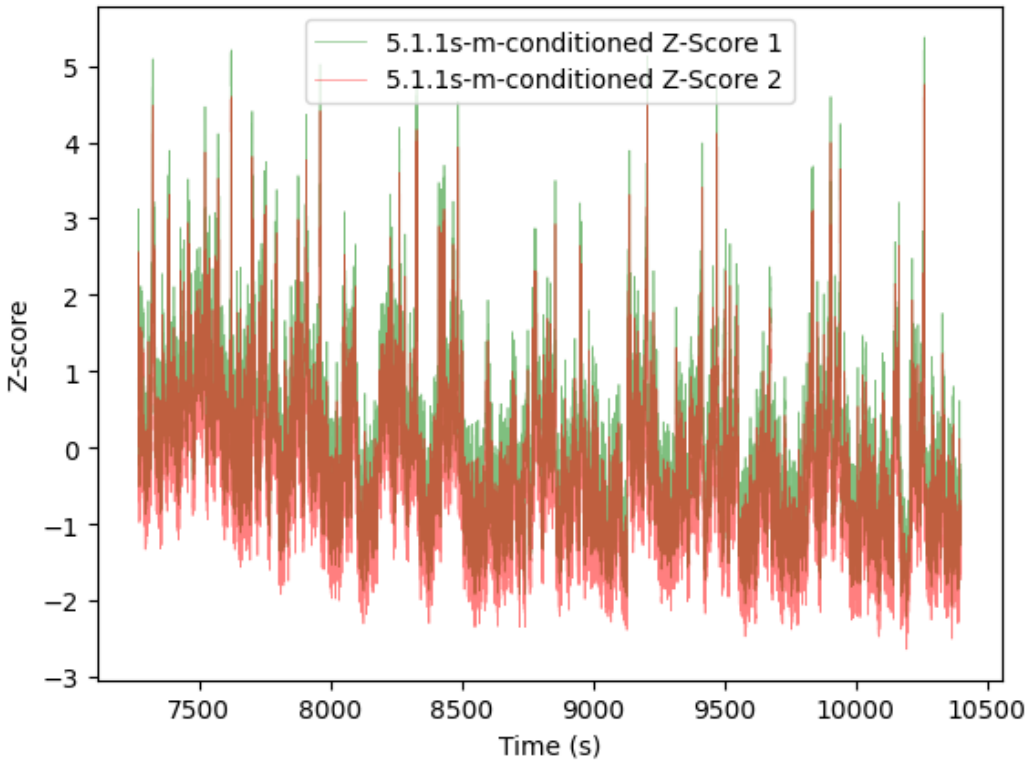
*# calcs mean and SD of the baseline period, for using in ZScore calcs*

baseline_mean_forZ2 = baseline_df["norm"].mean()

```
baseline_std_forZ2 = baseline_df["norm"].std()

# calcs Z-score of dfminusbaseline relative to the mean/std of the baseline period
signals_df["ZdF2"] = (signals_df["norm"]-baseline_mean_forZ2)/baseline_std_forZ2

# Define the figure and axis objects
fig, ax = plt.subplots()
x = signals_df['Timestamp']
y1 = signals_df["ZdF"]
y2 = signals_df["ZdF2"]
# Plot the 'Z-score' column of ZdF1 against the 'Timestamp' column of combinedthree
ax.plot(x, y1, color='green', linewidth=0.5, alpha = 0.5, label=f"{animal_id} Z-Score 1")
ax.plot(x, y2, color='red', linewidth=0.5, alpha = 0.5,  label=f"{animal_id} Z-Score 2")
# Set the axis labels and legend
ax.set_xlabel('Time (s)')
ax.set_ylabel('Z-score')
ax.legend()
# Show the plot
plt.show()
```




**Bonus section- adds the baseline period shading to the Z-score graph**

In [ ]:

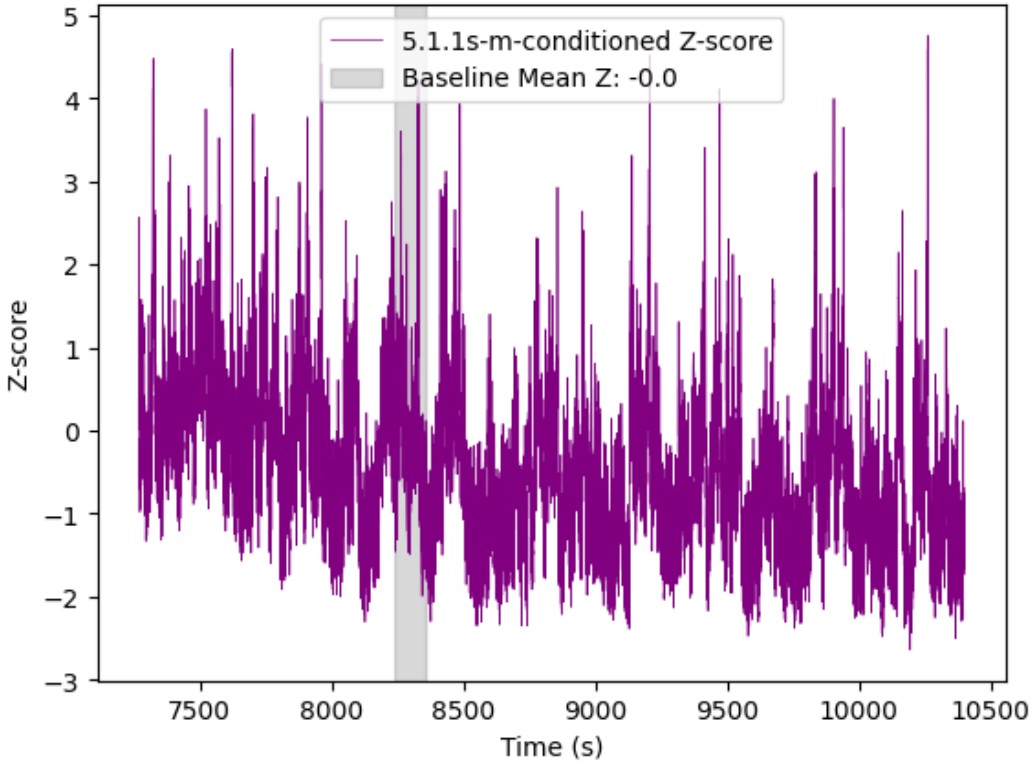
```
x = signals_df['Timestamp']
y1 = signals_df["ZdF2"]
```

```
# Create a figure and axis object
fig, ax = plt.subplots()

# Plot the 'pctdFF' column of the DataFrame against the x-axis
ax.plot(x, y1, color='purple', linewidth=0.5, label=f"{animal_id} Z-score")

#Calculates Median Z score during the baseline
baseline_df = signals_df.loc[BaselineStartFrame:BaselineFinalFrame]
ZdF2_mean_baseline_Z = baseline_df["ZdF2"].mean()
print("ZdF2_mean_baseline_Z: ", ZdF2_mean_baseline_Z)

# # Shade the area between the two X coordinates
ax.axvspan(x[BaselineStartFrame], x[BaselineFinalFrame], color='gray', alpha=0.3, label=f"Baseline Mean Z:
{round(ZdF2_mean_baseline_Z,4)}")
# # Set the axis labels and legend
ax.set_xlabel('Time (s)')
ax.set_ylabel('Z-score')
ax.legend()
# # Show the plot
plt.show()
ZdF2_mean_baseline_Z:  -1.1837446659893381e-17
```




**saves final output for peri-event analyses**

In [ ]:

```
# Formatting signals_df to save as csv
formatted_signals_df = signals_df.copy()
formatted_signals_df.index.name = "Frame"
formatted_signals_df = formatted_signals_df[["Timestamp", "ZdF2"]]
```

```
formatted_signals_df = formatted_signals_df.rename(columns={"ZdF2": "signal"})


# Saving as csv
formatted_signals_df.to_csv(os.path.join(savepath, f"{animal_id}_SignalZScoreFormatted.csv"), encoding='utf-8')
print('Success! Saved as {}'.format(os.path.join(savepath, f"{animal_id}_SignalZScoreFormatted.csv")))


formatted_signals_df
```

```
Success! Saved as Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Pre-processing\5.1.1s-m-conditioned_SignalZScoreFormatted.csv
```

Out[ ]:

| | Timestamp | signal |
|---|---|---|
| **Frame** | | |
| **10801** | 7268.959456 | 0.393348 |
| **10803** | 7269.009504 | 0.362139 |
| **10805** | 7269.059488 | 0.129380 |
| **10807** | 7269.109504 | 0.076251 |
| **10809** | 7269.159488 | 0.013546 |

| Frame | Timestamp | signal |
|---|---|---|
| ... | ... | ... |
| **136035** | 10399.864768 | -1.653556 |
| **136037** | 10399.914752 | -1.523949 |
| **136039** | 10399.964768 | -1.339281 |
| **136041** | 10400.014784 | -1.444821 |
| **136043** | 10400.064800 | -1.247207 |

62622 rows × 2 columns

In [ ]:

signals_df

Out[ ]:

| | Timestamp | Flags | raw_signal | raw_reference | arr | norm | dfminusbaseline | pctdFF | ZdF | ZdF2 |
|---|---|---|---|---|---|---|---|---|---|---|
| **FrameCounter** | | | | | | | | | | |
| **10801** | 7268.959456 | 18 | 0.264708 | 0.139103 | 0.254392 | 0.040553 | 0.021933 | 1.177939 | 0.897238 | 0.393348 |
| **10803** | 7269.009504 | 18 | 0.264442 | 0.139208 | 0.254453 | 0.039258 | 0.020638 | 1.108375 | 0.865208 | 0.362139 |
| **10805** | 7269.059488 | 18 | 0.261728 | 0.138779 | 0.254205 | 0.029598 | 0.010978 | 0.589572 | 0.626331 | 0.129380 |
| **10807** | 7269.109504 | 18 | 0.261360 | 0.139103 | 0.254392 | 0.027393 | 0.008773 | 0.471154 | 0.571807 | 0.076251 |
| **10809** | 7269.159488 | 18 | 0.260737 | 0.139168 | 0.254430 | 0.024790 | 0.006170 | 0.331388 | 0.507453 | 0.013546 |
| **...** | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| **136035** | 10399.864768 | 18 | 0.238212 | 0.130255 | 0.249280 | -0.044398 | -0.063018 | -3.384456 | -1.203463 | -1.653556 |
| **136037** | 10399.914752 | 18 | 0.239415 | 0.130007 | 0.249137 | -0.039019 | -0.057639 | -3.095573 | -1.070450 | -1.523949 |
| **136039** | 10399.964768 | 18 | 0.241580 | 0.130462 | 0.249399 | -0.031355 | -0.049975 | -2.683961 | -0.880928 | -1.339281 |
| **136041** | 10400.014784 | 18 | 0.240151 | 0.129860 | 0.249051 | -0.035735 | -0.054355 | -2.919201 | -0.989242 | -1.444821 |
| **136043** | 10400.064800 | 18 | 0.242001 | 0.129516 | 0.248853 | -0.027534 | -0.046154 | -2.478735 | -0.786434 | -1.247207 |

62622 rows × 10 columns

In [ ]:

```
# ## SAVES FINAL OUTPUT CSV to be used with other tools
# # f = final

# fTimestamp = combinedthree['Timestamp']
# fFrame = combinedthree['Frame']
# f470 = combinedthree['470nm']
# f415 = combinedthree['415nm']

# # Write FinalOutput to csv, using animal ID
# #FullOutput=pd.concat([fFrame, fTimestamp, f470, f415, pctdFF, ZdF], axis=1)
# #FullOutput.to_csv(savepath+'/'+AnimalID + "_FullOutput.csv", encoding='utf-8', index=False)
# #print('Success! Saved as {}'.format(savepath+'/'+AnimalID + "_FullOutput.csv"))

# # Write zscore, timestamp, frame to csv for peri-event
# #ZdF = ZdF.rename(columns={'Z-score': 'signal'})
# SignalZscore=pd.concat([fFrame, fTimestamp, ZdF2], axis=1)
# SignalZscore.to_csv(savepath+'/'+AnimalID + "_SignalZscore.csv", encoding='utf-8', index=False)
# print('Z-score signal saved as {}'.format(savepath+'/'+AnimalID + "_SignalZscore.csv"))
```

*# # Write zscore, timestamp, frame to csv for peri-event*

*# pctdFF = pctdFF.rename(columns={'pctdFF': 'signal'})*

*# SignalPercentDelta=pd.concat([fFrame, fTimestamp, pctdFF], axis=1)*

*# SignalPercentDelta.to_csv(savepath+'/'+AnimalID + "_SignalPercentDelta.csv", encoding='utf-8', index=False)*

*# print('Percent dFF signal saved as {}'.format(savepath+'/'+AnimalID + "_SignalPercentDelta.csv"))*

**5) This combines your behaviour and cameracsv into one sheet for the peri-event**

**GETS 470nm and 415nm in separate dataframes**

behaviourpath = glob.glob(path + "/*behaviour*.csv") print("Behaviour path: ", behaviourpath) camerapath = glob.glob(path + "/*csvforvideo*.csv") print("Camera path: ", camerapath)

**Gets info from behaviour sheet, and camera sheet, and collates them**

behaviourcols = ['frame', 'behaviour', 'hits'] behaviourcsv = pd.read_csv(behaviourpath[0], usecols=behaviourcols)

**puts that into a dataframe**

dfbehaviour=pd.DataFrame(behaviourcsv.values, columns = ["frame", "behaviour", "hits"])

**Gets info from behaviour sheet, and camera sheet, and collates them**

cameratimestamps = pd.read_csv(camerapath[0], header=None, usecols=[0])

**puts that into a dataframe**

dfcameratimestamps=pd.DataFrame(cameratimestamps.values, columns = ["Timestamp"])

**Combines the two dataframes so behaviour frames and camera timestmaps are side-by-side**

behaviourtimestamped=pd.concat([dfbehaviour,dfcameratimestamps], axis=1)

**Write combinedone df to csv, using animal ID**

behaviourtimestamped.to_csv(savepath+'/'+AnimalID + "_BehaviourTimeStamped.csv", encoding='utf-8', index=False) print('Files combined as {}'.format(savepath+'/'+AnimalID + "_BehaviourTimeStamped.csv"))

In [ ]:

```
#GETS 470nm and 415nm in separate dataframes

# morlogpath = glob.glob(path + "/*-scrubbed_scored_behaviour*.csv")
morlogpath = glob.glob(path + "/*_AnalysedBehaviour-RScrubbed.csv")
print("morlog file is: ", morlogpath)
camerapath = glob.glob(path + "/*csvforvideo*.csv")
print("camera timestamps file is: ", camerapath)

## Gets info from morlog behaviour sheet, and camera sheet, and collates them
# dfbehaviour = pd.read_csv(morlogpath[0], header=0, usecols=['frame', 'behaviour', 'hits'])
dfbehaviour = pd.read_csv(morlogpath[0], header=0)
dfbehaviour = dfbehaviour[["frame", "new_behaviour", "new_hits"]]
dfbehaviour = dfbehaviour.rename(columns={"new_behaviour": "behaviour", "new_hits": "hits"})

# puts that into a dataframe
# dfbehaviour=pd.DataFrame(behaviourcsv.values, columns = ["frame", "duration", "hits"])

# Gets info from morlog behaviour sheet, and camera sheet, and collates them
dfcameratimestamps = pd.read_csv(camerapath[0], header=None, usecols=[0])
```

```
dfcameratimestamps.columns = ["Timestamp"]

# puts that into a dataframe
# dfcameratimestamps=pd.DataFrame(cameratimestamps.values, columns = ["Timestamp"])

# Trimming the dataframes so they are the same size (sometimes, the csvforvideo dataframe has more rows than the scored_behaviour)
dfcameratimestamps = dfcameratimestamps.iloc[:dfbehaviour.shape[0]]

# Combines the two dataframes so behaviour frames and camera timestmaps are side-by-side
behaviourtimestamped = pd.concat([dfbehaviour,dfcameratimestamps], axis=1)
behaviourtimestamped = behaviourtimestamped.set_index("frame")

# Write combinedone df to csv, using animal ID
behaviourtimestamped.to_csv(os.path.join(savepath, f"{animal_id}_BehaviourTimeStamped.csv"))
print('Success! Saved as {}'.format(os.path.join(savepath, f"{animal_id}_BehaviourTimeStamped.csv")))
behaviourtimestamped
morlog file is:  ['Z:\\PRJ-BowenLab\\TimLee\\resources\\photometry\\5.1.1s-m-conditioned\\5.1.1s-m-conditioned_AnalysedBehaviour-RScrubbed.csv']
camera timestamps file is:  ['Z:\\PRJ-BowenLab\\TimLee\\resources\\photometry\\5.1.1s-m-conditioned\\5.1.1s-m-conditioned_csvforvideo_2022-10-22T16_52_38.csv']
Success! Saved as Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Pre-processing\5.1.1s-m-conditioned_BehaviourTimeStamped.csv
```

Out[ ]:

| | behaviour | hits | Timestamp |
|---|---|---|---|
| **frame** | | | |
| **1** | Nothing | NaN | 6998.954528 |
| **2** | Nothing | NaN | 6999.054528 |
| **3** | Nothing | NaN | 6999.104544 |
| **4** | Nothing | NaN | 6999.154528 |
| **5** | Nothing | NaN | 6999.254528 |
| **...** | ... | ... | ... |
| **51052** | othernonstim | NaN | 10402.414816 |
| **51053** | othernonstim | NaN | 10402.514816 |
| **51054** | othernonstim | NaN | 10402.564832 |
| **51055** | othernonstim | NaN | 10402.664832 |
| **51056** | othernonstim | NaN | 10402.714816 |

51056 rows × 3 columns

## Appendix A. 2 Peri-event analysis

**Photometry analysis**

In [ ]:

```
import pandas as pd
import numpy  as np
import glob
import os
```

**All inputs for the entire sheet can be put in here, and then you should be able to run the whole notebook**

In [ ]:

```
## Define your peri-event window
pre_window_secs = 5.0 ### How long *before* the behaviour onset do you want to extract?
post_window_secs = 5.0 ### How long *after* the behaviour onset do you want to extract?

####### The above input MUST be to one decimal point, e.g. 5.0, 3.5, 2.1. It will not work as "5"########

# What is the folder of your initial 5 files? This is the same folder input as the pre-processing book, and it will then look for your "pre-processing" folder which was created
path = r"Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned" # use your path

print("Source folder is: ", path)
```

*# What is the capture rate of the photometry system?*

*# If NPM is capturing at 40fps, and you're 1:1 interleaved with 470/415, then put in 20. This is only used to convert your peri-event seconds into # of frames.*

photom_hz = 20

Source folder is:  Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned

**1) Open and combine pre-processed data**

Here we will combine photometry and behaviour analysis files and pull out the information we need for analysis

Open your data files

In [ ]:

*# finds the pre-procesisng folder created in the pre-processing notebook, and uses the files here*

```
preprocessingpath = os.path.join(path,"Pre-processing")
print("Files being used are from: ", preprocessingpath)

#Makes a new folder to save files in to
savepath = os.path.join(path,"Peri-Event-Analysed")
if not os.path.exists(savepath):
    os.mkdir(savepath)

print("Peri-event files will be saved to: ", savepath)
```

Files being used are from:  Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Pre-processing

Peri-event files will be saved to:  Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Peri-Event-Analysed

In [ ]:

```
behaviourpath = glob.glob(preprocessingpath + "/*BehaviourTimestamped.csv")
print("Behaviour file is: ", behaviourpath)
photompath = glob.glob(preprocessingpath + "/*SignalZscoreFormatted.csv")
print("Signal file is: ", photompath)
##open the csv of your behaviour file and remove rows that are only nan (added in by excel sometimes)
behaviour = pd.read_csv(behaviourpath[0]).dropna(axis=0, how='all')
#print("behaviour is: ", behaviour)
photom = pd.read_csv(photompath[0]).dropna(axis=0, how='all')
#print("photom is: ", photom)
#Extract Animal ID from file name
concat_str = ''.join(behaviourpath)
AnimalID = concat_str.split('\\')[-1].split('_')[0]
print("Animal Name: ", AnimalID)
```

```
Behaviour file is:  ['Z:\\PRJ-BowenLab\\TimLee\\resources\\photometry\\5.1.1s-m-conditioned\\Pre-processing\\5.1.1s-m-conditioned_BehaviourTimeStamped.csv']
Signal file is:  ['Z:\\PRJ-BowenLab\\TimLee\\resources\\photometry\\5.1.1s-m-conditioned\\Pre-processing\\5.1.1s-m-conditioned_SignalZScoreFormatted.csv']
Animal Name:  5.1.1s-m-conditioned
```

In [ ]:

```
photom
```

Out[ ]:

| | Frame | Timestamp | signal |
|---|---|---|---|
| **0** | 10801 | 7268.959456 | 0.393348 |
| **1** | 10803 | 7269.009504 | 0.362139 |
| **2** | 10805 | 7269.059488 | 0.129380 |
| **3** | 10807 | 7269.109504 | 0.076251 |
| **4** | 10809 | 7269.159488 | 0.013546 |
| **...** | ... | ... | ... |
| **62617** | 136035 | 10399.864768 | -1.653556 |
| **62618** | 136037 | 10399.914752 | -1.523949 |
| **62619** | 136039 | 10399.964768 | -1.339281 |
| **62620** | 136041 | 10400.014784 | -1.444821 |
| **62621** | 136043 | 10400.064800 | -1.247207 |

62622 rows × 3 columns

**Here we create a function that combines a photometry trace and morlog file, since we have timestamp information in both files nothing else is needed**

In [ ]:

```
## This code combines photometry trace and behaviour file, and theiri respective timestamps.
## Due to these timestamps, we don't actually need the Photometry or Camera frame rates from above.Photometry hz is used for peri-event conversions though.
photom = photom.rename(columns={'Frame':'photom_frame'})
behaviour.set_index('Timestamp', inplace=True)
photom.set_index('Timestamp', inplace=True)
photom = behaviour.join(photom, how='outer')
photom = photom.rename(columns={'duration':'behaviour'})
photom.behaviour = photom.behaviour.fillna('Nothing')
##photom variable is now a combined dataframe with synced traces, behaviours and hits
##display is an alternative for print that keeps pandas dataframes looking pretty
#display(photom)
print("Success! Photometry trace and behaviour file and their timestamps have been combined in to one data frame!")
```

```
Success! Photometry trace and behaviour file and their timestamps have been combined in to one data frame!
```

**2) Peri-Event extraction**

Extract the photometry traces X seconds either side of the onset of each behaviour ("hits")

**Get pre and post window length in seconds**

In [ ]:

```
##convert secs to photometry capture frames
pre_window_frames  = pre_window_secs * photom_hz
post_window_frames = post_window_secs * photom_hz
print("Pre (s): ", pre_window_secs)
print("Post (s): ", post_window_secs)
print("Pre (frames): ", pre_window_frames)
print("Post (frames): ", post_window_frames)
pre_str = str(pre_window_secs)
post_str = str(post_window_secs)
##Create output file name
outputname = savepath+'/'+AnimalID+'_PeriEvent_'+pre_str+'s-pre_'+post_str+'s-post'+'.xlsx'
```

```
Pre (s):  5.0
Post (s):  5.0
Pre (frames):  100.0
Post (frames):  100.0
```

In [ ]: photom

Out[ ]:

| | frame | behaviour | hits | photom_frame | signal |
|---|---|---|---|---|---|
| **Timestamp** | | | | | |
| **6998.954528** | 1.0 | Nothing | NaN | NaN | NaN |
| **6999.054528** | 2.0 | Nothing | NaN | NaN | NaN |
| **6999.104544** | 3.0 | Nothing | NaN | NaN | NaN |
| **6999.154528** | 4.0 | Nothing | NaN | NaN | NaN |
| **6999.254528** | 5.0 | Nothing | NaN | NaN | NaN |
| **...** | ... | ... | ... | ... | ... |
| **10402.414816** | 51052.0 | othernonstim | NaN | NaN | NaN |
| **10402.514816** | 51053.0 | othernonstim | NaN | NaN | NaN |
| **10402.564832** | 51054.0 | othernonstim | NaN | NaN | NaN |
| **10402.664832** | 51055.0 | othernonstim | NaN | NaN | NaN |
| **10402.714816** | 51056.0 | othernonstim | NaN | NaN | NaN |

66712 rows × 5 columns

**Extract photometry signal for every behaviour, based on pre- and post-frames, and save as multi-sheet excel**

In [ ]:

```
def df_style(val):
    return 'font-weight: bold'


##create an empty dict to store photometry data for later visualisation
photom_hit_window = dict()
##create a sequence from prewindow photom frames to post window photom frames
pre_post_secs = np.arange(-pre_window_frames, post_window_frames)/(photom_hz)
##convert to ms
pre_post_secs = pre_post_secs*1000
##Find all the unique behaviours present in the file
unique_behaviours = photom['behaviour'].unique()
##remove Nothing
unique_behaviours = unique_behaviours[unique_behaviours!='Nothing']
##write each behaviour as an excel sheet
with pd.ExcelWriter(outputname) as writer:
    for beh in unique_behaviours:
        ##get index of current behaviour
        indexes = np.where(photom.hits==beh)[0]
        time_seconds = (indexes/photom_hz)
```

```
        onset_mins = time_seconds/60
        ##get create indexes for each window
        photom_hit_indexes = [np.arange(np.max([i-pre_window_frames, 0]), np.min([i+post_window_frames, photom.shape[0]])) for i in
indexes]
        ##get photom signal for each window (keeping windows seperate)
        photom_traces = [photom.signal.iloc[i].values for i in photom_hit_indexes]
        ##organise each photom signal into rows with columns labeled as ms pre/post behaviour
        trace_df = pd.DataFrame(photom_traces, columns = ['{} ms'.format(i) for i in pre_post_secs])
        trace_df.insert(0,"onset_seconds", time_seconds)
        trace_df.insert(1,"onset_mins", onset_mins)
        #row title should be Event no. #1, #2, etc
        trace_df.index+=1
        trace_df.index.name=None
        photom_hit_window[beh] = trace_df
        trace_df.index.name = 'Event No.'
        ##write new sheet
        trace_df = trace_df.style.applymap(df_style, subset=["onset_seconds", "onset_mins"])
        trace_df.to_excel(writer, beh)
        ##add to dictionary for later visualisation

print("Saved as: ", outputname)
```

Saved as: Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Peri-Event-Analysed/5.1.1s-m-conditioned_PeriEvent_5.0s-pre_5.0s-post.xlsx

In [ ]:

*## Check what behaviorus you have in your file. This list is used in the above script*

```
unique_behaviours
```

Out[ ]:

```
array(['phase', 'cornerimmp', 'othernonstim', 'proxapp', 'explore',
       'proxflee', 'distapp', 'distflee', 'retreat'], dtype=object)
```

**Plot your behaviours here and save as svg**

In [ ]:

```
import matplotlib.pyplot as plt
import numpy as np
import matplotlib
import seaborn as sns
import emoji

matplotlib.rcParams['figure.dpi']= 500
sns.set()

def plot_behaviour_trace(photom_hit_window, cur_behaviour, pre_post_secs):
    ##get index values (negative to positive ms)
```

```
    x = photom_hit_window[cur_behaviour].columns
    timestampnames = [str(i)+" ms" for i in pre_post_secs]

    ##get the mean line of all traces
    mean = photom_hit_window[cur_behaviour][timestampnames].mean()
    ##get the standard error of the mean
    sem = photom_hit_window[cur_behaviour][timestampnames].sem()

    # Make new plot
    fig, ax = plt.subplots()
    ##plot each individual trace
    for i in photom_hit_window[cur_behaviour][timestampnames].values:
        ax.plot(pre_post_secs, i, 'lightblue', label='mean_1', linewidth=1,  zorder=1)
    # [fig.plot(pre_post_secs, i, 'lightblue', label='mean_1', linewidth=1,  zorder=1) for i in
photom_hit_window[cur_behaviour][timestampnames].values]
    ##shade the SEM area
    ax.fill_between(pre_post_secs, mean - sem, mean + sem, color='orange', alpha=0.4, zorder=2)
    ##plot the mean trace
    ax.plot(pre_post_secs, mean, 'darkblue', label='mean_1', zorder=3)
    ##get current axis, i dont really get it but it lets you change chart settings
    # ax = plt.gca()
    ##make the chart background white
```

```
ax.set_facecolor('white')
##set the little ticks to be black
ax.spines['bottom'].set_color('0.5')
ax.spines['top'].set_color('0.5')
ax.spines['right'].set_color('0.5')
ax.spines['left'].set_color('0.5')
##remove the background grid
ax.grid(False)
##remove unneccessary white space
fig.tight_layout(pad=0)
##get the largest value in the dataset
max_of_all = photom_hit_window[cur_behaviour][timestampnames].values.max()
##draw a line to this point (though im pretty sure this draws an infinite line anyway)
ax.axline((0,0), (0, max_of_all), linestyle='dotted', color='red')
##make sure ticks are on the bottom and left
ax.tick_params(bottom=True, left=True)
##set the plot title to the current behaviour in all caps
ax.set_title(cur_behaviour.upper(), fontweight='bold')
##manually create a legend using the same lines i used for each type of trace
lines = [
    matplotlib.lines.Line2D((0,1),(0,1),linestyle = 'dotted', color='red'),
```

```
    matplotlib.lines.Line2D((0,1),(0,1), color='darkblue'),
    matplotlib.lines.Line2D((0,1),(0,1), color='orange'),
    matplotlib.lines.Line2D((0,1),(0,1), color='lightblue'),
  ]
  ##draw the legend and place it in the top right
  ax.legend(lines, ['Behaviour', 'Mean', 'SEM', 'Trace'], loc='upper right')
  ##X axis label
  ax.set_xlabel('Time (ms) from behaviour onset',  fontweight='bold')
  ##Y axis label
  ax.set_ylabel('Z-score',  fontweight='bold') #'% Δf / f'
  ##Save figure as an SVG
  plt.savefig(savepath+'/'+AnimalID+"_"+cur_behaviour+"_trace.svg", bbox_inches='tight')
  print("Peri-event trace figure saved as: ", savepath+'/'+AnimalID+"_"+cur_behaviour+"_trace.svg")
  ##Display current figure in the notebook
  # fig.show()


for beh in unique_behaviours:
  plot_behaviour_trace(photom_hit_window, beh, pre_post_secs)
```

Peri-event trace figure saved as:  Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Peri-Event-Analysed/5.1.1s-m-conditioned_phase_trace.svg

Peri-event trace figure saved as:  Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Peri-Event-Analysed/5.1.1s-m-conditioned_cornerimmp_trace.svg

Peri-event trace figure saved as: Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Peri-Event-Analysed/5.1.1s-m-conditioned_othernonstim_trace.svg

Peri-event trace figure saved as: Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Peri-Event-Analysed/5.1.1s-m-conditioned_proxapp_trace.svg

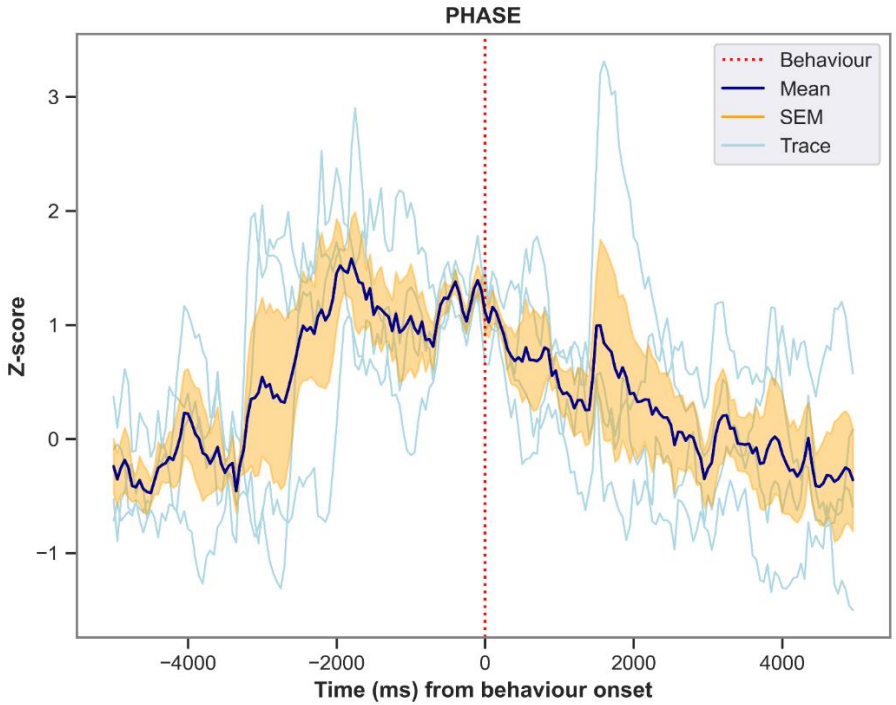
Peri-event trace figure saved as: Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Peri-Event-Analysed/5.1.1s-m-conditioned_explore_trace.svg

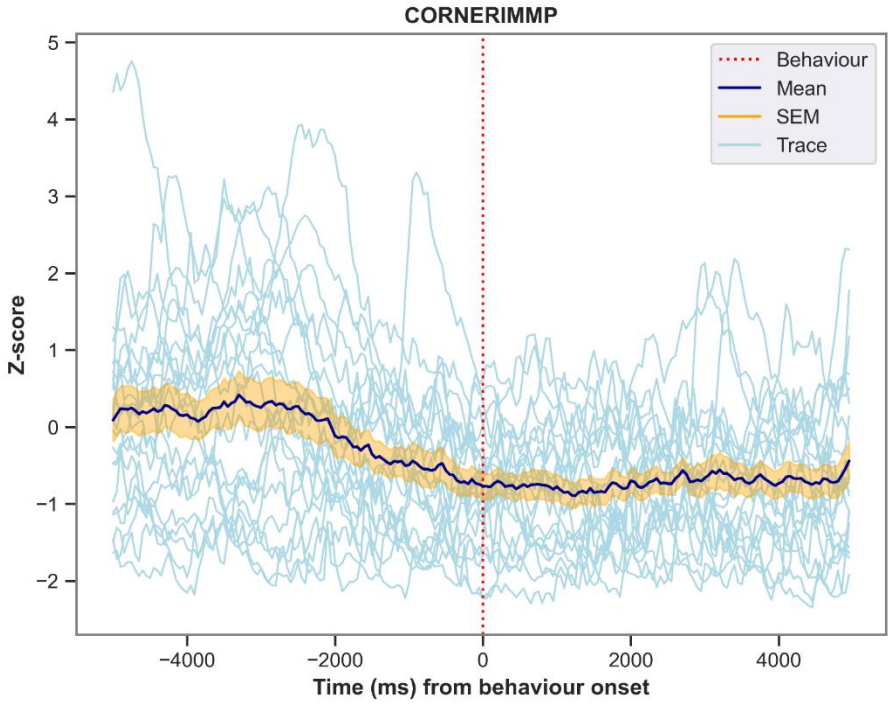
Peri-event trace figure saved as: Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Peri-Event-Analysed/5.1.1s-m-conditioned_proxflee_trace.svg

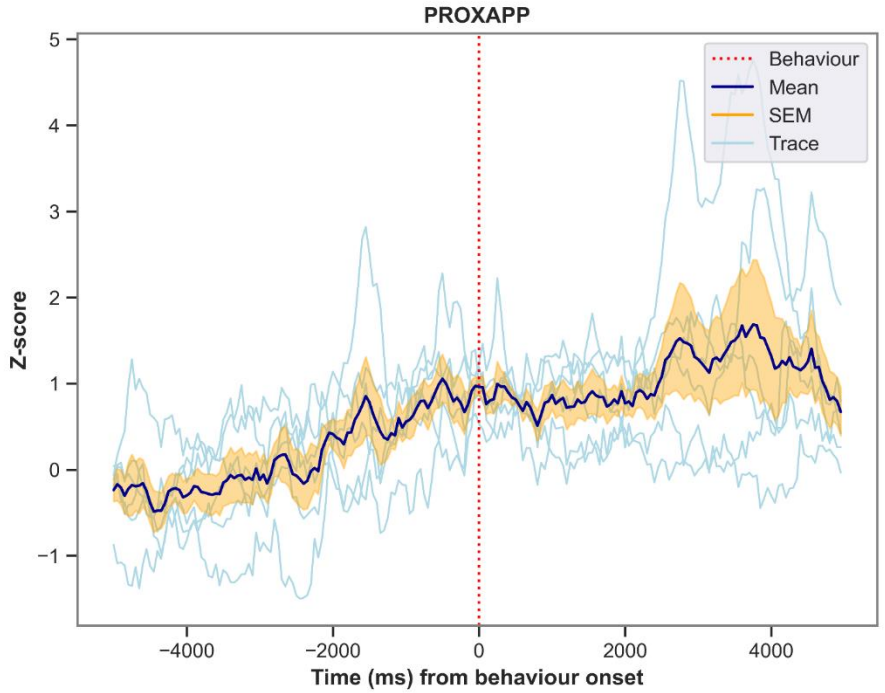
Peri-event trace figure saved as: Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Peri-Event-Analysed/5.1.1s-m-conditioned_distapp_trace.svg

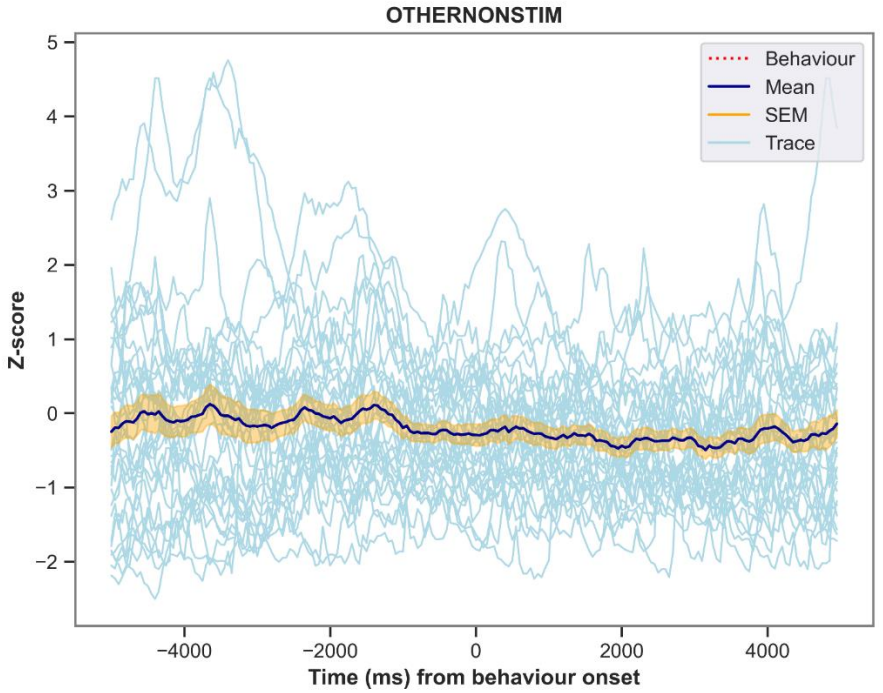
Peri-event trace figure saved as: Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Peri-Event-Analysed/5.1.1s-m-conditioned_distflee_trace.svg

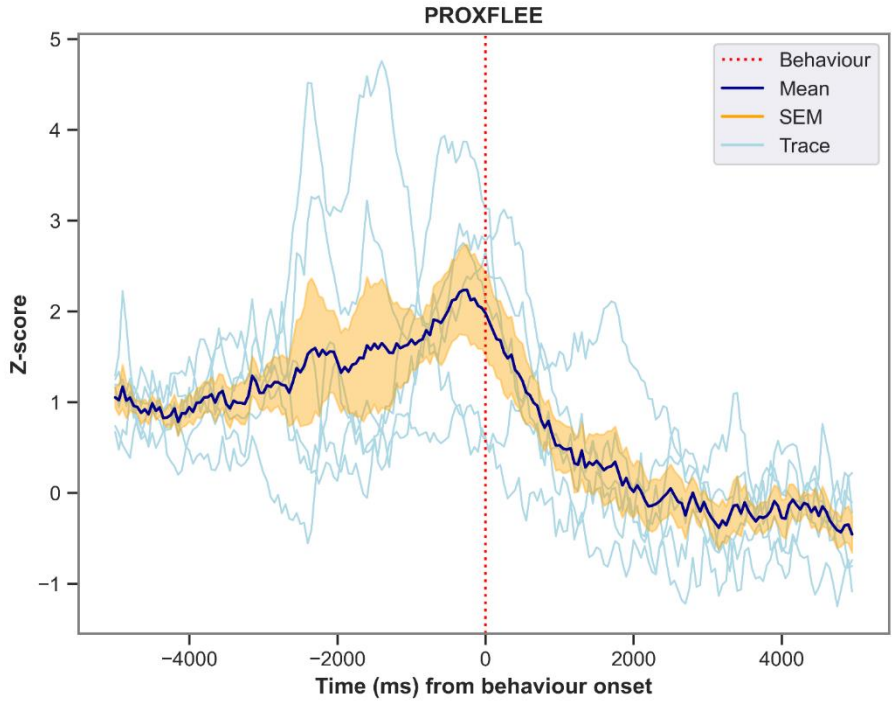
Peri-event trace figure saved as: Z:\PRJ-BowenLab\TimLee\resources\photometry\5.1.1s-m-conditioned\Peri-Event-Analysed/5.1.1s-m-conditioned_retreat_trace.svg

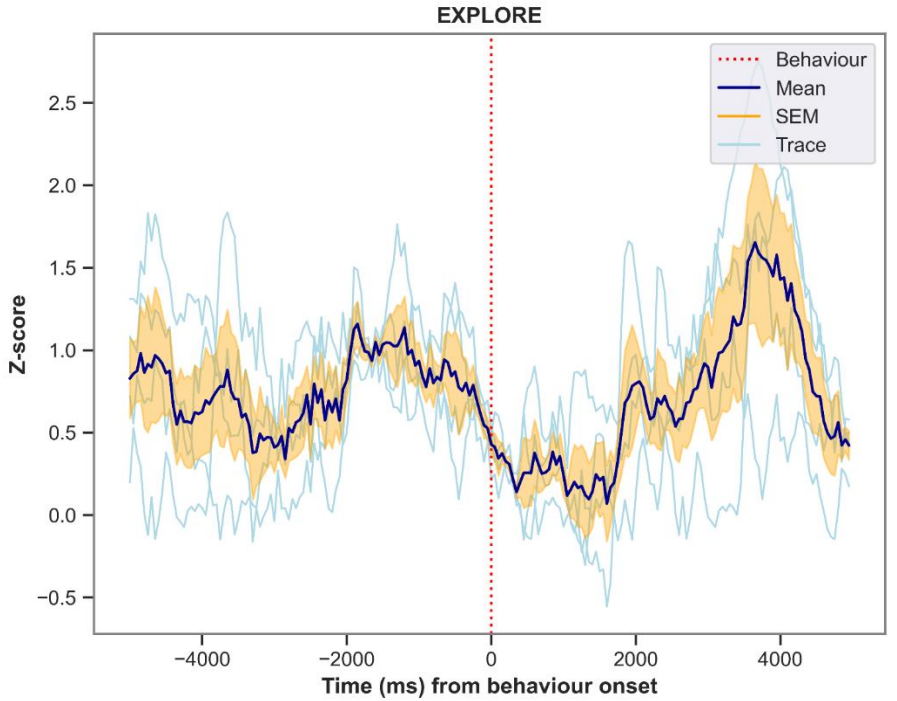

PHASE
Behaviour
Mean
SEM
Trace
Z-score
3
2
1
0
−1
−4000
−2000
0
2000
4000
Time (ms) from behaviour onset

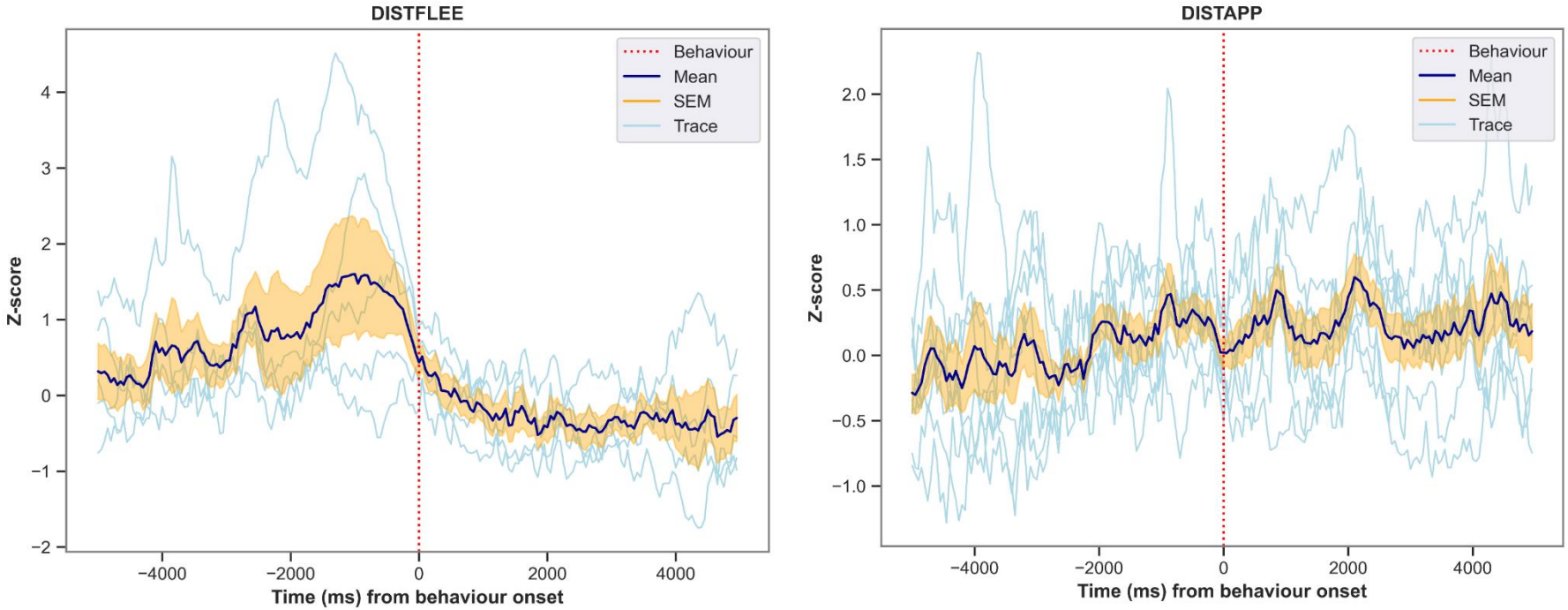

CORNERIMMP
Behaviour
Mean
SEM
Trace
Z-score
5
4
3
2
1
0
−1
−2
−4000
−2000
0
2000
4000
Time (ms) from behaviour onset

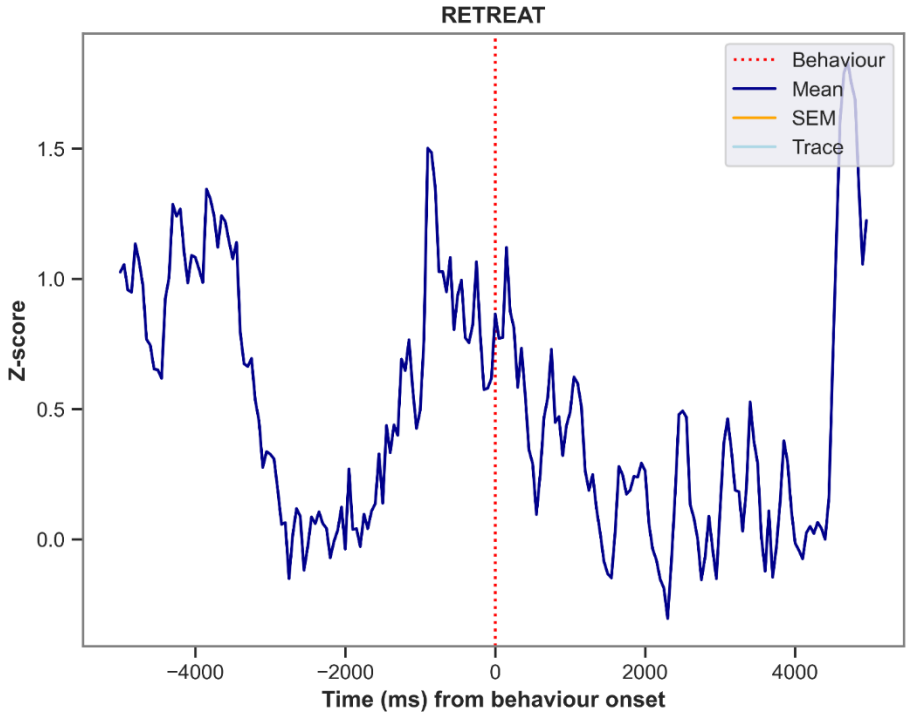

PROXAPP
Behaviour
Mean
SEM
Trace
Z-score
Time (ms) from behaviour onset


OTHERNONSTIM
Behaviour
Mean
SEM
Trace
Z-score
Time (ms) from behaviour onset


PROXFLEE
Behaviour
Mean
SEM
Trace
Z-score
5
4
3
2
1
0
−1
−4000
−2000
0
2000
4000
Time (ms) from behaviour onset


EXPLORE
Behaviour
Mean
SEM
Trace
Z-score
2.5
2.0
1.5
1.0
0.5
0.0
−0.5
−4000
−2000
0
2000
4000
Time (ms) from behaviour onset


DISTFLEE
Behaviour
Mean
SEM
Trace
Z-score
Time (ms) from behaviour onset
DISTAPP
Behaviour
Mean
SEM
Trace
Z-score
Time (ms) from behaviour onset


RETREAT
Behaviour
Mean
SEM
Trace
Z-score
1.5
1.0
0.5
0.0
−4000
−2000
0
2000
4000
Time (ms) from behaviour onset

# Appendix B

*Area under the curve of dLS signal before and after the onset of individually characterised behaviours.*

## Appendix B. 1 Experiment 1a: Stimulus Interaction Test

**Table 3. 1**

Area under the curve of dLS signal before and after the onset of stimulus investigation during the social interaction task.

| | -3 to 0s (pre-onset) | | | 0 to 3s (post-onset) | | |
|---|---|---|---|---|---|---|
| | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** |
| **Males** | | | | | | |
| ***Opposite-Sex*** | -592 ± 605 | 9.89 | [-1941, 758] | -1595.4 ± 550 | 10.1 | [-2820, -371] |
| ***Same-Sex*** | 261 ± 606 | 10.00 | [-1090, 1612] | -157.8 ± 552 | 10.2 | [-1384, 1069] |
| ***Non-Social*** | 323 ± 626 | 11.33 | [-1049, 1695] | -506.5 ± 572 | 11.8 | [-1756, 743] |
| **Females** | | | | | | |
| ***Opposite-Sex*** | 1354 ± 669 | 10.30 | [-131, 2839] | 1059.0 ± 610 | 10.6 | [-290, 2408] |
| ***Same-Sex*** | 586 ± 667 | 10.18 | [-897, 2069] | 79.9 ± 608 | 10.4 | [-1267, 1426] |
| ***Non-Social*** | 576 ± 682 | 11.10 | [-923, 2075] | 177.5 ± 623 | 11.5 | [-1186, 1541] |

***Note.*** Male and female mice underwent a novel social interaction task to examine preference for social versus non-social stimuli. This table illustrates the estimated marginal means (EMM) ± SEM of the area under the curve (AUC) for stimulus investigation, derived from behavioural scoring of fibre photometry data, during the 3 seconds before (pre-onset) and after (post-onset) behaviour initiation. The EMM was calculated using a hierarchical statistical model analysing the AUC of the dLS signal for each behavioural event, nested within subjects. No condition had an AUC greater than baseline (defined as the 95% CI not containing 0) before or after onset of stimulus investigation.

**Table 3. 2**

Area under the curve of dLS signal before and after the onset of stimulus approach during the social interaction task.

| | **-3 to 0s (pre-onset)** | | | **0 to 3s (post-onset)** | | |
|---|---|---|---|---|---|---|
| | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** |
| **Males** | | | | | | |
| ***Opposite-Sex*** | 195.0 ± 383 | 16.0 | [-617, 1007] | -142 ± 601 | 12.3 | [-1448, 1165] |
| ***Same-Sex*** | 490.0 ± 429 | 23.7 | [-396, 1376] | 906 ± 649 | 16.4 | [-467, 2278] |
| ***Non-Social*** | -76.8 ± 435 | 24.7 | [-973, 819] | 191 ± 655 | 16.9 | [-1190, 1573] |
| **Females** | | | | | | |
| ***Opposite-Sex*** | 1491.4 ± 462 | 22.6 | **[535, 2448]** | 2628 ± 702 | 15.7 | **[1138, 4118]** |
| ***Same-Sex*** | 1366.3 ± 502 | 30.1 | **[342, 2391]** | 1767 ± 743 | 19.4 | **[214, 3320]** |
| ***Non-Social*** | 1397.3 ± 479 | 25.9 | **[412, 2382]** | 1404 ± 719 | 17.2 | [-112, 2920] |

***Note.*** Male and female mice underwent a novel social interaction task to examine preference for social versus non-social stimuli. This table illustrates the estimated marginal means (EMM) ± SEM of the area under the curve (AUC) for stimulus approach, derived from behavioural scoring of fibre photometry data, during the 3 seconds before (pre-onset) and after (post-onset) behaviour initiation. The EMM was calculated using a hierarchical statistical model analysing the AUC of the dLS signal for each behavioural event, nested within subjects. The AUC was greater than baseline (defined as the 95% CI not containing 0) for females across all conditions but not for male mice. In the 3s following approach, only females interacting with opposite-sex and same-sex had AUC greater than baseline.

**Table 3. 3**

Area under the curve of dLS signal before and after the onset of disengagement and re-engagement of the stimulus during the social interaction task.

| | -3 to 0s (pre-onset) | | | 0 to 3s (post-onset) | | |
|---|---|---|---|---|---|---|
| | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** |
| **Males** | | | | | | |
| ***Opposite-Sex*** | -483.0 ± 683 | 10.2 | [-2000, 1034] | -1762 ± 651 | 10.2 | [-3208, -315] |
| ***Same-Sex*** | 404.7 ± 683 | 10.2 | [-1113, 1923] | -885 ± 652 | 10.2 | [-2333, 562] |
| ***Non-Social*** | 168.9 ± 762 | 15.7 | [-1448, 1786] | -1088 ± 721 | 15.3 | [-2623, 447] |
| **Females** | | | | | | |
| ***Opposite-Sex*** | 1479.4 ± 770 | 11.3 | [-210, 3168] | 462 ± 733 | 11.3 | [-1147, 2070] |
| ***Same-Sex*** | 398.9 ± 754 | 10.5 | [-1270, 2068] | -589 ± 719 | 10.5 | [-2180, 1002] |
| ***Non-Social*** | 85.6 ± 789 | 12.6 | [-1626, 1797] | -491 ± 750 | 12.4 | [-2120, 1137] |

***Note.*** Male and female mice underwent a novel social interaction task to examine preference for social versus non-social stimuli. This table illustrates the estimated marginal means (EMM) ± SEM of the area under the curve (AUC) for stimulus disengagement and immediate re-engagement of the stimulus, derived from behavioural scoring of fibre photometry data, during the 3 seconds before (pre-onset) and after (post-onset) behaviour initiation. The EMM was calculated using a hierarchical statistical model analysing the AUC of the dLS signal for each behavioural event, nested within subjects. No condition had an AUC greater than baseline (defined as the 95% CI not containing 0) before or after onset of stimulus investigation.

**Table 3. 4**

Area under the curve of dLS signal before and after the onset of proximal stimulus flee during the social interaction task.

| | -3 to 0s (pre-onset) | | | 0 to 3s (post-onset) | | |
|---|---|---|---|---|---|---|
| | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** |
| **Males** | | | | | | |
| ***Opposite-Sex*** | -866.2 ± 713 | 10.5 | [-2446.1, 714] | -1118 ± 582 | 12.3 | [-2384, 147] |
| ***Same-Sex*** | 785.6 ± 766 | 13.9 | [-859.0, 2430] | -588 ± 631 | 16.8 | [-1921, 745] |
| ***Non-Social*** | 1761.0 ± 909 | 25.6 | [-108.7, 3631] | -767 ± 761 | 31.6 | [-2318, 784] |
| **Females** | | | | | | |
| ***Opposite-Sex*** | 1865.5 ± 832 | 13.5 | **[74.9, 3656]** | 658 ± 685 | 16.3 | [-792, 2107] |
| ***Same-Sex*** | -250.7 ± 984 | 24.6 | [-2278.7, 1777] | 147 ± 823 | 30.2 | [-1533, 1827] |
| ***Non-Social*** | 46.2 ± 899 | 17.0 | [-1849.5, 1942] | 194 ± 745 | 20.6 | [-1356, 1744] |

***Note.*** Male and female mice underwent a novel social interaction task to examine preference for social versus non-social stimuli. This table illustrates the estimated marginal means (EMM) ± SEM of the area under the curve (AUC) for proximal flee, derived from behavioural scoring of fibre photometry data, during the 3 seconds before (pre-onset) and after (post-onset) behaviour initiation. The EMM was calculated using a hierarchical statistical model analysing the AUC of the dLS signal for each behavioural event, nested within subjects. Only females fleeing an opposite-sex stimulus had an AUC greater than baseline (defined as the 95% CI not containing 0) in the 3s prior to stimulus flee. However, in the 3s following stimulus flee, no condition had an AUC greater than baseline.

## Appendix B. 2 Experiment 1b: Social Fear Conditioning Pilot

**Table 3. 5**

Area under the curve of dLS signal before and after the onset of stimulus investigation during the SFC task.

| | **-3 to 0s (pre-onset)** | | | **0 to 3s (post-onset)** | | |
|---|---|---|---|---|---|---|
| | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** |
| ***S1-*** | 831 ± 253 | 4.41 | **[154, 1507]** | 720 ± 247 | 4.61 | **[69.4, 1370]** |
| ***S1+*** | 2375 ± 295 | 8.17 | **[1697, 3053]** | 2458 ± 302 | 10.29 | **[1787.5, 3129]** |
| ***S2+*** | 1092 ± 267 | 5.50 | **[423, 1760]** | 841 ± 266 | 6.18 | **[195.2, 1487]** |
| ***S3+*** | 899 ± 265 | 5.34 | **[231, 1567]** | 488 ± 263 | 5.96 | [-156.6, 1133] |

***Note.*** Mice with jGCamP8f in the dLS underwent social fear condition (SFC). During extinction, fibre photometry was used to record dLS activity during social fear extinction. This table illustrates the estimated marginal means (EMM) ± SEM of the area under the curve (AUC) for stimulus investigation, derived from behavioural scoring of fibre photometry data, during the 3 seconds before (pre-onset) and after (post-onset) behaviour initiation. The EMM was calculated using a hierarchical statistical model analysing the AUC of the dLS signal for each behavioural event, nested within subjects. The AUC was greater than baseline (defined as the 95% CI not containing 0) for all conditions prior to stimulus approach however in the 3s following stimulus approach S3+ did not have an AUC greater than baseline yet all other stimulus exposures did.

**Table 3. 6**

Area under the curve of dLS signal before and after the onset of stimulus approach during the SFC task.

| | **-3 to 0s (pre-onset)** | | | **0 to 3s (post-onset)** | | |
|---|---|---|---|---|---|---|
| | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** |
| ***S1-*** | 1767 ± 320 | 26.2 | **[1109, 2424]** | 1917 ± 352 | 34.7 | **[1203, 2631]** |
| ***S1+*** | 1782 ± 303 | 21.2 | **[1153, 2411]** | 2822 ± 331 | 27.8 | **[2144, 3499]** |
| ***S2+*** | 1710 ± 297 | 19.0 | **[1089, 2332]** | 1771 ± 323 | 24.2 | **[1104, 2439]** |
| ***S3+*** | 1256 ± 324 | 26.8 | **[592, 1920]** | 919 ± 356 | 35.5 | **[197, 1641]** |

***Note.*** Mice with jGCamP8f in the dLS underwent social fear condition (SFC). During extinction, fibre photometry was used to record dLS activity during social fear extinction. This table illustrates the estimated marginal means (EMM) ± SEM of the area under the curve (AUC) for stimulus approach, derived from behavioural scoring of fibre photometry data, during the 3 seconds before (pre-onset) and after (post-onset) behaviour initiation. The EMM was calculated using a hierarchical statistical model analysing the AUC of the dLS signal for each behavioural event, nested within subjects. The AUC was greater than baseline (defined as the 95% CI not containing 0) for all conditions in the 3s prior to and the 3s following stimulus approach.

**Table 3. 7**

Area under the curve of dLS signal before and after the onset of disengagement and re-engagement of the stimulus during the SFC task.

| | **-3 to 0 (pre-onset)** | | | **0 to 3s (post-onset)** | | |
|---|---|---|---|---|---|---|
| | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** |
| ***S1-*** | 1109 ± 261 | 4.57 | **[419, 1798]** | 184 ± 189 | 12.1 | [-227, 594] |
| ***S1+*** | 2596 ± 565 | 63.52 | **[1467, 3725]** | 1917 ± 620 | 114.2 | **[689, 3146]** |
| ***S2+*** | 1337 ± 371 | 16.88 | **[554, 2120]** | 53 ± 360 | 59.7 | [-667, 773] |
| ***S3+*** | 1300 ± 351 | 14.10 | **[547, 2053]** | 1181 ± 333 | 53.6 | **[513, 1850]** |

***Note.*** Mice with jGCamP8f in the dLS underwent social fear condition (SFC). During extinction, fibre photometry was used to record dLS activity during social fear extinction. This table illustrates the estimated marginal means (EMM) ± SEM of the area under the curve (AUC) for disengagement and re-engagement of the stimulus. These were derived from behavioural scoring of fibre photometry data during the 3 seconds before (pre-onset) and after (post-onset) behaviour initiation. The EMM was calculated using a hierarchical statistical model analysing the AUC of the dLS signal for each behavioural event, nested within subjects. The AUC was greater than baseline (defined as the 95% CI not containing 0) for all conditions prior to disengagement/re-engagement, but only greater for S1+ and S3+ in the 3 s following the behaviour.

**Table 3. 8**

Area under the curve of dLS signal before and after the onset of stimulus flee during the SFC task.

| | **-3 to 0s (pre-onset)** | | | **0 to 3s (post-onset)** | | |
|---|---|---|---|---|---|---|
| | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** |
| ***S1-*** | 2476 ± 563 | 24.25 | **[1315, 3637]** | 1192 ± 593 | 15.40 | [-69.5, 2453] |
| ***S1+*** | 4003 ± 438 | 9.67 | **[3023, 4984]** | 1953 ± 487 | 7.22 | **[808.9, 3098]** |
| ***S2+*** | 2619 ± 493 | 14.88 | **[1568, 3670]** | 934 ± 533 | 10.20 | [-250.8, 2119] |
| ***S3+*** | 812 ± 504 | 15.62 | [-258, 1881] | -107 ± 543 | 10.77 | [-1305.1, 1092] |

**Note.** Mice with jGCamP8f in the dLS underwent social fear condition (SFC). During extinction, fibre photometry was used to record dLS activity during social fear extinction. This table illustrates the estimated marginal means (EMM) ± SEM of the area under the curve (AUC) for stimulus flee, derived from behavioural scoring of fibre photometry data, during the 3 seconds before (pre-onset) and after (post-onset) behaviour initiation. The EMM was calculated using a hierarchical statistical model analysing the AUC of the dLS signal for each behavioural event, nested within subjects. The AUC was greater than baseline (defined as the 95% CI not containing 0) for all conditions except S3+ prior to the onset of fleeing, however in the 3s after stimulus flee only S1+ had an AUC greater than baseline.

## Appendix B. 3 Experiment 2: Social and Non-Social Fear Extinction

**Table 3. 9**

Area under the curve of dLS signal before and after the onset of proximal approach during social and non-social fear extinction.

| | -3 to 0s (pre-onset) | | | 0 to 3s (post-onset) | | |
|---|---|---|---|---|---|---|
| | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** |
| **Social** | | | | | | |
| ***S1-*** | 3541 ± 818 | 27.5 | **[1864, 5218]** | 3675 ± 737 | 26.4 | **[2161, 5189]** |
| ***S1+*** | 2406 ± 872 | 35.4 | **[638, 4175]** | 5755 ± 811 | 38.3 | **[4114, 7396]** |
| ***S2+*** | 1865 ± 860 | 33.8 | **[116, 3614]** | 3296 ± 796 | 36.2 | **[1681, 4910]** |
| ***S3+*** | 1925 ± 868 | 35.3 | **[164, 3687]** | 2454 ± 808 | 38.9 | **[820, 4087]** |
| ***S6+*** | 3409 ± 839 | 30.6 | **[1696, 5122]** | 2370 ± 767 | 31.0 | **[805, 3935]** |
| **Non-Social** | | | | | | |
| ***S1-*** | 2178 ± 703 | 32.3 | **[746, 3609]** | 1654 ± 647 | 33.4 | **[339, 2969]** |
| ***S1+*** | 2477 ± 713 | 33.0 | **[1027, 3927]** | 4068 ± 657 | 33.9 | **[2733, 5403]** |
| ***S2+*** | 2011 ± 732 | 38.5 | **[529, 3493]** | 3235 ± 687 | 43.3 | **[1849, 4621]** |
| ***S3+*** | 1845 ± 780 | 46.9 | **[276, 3414]** | 3267 ± 746 | 55.4 | **[1772, 4762]** |
| ***S6+*** | 2995 ± 754 | 41.0 | **[1473, 4517]** | 3084 ± 712 | 46.1 | **[1652, 4516]** |

***Note.*** Mice underwent a social (SFC) and non-social (nSFC) fear conditioning and extinction. This table illustrates the estimated marginal means (EMM) ± SEM of the area under the curve (AUC) for proximal approach, derived from behavioural scoring of fibre photometry data, during the 3 seconds before (pre-onset) and after (post-onset) behaviour initiation. The EMM was calculated using a hierarchical statistical model analysing the AUC of the dLS signal for each behavioural event, nested within subjects. In the 3s preceding and proceeding proximal approach, all conditions had an AUC for dLS activity greater than baseline (defined as the 95% CI not containing 0).

**Table 3. 10**

Area under the curve of dLS signal before and after the onset of proximal flee during social and non-social fear extinction.

| | -3 to 0s (pre-onset) | | | 0 to 3s (post-onset) | | |
|---|---|---|---|---|---|---|
| | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** |
| **Social** | | | | | | |
| ***S1-*** | 4695 ± 990 | 16.6 | **[2601, 6788]** | 2074 ± 793 | 16.5 | **[397, 3752]** |
| ***S1+*** | 8639 ± 1037 | 19.6 | **[6474, 10805]** | 2078 ± 834 | 19.8 | **[336, 3820]** |
| ***S2+*** | 4236 ± 1034 | 19.5 | **[2075, 6396]** | -244 ± 832 | 19.6 | [-1982, 1494] |
| ***S3+*** | 3241 ± 1041 | 20.0 | **[1069, 5414]** | -1097 ± 839 | 20.2 | [-2845, 651] |
| ***S6+*** | 1536 ± 1023 | 18.6 | [-607, 3679] | -1207 ± 822 | 18.7 | [-2929, 515] |
| **Non-Social** | | | | | | |
| ***S1-*** | 2309 ± 909 | 19.5 | **[409, 4209]** | 949 ± 732 | 19.7 | [-579, 2477] |
| ***S1+*** | 7506 ± 883 | 17.2 | **[5645, 9366]** | 2598 ± 708 | 17.1 | **[1106, 4090]** |
| ***S2+*** | 5566 ± 901 | 18.8 | **[3679, 7453]** | 1814 ± 724 | 18.9 | **[298, 3330]** |
| ***S3+*** | 4565 ± 950 | 23.0 | **[2600, 6529]** | 833 ± 768 | 23.7 | [-752, 2419] |
| ***S6+*** | 4226 ± 926 | 20.9 | **[2299, 6153]** | 946 ± 747 | 21.2 | [-606, 2499] |

***Note.*** Mice underwent a social (SFC) and non-social (nSFC) fear conditioning and extinction. This table illustrates the estimated marginal means (EMM) ± SEM of the area under the curve (AUC) for proximal flee, derived from behavioural scoring of fibre photometry data, during the 3 seconds before (pre-onset) and after (post-onset) behaviour initiation. The EMM was calculated using a hierarchical statistical model analysing the AUC of the dLS signal for each behavioural event, nested within subjects. In the 3s preceding flee, all conditions had an AUC for dLS activity greater than baseline (defined as the 95% CI not containing 0) except for S6+, however in the 3s proceeding proximal flee only social S1- and S1+ stimulus types and non-social S1+ and S2+ had AUC greater than baseline.

**Table 3.11**

Area under the curve of dLS signal before and after the onset of proximal flee in extinguishers versus non-extinguishers during conditioned social fear extinction.

| | **-3 to 0s (pre-onset)** | | | **0 to 3s (post-onset)** | | |
|---|---|---|---|---|---|---|
| | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** | **EMM ± SEM** | ***df*** | **95% CI [LL, UL]** |
| ***Extinguishers*** | | | | | | |
| ***S1-*** | 4634 ± 1478 | 12.1 | **[1417, 7851]** | 1357 ± 863 | 13.0 | [-507.3, 3221.3] |
| ***S1+*** | 8409 ± 1482 | 12.2 | **[5187, 11631]** | 1935 ± 866 | 13.1 | **[66.1, 3803.4]** |
| ***S2+*** | 4258 ± 1506 | 13.1 | **[1007, 7510]** | -414 ± 888 | 14.6 | [-2311.0, 1483.3] |
| ***S3+*** | 3335 ± 1491 | 12.6 | **[103, 6568]** | -1180 ± 875 | 13.7 | [-3059.5, 698.9] |
| ***S6+*** | 481 ± 1481 | 12.2 | [-2739, 3702] | -1776 ± 865 | 13.1 | [-3643.5, 92.2] |
| ***Non-Extinguishers*** | | | | | | |
| ***S1-*** | 6293 ± 1536 | 13.6 | **[2991, 9595]** | 1843 ± 909 | 14.8 | [-95.9, 3781.6] |
| ***S1+*** | 7144 ± 1685 | 20.1 | **[3630, 10657]** | 679 ± 1043 | 26.5 | [-1462.3, 2821.0] |
| ***S2+*** | 5722 ± 1751 | 22.4 | **[2096, 9349]** | 782 ± 1093 | 29.0 | [-1452.9, 3017.0] |
| ***S3+*** | 3948 ± 1806 | 25.5 | **[231, 7664]** | -532 ± 1141 | 34.7 | [-2848.9 1785.2] |
| ***S6+*** | 4517 ± 1679 | 19.6 | **[1011, 8024]** | 612 ± 1036 | 25.1 | [-1520.7 2743.8] |

***Note.*** Examination of the social fear conditioned (SFC+) group mice, showed a subset of mice in the social fear conditioned group were resistant to social fear extinction and spent less than 1% of the trial investigating the social stimulus (non-extinguishers). We examined the dLS signal in mice sensitive to fear extinction (extinguishers) and non-extinguishers. This table illustrates the estimated marginal means (EMM) ± SEM of the area under the curve (AUC) for proximal flee, derived from behavioural scoring of fibre photometry data, during the 3 seconds before (pre-onset) and after (post-onset) behaviour initiation. The EMM was calculated using a hierarchical statistical model analysing the AUC of the dLS signal for each behavioural event, nested within subjects. In the 3s preceding flee, all conditions had an AUC for dLS activity greater than baseline (defined as the 95% CI not containing 0) except for S6+, however in the 3s proceeding proximal flee in extinguishers and non-extinguishers, only extinguishers during S1+ and S1+ had AUC greater than baseline.