STRESS AND GABA_\text{A} RECEPTOR REGULATION

A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy

by

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Finally, a heartfelt thanks goes out to the people closest to me in my life for their support, advice, encouragement, and unconditional love - my parents (Marty and Greg Skilbeck) and my partner Charlie Rowda.
DECLARATION

“I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institute of higher learning, except where due acknowledgement is made in the text.”

CODE OF ETHICS

All experiments were performed in accordance with the “Australian code of practice for the care and use of animals for scientific purposes (7th edition)”. Australian Government Publishing Service, Canberra, 2004.
SUMMARY

1. GABA\textsubscript{A} receptors are implicated in the pathology of psychiatric disorders such as schizophrenia and depression. They are rapidly affected by stress in a sex-dependent fashion, suggesting that GABA\textsubscript{A} receptors may be relevant to understanding the association between stress and psychiatric disorders. Thus, this thesis examined how GABA\textsubscript{A} receptors are affected in both male and female mice exposed to stress in adulthood (Chapter 2), early-life (Chapter 3-5) and a combination of both early-life and adulthood stress (Chapter 6).

2. The effects of acute adulthood stress (3 minute warm swim stress) on GABA\textsubscript{A} receptor binding in the brains of male and female mice were examined using quantitative receptor autoradiography. The total number of GABA\textsubscript{A} receptor \[^{3}H\]GABA binding sites was increased following swim stress in specific forebrain cortical regions of female mice swum individually or in a group, but decreased in male mice when swum in a group only. These findings confirm and extend previous studies, identifying the cortical regions involved in rapid stress-induced changes in GABA\textsubscript{A} receptors.

3. Post-natal handling models in rodents comparing control (brief handling sessions; EH) with no intervention stress conditions (NH), indicate that the NH condition results in an anxious adulthood phenotype and this was confirmed in the present thesis using the elevated plus-maze behavioural test. Using this model the effects of early-life stress on adulthood GABA\textsubscript{A} receptors were then examined.

4. Regional densities of GABA\textsubscript{A} receptor $\alpha_1$ and $\alpha_2$ subunit proteins were observed in the adult brain of male and female mice using immunoperoxidase histochemistry. NH males showed a loss of the $\alpha_2$ subunit from the thalamus and the lower layers
(IV-VI) of the primary somatosensory cortex, whilst NH females showed a reduction of α₂ but an increase in α₁ protein in the lower layers of the primary somatosensory cortex only. These regionally specific alterations in the α₁:α₂ subunit ratio suggest that early-life stress disrupts the developmental α subunit switch, which occurs in a regionally-dependent fashion over the first two weeks of rodent life.

5. Double-labelling immunofluorescence and confocal microscopy were used to examine the effects of sex and early-life stress on GABA_A receptor synaptic clustering. Regardless of sex, mice exposed to early-life stress (NH) showed reduced colocalisation of the GABA_A receptor α₂ subunit with the synaptic marker protein gephyrin relative to the control condition (EH). This suggests that early-life stress impairs adulthood inhibitory synaptic strength and is consistent with the increased anxiety of the stressed relative to control mice.

6. Finally, the effects of early-life stress on adulthood swim stress-induced changes in GABA_A receptor binding were examined using quantitative receptor autoradiography in forebrain cortical regions. Findings showed that the effect of adulthood stress on the total number of GABA_A receptor binding sites for [³H]GABA in forebrain cortical regions was altered by early-life stress in both male and female mice, suggesting that the rapid adulthood stress response of GABA_A receptors is affected by early-life experience.

7. Together these results show that GABA_A receptors are sensitive to subtle changes in the environment in both early-life and adulthood and that these neurochemical responses to stress in adulthood are sex-dependent. The short and long-term stress-sensitivity of the GABAergic system implicates GABA_A receptors in the non-genetic aetiology of psychiatric illnesses in which sex and stress are important factors.
**Publications and Communications**

**Refereed Publications**


**Presentations**


† Represents Publications and Communications arising from this thesis
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<td>Serotonin</td>
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<td>ACh</td>
<td>Acetylcholine</td>
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<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<td>AFR</td>
<td>Animal facility reared early life manipulation condition</td>
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<td>AMYG</td>
<td>Amygdala</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>AP</td>
<td>Allopregnanalone / 3α-hydroxy-5α-pregnane-20-one</td>
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<tr>
<td>APMPA</td>
<td>3-Aminopropyl-(methyl)phosphinic acid</td>
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<td>BDNF</td>
<td>Brain Derived Neurotrophic Factor</td>
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<td>BLa</td>
<td>Basolateral nucleus of the amygdala</td>
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<td>B&lt;sub&gt;MAX&lt;/sub&gt;</td>
<td>Saturation binding maximum constant</td>
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<td>BNST</td>
<td>Bed nucleus of the stria terminalis</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CA</td>
<td>Cornu Ammonis</td>
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<td>CeA</td>
<td>Central nucleus of the amygdala</td>
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<td>CING</td>
<td>Cingulate cortex</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CRH / CRF</td>
<td>Corticotropin-releasing hormone / Corticotropin-releasing factor</td>
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<td>DAB</td>
<td>Diaminobenzidine</td>
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<td>DG</td>
<td>Dentate gyrus of the hippocampus</td>
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<td>DPSS</td>
<td>Diode-pumped solid-state laser</td>
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<td>GAT-1 / GAT-3</td>
<td>GABA transporter type 1 / GABA transporter type 3</td>
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<td>GR</td>
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<td>HIPP</td>
<td>Hippocampus</td>
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<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
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<td>HRP</td>
<td>Horse-radish peroxidase</td>
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<td>IC50</td>
<td>Concentration of drug that inhibits 50% of response</td>
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<td>2-Methylbutane</td>
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<td>iv</td>
<td>Intravenous</td>
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<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>La</td>
<td>Lateral nucleus of the amygdala</td>
</tr>
<tr>
<td>Laser</td>
<td>Light Amplification by Stimulated Emission of Radiation</td>
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<tr>
<td>LC</td>
<td>Locus Coeruleus</td>
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<tr>
<td>LD</td>
<td>Lateral dorsal nucleus of the thalamus</td>
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<td>LS</td>
<td>Lateral septum</td>
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M1  Primary motor cortex
M2  Secondary motor cortex
mIPSC  Miniature inhibitory postsynaptic current
mPFC  Medial prefrontal cortex
mRNA  Messenger ribonucleic acid
MS  Maternal separation early-life condition
NGS  Normal goat serum
NH  Non-handled early-life manipulation condition
NTS  Nucleus of the solitary tract
OD  Optical density
PAG  Periaqueductal gray
PBS  Phosphate-buffered saline
PET  Positron emission tomography
PFC  Prefrontal cortex
PKA / PKC  Protein kinase A / Protein kinase C
PND  Postnatal day
POMC  Pro-opiomelanocortin
PPI  Prepulse inhibition
PTSD  Post-traumatic stress disorder
PTZ  Pentylenetetrazol
PVN  Paraventricular nucleus
QS  Quackenbush Swiss
RNA  Ribonucleic acid
rt PCR  Reverse transcriptase polymerase chain reaction
SAM  Sympathetic-adrenal-medullary
SEM  Standard error of the mean
silane  3-Amino-propyltriethoxysilane
SPECT  Single photon emission computed tomography
SS  Somatosensory cortex
TBOB  t-Butylbicycloorthobenzoate
TBPS  t-Butylbicyclophosphorothionate
TBS  Tris-buffered saline
THAL  Thalamus
THDOC  Tetrahydrodeoxy corticosterone
THIP  4, 5, 6, 7-Tetrahydroisoxazolo[5, 4-c]pyridin-3-ol
TPMPA  (1, 2, 5, 6-Tetrahydropyridin-4-yl)methylphosphinic acid
Tris  Tris(hydroxymethyl)aminomethane / 2-Amino-2-hydroxymethyl-propane-1,3-diol
TX-100  t-Octylphenoxypolyethoxyethanol
VL  Ventral-lateral nucleus of the thalamus
ZAPA  Z-3-[(aminoiminomethyl)thio]prop-2-enoic acid
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PART A:

REVIEW OF LITERATURE
CHAPTER 1:  
GABA_\text{A} Receptors, Sex-Differences and Stress

1.1 The GABAergic system

1.1.1. GABA as a neurotransmitter

\gamma\text{-Aminobutyric acid (GABA)} is an amino acid neurotransmitter that is important during development and adulthood. GABA was first discovered as a transmitter at inhibitory synapses in 1950 (Awapara et al., 1950; Roberts and Frankel, 1950; Roberts et al., 1950). In the adult mammalian brain, between 20-30% of neurons synthesise GABA, 25-50% of synapses contain GABA and every neuron expresses GABA receptors, thus GABA is an important neurotransmitter in adulthood brain function (Curtis and Johnston, 1970; Koella, 1981).

1.1.2. GABA synthesis, release, re-uptake and metabolism

Functioning of the GABAergic system relies on numerous proteins involved in its synthesis, release, reuptake and metabolism. GABA is synthesised in neuronal terminals via \( \alpha \)-decarboxylation of L-glutamate (Roberts and Frankel, 1950) in a rate-limiting step by the enzyme glutamate decarboxylase (GAD) for which there are two protein isoforms GAD_{65} and GAD_{67} (Erlander et al., 1991). GABA is packaged into vesicles in the neuronal terminal where it is stored until neuronal depolarisation induces Ca^{2+} dependent vesicular exocytosis from the presynaptic terminal. High affinity Na^{+} dependent GABA reuptake transporters terminate the activity of GABA. In the brain GABA reuptake is primarily dependent on the GAT-1 and GAT-3 transporters (Dalby, 2003). GAT-1 appears to be expressed in presynaptic neuronal terminals, astrocytic processes and possibly postsynaptic terminals (Dalby, 2003; Pow et al.,
2005), whilst GAT-3 is found in astrocytic processes surrounding synapses (Dalby, 2003, Pow et al., 2005) and oligodendrocytes in the human, cat and monkey brain (Pow et al., 2005). Once removed from the synaptic cleft GABA may be recycled for re-release or it may be metabolised by a mitochondrial enzyme, GABA-aminotransferase (GABA-T) in either the terminal or neighbouring astrocytes. GABA-T transfers the amino group from GABA to α-oxoglutaric acid to yield glutamate and succinic-semialdehyde. Succinic-semialdehyde is oxidised by succinic-semialdehyde dehydrogenase to succinic acid, which enters the KREBS cycle. A typical GABAergic synapse is shown in figure 1.1, with pharmacological agents acting on different components of synthesis, release, reuptake and metabolism given in italics.

Figure 1.1: Physiology and pharmacology of GABA_A receptor transmission
1.1.3. GABA receptors

GABA is a flexible compound that can assume a number of low energy conformations that bind to reuptake transporters as well as receptors (Johnston, 1996). Synaptic GABA mediates neuronal inhibition via receptors, which are found on almost all cortical neurons (Silvotti and Nistri, 1991). There are three different classes of GABA receptors; GABA_A, GABA_B and GABA_C receptors. GABA_A and GABA_C receptors are pentameric ligand-gated chloride channels and GABA_B receptors are 7-transmembrane G-protein coupled metabotropic receptors. GABA_A receptors are pharmacologically defined on the basis of selective antagonism by bicuculline and insensitivity to baclofen (Johnston, 2005). They are distinguished from GABA_B receptors, which are selectively stimulated by baclofen and insensitive to bicuculline, and GABA_C receptors, which are insensitive to bicuculline and baclofen but selectively antagonised by TPMPA (Johnston, 2005).

1.2 GABA_A receptors

1.2.1. GABA_A receptor complexity

GABA_A receptors are widespread throughout the brain. These receptors are structurally and pharmacologically complex with a number of different receptor subtypes being expressed in the adult mammalian brain. Subtypes vary in their regional and cellular distributions, pharmacological sensitivities and the behavioural effects they mediate. An understanding of the complexity of GABA_A receptors is highly relevant to an examination of alterations in GABA_A receptor expression.
1.2.2. GABA_\text{A} receptor structure

GABA_\text{A} receptors belong to the cys-loop or nicotinoid family of ligand-gated ion channels, which also includes the nicotinic acetylcholine (nAChR), 5-HT_3, and glycine receptors (Barnard, 1996). Based on sequence homology with the nACh receptor, all receptors of the nicotinoid family are considered to be a combination of 5-membrane spanning protein subunits around a central ion channel (Nayeem et al., 1994; Le Novere and Changeux, 1995; Unwin, 1989). GABA_\text{A} receptors are heteromeric receptors as more than one type of subunit is required for expression of functional receptors (Schofield et al., 1987; Sieghart et al., 1999). In contrast, ionotropic GABA_\text{C} receptors are homomeric because functional receptors form from a single subunit protein.

![Diagram of GABA_A receptor subunit structure and arrangement](image)

Figure 1.2 shows the postulated structure of nicotinoid family receptors based on the structure of the nicotinic ACh receptor (Unwin, 2000). Each subunit has an extracellular N-terminal region containing a cysteine-cysteine bridge (cys-loop), 4 membrane spanning hydrophobic domains (M1-4) and an extracellular carboxyl terminal (Le Novere and Changeux, 1995). The cys-loop contains agonist / antagonist
binding sites (Johnston, 2005) whilst the cytoplasmic loop between the third and fourth transmembrane domains contains sites for intracellular mediators including serine, threonine and tyrosine kinases (Moss and Smart, 1996) and microtubule binding elements (Johnston, 2005). These protein subunits are arranged such that the M2 domain lines the central channel pore (Schofield et al., 1987).

1.2.3. Effects on membrane potential

For GABA\textsubscript{A} receptors the central pore conducts chloride ions when the ion channel is in the ‘open-state’. Presumably a rapid conformational change underlies the transition from the closed to the open state. This transition may involve removal of an entity masking the pore perhaps from regions of the protein itself as is suggested for nACh receptors, or by membrane lipids which appear important for GABA\textsubscript{A} receptors. However, the molecular basis of channel gating remains poorly understood, in part due to the lack of a high-resolution structure of the entire receptor (Kash et al., 2004).

GABA is the primary source of inhibition in the brain but also a source of excitation. GABA can induce hyperpolarizing or depolarizing potentials via the GABA\textsubscript{A} receptor (Cherubini et al., 1991; Gao et al., 2001) depending on the transmembrane chloride concentration gradient, which determines whether inward or outward chloride currents arise upon channel opening (Luhmann and Prince, 1991; Rivera et al., 1999). Excitatory actions of GABA are most prominent during brain development prior to postnatal day (P) 4-10 in rodents (Ben-Ari et al., 1989; Gao et al., 2001; Obrietan and van den Pol, 1995) by which time chloride ion transporter maturation (Lee et al., 2005; Plotkin et al., 1997) results in a negative chloride ion membrane reversal potential. However, GABA\textsubscript{A} receptors can also mediate membrane depolarisation in certain parts of the adult brain such as the hippocampus (Ben-Ari et
al., 1989; Ben-Ari et al., 1997; Cherubini et al., 1998; Cherubini et al., 1990; Michelson and Wong, 1991; Otis and Mody, 1992), hypothalamus (Gao and Van den Pol, 2001) neocortex (Owens et al., 1996), and brainstem (Marchetti et al., 2002; Ritter and Zhang, 2000). During development outward chloride current-induced membrane depolarisation is sufficient to result in opening of voltage-gated calcium channels leading to a rise in intracellular calcium, which has trophic effects on neurons (Barbin et al., 1993; Barker et al., 1998; Behar et al., 1996; Cherubini et al., 1998; Maric et al., 2001; Meier et al., 1987; Meier and Jorgensen, 1986; Spoerri, 1988), and may be involved in neuronal differentiation (Ben-Ari et al., 1994; Kullmann et al., 2002; Marty et al., 1996) and the expression of other growth factors such as brain derived neurotrophic factor (BDNF) (Berninger et al., 1995).

1.2.4. GABA_\text{A} receptor subtypes

1.2.4.1. Subunit diversity

GABA_\text{A} receptors are the most complex, both structurally and pharmacologically, of the ligand-gated ion-channel superfamily (Johnston, 1996). Combined affinity purification and cloning from cDNA libraries has identified 16 subunits from which GABA_\text{A} receptors may be assembled in the mammalian brain. These subunits are encoded by separate genes and classified by sequence identity into seven subunit classes, including six \( \alpha \) (\( \alpha_1-\alpha_6 \)), four \( \beta \) (\( \beta_1-\beta_4 \)), three \( \gamma \) (\( \gamma_1-\gamma_3 \), 2 splice variants; \( \gamma_{2\text{short}} \), \( \gamma_{2\text{long}} \)), one \( \delta \), one \( \varepsilon \), and one \( \theta \) subunit (Whiting, 2003). Splice variants also exist for the \( \alpha_5 \), \( \alpha_6 \), \( \beta_2 \), \( \beta_3 \) and \( \gamma_2 \) subunits (Barnard et al., 1998). Approximately 30\% amino acid sequence homology exists between, and 70-80\% exists within, the subunit classes (Costa, 1998).
1.2.4.2. Composition

Receptors assembled from different subunit protein combinations are considered different receptor subtypes. The diversity of subunits and a hetero-pentameric arrangement implies a large number of GABA_\text{A} receptor subtypes exist, yet no more than 20 have been clearly identified in the mammalian CNS (McKernan and Whiting, 1996). This is because the subunits cannot form functional receptors when expressed alone and not all subunits can co-assemble to give functional receptors (Verdoorn et al., 1990). Immunohistochemistry and in situ hybridisation studies measuring subunit colocalisation on membranes suggest that most subtypes contain \( \alpha, \beta \) and \( \gamma \) subunits (Fritschy & Mohler, 1995; Sieghart et al., 1999; Wisden et al., 1992), particularly in the ratio 2:2:1, although stoichiometry may vary (i.e. 2:1:2, 3:2:0) (Whiting et al., 1995). Functional receptors appear to be arranged only in the order \( \gamma \beta \alpha \beta \alpha \), substantially reducing the number of possible configurations (Baumann et al., 2002). However, \( \delta \) and \( \varepsilon \) subunits may be able to replace \( \gamma \), and \( \theta \) may replace \( \beta \) subunits in some subunit combinations (Sieghart et al., 1999). Neurons express from two to many subunit mRNAs (Sieghart et al., 1999) and protein subunit expression appears to vary over time (Zheng et al., 1994) and location (Fritschy et al., 1992) within a single neuron (Penschuck et al., 1999).

1.2.4.3. Regional distribution of GABA_\text{A} receptor subtypes

Immunohistochemical studies indicate that GABA_\text{A} receptor subtypes are differentially distributed in the CNS (Pirker et al., 2000). The most common subtype (~43%) contains \( \alpha_1, \beta_{2/3} \) and \( \gamma_2 \) subunits and is distributed throughout the brain, with
highest expression in the cortex and thalamus (Fritschy et al., 1992; Gao & Fritschy, 1994; McKernan and Whiting, 1996; Pirker et al., 2000). Interestingly, deletion of $\alpha_1$ and $\beta_2$ subunits is not lethal and does not cause seizures despite a loss of 50% of total GABA$_A$ receptors (Sur et al., 2001; Vicini et al., 2001). In contrast, deletion of $\beta_3$ and $\gamma_2$ subunits produces non-viable offspring which die shortly after birth, indicating the importance of these subunits (DeLorey et al., 1998; Gunther et al., 1995; Homanics et al., 1997).

Subtypes containing $\alpha_2\beta_2\gamma_2$ and $\alpha_4\beta_2\gamma_2$ subunit combinations are also common and are expressed mainly in regions where $\alpha_4$ is low, such as the striatum, internal granular layer of the olfactory bulb, reticular thalamic nucleus (Pirker et al., 2000; Waldvogel et al., 1999; Zimprich et al., 1991) and cholinergic and monoaminergic cells projecting to the cortex (Fritschy et al., 1992; Gao et al., 1993). Furthermore, whilst $\alpha_3$ subunits predominate in the inner layers of the cortex, $\alpha_2$ subunits predominate in the outer layers (Pirker et al., 2000; Zimprich et al., 1991). All three $\beta$ subunits are widely distributed in the brain with complementary expression in subcortical and cerebellar regions and a pattern of $\beta_2$ predominance on interneurons (Miralles et al., 1999; Pirker et al., 2000). In contrast to the widely distributed $\alpha_{1/2}$ and $\gamma_2$ subunits, the $\alpha_{5,6}$, $\gamma_1$ and $\delta$ subunits are largely confined to particular regions. For example, the $\alpha_6$ is present only in the granule cell layer of the cerebellum and comprises only 4% of GABA$_A$ receptors (Fritschy and Mohler, 1995; Gao et al., 1993; McKernan and Whiting, 1996; Pirker et al., 2000), whilst $\alpha_5$ is largely confined to the hippocampus (Fritschy and Mohler, 1995). Finally, immunohistochemical staining patterns show an overlap of $\alpha_1$ and $\beta_2$, $\alpha_2$ and $\beta_3$, $\alpha_{4,6}$ and $\delta$ subunit distributions, suggesting that in general, these subunit combinations are preferred (Jechlinger et al., 1998; Pirker et al., 2000; Sur et al., 1999).
1.2.5. GABA<sub>A</sub> receptor pharmacology

1.2.5.1. The orthosteric site

The orthosteric site of GABA<sub>A</sub> receptors is the site where GABA binds to induce chloride channel opening and membrane currents. The orthosteric site is selectively blocked by the antagonist bicuculline (Curtis et al., 1970) but no selective GABA<sub>A</sub> receptor agonist exists that does not act on GABA<sub>B</sub> or GABA<sub>C</sub> receptors (Johnston, 2005). For example, muscimol acts as an agonist at the GABA<sub>A</sub> receptor orthosteric site but also acts as a potent agonist on the GABA<sub>C</sub> receptor (Johnston, 2005). Partial agonists that have reduced maximal efficacy compared with GABA also exist such as THIP, which acts as an antagonist at GABA<sub>C</sub> receptors (Johnston, 2005).

The GABA<sub>A</sub> receptor orthosteric site is thought to exist at the interface of the α and β subunits of GABA<sub>A</sub> receptors (Baur and Siegel, 2003). The orthosteric binding site has been extensively studied using radiolabelled agonists such as [³H]GABA and [³H]muscimol and antagonists such as [³H]bicuculline and [³H]SR 95531. Analysis of Scatchard plots from such studies has lead to a general consensus that there exists both high affinity (nM) and low affinity (nM-μM) binding sites. Whether these different binding site populations represent different conformations of the same binding site, or distinct sites on the same or different macromolecular complexes is unknown (Baur and Siegel 2003; Cash and Subbarao, 1987; Edgar and Schwartz, 1992; Harris and Allan, 1985; Maksay, 1996; Smith and Olsen 1994; Yeung et al., 2003). However, electrophysiological studies on cerebellar neuronal patches (Maconochie et al., 1994) and recombinant receptors (Baur and Siegel, 2003) as well as studies of chloride uptake into brain vesicle preparations (Harris and Allan, 1985) all show that μM concentrations...
of GABA are required for channel opening, suggesting that the low affinity GABA binding site represents the functional site.

1.2.5.2. Abundance of allosteric sites

GABA_A receptors contain many allosteric modulatory sites that are presumably remote from the orthosteric site (Johnston, 2005). When these sites are occupied, binding of GABA or its ability to open the ion channel changes. Agents that act to enhance the action of GABA on GABA_A receptors are termed positive modulators and separate positive modulatory sites exist for a variety of compounds including therapeutic agents (benzodiazepines, barbiturates, anaesthetics), recreational agents (ethanol), cations (e.g. Zn^{2+}, Mg^{2+}, Ca^{2+}), endogenous neurosteroids (e.g. allopregnanalalone, THDOC) and dietary compounds (flavonoids, terpenes, sage) (Johnston, 2005). Conversely, those that reduce the action of GABA on GABA_A receptors are termed negative modulators or inverse agonists (Johnston, 2005). These compounds have anxiogenic and convulsant effects and so their clinical use is limited to cases of overdose with drugs of abuse such as GHB. Agents can also block the allosteric modulatory sites without exerting any effect on the chloride channel opening and these are termed neutralising allosteric modulators of which flumazenil is an example at the benzodiazepine site (Johnston, 2005). In addition, some compounds appear to bind directly within the ion-channel to block GABA_A receptor function such as picrotoxin, TBPS and TBOB (Squires et al., 1983).

1.2.5.3. Variations in pharmacological sensitivity according to receptor subtype

Different GABA_A receptor subtypes appear to vary in pharmacological sensitivity based on subunit composition. Studies in Xenopus oocytes suggest an α and β subunit
are required for GABA to exert an effect (Pritchett et al., 1989). Varying the β subunit of recombinant receptors does not affect GABA-induced responses, but varying the α subunit can produce a 70-fold difference in sensitivity to GABA ($\alpha_3 > \alpha_1 > \alpha_6 > \alpha_3$) (Ebert et al., 1994) and inclusion of a γ subunit results in reduced sensitivity to GABA.

The binding of allosteric modulators is also affected by subunit composition. Benzodiazepines are thought to act at the interface of α and γ₂ subunits to increase the frequency of ion-channel opening. Benzodiazepines produce high affinity (nM) modulation of GABA at subtypes containing γ₂ subunits, with only low affinity (mM) enhancement if γ₁ or no γ subunits are present (Pritchett et al., 1989; Walters et al., 2000), indicating that two separate or overlapping sites may exist. Varying the type of β subunit expressed in recombinant receptors does not change benzodiazepine enhancement of GABA-currents (Pritchett et al., 1989), but varying the α subunit does, with benzodiazepines like diazepam and flunitrazepam having greatly reduced affinity for α₄ and α₆ containing subtypes (Luddens et al., 1991). Similarly, ethanol enhancement of GABA chloride currents depends on δ subunit presence (Lobo and Harris, 2008) and the isoform of the β subunit influences the effects of the anaesthetic etomidate and the anticonvulsant loreclezole (Belelli et al., 1997).

Studies of subunit knockout mice largely agree with the subunit pharmacology established for GABAergic compounds from electrophysiological studies on recombinant receptors. Mice deficient in the γ₂L subunit show slightly greater sleep times in response to benzodiazepines and the α₁ subunit selective allosteric modulator zolpidem but responses to non-benzodiazepines like ethanol and barbiturate anaesthetics are unchanged (Quinlan et al., 2000; Homanics et al., 1999). β₃ subunit null mice show reduced sensitivity to etomidate but not pentobarbital or ethanol
(Quinlan et al., 1998). It has also been shown that mice deficient in the $\delta$ subunit show reduced sensitivity to neuroactive steroids (Mihalek et al., 1999).

1.2.6. GABA$_A$ receptors and behaviour

Compounds acting to enhance GABAergic transmission via GABA$_A$ receptors have widespread therapeutic use as anxiolytics, sedative-hypnotics, anticonvulsants and anaesthetics (Johnston, 2005). Mice lacking GABA$_A$ receptor subunits provide insight into the role of GABA$_A$ receptors in brain function and behaviour. Mice lacking the $\gamma_2$ or $\beta_3$ subunits die shortly after birth (Gunther et al., 1995), whereas mice deficient in all other subunits are viable (Blednov et al. 2003), although spontaneous seizures are observed in $\delta$ subunit deficient mice (Mihalek et al., 1999). Studies with knockout mice have suggested that different $\alpha$ subunit isoforms may be involved in different behavioural effects of drugs, with $\alpha_1$ mediating sedation and $\alpha_{2/3}$ subunits mediating anxiolysis resulting from benzodiazepine administration (McKernan, 2000; Reynolds et al., 2001; Rudolph et al., 1999) and $\alpha_5$ subunits mediating spatial memory (Johnston, 2005). Compounds developed with preferential affinities for $\alpha_1$ (zolpidem) and $\alpha_{2/3}$ subunits (L-838, 417) have confirmed this subtype selective sedation-anxiety effect (Crestani et al., 2000; McKernan, 2000). The $\gamma_2$ subunit has also been implicated in anxiety as mice heterozygous for the $\gamma_2$ subunit show enhanced fear conditioning and harm avoidance behaviours without alterations in spatial memory or sedation following benzodiazepine treatment (Chandra et al., 2005; Crestani et al., 1999). Given that $\alpha_2$ receptors are common in extrasynaptic regions and $\gamma_2$ deficiency leads to reduced synaptic clusters, susceptibility to stress and anxiety may be related to reduced synaptic clustering (Chandra et al., 2005; Crestani et al., 1999).
1.2.7. GABA_A receptors and human psychiatric illness: Schizophrenia, Anxiety disorders and Depression

1.2.7.1. Schizophrenia

In schizophrenia, one of the most consistently observed abnormalities post-mortem is an increase in GABA_A receptors. A number of studies have shown increased total [³H]muscimol binding at GABA_A receptors in various regions of the schizophrenic brain suggesting an upregulation of GABA_A receptors occurs in schizophrenia (Benes et al., 1992; Benes et al., 1996a; Benes et al., 1996b; Benes et al., 1997; Dean et al., 1999; Deng and Huang, 2006; Hanada et al., 1987). Such radioligand binding studies are further supported by studies showing increased GABA_A receptor α1, α2, α3, α4 and α5 subunit mRNAs (Impagnatiello et al., 1998; Onhuma et al., 1999; Pesold et al., 1998; Volk et al., 2002) and increased α1 and β2/3 subunit protein in the PFC of the schizophrenic brain (Ishikawa et al., 2004). However, whilst the total population of GABA_A receptors, labelled by [³H]muscimol, appear to be increased in schizophrenia, benzodiazepine-sensitive GABA_A receptors, measured by benzodiazepine-site specific radioligands, appear to be either unchanged (Benes et al., 1997; Owen et al., 1981; Reynolds & Stroud, 1993) or reduced (Squires et al., 1993) in the schizophrenic brain. Furthermore, mRNA and protein expression for the γ2 subunit that is required for high affinity benzodiazepine binding is also reduced (Huntsman et al., 1998), or unchanged (Akbarian et al., 1995) in the PFC of schizophrenic brains. Thus, whilst GABA_A receptors are upregulated in schizophrenia, only a subset of GABA_A receptors appear to be affected.
The changes in GABA\textsubscript{A} receptors that are observed in schizophrenia do not appear to be a result of antipsychotic drug treatment. Studies in rats have indicated that long-term antipsychotic drug administration does not produce the increases in $[^3]$Hmuscimol binding that are observed post-mortem in schizophrenia but rather, result in no change, or reductions in $[^3]$Hmuscimol binding in the PFC (Skilbeck et al., 2007; Skilbeck et al., 2008b), temporal cortex, hippocampus (Farnbach-Pralong et al., 1998), striatum (Dean et al., 2001) and thalamus (McLeod et al., 2008). Furthermore, combined treatment of haloperidol and diazepam over 12 days does not appear to produce the increases in $[^3]$Hmuscimol binding that are observed post-mortem in schizophrenia (McLeod et al., 2008). Similarly, antipsychotic drug treatment alters benzodiazepine-sensitive receptors in a fashion that is inconsistent with the changes observed post-mortem in schizophrenia with studies showing increased $[^3]$Hflunitrazepam binding in the PFC following prolonged administration of antipsychotic drugs (Skilbeck et al., 2007; Skilbeck et al., 2008b). Thus, GABA\textsubscript{A} receptor changes observed in the schizophrenic brain do not appear to arise from antipsychotic drug treatment for the disorder.

It is unknown what the significance of altered GABA\textsubscript{A} receptors in schizophrenia holds. For example, alterations in GABA\textsubscript{A} receptors may result from an adaptation to impaired presynaptic GABA\textsubscript{ergic} function, or an adaptation to changes in other neurotransmitter systems. However, in support of a role for GABA\textsubscript{A} receptors in the disease symptoms, certain studies have shown a correlation between symptom severity and reduced \textit{in vivo} binding at the benzodiazepine site of GABA\textsubscript{A} receptors (Asai et al., 2008; Ball et al., 1998; Busatto et al., 1997). Furthermore, recent studies showing that GABA\textsubscript{A} receptor $\alpha_3$ and $\alpha_5$ subunit knockout mice show specific deficits in sensorimotor gating, measured using the pre-pulse inhibition (PPI) test, suggest that a
loss of specific GABA$_A$ receptor subtypes (i.e: $\alpha_3$ and $\alpha_5$ – subunit containing subtypes), or a compensatory increase in remaining GABA$_A$ receptor subunits in these knockout mice, may be responsible for sensorimotor gating impairments in schizophrenia (Hauser et al., 2005; Yee et al., 2005).

### 1.2.7.2. Anxiety disorders

Several lines of evidence support a role for GABA$_A$ receptors in anxiety disorders including panic disorder, generalised anxiety disorder and post-traumatic stress disorder. For example, PET (positron emission tomography) and SPET (single photon emission tomography) studies show that in vivo binding at the benzodiazepine site measured using benzodiazepine site ligands such as $[^{11}\text{C}]$flumazenil and $[^{123}\text{I}]$iomazenil, is reduced in patients suffering from panic disorder (Malizia et al., 1998; Nutt and Malizia 2001; Tokunaga et al., 1997) and generalised anxiety disorders (Tiihonen et al., 1997). Furthermore, reduced $[^{3}\text{H}]$flunitrazepam binding is observed in the cortex and hippocampus of rats displaying anxiety-type behaviours such as a bias towards threatening cues in the environment that are similar to those observed in human anxiety disorders (Crestani et al., 1999). Thus, alterations in GABA$_A$ receptors are thought to be of primary importance in the pathophysiology of anxiety disorders (Mohler, 2006).

### 1.2.7.3. Depression

The overlap of symptoms and clinical treatments for depression and anxiety disorders has resulted in GABA$_A$ receptors being implicated in the pathophysiology of major depressive disorder. The most compelling evidence comes from animal models of depression which show that depressive type behaviours such as immobility in the forced swim test and escape failure in the learned helplessness model are reduced by the
administration of GABA_A receptor agonists muscimol and THIP (Borsini et al., 1986; Borsini et al., 1988; Poncelet et al., 1987; Sherman and Petty, 1980), but enhanced by the GABA_A receptor antagonist bicuculline (Sherman and Petty, 1980) and the benzodiazepine inverse agonist FG 7142 (Corda et al., 1983; Drugan et al., 1985; Guidotti et al., 1985). Furthermore, in rats that develop learned helplessness, the total number of GABA_A receptor binding sites is largely down-regulated in the frontal cortex, hippocampus and striatum (Drugan et al., 1989). Thus, animal studies support a deficit in GABAergic function in depression.

Despite evidence from animal studies suggesting GABA_A receptors may be relevant to depression, neuropathological studies of GABA_A receptors in people with depression are limited. For example, radioligand binding studies of GABA_A receptors in the depressed brain have only examined benzodiazepine-sensitive GABA_A receptors and observed no change in the maximum number of benzodiazepine sites in most brain regions (Cheetham et al., 1988; Crow et al., 1984; Manchon et al., 1987; Stocks et al., 1990), except the frontal cortex, where either no change (Crow et al., 1984), or increases (Cheetham et al., 1988; Pandey et al., 1997) are observed. However, one more recent study has shown that GABA_A receptor α1, α3, α4 and δ subunit mRNA expression is reduced in post-mortem tissue from depressed suicides relative to controls in the frontopolar cortex (Merali et al., 2004). Thus, a deficit in GABAergic transmission via certain GABA_A receptor subtypes may be of importance in depression.

Changes in GABA_A receptors that are observed in depression do not appear to be a result of chronic antidepressant drug treatment. For example, treatment for a minimum of 21 days with tricyclic, monoamine-oxidase inhibitor (MAOI) and selective serotonin reuptake-inhibitor (SSRI) antidepressant drug classes in rats has been shown to reduce the number of benzodiazepine binding sites in most brain regions (McKenna
et al., 1994; Suranyi-Cadotte et al., 1984; Tunnicliff et al., 1999), although not all studies have shown changes in benzodiazepine binding (Kimber et al., 1987; Przegalinski et al., 1987; Todd et al., 1995). Nonetheless, antidepressant-induced reductions in benzodiazepine binding are supported by observations of reduced flurazepam efficacy in the rat brain following chronic treatment with antidepressants but only after two weeks of drug treatment suggesting a potential role for altered benzodiazepine binding in the delayed therapeutic efficacy of antidepressants (Bouthillier and deMontigny, 1987). Furthermore, infusion of imipramine over 21 days has been shown to increase $\beta_2$, $\alpha_2$ and $\gamma_2$ but decrease $\alpha_1$ subunit mRNA expression in the rat brainstem (Tanay et al., 1996; Tanay et al., 2001), suggesting chronic antidepressant administration has subtype dependent effects on GABA$_A$ receptors.

1.2.8. Summary

From the above review of the literature it is clear that GABA$_A$ receptor ionophores are a complex receptor class. There are a number of receptor subtypes distinguished by the molecular composition of subunits contributing to the pentameric structure. Despite the potential for a number of subtypes given the subunit molecular diversity, only about 20 appear to exist in the mammalian brain. These receptor subtypes vary in regional distributions and pharmacological sensitivities, with evidence suggesting a greater relative importance of certain subtypes for certain behaviours and in psychiatric disorders such as anxiety disorders, schizophrenia and depression.
1.3 GABA_\text{A} receptor regulation

1.3.1. GABA_\text{A} receptor trafficking and membrane expression

The mechanisms involved in GABA_\text{A} receptor trafficking that underlie the actual expression of a functional receptor on the plasma membrane are becoming increasingly understood (see figure 1.3). When examining protein expression, possible subcellular locations of the protein must be understood, as only receptors expressed on the membrane surface are likely to affect membrane potential (Brunig et al., 2001; Kittler et al., 2001; Nusser et al., 1997; Wan et al., 1997). Altered expression of protein subunits on the plasma membrane may arise from quite rapid (3-10 minutes) trafficking processes (up/down regulation) (Thomas et al., 2005; Wan et al., 1997; Washbourne et al., 2004) resulting in an altered subcellular distribution of receptors, or over longer periods (hours), may arise from alterations in protein synthesis (Connolly et al., 1999a).

Figure 1.3: GABA_\text{A} receptor trafficking. Adapted from Lusher and Keller, 2001.
1.3.2. Receptor assembly

GABA_\text{A} receptors are synthesised in the endoplasmic reticulum of the neuronal cell body. Following translation of GABA_\text{A} receptor subunit mRNA to protein in the endoplasmic reticulum, GABA_\text{A} receptors are assembled into pentameric ion channels. Immunohistochemistry experiments measuring the subcellular distribution of epitope tagged subunit proteins in cell expression systems have indicated that subunits are assembled into pentamers in the endoplasmic reticulum (ER) (Connolly et al., 1996a). Assembled subunits are then transported via the Golgi apparatus to the plasma membrane (Connolly et al., 1996a). Intracellular transport proteins including GABARAP, catalytically inactive phospholipase C (p130), Plic-1 and N-ethylmaleimide-sensitive factor (NSF) are then responsible for the movement of the assembled pentamer to the plasma membrane (Kittler and Moss, 2001).

Only certain subunit combinations may form functional receptors that reach the plasma membrane. When expressed alone, only \( \gamma_{2S}, \beta_1, \beta_3 \) and the chick \( \beta_4 \) subunits can reach the plasma membrane (Barnes, 2000; Kittler et al., 2002) and in mammals only \( \beta_1 \) and \( \beta_3 \) subunits may produce homomeric channels at the surface but are sensitive only to pentobarbital and picrotoxin (Connolly et al., 1996b; Davies et al., 1997; Krishek et al., 1996; Wooltorton et al., 1997). In contrast, when \( \alpha \) and \( \beta_2 \), subunits are expressed alone in neuronal cultures they are retained in the ER and rapidly degraded, but when expressed together and in the presence of \( \gamma_2 \) subunits they access the membrane as functional GABA-gated channels that are blocked by bicuculline (Connolly et al., 1996b; Connolly et al., 1999b; Gorrie et al., 1997, Kittler et al., 2000; Pritchett et al., 1989). Thus, access of translated protein to the cell surface requires formation of a
pentamer in the ER of which only a few subunit combinations, including $\alpha\beta$, $\alpha\beta\gamma\delta$, may actually be expressed on the membrane as functioning receptors.

**1.3.3. Distribution of GABA$\sub A$ receptor subtypes on the membrane**

GABA$\sub A$ receptors are usually found on post-synaptic densities, dendrites and cell bodies but studies suggest variations in the membrane locations between different GABA$\sub A$ receptor subunit combinations (Connolly et al., 1996a; Fritschy et al., 1998; Nusser et al., 1996). For example, studies of hippocampal pyramidal cells using immunogold electron microscopy and immunofluorescence suggest that $\alpha_5$ subunits are found almost exclusively on soma and dendrites, whilst $\alpha_2$ subunits are preferably located on the axon-initial segment of mainly somato-dendritic synapses (Connolly et al., 1996a; Fritschy et al., 1998; Nusser et al., 1996). Additionally, studies of colocalisation of GABA$\sub A$ receptors subunits with the putative GABAergic synaptic marker gephyrin indicate certain subunits may be preferentially located in synaptic positions (see below). In contrast, extrasynaptic receptors may be formed by any subunits except $\gamma_2$ (Essrich et al., 1998) and certain subunits such as the $\alpha_4$ and $\delta$ subunits only appear to form extrasynaptic receptors. Receptors at extrasynaptic sites provide tonic inhibition as demonstrated by the slow decay kinetics and high affinity for GABA of $\delta$-subunit containing GABA$\sub A$ receptors, allowing for sensitivity to GABA that spills over from the synapse (Banks et al., 2000). This tonic inhibition appears to serve an important role in brain function given that $\delta$-subunit knockout mice display spontaneous seizures indicative of a drastic loss of inhibitory tone (Mihalek et al., 1999).
1.3.4. GABA$_A$ receptor synapses and gephyrin

Fast-synaptic or phasic transmission between neurons requires close alignment of the presynaptic terminal with a high density of post-synaptic receptors. A number of proteins have been identified that appear to serve a role in the movement and membrane stability of GABA$_A$ receptors. Several lines of evidence suggest that gephyrin, a 93kDa protein that is necessary for glycine receptor clustering, contributes to GABA$_A$ receptor synaptic clustering. In the brain gephyrin IR is enriched on the cytoplasmic side of GABA$_A$ receptor synapses and largely overlaps with the 3 most predominant $\alpha$ subunit variants (1-3) as well as the $\gamma_2$ subunit (Sassoe-Pognetto et al., 1995). Gephyrin is observed at GABAergic synapses throughout the CNS (Bohlhalter et al., 1994; Cabot et al., 1995; Crestani et al., 1999; Giustetto et al., 1998; Sassoe-Pognetto et al., 1995; Todd et al., 1996; Triller et al., 1985) as well as in cultured hippocampal (Craig et al., 1996; Essrich et al., 1998) and cortical neurons (Essrich et al., 1998). Thus, gephyrin is used as a marker of GABA$_A$ receptor synaptic clusters (Yu et al., 2006).

The function of gephyrin at this post-synaptic location is still under investigation. The contribution of gephyrin to GABA$_A$ receptor clustering has been confirmed in both gephyrin knockout mice, which show a loss of post-synaptic $\alpha_2$ and $\gamma_2$ subunit clusters (Kneussel et al., 1999b), and in experiments showing reduced $\alpha_2$ and $\gamma_2$ subunit clusters when gephyrin expression is inhibited (Essrich et al., 1998). However, gephyrin does not appear to be involved in either cluster assembly, trafficking or membrane insertion as gephyrin-deficient mice retain small GABA$_A$ receptor clusters (that are likely extrasynaptic or intracellular) (Fischer et al., 2000; Kneussel et al., 2001; Levi et al., 2004), and receptor function is only marginally reduced (Kneussel et al., 1999b; Betz, 1998). Instead, gephyrin is thought to facilitate the accumulation of GABA$_A$ receptors.
at synaptic sites (Levi et al., 2004) as studies have shown that lateral movements of synaptic receptors are reduced compared with extrasynaptic receptors (Thomas et al., 2005) and gephyrin reduces the diffusion rate of GABA\textsubscript{A} receptors (Jacob et al., 2005). Thus, when gephyrin auto-oligimerises it is thought to provide a scaffold that facilitates GABA\textsubscript{A} receptor clustering beneath the synapse (Jacob et al., 2005; Levi et al., 2004; Studler et al., 2005; Yu et al., 2007).

Interestingly, different GABA\textsubscript{A} receptors appear to vary in terms of the extent to which they colocalise with gephyrin. For example, in contrast to \(\alpha_{1-3}, \beta\) and \(\gamma_2\) subunits, the extrasynaptically located \(\alpha_4, \alpha_5\) and \(\delta\) subunits fail to colocalise with gephyrin (Kralic et al., 2006, Crestani et al., 2002, Serwanski et al., 2006; Sassoe-Pognetto et al., 1995). Furthermore, recent studies have observed that gephyrin binds directly to a hydrophobic motif of the \(\alpha_2\) subunit intracellular loop to regulate the synaptic localisation of \(\alpha_2\) containing GABA\textsubscript{A} receptors in cultured cortical neurons (Tretter et al., 2008). Despite this, as the \(\alpha_{2,3}\) subunits show diffuse IR in addition to the clustered punctate staining that colocalises with gephyrin, it appears that \(\alpha\) subunits may occupy synaptic, extrasynaptic (Essrich et al., 1998; Fritschy et al., 1998; Nusser et al., 1995; Somogyi et al., 1996) or intracellular locations. Staining for the \(\gamma_2\) subunit overlaps more closely with that of gephyrin, indicating a preferential synaptic location for this subunit (Kneussel and Betz, 2000). Whilst no direct binding motif has been observed for gephyrin on the \(\gamma_2\) subunit (Alldred et al., 2005; Meyer et al., 1995; Fritschy et al., 2008), there appears to be an interdependence of these two proteins as \(\gamma_2\) subunit-deficient mice lose both gephyrin and \(\gamma_2\) receptor clusters (Alldred et al., 2005; Essrich et al., 1998; Yu et al., 2007).
Whilst the role of gephyrin in GABA\textsubscript{A} receptor synaptic clustering is still under investigation, studies indicate that reductions in the colocalisation of gephyrin with GABA\textsubscript{A} receptor subunit proteins affects GABAergic function. For example, reduced GABA\textsubscript{A} receptor colocalisation with gephyrin results in alterations in single channel conductance times (Crestani \textit{et al.}, 1999) and in the mean amplitude, but not the frequency of whole cell mIPSCs (Levi \textit{et al.}, 2004; Kneussel \textit{et al.}, 1999b). Such findings indicate a change in GABAergic synaptic strength and a redistribution of receptors to extrasynaptic sites on the plasma membrane arises following loss of receptors in the synapse (Crestani \textit{et al.}, 1999; Levi \textit{et al.}, 2004).

Functional alterations in GABAergic synaptic function appear to translate into behavioural differences. For example, a loss of \(\alpha_1\) and \(\alpha_2\) receptor clusters in hippocampus without alteration in gephyrin clusters is observed in mice lacking dystrophin in which animals show severe cognitive deficits (Kneussel \textit{et al.}, 1999b). Furthermore, a loss of GABA\textsubscript{A} receptor synaptic clusters in the hippocampus and cortex of heterozygous \(\gamma_2\) deficient mice is associated with increased bias for learning negative associations (trace fear conditioning; ambiguous cue discrimination), enhanced reactivity to aversive stimuli and increased anxiety on several behavioural measures (Crestani \textit{et al.}, 1999). Thus, deficits in GABA\textsubscript{A} receptor synaptic clustering appear to translate into a more anxious, behaviourally reactive phenotype reminiscent of anxiety disorders and depression in humans (Crestani \textit{et al.}, 1999).

### 1.3.5. Endocytosis, degradation and recycling

GABA\textsubscript{A} receptors undergo constitutive endocytosis under basal conditions to facilitate receptor turnover. GABA\textsubscript{A} receptors on the plasma membrane cluster in Clathrin-coated pits which endocytose to form clathrin-coated vesicles, the main vehicle
for receptor internalisation in the CNS (Barnes, 2000). Endocytosis of GABA$_A$ receptors into clathrin-coated pits is dependent on the GTPase dynamin, its binding partner amphiphysin and the adaptin AP2 (Kittler et al., 2000). Once in clathrin-coated vesicles receptors are returned to the endosomal system where they are degraded via proteolysis in late endosomes or lysosomes or recycled to the plasma membrane.

Interestingly, large pools of GABA$_A$ receptors appear to reside in clathrin-coated vesicles from where they may be rapidly expressed on the surface (Tehrani and Barnes, 1997; Tehrani et al., 1997). Receptors in clathrin-coated vesicles are labelled by orthosteric site agonists in ex vivo studies, but have impaired allosteric coupling with benzodiazepines and picrotoxin (Tehrani et al., 1997). Whilst the major subunit identities of GABA$_A$ receptors residing in coated-coated vesicles have not been characterised, receptors containing only $\alpha$ and $\beta$ subunits appear to be targeted to peripheral endosomes whilst $\alpha\beta\gamma$ subtypes are targeted to late endosomes (Connolly et al., 1999b) suggesting that $\alpha\beta$ subtypes are more likely involved in rapid up and down regulation of receptors.

Orthosteric and allosteric site agonists induce ligand-dependent endocytosis or receptor downregulation. For example, receptor endocytosis is observed when GABA or benzodiazepines are incubated with cortical neurons for 2 hours at 37°C, a process that is blocked by benzodiazepine antagonists (Johnston et al., 1998). Chronic administration of benzodiazepines (Tehrani and Barnes, 1997) and ethanol (Poisbeau et al., 1997) in rats also results in enhanced GABA$_A$ receptor subunit immunoreactivity in clathrin-coated vesicles. Ligand-dependent endocytosis likely provides a mechanism via which surface receptors are controlled by tonic levels of GABA and may underlie tolerance to GABAergic compounds (Barnes 2000; Kittler et al., 2002).
1.3.6. Phosphorylation and GABA<sub>A</sub> receptor regulation

GABA<sub>A</sub> receptor phosphorylation is a complex topic. A number of residues within the β and γ<sub>2</sub> subunits are capable of binding known kinases. The β<sub>1-3</sub> subunits may be phosphorylated by protein kinase A, C, G (PKA; PKC, PKG) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) (McDonald and Moss, 1997; Moss et al., 1992). The γ<sub>2</sub> subunit is phosphorylated by both PKC and CaMKII (Brandon et al., 2002; McDonald and Moss, 1997; Moss et al., 1992). Kinase induced alterations of the GABA<sub>A</sub> receptor phosphorylation state via the β or γ<sub>2</sub> subunits may affect channel opening as suggested by effects of PKA, PKC and tyrosine kinase on GABA-induced chloride currents (Brandon et al., 2000; Brandon and Moss, 2000; Brandon et al., 2002; McDonald et al., 1998; Moss et al., 1995; Moss and Smart, 1996). Phosphorylation also appears to play a role in receptor trafficking with findings that the AP2 protein found in clathrin-coated pits colocalises with unphosphorylated β and γ<sub>2</sub> subunits in cultured hippocampal neurons, and manipulation of the function of PKC in vitro alters membrane expression of receptors (Connolly et al., 1999a; Filippova et al., 2000). Thus, it is likely that phosphorylation of GABA<sub>A</sub> receptors is important for both receptor trafficking and chloride ion conductance both of which are highly important to receptor function.

Phosphorylation also appears to play a role in allosteric modulation of the GABA<sub>A</sub> receptor channel but whether these effects relate to direct effects on receptor conformation or altered trafficking are unknown. For example, PKC has been shown to potentiate benzodiazepine and TBPS binding but inhibit muscimol binding in a region specific manner in the brain (Oh et al., 1999) and activation of PKC reduces benzodiazepine potency at GABA<sub>A</sub> receptors. Constitutive PKC function appears
necessary for the neurosteroid allopregnanalone to positively modulate GABA\(_A\) receptors (Brandon et al., 2002), and allopregnanolone binding at GABA\(_A\) receptors prevents PKC induced inhibition of GABA\(_A\) receptor currents (Brussard et al., 2000). CAMKII has also been shown to affect benzodiazepine activity by inducing enhanced benzodiazepine binding to GABA\(_A\) receptors via interactions with the \(\alpha_1\) subunit (Churn et al., 2002). Phosphorylation has also been implicated in behavioural effects of allostERIC modulators with PKC\(\varepsilon\) (Hodge et al., 1999) but not PKC\(\gamma\) (Harris et al., 1995) knockout mice showing an increased sensitivity to benzodiazepines (Gao and Greenfield, 2005).

1.3.7. Summary

In summary, the subcellular location of GABA\(_A\) receptors on the plasma membrane is regulated by complex trafficking mechanisms. A number of proteins including protein kinases and gephyrin have been identified for their involvement in the movement and stabilisation of pentameric subunit combinations from the endoplasmic reticulum, to the membrane, and consequently from the membrane to Clathrin-coated vesicles where they may be recycled or destroyed. Only certain subunit combinations may be expressed on the membrane and there appears to be preferential membrane locations for a number of subunits. The location of receptors as synaptic or extrasynaptic provides an indication as to the type of inhibitory function within a neuron and variations in the expression at such locations may induce electrophysiological and behavioural changes.
1.4 Developmental changes in GABA<sub>A</sub> receptor expression

1.4.1. GABA<sub>A</sub> receptor onset and maturation

The developmental onset and maturational changes in brain GABA<sub>A</sub> receptor expression is of interest given the role of the GABAergic system in normal brain development (see sections 1.1.1. and 1.1.2). Studies measuring GABA<sub>A</sub> receptor binding sites (Schlumpf <i>et al.</i>, 1983; Shaw <i>et al.</i>, 1991), subunit mRNA expression (Laurie <i>et al.</i>, 1992; MacLennan <i>et al.</i>, 1991; Poulter <i>et al.</i>, 1992; Poulter <i>et al.</i>, 1993; Zhang <i>et al.</i>, 1991) and electrophysiological responses (Kellogg and Pleger, 1989) all show that GABA<sub>A</sub> receptors are abundant and functional in early brain development appearing by 15-18 weeks gestation in human cortex (Aaltonen <i>et al.</i>, 1983; Brooksbank <i>et al.</i>, 1982), foetal day 60 in the developing macaque cortex (Hendrickson <i>et al.</i>, 1994; Shaw <i>et al.</i>, 1991) and around gestational day 14 in rat brainstem (Poulter <i>et al.</i>, 1992; Schlumpf <i>et al.</i>, 1983). Radioligand binding studies suggest that the total population of GABA<sub>A</sub> receptors (both high and low affinity) measured by [³H]GABA or [³H]muscimol in the rat (Coyle and Enna, 1976; Frostholm and Rotter, 1987; Rothe and Bigl, 1989; Skerritt and Johnston, 1982; Xia and Haddad, 1992) and primate (Lidow <i>et al.</i>, 1991) brain increases dramatically after birth. The subset of GABA<sub>A</sub> receptors containing the γ<sub>2</sub> subunit that are labelled by benzodiazepines are highly expressed early in cortical development but they decrease during development to reach adult levels by PND 14 in rats (McKernan <i>et al.</i>, 1991) and by birth in primates (Shaw <i>et al.</i>, 1991), consistent with the loss of γ<sub>2</sub> mRNA from PND 14 in rats (Gambarana <i>et al.</i>, 1991).
1.4.2. Developmental ‘switch’ in GABA_A receptor α subunits

Interestingly, all species examined show developmental changes in GABA_A receptor subunit protein and mRNA expression. The most striking change is the decrease in α_2 subunit expression, the predominant α subunit in early development, and maturational increase in α_1 subunit expression, the predominant adult form of α subunit (Araki et al., 1992; Bosman et al., 2002; Fritschy et al., 1994; Fuchs and Sieghart, 1989; Gambarana et al., 1990; Gambarana et al., 1991; Heinen et al., 2004; Hendrickson et al., 1994; Hornung and Fritschy, 1996; Laurie et al., 1992; Lopez-Tellez et al., 2004; MacLennan et al., 1991; McKernan et al., 1991; Okada et al., 2000; Paysan et al., 1994; Poulter et al., 1992; Poulter et al., 1993; Sato and Neale, 1989; Vitorica et al., 1990; Zhang et al., 1992). These maturational changes in α subunit expression are also supported by binding studies examining type I (α_1/5 subunit-containing) and type II (α_2/3 subunit-containing) benzodiazepine sites which show developmental decreases in type II sites and increases in [^3^H]zolpidem labelling of type-I sites (Hendrickson et al., 1994; March and Shaw, 1993; Sato and Neale, 1989; Vitorica et al., 1990). This developmental change in α subunit expression is termed the α_1/α_2 subunit ‘switch’ (McKernan et al., 1991).

The α subunit ‘switch’ appears to be largely conserved across species and sexes (Davis et al., 2000) despite variations in the timecourse (Hornung and Fritschy, 1996). Immunoreactivity for the α_1 subunit is mostly absent from the foetal brain of humans (Brooks-Kayal and Pritchett, 1993; Kananumi et al., 2006; Reichelt et al., 1991), non-human primates (Hendrickson et al., 1994; Hornung and Fritschy, 1996) and rodents (Fritschy et al., 1994; Lopez-Tellez et al., 2004; McKernan et al., 1991) whilst α_2 immunoreactivity is prominent and widespread prenatally (Fritschy et al., 1994;
Subunit mRNAs are first detected in rat brain at E15 with $\alpha_5$ appearing at E17. $\alpha_1$ Subunit mRNA appears in the rat cortex at E19 and PND 5 in the hippocampus (Lopez-Tellez et al., 2004; Poulter et al., 1992).

1.4.3. Regional variations in the developmental ‘switch’

Table 1.1 shows regional variations in the $\alpha_{1/2}$ developmental ‘switch’. Immunoreactivity for the $\alpha_1$ subunit is first seen in regions of the brainstem, cerebellum, basal forebrain, primary sensory cortices (visual and somatosensory) and pallidum during the last weeks of gestation in primates (where prenatal expression has been examined), whilst regions such as the thalamus, and remaining neocortex appear to have delayed onset of $\alpha_1$ immunoreactivity (Brooks-Kayal and Pritchett, 1993; Hendrickson et al., 1994; Hornung and Fritschy, 1996; Kananumi et al., 2006; Lopez-Tellez et al., 2004; Paysan et al., 1994; Reichelt et al., 1991) or just after birth in rodents (Fritschy et al., 1994; Lopez-Tellez et al., 2004; McKernan et al., 1991; Paysan et al., 1994). These findings are consistent with the appearance of $[^3]H$zolpidem binding in the macaque (Hendrickson et al., 1994) and $[^3]H$flunitrazepam displacement by $\alpha_1$ selective ligand CL218872 in the human brain (March and Shaw, 1993), as well as studies of mRNA expression (Gambarana et al., 1990; Laurie et al., 1992). Disappearance of $\alpha_2$ immunoreactivity occurs initially in similar regions of the basal forebrain, substantia nigra, primary sensory cortices (visual and somatosensory) and pallidum usually just after the appearance of $\alpha_1$ immunoreactivity (Fritschy et al., 1994; Hornung and Fritschy, 1996; Lopez-Tellez et al., 2004; McKernan et al., 1991). The adult $\alpha$ subunit regional immunoreactivity pattern is generally observed by the onset of
behavioural and sexual maturity for rats (21 days) (Fritschy et al., 1994) and marmosets (3 years) (Hornung and Fritschy, 1996).

Variations in the prominence of the ‘switch’ are observed amongst brain regions. The ‘switch’ appears to be most evident in the thalamus and pallidum where $\alpha_2$ subunit immunoreactivity is intense in foetal brain and lacking in the adult (Fritschy et al., 1994; Hornung and Fritschy, 1996). It is noted, however that some amygdalar, hippocampal and hypothalamic regions do not appear to show the $\alpha$ subunit ‘switch’ during brain maturation (Davis et al., 2000; Kanaumi et al., 2006) and certain nuclei of the brainstem show constant $\alpha_2$ expression but a ‘switch’ from $\alpha_3$ to $\alpha_1$ subunit expression during development (Liu and Wong-Riley, 2004; Liu and Wong-Riley, 2006) (see table 1.1). Furthermore, regions such as the granule cell layer of the hippocampus, striatum and outer cortical layers have little to no $\alpha_1$ immunoreactivity at any age and maintain intense $\alpha_2$ immunoreactivity (Fritschy et al., 1994; Hornung and Fritschy, 1996) into adulthood, whilst regions such as the reticular nucleus of the thalamus and superior olivary complex never express either $\alpha_1$ or $\alpha_2$ subunits (Fritschy et al., 1994).

The pattern of the developmental ‘switch’ in GABA$_A$ receptor $\alpha$ subunits shows an area and lamina specific pattern however, it is unknown what signals trigger its onset. The onset of $\alpha_1$ subunit immunoreactivity is marked by sharp regional boundaries seen particularly in the primary sensory cortices. The primary visual and somatosensory cortex are the first cortical regions that show $\alpha_1$ immunoreactivity (Fritschy et al., 1994; Hendrickson et al., 1994; Hornung and Fritschy, 1996; Paysan et al., 1994) and displacement of $[^3]$Hflunitrazepam binding by $\alpha_1$-selective ligands (Hendrickson et al., 1994) both of which are evident first in the major thalamic input
layers (layer III-IV and VI). Other cortical regions and layers only become apparent postnatally with staining appearing first in lamina V (Fritschy et al., 1994; Hornung and Fritschy, 1996). In contrast, \( \alpha_2 \) is lost from layer III of primary sensory cortices first, with loss from other layers occurring later in development, with the exception of outer cortical layers which retain intense \( \alpha_2 \) staining in the adult brain.
Table 1.1: Maturational changes in \( \alpha_1 \) and \( \alpha_2 \) subunit protein expression by region

<table>
<thead>
<tr>
<th>Region</th>
<th>( \alpha_1 )</th>
<th>( \alpha_2 )</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>↑</td>
<td>↓</td>
<td>Rat</td>
<td>McKernan et al., 1991</td>
</tr>
<tr>
<td>Primary sensory (BA17, S1)</td>
<td>↑</td>
<td>↑</td>
<td>Marmoset</td>
<td>Hornung and Fritschy, 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>Fritschy et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Macaque</td>
<td>*Paysan et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hendrickson et al., 1994</td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temporal</td>
<td>^↑</td>
<td>0</td>
<td>Human</td>
<td>Kanaumi et al., 2006</td>
</tr>
<tr>
<td>Motor, association areas</td>
<td>↑</td>
<td>↓</td>
<td>Marmoset</td>
<td>Hornung and Fritschy, 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>Fritschy et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Macaque</td>
<td>Paysan et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hendrickson et al., 1994</td>
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<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentate Gyrus</td>
<td>^↑</td>
<td>↓</td>
<td>Human</td>
<td>Kanaumi et al., 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>Lopez-Tellez et al., 2004</td>
</tr>
<tr>
<td>CA1</td>
<td>↑</td>
<td>↑</td>
<td>Human</td>
<td>Kanaumi et al., 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>Davis et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lopez-Tellez et al., 2004</td>
</tr>
<tr>
<td>CA3</td>
<td>0 (Human)</td>
<td>0</td>
<td>Human</td>
<td>Kanaumi et al., 2006</td>
</tr>
<tr>
<td></td>
<td>↑ (Rat)</td>
<td></td>
<td>Rat</td>
<td>Davis et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lopez-Tellez et al., 2004</td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td>↑</td>
<td>↓</td>
<td>Marmoset</td>
<td>Hornung and Fritschy, 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>Fritschy et al., 1994</td>
</tr>
<tr>
<td>Ventrolateral nucleus</td>
<td>↑</td>
<td>↓</td>
<td>Rat</td>
<td>Davis et al., 2000</td>
</tr>
<tr>
<td>Laterodorsal nucleus</td>
<td>↑</td>
<td>↓</td>
<td>Rat</td>
<td>Okada et al., 2000</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POA</td>
<td>↑</td>
<td></td>
<td>Rat</td>
<td>Davis et al., 2000</td>
</tr>
<tr>
<td>VMN</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amygdala</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td>↓</td>
<td>↓</td>
<td>Rat</td>
<td>Davis et al., 2000</td>
</tr>
<tr>
<td>Basal Forebrain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>↑</td>
<td></td>
<td>Marmoset</td>
<td>Hornung and Fritschy, 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>Fritschy et al., 1994</td>
</tr>
<tr>
<td>Medial septum</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palidum</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td>↑</td>
<td>↓</td>
<td>Rat</td>
<td>McKernan et al., 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fritschy et al., 1994</td>
</tr>
<tr>
<td>Brainstem</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-botzinger complex</td>
<td>↑</td>
<td>0</td>
<td>Rat</td>
<td>Liu and Wong-Riley, 2004</td>
</tr>
<tr>
<td>NTS</td>
<td>↑</td>
<td></td>
<td>Rat</td>
<td>Liu and Wong-Riley, 2006</td>
</tr>
<tr>
<td>Cuneate</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Denotes references that only apply to \( \alpha_1 \) subunit changes, ^denotes transient change
1.4.4. Cellular expression of the subunit ‘switch’

At the cellular level, the subunit ‘switch’ appears to represent a gradual replacement of $\alpha_2$ subunits with $\alpha_1$, with all neurons in a given region being affected by the subunit ‘switch’ (Fritschy et al., 1994; Hornung and Fritschy, 1996). Disappearance of $\alpha_2$ occurs first from dendrites then cell bodies and neuropil whilst $\alpha_1$ immunoreactivity progresses from cell bodies and dendrites to the neuropil (Fritschy et al., 1994; Hendrickson et al., 1994; Hornung and Fritschy, 1996). Death of $\alpha_2$ subunit-containing neurons is unlikely as it has been shown that the increase in $\alpha_1$ precedes the loss of $\alpha_2$ by several days resulting in coexpression of both subunits during a limited time window (Fritschy et al., 1994; Hornung and Fritschy, 1996).

Throughout development, both $\alpha_1$ and $\alpha_2$ subunits largely overlap with $\beta_{2/3}$ subunits, which in turn are widespread in neonatal and adult brain of rodents and primates (Fritschy et al., 1994; Hendrickson et al., 1994; Hornung and Fritschy, 1996; Meinecke and Rakic, 1992). Protein expression of the $\beta_{2/3}$ subunit is fairly constant in comparison to the $\alpha$ subunits, however, maturational increases are observed in the striatum, pallidum, substantia nigra, cerebellum and reticular formation and decreases with age in the superior and inferior olivary complexes and the reticular nucleus of the thalamus (Fritschy et al., 1994) and primary visual cortex (Hendrickson et al., 1994). Interestingly, it has been suggested that another $\beta$ subunit may be prominent in very early development where immunoreactive colocalisation is not as evident as the adult pattern of $\alpha_1-\beta_{2/3}$ and $\alpha_2-\beta_{2/3}$ colocalisation (Hendrickson et al., 1994; Hornung and Fritschy, 1996). However, studies of $\beta$ subunit mRNA expression suggest limited $\beta_1$ expression throughout development while reports on the $\beta_2$ and $\beta_3$ subunit genes are conflicting. Some reports suggest both $\beta_2$ and $\beta_3$ mRNAs are highly expressed at birth.
(Gambarana et al., 1991; Poulter et al., 1993), whilst other studies observed that $\beta_3$ predominates in earlier development and remains constant, whilst $\beta_2$ subunit mRNA shows a delayed increase in expression (Laurie et al., 1992; Zhang et al., 1991).

1.4.5. Significance of the subunit ‘switch’ for brain function

The ‘switch’ in $\alpha$ subunit expression alters $\text{GABA}_A$ receptor function. In terms of pharmacological function, there is a change in the sensitivity to GABAergic compounds during brain development in rodents and primates (Brooks-Kayal and Pritchett, 1993; Candy and Martin, 1979; Hendrickson et al., 1994; Kapur and MacDonald, 1999; Lippa et al., 1981; March and Shaw, 1993; Reichelt et al., 1991; Shaw et al., 1991) with $\alpha_2$ subunit containing receptors, predominant in early life, showing greater sensitivity to GABA and the neurosteroid allopregnanalone (Brussard et al., 1997). In studies of recombinant receptors, $\alpha_1$ subunit containing receptors have reduced sensitivity to diazepam and clonazepam compared with the $\alpha_2$ and $\alpha_3$ containing subtypes (Puia et al., 1991). Furthermore, in $\alpha_1$ subunit knockout mice, the absence of the subunit ‘switch’ results in altered behavioural sensitivities in response to a number of compounds such as zolpidem, ethanol, THIP and flurazepam (Blednov et al., 2003). Such changes in pharmacological sensitivity may be associated with the marked reductions in the number of adult $\text{GABA}_A$ receptor sites that are measured by $[^3H]\text{muscimol}$, $[^3H]\text{flumazenil}$, $[^35S]\text{TBPS}$ (Sur et al., 2001), and the reduced muscimol-stimulated chloride uptake (Blednov et al., 2003) observed in these mice. Thus, the $\alpha$ subunit ‘switch’ appears to alter adulthood receptor expression and pharmacology.

The ‘switch’ from $\alpha_2$ to $\alpha_1$ also leads to alterations in channel gating properties and such changes are associated with certain behaviours. $\alpha_2$ and $\alpha_3$ subunit-containing receptors show slower decay times and greater current amplitudes of mIPSPs than $\alpha_1$.
receptors (Bosman et al., 2002; Heinen et al., 2004; Hollrigel and Soltesz, 1997; Hutcheon et al., 2000; Juttner et al., 2001; Okada et al., 2000; Ortinski et al., 2004; Taketo and Yoshioka, 2000; Vicini et al., 2001). Predictably then, the onset of $\alpha_1$ expression coincides with the onset of faster decay time constants for mIPSPs (Bosman et al., 2002; Juttner et al., 2001; Okada et al., 2000), which are not observed at any developmental stage in $\alpha_1$ subunit knockout mice (Barberis et al., 2005; Bosman et al., 2005; Goldstein et al., 2002; Heinen et al., 2003; Lagier et al., 2007; Vicini et al., 2001). Longer decay times support enhanced synaptic efficacy and lead to sedation (Franks and Lieb, 1994; Tanelian et al., 1993), whereas shorter decay times can produce anxiety and seizures (Worms and Lloyd, 1981). Interestingly, it has been suggested that developmental shortening of decay time may support fast rhythmic oscillations required for high-level consciousness seen in adulthood (Okada et al., 2000). Thus, evidence suggests that the $\alpha$ subunit ‘switch’ has a significant impact on normal and pharmacologically-manipulated brain function.

1.4.6. Significance of the subunit ‘switch’ in brain development

The change in the predominant $\alpha$ subunit coincides with important developmental changes leading to the hypothesis that the subunit ‘switch’ plays a role in brain development. One suggestion is that the $\alpha_{1/2}$ subunit ‘switch’ plays a role in the ‘switch’ from excitatory to inhibitory GABAergic currents. Whilst the immediate cause of the onset of hyperpolarizing currents involves changes in the internal chloride concentration, the subunit ‘switch’ coincides with the onset of GABAergic inhibition (Lin et al., 1994) and the immature $\alpha_{2/3}$ subunits are expressed predominantly in neurons where excitatory GABA$\alpha_3$ receptors have been observed in the adult brain.
(Cherubini et al., 1990, Isomura et al., 2003; Laurie et al., 1992; Mercuri et al., 1991; Michelson and Wong, 1991; Reichling et al., 1994). Thus, it is possible that some form of signalling triggers both hyperpolarizing currents and subunit expression changes, yet it is unknown which precedes the other.

The GABA$_A$ receptor $\alpha$ subunit ‘switch’ has also been implicated in the onset of synaptogenesis. The onset of $\alpha_1$ and disappearance of $\alpha_2$ immunoreactivity (Hornung and Fritschy, 1996) coincides with the period of synaptogenesis in marmoset primary visual cortex (after embryonic day (ED) 100 - post-natal day (PND) 60) (Missler et al., 1993). However, in the macaque, the subunit ‘switch’ occurs much later than synaptogenesis (Zielinski et al., 1992) limiting the importance of the subunit ‘switch’ for the onset of synaptogenesis (Hendrickson et al., 1994). Alternatively, it has been suggested that the subunit ‘switch’ is associated with axonal sorting and area specification in the macaque (Hendrickson et al., 1994). Consistent with this, $\alpha_1$ subunit knockout mice show impaired maturation of dendritic spines and reduced adulthood spine density, suggesting an impairment in synaptic consolidation (Heinen et al., 2003). However, future studies are required to confirm the role of $\alpha$ subunit maturational changes in brain development.

1.4.7. Summary

In summary, there are well known variations in the expression of GABA$_A$ receptor $\alpha$ subunits in the developing brain with the $\alpha_2$ subunit predominating in the immature brain and the $\alpha_1$ subunit in the adult brain. This subunit ‘switch’ is likely important given the different pharmacological and electrophysiological profiles for the $\alpha_1$ and $\alpha_2$ subunit containing GABA$_A$ receptors. Important developmental changes
such as synapse formation and the ‘switch’ of GABA from excitatory to inhibitory show temporal correlation with the subunit ‘switch’, which may be due to a causal relationship or triggered by the same pathways of molecules. It is hypothesised then that disruptions to the subunit ‘switch’ would alter behavioural, electrophysiological, and pharmacological properties of the GABAergic system.

1.5. Sex differences and GABA\(_\alpha\) receptors

1.5.1. Sexual differentiation of the brain

Investigation into sex-differences in the brain is highly relevant given the observed sex-differences in psychiatric disorders. For example, research has consistently shown that women are about twice as likely as men to develop depression, regardless of culture or ethnicity (Nolen-Hoeksema, 2001; Weissman et al., 1996). For anxiety disorders such as agoraphobia, panic disorder, post-traumatic stress disorder (PTSD) and generalised anxiety disorders, but not social phobia and obsessive compulsive disorder (OCD), females have substantially higher lifetime prevalence and symptom severity than do males (reviewed in Bekker and van Mens-Verhulst, 2007). In contrast, epidemiological studies of schizophrenic patient populations show that, females have a later age of symptom onset and generally a better course of illness than males (Angermeyer et al., 1989; Holden, 2005; Goldstein, 1988; Grossman et al., 2008; Grossman et al., 2006; Seeman, 1986). Thus, an improved understanding of the neurobiological differences between males and females may improve understanding of the causes and treatments of such diseases.

Recent microarray studies show that approximately 650 genes are differentially expressed in the brains of male and female mice (around 15% of all genes expressed) with 50% being more abundant in males and 50% in females (Yang et al., 2006). Not
surprisingly then, sex differences are observed at many levels of investigation including neuropsychology, brain anatomy, brain function and neurochemistry (Cahill et al., 2006; Davies and Wilkinson, 2006). In terms of brain anatomy, differences in neuronal density and regional volume are observed in regions that are important for reproductive behaviour such as the sexually dimorphic nucleus of the medial preoptic area which is larger in males (Gorski et al., 1980; Simmerly, 2002) and the anteroventral periventricular nucleus of the hypothalamus which is larger in females. Regions that are not linked directly with reproductive behaviour also show sexual dimorphism, for example the cortex (anterior cingulate and posterior temporal) (Markham and Juraska, 2002; Witelson et al., 1995) and hippocampus (Madeira et al., 1991; Nunez et al., 2000), particularly CA1 (Isgor et al., 1998) and dentate gyrus (Juraska et al., 1989). Sex differences in brain function are supported by differences in the connectivities between regions (Simerly, 2002) and in neurotransmitter systems such as the monoaminergic, GABAergic (see below) and opioid systems (Cahill et al., 2006). Undoubtedly, such sex-differences in brain anatomy and function may lead to behavioural sex-differences or may allow for compensation to prevent differences arising from both hormonal and genetic sex-differences (DeVries et al., 2004).

1.5.2. Causes of brain sex-differences

The role of hormones in sexual differentiation has been recognised for many years and traditionally hormonal signalling was considered the basis for brain sexual differentiation. For example, some regional differences may be reversed by treating females with testosterone or blocking the effects of testosterone in males (Arnold and Gorski, 1984; Nordeen et al., 1985; Phoenix et al., 1959). In other cases sex-differences precede or are only partially explained by testosterone or oestrogen.
(Arnold et al., 2003) implicating other gonadal hormones such as MIS (Wang et al., 2005) as well as chromosomal differences (Arnold et al., 2003; Arnold et al., 2004; Davies and Wilkinson, 2006) in the expression of brain sex-differences.

1.5.3. Sex-differences and behavioural sensitivity to GABA_A receptor ligands

In general, most studies have indicated that where sex-differences exist, males are more sensitive to the behavioural effects of compounds acting at GABA_A receptors than females (see table 1.2 and table 1.3). As shown in table 1.2, literature examining sex-differences in the effects of different allosteric modulators such as benzodiazepine site agonists and inverse agonists as well as ethanol and the neurosteroid allopregnanalone, is complex. For example, observations of sex-differences depend on the dose of ethanol (Crippens et al., 1999; Tayyabkhan et al., 2002; Webb et al., 2002) and diazepam (Bitran et al., 1991; Fernandez-Guasti and Picazo, 1990; Fernandez-Guasti and Picazo, 1997; Fernandez-Guasti and Picazo, 1999; Wilson et al., 2004) and the behavioural parameter that is examined for the neurosteroid allopregnanalone (Fernandez-Guasti and Picazo, 1990; Fernandez-Guasti and Picazo, 1997; Fernandez-Guasti and Picazo, 1999). As shown in table 1.3 convulsant effects of agents administered intravenously (i.v.) in rats, such as pentylenetetrazol (Kokka et al., 1992), picrotoxin (Pericic and Bujas, 1997a) and bicuculline (Bujas et al., 1997; Guillet and Dunham, 1995; Pericic and Bujas, 1997a; Pericic and Bujas, 1997b; Pericic et al., 1999; Manev et al., 1987; Wilson 1992) are more apparent in males, however, both drug administration route (Pericic and Bujas, 1997a; Pericic et al., 1985; Pericic et al., 1986) and species (Manev et al., 1987) appear to be complicating factors, suggesting that sex-differences in sensitivity to GABAergic compounds are affected by pharmacokinetic sex-differences (Webb et al., 2002).
Table 1.2: Sex-differences in behavioural sensitivities to GABA<sub>A</sub> receptor allosteric modulators

<table>
<thead>
<tr>
<th>Drug</th>
<th>Behavioural Effect of drug (test)</th>
<th>Species</th>
<th>More sensitive sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ro 15-4513 FG7142</td>
<td>Learning Impairment (operant conditioning)</td>
<td>Mice</td>
<td>Males (Bao et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Reduced Activity (Open Field)</td>
<td>Rats</td>
<td>Males (Meng and Drugan, 1993)</td>
</tr>
<tr>
<td>Diazepam</td>
<td>Anxiolysis (Light-dark transitions)</td>
<td>Mice</td>
<td>Males (depends on female oestrous cycle) (Carey et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Protection from PTZ-induced seizure</td>
<td>Rats</td>
<td>No difference (Kokka et al., 1992; Wilson and Biscardi, 1992)</td>
</tr>
<tr>
<td></td>
<td>Protection from bicuculline-induced seizure</td>
<td>Rats</td>
<td>No difference (Wilson, 1992)</td>
</tr>
<tr>
<td></td>
<td>Anxiolysis (plus-maze)</td>
<td>Rats</td>
<td>Males - Low dose (Wilson et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Anxiolysis (defensive prod burying)</td>
<td>Rats</td>
<td>Males - Low dose (Fernandez-Guasti and Picazzo, 1990; 1997; 1999)</td>
</tr>
<tr>
<td></td>
<td>Reduced conflict behaviour (punished vs. unpunished drinking)</td>
<td>Rats</td>
<td>No difference – High dose (Wilson et al., 2004; Boehm et al., 2002)</td>
</tr>
<tr>
<td>Allopregnanalone</td>
<td>Anxiolysis (plus-maze)</td>
<td>Rats</td>
<td>No difference (Fernandez-Guasti and Picazo, 1999)</td>
</tr>
<tr>
<td></td>
<td>Anxiolysis (defensive prod burying)</td>
<td>Rats</td>
<td>Males (Fernandez-Guasti and Picazo, 1997; 1999)</td>
</tr>
<tr>
<td></td>
<td>Anxiolysis (acoustic startle)</td>
<td>Rats</td>
<td>Males (Guinello and Smith, 2003)</td>
</tr>
<tr>
<td></td>
<td>Anxiolysis (grooming)</td>
<td>Rats</td>
<td>Females (Zimmerberg et al., 1999)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Protection from PTZ-induced seizure</td>
<td>Rats</td>
<td>No difference (Kokka et al., 1992; Wilson and Biscardi, 1992)</td>
</tr>
<tr>
<td></td>
<td>Anxiolysis (plus maze)</td>
<td>Rats</td>
<td>No difference – low / high dose (Wilson et al., 2004; 1992; Stock et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Defensive prod-burying</td>
<td>Rats</td>
<td>No difference – low dose (Wilson et al., 2004; Boehm et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Operant Conditioning</td>
<td>Mice</td>
<td>No difference (Bao et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Sedation (high dose)</td>
<td>Rats</td>
<td>Males (Webb et al., 2002; Tayyabkhan et al., 2002; Crippens et al., 1999; Wilson et al., 2004)</td>
</tr>
</tbody>
</table>
Support that sex-differences arise from pharmacodynamic as opposed to pharmacokinetic parameters has come from several studies. For example, it has been shown that males are more sensitive than females to the behavioural effects of ethanol and diazepam when there is no sex-difference in the brain concentration of these drugs (Crippens et al., 1999). Furthermore, there appears to be a physiological sex-difference in GABA<sub>A</sub> receptor sensitivity to ethanol as female pyramidal neurons are less sensitive to the ethanol induced spontaneous GABAergic activity (Cha et al., 2006) and female hippocampal neurons take longer to alter protein subunit expression (9 days) following ethanol administration than male hippocampal neurons (3 days) (Devaud and Alele, 2004). Sex-differences at the level of neuronal function are also observed for channel blocking agents with female spinal motor neuron discharge frequency being more sensitive to picrotoxin (Pericic et al., 1986). However, no sex-differences were observed in allopregnanalone effects on GABA activated chloride flux (Wilson and Biscardi, 1997). Thus, sex-differences occur in neuronal sensitivity to GABAergic compounds suggesting that sex-differences in the GABAergic system are at least partially responsible for sex-differences in the behavioural sensitivities to compounds acting via GABA<sub>A</sub> receptors.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Species</th>
<th>More sensitive sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTZ (i.v)</td>
<td>Rats</td>
<td>Males (Kokka et al., 1992; Pericic and Bujas, 1997a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Males (Pericic et al., 1999; Guillet and Dunham, 1995; Pericic and Bujas, 1997a &amp; b; Wilson 1992; Bujas et al., 1997; Manev et al., 1987)</td>
</tr>
<tr>
<td>Bicuculline (i.v)</td>
<td>Rats</td>
<td>No difference (Wilson and Biscardi, 1992; Devaud et al., 1995)</td>
</tr>
<tr>
<td>Bicuculline (i.p)</td>
<td>Rats</td>
<td>No difference (Pericic et al., 1986)</td>
</tr>
<tr>
<td>Picrotoxin (i.v)</td>
<td>Rats</td>
<td>Males (Pericic and Bujas, 1997a)</td>
</tr>
<tr>
<td>Picrotoxin (i.v)</td>
<td>Mice</td>
<td>No difference (Pericic and Bujas, 1997a)</td>
</tr>
<tr>
<td>Picrotoxin (i.p)</td>
<td>Rats</td>
<td>Females (Pericic et al., 1985)</td>
</tr>
<tr>
<td>Picrotoxin (i.p)</td>
<td>Cats</td>
<td>Females (Pericic et al., 1986)</td>
</tr>
<tr>
<td>Picrotoxin (i.p)</td>
<td>Mice</td>
<td>Males (Pericic et al., 1986)</td>
</tr>
</tbody>
</table>
1.5.4. Effects of gonadal hormones on drug sensitivity

Sex-differences in responses to drugs acting on GABA\textsubscript{A} receptors appear to be affected by gonadal hormones. Sex-differences in ethanol-induced sedation (Silveri and Spear, 1998) and seizure induction following picrotoxin (Manev et al., 1987) and bicuculline administration (Bujas et al., 1997; Schwarz-Giblin et al., 1989; Wilson, 1992) are absent in sexually immature animals. Female gonadectomy also removes sex-differences in picrotoxin, bicuculline (Bujas et al., 1997; Schwarz-Giblin et al., 1989; Wilson, 1992) and PTZ-induced (Kokka et al., 1992) seizure thresholds. Interestingly, gonadectomy appears to exaggerate sex-differences in diazepam-induced anxiolysis by decreasing female (Bitran et al., 1991) and increasing male sensitivity (Fernandez-Guasti and Mota, 2003) relative to proestrous females or intact males, respectively. Furthermore, oestrogen and progesterone administration reinstate diazepam-induced anxiolysis in ovariectomised females (Bitran et al., 1991) and testosterone reduces diazepam sensitivity of the gonadectomized male (Fernandez-Guasti and Mota, 2003). Thus, the role of gonadal steroids in GABA\textsubscript{A} receptor pharmacological sensitivity is complex and appears less important for some drugs such as diazepam, than others such as the cage convulsants.

The female oestrous cycle stage also alters drug sensitivity, highlighting the importance of oestrogen and progesterone. Sex-differences in the anxiolytic actions of low dose diazepam are only observed in metoestrous and proestrous (Carey et al., 1992; Fernandez-Guasti and Picazo, 1997) and sex-differences in sedative effects of high dose ethanol are only observed in proestrous or diestrous phases (Crippens et al., 1999) suggesting that subtype selectivity of circulating oestrogen and progesterone are important in the actions of each of these drugs.
1.5.5. Sex-differences in the GABAergic system

Sex differences are apparent in the GABAergic system. Human males have a higher GABA concentration in CSF (Hare et al., 1980) and male rats have a higher concentration of GABA in the medial preoptic area (MPA), diagonal band, ventromedial hypothalamus (Frankfurt et al., 1984) and cingulate cortex (Manev et al., 1985), but a lower GABA concentration in the hypophysis (Manev et al., 1985). Male rats also appear to have a higher rate of GABA synthesis in the substantia nigra (Manev et al., 1986; Manev and Pericic, 1987) despite no difference being observed in GABA concentration (Manev and Pericic, 1987). Gonadal hormones appear to play a role in such sex differences in the GABAergic system as adulthood gonadectomy affects GABA turnover (Grattan and Selmanoff, 1993), neuronal activity and concentration (Earley and Leonard, 1978; Grattan and Selmanoff, 1993; Yoo et al., 2000) in males, and GABA concentration (Ondo et al., 1982; Saad, 1970), neuronal activity (Yoo et al., 2000) and GAT expression (Herbison et al., 1995) in females, with most changes being observed in striatal and hypothalamic regions. Furthermore, in various striatal, hypothalamic and amygdalar regions oestrogen administration has been shown to alter GAD expression (Leigh et al., 1990; Weiland, 1992), reduce GAD activity (Gordon et al., 1977; McGinnis et al., 1980; Nicoletti et al., 1982; Nicoletti and Meek, 1985; Wallis and Luttge, 1980) and alter basal GABA concentrations (Demling et al., 1985; Daabees et al., 1981; Herbison et al., 1991; Mansky et al., 1982; Nicoletti and Meek, 1985) of both intact males and ovariectomised females as has progesterone in females (Wallis and Luttge, 1980). Testosterone administration appears to produce similar effects to oestrogen raising the possibility that the effects of testosterone arise from its conversion to oestrogen (Earley and Leonard, 1978). Thus, in many brain regions
males appear to have more GABA and a more active GABA system with evidence suggesting a role for gonadal hormones, particularly oestrogen, in such sex-differences.

1.5.6. Sex-differences in GABA\textsubscript{A} receptors

Whilst it is unclear if sex-differences exist in GABA\textsubscript{A} receptor expression, there appear to be sex-differences in receptor sensitivity. No sex-differences were observed in the number (Wilson, 1992; Wilson and Biscardi, 1992) or affinity (Bujas \textit{et al.}, 1997; Wilson, 1992; Wilson and Biscardi, 1992) of whole cortical and cerebellar \textsuperscript{[\textit{3}H]}bicuculline sites (Kokka \textit{et al.}, 1992), however, regional information is not available. Interestingly, intact females do show a lower GABA IC\textsubscript{50} than males for displacement of \textsuperscript{[\textit{3}H]}bicuculline binding, suggesting that overall female GABA\textsubscript{A} receptors are more sensitive to the effects of GABA. This difference was eliminated by gonadectomy (Bitran \textit{et al.}, 1991; Wilson, 1992) and both testosterone (Bitran \textit{et al.}, 1993) and oestrogen (Perez \textit{et al.}, 1988) administration have been observed to affect chloride influx to cortical synaptosomes (Bitran \textit{et al.}, 1993) and cortical TBPS / TBOB binding (Perez \textit{et al.}, 1988). Thus, the presence of gonadal hormones in adulthood likely affects the functional state of the GABA\textsubscript{A} receptor resulting in females having greater receptor sensitivity to orthosteric site agonists.

Information regarding the number of high affinity binding sites varies according to region. \textsuperscript{[\textit{3}H]}muscimol binding is increased in the substantia nigra, ventrolateral thalamus, bed nucleus of the stria terminalis and caudate putamen of the female woodland rodent brain (Canonaco \textit{et al.}, 1996). However, these sex differences depend on the brain region as males appear to have higher \textsuperscript{[\textit{3}H]}muscimol binding in hypothalamic brain regions such as the preoptic area and mediobasal hypothalamus of the rat (Juptner and Hiemke, 1990) and the anterior hypothalamus and ventromedial
hypothalamus of the woodland rodent (Canonaco et al., 1996). Sexually dimorphic $[^3]$Hmuscimol binding may be mediated by ovarian hormones, given that both oestrogen alone (Maggi and Perez, 1984; McCarthy et al., 1991; Perez et al., 1986; Perez et al., 1988; Schumacher et al., 1989a) and in combination with progesterone (Maggi and Perez, 1984; McCarthy et al., 1991; Schumacher et al., 1989a; Weiland, 1992) increases muscimol binding in many brain regions, except various hypothalamic regions where oestrogen decreases muscimol binding (reversed by progesterone) in ovariectomised female rats (O’Connor et al., 1988; Schumacher et al., 1989b). Finally, it is unknown if sex-differences in $[^3]$Hmuscimol binding occur in the cortex as slightly more (Juptner and Hiemke, 1990), less (Kokka et al., 1992) or the same (Bujas et al., 1997) number of $[^3]$Hmuscimol binding sites have been observed in females compared with males. Thus, $[^3]$Hmuscimol binding appears to be sexually dimorphic in a number of regions, although whether males or females have a greater number of sites depends on the brain region.

Sex-differences in benzodiazepine binding sites are not well understood. It has been shown that there are no sex differences (Kokka et al., 1992) or higher $[^3]$Hflunitrazepam binding in the female cortex, but lower benzodiazepine sites than males have been observed in the striatum and hippocampus (Shephard et al., 1982). Females have also been observed to have a higher binding affinity for flunitrazepam in the cortex compared with males and gonadectomized groups (Wilson, 1992). Thus sex differences in flunitrazepam binding are likely dependent on brain region.

1.5.7. Gonadal hormone effects on GABA$_A$ receptors

Ovarian steroids appear to affect certain GABA$_A$ receptor subtypes preferentially. For example, in regions where there is a number of type II GABA$_A$ ($\alpha_2$- and $\alpha_3$-
subunit containing) receptors such as the spinal cord, cortex and hippocampus, oestrogen produces an increase in high affinity GABA_\(A\) receptor binding, but the opposite occurs in regions where there is a predominance of type I (\(\alpha_1\)-containing) GABA_\(A\) receptors (McCarthy et al., 1991). It has also been observed that GABA receptor subunit expression changes over the oestrous cycle in Wistar rats, with falling progesterone levels (late diestrous) being associated with increased \(\alpha_4\) (Gallo and Smith, 1993; Guinello et al., 2003; Lovick et al., 2005; Smith et al., 1998; Sundstrom-Poromaa et al., 2003) and \(\partial\) subunit (Gallo and Smith, 1993; Smith et al., 1998; Guinello et al., 2003; Sundstrom-Poromaa et al., 2003) labelling in the PAG (Lovick et al., 2005), hippocampus and amygdala (Gallo and Smith, 1993; Guinello et al., 2003; Smith et al., 1998; Sundstrom-Poromaa et al., 2003), whilst in other phases of oestrous cycle females are similar to males (Lovick et al., 2005). These changes in subunit expression are thought to be due to underlying changes in the levels of the potent GABA_\(A\) receptor modulator allopregnanalone (Smith et al., 1998) that accompanies changes in progesterone levels. Effects of oestrogen and progesterone on these subunits likely results in functional changes given that the \(\partial\) subunits are extrasynaptic and their absence results in spontaneous seizures (Nusser et al., 1998).

1.5.8. Summary

In conclusion, sex-differences are observed in the GABAergic system but literature concerning this issue is complex. Studies examining behavioural effects of GABA_\(A\) receptor compounds are affected by a number of factors such as drug dose, behavioural parameter examined, drug administration route and species examined, thus conclusions from this information about sex-differences in GABA_\(A\) receptors are
difficult to make. Whilst males often appear to be more sensitive to the behavioural
effects of compounds acting on GABA$\text{A}$ receptors, this is by no means a robust finding.
*Ex vivo* investigations provide more convincing results regarding sex-differences in
GABA$\text{A}$ receptors, with evidence to suggest that GABA$\text{A}$ receptors in females are more
sensitive to orthosteric site ligands and channel blocking drugs whilst male neurons are
more sensitive to the allosteric modulator ethanol. However, given knowledge of sex-
differences in GABA turnover, basal levels of GABA and GABA$\text{A}$ receptor sensitivity,
without information on sex differences in GABA$\text{A}$ receptor expression, it is difficult to
predict how GABAergic function varies between the sexes.

1.6. Acute stress and GABA$\text{A}$ receptors

1.6.1. Defining stress

The physiologist Walter Cannon (Cannon, 1929) borrowed the word “stress” from
engineering to refer to the physiological reaction that is a universal biological
phenomenon caused by the perception of aversive or threatening situations (Cannon,
1929). Hans Seyle (1956), a pioneer in stress research defined stress as the non-specific
response of the body to any demand whether it is caused by or results in pleasant or
unpleasant conditions. Seyle’s definition of stress is helpful as it does not require that
stress only be applied to negative circumstances. Thus, the term eustress is used to refer
to stress evoked by positive events and distress to refer to stress evoked by negative
events, allowing the concept of stress itself to remain neutral. For the purposes of this
thesis stress is defined in a manner consistent with the original and current definitions of
the term in medical research, as the integrated bodily response that is produced to deal
with extraordinary circumstances (Herman *et al.*, 2003).
A stressor is anything that provides a real or predicted threat to an organism. Stressors may be associated with positive or negative events of varying intensities and may include both physical (e.g. pain) and / or psychological (e.g. exposure to uncontrollable environment, learned fear) events. Each of these categories underlies different physiological and behavioural response patterns. For example, stressors that present a genuine homeostatic threat to the organism (changes in body temperature, haemorrhage or immunological challenges) activate different brain regions to purely psychological or anticipatory stressors (learned response to an impending adverse condition or a species specific fear) (Herman et al., 2003). Importantly, this distinction between physical and psychological stressors is not necessarily mutually exclusive with some stressors consisting of a physical stimulus with a psychological component such as pain, footshock, immobilisation and swim stress (Van de Kar and Blair, 1999).

1.6.2. Physiology of stress

The stress response encompasses neuronal and hormonal activity and results in physiological and behavioural changes organised to preserve homeostasis. The major systems involved in stress include the sympathetic-adrenal-medullary system (SAM) and the hypothalamic-pituitary adrenal (HPA) axis.

1.6.2.1. The hypothalamic-pituitary-adrenal axis

The HPA axis triggers the release of glucocorticoids, which provide tissue with the fuel for emergency situations by shutting down energy expensive systems (growth, reproduction, immune system), initiating glycogenolysis, proteolysis and lipolysis (Munck et al., 1984) and increasing blood pressure and cardiac output (Sambhi et al., 1965). HPA axis activity is initiated by the paraventricular nucleus (PVN) of the
hypothalamus, which releases oxytocin, vasopressin and the peptide corticotropin-releasing factor (CRF) within seconds of being exposed to a stressor (Swanson and Sawchenko 1983; Carrasco and Van de Kar, 2003; Brownstein et al., 1980). CRF is released directly into portal blood at the median eminence and travels in the hypothalamo-hypophyseal portal system to the anterior lobe of the pituitary where it induces proteolytic cleavage of pro-opiomelanocortin (POMC) products including adrenal corticotrophin releasing-hormone (ACTH) and β-endorphin (Seidah et al., 1999). ACTH is released into the systemic circulation from the anterior pituitary, and acts on the adrenal cortex to trigger the synthesis and secretion of glucocorticoids (Jacobson, 2005) in a species-specific fashion (e.g. cortisol in primates, swine and canines and corticosterone in rodents which lack 17α-hydroxylase) (Dallman et al., 1987). Glucocorticoids (long-feedback loop) and possibly ACTH (short-feedback loop) also exert negative feedback inhibition on the HPA axis (Aguilera, 1998).

1.6.2.2. The sympathetic-adrenal-medullary system

When exposed to real or perceived threats the sympathetic nervous system underlies the expression of the “fight-flight” response first described by Walter Cannon over 75 years ago (Cannon, 1929) which serves to enhance skeletal muscle function and provide readily available sources of energy. Activation of the sympathetic nervous system involves the activity of noradrenaline in central neuronal circuits and adrenaline as an endocrine messenger in the periphery. In the periphery, the medulla of the adrenal gland receives preganglionic sympathetic fibers from the spinal cord, which release ACh and cause the secretion of adrenaline and noradrenaline from chromaffin cells into the blood where adrenaline acts as a hormone (al’Absi and Arnett, 2000). In the brainstem the major noradrenergic projections from the locus coeruleus project to both
the spinal cord and the cortex and the release of noradrenaline to these central regions is thought to be involved in heightened attention and vigilance during stress or its anticipation (anxiety) (Redmond and Huang, 1979).

1.6.2.3. Neuroscience of stress responses

CRF neuronal responses to stress are triggered by either direct or indirect innervation that may arise from various forebrain and brainstem regions as shown in figure 1.4. The medial parvocellular PVN, which acts as a gatekeeper of the HPA response receives monosynaptic input from the nucleus of the solitary tract (NTS), the raphe nuclei, the parabrachial nucleus, the periaqueductal grey (PAG), the bed nucleus of the stria terminalis (BNST), the thalamus and the hypothalamus. Many of these regions such as the NTS, PAG, raphe and parabrachial nuclei of the brainstem receive information on primary sensory modalities including cardiovascular tone, respiration and pain, such that physical or reactive stressors can directly activate PVN neurons (Herman et al., 2003; Herman et al., 2005). These regions may also interact with each other or with higher brain structures such as the amygdala, prefrontal cortex (PFC) and hippocampus (Herman et al., 2003; Herman et al., 2005). The PVN itself may also provide direct input via axosomatic interactions of corticotropin releasing hormone (CRH) and non-CRH neurons, dendritic release of peptides and release of nitric oxide from magnocellular neurons of the PVN (Herman et al., 2003). The PVN also receives direct input from soluble factors in the blood (steroid hormones, aldosterone, cytokines) via a dense capillary plexus and possibly from CSF-borne substances (Herman et al., 2003).

 Whilst stressors that induce physical homeostatic challenges activate regions that synapse directly on CRF releasing cells in the PVN, psychological or emotional
stressors activate limbic and cortical areas that do not project directly to the PVN (Herman et al., 2003; Herman et al., 2005). Sensory cortices, which receive incoming information from the thalamus project to the perirhinal cortex, which can communicate with the lateral amygdala directly or via the hippocampus (Van de Kar and Blair, 1999; Herman et al., 2005). Input from forebrain regions is largely mediated via the BNST and the other nuclei of the hypothalamus such as the peri-PVN region, the dorsomedial, posterior, arcuate and lateral nuclei and the medial preoptic area (Herman et al., 2003; Herman et al., 2005). Forebrain projections come from limbic areas including the infralimbic / prelimbic neurons of the PFC, the ventral subiculum of the hippocampus, the central amygdala and lateral septum (Cullinan et al., 1993; Hurley et al., 1991; Canteras and Swanson, 1992; Prewitt and Herman, 1998; Canteras et al., 1995).

**Figure 1.4:** Diagrammatic representation of brain regions involved in stress responses. Adapted from Herman et al., 2005.
1.6.2.4. Sex differences and stress responses

A number of studies suggest males and females vary in their stress responses. The pattern of circadian rhythm in female rats is the reverse of that in males but resting levels of plasma corticosterone do not show sexual dimorphism (Hiroshige et al., 1973). Females produce more corticosterone (Akinci and Johnston, 1993; Kitay, 1961; Shors et al., 2001) and more CRF than males following stress (Hiroshige et al., 1973). This enhanced HPA axis stress reactivity in females appears to be at least partially mediated by gonadal hormones as female rats display HPA axis hyperactivity particularly during proestrus (Carey et al., 1995) and oestrogen-replaced ovariectomised females show enhanced stress-induced corticosterone release and CRF mRNA levels (Carey et al., 1995; Viau et al., 1999). In contrast, testosterone injections inhibit HPA responses to stress (Viau et al., 1999). Such sex differences may be affected by the stressor environment. For example, male rodents exhibit impaired stress responses following crowded housing rather than isolation (Brown and Grunberg, 1995), presumably due to increased aggression, but in females social instability in primates and isolation in rodents (Haller et al., 1998) appear to be stressful.

1.6.3. Effects of acute stress on GABA<sub>A</sub> receptors

1.6.3.1. Overview

Not surprisingly, studies of the effects of acute adulthood stress on GABA<sub>A</sub> receptors have focused on rapid changes in binding site expression, affinity and function rather than the delayed effects on protein and mRNA expression of subunits. Such radioligand binding studies (as reviewed below) suggest rapid alterations in the GABAergic system occur in response to stress. These rapid alterations are of particular interest as they provide an example of fast neurotransmitter system plasticity in
response to experience that may be mediated by alterations in the expression of endogenous GABAergic ligands and / or rapid trafficking of GABA_\text{A} receptors.

1.6.3.2. Orthosteric binding sites

Studies measuring $[^3]\text{H}GABA$ binding suggest that the availability of low-affinity binding sites ($B_{\text{MAX}}$) is rapidly affected following stress in a sex and paradigm specific manner (see table 1.4), whilst the affinity ($K_D$) is not affected. Studies in males suggest that different stressors produce different effects, with acute swim stress producing no changes (Skerritt et al. 1981; Motohashi et al., 1993) while footshock stress (Biggio et al., 1981; Concas et al., 1985; Corda et al., 1985; Cuadra and Molina, 1993) and stress from guillotine in handling-naïve rats (Biggio et al., 1981; Biggio et al., 1984; Biggio et al., 1987; Concas et al., 1985) reduced forebrain low affinity $[^3]\text{H}GABA$ binding. Apparent differences between different stressors may also arise from different laboratory stress protocols given that the presence of conspecifics during stress (Cuadra and Molina, 1993) and habituation of animals to experimenter handling (Biggio et al., 1981; Concas et al., 1985; Corda et al., 1985; Cuadra and Molina, 1993) have been shown to affect GABA_\text{A} receptor binding even in the same stress paradigm.

There appear to be sex differences in the effects of stress on GABA_\text{A} receptors. Studies have shown rapid increases in female but no change in male low-affinity ($B_{\text{MAX}}$) $[^3]\text{H}GABA$ binding sites following acute swim stress (Akinci and Johnston, 1997; Akinci and Johnston, 1993; Skerritt et al., 1981). Interestingly, comparisons of unwashed and well washed crude membrane preparations used for $[^3]\text{H}GABA$ binding show that female mice appear to contain higher concentrations of endogenous inhibitors of $[^3]\text{H}GABA$ binding compared with male mice (Akinci and Johnston, 1993). Thus, in general stress appears to induce an increase in functional binding sites in females and
various changes in males, apparently dependent on the stress-paradigm used. However it is unknown if any of these effects are regionally specific.

Binding of channel blocking agents is also affected by acute swim stress, suggesting alterations in functional GABA binding sites consistent with altered low-affinity GABA binding (Havoundjan et al., 1986). $[^{35}\text{S}]$TBPS binds within the channel domain of the GABA_A receptor. Reduced binding of $[^{35}\text{S}]$TBPS is observed in the presence of orthosteric and allosteric agonists and enhanced binding is observed in the presence of orthosteric and allosteric site antagonists (Concas et al., 1987; Concas et al., 1988b). The authors of many studies examining $[^{35}\text{S}]$TBPS binding speculate that changes in binding of this radioligand reflect changes in the availability of GABA_A receptor binding-sites, and receptors that are bound by $[^{35}\text{S}]$TBPS are thought to be in an antagonist-preferring conformation with reduced ability to conduct chloride ions (Concas et al., 1986; Concas et al., 1987; Havoundjan et al., 1986). Thus, the consistently observed increase in the number and affinity of $[^{35}\text{S}]$TBPS sites in the brain following various stressors such as footshock (Concas et al., 1987; Concas et al., 1988a; Concas et al., 1993), exposure to carbon dioxide gas (Concas et al., 1993), restraint stress (McIntyre et al., 1988), swim stress (Havoundjian et al., 1986) and learned helplessness (Drugan et al., 1994) is thought to represent an increase in non-functional receptors and correlates with the reduced binding at the low-affinity orthosteric site observed by the same groups in separate studies. However studies by other groups directly examining the function of the ion channel through measurement of chloride uptake into rat brain synaptosomes contradict these findings as they have found either no change (Drugan et al., 1989) or increased (Schwartz et al., 1987) muscimol-stimulated chloride uptake following footshock and swim stress respectively.
1.6.3.3. Allosteric binding sites

As was the case for agents binding to the orthosteric binding site, the effects of stress on benzodiazepine binding in rodents vary depending on the stress paradigm (see table 1.5) and are typically of smaller magnitude than changes observed in the orthosteric site (Braestrup et al., 1979). For example, whilst male mice show no changes in benzodiazepine site binding following swim stress and isolation (Braestrup et al., 1979; Park et al., 1993; Skerritt et al., 1981), footshock and social immobilisation resulted in increased and decreased benzodiazepine binding respectively in forebrain cortical regions (Braestrup et al., 1979). Regional information is available for stress-induced changes in benzodiazepine binding but is largely inconsistent as some studies show increased binding at the benzodiazepine site compared with controls in the cortex (Motohashi et al., 1993; Rago et al., 1989; Soubrie et al., 1980) but not the hippocampus or cerebellum (Motohashi et al., 1993) following swim stress, whereas others have consistently found decreases in $[^3H]flunitrazepam$ and $[^3H]\beta CCE$ binding in the cortex of male rats following swim stress (Medina et al., 1983a; Medina et al.,
Differences in stress-induced changes in benzodiazepine binding also appear to depend on the radioligand examined as no change was observed in the binding of benzodiazepine agonists in males subject to social defeat (Miller et al., 1987) and swim stress (Park et al., 1993), but changes were observed in binding of a benzodiazepine-site antagonist ([³H]Ro 15-1788) in the same mice. Thus, changes in both the number of sites and the preferred conformation of the benzodiazepine site (Miller et al., 1997; Park et al., 1993) may result from stress but the effects are not as large or consistent as those seen for the orthosteric site.

Studies in chicks subjected to swim stress, have found more consistent increases in forebrain benzodiazepine sites (Benavidez and Arce, 2002; Martijena et al., 1992; Salvatierra et al., 1994). Interestingly, these increases in benzodiazepine binding appear to be explained by a rapid recruitment of the benzodiazepine receptor from a pool that is unmasked using triton-X solubilisation in controls (Benavidez and Arce, 2002). Furthermore, disruption of microtubules and phosphorylation prevents stress-induced increases in the benzodiazepine-site (Martijena et al., 1992) suggesting a role for receptor trafficking in the rapid alterations of GABA₆ receptors following acute stress.

In contrast to the orthosteric site, sex differences in benzodiazepine-site binding following stress have not been observed. In mice, only one study looked at females and found no change in [³H]diazepam binding in the forebrain following warm water swim stress in males or females. This study also suggests that the large changes observed in binding at the orthosteric site in stressed females are not accompanied by changes in allosteric site binding (Skerritt et al., 1981) suggesting stress has greater effects on non γ₂-containing GABA₆ receptor subtypes.
### Table 1.5: Stress-induced changes in GABA$_A$ receptor benzodiazepine site binding maximum

<table>
<thead>
<tr>
<th>Stress</th>
<th>Animal</th>
<th>Radioligand</th>
<th>Change</th>
<th>Region</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swim (25°C, 15 min)</td>
<td>Male rats</td>
<td>[³H]Flunitrazepam</td>
<td>Increase</td>
<td>No change</td>
<td>Cortex</td>
</tr>
<tr>
<td>Swim (6°C, 3 min)</td>
<td>Male rats</td>
<td>[³H]Flunitrazepam</td>
<td>Increase</td>
<td>No change</td>
<td>Cortex</td>
</tr>
<tr>
<td>Swim (18°C, 15 min)</td>
<td>Male rats</td>
<td>[³H]Flunitrazepam</td>
<td>Increase</td>
<td>No change</td>
<td>Cortex</td>
</tr>
<tr>
<td>Swim (18°C, 15 min)</td>
<td>Rats</td>
<td>[³H]Flunitrazepam [³H]jCCE</td>
<td>Decrease</td>
<td>No change</td>
<td>Cortex</td>
</tr>
<tr>
<td>Swim (6°C, 10 min)</td>
<td>Male mice</td>
<td>[³H]Flunitrazepam</td>
<td>No change</td>
<td>No change</td>
<td>Cortex</td>
</tr>
<tr>
<td>Swim (32°C, 3 min)</td>
<td>Male mice</td>
<td>[³H]Flunitrazepam [³H]jRo 15-1788</td>
<td>No change</td>
<td>No change</td>
<td>Cortex</td>
</tr>
<tr>
<td>Swim (25°C) Footshock</td>
<td>Male mice Female mice</td>
<td>[³H]Diazepam</td>
<td>No change</td>
<td>No change</td>
<td>Forebrain</td>
</tr>
<tr>
<td>Social Defeat</td>
<td>Male mice</td>
<td>[³H]Flunitrazepam</td>
<td>No change</td>
<td>No change</td>
<td>Cortex</td>
</tr>
<tr>
<td>Conflict Footshock</td>
<td>Male rats</td>
<td>[³H]Diazepam</td>
<td>Decrease</td>
<td>No change</td>
<td>Cortex</td>
</tr>
<tr>
<td>Swim (38°C, 15 min)</td>
<td>Male Chicks Female Chicks</td>
<td>[³H]Flunitrazepam</td>
<td>Increase</td>
<td>No change</td>
<td>Cortex</td>
</tr>
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<td>Learned helplessness</td>
<td>Male rats</td>
<td>[³H]Ro15-1788</td>
<td>Decrease</td>
<td>Decrease</td>
<td>Cortex</td>
</tr>
<tr>
<td>Footshock</td>
<td>Male rats</td>
<td>[³H]Ro15-1788</td>
<td>No Change</td>
<td>Cortex</td>
<td>Hippocampus</td>
</tr>
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</table>
1.6.4. Endogenous mediators of GABA_\textsubscript{A} receptors and stress

Steroids that influence receptors in the brain via non-genomic mechanisms are termed neuroactive steroids. Potent positive modulation (nM) of GABAergic currents is observed with numerous steroids including the anaesthetic alphaxalone (Harrison and Simmonds, 1984) and endogenous metabolites of progesterone (allopregnanolone, pregnenolone) and deoxycorticosterone (allotetrahydrodeoxycorticosterone; THDOC) (Barker et al., 1987; Majewska et al., 1986) and at higher concentrations, these endogenous steroids act as direct agonists on the GABA_\textsubscript{A} receptor (Cottrell et al., 1987). Cortisol acts as a bi-directional modulator of GABA function with enhancement at low concentrations (pM) and inhibition at higher concentrations (nM) and cortisone inhibits GABA function at low concentrations (pM) in guinea pig ileum preparations (Ong et al., 1987; Ong et al., 1990). In contrast, sulphated steroids such as pregnenolone sulphate and dehydroepiandrosterone sulphate (DHEAS) are low potency (\mu M) negative modulators of GABA_\textsubscript{A} receptors (Majewska et al., 1990; Majewska and Schwartz, 1987). As mentioned above, steroid action is affected by phosphorylation state and subunit composition, with the \( \delta \) subunit appearing necessary for steroid enhancement (Belelli et al., 2002; Belelli and Lambert, 2005; Mihalek et al., 1999).

Endogenous steroids are synthesised from cholesterol by enzymes in the adrenals (e.g. THDOC) and enzymes in the brain. Steroids synthesised in the brain are termed neurosteroids (e.g. allopregnanolone) (Robel et al., 1999). Following stress neurosteroids are rapidly elevated in the brain but not in plasma of adrenalectomised rats (Purdy et al., 1991). In intact animals, increases in brain and plasma concentrations of neurosteroids have been observed following swim stress (Mele et al., 2004; Purdy et al., 1991), exposure to footshock and carbon dioxide inhalation (Barbaccia et al., 1996a; Barbaccia et al., 2001). Progesterone and deoxycorticosterone show maximal
increases in rat cortex 10 minutes after stress with return to basal values by 30 and 60 minutes respectively. In contrast, pregnanolone and allopregnanolone concentrations are maximally increased 30 minutes after stress and return to baseline 120 minutes later (Barbaccia et al., 1996a; Barbaccia et al., 1996b). Whilst rapid stress-induced increases in steroids that alter GABA_\text{A} receptor function may contribute to observations of rapid changes in GABA_\text{A} receptor binding following stress, they are not sufficient to explain them, as altered [\textsuperscript{3}H]GABA binding occurs in the absence of endogenous mediators (Akinci and Johnston, 1993). Thus the effects of neurosteroids may be mediated by their effects on receptor trafficking, which in turn may occur via effects on receptor phosphorylation state.

1.6.5. GABA_\text{A} receptors and behavioural changes following acute stress

Consistent with stress-induced changes in GABA_\text{A} receptors, acute stress alters behavioural sensitivities to GABA_\text{A} receptor ligands. In males forced swim stress has been observed to remove anxiolytic effects of diazepam on the dark-light exploratory behaviour test (Briones-Aranda et al., 2005), reduce the anti-seizure efficacy of benzodiazepines (Deutsch et al., 1990) and reduce the seizure-threshold for bicuculline and picrotoxin (Abel and Berman, 1993; Drugan et al., 1985; Pericic et al., 2001; Soubrie et al., 1980) suggesting impaired sensitivity of GABA_\text{A} receptors, consistent with a loss of functional GABA binding sites in stressed males. Consistent with sex differences in the effects of stress on GABA_\text{A} receptors, stress has been observed to eliminate sex differences in behavioural responses to diazepam and ethanol (Wilson et al., 2004). Interestingly, stress-induced reductions in the convulsive activity of GABA_\text{A} receptor antagonists is blocked by finasteride inhibition of THDOC synthesis.
(Barbaccia et al., 1998) implicating neurosteroids in the effects of stress on GABA\textsubscript{A} receptors.

1.6.6. Summary

Stress, defined as an integrated bodily response that is produced to deal with extraordinary circumstances, involves recruitment of the hypothalamic pituitary adrenal axis and the sympathetic-adrenal medullary system and results in both rapid (mins) and more delayed effects on target tissues. The PVN of the hypothalamus acts as a ‘gatekeeper’ of such stress systems and itself is activated by various, limbic and brainstem structures. Acute stress induces rapid changes in binding at the GABA\textsubscript{A} receptor, particularly the orthosteric site, with the direction of the changes varying according to sex and stress paradigm but likely resulting in altered behavioural sensitivity to GABAergic ligands. These rapid alterations are of particular interest as they provide an example of fast neurotransmitter system plasticity that may be mediated by stress-induced increases in neurosteroids, perhaps via effects on phosphorylation and / or receptor trafficking.
1.7. Early-life stress and GABA$_A$ receptors

1.7.1. Early-life environment: Impact in adulthood?

Clinical and epidemiological studies are increasingly showing a relationship between the early post-natal environment and long-term neurobiological and psychological development. Indeed early loss of a parent, parental neglect or abuse or being cared for by a parent with psychiatric concerns results in increased vulnerability to a number of medical concerns in adulthood (Heim et al., 2001; Heim et al., 2000a; Heim et al., 2000b; Heim and Nemeroff, 2001) irrespective of genetic predisposition. Whilst genetics is of great importance in developing psychiatric illnesses, in humans early postnatal environmental factors can increase the risk of developing psychiatric disorders, cardiovascular disorders, adult obesity and diabetes (Canetti et al., 1997; Felitti et al., 1998; Lissau and Sorensen, 1994; McCauley et al., 1997; Russak and Schwartz, 1997). Thus, an understanding of the long-term changes in physiology, behaviour and stress reactivity incurred following post-natal environmental disturbances is highly relevant to a number of human diseases.

1.7.2. Models of early-life environmental manipulations in rodents

Models of interrupted early-life environment have been examined for over 50 years (see Levine, 1957) leading to the development of a number of animal models to examine the effects of early-life stress on adulthood physiology and behaviour (see table 1.6). Table 1.6 outlines the number of models in use and the nomenclature proposed by Pryce (Pryce and Feldon, 2003) in an attempt to provide a universal framework amongst researchers.
Table 1.6: Early-life environmental manipulation protocols in rodents (Adapted from Pryce and Feldon, 2003)

<table>
<thead>
<tr>
<th>Classification</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early-life handling (EH)</td>
<td>Experimenter removes pups from home cage, mother and siblings for several minutes daily over early post-natal life</td>
</tr>
<tr>
<td>Non-Handled (NH)</td>
<td>No handling, cage cleaning e.t.c from experimenters or animal house staff</td>
</tr>
<tr>
<td>Maternal Separation (MS)</td>
<td>Separation of litter from dam for at least 1 hour per day over several postnatal days</td>
</tr>
<tr>
<td>Single MS</td>
<td>Separation of litter from mother for 1-24 hour period</td>
</tr>
<tr>
<td>Early-life deprivation (ED)</td>
<td>Separation of pups from mother and litter for more than 1 hour over several post-natal days (more than normal bouts of mother leaving the nest)</td>
</tr>
<tr>
<td>Animal Facility Rearing (AFR)</td>
<td>Varies but involves normal cage-cleaning</td>
</tr>
</tbody>
</table>

The most commonly used experimental designs providing the most robust adulthood differences are comparisons of EH and NH groups. The EH group is better identified as the ‘control’ condition despite the natural assumption that the ‘no-intervention’ condition, that is the NH condition, would represent the baseline. The EH group represents a standardised ‘normal’ rearing condition for laboratory rodents not achieved in the AFR group due to variations amongst breeding facilities (Pryce et al., 2002). EH laboratory rodents receive minimal amounts of stress and human stimulation not provided in the NH group (Pryce and Feldon, 2003), and this situation is thought to best represent that in the wild, where the mother leaves the nest and pups briefly every day to forage (Calhoun, 1963). EH procedures result in enhanced maternal attention to the offspring in the form of licking, grooming and arched-back nursing, behaviours, which are not observed as readily in the NH group, perhaps due to the stress of prolonged confinement of the mother with the pups (Anisman et al., 2001; Cadji et al., 1998; Francis et al., 1999; Hennessy et al., 1982; Lee and Williams, 1975; Liu et al., 1997; Smotherman and Bell, 1980). However, whether the robust and long-lasting differences between NH and EH groups arise from enhanced maternal care, altered behaviour amongst siblings, changes in body temperature or brief periods of human stimulation, remains uncertain (Pryce and Feldon, 2003; Denenberg, 1999).
What is clear is that in adulthood the NH group performs in a less ‘adaptive’ fashion than the EH group.

There are clear differences in terms of behavioural responses to novelty, learning acquisition and HPA axis stress reactivity between EH and NH groups (see below). Interestingly, the other paradigms of early-life manipulation presented in table 1.6 are largely similar on such measures to either the EH or NH groups. For example, the majority of studies the AFR group do not vary from the EH group in HPA axis responses or behaviours in adulthood (Ladd et al., 2000; Pryce et al., 2001; Parfitt et al., 2007; Millstein and Holmes, 2007). ED groups are different from NH groups, but surprisingly, resemble EH and AFR groups in stress-induced corticosterone responses, behavioural responses to novelty (Pryce et al, 2001) and a variety of learning paradigms (Lehmann and Feldon, 2000; Pryce et al., 2003). The MS group appears to be different to the AFR and EH groups (Huot et al., 2001; Ladd et al., 2000) but largely similar to the NH group in behaviour (Caldji et al., 2000b; Moffett et al., 2006; Parfitt et al., 2004) and stress reactivity (Liu et al., 2000; Plotsky et al., 2005; Plotsky et al., 1993).

However, there are a number of discrepancies in the literature regarding the behavioural outcome of MS in early-life, perhaps given the variety of separation periods that are used and the variability between studies in the post-natal days on which such separations are performed. Furthermore, a recent study has shown that features such as the ambient temperature and the light phase during which the MS procedure is carried out affects the behavioural outcome in these rodents (Ruedi-Bettschen et al., 2005). Longer periods of maternal separation (i.e. at least 6 hours given that mothers may leave the nest for up to 3 hours) appear to be required to differentiate MS and NH groups (Huot et al., 2001) and 24 hour MS does appear to produce different patterns of behaviour and stress-reactivity to NH mice (De Kloet et al., 1998; Macri and Laviola,
2004; MacQueen et al., 2003; Venerosi et al., 2003). Nonetheless, EH and NH have been studied the most extensively in the literature and provide distinct adulthood behavioural and stress reactive phenotypes with the other experimental groups not greatly adding to our understanding of how early-life environment impacts on development.

1.7.3. Effects of early-life environment on behaviour

A number of differences in adulthood behaviour and physiological stress reactivity are observed between EH and NH groups (reviewed in Chappillon et al., 2002; Meaney, 2001; Pryce and Feldon, 2003; Pryce et al., 2002). The NH group has a well defined behavioural phenotype in that NH animals are more anxious and behaviourally reactive than their EH counterparts. NH rats (McIntosh et al., 1999; Meerlo et al., 1999; Nunez et al., 1995; Vallee et al., 1997; Ploj et al., 1999) and mice (Cabib et al., 1993; D’Amato et al., 1998; Pryce et al., 2001) of both sexes show increased anxiety-type behaviour on the elevated plus maze (EPM) relative to EH rodents. Similarly, studies using the open field test to measure locomotor exploration and fear of novel open spaces, have shown that NH and MS rodents have reduced exploration, more defecation and spend less time in the central squares compared with EH groups (Caldji et al., 2000b; Meerlo et al., 1999; Vallee et al., 1997; Weizman et al., 1999; Ader and Grota, 1969; Denenberg, 1964; Levine, 1957; Pihoker et al., 1993; Plotsky and Meaney, 1993; Pryce et al., 2001, Pryce et al., 2003). Behavioural reactivity is also observed in the NH group in that they show increased acoustic startle responses (Caldji et al., 2000b; Pryce et al., 2001, Pryce et al., 2003) and increased behavioural inhibition in response to a predator (Padoin et al., 2001).
Of relevance to diseases such as depression, studies also indicate a reduction in reward seeking behaviours occurs in adult rats exposed to an early-life NH protocol compared to an EH protocol. For example, NH male and female rats consume less of a palatable reward snack over 10 days (Graham wafer), than their EH and female MS counterparts (McIntosh et al., 1999). NH rats (Bodnoff et al., 1987; Fernandez-Teruel et al., 1991; Levine, 1962, Levine, 1967, Levine, 1957; Meerlo et al., 1999; Denenberg 1964, Caldji et al., 2000b) and mice (Ferre et al., 1995) also show enhanced novelty induced suppression of appetitive behaviours such as feeding in a novel area (neophagia test) relative to EH, perhaps indicative of both neophobia as well as reduced motivation to seek reward.

Studies of adulthood learning acquisition are enlightening in supporting the EH group as the ‘normal’ situation and thus the control condition in the EH-NH comparison. NH adult males show impaired learning in two-way active avoidance (Escorihuela et al., 1992; Pryce et al., 2003), passive avoidance (Nunez et al., 1996) and latent inhibition tasks (Weiner et al., 1985), which measure the ability to ignore irrelevant information by ‘unlearning’ an association between a neutral and noxious stimulus when the temporal association no longer exists. Such abnormalities in learning may result from either the apparent increase in fearfulness observed in response to innately noxious stimuli e.g. open field, acoustic startle or from impairments in making associations between stimuli, or even a combination of the two. Nonetheless, as stated by Pryce and Feldon (2003), such impairments in adaptive and ubiquitous behavioural phenomena such as latent inhibition suggest that the NH group represent a behaviourally abnormal adulthood phenotype relative to the EH group. Interestingly, latent inhibition (e.g. pre-pulse inhibition test) is also disrupted in psychiatric disorders such as schizophrenia (Pryce and Feldon, 2003).
Thus, the NH condition, when compared with the EH condition, appears to produce a more anxious, behaviourally reactive, neophobic and less reward motivated adulthood phenotype which is of relevance to psychiatric diseases where anhedonia, anxiety, behavioural reactivity and fear are prominent.

1.7.4. Effects of early-life environment on stress reactivity

Given that adulthood stress has been related to the symptom onset in a number of affective disorders (major depression) and psychotic disorders (schizophrenia), it is highly relevant to investigate experimental paradigms that produce lasting changes on stress reactivity, and early-life interventions provide an example of this. There are no differences between EH and NH animals in basal diurnal corticosterone (Levine, 1957; Levine, 1962; Meaney et al., 1985; Meaney et al., 1989), ACTH (Meaney et al., 1989; Meaney et al., 1991) nor sensitivity to these hormones (Meaney et al., 1989). However, EH and NH males do differ in adulthood HPA axis responses to a variety of stressors including restraint (Meaney et al., 1989; Plotsky and Meaney, 1993) exposure to an open field (Levine, 1967), air puff, startle and electric shock (Meaney et al., 1996). EH have less CRF released into the hypophyseal system (Plotsky et al., 1993), and lower peak plasma ACTH (Meaney et al., 1989) and corticosterone (Meaney et al., 1989; Pryce et al., 2001; Zaharia et al., 1996) levels, with faster returns of each hormone to baseline following stress (Levine, 1962; Meaney et al., 1989). Baseline CRF mRNA and immunoreactivity in the hypothalamus (Plotsky and Meaney, 1993) particularly the PVN (Plotsky et al., 2005) is reduced and glucocorticoid (GR) receptor expression and sensitivity is increased in the hippocampus of EH rats suggesting that HPA axis differences arise from differences in negative feedback capabilities (Meaney et al., 1996; Meaney et al., 1989; Meaney et al., 1985; O’Donnell et al., 1994). However,
altered neuronal circuitry, particularly enhanced GABAergic inhibition in the amygdala, locus coeruleus and NTS of EH vs. NH animals may also play a role (Caldji et al., 2000b; Caldji et al., 1998).

In contrast to the reduced stress-induced HPA activity observed in EH males, little is known about the effects of EH on stress responsivity in females. EH and NH females do not differ in their plasma corticosterone levels during stress (Ader, 1975). However, EH females, like EH males have a faster return to baseline levels of corticosterone following stress and increased glucocorticoid binding sites in the hippocampus (Meaney et al., 1985; Meaney et al., 1991) suggesting that early-life intervention produces long-term changes in the HPA axis and its ability to respond to stress. Further study is required to better understand sex differences in effects of early-life manipulations on HPA stress responses.

1.7.5. Early-life stress and GABA$_A$ receptors

Several lines of evidence have suggested long-lasting changes in GABA$_A$ receptors arise in animal models of early-life stress. For example, adult rats exposed to early-life stress (NH condition) display decreased numbers of high affinity GABA binding sites in the mPFC, NTS and locus coeruleus (LC) (Caldji et al., 2000b) as well as decreased numbers of forebrain and amygdala benzodiazepine sites compared with EH controls (Bodnoff et al., 1987; Bolden et al., 1990). Consistent with early-life stress inducing long-term decreases in benzodiazepine receptors, are observations of decreased $\gamma_2$ subunit expression in the amygdala, NTS and LC in NH and MS groups relative to EH controls (Caldji et al., 2000b). Thus it appears that early-life stress results in long-term decreases in benzodiazepine receptors and their requisite $\gamma_2$ subunit
mRNA, with the amygdala having been identified as a forebrain region relevant to such changes.

Early-life stress also appears to influence the $\alpha_{1/2}$ subunit ‘switch’ that occurs in rodents during the early post-natal period suggesting environmental manipulations may affect GABAergic system development. Hippocampal dentate gyrus cells from adult rats given two handling separations (30 minutes / 6 hours) (MS) before P10 were less sensitive to zolpidem enhancement of GABAergic currents and showed longer current decay times relative to AFR controls (Hsu et al., 2003) indicative of a reduced $\alpha_1$ subunit contribution. These findings were confirmed by observations of decreased $\alpha_1$ and increased $\alpha_2$ subunit mRNA without evidence of cell loss in the dentate gyrus of the MS group (Hsu et al., 2003). Consistent with these findings for MS animals, following the NH early-life stress condition, a reduction in binding sites for the $\alpha_{1}$-subunit selective compound $[^3]$H]zolpidem was observed in the amygdala (Caldji et al., 2000b). Thus, whilst examination of other brain regions is required to confirm this hypothesis, there is evidence that the developmental subunit ‘switch’ may be disrupted by early-life stress resulting in an alteration of the GABA$_A$ receptor phenotype that prevails into adulthood (Hsu et al., 2003).

1.7.6. Summary

Early-life intervention models in rodents produce changes in adulthood stress reactivity and behaviour. The best examined models showing the most robust differences are the EH and NH protocols, where the NH protocol appears to produce the more anxious and stress-reactive phenotype that is reflective of psychiatric disorders such as schizophrenia. Few studies have examined the effects of early-life intervention
protocols on GABA\(_A\) receptors however from those that have it is apparent there are long-lasting changes in \([^3]H\)flunitrazepam binding and alterations in GABA\(_A\) receptor subunit expression. Alterations in \(\alpha_1\) and \(\alpha_2\) subunit expression are consistent with disruptions in the development of the GABAergic system, in that there appears to be an impairment in the \(\alpha_1/\alpha_2\) subunit developmental ‘switch’ in certain brain regions of animals that display abnormal behavioural and stress-reactive phenotypes in adulthood. Evidence of disruptions in brain development leading to alterations in adulthood behaviour and HPA-axis stress-reactivity are highly relevant to neurodevelopmental psychiatric disorders such as schizophrenia. However, it remains to be investigated whether other brain regions may be implicated. Furthermore, given the diathesis-stress models of psychiatric disorders such as schizophrenia and depression in which adulthood stress is hypothesised to precipitate the expression of disease symptoms in individuals with impairments in brain development, it will be interesting to ascertain if adulthood stress reactivity in the GABAergic system is affected following early-life stress.
1.8. Thesis aims

The aims of this thesis were to define the brain regions in which adulthood and early-life stress affect GABA$_A$ receptor binding site availability and the $\alpha$ protein subunits associated with early brain development and adulthood behaviour. Following on from this, as early-life stress affects adulthood behavioural and neuroendocrine stress responses, this thesis also aims to examine if early-life stress affects the adulthood stress responses of GABA$_A$ receptors. This research is relevant to our understanding of the neurophysiology of stress and the role of the environment in contributing to GABA$_A$ receptor pathologies observed in psychiatric illnesses such as schizophrenia, anxiety disorders and depression.
PART B:

$\text{GABA}_A$ RECEPTORS AND ACUTE ADULTHOOD STRESS
CHAPTER 2:
Effects of Adulthood Stress on GABA<sub>A</sub> Receptor Binding Sites by Region

2.1. Introduction

2.1.1. Background

An understanding of the differences between males and females and their responses to stress is of importance given that a number of clinical conditions, from cardiovascular disease and diabetes through to psychiatric conditions such as anorexia, schizophrenia and depression, have both stress and sex as predisposing factors.

Previous studies have shown that acute stress in adulthood induces rapid changes in GABA<sub>A</sub> receptor binding sites in a sex dependent fashion. Radioligand binding studies measuring high and low-affinity sites for $[^3]$H]GABA show a rapid increase in the availability of low-affinity binding sites ($B_{\text{MAX}}$), and a smaller decrease in the number of high-affinity binding sites, following acute swim stress in females (Akinci and Johnston, 1993; Akinci and Johnston, 1997; Skerritt et al., 1981; Wilson and Biscardi, 1994). In contrast, male rats and mice exposed to swim stress show no changes in either the high or low-affinity GABA binding sites (Motohashi et al., 1993; Skerritt et al. 1981), and in other stress paradigms, show large reductions in forebrain low-affinity $[^3]$H]GABA binding sites (Biggio et al., 1981; Concas et al., 1985; Cuadra and Molina, 1993). As electrophysiological studies indicate that micromolar concentrations of GABA are required for channel conductance, stress-induced alterations in low-affinity (1µM) $[^3]$H]GABA binding are indicative of alterations in GABA<sub>A</sub> receptor function (Baur and Siegel, 2003; Harris and Allan, 1985; Maconochie et al., 1994). Thus, stress rapidly alters the availability of functional (low-affinity) GABA<sub>A</sub> receptor sites in a sex-dependent fashion, with females showing an increase
and males showing a decrease or no change in functional GABA binding sites. However, as these previous studies have only examined brain homogenate preparations, it is unknown whether such rapid alterations in the availability of GABA binding sites are specific to certain brain regions activated during stress, or are a more generalised stress response affecting all GABA_A receptors in the brain.

Sex differences apparent in control animals are reduced following stress. Stress has been shown to reduce sex differences observed in unstressed mice in the number of low-affinity GABA binding sites (Akinci and Johnston, 1993; Skerritt et al., 1981; Wilson and Biscardi, 1994) and the sensitivity to GABA_A receptor modulators (Wilson et al., 2004). However, baseline sex differences in the GABAergic system are not found in all studies. For example, whilst males are often found to be more sensitive to compounds that act on GABA_A receptors (Bujas et al., 1997; Crippens et al., 1999; Fernandez-Gausti and Picazo, 1997; Fernandez-Gausti and Picazo, 1999; Guiliet and Dunham, 1995; Gulinello and Smith, 2003; Kokka et al., 1992; Manev et al., 1987; Pericic and Bujas, 1997; Pericic et al., 1999; Tayyabkhan et al., 2002; Webb et al., 2002; Wilson 1992; Wilson et al., 2004), findings vary according to species (Manev et al., 1987; Pericic and Bujas, 1997), route of drug administration (Pericic et al., 1986), drug dose (Wilson et al., 2004) and the behavioural parameter measured. Sex differences in GABA_A receptor binding sites are also variable between studies with some studies suggesting no sex differences in low-affinity binding sites (Bujas et al., 1997; Wilson, 1992; Wilson and Biscardi, 1992), in contrast to the reports mentioned above, where unstressed males had a greater number of low-affinity sites than unstressed females (Akinci and Johnston, 1993; Skerritt et al., 1981; Wilson and Biscardi, 1994). Interestingly, for high-affinity GABA binding sites it has been
observed that sex differences in GABA binding are regionally dependent (Juptner and Hiemke, 1990; Kokka et al., 1992). Thus, regional information on sex differences in low-affinity GABA binding is likely to provide a greater understanding of GABA$_A$ receptor sex differences.

### 2.1.2. Overview of the quantitative receptor autoradiography technique

Quantitative receptor autoradiography is a method of determining both the quantity and anatomical location of receptor binding sites. The procedure involves exposure of tissue sections to a radiolabelled compound that binds selectively to a site on a protein of interest. Tissue containing the bound radioligand is then exposed to a silver halide photographic emulsion (Keen & MacDermot, 1993). Energy emitted from the radioactive specimen disrupts the silver halide lattice of the emulsion producing deposits of silver (Keen & MacDermot, 1993). The deposits of silver produce an image of the radio-labelled binding sites in the tissue allowing for quantification of binding sites by region.

Radioligand binding at a receptor is theoretically described by the law of mass action for the association of a diffusible ligand [L] and receptor [R] to form a complex [LR] of ligand bound to the receptor:

$$[L] + [R] \xrightleftharpoons{\kappa_+}{\kappa_-} [LR]$$

where k$_+$ and k$_-$ are the rates for the forward and reverse reactions respectively (Keen & MacDermot, 1993). Forward (association) and reverse (dissociation) rates of the reaction are dependent on a number of factors including temperature, pH and drug
concentration (Enna and Snyder, 1977). When equilibrium is reached under constant conditions, the association and dissociation rates are stable. Thus the equilibrium dissociation constant $K_D = k_{-1}/k_1$ is a measure of the affinity of the ligand for the receptor. $B_{\text{MAX}}$ provides a measure of the number of available binding sites for a ligand and is equal to the asymptote for the hyperbolic relationship between radioligand concentration exposed to the tissue and radioactivity remaining in the tissue when unbound radioligand is removed. Radioligand binding assays performed in this thesis are based on the law of mass action at equilibrium and use concentrations of GABA known to produce $B_{\text{MAX}}$ for the high- and low-affinity binding sites.

### 2.1.3. Aims

The aim of this study was to determine the brain regions where baseline sex differences and stress-induced alterations in the number of GABA binding sites occur. To determine these brain regions, the density of both high and low-affinity $[^3\text{H}]$GABA binding sites was measured using quantitative receptor autoradiography in several brain regions of male and female mice that were exposed to no stress or a 3 minute swim stress immediately prior to brain removal. As it has been suggested that the presence of conspecifics may influence stress responses differently between sexes (Cuadra and Molina, 1993; Taylor et al., 2000; Troisi, 2001), mice were swum either individually or with cage-mates in order to examine the potential influence of the social environment of the stressor. This study was performed to better clarify the literature regarding sex differences in GABA$_\Lambda$ receptor binding, and to provide a better understanding of the mechanism of rapid GABA$_\Lambda$ receptor alterations in response to stress.
2.2. Methods: Acute stress in adult mice

2.2.1. Subjects

Eighteen female and eighteen male Quackenbush Swiss (QS) albino mice aged 8 weeks (Laboratory Animal Services, Perth, WA) were housed in groups of three upon arrival at the animal house. All mice were housed under a 12hr/12hr light/dark cycle with constant temperature (21°C) and permitted food and water *ad libitum*. Minimising animal stress in housing and immediately prior to the procedure was considered crucial to distinguishing between control and stressed groups, thus animals were allowed to habituate to the environment of the animal house for 1 week and were then handled by experimenters for an additional 2 weeks prior to acute stress protocols. The experiment protocols were approved by the Animal Ethics Committee of the University of Sydney.

2.2.2. Subject allocation

On the day of experimentation, cages of mice (n=3) were randomly assigned to either individual or group conditions. Within the cages assigned to individual conditions, mice were randomly assigned to either control or acute stress conditions. As a result there were 6 experimental groups with n=6 subjects per group; male control, male individual stress, male group stress, female control, female individual stress, female group stress. The 12 cages were processed on 2 consecutive days with 6 cages and n=3 per group, tested in a random order, on each day.

2.2.3. Acute swim stress

This protocol has been designed to produce a mild, painless stress that is effective in producing rapid release of adrenal hormones and non-opioid mediated analgesia
(Akinci and Johnston, 1993; Skerritt et al., 1981), which indicate a physiological stress response. Six (6) cages of 3 single sex QS mice aged 11 weeks were carried to the experimental room at 10am and remained there unhandled for 1 hour under normal housing conditions. Mice assigned to a stress condition were swum either individually or in groups of 3 between 11am and 1pm. All animal procedures were performed in this 2 hour time period to minimise between group effects of diurnal hormone variations (Jacobson, 2005). The swim stress procedure involved mice being placed in 32±1°C water at 10cm depth in a 39 x 20 x 15 cm container. Water was changed between sessions and temperature measured immediately before mice were exposed to it to ensure consistency between groups. Mice were swum for 3 minutes then immediately killed via cervical dislocation in isolation from other animals. The immediacy of death following swim-stress was important as adrenal steroid release and GABA binding both decline over time following separation from the stressor (Skerritt et al., 1981). Control mice remained in their home cage until they were carried in the arms of the experimenter to the adjacent room where they were immediately euthanased.

2.3. Materials and methods: Quantitative receptor autoradiography

2.3.1. Materials

2.3.1.1. Radioligand binding materials

[^3]H)GABA (87 Ci/mmol) was purchased from G.E Healthcare (Castle Hill, NSW, Australia). The concentration of radioactivity was corrected from the date of purchase to the time of experiments using the tritium decay equation; Fraction remaining = e^{0.056t}. Hydrochloric acid for pH adjustments was purchased from APS Finechem
(Seven Hills, Australia). TRIZMA base was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3.1.2. Materials for tissue preparation

3-Aminopropyltriethoxy silane (silane) and 2-methylbutane (isopentane) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tissue-Tek- OCT (optimal cutting temperature) embedding compound was purchased from Sakura Finetechical (Tokyo, Japan.).

2.3.1.3. Materials for autoradiogram generation

Kodak Biomax MS film, autoradiography cassettes and tritium microscale standards (2.0-110.0 nCi/mg and 0.1-15.4 nCi/mg) were all purchased from G.E Healthcare (Castle Hill, NSW, Australia). Phenisol developer and Hypam Rapid Fixer were obtained from Ilford (Mt Waverley, NSW, Australia).

2.3.2. Tissue acquisition and preparation

Because perfusion and tissue fixation can affect radioligand binding (Young & Kuhar, 1979) fresh tissue was used with immediate freezing to maintain the anatomical and chemical environment of receptors (Keen & MacDermot, 1993). Mice were sacrificed by cervical dislocation and immediately decapitated with scissors. Brains were removed from the cranium over ice then immediately immersed in liquid isopentane on dry ice (-30°C) for 30 seconds to ensure rapid freezing. Frozen tissue was then stored at -70°C in OCT embedding compound until sectioning.

Coronal sections were cut at 12 μm thickness in a cryostat (Damon/IEC Division, Nedham Heights, MA, USA) maintained at -14°C then thaw-mounted onto slides pre-
treated with 2% silane in acetone. As tritium is a weak $\beta$ emitter, binding would only be expected to occur in the top 5$\mu$m of tissue exposed to $[^3]$H]GABA (Kuhar and Unnerstall, 1985). Sections were cut from three blocks at levels of bregma; 1.1-0.62 mm, -1.0-1.34 mm, -1.7-2.18 mm (Paxinos and Franklin, 2001). Three sections were mounted per slide and slides were stored for a maximum of 12 days at -70°C prior to receptor binding assays.

2.3.3. Cresyl violet staining

Cresyl violet stains blue or violet the nucleoli, Nissl bodies and nuclear membrane of each cell body including neurons and glia. Representative adjacent cryosections were Nissl stained to allow delineation of anatomical regions during analysis. Before staining, tissue was baked at 60°C for 40 minutes to ensure adherence to the slide. Tissue was then dehydrated and rehydrated to extract lipids and facilitate stain penetration, by dipping (5 x each) in increasing then decreasing concentrations of ethanol (70%, 95%, 95%, 100%). Tissue was stained by incubation in filtered 0.1% cresyl violet solution for 1-2 minutes. Following incubation, slides were rinsed in water, then transferred through increasing concentrations of ethanol (70, 95%, 95%, 100%) to reduce background staining of cytoplasm until clear differentiation of nucleoli was possible when viewed under a light microscope. Slides were placed in the clearing agent histoclear for a minimum of 1 minute before mounting a coverslip using DEPX (Pentex, Medite, Germany).
2.3.4. Receptor binding assays

2.3.4.1. Buffer

50mM Tris-HCl buffer (50mM TRIZMA base in deionised water) pH 7.4 (using 10M HCl) was used for tissue incubations. Tris-HCl buffer is a salt solution, which mimics neuronal extracellular solution for facilitation of binding (Keen & Macdermot, 1993). Care was taken to ensure that ions such as Na\(^+\) and Ca\(^{2+}\) were not present in the buffer to remove binding to reuptake transporters (Enna and Snyder, 1975) and GABA\(_B\) receptors (Bristow and Martin, 1989), respectively.

2.3.4.2. Radioligand incubation conditions

For quantitative analysis of the number of binding sites at a given concentration to be unaffected by binding kinetics, it is essential that equilibrium between free and bound ligand is reached over the period of incubation. As the rates of the forward and reverse reactions are temperature dependent, incubation and washing steps were performed at 0\(^\circ\)C to retard the dissociation of the radioligand from the receptor (Keen & MacDermot, 1993). The incubation time of 60 minutes for [\(^3\)H]GABA was based on and previous experiments measuring the association profile of specific [\(^3\)H]GABA binding in autoradiography experiments (Bristow and Martin, 1989).

2.3.4.3. Radioligand concentrations used

For experiments examining the high-affinity [\(^3\)H]GABA site, 30nM [\(^3\)H]GABA (87 Ci/mmoll) was used as this is the experimentally derived concentration of GABA at which \(B_{\text{MAX}}\) (saturation of high-affinity sites) occurs for the high-affinity site (see table 2.1) and thus provides the best estimate of the number of high-affinity binding sites.
For experiments examining the low-affinity \[^3\text{H}\]GABA site 1000nM \[^3\text{H}\]GABA was used as this is the experimentally derived concentration of GABA at which $B_{\text{MAX}}$ (saturation of low-affinity sites) occurs for the low-affinity site (see table 2.1) and thus provides the best estimate of the number of low-affinity binding sites. For cost effectiveness and to enable the same film exposure period for high and low-affinity GABA binding sites, the technique of homoisotopic dilution of the radioligand (Akinci and Johnston, 1993; Bylund and Murrin, 2000; Cuadra and Molina, 1993; Skerritt et al., 1981; Toffano et al., 1978) was employed. Thus, \[^3\text{H}\]GABA stock (87 Ci/mmol) was diluted 1/10 with unlabelled GABA such that the final specific activity of \[^3\text{H}\]GABA in experiments with 1000nM \[^3\text{H}\]GABA was 8.7 Ci/mmol. Non-specific binding of \[^3\text{H}\]GABA was determined on additional sections at each concentration by adding 100\text{\mu}M\text{ GABA to the radioligand incubation medium.}

As \[^3\text{H}\]GABA binding fits a two site binding curve it is not necessarily possible to measure only one site independently of the other site using currently available techniques. Thus, the sites measured at 30nM GABA may indeed represent a good proportion of high-affinity binding sites plus a small proportion of low-affinity binding sites. Conversely, the sites measured at 1000nM GABA may represent a combination of high- and low-affinity binding sites. However, it is uncertain from the literature whether the high and low-affinity sites represent the same site in different confirmations or two different sites acting independently and thus it is impossible to define the separate the proportions of high and low-affinity sites that are measured at each concentration. Nonetheless, the assays performed in this study at concentrations representing the $B_{\text{MAX}}$ values of the two-site GABA binding curve do provide the best available means of estimating how stress affects the availability of each site. For this
reason, binding measured at 30nM \[^3\text{H}\text{GABA}\] is referred to as ‘high-affinity binding’, whilst that measured at 1000nM GABA is referred to as ‘low-affinity binding’.

| Table 2.1: High- and low-affinity binding sites for \[^3\text{H}\text{GABA}\] |
|---|---|---|
| **K_d (nM)** | High-affinity | Low-affinity |
| | 5-20nM | 100-400nM |
| \([\text{GABA}] \text{ for } B_{\text{MAX}} \text{(nM)}\) | 30nM | 1000nM |
| **References** | Olsen et al., 1981 | Olsen et al., 1981 |
| | Guidotti et al., 1979 | Guidotti et al., 1979 |
| | Enna and Snyder, 1975 | Enna and Snyder, 1975 |

### 2.3.4.4. Procedure

The radioligand binding procedure is outlined in figure 2.1. Sections were thawed for 20 minutes at room temperature. Two 15-minute pre-incubations at room temperature were performed in 50mM tris-HCl buffer to remove endogenous ligands (such as GABA) that may compete for the radioligand binding site. Sections from the brains of each subject were incubated for 60 minutes at 0°C in 50 mM Tris-HCl (pH 7.4) containing the concentration of radioligand under investigation. The incubation was terminated by a rapid dip wash in four separate flasks of ice cold 50mM Tris-HCl (pH 7.4). Slides were then dipped in distilled water to remove excess salts before rapid drying under a stream of cool air to prevent radioligand diffusion from the binding site. Slides were stored overnight at 4°C. Sections from all 36 mice were processed simultaneously in each experiment to minimise variability between subjects.
2.3.5. Generation of autoradiograms

Slides were placed in an autoradiography cassette with two tritium microscale standards (2.0–110.0nCi/mg and 0.1–15.4nCi/mg protein) and exposed to Kodak Biomax-MS film at -20°C. In pilot studies sections were exposed to films for variable times (2, 4 or 6 weeks) to determine the optimal exposure period for later studies. In all other studies sections were exposed to film for 6 weeks as this was determined as a sufficient period to produce a signal in the required dynamic range of the film.

After 6 weeks, films were developed for 5 minutes in Ilford Phenisol then immediately placed in a 0.5% glacial acetic acid for 30 secs to stop the film development. Following fixation for 7 minutes in Ilford Hypam Rapid Fixer, films were thoroughly rinsed under running water then air dried overnight. All films were exposed and developed without any light sources then scanned using a BIO-RAD densitometry scanner (GS-800 Imaging Densitometer, School of Molecular and Microbial Sciences, University of Sydney).
2.3.6. Brain regions examined

Brain regions were defined by the experimenter circling a brain region on the digital autoradiograph with reference to cresyl violet stained slides and the mouse brain atlas (Paxinos & Franklin, 2001). Brain regions were selected based on ease of delineation of boundaries on the autoradiograph as well as relevance to stress physiology. On sections taken from between bregma 1.10 - 0.50 mm the following regions were examined; frontal cortex (layers I-VI), upper cortical layers (I-III), lower cortical layers (IV-VI), cingulate cortex, lateral septum, caudate-putamen. On sections taken from between bregma -1.70 and -2.30 mm the following regions were examined; temporal cortex, hippocampus, CA1-CA2, CA3, dentate gyrus, medial amygdala, basolateral amygdala. Thus thirteen brain regions were examined per animal as shown in figure 2.2.

Figure 2.2: Brain regions examined in acute stress autoradiography. Images are reproduced with permission from "The Mouse Brain Atlas in Stereotaxic Coordinates" (Paxinos & Franklin, 2001). Regions examined in this experiment are labelled with the following abbreviations: cingulate cortex (Cg); caudate putamen (CPu); lateral septum (LS); basolateral amygdala (BLa); medial amygdala (Me); dentate gyrus (DG); cortical layers (I-VI).
2.3.7. Quantitative analysis of binding

2.3.7.1. Optical density measurements

Mean optical density (sum of pixel values / number of pixels) was measured in each brain region of interest on 8 bit greyscale digital images using the program Image Quant v1.1 software (Molecular Dynamics, ITC-Academic Computing Health Science, University of Virginia, USA). For each brain region examined four optical density measurements were made (2 per hemisphere) on each section. Slide background optical density was subtracted from the mean optical density measured for each brain region.

2.3.7.2. Calibration of optical density measurements

Optical density measurements were converted to concentration of radioactivity per weight of tissue equivalent (nCi/mg) using [3H]microscales. Tritium concentrations in the microscale standards were corrected for radioactive decay using the radioactive decay equation (see 2.1.3.1). The relationship between measured optical density for microscale standards and the known concentration of tritium per weight of tissue equivalent was plotted using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Using Prism, first-order \((Y=A+BX)\) and second order \((Y=A+BX+CX^2)\) polynomials were compared for best fit. If the second order polynomial fit best then higher data points were successively separated from lower data points until first order polynomials could be fit to all sets of points. All optical density measurements were then substituted for \(Y\) in the first order polynomial established for their optical density range and defined by the slope constant, \(A\), and \(Y\)-intercept, \(B\): \(X=(Y-A)/B\), where \(X\) is the optical density transformed into nCi/mg.
2.3.7.3. Determination of specific binding

Specific binding was determined per animal in each experiment by subtracting non-specific binding (mean nCi/mg value in the presence of 100µm unlabelled GABA) from total binding (mean nCi/mg value). Graphs of specific binding for each brain region were then compiled in Prism 4.0.

2.3.8. Statistical analysis

All statistical analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, Ill., USA). To examine the effects of sex on stress-induced differences in high and low-affinity [³H]GABA binding, between-subjects type-III two-way ANOVA was conducted using pairwise Bonferroni’s planned contrasts to determine the source of significant main effects. Means comparison contrasts were used to examine the source of differences for significant sex by stress interactions.
2.4. Results: Regional changes in GABA\textsubscript{A} receptor binding sites

2.4.1. Low-affinity (1000nM) $[^3]$H[GABA binding sites

2.4.1.1. Cortical regions

Low-affinity binding site regional distributions were consistent with those reported in the literature (Olsen et al., 1990). Figures 2.3 and 2.4 show results from experiments measuring the number of low-affinity GABA binding sites in cortical regions from males and females exposed to different stress conditions. Results from the two-way ANOVA by region are presented in table 2.2. No significant effects of stress or sex were observed in the temporal cortex (see table 2.2). There were significant main effects of sex in the whole frontal cortex ($F_{1,27}=8.91$, $p=0.006$) and both the upper ($F_{1,27}=8.94$, $p=0.006$) and lower ($F_{1,27}=7.88$, $p=0.009$) layers of the frontal cortex indicating that regardless of stress condition, females have reduced $[^3]$H[GABA binding site density relative to males in these regions. There were significant interaction effects (sex x stress) in the frontal cortex ($F_{2,27}=4.33$, $p=0.023$), the upper layers of the frontal cortex ($F_{2,27}=8.30$, $p=0.002$) and the cingulate cortex ($F_{2,27}=6.56$, $p=0.005$) indicating that the effects of stress on $[^3]$H[GABA binding site density vary according to sex.

*Sex differences*

Post-hoc contrast analysis, showed that control males had significantly higher $[^3]$H[GABA binding densities than control females in the frontal cortex ($p<0.001$), the upper layers of the cortex ($p<0.001$) and the cingulate cortex ($p=0.009$) but no differences were observed between the sexes after exposure to either individual (frontal $p=0.547$; upper $p=0.371$; cingulate $p=0.307$) or group stress (frontal $p=0.665$; upper $p=0.931$; cingulate $p=0.075$) in these regions.
Effects of stress

Post-hoc contrast analysis, showed that stress-induced increases in female low-affinity \[^3\text{H}\]GABA binding were significant in the upper layers of the cortex (individual stress \(p=0.037\); group stress \(p=0.027\)) and the cingulate cortex (individual stress \(p=0.017\); group stress \(p=0.019\)) but not the whole frontal cortex (individual stress \(p=0.547\); group stress \(p=0.665\)). Stress-induced decreases in male \[^3\text{H}\]GABA binding density were only significant in the group-stressed males in both the frontal cortex measured as a whole (\(p=0.046\)) and the upper layers of the frontal cortex (\(p=0.029\)) relative to controls but not the cingulate cortex (\(p=0.185\)). In contrast, individual stress did not alter \[^3\text{H}\]GABA binding sites in males relative to controls (frontal \(p=0.285\); upper \(p=0.444\); cingulate \(p=0.998\)).

Thus, as demonstrated in figure 2.3, for regions of the frontal cortex, stress reduced the number of low-affinity sites for males but increased binding to these sites for females, such that sex differences between control groups in low-affinity \[^3\text{H}\]GABA binding (male>female) were removed by stress (male=female).

![Figure 2.3: Representative autoradiographs of forebrain 1000nM \[^3\text{H}\]GABA binding sites. Pictures are from male (a, b, c) and female (d, e, f) mice exposed to no adulthood stress (a, d), individual 3 minute adulthood swim stress (b, e) and group stress in adulthood (c, f). Scale bar represents 0.5cm.](image)
Figure 2.4: Effects of sex and adulthood stress on 1000nM [³H]GABA binding sites in cortical regions. Data are expressed as mean ± SEM for a) cingulate b) frontal c) temporal cortical regions and d) upper e) lower cortical layers. *=p<0.05, **=p<0.01 for significant stress induced differences relative to control mice of the same sex. ^=p<0.05, ^^=p<0.01 for significant sex differences relative to male controls of the same adulthood stress condition. Grouped bars represent significant main effects where the overall interaction was not significant.

Table 2.2: Results of 2-way ANOVA tests for 1000nM [³H]GABA binding in cortical regions. Tests reaching significance with p<0.05 are highlighted.

<table>
<thead>
<tr>
<th></th>
<th>Cingulate</th>
<th>Frontal</th>
<th>Temporal</th>
<th>Upper</th>
<th>Lower</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main Effects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>$F_{(1,27)}=3.03$, p=0.087</td>
<td>$F_{(1,27)}=8.91$, p=0.006</td>
<td>$F_{(1,27)}=0.26$, p=0.613</td>
<td>$F_{(1,27)}=8.94$, p=0.006</td>
<td>$F_{(1,27)}=7.88$, p=0.009</td>
</tr>
<tr>
<td>Stress</td>
<td>$F_{(2,27)}=0.89$, p=0.424</td>
<td>$F_{(2,27)}=0.33$, p=0.720</td>
<td>$F_{(2,27)}=0.84$, p=0.442</td>
<td>$F_{(2,27)}=0.41$, p=0.666</td>
<td>$F_{(2,27)}=0.29$, p=0.752</td>
</tr>
<tr>
<td><strong>Interaction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex x Stress</td>
<td>$F_{(2,27)}=6.56$, p=0.005</td>
<td>$F_{(2,27)}=4.33$, p=0.023</td>
<td>$F_{(2,27)}=0.08$, p=0.927</td>
<td>$F_{(2,27)}=8.30$, p=0.002</td>
<td>$F_{(2,27)}=2.68$, p=0.087</td>
</tr>
</tbody>
</table>
2.4.1.2. Hippocampal regions

Figure 2.5 shows low-affinity GABA binding in hippocampal regions of male and female mice exposed to different stress conditions. Results from the two-way ANOVA by hippocampal region are presented in table 2.3. No significant main or interaction effects were observed in the hippocampus, CA1-CA2 or CA3 regions. A significant interaction effect was observed in the dentate gyrus ($F_{2,27}=3.49$, $p=0.046$) indicating that the effects of stress varied according to sex. Post-hoc contrast analysis showed that control males had significantly higher 1000nM $[^3]$H[GABA binding than control females in the dentate gyrus ($p=0.028$), but following exposure to individual ($p=0.334$) and group ($p=0.187$) stress no sex difference was observed. In females, individual stress caused significant increases ($p=0.041$) in low-affinity GABA binding relative to controls whilst group stress did not affect low-affinity GABA binding sites in females ($p=1.000$). In males, group stress caused significant decreases ($p=0.039$) in GABA binding sites relative to controls but individual stress ($p=0.635$) did not affect low-affinity $[^3]$H[GABA binding.
Figure 2.5: Effects of sex and adulthood stress on 1000nM [³H]GABA binding sites in the hippocampus. Data are expressed as mean ± SEM for a) whole hippocampus and b) CA1-CA2 c) CA3 d) dentate gyrus subregions of the hippocampus. *=p<0.05 for significant stress induced differences relative to control mice of the same sex. ^=p<0.05 for significant sex differences relative to male controls of the same adulthood stress condition.

Table 2.3: Results of 2-way ANOVA tests for 1000nM [³H]GABA binding in hippocampal regions. Tests reaching significance with p<0.05 are highlighted

<table>
<thead>
<tr>
<th></th>
<th>Hippocampus</th>
<th>CA1-CA2</th>
<th>CA3</th>
<th>Dentate Gyrus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>F(1,27)=0.05, p&gt;0.05</td>
<td>F(1,27)=0.77, p&gt;0.05</td>
<td>F(1,27)=1.02, p&gt;0.05</td>
<td>F(1,27)=0.34, p&gt;0.05</td>
</tr>
<tr>
<td><strong>Stress</strong></td>
<td>F(2,27)=1.14, p&gt;0.05</td>
<td>F(2,27)=2.51, p&gt;0.05</td>
<td>F(2,27)=0.58, p&gt;0.05</td>
<td>F(2,27)=1.96, p&gt;0.05</td>
</tr>
<tr>
<td><strong>Sex x Stress</strong></td>
<td>F(2,27)=0.10, p&gt;0.05</td>
<td>F(2,27)=0.36, p&gt;0.05</td>
<td>F(2,27)=1.23, p&gt;0.05</td>
<td>F(2,27)=3.49, p&lt;0.05</td>
</tr>
</tbody>
</table>
2.4.1.3. Other subcortical regions

Figure 2.6 shows the densities of low-affinity $[^3]$H]GABA binding sites in various subcortical regions. As seen in Table 2.4, there were no significant main or interaction effects in the amygdalar regions examined (basolateral and medial amygdala) or the caudate-putamen. In the lateral septum, there was a significant interaction effect meaning stress-induced changes depended on sex ($F_{2,27}=3.40$, $p=0.038$). Post-hoc contrast analysis showed that control males had greater 1000nM $[^3]$H]GABA binding sites than control females ($p=0.042$) with no sex differences being observed in the individually ($p=0.155$) nor group-stressed ($p=0.482$) groups. For males, stress-induced decreases in low-affinity $[^3]$H]GABA binding relative to the control group resulted from exposure to the group stress condition ($p=0.017$), whilst neither individually stressed males ($p=1.000$), individually ($p=1.000$) nor group ($p=1.000$) stressed females varied significantly from controls.
Figure 2.6: Effects of sex and adulthood stress on 1000nM $[^3]$H[GABA binding sites in various subcortical regions. Data are expressed as mean ± SEM for the a) caudate putamen b) lateral septum c) baolateral amygdala and d) medial amygdala. *=p<0.05, for significant stress induced differences relative to control mice of the same sex.

Table 2.4: Results of 2-way ANOVA tests for 1000nM $[^3]$H[GABA binding in subcortical regions. Tests reaching significance with p<0.05 are highlighted

<table>
<thead>
<tr>
<th></th>
<th>Caudate-Putamen</th>
<th>Lateral Septum</th>
<th>Basolateral Amygdala</th>
<th>Medial Amygdala</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>$F_{(1,27)}=3.01$, p=0.914</td>
<td>$F_{(1,27)}=1.57$, p=0.221</td>
<td>$F_{(1,27)}=0.00$, p=0.963</td>
<td>$F_{(1,27)}=1.48$, p=0.235</td>
</tr>
<tr>
<td>Stress</td>
<td>$F_{(2,27)}=0.52$, p=0.603</td>
<td>$F_{(2,27)}=2.85$, p=0.075</td>
<td>$F_{(2,27)}=0.22$, p=0.805</td>
<td>$F_{(2,27)}=0.08$, p=0.925</td>
</tr>
<tr>
<td>Sex x Stress</td>
<td>$F_{(2,27)}=1.55$, p=0.230</td>
<td>$F_{(2,27)}=3.40$, p=0.038</td>
<td>$F_{(2,27)}=0.20$, p=0.823</td>
<td>$F_{(2,27)}=0.19$, p=0.830</td>
</tr>
</tbody>
</table>
2.4.2. High-affinity (30nM) $[^3]$H]GABA binding sites

2.4.2.1. Cortical regions

High-affinity binding site regional distributions were consistent with those reported previously for $[^3]$H]GABA binding at 30nM (Hechler et al., 1987; Palacios et al., 1981). Figures 2.7 and 2.8 show results from experiments measuring 30nM GABA binding at high-affinity sites in cortical regions from males and females exposed to different stress conditions. Table 2.5 shows results from the two-way ANOVA by region. No significant effects of stress or sex were observed in the temporal cortex or lower cortical layers (see table 2.5). There was a significant sex x stress interaction in the frontal cortex ($F_{2,25}=4.93$, $p=0.016$), the upper layers of the frontal cortex ($F_{2,25}=4.09$, $p=0.030$) and the cingulate cortex ($F_{2,27}=4.30$, $p=0.025$) indicating that the effects of stress on high-affinity $[^3]$H]GABA binding density varied according to sex in these forebrain regions. Post-hoc interaction means comparison contrasts were examined to determine the source of the interactions.

Sex differences

In the frontal cortex ($p=0.045$), upper cortical layers ($p=0.048$) and cingulate cortex ($p=0.048$), control males had fewer high-affinity sites than control females. Following individual stress, this sex difference was reversed in each of these regions (frontal cortex $p=0.050$, upper cortical layers $p=0.050$, and cingulate cortex $p=0.019$) with stressed males having more high-affinity GABA binding sites than stressed females. Following group stress, no sex differences were observed (frontal cortex $p=0.105$, upper cortical layers $p=0.194$, and cingulate cortex $p=0.092$).
Effects of stress

Despite the stress-induced reversal of sex differences, individual stress did not induce significant changes relative to controls in any of the brain regions examined for males (frontal cortex p=0.289, upper cortical layers p=0.401, and cingulate cortex p=0.201) or females (frontal cortex p=0.166, upper cortical layers p=0.179, and cingulate cortex p=0.237). Similarly, group stress did not induce significant changes relative to controls in any of the brain regions examined for males (frontal cortex p=0.220, upper cortical layers p=0.272, and cingulate cortex p=0.814) or females (frontal cortex p=0.396, upper cortical layers p=0.893, and cingulate cortex p=0.217).

In summary and as displayed in figure 2.7, exposure to stress resulted in an increase in high-affinity sites for males but a decrease for females, with a net individual stress-induced sex difference such that stressed males became similar to control females and stressed females became similar to control males.

Figure 2.7: Representative autoradiographs of forebrain 30nM [^3^H]GABA binding sites. Images are from male (a, b) and female (c, d) mice exposed to no adulthood stress (a, c) and individual 3 minute adulthood swim stress (b, d). Scale bar represents 0.5cm.
Table 2.5: Results of 2-way ANOVA test for 30nM [³H]GABA binding in cortical regions. Tests reaching significance with $p<0.05$ are highlighted.

<table>
<thead>
<tr>
<th>Main Effects</th>
<th>Cingulate</th>
<th>Frontal</th>
<th>Temporal</th>
<th>Upper</th>
<th>Lower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>$F_{(1,27)}=3.27$, $p&gt;0.05$</td>
<td>$F_{(1,27)}=1.35$, $p&gt;0.05$</td>
<td>$F_{(1,27)}=0.26$, $p&gt;0.05$</td>
<td>$F_{(1,27)}=1.19$, $p&gt;0.05$</td>
<td>$F_{(1,27)}=0.22$, $p&gt;0.05$</td>
</tr>
<tr>
<td>Stress</td>
<td>$F_{(2,27)}=0.20$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=0.09$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=0.46$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=0.32$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=0.50$, $p&gt;0.05$</td>
</tr>
<tr>
<td>Interaction</td>
<td>$F_{(2,27)}=4.30$, $p&lt;0.05$</td>
<td>$F_{(2,27)}=4.93$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=1.56$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=4.09$, $P&lt;0.05$</td>
<td>$F_{(2,27)}=2.70$, $p&gt;0.05$</td>
</tr>
</tbody>
</table>

Figure 2.8: Effects of sex and adulthood stress on 30nM [³H]GABA binding sites in cortical regions. Data are expressed as mean ± SEM for the a) cingulate b) frontal c) temporal cortical regions and d) upper e) lower cortical layers. ^=p<0.05 for significant sex differences relative to male controls of the same adulthood stress condition. Grouped bars represent significant main effects where the overall interaction was not significant.
2.4.2.2. Hippocampal regions

Figures 2.9 and 2.10 show high-affinity GABA binding sites in hippocampal regions. Two-way ANOVA of this data (see table 2.6) showed no significant main or interaction effects in the dentate gyrus. However, there was a significant main effect of sex in the whole hippocampus ($F_{1,27}=3.88, p=0.041$) indicating that regardless of stress condition, females had fewer high-affinity GABA binding sites in the hippocampus compared with males. There were also significant interaction effects in the hippocampus (measured as a single region) ($F_{2,27}=3.12, p=0.045$), CA1-CA2 ($F_{2,27}=3.41, p=0.039$) and CA3 ($F_{2,27}=3.47, p=0.048$) indicating that the effects of stress on high-affinity GABA binding sites varied according to sex in these regions.

Sex differences

Post-hoc means comparison contrasts showed that, no sex differences were apparent in any of the hippocampal regions examined in control mice (hippocampus $p=0.669$; CA1-CA2 $p=0.311$, CA3 $p=0.212$). Individually stressed females showed reduced 30nM GABA binding sites relative to individually stressed males in the hippocampus ($p=0.001$) and CA1-CA2 subregion ($p=0.034$) but not the CA3 subregion ($p=0.483$). Group stressed females also showed reduced GABA binding relative to group stressed males in the whole hippocampus ($p=0.037$) but not the CA1-CA2 ($p=0.319$) nor CA3 ($p=0.483$) subregions.

Effects of stress

Post-hoc means comparison contrasts showed that for females, individually stressed mice had reduced high-affinity $[^{3}H]$GABA binding density in the hippocampus.
(p=0.043) including CA1-CA2 (p=0.009) and CA3 (p=0.016) regions relative to controls. Individual stress did not affect 30nM GABA binding in males (hippocampus p=0.999; CA1-CA2 p=0.999, CA3 p=0.999). Group stress did not affect 30nM GABA binding in neither the male hippocampus (p=0.999; CA1-CA2 p=0.999, CA3 p=0.999) nor the female (hippocampus p=0.694; CA1-CA2 p=0.199, CA3 p=0.376) hippocampal regions examined.

In summary, as shown in figure 2.9, reduced high-affinity GABA binding sites were observed in individually stressed female hippocampi relative to controls and stressed males, whilst no stress differences occurred in the male hippocampus.

![Figure 2.9: Representative autoradiographs of hippocampal 30nM [³H]GABA binding sites. Images are from male (a, b) and female (c, d) mice exposed to no adulthood stress (a, c) and individual 3 minute adulthood swim stress (b, d). Scale bar represents 0.5cm.](image)
Figure 2.10: Effects of sex and adulthood stress on 30nM \(^{3}H\)GABA binding sites in hippocampal regions. Data are expressed as mean ± SEM for the a) whole hippocampus and b) CA1-CA2 c) CA3 d) dentate gyrus subregions of the hippocampus. *\(p<0.05\), **\(p<0.01\) for significant stress-induced differences relative to control mice of the same sex. \(\wedge\)=\(p<0.05\) for significant sex differences relative to male controls of the same adulthood stress condition.

Table 2.6: Results of 2-way ANOVA tests for 30nM \(^{3}H\)GABA binding in hippocampal regions. Tests reaching significance with \(p<0.05\) are highlighted.

<table>
<thead>
<tr>
<th></th>
<th>Hippocampus</th>
<th>CA1-CA2</th>
<th>CA3</th>
<th>Dentate Gyrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>(F_{(1,27)}=3.88, p&lt;0.05)</td>
<td>(F_{(1,27)}=1.91, p&gt;0.05)</td>
<td>(F_{(1,27)}=0.13, p&gt;0.05)</td>
<td>(F_{(1,27)}=0.13, p&gt;0.05)</td>
</tr>
<tr>
<td>Stress</td>
<td>(F_{(2,27)}=0.62, p&gt;0.05)</td>
<td>(F_{(2,27)}=2.12, p&gt;0.05)</td>
<td>(F_{(2,27)}=3.01, p&gt;0.05)</td>
<td>(F_{(2,27)}=1.11, p&gt;0.05)</td>
</tr>
<tr>
<td>Sex x Stress</td>
<td>(F_{(2,27)}=3.12, p&lt;0.05)</td>
<td>(F_{(2,27)}=3.41, p&lt;0.05)</td>
<td>(F_{(2,27)}=3.47, p&lt;0.05)</td>
<td>(F_{(2,27)}=0.80, p&gt;0.05)</td>
</tr>
</tbody>
</table>
2.4.2.3. Other subcortical regions

30nM $[^3]H$GABA binding values for various subcortical regions are given in figure 2.11. Results of the two-way ANOVA in these regions given in table 2.7 show there were no significant effects of stress or sex on high-affinity GABA binding in the caudate putamen, lateral septum or the basolateral and medial amygdalar regions.

![Figure 2.11: Effects of sex and adulthood stress on 30nM $[^3]H$GABA binding sites in subcortical regions.](image)

Data are expressed as mean ± SEM for the a) caudate putamen b) lateral septum c) basolateral amygdala and d) medial amygdala.

**Table 2.7: Results of 2-way ANOVA tests for 30nM $[^3]H$GABA binding in subcortical regions.** Tests reaching significance with $p<0.05$ are highlighted

<table>
<thead>
<tr>
<th></th>
<th>Caudate-Putamen</th>
<th>Lateral Septum</th>
<th>Basolateral Amygdala</th>
<th>Medial Amygdala</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>$F_{(1,27)}=0.06$, $p&gt;0.05$</td>
<td>$F_{(1,27)}=0.01$, $p&gt;0.05$</td>
<td>$F_{(1,27)}=0.00$, $p&gt;0.05$</td>
<td>$F_{(1,27)}=0.91$, $p&gt;0.05$</td>
</tr>
<tr>
<td><strong>Stress</strong></td>
<td>$F_{(2,27)}=0.59$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=0.13$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=0.05$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=0.12$, $p&gt;0.05$</td>
</tr>
<tr>
<td><strong>Sex x Stress</strong></td>
<td>$F_{(2,27)}=0.10$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=0.06$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=0.47$, $p&gt;0.05$</td>
<td>$F_{(2,27)}=0.19$, $p&gt;0.05$</td>
</tr>
</tbody>
</table>
2.5. Discussion

2.5.1. Baseline sex differences in regional $[^3H]$GABA binding

2.5.1.1. Low-affinity binding sites (1000nM GABA)

Results of the present study indicate that males have a greater number of GABA$_A$ receptor low-affinity binding sites than females in particular forebrain cortical regions. Few studies have previously examined sex differences in binding at the low-affinity GABA binding site, and some of these previous studies observed no sex differences in cortical membrane preparations (Wilson, 1992; Wilson and Biscardi, 1992). This suggests that the regional differences that were observed in the present study are masked when the net effects on the whole cortex are examined. This increase in the number of low-affinity GABA binding sites, those that are found in electrophysiological studies to correspond to sites of channel conductance, may contribute to an explanation as to why a number of studies have indicated that males are more sensitive to the behavioural effects of compounds that act on GABA$_A$ receptors.

Interestingly, a study that examined different membrane washing procedures (Akinci and Johnston, 1993) found that the number of GABA binding sites and the proportion of low-affinity binding sites in crude forebrain homogenates were greatly increased in males compared with females, as was observed in the present study. This earlier finding suggests that the presence of endogenous mediators such as neurosteroids that are often lost or extracted with more vigorous membrane washing procedures may be relevant to the increased number low-affinity GABA binding sites in males (Akinci and Johnston, 1993). Thus, it appears that sex differences in low-affinity binding sites are observed only in tissue that undergoes limited post-mortem manipulation and are
restricted to certain forebrain cortical regions where perhaps such endogenous mediators are most abundant.

2.5.1.2. High-affinity binding sites (30nM GABA)

Binding sites labelled by 30nM GABA in the present experiments are representative of high-affinity GABA$_A$ receptor orthosteric sites. In contrast to 1000nM GABA binding sites, females showed a greater number of binding sites labelled by 30nM GABA compared with males. This finding was observed in the frontal cortex, particularly the upper layers and the cingulate cortex, and is consistent with previous work showing increased [$^3$H]muscimol binding in cortical homogenates from females (Juptner and Hiemke, 1990) and the finding that ovarian steroids increase [$^3$H]muscimol binding in the cortex (Maggi and Perez, 1984; Perez et al., 1986) without variation over the oestrus cycle (Hamon et al., 1983).

The relevance of increased high-affinity GABA binding sites in certain forebrain cortical regions of the female brain is difficult to interpret. Whilst analysis of Scatchard plots from [$^3$H]GABA binding studies has lead to a general consensus that there exists both high-affinity (nM) and low-affinity (nM-μM) binding sites, and that high GABA concentrations are required for opening of the central chloride channel (Baur and Siegel, 2003; Harris and Allan, 1985; Maconochie et al., 1994), whether the different binding site populations represent different conformations of the same binding site, or distinct sites on the same or different macromolecular complexes is unknown (Baur and Sigel 2003; Cash and Subbarao, 1987; Edgar and Schwartz, 1992; Harris and Allan, 1985; Maksay, 1996; Smith and Olsen 1994; Yeung et al., 2003). Perhaps of importance are findings from electrophysiological studies that have observed extrasynaptic GABA$_A$
receptors with a higher affinity for GABA (Yeung et al., 2003). Thus it is possible that the present findings of sex differences in high-affinity sites represent sex differences in the subset of extrasynaptic GABA_\textsubscript{A} receptors responsible for mediating tonic non-densensitising GABAergic currents in the brain.

2.5.2. Stress-induced changes in [\textsuperscript{3}H]GABA binding sites

2.5.2.1. Effects of stress in males

Males exposed to group stress showed no change in the number of binding sites labelled by 30nM GABA but a reduced number of sites labelled by 1000nM GABA in the frontal cortex, upper layers of the frontal cortex, cingulate cortex, dentate gyrus and lateral septum. Similarly previous studies have observed no stress-induced changes in cortical [\textsuperscript{3}H]muscimol labelling of the high-affinity GABA binding site (Motohashi et al., 1993). As well, reductions in the density of cortical low-affinity GABA binding sites have been observed previously in rats following both footshock stress (Biggio et al., 1981; Concas et al., 1985; Corda et al., 1985; Cuadra and Molina, 1993) and stress from guillotine in handling-naïve rats (Biggio et al., 1981; Biggio et al., 1984; Concas et al., 1985; Biggio et al., 1987). However, no change (Akinci and Johnston, 1993; Skerritt et al., 1981) and increased (Wilson and Biscardi, 1992) cortical low-affinity GABA_\textsubscript{A} receptor binding sites have also been reported in males following swim stress and handling stress, respectively. Such discrepancies in the literature may arise from differences between studies in habituation of animals to experimenter handling as suggested previously (Biggio et al., 1981; Concas et al., 1985; Corda et al., 1985; Cuadra and Molina, 1993). The presence of cage-mates during male stress, may also be relevant to such discrepancies in the literature as our findings show that only
group-stressed and not individually stressed males undergo changes in GABA binding sites.

Loss of cortical low-affinity GABA<sub>A</sub> binding sites suggests a stress-induced loss of functional GABA<sub>A</sub> receptor sites in male frontal cortical regions. Interestingly, forced swim stress has been observed to remove anxiolytic effects of diazepam (Briones-Aranda <i>et al.</i>, 2005), reduce the anti-seizure efficacy of benzodiazepines (Deutsch <i>et al.</i>, 1990) and reduce the convulsive activity of GABA<sub>A</sub> receptor antagonists (Drugan <i>et al.</i>, 1985; Pericic <i>et al.</i>, 2000; Pericic <i>et al.</i>, 2001; Soubrie <i>et al.</i>, 1980), suggesting impaired sensitivity of GABA<sub>A</sub> receptors following stress and consistent with the findings of a loss of functional GABA binding sites in stressed males that were observed here.

### 2.5.2.2. Effects of stress in females

Females exposed to individual stress experienced a stress-induced increase in the number of binding sites labelled by 1000nM GABA in the upper layers of the frontal cortex, the cingulate cortex and the dentate gyrus. This finding is consistent with previous studies that also found stress-induced increases in the density of cortical low-affinity GABA binding sites in females (Akinci and Johnston, 1993; Skerritt <i>et al.</i>, 1981; Wilson and Biscardi, 1994). High-affinity binding sites in females were also affected by stress with a net reduction in these sites in hippocampal regions suggesting a difference between males and females in the recruitment of the hippocampus for stress. Thus there appears to be an increase in the number of low-affinity sites for GABA in the cortex but a reduction in the number of hippocampal high-affinity GABA binding sites in stressed females.
2.5.2.3. *Stress-induced sex differences*

The present study shows that a brief 3 minute swim stress affects \[^3\text{H}]\text{GABA}\) binding differently in males and females. Stress reversed the sex differences in high-affinity GABA binding sites such that the stressed males had a greater number of cortical high-affinity sites than stressed females. In contrast, stress eliminated sex differences in cortical low-affinity \[^3\text{H}]\text{GABA}\) binding sites. A stress-induced elimination of sex differences in the number of functional GABA binding sites suggests that stress rapidly alters the availability of GABA binding sites in a regional and sex dependent fashion. Consistent with the present study, previous work has observed that stress eliminates sex differences in low-affinity cortical GABA binding (Wilson and Biscardi, 1994) and in behavioural responses to GABA\_A receptor modulators diazepam and ethanol (Wilson *et al.*, 2004). These findings suggest that following stress, sex differences in GABAergic signalling and behaviours mediated via this neurotransmitter system would be reduced.

2.5.3. *Potential mechanism of rapid stress-induced changes in GABA\_A receptors*

The rapid alterations in male and female GABA binding observed in this study suggest a mechanism for rapid plasticity of neurochemical signalling systems in response to stress. It is important to recognise that changes in maximum \[^3\text{H}]\text{GABA}\) binding site availability, which are observed immediately following a 3 minute stressor, are unlikely to represent changes in the total number of GABA\_A receptors. For example, alterations in protein and mRNA synthesis take at least hours to occur (Connolly *et al.*, 1996a; Kang *et al.*, 1991; Orchinik *et al.*, 1995). However, rapid changes in binding site availability may occur via effects on receptor surface expression.
as is proposed to explain the rapid modifications of GABA_{A} receptors observed during seizures (Goodkin et al., 2007). Thus alterations in GABA_{A} receptor binding site exposure may be observed following stress due to the presence of intracellular transporter proteins (Thomas et al., 2005; Wan et al., 1997; Washbourne et al., 2004) or membrane lipids.

Rapid post-translational modifications of the GABA_{A} receptor population may be mediated by neurosteroids and corticosteroids released in the brain during stress (Akinci and Johnston, 1993; Purdy et al., 1991). As altered [^{3}H]GABA binding occurs in the absence of endogenous mediators (Akinci and Johnston, 1993), effects of such endogenous mediators are not a result of direct ligand-receptor interactions. However, neurosteroids may be responsible for rapid post-translational modifications of GABA_{A} receptors following stress via effects on receptor trafficking, which in turn appears to involve effects on receptor phosphorylation state. Future studies should examine if a brief incubation of brain tissue with different neurosteroids may produce changes in GABA binding site availability that can be observed following their removal from the tissue.

Of particular interest in this study is the discovery of regional differences in the stress-induced alterations in [^{3}H]GABA binding sites. In both males and females predominantly forebrain cortical regions were affected by stress, despite the fact that changes were in opposite directions in either sex. Relative to other brain regions forebrain cortical preparations show the greatest increases in concentrations of endogenous neurosteroids such as allopregnanalone and THDOC during stress (Purdy et al., 1993) and this may explain the regional specificity of the stress-induced changes in GABA_{A} receptors that were observed in the present study. Alternatively, regional
differences in GABA$_A$ receptor subunit composition may explain the varied sensitivity of different brain regions to stress. For example, regions such as the outer cortical layers and the dentate gyrus of the hippocampus which have abundant $\alpha_2$ subunit expression (Fritschy and Mohler, 1995) appear to be more affected by acute swim stress than other brain regions in both males and females. Variations in pharmacological sensitivity amongst GABA$_A$ receptor subtypes to endogenous steroids released in the brain during stress may thus explain the regional differences in the effects on [$^3$H]GABA binding observed in the present study.

2.5.4. Relevance of GABA$_A$ receptor stress responses

The present study confirmed previous findings which showed rapid stress-induced alterations in [$^3$H]GABA binding sites, and extended upon this work to show the specific forebrain cortical regions that are involved in this stress response. The rapid upregulation of GABA$_A$ receptor binding sites may impact both endocrine and behavioural responses to stress. For example, rapid changes in GABA binding with stress may contribute to behavioural effects, associated with GABAergic signalling that are observed immediately following stress such as opioid resistant analgesia (Skerritt et al., 1981) and anxiolysis (Briones-Aranda et al., 2005; Johnston and File, 1991).

2.5.5. Conclusions

These data show that both sex and stress affect the number of functional GABA binding sites in a regionally specific manner. Forced swim stress induced rapid changes in forebrain GABA binding sites in females and group stressed males suggesting a mechanism for rapid GABAergic plasticity and potential alterations in inhibitory tone
perhaps via receptor trafficking or changes in endogenous GABAergic substances. However the number of functional binding sites for GABA in certain forebrain regions was altered by stress in opposite directions in males and females, such that following stress baseline sex differences were removed. These results exemplify sex differences in brain chemical function and stress responses and disruptions to such responses may be relevant to disorders in which stress is a predisposing factor such as schizophrenia and depression.
PART C:

Effects of Early-Life Stress on GABA<sub>α</sub> Receptors in Adulthood
CHAPTER 3:
Early-Life Stress Models and Adulthood Behaviour

3.1. Introduction

3.1.1. Background

Aversive early-life experiences are thought to affect long-term neurobiological and psychological development and can lead to increased vulnerability to a number of diseases such as psychiatric disorders, cardiovascular disorders, adult obesity and diabetes (Canetti et al., 1997; Felitti et al., 1998; Lissau and Sorensen, 1994; McCauley et al., 1997; Russak and Schwartz, 1997). Early post-natal environmental manipulations in rodents have been observed to produce long-lasting changes in adulthood behaviour (Moffett et al., 2007), immune function (Avistur et al., 2006) stress reactivity, and neurophysiology (Blaise et al., 2008; Vicentic et al., 2006), thus offering insight into the relationship between early-life-environment and susceptibility to illness in adulthood.

Models of early-life experience involving maternal separation are complex. The EH-NH model has been used most consistently throughout the literature and produces the most robust between-group differences (see section 1.7). Rodents that are separated briefly (15 minutes) every day from the dam, home cage and siblings over post-natal day (PND) 1-14, are termed early-life handled (EH). Despite the fact that the EH condition undergoes active experimenter interaction, these animals are considered the control group as they best represent the ‘normal’ early-life experience of rodents in an animal house and in the wild where pups are briefly separated from the dam and litter during cage cleaning and in bouts of maternal foraging (Calhoun, 1963). The NH, or
non-handled, group is left undisturbed by both experimenters and animal house staff over PND1-14. This prolonged uninterrupted confinement of the dam and litter in their cage results in large reductions in dam-pup interactions and maternal stress, both of which are expected to act as aversive early-life events and result in a more anxious, reactive and fearful adulthood behavioural phenotype. Thus, the NH group is considered the experimental ‘early-life stress’ condition (Anisman et al., 2001; Cadji et al., 1998; Francis et al., 1999; Hennessy et al., 1982; Lee and Williams, 1975; Liu et al., 1997; Smotherman and Bell, 1980).

When compared with the EH condition, NH animals show consistent behavioural differences in adulthood across studies. For example, increased anxiety-type behaviour has been observed by NH animals on the elevated plus maze (EPM) (Cabib et al., 1993; D’Amato et al., 1998; McIntosh et al., 1999; Meerlo et al., 1999; Moles et al., 2004; Nunez et al., 1995; Ploj et al., 1999; Pryce et al., 2001; Vallee et al., 1997), and the light-dark box test (Fernandez-Teruel et al., 1991; Steimer et al., 1998). Associated with these increased anxiety behaviours are findings that NH rodents also show increased behavioural responsivity, interpreted as increased fearfulness, in response to an acoustic stimulus (Caldji et al., 2000b; Pryce et al., 2001, Pryce et al., 2003) or the presence of a predator (Padoin et al., 2001) relative to the EH condition. Thus, the EH-NH model consistently produces mice with different adulthood anxiety profiles. Studies such as the present one that employ the EH-NH model therefore benefit from confirmation of the adulthood behavioural effects of the early-life environmental manipulation prior to post-mortem analysis.
3.1.2. Overview of the elevated plus maze

The elevated plus maze (EPM) has been extensively validated as a test of anxiety in both mice and rats and thus is considered one of the most robust behavioural indicators of anxiety in rodents (File, 2001). It is used in screening for anxiolytic drugs as well as a post-hoc tool for providing evidence of altered emotionality in animals (Carobrez and Bertoglio, 2005). It is of particular advantage with respect to the present study that unlike other behavioural measurements of anxiety, the EPM does not greatly interfere with animals through requirements of training, food or water deprivation, or exposure to stress in the form of predators, restraint or electric shock (Rodgers and Johnson, 1995).

The maze is designed to exploit the natural fear rodents have of open space, unfamiliarity and elevation (File, 2001). It is comprised of four elevated intersecting arms of equal size separated by a central platform. Two opposing arms are bounded by walls (“closed arms”) and the other two opposing arms, at right angles to the closed arms, are unbounded (“open arms”) (File, 2001). The EPM is most useful in providing measures of two independent factors: anxiety and locomotor activity (Lister, 1987). Measures of anxiety that are largely independent of other behavioural parameters (factor loadings >0.9), are the open arm entries expressed as a percentage of total entries, and the time spent on the open arms expressed as a percentage of total time spent on either the open or closed arms (Espejo, 1997; File, 2001; Lister, 1987; Rodgers and Johnson, 1995). Importantly, percentages are not expressed with respect to the 5 minute test duration as it is uncertain exactly what the time spent on the central platform represents (File, 2001). Locomotor activity is best represented by number of closed arm entries (Fernandes and File, 1996; Rodgers and Johnson, 1995) with factor analysis studies.
showing that the total number of arm entries, the parameter often used to indicate motor activity, is affected by both anxiety and locomotion. Interestingly, factor analysis reveals sex differences in the contribution of different factors on the EPM in rats. For male rats the test is most sensitive to variability in anxiety, while for female rats the test is most sensitive to differences in motor activity (Fernandes et al., 1999). This may suggest it is more difficult to identify changes in anxiety in females on the plus maze, however it is evident that this sex-difference in factor loadings is not observed in mice as it is for rats (File, 2001; Miyakawa et al., 1996).

3.1.3. Aims

In the current study the EH-NH model was used to examine the effects of early-life environmental stress on GABA\textsubscript{A} receptor subunit expression and synaptic clustering. In order to validate the use of this early-life stress model in our animal house and to ensure the expected adulthood behavioural phenotype could be produced, behavioural testing was carried out prior to brain removal. Thus, the aims of this study were to investigate anxiety of male and female mice exposed to EH and NH early-life conditions using the EPM.
3.2. Materials and Methods:

3.2.1 Materials

3.2.1.1. EPM apparatus

The elevated plus maze (EPM) comprised two open arms (30x5 cm), and two closed arms (10x5 cm, surrounded by 15 cm high walls) extending from a common central platform (5x5 cm). The apparatus was constructed from plexiglass (black floor, clear walls covered with black cardboard) elevated to a height of 60 cm. A video camera containing a DVD burner and mounted on a tripod was positioned such that the entire maze could be recorded in the field of view.

3.2.2 Methods: Animal Model

3.2.2.1. Subjects

Six female Quakenbush Swiss (QS) albino mice from a single litter (9 weeks) and one male QS albino mouse (9 weeks) (Laboratory Animal Services, Perth, WA) were housed together upon arrival at the animal house for 24 hours, allowing for impregnation of the females. Pregnant female mice were then housed individually in solid-bottomed breeding cages with free access to food and water. Litters born 18-22 days later were immediately culled to a maximum of 8 pups each and in all but one litter (where only 3 females were present), 4 males and 4 females remained. It was considered important that mice were born in the animal house from mothers impregnated in the animal house to avoid exposure to stress of transport during the gestational and post-natal periods. All mice were housed under a 12hr/12hr light/dark cycle with constant temperature (21°C) and permitted food and water ad libitum. The
Animal Ethics Committee of the University of Sydney approved all animal experiment protocols.

3.2.2.2. **Timeline**

The timeline of live animal work is given in table 3.1 Active experimental interventions occurred on post-natal day (PND) 1-14 (see section 3.2.2.4. for details), and in adulthood (age 13 weeks) when an adulthood acute stress protocol was performed (see section 3.2.2.6.).

Adulthood behavioural testing was performed to ensure the EH-NH model of early-life stress carried out in our animal house produced similar behavioural effects to those performed elsewhere. At age 11 weeks the elevated plus-maze test for anxiety was performed. This test was chosen on the basis that it is less likely to act as a stressor than alternative procedures by avoiding shock administration, as well as food and water deprivation (Lister, 1987; Stephens and Andrews, 1991). Furthermore, the EPM and the acute stress protocol were each carried out with two weeks between each procedure to minimise carry over effects between tests.

<table>
<thead>
<tr>
<th><strong>Time Period</strong></th>
<th><strong>Stage of animal model</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>ED  1-21</td>
<td>Gestation period</td>
</tr>
<tr>
<td>PND 0-14</td>
<td>EH-NH model</td>
</tr>
<tr>
<td>PND 14-21</td>
<td>Routine monitoring</td>
</tr>
<tr>
<td>PND 21</td>
<td>Weaning</td>
</tr>
<tr>
<td>PND 22-57</td>
<td>Routine Monitoring</td>
</tr>
<tr>
<td>PND 58-77</td>
<td>Routine monitoring &amp; experimenter handling</td>
</tr>
<tr>
<td>PND 78-79</td>
<td>Elevated Plus Maze</td>
</tr>
<tr>
<td>PND 90-91</td>
<td>Adulthood stress protocol and euthanasia</td>
</tr>
</tbody>
</table>

**Abbreviations:** Post-natal day (PND), ED (embryonic day)
3.2.2.3 Subject assignment

On PND1 (with date of birth considered PND0), litters were randomly assigned to either non-handled (NH: stress) or early-life handled (EH: control) conditions such that there were n=3 litters (24 mice; 12 male, 12 female) in each condition. Whole litters were assigned to the same early-life condition as the NH condition required complete absence of experimenter intervention.

Animals remained in their litters until PND21 when they were weaned from their mother. Weaning involved removal from the dam and separation into cages of 4-6 mice. Each cage contained only one sex (male or female) and one early-life condition (EH or NH). To prevent behavioural differences between EH and NH mice being transmitted between groups, animals that experienced the same early-life conditions were housed together. The cage assignments at weaning were counterbalanced between litters of the same early-life condition, thus mice were housed with at least one full sibling (same mother and father) and two half siblings (same father only).

3.2.2.4 Early-life manipulation procedure

The early-life manipulation procedure was carried out to establish EH and NH groups according to the nomenclature of Pryce and Feldon (2003). Litters allocated to the early-life handling (EH) group were separated from the dam and siblings for 15 minutes a day at room temperature on PND1-14. During the EH procedure, dams were removed from the home cage and placed into individual cages for the duration of the separation. Pups were placed individually in plastic cages with tissue bedding for 15 minutes. At the conclusion of the separation period pups were returned to the nest
before the dam was replaced in the cage. NH litters were left undisturbed from PND1-14.

3.2.2.5. Elevated plus maze behavioural testing

i) Procedure

The elevated plus maze test was carried out when mice were 11 weeks of age. Subjects were tested between the hours of 11 am - 1 pm to minimise effects of diurnal hormonal variations. Cages were transported to a room adjacent to the testing room and left undisturbed for 1 hour.

The testing procedure involved mice being individually carried to the plus maze by the experimenter and placed on the central platform facing an open arm. Mice were then allowed to freely explore the maze for 5 minutes whilst being videotaped. The apparatus was thoroughly cleaned with detergent and dried between subjects and prior to the first animal of the day being tested. To avoid scent or movement distraction, the experimenter waited in a separate room behind a closed door during the 5 minute exploration period.

ii) Behavioural measures

Parameters measured from video recordings were: the total number of arm entries, number of closed arm entries, number of open arm entries (expressed as a % of total entries), time spent on the open arm (expressed as a % of time spent on open + closed arms), time spent on the closed arm (expressed as a % of time spent on open + closed arms) and latency to enter the open arm. For all of these parameters, an arm entry occurred when all 4 paws were present in a single arm.
iii) Data analysis

All graphs were compiled in PRISM 4.0 and groups were compared on all parameters by two-way ANOVA (sex by early-life condition) using SPSS 15.0. Means comparison contrast analysis was performed in the case of significant interaction effects.

3.2.2.6. Adulthood acute stress procedure

At 13 weeks of age mice were exposed to the acute adulthood stress procedure immediately prior to euthanasia. Mice assigned to the control (no stress) condition were used as subjects for *ex vivo* studies in chapters 4 and 5. In chapter 6, both stressed and control mice were compared.

i) Subject assignment

For adulthood stress, animals within a cage were assigned to either individual swim stress or control conditions. In each of the two pairs of siblings per cage, one would be assigned to the stress condition and another to the control condition. As a result control and stress conditions of the adulthood acute swim stress procedure were each comprised of equal numbers of mice from different litters and post-weaning cage environments.

ii) Procedure

The procedure took place over two separate days between 11am and 1pm with eight cages being processed per day. On each day four male and four female cages
were processed, two from each early-life condition for each sex. Cages were processed in a random order and the order of control and stress treatments was randomised within a cage. The procedure followed that already described in section 2.2.3. In brief, mice assigned to a stress condition were swum individually for 3 minutes in 32±1°C water at 10 cm depth in a 39 x 20 x 15 cm container, then immediately euthanased. Control mice remained in their home cage until euthanasia.

### 3.2.2.7. Tissue preparation

Mice were killed by cervical dislocation and decapitated. Brains were removed from the cranium on ice and snap frozen in isopentane on dry ice. Frozen whole brains were then embedded in OCT embedding compound and stored at -70°C until sectioning. Coronal sections were cut rostral-caudally using a cryostat (Reichert-Jung, Vienna, Austria) maintained at -20°C. Sections were thaw mounted onto silane-coated slides to give a 1:20 parallel series with six 10 μm sections per slide (every second section was collected). Slides were then stored at -70°C until use in either immunohistochemistry (see chapter 4 and chapter 5) or autoradiography experiments (see chapter 6).

### 3.2.2.8. Cresyl violet staining

Representative adjacent cryosections were Nissl stained to allow delineation of anatomical regions during analysis. Staining was performed as described in section 2.3.3.
3.3. Results: Elevated plus maze behavioural testing

Figure 3.1 shows the effects of sex and early-life experience on EPM behaviours. NH mice showed a decreased % of open arm entries ($F_{1,44}=4.58, p<0.05$), and decreased % of time spent on the open arm ($F_{1,44}=4.28, p<0.05$). There were no significant main effects of sex ($p>0.05$) or significant interactions ($p>0.05$) between sex and early-life on either the % of open arm entries or % of time spent on the open arms, indicating that sex did not affect the impact of early-life on the preference for the open arms. Additionally, there was no significant main effect of sex ($F_{1,44}=0.42, p>0.05$), early-life condition ($F_{1,44}=2.10, p>0.05$) or sex x early-life interaction ($F_{1,44}=1.19, p>0.05$) on the number of closed arm entries.

![Figure 3.1: The effects of sex and early-life condition on elevated plus-maze behaviours.](image)

Figures represent % of entries into the open arms (A), the time spent on the open arms (B), and the total number of closed arm entries (C) over 5 minutes of free exploration of the elevated plus maze. Data are expressed as mean±SEM. Bars represent a significant main effect, where *$p<0.05$ denotes significant differences of NH relative to EH.
3.4. Discussion

3.4.1. Effects of early-life stress on adulthood anxiety

Results from the EPM showed that both male and female mice exposed to the NH early-life condition spent less time and made fewer entries onto the open arms than their EH counterparts. This finding indicates that NH mice display more anxious behaviour on the EPM, and is consistent with previous reports using the same early-life stress model in rats (Bodnoff et al., 1987; D’Amato et al., 1998; Fernandez-Teruel et al., 1990; Ferre et al., 1995; McIntosh et al., 1999; Meerlo et al., 1999; Nunez et al., 1995; Ploj et al., 1999; Pryce et al., 2001; Vallee et al., 1997) and mice (Cabib et al., 1993; Moles et al., 2004). Studies using other tests to measure anxiety are also in accordance with these findings showing that NH rodents are more anxious in light-dark box tests (Fernandez-Teruel et al., 1991; Steimer et al., 1998) and tests of behavioural reactivity (Caldji et al., 2000b; Padoin et al., 2001). Furthermore, previous studies that have examined the effects of early-life stress on the EPM behaviour of both males and females have also observed no sex differences in the effect of the NH procedure on anxiety (McIntosh et al., 1999; Severino et al., 2004). Thus, as expected, the NH early-life stress condition produced a more anxious adulthood phenotype in both males and females than the EH condition.

Results of this study also showed that early-life experience had no effect on the number of closed arm entries in the EPM. This indicates that locomotor activity does not vary between sexes or different early-life conditions and that the increased anxiety observed in NH mice occurs without effects on locomotor activity. Consistent with this finding, previous studies have also indicated that EH and NH early-life conditions do not affect locomotor activity on the EPM for either sex (McIntosh et al., 1999; Severino
et al., 2004). Interestingly, studies using the open field test to examine locomotion have suggested that NH mice of both sexes show a locomotor deficit (Arnold and Siviy, 2002; Ader and Grota, 1969; Caldji et al., 2000b; Denenberg, 1964; Levine, 1957; Meerlo et al., 1999; Pihoker et al., 1993; Plotsky and Meaney, 1993; Pryce et al., 2001, Pryce et al., 2003; Vallee et al., 1997; Weizman et al., 1999). However, given that factor analysis studies have shown that activity measurements using the open field test are confounded by anxiety and/or exploration (Fernandes et al., 1999; File, 1985; File, 2001), and that adulthood anxiety is consistently shown to be affected in the NH vs. EH model in several different anxiety tests, findings from the open field test regarding locomotor activity are inconclusive. In contrast, the EPM has been shown to measure locomotion independently of anxiety in both males and females from various strains of rats and mice (File et al., 2001). Therefore it seems more likely that NH and EH mice do not vary in locomotor activity. Confirmation of this finding should be attained via examination of the effects of the EH and NH conditions on the holeboard test which, like the EPM, is thought to measure motor activity independently of other behavioural parameters (File, 2001).

3.4.2. Conclusions

The current investigation aimed to reproduce findings from previous studies measuring adulthood anxiety following early-life manipulation using the EH-NH model. Behavioural testing showed that regardless of sex, NH mice are more anxious on the elevated plus maze compared with EH mice, with no between-group differences in locomotor activity. Thus, the early-life model used in the current study produced adulthood behavioural changes consistent with previous work. Whilst specific early-life
environmental factors identified in animal models cannot be directly extrapolated to human rearing conditions, the EH-NH model of early-life stress in rodents provides a model by which we can systematically examine the long-term effects of early-life environment on neurochemical systems and behaviour. Given the role of GABA_A receptors as targets for anxiolytic drugs, differences in adulthood behaviour in animals exposed to different early-life conditions may be related to alterations in GABA_A receptors. In the following chapters the effects of early-life manipulation on adulthood GABA_A receptors (chapter 4 and 5) and stress-induced changes in GABA_A receptors (chapter 6) are addressed.
CHAPTER 4:
Effects of Early-Life Stress on GABA$_\alpha$ Receptor $\alpha$ Subunit Expression

4.1. Introduction

4.1.1. Background

Characterisation of the long-term effects of early-life environment on neurochemical functioning is important for understanding factors contributing to proposed neurodevelopmental disorders such as schizophrenia where genetics do not completely explain the disease etiology. Long-lasting behavioural changes that were observed following different early-life conditions in chapter 3 suggest that the developing nervous system is sensitive to subtle changes in the environment, however neurochemical changes underlying such behaviours are not fully understood. Whilst previous studies have indicated long-lasting effects of early-life environment on multiple neurotransmitter systems (Arborelius and Eklund, 2007; Heim et al., 2001), the GABAergic system has largely been ignored despite its involvement in mediating anxiety and behavioural reactivity. Thus, an improved understanding of the effects of early-life environment on the adulthood GABAergic system is required.

GABA$_\alpha$ receptors undergo marked changes in their subunit composition during development, involving the gradual replacement of the $\alpha_2$ subunit with the $\alpha_1$ subunit (Fritschy et al., 1994; Laurie et al., 1992; Lopez-Tellez et al., 2004; MacLennan et al., 1991; McKernan et al., 1991; Okada et al., 2000; Paysan et al., 1994; Poulter et al., 1992; Poulter et al., 1993). This switch from $\alpha_2$ to $\alpha_1$ subunit dominance is regionally-dependent, being most evident in regions such as the thalamus and lower cortical layers of primary sensory cortices (Fritschy et al., 1994), but almost non-existent in regions which maintain high $\alpha_2$ expression throughout maturation such as the outer cortical
layers, the pyramidal and granule cell layers of the hippocampus and certain amygdalar nuclei (Fritschy et al., 1994). The gradual replacement of $\alpha_2$ subunits with the $\alpha_1$ subunit occurs largely over the first two post-natal weeks in rodents and so it is feasible that early-life environmental manipulations over this time period may disrupt this developmental process. Given that the $\alpha_1$ and $\alpha_2$ subunits are thought to be responsible for mediating different behaviours via GABA$_A$ receptors (Bosman et al., 2002; Brooks-Kayal and Pritchett, 1993; Juttner et al., 2001; Kapur and MacDonald, 1999; Okada et al., 2000), disruptions in the developmental ‘switch’ may provide a molecular basis for the effects of early-life stress on adulthood anxiety. Thus, an understanding of whether early life stress has long-term effects on GABA$_A$ receptor $\alpha_1$ and $\alpha_2$ receptor subunits is highly relevant.

Previous studies have suggested early-life environment can have long-lasting effects on GABA$_A$ receptors. For example, compared with EH rats, NH rats have been observed to show reduced high-affinity [$^3$H]GABA binding sites in brainstem nuclei (Caldji et al., 2000b), reduced benzodiazepine binding sites in forebrain and amygdalar regions (Bodnoff et al., 1987; Bolden et al., 1990; Caldji et al., 2000b) and reduced $\gamma_2$ subunit mRNA in the amygdala (Caldji et al., 2000b; Caldji et al., 2003; Caldji et al., 2004). As well, previous studies have provided support for long-term effects of early-life environment on the $\alpha_1$ subunit in the dentate gyrus and amygdala (Caldji et al., 2000b; Hsu et al., 2003), and the $\alpha_2$ subunit in the dentate gyrus (DG) (Hsu et al., 2003) of male rats. Surprisingly, no previous studies have examined changes in $\alpha$ subunit expression in regions such as the primary sensory cortices and the thalamus, where the developmental subunit switch in $\alpha$ subunits is most prominent. Thus, the present study investigated the effects of early-life stress on $\alpha_1$ and $\alpha_2$ subunit protein
expression in various brain regions, including those where the subunit switch is most prominent, using immunohistochemistry. Furthermore, given the abundant evidence indicating sex differences in GABA<sub>A</sub> receptors and how they are affected by stress (see chapter 2), both male and female mice were examined.

4.1.2. Overview of immunohistochemistry

Immunohistochemistry allows the observation of the anatomical distribution of proteins at a microscopic scale. Immunohistochemical staining occurs when an antibody directed against an immunogenic substance, termed an antigen, binds specifically to a small portion of that antigen, termed an epitope, in a tissue section, to form an antibody-antigen complex (Hudson and Hay, 1989). The formation of an antigen-antibody complex may occur via hydrogen bonds, hydrophilic bonds and Van der Waals forces (Chemicon, 2005).

Antibodies are glycoproteins synthesised as part of the body’s humoral response following exposure to an antigen (Benjamin and Leskowitz, 1991). Structurally, antibodies have two heavy and two light chain polypeptides arranged in a Y shape (Benjamin and Leskowitz, 1991). The tail of the Y forms the Fc binding site for immune cells, and the arms of the Y give rise to two F(ab) variable regions, which provide the antigen binding sites (Benjamin and Leskowitz, 1991). The most concentrated serum antibodies are the IgG class (secondary humoral response), which are typically used in immunohistochemistry (Pearse, 1980; Radford et al., 2005). Antibodies are commercially available as either polyclonal (mixture of antibodies that react with a variety of epitopes on the immunising antigen) or monoclonal (copies of a single antibody directed against a single epitope) preparations, which may be purified of
non-specific serum proteins and immunoglobulins using protein A, protein G or antigen-affinity purification techniques (Chemicon, 2005).

Visualisation of the antibody-labelled antigen occurs using an enzyme (immunohistochemistry) or fluorochrome reporter molecule (immunofluorescence) (Hudson and Hay, 1989; Radford et al., 2005). The present chapter deals with visualisation of antibodies using enzymes while the following chapter deals with visualisation via fluorophores, which is preferred when cellular and subcellular staining is of interest.

Enzymes used in immunohistochemistry catalyse the formation of coloured end-products that can be visualised with light microscopy. In the present study the enzyme horse-radish peroxidase was used to catalyse the reaction between substrates hydrogen peroxide and diaminobenzidine (DAB) to give a brown coloured end-product. Enzymes may be directly conjugated to the primary antibody (direct method) or conjugated to a secondary antibody (indirect method). The secondary antibody binds to one or more Fc receptors on the primary antibody allowing a greater number of enzyme molecules per antigen, resulting in an increased signal (Pearse, 1980; Radford et al., 2005). Other signal amplification techniques may result in the enzyme being conjugated to the secondary antibody in a polymerised enzyme complex (used in section 4.2.4 $\alpha_1$ staining procedure), or in immune (PAP method) or non-immune avidin- or streptavidin-biotin complexes (see section 4.2.5 - $\alpha_2$ staining procedure) (Chemicon, 2005; Radford et al., 2005).
4.1.3. Aims

The aim of this study was to examine the effects of early-life stress on regional and laminae patterns of adulthood GABA<sub>A</sub> receptor α<sub>1</sub> and α<sub>2</sub> subunit expression in a variety of brain regions. Immunoperoxidase histochemistry was used to examine the relative density of each of the α<sub>1</sub> and α<sub>2</sub> subunit proteins in male and female mice exposed to either EH or NH early-life conditions. This study will thus aid in understanding whether the development of the GABAergic system is affected by early-life environment and is of relevance to neurodevelopmental disorders such as schizophrenia.
4.2. Materials and methods

4.2.1. Materials

4.2.1.1. General immunohistochemistry materials

Slides (76 x 36 mm) were obtained from Starfrost (Berlin, Germany). Liquid blocker super pap-pen and Tissue-Tek® OCT mounting media were purchased from ProSciTech (Thuringowa, QLD, Australia). Isopentane (2-methylbutane), paraformaldehyde powder, sodium chloride, sodium phosphate monobasic (anhydrous), sodium phosphate dibasic (anhydrous), TRIZMA base, tris-HCl, bovine serum albumin (BSA, fraction V) and triton-X 100 (t-octylphenoxypolyethoxyethanol) were all purchased from Sigma Aldrich (St Louis, MO, USA). Cresyl violet acetate was obtained from BDH Laboratory supplies (Poole, England).

4.2.1.2. Immunoperoxidase staining materials

Hydrogen peroxide was obtained from Biolab (Clayton, VIC, Australia). Normal goat serum was purchased from Sigma Aldrich (St Louis, MO, USA). DEPX mountant was obtained from Pentex (Medite, Germany). A Liquid DAB (3,3’-Diaminobenzidine) and Substrate Chromogen Visualisation System, rabbit IgG isotype control solution and serum-free protein block were all purchased from Dako (Carpenteria, CA, USA). A Standard Vectastain Elite® ABC kit was purchased from Vector Laboratories (Burlighame, CA, USA).

4.2.2. Tissue acquisition and preparation

4.2.2.1. Subjects

Subjects were those described in section 3.2.2.1. In brief, male (n=13) and female (n=11) Quackenbush Swiss (QS) albino mice were born in the animal house and
exposed to either EH or NH early-life conditions on PND1-14 as described in section 3.2.2.4 (males NH n=7, EH n=6; females NH n=5, EH n=6). Mice examined in immunohistochemistry experiments were not exposed to adulthood swim stress and were those described as controls in section 3.2.2.6.

4.2.2. Tissue preparation and fixation

Fresh frozen tissue was prepared and cryosectioned as described in section 3.2.2.7. Slides devoted to immunohistochemistry were post-fixed within 10 days of sectioning to preserve tissue morphology and prevent breakdown of structures during storage. A post-fixation method was used on all slides as previous studies examining GABA<sub>A</sub> receptor immunohistochemistry have revealed a reduction in background staining when fresh-frozen cryostat sectioned tissue is used with minimal fixation as opposed to perfusion fixed tissue (Fritschy et al., 1998). Fixation involved 5 minute immersion of slides in 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) at room temperature followed by 5 dips in 0.01 M PBS to remove excess fixative. Sections were air-dried in a fumehood overnight, then stored at -20°C until immunohistochemistry experiments.

4.2.3. General immunohistochemistry methods

4.2.3.1. Buffers for immunohistochemistry

Buffers were compared in pilot studies in an attempt to reduce background staining. 0.1 M Tris-buffered saline (TBS) pH 7.4 was found to increase the signal : noise ratio of staining compared with 0.01 M phosphate buffered saline (PBS) pH 7.4. A variety of NaCl concentrations (4-15g/L) for the 0.1 M TBS buffer were also investigated to further reduce background staining produced by unwanted ionic
interactions. Increasing the salt strength in the buffer to 15 g/L of NaCl was found to provide optimal staining for the \( \alpha_1 \) primary antibody but did not affect the \( \alpha_2 \) subunit staining. Thus, 0.1 M TBS\(^+\) pH 7.4 was used for washing slides and reagent dilution throughout the \( \alpha_1 \) staining procedure whilst standard 0.1 M TBS pH 7.4 was used for the \( \alpha_2 \) staining procedure.

4.2.3.2. Staining sections on slides

All staining was performed on sections thaw-mounted onto slides. Washes were performed in Coplin staining jars. For incubation of solutions (300 \( \mu \)L per slide) sections on each slide were circled with the liquid blocker super pap-pen, which repels water and thus prevents solutions running off the slide. All incubations were performed in Nunc bioassay dishes (Nalge Nunc International, Naperville, Ill, USA) with raised grids upon which sections were placed to ensure even and complete distribution of solution across the tissue. To prevent tissue drying out and resultant staining artefacts, humidity was maintained during incubation by placing dampened tissue in the bottom of the bioassay dish and the dish was then sealed during incubation (Costa and Furness, 1983).

4.2.3.3. Experimental design

i) Replicates

As each brain was sectioned in a 1:20 series with 6 sections per slide, for each animal, there were two 1:20 series; one comprising sections between bregma 2.0 mm and 0.0 mm and the other comprising sections between bregma -0.55 mm and -2.54 mm (Paxinos and Franklin, 2001). For each animal two replicate slides from each
of the two blocks were stained in separate experiments for each antibody. Thus, there were four separate experiments for each antibody.

ii) Controls

Negative controls were included in every experiment to ensure the secondary antibody did not cross-react with non-antigenic proteins. For $\alpha_1$ subunit staining, negative isotype controls were used where the primary antibody was replaced with rabbit IgG (Dako, Carpinteria, CA, USA) at the same protein concentration. For $\alpha_2$ subunit staining, the primary antibody solution was replaced with the antibody diluent. No staining was observed on negative control slides included in final experiments.

4.2.4. $\alpha_1$ Immunoperoxidase staining

4.2.4.1. Antibodies

i) Primary antibody

A polyclonal rabbit IgG directed against the GABA$_A$ receptor $\alpha_1$ subunit protein (batch # 31775) was obtained from Millipore (Billerica, MA, USA). The protein-A purified IgG was raised in rabbits immunised with the synthetic peptide sequence (QPSQDELKDNTTV FT-C) corresponding to amino acids 1-15 at the C-terminal of the rat GABA$_A$ receptor $\alpha_1$ subunit. This sequence is identical in mice. This antibody has been characterised by Western blot analysis on rat brain microsomal preparations where it recognises a protein band at 51 kDa representing the $\alpha_1$ subunit protein (company product details). This antibody produces a similar staining distribution pattern in mouse brain (see section 4.3.1.1) as a previously characterised antibody for the $\alpha_1$ subunit (Fritschy and Mohler, 1994).
ii) Secondary antibody

The secondary antibody used was an anti-rabbit IgG raised in goat and conjugated to a HRP (horse radish peroxidase)-labelled polymer (Envision+ DakoCytomation, Carpenteria, CA, USA). This antibody was used to enhance the signal of the primary antibody as it has an increased number of peroxidase molecules attached to each secondary antibody IgG molecule compared with a streptavidin-peroxidase labelled biotinylated secondary antibody.

4.2.4.2. α1 Immunoperoxidase staining procedure

i) Endogenous peroxidase activity blocking

As immunoperoxidase techniques rely on the peroxidase catalysed conversion of DAB and hydrogen peroxide to a brown coloured precipitant, endogenous peroxidase in tissue can result in non-specific staining. Thus, after slides were thawed for 20 minutes at room temperature, endogenous peroxidase was blocked by incubating sections for 15 minutes at room temperature in 0.3% H₂O₂ in TBS. Sections were then washed three times for 5 minutes each in 0.1 M TBS.

ii) Non-immune protein blocking

Tissue was exposed to an innocuous protein solution to mask charged proteins in the tissue and thus reduce background staining. Pilot studies indicated significantly reduced background with a serum-free protein block (Dako) compared with 2-10% normal goat serum. Thus, 300 μL of serum free protein block was added to each slide and incubated for 40 minutes at room temperature. After 40 minutes the blocking solution was tipped off the slide before the primary antibody solution was added.
iii) Primary antibody incubation

In pilot studies, the primary antibody was titrated 1:50-1:1000 (0.02 mg/mL - 0.001 mg/mL) against the secondary antibody. The minimum concentration that provided sufficient antibody signal 1:100 v/v (0.01 mg/mL) was used and diluted in 0.1 M TBS containing 3% v/v normal goat serum (NGS) and 0.025% v/v triton-X 100 to aid antibody penetration. The primary antibody solution was incubated with tissue sections at 4°C for 16 hours in a humidity chamber. At the end of the incubation, unbound primary antibody was removed by three 10 minute washes in 0.1 M TBS.

iv) Secondary antibody incubation

The anti-rabbit secondary antibody conjugated to a HRP-labelled polymer described in section 4.2.4.1(ii) was a ‘ready to use’ solution. Thus, as per the manufacturer’s instructions 300 µL of this solution was added to each slide and incubated at room temperature for 40 minutes. The solution was removed by three 10 minute washes in 0.1 M TBS.

v) DAB reaction

DAB and hydrogen peroxide are converted to an insoluble brown precipitant in a peroxidase catalysed reaction. Thus, a brown precipitant is formed at the site of peroxidase-labelled secondary antibodies upon addition of DAB and hydrogen peroxide substrates. Peroxidase catalysed visualisation was performed using the Liquid DAB and Substrate Chromogen System according to the manufacturer’s instructions (Dako, Carpenteria, CA, USA). Liquid DAB was diluted in hydrogen peroxide buffer (20 µL liquid DAB per 1 mL H₂O₂ buffer) as per the manufacturer’s instructions. Pilot studies
examining the optimal time for DAB incubation (1-20 minutes) showed 3 minutes gave the most intense staining with the least background. Thus, 300 µL of DAB-H₂O₂ was added per slide and incubated at room temperature for exactly 3 minutes before the slide was rinsed thoroughly in 0.1 M TBS for 3 lots of 10 minutes.

vi) Coverslipping slides

Sections were dehydrated by immersion through a series of increasing concentrations of ethanol (70%, 80%, 95%, 100%) for 30 seconds each. Slides were then cleared in histoclear for 5 minutes before mounting coverslips (76 x 30 mm) using DEPX mountant (Pentex, Medite, Germany). Coverslipped slides were then air-dried in a fumehood overnight.
4.2.5. $\alpha_2$ Immunoperoxidase staining

4.2.5.1. Antibodies

i) Primary Antibody

A polyclonal guinea-pig antibody directed against the $\alpha_2$ subunit was kindly provided by Dr Jean-Marc Fritschy (Institute of Pharmacology and Toxicology, University of Zurich, Switzerland). The affinity-purified antisera came from guinea pigs immunised with a synthetic peptide sequence specific for the $\alpha_2$ subunit N-terminal (extracellular) residue 1-9 (Fritschy and Mohler, 1995). This antisera has been previously characterised by immunoreactivity on rat (Fritschy and Mohler, 1995; Fritschy et al., 1998) and mouse (Crestani et al., 1999; Crestani et al., 2002) brain, and by Western blotting on rat and mouse brain where it recognises a single protein band at 52 kDa (McKernan et al., 1991; Marksitzer et al., 1993). Immunoperoxidase staining throughout the cortex, hippocampus and thalamus (see section 4.3.2.1) produced a pattern of $\alpha_2$ subunit immunoreactivity that was identical with previous descriptions.

ii) Secondary Antibody

A biotinylated anti-guinea pig IgG (H+L) (Vector Labs, Burlingame, CA, USA) was used as the secondary antibody for these experiments. This antibody was raised in goats against guinea pig serum IgG, then conjugated to biotin. This secondary antibody was chosen as it has been used successfully with this primary antibody in previous studies (Fritschy and Mohler, 1995). Furthermore, no HRP-polymer labelled anti-guinea-pig secondary antibody (see section 4.2.4.1), which would be expected to reduce the required amount of primary antibody, was available at the time of experimentation.
4.2.5.2. \( \alpha_2 \) Immunoperoxidase staining procedure

i) Antigen retrieval

Antigen retrieval is a procedure carried out to unmask antigens in tissue using either proteolytic digestion or exposure to heat prior to immunostaining. The exact mechanism underlying these procedures is not well understood but is thought to involve removal of crosslinks formed during formaldehyde fixation and/or reversal of protein denaturation that presumably occurs during fixation (Fritschy et al., 1998). The most effective antigen retrieval method varies for a given antigen and depends on pH and temperature (Fritschy et al., 1998). In the case of the GABA\(_A\) receptor \( \alpha_2 \) subunit, it has been shown that antigen retrieval via exposure to heat (microwave irradiation) under acidic conditions improves the signal to noise ratio of staining for GABA\(_A\) receptor subunits (Fritschy et al., 1998), even in tissue exposed to minimal fixation. In pilot experiments of the current study, microwave irradiation methods designed for free-floating section immunohistochemistry were originally attempted but abandoned due to compromised tissue morphology, with some of the tissue appearing to come off the slide. Thus a gentler method of antigen retrieval developed for sections on slides was used (Dixon and Harper, 2001) and found to substantially improve the signal to noise ratio of \( \alpha_2 \) subunit staining. Of note, \( \alpha_1 \) staining seemed largely unaffected by this antigen retrieval method suggesting limited epitope masking occurs during fixation with this antibody. Thus no antigen retrieval was used in the \( \alpha_1 \) staining protocol described in section 4.2.4.

The gentle antigen retrieval method involved thawing slides for 20 minutes at room temperature then incubating in a polyacetyl staining box (HD Scientific,
Blacktown, NSW, Australia) containing 0.01 M citric acid buffer (pH 6.0) and suspended in boiling water. The temperature of the citric-acid buffer was maintained at 90°C over a 90 minute incubation period and then the polyacetyl staining box was removed from the boiling water and cooled to room temperature. Slides were then removed from the citric acid buffer and washed for 3 x 10 minutes in 0.1 M TBS.

**ii) Blocking**

Previous studies using the α2 antibody provided by The Institute of Pharmacology and Toxicology, University of Zurich, Switzerland have not used blocking steps prior to primary antibody incubation (Fritschy and Mohler, 1995; Fritschy et al., 1998). Pilot studies indicated no significant effect of peroxidase, serum (2-10% NGS) or non-serum (Dako) protein blocking on α2 subunit staining. Thus no blocking step was performed for the α2 subunit immunoperoxidase procedure in the present study.

**iii) Primary antibody incubation**

In pilot studies, the primary antibody (0.2 mg/mL stock) was titrated 1:200-1:2000 against the secondary antibody (1:100, 1:200 and 1:500). The minimum concentration that provided sufficient antibody signal 1:300 v/v was used. The primary antibody was diluted in 0.1 M TBS containing 2% v/v NGS and 0.2% v/v triton-X 100 as described previously for this antibody (Fritschy and Mohler, 1995). The primary antibody solution (300 μL per slide) was then incubated with tissue sections at 4°C for 22 hours in a humidity chamber. At the end of the incubation, the primary antibody solution was tipped off the slides and unbound primary antibody was removed by three 10 minute washes in 0.1 M TBS.
iv) Secondary antibody incubation

The optimal dilution factor of the biotinylated secondary antibody was established in pilot experiments to be 1:200. Thus, the secondary antibody was diluted 1:200 in 0.1M TBS with 2% (v/v) NGS. This secondary antibody solution was then incubated with tissue sections at room temperature for 1.5 hours as per the manufacturer’s instructions. The secondary antibody solution was then tipped from the slides and excess solution removed by three 10 minute washes in 0.1 M TBS.

v) Streptavidin-peroxidase reaction

Avidin-biotin and streptavidin-biotin methods for peroxidase labelling of secondary antibodies rely on the high affinity interaction of the vitamin biotin with the glycoproteins avidin and streptavidin (Harlow and Lane, 1999). Streptavidin is neutral at physiological pH and thus is used preferentially to avidin to avoid background from unwanted ionic interactions with charged proteins (Harlow and Lane, 1999). In streptavidin-biotin techniques a streptavidin-biotin peroxidase complex acts as a tertiary label of the antigen providing a number (16) of peroxidase molecules per biotinylated secondary antibody (Harlow and Lane, 1999).

The streptavidin-peroxidase conjugate was prepared according to the manufacturer’s instructions for the Standard Vectastain Elite ABC kit. Briefly, components A and B were each diluted 1:50 in 0.1 M TBS and this solution was incubated for 30 minutes at room temperature to allow conjugation of the two components. This streptavidin-peroxidase solution was then incubated with the tissue for 45 minutes at room temperature as per the manufacturer’s instructions. The solution
was then tipped from the slides and excess removed by three 10 minute washes in 0.1 M TBS.

vi) **DAB reaction**

Colorimetric visualisation using DAB was performed as described in section 4.2.4.2 (see part v).

vii) **Coverslapping slides**

Slide coverslapping was performed as described in section 4.2.4.2 (see part vi).
4.2.6. Image acquisition

4.2.6.1. Brightfield microscopy image capture

Immunostained images were collected using a binocular Olympus (BX51, Olympus Optical Ltd, Mount Waverley, Victoria, Australia) light microscope set up for brightfield microscopy under Köhler illumination conditions. The microscope was fitted with a DC500 digital colour camera connected to a PC, using the Leica image capture software IM1000 (Leica Biosystems, Mount Waverley, Victoria, Australia). Digital shading corrections were performed using the IM1000 software to ensure even illumination across the captured field of view. Images for analysis were then captured as 8 bit greyscale tiff images (1024 x 1024) on a 4X, numerical aperture 0.16 plan apochromat objective. Digital images of each region to be analysed were captured from 8 sections per animal per antibody in each hemisphere.

Prior to image capture a number of sections were viewed such that the microscope light intensity could be adjusted to provide maximal signal range whilst ensuring no signal was lost through over- or under-saturation. All images were taken for a given antibody in a given region on the same day under constant conditions of exposure time (684 ms) and gamma (=1). Despite all precautions to ensure consistency in imaging, it is expected that the illumination provided by the microscope light source will vary over a given session from variations in the voltage supplying the light source. Such variations were accounted for by randomising the order of imaging across groups and making background corrections in the final image analysis. Representative captured images from each group were then compiled in Adobe Photoshop V7.0 (Adobe Systems Incorporated, San Jose, USA) for presentation.
4.2.6.2. Brain regions examined

Figure 4.1 shows the brain regions examined. Brain regions were defined by the experimenter circling a the region on the digital image with reference to cresyl violet stained slides and the mouse brain atlas (Paxinos & Franklin, 2001). Brain regions selected were those where the developmental subunit switch was prominent, well characterised and occurred in late gestation or early postnatal life of rodents. The amygdala (central, basal and lateral nuclei) was examined as it has previously been shown to have altered α subunit expression in adulthood when exposed to different early-life manipulations (Caldji et al., 2000b). On sections taken from between bregma 2.00 and 0.00 mm the following regions were examined; cingulate cortex, frontal cortex (M1, M2), somatosensory cortex (SS, layers I-VI) as illustrated in figure 4.1. On sections taken from between bregma 0.00 and -2.00 mm the following regions were examined; somatosensory cortex (SS, layers I-VI), hippocampus (CA1-CA2, CA3, dentate gyrus (DG)), amygdala (lateral, basolateral, central) and hippocampal layers of the CA1 (striatum oriens, pyramidal cell layer, striatum radiatum) and dentate gyrus (molecular cell, granule cell, polymorphic cell), as shown in figure 4.1.
4.2.7. Data analysis

4.2.7.1. Semi-quantitative image analysis

Immunohistochemistry is most often used to determine cellular location of proteins. Under controlled conditions, this method is also used with computer aided image analysis to examine amounts of reaction product (Auger et al., 1995; Benno et al., 1982a; Benno et al., 1982b; Huang et al., 1996; Mize et al., 1994). In the absence of calibrated standards, the non-linear nature of the peroxidase reaction precludes statements relating immunoreactivity to absolute protein quantities, however differences in the regional optical densities of the reaction product may be used to make conclusions regarding changes in the protein density between groups in certain brain regions. This semi-quantitative method for immunohistochemistry data analysis is particularly applicable to comparing different treatment groups in levels of GABAₐ receptor staining (Yu et al., 2006) which is typically diffuse, including membrane and
subcellular staining amongst neuropil, making delineation of individual cells for stereological counts or cellular protein densities highly subjective.

Optical density scores in arbitrary units were calculated using similar methods to that of Yu et al. (2006) in a GABA$\alpha$ receptor subunit immunohistochemistry study. Mean optical density (sum of pixel values / number of pixels) was measured in each brain region of interest on 8 bit greyscale digital images (1024 x 1024 pixels) using the program Image Quant v1.1 software (Molecular Dynamics, ITC-Academic Computing Health Science, University of Virginia, USA). For each brain region examined four optical density measurements were made (2 per hemisphere) on each section. Background optical density was measured from white matter on the same section and this was subtracted from the mean optical density measured for each brain region.

4.2.7.2. Statistical analysis

All statistical analyses were performed using SPSS V15.0 (SPSS, Inc., Chicago, Ill., USA). To examine the effects of sex and early-life stress on $\alpha_1$ or $\alpha_2$ subunit density in a given brain region, between-subjects type III two-way ANOVA was conducted followed by pairwise Bonferroni’s planned contrasts to determine the source of significant main effects. Means comparison contrasts were used to examine the source of differences for significant sex x early-life environment interactions.
4.3. Results: Early-life environment effects on GABA<sub>Α</sub> receptors

4.3.1. α<sub>1</sub> Subunit immunoreactivity

4.3.1.1. α<sub>1</sub> Subunit distribution

α<sub>1</sub> Subunit immunohistochemistry (IHC) revealed a selective pattern of distribution (see table 4.1). In general, the α<sub>1</sub> subunit expression was greatest in the cortex with abundant expression throughout the frontal (motor), somatosensory and cingulate cortices of all animals. α<sub>1</sub> subunit immunoreactivity (IR) in the cortex showed a particularly strong band of staining in layer IV throughout the neocortex and a somewhat weaker band of staining in layer V. In the hippocampus, α<sub>1</sub> IR was greatest in the CA1 region with only weak to moderate staining in the DG. Furthermore, the α<sub>1</sub> subunit was not expressed in the layers with the greatest α<sub>2</sub> IR - the cell body layers (pyramidal layers of CA1-CA3 and the granule cell layer of the dentate gyrus). α<sub>1</sub> staining was moderate in the amygdala, being particularly weak in the central nucleus where α<sub>2</sub> staining was strong. In the thalamus, the α<sub>1</sub> subunit was abundantly expressed in most nuclei, particularly the lateral dorsal and the ventral-lateral nuclei, although it was absent in the reticular nucleus and weak in the periventricular nucleus. In the more medially positioned thalamic nuclei α<sub>1</sub> expression was moderate.
4.3.1.2. $\alpha_1$ Subunit regional IR

Table 4.1 shows the optical density of $\alpha_1$ subunit IR in male and female mice exposed to either EH or NH early-life conditions in various brain regions. Two-way ANOVA (sex x early-life) of this data for each of the brain regions examined showed there were no significant main effects of sex or early-life condition in any of the brain regions examined. No significant sex x early-life environment interaction effects were observed in any of the brain regions examined either.

Table 4.1: Regional optical density scores for the GABA$_A$ receptor $\alpha_1$ subunit immunoreactivity by brain region in males and females exposed to EH and NH early-life conditions. Data are given as mean relative OD±SEM (n).

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EH</td>
<td>NH</td>
</tr>
<tr>
<td>CING</td>
<td>88.92±7.4 (5)</td>
<td>87.43±7.8 (6)</td>
</tr>
<tr>
<td>M1</td>
<td>84.20±11.3 (5)</td>
<td>84.95±6.1 (6)</td>
</tr>
<tr>
<td>M2</td>
<td>82.46±14.5 (5)</td>
<td>80.57±7.0 (6)</td>
</tr>
<tr>
<td>SS</td>
<td>85.49±7.7 (5)</td>
<td>72.74±8.0 (6)</td>
</tr>
<tr>
<td>CA1</td>
<td>105.70±8.9 (5)</td>
<td>100.67±11.9 (6)</td>
</tr>
<tr>
<td>CA3</td>
<td>48.25±3.8 (5)</td>
<td>46.32±5.3 (6)</td>
</tr>
<tr>
<td>DG</td>
<td>68.96±5.4 (5)</td>
<td>61.59±6.1 (6)</td>
</tr>
<tr>
<td>VL</td>
<td>87.06±9.1 (5)</td>
<td>83.74±8.2 (6)</td>
</tr>
<tr>
<td>LD</td>
<td>96.32±6.5 (5)</td>
<td>94.00±7.7 (6)</td>
</tr>
<tr>
<td>Lateral</td>
<td>77.28±10.7 (4)</td>
<td>86.2±3.4 (5)</td>
</tr>
<tr>
<td>BLa</td>
<td>53.28±11.8 (4)</td>
<td>57.93±13.0 (5)</td>
</tr>
<tr>
<td>CeA</td>
<td>17.13±2.2 (4)</td>
<td>12.46±6.4 (4)</td>
</tr>
</tbody>
</table>

Abbreviations: Cingulate cortex (CING), primary motor cortex (M1), secondary motor cortex (M2), somatosensory cortex (SS), hippocampus (HIPP), dentate gyrus (DG), thalamus (THAL), ventrolateral thalamic nucleus (VL), lateral-dorsal thalamic nucleus (LD), amygdala (AMYG), basolateral amygdaloid nucleus (BLa), central amygdaloid nucleus (CeA).
4.3.1.3. $\alpha_1$ Subunit IR in cortical laminae

Figures 4.2 and 4.3 show the effects of sex and early-life environment on $\alpha_1$ subunit IR in the cortical laminae of region S1 of the somatosensory cortex. Results of the two-way ANOVA shown in table 4.2 indicated $\alpha_1$ subunit IR is significantly different between sexes in layers II-III. This means that regardless of early-life condition, males showed greater $\alpha_1$ IR compared with females (mean difference EH: 6.3±4.5; NH: 14.4±6.6). No other main effects of sex or early-life condition were observed for $\alpha_1$ subunit IR. As shown in table 4.2, significant sex x early-life interactions were observed for $\alpha_1$ subunit IR in layers IV, V and VI, indicating that the effects of early-life stress depend on sex in each of these cortical layers. Post-hoc contrast analysis showed that in layers IV, V and VI, NH females had increased $\alpha_1$ IR relative to EH females (layer IV mean difference 17.7±8.2, p=0.049; layer V mean difference: 16.0±7.2, p=0.046; layer VI mean difference: 15.0±6.4, p=0.047) whilst NH and EH males did not vary significantly (layer IV p=0.290; layer V p=0.582; layer VI p=0.870). In layers V and VI, no sex differences were observed in EH (layer V p=0.132; layer VI p=0.161) or NH groups (layer V p=0.307; layer VI p=0.612). In layer IV EH males had increased $\alpha_2$ IR relative to EH females (mean difference: 22.6±9.2, p=0.029), whilst no sex difference occurred in NH mice (p=0.559).

<table>
<thead>
<tr>
<th>Table 4.2: Results of 2-way ANOVA tests for $\alpha_1$ subunit immunoreactivity in cortical laminae.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tests reaching significance with p&lt;0.05 are highlighted.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortical Layer</th>
<th>Sex</th>
<th>Early-life environment</th>
<th>Sex x Early-life environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>F(1,17)=0.02, p&gt;0.05</td>
<td>F(1,17)=0.86, p&gt;0.05</td>
<td>F(1,17)=0.26, p&gt;0.05</td>
</tr>
<tr>
<td>II-III</td>
<td>F(1,17)=4.70, p&lt;0.05</td>
<td>F(1,17)=4.14, p&lt;0.05</td>
<td>F(1,17)=4.50, p&lt;0.05</td>
</tr>
<tr>
<td>IV</td>
<td>F(1,17)=1.56, p&gt;0.05</td>
<td>F(1,17)=0.27, p&gt;0.05</td>
<td>F(1,17)=4.44, p&lt;0.05</td>
</tr>
<tr>
<td>V</td>
<td>F(1,17)=1.79, p&gt;0.05</td>
<td>F(1,17)=0.42, p&gt;0.05</td>
<td>F(1,17)=4.56, p&lt;0.05</td>
</tr>
<tr>
<td>VI</td>
<td>F(1,17)=0.56, p&gt;0.05</td>
<td>F(1,17)=3.36, p&gt;0.05</td>
<td>F(1,17)=4.56, p&lt;0.05</td>
</tr>
</tbody>
</table>
**PART C: EARLY-LIFE STRESS**

**CHAPTER 4**

Figure 4.2: Representative images of $\alpha_1$ subunit immunoreactivity in the somatosensory cortex. Images are taken from male (a,b) and female (c,d) mice exposed to EH (a,c) and NH (b, d) early-life environmental conditions. Scale 1 mm.

Figure 4.3: The effects of sex and early-life condition on $\alpha_1$ subunit expression by cortical layer. Data represent mean relative optical density±SEM in optical density units (ODU). *p<0.05 denotes significant effects of early-life relative to EH group of same sex following a significant interaction. ^p<0.05 denotes significant effects of sex relative to males of the same early-life condition following a significant interaction. Bars represent significant main effects at p<0.05 for * early-life and ^ sex.
4.3.1.4. $\alpha_1$ Subunit IR in hippocampal laminae

Figures 4.4 and 4.5 show the effects of sex and early-life condition on $\alpha_1$ subunit IR in hippocampal layers. Results of the two-way ANOVA shown in table 4.3 indicate a significant reduction in $\alpha_1$ IR in the molecular cell layer and polymorphic cell layer of the dentate gyrus of NH mice compared with EH mice when data were averaged for sex. No significant main effects of sex or sex x early-life condition interactions were observed in any hippocampal layers (see table 4.3).

<table>
<thead>
<tr>
<th>Hippocampal Layer</th>
<th>Sex</th>
<th>Early-life environment</th>
<th>Sex x Early-life environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stratum oriens</td>
<td>$F(1,19)=1.24, p&gt;0.05$</td>
<td>$F(1,19)=1.12, p&gt;0.05$</td>
<td>$F(1,19)=0.45, p&gt;0.05$</td>
</tr>
<tr>
<td>Pyramidal cell</td>
<td>$F(1,19)=0.01, p&gt;0.05$</td>
<td>$F(1,19)=0.00, p&gt;0.05$</td>
<td>$F(1,19)=1.02, p&gt;0.05$</td>
</tr>
<tr>
<td>Stratum radiatum</td>
<td>$F(1,19)=1.59, p&gt;0.05$</td>
<td>$F(1,19)=0.38, p&gt;0.05$</td>
<td>$F(1,19)=0.80, p&gt;0.05$</td>
</tr>
<tr>
<td>Molecular cell</td>
<td>$F(1,19)=0.00, p&gt;0.05$</td>
<td>$F(1,19)=4.22, p&lt;0.05$</td>
<td>$F(1,19)=0.55, p&gt;0.05$</td>
</tr>
<tr>
<td>Granule cell</td>
<td>$F(1,19)=0.40, p&gt;0.05$</td>
<td>$F(1,19)=0.31, p&gt;0.05$</td>
<td>$F(1,19)=0.24, p&gt;0.05$</td>
</tr>
<tr>
<td>Polymorphic</td>
<td>$F(1,19)=0.04, p&gt;0.05$</td>
<td>$F(1,19)=4.12, p&lt;0.05$</td>
<td>$F(1,19)=0.97, p&gt;0.05$</td>
</tr>
</tbody>
</table>

*Tests reaching significance with $p<0.05$ are highlighted.*
Figure 4.4: Representative images of $\alpha_1$ subunit IR in the hippocampus. Images are taken from male (a,c) and female (b, d) mice exposed to EH (a,b) and NH (c,d) early-life environmental conditions. Scale 1 mm.

Figure 4.5: The effects of sex and early-life condition on $\alpha_1$ subunit expression by layer in the CA1 and dentate gyrus hippocampal subregions. Data represent mean relative optical density±SEM in optical density units (ODU). Bars represent significant main effects at $p<0.05$ for * early-life condition.
4.3.2. \( \alpha_2 \) Subunit immunoreactivity

4.3.2.1. \( \alpha_2 \) Subunit distribution

Similar to \( \alpha_1 \) subunit expression, \( \alpha_2 \) IR was high in the cortex with expression throughout the frontal (motor), somatosensory and cingulate cortices (see table 4.4). However, the pattern of \( \alpha_2 \) staining was distinguished by its preferential distribution in the outer cortical layers (I-IV) with weaker IR in the deeper cortical layers (V-VI) (see figure 4.6). The \( \alpha_2 \) subunit was abundant in the hippocampus with slightly stronger staining in the dentate gyrus compared with the CA1-CA3 regions. A clear laminar pattern was also observed in the hippocampus for the \( \alpha_2 \) subunit (see figure 4.8) with strong bands of staining distinguishing the cell body layers where \( \alpha_1 \) immunostaining was largely absent (pyramidal cell layers of CA1-CA3 and the granular cell layer of the dentate gyrus), from the more moderately stained dendritic layers. The only layer showing weak \( \alpha_2 \) immunostaining in the hippocampus was the polymorphic cell layer of the dentate gyrus. In the amygdala, \( \alpha_2 \) subunit IR was also more abundant than \( \alpha_1 \) with strong staining in nuclei where \( \alpha_1 \) was weak such as the central nucleus of the amygdala. In the thalamus, the \( \alpha_2 \) subunit was only weakly expressed with the exception of nuclei such as the reticular and the periventricular nuclei where the \( \alpha_1 \) expression was low.

4.3.2.2. \( \alpha_2 \) Subunit regional IR

Table 4.4 shows the effects of early-life condition and sex on the mean \( \alpha_2 \) subunit relative optical density values in various brain regions. Table 4.5 shows the results of the two-way ANOVA (sex x early-life) of this data for each of the brain regions examined. In the cortex, no main effects were observed with the exception of a
significant main effect of early-life stress in the somatosensory cortex where NH mice showed reduced $\alpha_2$ subunit IR relative to EH mice. However, a significant interaction in the somatosensory cortex indicated that effects of early-life stress varied for males and females. Post-hoc analysis showed that whilst NH males had reduced $\alpha_2$ subunit IR compared with EH males (mean difference $25.2\pm 9.4$; $p=0.016$), early-life environment did not affect $\alpha_2$ IR in females ($p=0.763$). EH females also showed reduced $\alpha_2$ IR relative to EH males (mean difference $21.3\pm 9.4$; $p=0.037$) but NH males and females did not vary ($p=0.934$).

In the hippocampus and amygdala early life condition did not affect $\alpha_2$ subunit regional IR (see table 4.5). However, there was a significant main effect of sex in the dentate gyrus with females showing increased $\alpha_2$ subunit IR in this region compared with males regardless of early-life environment (mean difference: EH $13.6\pm 5.5$; NH $11.0\pm 6.7$). There were no other significant main effects of sex in any of the hippocampal or amygdalar regions examined (see table 4.5).

In the thalamus, there was a significant interaction in the lateral-dorsal nucleus but not the ventrolateral nucleus (see table 4.5) indicating that the effects of early-life stress were sex-dependent in this nucleus. Post-hoc analysis showed that whilst NH females did not vary from EH females ($p=0.494$), NH males showed reduced $\alpha_2$ subunit IR relative to EH males (mean difference $14.2\pm 5.3$; $p=0.046$). There were no other sex differences in the EH ($p=0.133$) or NH mice ($p=0.252$).
Table 4.4: Regional optical density scores for the GABA<sub>A</sub> receptor α<sub>2</sub> subunit immunoreactivities by brain region in males and females exposed to EH and NH early-life conditions. Data are given as mean relative OD±SEM (n).

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EH</td>
<td>NH</td>
</tr>
<tr>
<td>CING</td>
<td>90.06±7.4(6)</td>
<td>83.94±4.6(6)</td>
</tr>
<tr>
<td>M1</td>
<td>97.02±7.3(6)</td>
<td>81.79±6.9(6)</td>
</tr>
<tr>
<td>M2</td>
<td>85.42±6.6(6)</td>
<td>80.97±4.1(6)</td>
</tr>
<tr>
<td>S ♠</td>
<td>96.98±7.8(6)*</td>
<td>71.79±2.4(6)</td>
</tr>
<tr>
<td>CA1</td>
<td>77.25±3.7(6)</td>
<td>84.22±3.5(6)</td>
</tr>
<tr>
<td>CA3</td>
<td>89.12±3.6(6)</td>
<td>92.18±8.8(5)</td>
</tr>
<tr>
<td>DG †</td>
<td>98.01±3.8(6)</td>
<td>95.11±8.1(5)</td>
</tr>
<tr>
<td>THAL</td>
<td>55.87±2.4(6)</td>
<td>56.01±1.9(5)</td>
</tr>
<tr>
<td>Lateral</td>
<td>58.97±2.0(6)</td>
<td>45.81±5.1(5)*</td>
</tr>
<tr>
<td>BLa</td>
<td>82.18±3.1(6)</td>
<td>86.57±9.8(6)</td>
</tr>
<tr>
<td>CeA</td>
<td>101.83±6.0(6)</td>
<td>100.34±10.3(6)</td>
</tr>
</tbody>
</table>

Data are given as mean±SEM (n). ♠ p<0.05 for a main effect of sex; † p<0.05 for a main effect of early life; * p<0.05 for an effect of early-life following a significant interaction effect. Abbreviations: Cingulate cortex (CING), primary motor cortex (M1), secondary motor cortex (M2), somatosensory cortex (SS), hippocampus (HIPP), dentate gyrus (DG), thalamus (THAL), ventrolateral thalamic nucleus (VL), lateral-dorsal thalamic nucleus (LD), amygdala (AMYG), basolateral amygdaloid nucleus (BLa), central amygdaloid nucleus (CeA).

Table 4.5: Results of 2-way ANOVA tests for α<sub>2</sub> subunit immunoreactivity in various brain regions. Tests reaching significance with p<0.05 are highlighted.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Sex</th>
<th>Early-life environment</th>
<th>Sex x Early-life environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CING</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=2.49, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=1.47, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=0.37, p&gt;0.05</td>
</tr>
<tr>
<td>M1</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=1.00, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=1.88, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=0.27, p&gt;0.05</td>
</tr>
<tr>
<td>M2</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=0.41, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=0.01, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=0.45, p&gt;0.05</td>
</tr>
<tr>
<td>SS</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=2.20, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=4.52, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=5.58, p&lt;0.05</td>
</tr>
<tr>
<td>CA1</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=2.86, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=2.28, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=0.15, p&gt;0.05</td>
</tr>
<tr>
<td>CA3</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=3.27, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=0.08, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=0.60, p&gt;0.05</td>
</tr>
<tr>
<td>DG</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=6.42, p&lt;0.05</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=0.54, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=0.54, p&gt;0.05</td>
</tr>
<tr>
<td>VL</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=3.19, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=0.14, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=0.18, p&gt;0.05</td>
</tr>
<tr>
<td>LD</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=0.06, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=0.88, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,17)&lt;/sub&gt;=4.42, p&lt;0.05</td>
</tr>
<tr>
<td>Bla</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=0.57, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=0.00, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=0.23, p&gt;0.05</td>
</tr>
<tr>
<td>CeA</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=0.46, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=0.21, p&gt;0.05</td>
<td>F&lt;sub&gt;(1,19)&lt;/sub&gt;=0.20, p&gt;0.05</td>
</tr>
</tbody>
</table>

Abbreviations: Cingulate cortex (CING), primary motor cortex (M1), secondary motor cortex (M2), somatosensory cortex (SS), dentate gyrus (DG), ventrolateral thalamic nucleus (VL), lateral-dorsal thalamic nucleus (LD), amygdala (AMYG), basolateral amygdaloid nucleus (BLa), central amygdaloid nucleus (CeA).
4.3.2.3. \( \alpha_2 \) Subunit IR in cortical laminae

Figures 4.6 and 4.7 show the effects of sex and early-life condition on \( \alpha_2 \) subunit IR in somatosensory cortical layers. Results of the two-way ANOVA are shown in table 4.6. There was a significant main effect of early-life condition on \( \alpha_2 \) subunit IR in layer IV, V and VI, indicating that regardless of sex, NH mice have reduced \( \alpha_2 \) subunit IR compared with EH mice in these layers. The difference between EH and NH mice was of greater magnitude in layer IV of males (layer IV: 21.0±8.7; layer V: 15.9±7.6; layer VI 13.5±8.7) compared with females (layer IV 12.7±9.1; layer V 13.5±6.5; layer VI 10.9±7.0), however, the interaction was not significant. No other main or interaction effects were observed in any cortical laminae. Thus, NH mice showed a reduction in \( \alpha_2 \) subunit IR in cortical layers IV and V and VI relative to EH mice.

![Table 4.6: Results of 2-way ANOVA tests for \( \alpha_2 \) subunit immunoreactivity in cortical laminae.](https://example.com/table4.6.png)

Tests reaching significance with \( p<0.05 \) are highlighted.

<table>
<thead>
<tr>
<th>Cortical Layer</th>
<th>Sex</th>
<th>Early-life environment</th>
<th>Sex x Early-life environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>( F_{(1,17)}=1.97, p&gt;0.05 )</td>
<td>( F_{(1,17)}=4.08, p&gt;0.05 )</td>
<td>( F_{(1,17)}=4.17, p&gt;0.05 )</td>
</tr>
<tr>
<td>II-III</td>
<td>( F_{(1,17)}=0.66, p&gt;0.05 )</td>
<td>( F_{(1,17)}=3.89, p&gt;0.05 )</td>
<td>( F_{(1,17)}=2.53, p&gt;0.05 )</td>
</tr>
<tr>
<td>IV</td>
<td>( F_{(1,17)}=3.23, p&gt;0.05 )</td>
<td>( F_{(1,17)}=7.68, p&lt;0.05 )</td>
<td>( F_{(1,17)}=0.47, p&gt;0.05 )</td>
</tr>
<tr>
<td>V</td>
<td>( F_{(1,17)}=3.49, p&gt;0.05 )</td>
<td>( F_{(1,17)}=5.80, p&lt;0.05 )</td>
<td>( F_{(1,17)}=0.04, p&gt;0.05 )</td>
</tr>
<tr>
<td>VI</td>
<td>( F_{(1,17)}=2.61, p&gt;0.05 )</td>
<td>( F_{(1,17)}=4.43, p&lt;0.05 )</td>
<td>( F_{(1,17)}=0.04, p&gt;0.05 )</td>
</tr>
</tbody>
</table>
Figure 4.6: Representative images of α2 subunit IR in the somatosensory cortex. Images are taken from male (a,b) and female (c,d) mice exposed to EH (a,c) and NH (b, d) early-life environmental conditions. Scale 1mm.

Figure 4.7: The effects of sex and early-life condition on α2 subunit expression by cortical layer. Data represent mean relative optical density±SEM in optical density units (ODU). *p<0.05, **p<0.01 denote significant effects of early-life relative to EH group of same sex following a significant interaction. ^ p<0.05 denotes significant sex difference relative to males from the same early-life condition following a significant interaction. Grouped bars represent significant main effects at p<0.05 for individual layers.
4.3.2.4. $\alpha_2$ Subunit IR in hippocampal laminae

Figures 4.8 and 4.9 show the effects of sex and early-life condition on $\alpha_2$ subunit IR in hippocampal layers. Results of the two-way ANOVA shown in table 4.7 indicated significant main effects of sex in the molecular and polymorphic cell layers of the DG. Thus, regardless of early-life condition, male mice have a small but significant reduction of $\alpha_2$ subunit IR in the molecular (mean sex difference EH 13.1±8.7; NH 20.8±9.8) and polymorphic (mean sex difference EH 14.7±7.2; NH 10.1±6.3) cell layers of the DG compared with females. No main effects of sex were observed in CA1 or the granule cell layer of the DG. Furthermore, no main effects of early-life condition or sex x early-life condition interactions were observed in any hippocampal layers.

<table>
<thead>
<tr>
<th>Hippocampal Layer</th>
<th>Sex</th>
<th>Early-life environment</th>
<th>Sex x Early-life environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stratum oriens</td>
<td>$F_{(1,19)}=5.35$, $p&lt;0.05$</td>
<td>$F_{(1,19)}=0.30$, $p&gt;0.05$</td>
<td>$F_{(1,19)}=0.19$, $p&gt;0.05$</td>
</tr>
<tr>
<td>Pyramidal cell</td>
<td>$F_{(1,19)}=2.26$, $p&gt;0.05$</td>
<td>$F_{(1,19)}=0.00$, $p&gt;0.05$</td>
<td>$F_{(1,19)}=0.03$, $p&gt;0.05$</td>
</tr>
<tr>
<td>Stratum radiatum</td>
<td>$F_{(1,19)}=10.27$, $p&lt;0.05$</td>
<td>$F_{(1,19)}=0.54$, $p&gt;0.05$</td>
<td>$F_{(1,19)}=0.00$, $p&gt;0.05$</td>
</tr>
<tr>
<td>Molecular cell</td>
<td>$F_{(1,19)}=7.10$, $p&lt;0.05$</td>
<td>$F_{(1,19)}=0.65$, $p&gt;0.05$</td>
<td>$F_{(1,19)}=0.76$, $p&gt;0.05$</td>
</tr>
<tr>
<td>Granule cell</td>
<td>$F_{(1,19)}=0.79$, $p&gt;0.05$</td>
<td>$F_{(1,19)}=0.02$, $p&gt;0.05$</td>
<td>$F_{(1,19)}=0.06$, $p&gt;0.05$</td>
</tr>
<tr>
<td>Polymorphic</td>
<td>$F_{(1,19)}=1.67$, $p&gt;0.05$</td>
<td>$F_{(1,19)}=0.03$, $p&gt;0.05$</td>
<td>$F_{(1,19)}=0.09$, $p&gt;0.05$</td>
</tr>
</tbody>
</table>
Figure 4.8: Representative images of $\alpha_2$ subunit IR in the hippocampus. Images are taken from male (a, c) and female (b, d) mice exposed to EH (a, b) and NH (c, d) early-life environmental conditions. Scale 1mm.

Figure 4.9: The effects of sex and early-life condition $\alpha_2$ subunit expression in the CA1 and DG hippocampal subregions. Data represent mean relative optical density±SEM in optical density units (ODU). Grouped bars represent significant main effects of sex for individual layers of the hippocampus at $^\wedge$ p<0.05.
4.4. Discussion

4.4.1. Sex differences in GABA\_A receptor $\alpha$ subunit expression

Results from the present study showed sex differences in GABA\_A receptor $\alpha_1$ and $\alpha_2$ subunit density and distribution, which occurred regardless of early-life condition. In layer II-III of the SS cortex, males were observed to have increased IR for the $\alpha_1$ subunit compared with females. Consistent with this finding, previous work has observed increased $\alpha_1$ subunit mRNA in the SS cortex of male relative to female rats (Li et al., 2007). Findings from the present study using immunohistochemical procedures extend upon the previous RT-PCR study (Li et al., 2007) to suggest that increased $\alpha_1$ subunit in the male SS cortex is either specific to layer II-III of the SS cortex or alternatively, is only translated to differences in protein expression in these outer cortical layers. That sex differences in GABA\_A receptor protein subunit expression may be confined to the outer cortical layers is also consistent with findings in chapter 2, where sex differences in $[^3]$H\_GABA receptor binding sites at GABA\_A receptors were most prominent in the outer cortical layers.

The present study also observed increased $\alpha_2$ subunit IR in the dentate gyrus of females relative to males. Interestingly, findings in chapter 2 suggested that whilst sex differences occurred in the dentate gyrus of the hippocampus, males had a greater number of $[^3]$H\_GABA binding sites at GABA\_A receptors compared with females. In order to consolidate this finding, sex differences in other GABA\_A receptor subunits require investigation, such as the $\alpha_5$ subunit, which is also highly expressed in this region (Crestani et al., 2002).
It is important to note that most regions examined in the present study showed no sex differences in the IR for either the \( \alpha_1 \) or \( \alpha_2 \) GABA\(_A\) receptor subunits. There are limited studies examining \( \alpha_1 \) and \( \alpha_2 \) subunit expression in both adult males and females, with most previous studies of sex differences focussing on the less abundant subunits GABA\(_A\) receptor subunits such as the \( \alpha_4 \) and \( \delta \) subunits (Gallo and Smith, 1993; Guinello et al., 2003; Lovick et al., 2005; Smith et al., 1998; Sundstrom-Poromaa et al., 2002). Despite this, one previous study also observed no sex differences in \( \alpha_1 \) or \( \alpha_2 \) immunoreactivity in the CA1 region of the hippocampus, the amygdala and the thalamus (Davis et al., 2000), which is consistent with findings of the present study. Thus, the density of the \( \alpha_1 \) and \( \alpha_2 \) subunits appear to be largely conserved across most brain regions.

Whilst the sex differences in \( \alpha_1 \) and \( \alpha_2 \) subunit expression were limited, any differences in the expression of these subunits would be important to brain function and behaviour. Previous studies have demonstrated that \( \alpha_1 \) and \( \alpha_2 \) subunits mediate different behavioural effects of benzodiazepines (Fritschy and Brunig, 2003; Mohler et al., 2001; Rudolph et al., 1999) and affect both GABA\(_A\) receptor pharmacological sensitivities and channel conductance times (Bosman et al., 2002; Brooks-Kayal and Pritchett, 1993; Juttner et al., 2001; Kapur and MacDonald, 1999; Okada et al., 2000). Thus, sex differences in the relative amounts of the \( \alpha_1 \) and \( \alpha_2 \) subunit proteins may contribute to the sex differences observed in the behavioural effects of GABA\(_A\) receptor compounds such as ethanol (Crippens et al., 1999; Tayyabkhan et al., 2002; Webb et al., 2002; Wilson et al., 2004), allopregnanalone (Fernandez-Gausti and Picazo, 1997; 1999; Guinello and Smith, 2003) and diazepam (Fernandez-Gausti and Picazzo, 1990; 1997; Fernandez-Gausti and Picazzo, 1999; Wilson et al., 2004). Furthermore, future
work should examine whether males and females vary in their responses to the α₁-selective compounds such as zolpidem.

4.4.2. Effects of early-life stress on GABA<sub>A</sub> receptor α subunit IR

4.4.2.1. Effects of early-life stress on adult male GABA<sub>A</sub> receptor α subunit IR

Findings of the present study indicate long-term effects of early life stress on GABA<sub>A</sub> receptor α subunit protein regional densities in males. In adulthood, NH males showed reduced α₂ subunit expression in layers I, IV, V and VI of the SS cortex and the lateral-dorsal thalamic nucleus relative to EH males. This is the first study to show an effect of early-life environment on α subunit expression in the male SS cortex and thalamus. Additionally, reduced α₁ subunit expression in the polymorphic and molecular cell layers of the dentate gyrus was also observed in NH males compared with EH males consistent with a previous study of α₁ subunit mRNA expression in the DG (Hsu et al., 2003). However, the authors of this previous study also observed effects of early-life stress on α₂ subunit mRNA in the adult male DG, which is in contrast to the present study where no differences according to early-life condition were observed for the α₂ subunit IR in this region. Hsu et al. (2003) also used a different early-life stress procedure to the present study, involving a comparison of facility-reared controls (AFR) with maternally separated (MS) rats. Given that it is difficult to anticipate the specific handling procedures of the AFR group and the maternal separations performed were on specific days (PND 9-10) during the post-natal period, whilst the present study involved variations across the entire first 2 post-natal weeks, the findings of Hsu et al. (2003) may not be directly comparable with those of the present study. However, it is also possible that changes in α₂ mRNA in the DG that
were observed by Hsu et al. (2003) are not translated to changes in protein density and thus not observed in the present study. Alternatively, the semi-quantitative analysis of the relative staining OD to compare subunit density between groups may not be as sensitive to subunit changes as the rt-PCR technique used by Hsu et al. (2003). Thus at least for an EH-NH early-life model it appears that $\alpha_2$ subunit protein expression is not affected in the DG.

No early-life environment-induced changes were observed in the $\alpha_1$ or $\alpha_2$ subunit protein density or distribution in any other brain regions. The regionally limited effects of early-life stress observed for the $\alpha_1$ subunit in the present study are consistent with a previous study showing that zolpidem binding at $\alpha_1$ subunit-containing GABA$_A$ receptors did not vary between EH and NH groups in the frontal cortex and amygdala (Caldji et al., 2000b). As previous studies have only examined $\alpha_2$ subunit changes following early-life environmental stressors in the DG (Hsu et al., 2003), the present study is the first to suggest that the effects of early-life stress on the $\alpha_2$ subunit are regionally dependent.

### 4.4.2.2. Effects of early-life stress on adult female GABA$_A$ receptor $\alpha$ subunit IR

Findings of the present study also indicated long-term effects of early-life stress on GABA$_A$ receptor $\alpha$ subunit protein densities in females. Similarly to males, adult NH females showed a reduction in $\alpha_2$ subunit density in the lower SS cortical layers as well as a reduction in $\alpha_1$ subunit density in the polymorphic and molecular cell layers of the DG relative to EH females. NH females also showed an increase in $\alpha_1$ subunit density in the lower layers (IV, V, VI) of the SS cortex. To our knowledge no previous studies have examined the effects of early-life environment on female $\alpha$ subunit
expression in adulthood and thus, this is the first study to indicate long-term changes occur in females. The changes observed here in the female brain occurred in similar regions to males, with the exception of the laterodorsal nucleus of the thalamus. Thus, the present study indicates subtle changes in the environment of early-life affect the adulthood expression of GABA_\text{A} receptor \( \alpha \) subunits in similar regions in both males and females.

4.4.3. Neurodevelopmental disruption and long-term effects of early-life stress

Whilst the present study did not examine GABA_\text{A} receptor subunit expression at different developmental time-points, and so cannot causally relate adulthood changes in \( \alpha \) subunit expression to a disruption of the developmental changes in GABA_\text{A} receptors, the regional dependence of the effects observed in the present study are consistent with disruptions of the \( \alpha \) subunit developmental reshuffling that occurs over the first few postnatal weeks. The \( \alpha_2 \) subunit was only affected in the adult male lateral-dorsal thalamus and lower cortical layers, the two major regions undergoing the most prominent reductions in this subunit during development (Fritschy et al., 1994). The fact that \( \alpha_2 \) expression in the supragranular layers of the SS cortex, which undergo only small reductions in \( \alpha_2 \) subunit expression during development (Fritschy et al., 1994), were not affected by early-life environment, supports the hypothesis that early-life effects on adulthood \( \alpha \) subunit expression may be mediated via a disruption of the developmental reshuffling of these subunits. In females the lower cortical layers of the SS cortex also showed changes in \( \alpha \) subunit expression with both the \( \alpha_1 \) and \( \alpha_2 \) subunits being affected but in opposite directions. This suggests that both the developmental increase in the \( \alpha_1 \) subunit and decrease in the \( \alpha_2 \) subunit may be disrupted in females.
Interestingly, if early-life effects on adulthood $\alpha$ subunit expression are mediated via a disruption of the normal developmental changes in these subunits, then results of the present study would then suggest that the early-life stress condition (NH group), exaggerates the developmental changes in the expression of these subunits that occur during development.

The subunit switch has been associated with important developmental properties and a developmental disruption of this process would suggest more extensive neurodevelopmental aberrations. For example, the $\alpha_2:\alpha_1$ subunit switch has been shown to occur in a similar fashion in a number of species including rodents, primates and humans, and across sexes (Brooks-Kayal and Pritchett, 1993; Davis et al., 2000; Fritschy et al., 1994; Hendrickson et al., 1994; Hornung and Fritschy, 1996; McKernan et al., 1991; Reichelt et al., 1991), suggesting that the occurrence of the switch is important in mammalian brain development. Indeed, studies have implicated the subunit switch in synapse formation (Hornung and Fritschy, 1996) and/or maturation (Hendrickson et al., 1994), with evidence from $\alpha_1$ subunit knockout mice indicating the switch aids the process of axonal sorting and synaptic consolidation (Heinen et al., 2003; Hendrickson et al., 1994). Thus, the different adulthood $\alpha_1:\alpha_2$ subunit ratios observed in the present work as a result of early-life stress, may represent disruptions to a range of important neurodevelopmental processes that have been associated with the developmental $\alpha$ subunit switch.

4.4.4. Relevance of altered GABA$_A$ receptor $\alpha$ subunit expression in adulthood

The present study expands on work of others suggesting that handling-induced behavioural changes may be mediated by alterations in GABA$_A$ receptors (Bodnoff et
C H A P T E R  4

al., 1987; Bolden et al., 1990; Caldji et al., 2000a; Caldji et al., 2000b). Unfortunately, it is difficult to differentiate between differences in GABA\textsubscript{A} receptor subunit expression and a loss in the number of GABAergic cells within a region using GABA\textsubscript{A} receptor immunohistochemistry (Yu et al., 2006). Nonetheless, it is thought that the expression ratio between different subunits is a more accurate predictor of inhibitory tone in a region (Brooks-Kayal et al., 1998; Brooks-Kayal et al., 2001).

The \( \alpha_1 \) and \( \alpha_2 \) subunits are abundant in the regions where they were affected by early-life environment and so it is likely that alterations in their expression would affect adulthood GABAergic function. In this study the NH group show an increased \( \alpha_1: \alpha_2 \) ratio in a regionally specific fashion whilst EH group showed a reduced ratio of \( \alpha_1: \alpha_2 \) subunits. It is well documented that different GABA\textsubscript{A} receptor subtypes exhibit distinct pharmacological and electrophysiological properties (Brooks-Kayal et al., 2001; Mohler et al., 2001). For example, previous studies have demonstrated that distinct GABA\textsubscript{A} receptor subunits are associated with different behavioural effects of benzodiazepines, with the \( \alpha_1 \) subunit being associated with the sedative, amnesic, and anticonvulsant actions, whilst \( \alpha_2, \alpha_3, \) and \( \alpha_5 \) subunits are thought to mediate the anxiolytic effects of these drugs (Fritschy and Brunig, 2003; Mohler et al., 2001; Rudolph et al., 1999). Furthermore, \( \alpha_2 \) subunit-containing receptors show slower decay times and greater mIPSP current amplitudes than \( \alpha_1 \) receptors (Bosman et al., 2002; Heinen et al., 2004; Hollrigel and Soltesz, 1997; Hutcheon et al., 2000; Juttner et al., 2001; Okada et al., 2000; Ortinski et al., 2004; Taketo and Yoshioka, 2000; Vicini et al., 2001). These longer decay times are thought to support enhanced synaptic efficacy that is associated with anxiolysis in animals with greater \( \alpha_2: \alpha_1 \) subunit ratios (Franks and Lieb, 1994). Thus, the increased \( \alpha_1: \alpha_2 \) subunit ratio observed in both the male and female groups
exposed to an NH relative to the EH early-life condition is consistent with the increased adulthood anxiety in NH groups compared with EH groups that was reported in chapter 3. Early-life induced alterations in the adulthood $\alpha_1: \alpha_2$ subunit ratios observed in the present study therefore support behavioural findings that the NH manipulation produces a more anxious adulthood behavioural phenotype than the EH manipulation.

4.4.5. Early-life stress and GABA$_A$ receptor disturbances in psychiatric disorders

The effects of early-life stress on GABA$_A$ receptor $\alpha_1$ and $\alpha_2$ subunit protein density observed in the present study may be relevant to understanding GABA$_A$ receptor changes that are seen in psychiatric illnesses. In schizophrenia, studies have shown increased $\alpha_1$ and $\alpha_2$ subunit protein expression in the PFC of schizophrenic subjects (Ishikawa et al., 2004; Pesold et al., 1998; Volk et al., 2002), whilst in depression reduced $\alpha_1$, $\alpha_3$, $\alpha_4$ and $\delta$ subunit mRNA expression is seen in the frontopolar cortex of depressed suicides (Merali et al., 2004). Given that the present study showed effects of early-life stress on $\alpha$ subunit expression were only evident in the SS cortex, laterodorsal thalamus and certain hippocampal laminae, it is evident that GABA$_A$ receptor pathologies observed in psychiatric illnesses are unlikely to arise solely from early-life stress. However, there are a number of discrepancies in the literature regarding how GABA$_A$ receptors are affected in depression, perhaps due to the fact that different studies come from subjects dying of varying methods of suicide (Pandey et al., 1997), meaning there are still uncertainties regarding the nature of GABA$_A$ receptor changes in the depressed brain. Furthermore, as diseases such as schizophrenia and depression occur on a background of genetic disturbances and are associated with not only early-life but also stress in early-adulthood prior to symptom manifestation (McGrath et al., 2003), and adulthood stress is known to affect GABA$_A$ receptors (see chapter 2), it
is likely that the changes in GABA_A receptors observed post-mortem in such diseases reflect a variety of these factors making it difficult to ascertain the contribution of any one factor alone.

Early-life stress may also be involved in changes in GABA_A receptors that occur in anxiety disorders. For example, given the role of the \( \alpha_2 \) subunit in mediation of anxiolytic effects of GABAergic compounds (Fritschy and Brunig, 2003; Mohler et al., 2001; Rudolph et al., 1999), it is interesting to speculate that a reduction in the \( \alpha_2 \) subunit in the brains of adult animals exposed to early-life stress may be relevant to the pathology of human anxiety type disorders. However, there is currently no information on how the \( \alpha_2 \) subunit is affected in anxiety disorders such as PTSD, panic disorder and generalised anxiety disorder. Furthermore, in animal models of anxiety-disorders, abnormal cue discrimination, which is associated with pathological anxiety in humans, has been better associated with a change in the subcellular locations of GABA_A receptors, namely, a loss of synaptic clustering of GABA_A receptors. Thus, in the next chapter (chapter 5) the effects of early-life stress on GABA_A receptor synaptic clustering shall be examined.

4.4.6. Conclusions

Findings of the present study indicated region-dependent sex differences and long-term effects of early-life stress on GABA_A receptor \( \alpha \) subunit expression. The \( \alpha_1 \) and \( \alpha_2 \) subunit expression in males and females was largely conserved across most brain regions with the exception of layer II-III of the SS cortex and the dentate gyrus. Early-life stress produced long term effects on the adult \( \alpha_1: \alpha_2 \) subunit ratios of the lower cortical layers where the NH group showed an increased \( \alpha_1: \alpha_2 \) subunit ratio as a
result of reduced $\alpha_2$ expression in males and a combined reduction of $\alpha_2$ and increase of $\alpha_1$ expression in females. The increased $\alpha_1:\alpha_2$ subunit ratio observed in NH animals is consistent with the enhanced behavioural anxiety reported for these animals in chapter 3, however it is surprising that this was observed in the somatosensory cortex, a region that is not traditionally associated with anxiety. Nonetheless, the regional dependence of alterations in the $\alpha_1:\alpha_2$ subunit ratio that was observed in the present study is consistent with the region-dependent variations in $\alpha_1:\alpha_2$ subunit expression during the first two post-natal weeks. Thus it is proposed that early-life environmental manipulations over the first two post-natal weeks exert long-term effects on GABA$_A$ receptors via disruptions of the $\alpha$ subunit developmental reshuffling that occurs during the same period.
CHAPTER 5: Effects of Early-Life Stress on GABA\(_A\) Receptor Synaptic Clustering

5.1. Introduction

5.1.1. Background

As shown in the previous study (chapter 4), early-life stress alters the ratio of \(\alpha_1:\alpha_2\) subunit protein expression in a region-dependent fashion, consistent with long-lasting changes in behaviour. However, both brain function and behaviour may be affected not only by changes in the regional protein expression of subunits for GABA\(_A\) receptors, but also by the sub-cellular distribution of these receptors (Chhatwal et al., 2005; Crestani et al., 1999; Levi et al., 2004). The aggregation of receptors beneath inhibitory terminals is required for fast or phasic signal transmission at synapses and variations in the amount of synaptic GABA\(_A\) receptors affect post-synaptic membrane currents (Levi et al., 2004). Thus, in order to gain better insight into the neurophysiological changes that accompany the behavioural differences between EH and NH mice that were reported in chapter 3, it is important to determine if these different early-life conditions may also exert long-lasting changes on subcellular distributions of GABA\(_A\) receptors.

GABA\(_A\) receptor clustering on the post-synaptic membrane is associated with the protein gephyrin (Fritschy et al., 2008; Fritschy et al., 2003; Kneussel and Betz; 2000; Sassoe-Pognetto and Fritschy, 2000). Whilst the role of this protein in GABA\(_A\) receptor synaptic clustering is not well understood (see section 1.3.4), several lines of evidence support the use of gephyrin as a synaptic marker in vivo. For example, gephyrin is enriched at post-synaptic sites of GABAergic synapses throughout the brain and spinal cord (Bolthaler et al., 1994; Cabot et al., 1995; Craig et al., 1996; Giustetto et al.,
1998; Sassoe-Pognetto et al., 1995; Todd et al., 1996; Triller et al., 1987), and a disruption in gephyrin expression via gene knockout or mRNA inhibition results in an impairment of GABA_\text{A} receptor \(\alpha_2\) and \(\gamma_2\) subunit post-synaptic clustering (Essrich et al., 1998; Fisher et al., 2000; Kneussel et al., 1999b; Levi et al., 2004; Yu et al., 2007).

Thus, gephyrin is presumed to provide an indication of the synaptic location of at least the \(\alpha_2\) and \(\gamma_2\) subunit-containing GABA_\text{A} receptors (Essrich et al., 1998; Jacob et al., 2005; Kneussel et al., 1999b; Levi et al., 2004), and alterations in the extent to which these proteins colocalise with gephyrin can indicate changes in GABA_\text{A} receptor synaptic clustering.

Previous studies have observed that alterations in GABA_\text{A} receptor synaptic clustering result in variations in brain function and behaviour. In particular, a reduction in colocalisation of the \(\alpha_2\) subunit with gephyrin in the hippocampus (CA1 and DG) of mice heterozygous for the GABA_\text{A} receptor \(\gamma_2\) subunit, was observed to result in enhanced anxiety, enhanced behavioural reactivity, a behavioural bias for threat cues and enhanced fear conditioning (Crestani et al., 1999). This behavioural phenotype is similar to that which has been well documented for the EH-NH early-life stress model where NH mice show increased behavioural reactivity and anxiety relative to the EH group (reviewed in section 1.7.3; and see Chapillon et al., 2002; Levine, 2000; Meaney et al., 2001; Pryce and Feldon, 2003; Pryce et al., 2002). Thus it is of interest to examine the effects of early-life environment on GABA_\text{A} receptor synaptic clustering.

GABA_\text{A} receptor synaptic clustering is particularly likely to be sensitive to early-life environmental manipulations. Recruitment of GABA_\text{A} receptors to clusters and the formation of synapses is largely post-natal in rodents, occurring at or about the same time as the protein switch from \(\alpha_2\) to \(\alpha_1\) subunits and the functional switch from
an excitatory to an inhibitory role of this neurotransmitter (Fritschy et al., 1994; Hutcheon et al., 2004; Laurie et al., 1992; Poulter et al., 1992; Viltono et al., 2008). An effect of early-life stress on such developmental processes would be of relevance to neurodevelopmental psychiatric disorders that have been associated with early-life environmental factors such as schizophrenia.

5.1.2. Aims

The aim of this study was to examine the effects of early-life stress and sex on the clustering of GABA<sub>A</sub> receptors and their cellular location. To examine GABA<sub>A</sub> receptor clustering the α<sub>2</sub> subunit and gephyrin proteins were immunofluorescently labelled on the same tissue sections. Individual protein cluster properties (size, number, area) and the extent of colocalisation of the two proteins was measured in male and female mice exposed to either EH or NH early-life conditions. Measurements were taken from confocal images of the hippocampus as this region has been investigated most rigorously in previous studies relating GABA<sub>A</sub> receptor synaptic clustering to behaviour. Furthermore, as we wanted to examine specific effects on receptor clustering that were not confounded by alterations in protein expression, measurements were taken from the granule cell layer of the dentate gyrus, a region observed in the previous study (see chapter 4) to have equivalent α<sub>2</sub> IR across sexes and early-life manipulation conditions. The present investigation will provide insight into the effects of early-life stress on another aspect of GABA<sub>A</sub> receptor expression that is associated with brain function and behaviour and thus may provide insight into the neurophysiological correlates of the long-term behavioural differences of the EH and NH groups (see chapter 3).
5.2. Materials and Methods: Double-labelling immunofluorescence

5.2.1. Materials

5.2.1.1. General materials

General materials used are those described in section 4.2.1.1.

5.2.1.2. Immunofluorescence materials

VECTASHIELD anti-fade fluorescent mounting medium was purchased from Vector Laboratories (Burlingame, CA, USA).

5.2.1.3. Primary antibodies

i) $\alpha_2$ primary antibody

At the time of experimentation no guinea pig raised anti-GABA$_A$ receptor $\alpha_2$ subunit antibody, as described in section 4.2.5.1 (part i), was available for use and the only commercially available source with proven specificity was a rabbit raised IgG.

The polyclonal rabbit anti-GABAAR $\alpha_2$ subunit antibody (batch # AN-01; 0.8mg/mL) was obtained from Alomone Labs (Jerusalem, Israel). The affinity purified IgG was raised in rabbits immunised with the synthetic peptide sequence ((C)TPEPNKKPENKPA) corresponding to amino acids 393-405 at the C-terminal (cytoplasmic region) of the rat GABA$_A$ receptor $\alpha_2$ subunit. This antibody has been characterized by Western blotting of rat brain membranes recognizing a single protein band with the appropriate molecular weight and by immunohistochemistry on mouse cerebellum (manufacturer's technical information). Antigen pre-absorption studies on our mouse tissue using a sample of the antigen supplied by the manufacturer resulted in the loss of specific immunoreactivity. Furthermore, immunofluorescent staining
throughout the cortex, hippocampus and thalamus produced a pattern of $\alpha_2$ subunit immunoreactivity that was identical with previous descriptions for the $\alpha_2$ subunit (Fritschy and Mohler, 1995).

**ii) Gephyrin primary antibody**

The monoclonal mouse antibody (clone mAB7a; 1$\mu$g/$\mu$L) was imported from Synaptic Systems (Göttingen, Germany). The affinity purified IgG$1$ was raised against purified rat gephyrin and is known to identify the brain specific 93 kDa splice variant of gephyrin in rat, mouse, human, pig and goldfish tissue (company data sheet). This antibody has been extensively characterized by immunohistochemistry on mouse (Baer *et al.*, 2000; Crestani *et al.*, 1999; Kneussel *et al.*, 1999a; Kneussel *et al.*, 1999b; Kralic *et al.*, 2006; Studer *et al.*, 2006) and rat (Fritschy *et al.*, 1998; Hermann *et al.*, 2001) tissue. The mAB7a clone is also observed to colocalise with the anti-gephyrin clone against the c-terminus of the gephyrin protein in mouse cerebellum (Sassoe-Pognetto company product page). The staining procedure described for the current experiments, produced a pattern of gephyrin immunoreactivity identical with previous descriptions (Crestani *et al.*, 1999; Fritschy *et al.*, 1998).

**5.2.1.4. Secondary antibodies**

Secondary antibodies conjugated to Alexa Fluor® fluorescent dyes were purchased from Molecular Probes (Eugene, Oregon, USA). Alexa Fluor® fluorescent dyes were chosen as they provide high absorbance and output, narrow spectral bandwidths (see section 5.2.2.5i - Visualisation of fluorescence) and are particularly photostable compared with other fluorescent dye conjugates (e.g. fluorescein, Texas
red, CY3) (Invitrogen Guide to Fluorescence Handbook). To label the rabbit primary antibody against the $\alpha_2$ subunit of the GABA$_A$ receptor an anti-rabbit IgG conjugated to the Alexa Fluor® 594 dye was used. To label the mouse primary antibody directed against gephyrin an anti-mouse IgG conjugated to the Alexa Fluor® 488 dye was used. Both secondary antibodies were raised in goats and directed against affinity purified IgG’s (from either rabbit or mice) resulting in specific reactivity with IgG heavy chains and all immunoglobulin light chains (H+L) of IgG’s from the species they were raised against. To minimise cross reactivity, experimenter contamination, or the binding of secondary antibodies to one another, each secondary antibody was highly cross-adsorbed. That is, they were adsorbed against human IgG and human serum, as well as goat, bovine, and rat serum. The anti-rabbit secondary antibody (intended to label the rabbit antibody against the $\alpha_2$ subunit) was also adsorbed against mouse IgG to reduce non-specific binding to endogenous mouse IgG or the mouse raised gephyrin antibody.

5.2.2. Methods

5.2.2.1. Subjects

Subjects are those described in section 3.2.2. Briefly, as described in section 4.2.2.1, subjects were male (n=13) and female (n=11) Quackenbush Swiss (QS) albino mice, born in the animal house and exposed to either EH or NH early-life conditions over PND1-14. Mice were not exposed to adulthood swim stress and were those described as controls in section 3.2.2.6. Thus, the following groups were examined in this chapter: Males NH n=7, EH n=6; Females NH n=5, EH n=6.
5.2.2.2. Tissue preparation and fixation

Tissue was collected and sectioned as described in section 3.2.2.7 and briefly post-fixed in 4% paraformaldehyde for 5 minutes as described in section 4.2.2.2.

5.2.2.3. Experimental design

i) Replicates

All staining was performed on sections thaw-mounted onto slides using the general principles described in the section 4.2.3.2 - Staining of sections on slides. For each animal 2 replicate slides (6 sections per slide) from between bregma -0.55 and -2.54 mm (Paxinos and Franklin, 2001), were double-labelled in separate experiments.

ii) Pilot experiments: Confirming specificity of double-labelling procedure

Pilot studies were first performed to optimise staining for each antibody individually. Negative isotype control experiments were performed concurrently to monitor secondary antibody specificity. In negative control experiments, the IgG concentration of the primary antibody was replaced with rabbit IgG (Dako, Carpenteria, CA, USA) for the α2 antibody, or mouse IgG1 (Dako, Carpenteria, CA, USA) for the gephyrin antibody. These negative control slides did not produce fluorescent signals above the background autofluorescence when each secondary antibody was used at a concentration of 1:1000.

Once single-labelling fluorescence was optimised for each antigen, simultaneous addition of primary antibodies then secondary antibodies was examined. Concurrent negative isotype control slides, were performed to determine if secondary antibodies were cross-reacting with primary antibodies raised in a different species. In these
experiments the primary antibody solution (containing both gephyrin and $\alpha_2$ subunit antibodies) was replaced with a negative isotype control solution, containing equal rabbit and mouse IgG concentrations as the $\alpha_2$ and gephyrin antibodies, respectively. Simultaneous addition of antibodies did not alter the staining properties observed in the single-labelling experiments. The specificity of the simultaneous double-labelling technique was confirmed by ensuring no staining occurred on single-labelled slides where the secondary antibody was replaced with that intended to label the other primary antibody. Thus, a simultaneous double-labelling procedure was used in final experiments.

5.2.2.4. Double-labelling immunofluorescence staining procedure

i) Buffer

Buffers were compared in pilot studies in an attempt to reduce background staining observed with both $\alpha_2$ and gephyrin immunofluorescence staining individually. 0.1 M Tris-buffered saline (TBS) pH 7.4 was found to provide optimal staining across all protocols compared with 0.01 M phosphate buffered saline (PBS) pH 7.4. Thus 0.1 M TBS pH 7.4 was used throughout the procedures for washing slides and reagent dilution.

ii) Antigen retrieval

Antigen retrieval was performed as described in section 4.2.5.2 (see part i).
iii) Incubation with primary antibodies

In pilot studies, the primary antibodies were individually titrated 1:50-1:2000 against their secondary antibody solution (both diluted 1:1000) to establish working concentrations that were then adjusted for the simultaneous addition of antibodies in the double-labelling procedure. The final dilution factor of the $\alpha_2$ primary antibody was 1:100 and for the gephyrin primary antibody it was 1:300. Primary antibodies were diluted in 0.1 M TBS containing 1% v/v BSA and 0.2% v/v triton-X 100. The primary antibody solution (300 $\mu$L per slide) was then incubated with tissue sections at 4°C for 22 hours in a humidity chamber. At the end of the incubation, the solution was tipped off and tissue was washed three times for 10 minutes each time in 0.1 M TBS.

iv) Incubation with secondary antibodies

The anti-rabbit and anti-mouse fluorophore-conjugated secondary antibodies were both diluted 1:1000 in TBS with 1% BSA and 0.2% v/v triton-X 100. The secondary antibody solution was then incubated with tissue sections at room temperature for 1 hour in a “light-tight” humidity chamber, as per the manufacturer’s instructions. Following incubation, the secondary antibody solution was tipped from the slides and excess solution removed by three 10 minute washes in 0.1 M TBS.

v) Coverslipping Slides

The aqueous VECTASHIELD anti-fade fluorescent mountant (Vector Laboratories, Burlinghame, CA, USA) was used as previous studies indicate it minimises photobleaching during fluorescence microscopy and slows the rate of fading during long term storage without quenching fluorescent emission (Florijn et al., 1995).
The anti-fade mountant was applied to each slide following the final washes and sections were coverslipped. Clear nail polish was then applied to edge of the coverslip on the slide to seal the water-soluble mountant. Once nail polish dried, sections were stored in microscopic slide folders at 4°C.

5.2.2.5. Image capture and analysis

i) Fluorescence visualisation and fluorophore selection

Visualisation of fluorescent molecules occurs in three stages. Fluorescent molecules absorb energy of a particular wavelength resulting in their excitation to a higher energy state. The excited state of the molecule exists only briefly before it returns to ground state, emitting energy at a particular wavelength in the process that is visualised as fluorescence. Due to loss of energy during the excitation state, emitted light has a longer wavelength than that which is initially absorbed. The excitation and emission process occurs continuously unless molecules are destroyed, usually when exposed to light of high intensity or over prolonged periods - referred to as photobleaching.

For a fluorescent molecule in solution, light is absorbed and emitted over a spectral bandwidth referred to as absorption and emission spectra which are provided in figure 5.1 for the Alexa fluor 594 and 488 dyes used in this experiment. Given these spectral bandwidths, the microscope optics used for visualisation and image capture were set-up such that the different photomultiplier tube detectors of the microscope received emission from only a single dye. For visualisation of the Alexa 488 dye an argon laser, producing laser lines at 458, 477, 488, 514 nm wavelengths (argon 2 458, 477, 488, 514), was directed to the specimen via a beamsplitter (HFT 405, 488, 561)
selecting for the 488 nm laser line, which excites only the Alexa 488 dye (see figure 5.1). Emission from the Alexa 488 dye was directed to a single detector channel via successive beamsplitters rejecting light of longer wavelength than 565 nm (NFT 565) and of shorter wavelength than 490 nm (NFT 490), followed by a 505-550 nm bandpass filter selecting for light in the range of the Alexa 488 emission spectra only. For visualisation of the Alexa 594 dye, a diode pumped solid state laser producing a laser line at 561 nm (DPSS 561), falling in the excitation bandwidth of the Alexa 594 dye, was directed to the specimen (see figure 5.1). Emission from the Alexa 594 dye was then directed to a different detector to that of the Alexa 488 dye via a beamsplitter accepting light of longer wavelength than 565 nm (NFT 565), and a bandpass filter selecting for light above 575 nm.

![Figure 5.1: Excitation and emission spectra for fluorescent dyes Alexa Fluor 488 and Alexa Fluor 594.](image)

Figure 5.1: Excitation and emission spectra for fluorescent dyes Alexa Fluor 488 and Alexa Fluor 594. Absorption spectra are represented as dashed lines, whilst emission spectra are represented by filled lines. Green lines show the Alexa 488 spectra, whilst red lines show the Alexa 594 spectra. Laser lines for excitation of dyes are shown as vertical lines at 488 nm and 561 nm. Detected bandwidths are shown as translucent bandwidths overlapping emission spectra, in green for Alexa Fluor 488, and red for Alexa Fluor 594. Image created using the Invitrogen fluorescence spectra viewer tool available online at www.invitrogen.com.
ii) Confocal microscopy

Confocal microscopy was used for fluorescence visualisation and image capture as it provides enhanced resolution in the lateral (x,y) and vertical (z) planes by reducing interference from out of focus light. Confocal microscopy is particularly useful for double-labelling experiments as the provision of optical sectioning along the z-axis reduces the incidence of overlapping signals being detected from fluorophores at different depths. Confocal microscopes reduce the detection of out-of-focus light through the use of pinholes to focus light beams produced by the laser, as well as scanning mirrors, which provide point by point illumination of the specimen at a particular depth. In comparison, conventional fluorescent microscopes use extended light sources, which broadly illuminate the entire specimen simultaneously resulting in interference from out-of-focus light in the lateral and vertical planes.

iii) Image capture

Images of the molecular cell layer of the dentate gyrus were captured on a Zeiss inverted confocal microscope (LSM 510) into the LSM 510 image capture software (Carl Zeiss, Thornwood, NY, USA). Prior to image capture a range of sections from each group was examined to establish the minimal laser intensity for each channel that was required to observe fluorescence in stained sections. The gain and offset of the photomultiplier tubes for each channel were then adjusted such that the brightest sections were not saturated (gain) and isotype controls provided no signal (offset). Once these conditions were established they were kept constant for the capture of all images.
Images were captured using a sequential acquisition procedure, where fluorophores were individually excited and detected, to avoid crosstalk between the fluorescent channels. Stacks of 6-10 confocal sections spaced by 380 nm at a 1 x optical zoom factor were acquired from the brightest portion of the section. All images were acquired at a depth of 8 bits with a resolution of 2048 x 2048 pixels and a magnification of 70 nm/pixel using a 63 x oil immersion lens (numerical aperture 1.4) and a pinhole set at 1 Airy unit. Criteria for pixel size (70 nm/pixel i.e. 1 pixel = 0.0049 \( \mu \text{m}^2 \)) was selected based on previous investigations investigating the size range of clusters for a similar experiment (Christie \textit{et al.}, 2002; Marty \textit{et al.}, 2004; Sassoe-Pognetto \textit{et al.}, 2000) and the Nyquist criterion, which requires 2.3 pixels to digitally sample a minimum resolved distance.

\textit{iv) Image analysis}

For quantification, single confocal images judged to be the brightest in each stack were processed using the Image J 1.40 software (National Institutes of Health, USA - available online at http://rsb.info.nih.gov/ij/). Four images from each hemisphere were analysed per animal as described previously (Crestani \textit{et al.}, 1999; Koksma \textit{et al.}, 2005). Overlaid images were split into two 8-bit greyscale images of the individual red and green channels. Pairs of these 8-bit greyscale images were then processed with a colocalisation algorithm provided as a plugin (“colocalization.class”) for the ImageJ program. This plugin produces a binary image displaying all the pixels above a user-defined segmentation threshold (30% of maximal intensity for each channel). The threshold for each channel was based on criteria of Koksma \textit{et al.} (2005) that is, to minimise the inclusion of single-labelled grainy structures in the analysis. These
thresholds were then applied to the images from each channel and the three resulting binary images (red channel, green channel, colocalised pixels) were converted to a stack. From this stack the molecular cell layer of the dentate gyrus was outlined as a region of interest and cluster properties (size, area and number) within this region were analysed using the Image J ‘particle analysis’ algorithm, where the minimal cluster size was defined as 150 nm² (3 adjacent pixels).

5.2.2.6. Statistical analysis

All statistical analyses were performed using SPSS V15.0 (SPSS, Inc., Chicago, Ill., USA). For each of the features of the α₂, gephyrin and colocalised gephyrin clusters (number, size, area covered) and the % area colocalised for each of the α₂ and gephyrin proteins, a between-subjects type-III two-way ANOVA was conducted to examine the effects of sex and early-life environment.
5.3. Results

5.3.1. $\alpha_2$ Subunit-containing GABA$_A$ receptor clusters

Figure 5.2 shows the effects of sex and early-life condition on $\alpha_2$ subunit cluster properties in the granule cell layer of the dentate gyrus of the hippocampus. Results of the two-way ANOVA shown in table 5.1 indicate that whilst the number of clusters counted per 1000 $\mu$m$^2$ was not significantly different between EH and NH conditions, there was a significant main effect of early-life stress on the size of $\alpha_2$ subunit clusters ($F_{(1,16)}=9.59$, $p<0.01$) and the area covered by $\alpha_2$ subunit clusters ($F_{(1,16)}=11.30$, $p<0.01$). Thus, regardless of sex, $\alpha_2$ subunit clusters of NH compared with EH mice are reduced in size (mean difference between early-life conditions for males: 0.22±0.08 $\mu$m$^2$; and females: 0.19±0.09 $\mu$m$^2$) and occupy a smaller area (mean difference between early-life conditions for males: 174.70±62.08 $\mu$m$^2$/1000 $\mu$m$^2$; and females: 128.46±65.43 $\mu$m$^2$/1000 $\mu$m$^2$). No significant effects of sex or sex x early-life interactions were observed for any of the $\alpha_2$ subunit cluster characteristics (see table 5.1).

5.3.2. Gephyrin protein clusters

Figure 5.3 shows the effects of sex and early-life condition on gephyrin subunit cluster properties in the granule cell layer of the dentate gyrus of the hippocampus. Results of the two-way ANOVA shown in table 5.2 indicate no significant main effects of sex or early-life, nor sex x early-life interactions for any of the gephyrin subunit cluster characteristics (see table 5.2).
Figure 5.2: Effects of sex and early-life condition on α₂ receptor subunit clusters in the granule cell layer of the dentate gyrus. Data points represent average (n=5) ± SEM (a) number of clusters / 1000 μm²; b) size of clusters (μm²); and c) area covered by clusters (μm²) / 1000 μm². Bars represent significant main effects at p<0.01 for **early-life environmental condition.

Table 5.1: Results of 2-way ANOVA tests examining between-group differences in features of α₂ subunit protein clusters in the hippocampal granule cell layer. Tests reaching significance with p<0.05 are highlighted.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Sex</th>
<th>Early-life condition</th>
<th>Sex x Early-life condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count / 1000 μm²</td>
<td>F(1,16)=1.05; P&gt;0.05</td>
<td>F(1,16)=4.22; P&gt;0.05</td>
<td>F(1,16)=0.04; P&gt;0.05</td>
</tr>
<tr>
<td>Size (μm²)</td>
<td>F(1,16)=0.32; P&gt;0.05</td>
<td>F(1,16)=9.59; P&lt;0.01</td>
<td>F(1,16)=0.08; P&gt;0.05</td>
</tr>
<tr>
<td>Area (μm²) / 1000 μm²</td>
<td>F(1,16)=0.13; P&gt;0.05</td>
<td>F(1,16)=11.30; P&lt;0.01</td>
<td>F(1,16)=0.26; P&gt;0.05</td>
</tr>
</tbody>
</table>

Figure 5.3: Effects of sex and early-life condition on gephyrin protein clusters in the granule cell layer of the dentate gyrus. Data points represent average (n=5) ± SEM (a) number of clusters / 1000 μm²; b) size of clusters (μm²); and c) area covered by clusters (μm²) / 1000 μm².

Table 5.2: Results of two-way ANOVA tests examining between group differences in features of gephyrin protein clusters in the hippocampal granule cell layer.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Sex</th>
<th>Early-life condition</th>
<th>Sex x Early-life condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count / 1000 μm²</td>
<td>F(1,16)=0.57; P&gt;0.05</td>
<td>F(1,16)=0.10; P&gt;0.05</td>
<td>F(1,16)=0.12; P&gt;0.05</td>
</tr>
<tr>
<td>Size (μm²)</td>
<td>F(1,16)=0.23; P&gt;0.05</td>
<td>F(1,16)=0.06; P&gt;0.05</td>
<td>F(1,16)=3.47; P&gt;0.05</td>
</tr>
<tr>
<td>Area (μm²) / 1000 μm²</td>
<td>F(1,16)=0.10; P&gt;0.05</td>
<td>F(1,16)=0.11; P&gt;0.05</td>
<td>F(1,16)=0.10; P&gt;0.05</td>
</tr>
</tbody>
</table>
5.3.3. \(\alpha_2\) Subunit and gephyrin colocalisation

Figure 5.4 and 5.5 show the effects of sex and early-life condition on \(\alpha_2\) subunit cluster properties in the granule cell layer of the dentate gyrus of the hippocampus. Results of the two-way ANOVA shown in table 5.3 indicate that whilst the size of colocalised clusters was not significantly different between early-life conditions, there was a significant main effect of early-life stress on the number \((F_{(1,16)}=5.60, p<0.05)\) and area covered \((F_{(1,16)}=8.15, p<0.05)\) by colocalised clusters. Thus, when male and female data is averaged, colocalised clusters of NH mice are reduced in number (mean difference for males: 269.35±128.73 /1000 \(\mu m^2\); and females: 332.28±149.73 /1000 \(\mu m^2\)) and occupy a smaller area (mean difference for males: 43.47±18.24 \(\mu m^2\)/1000 \(\mu m^2\); and females: 30.14±12.24 \(\mu m^2\)/1000 \(\mu m^2\)) compared with those of EH mice. Following from this there were significant main effects of early-life stress on the % of both gephyrin \((F_{(1,16)}=6.52, p<0.05)\) and \(\alpha_2\) subunit \((F_{(1,16)}=9.35, p<0.01)\) staining that was colocalised. This means that when data is averaged across sexes, NH mice have a reduced % of staining area colocalised for both gephyrin (mean difference for males: 9.90±4.73 %; and females: 10.41±4.68 %) and \(\alpha_2\) (mean difference for males: 18.25±8.22 %; and females: 23.86±9.22 %) proteins compared with EH mice. No significant main effects of sex or sex x early-life interactions were observed for any of the colocalised cluster characteristics, or proportion of colocalisation observed for either protein (table 5.3).

<table>
<thead>
<tr>
<th>Feature</th>
<th>Sex</th>
<th>Early-life condition</th>
<th>Sex x Early-life condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count / 1000 (\mu m^2)</td>
<td>(F_{(1,16)}=0.03; P&gt;0.05)</td>
<td>(F_{(1,16)}=5.60; P&lt;0.05)</td>
<td>(F_{(1,16)}=0.06; P&gt;0.05)</td>
</tr>
<tr>
<td>Size ((\mu m^2))</td>
<td>(F_{(1,16)}=0.04; P&gt;0.05)</td>
<td>(F_{(1,16)}=0.00; P&gt;0.05)</td>
<td>(F_{(1,16)}=0.09; P&gt;0.05)</td>
</tr>
<tr>
<td>Area ((\mu m^2)) / 1000 (\mu m^2)</td>
<td>(F_{(1,16)}=1.01; P&gt;0.05)</td>
<td>(F_{(1,16)}=8.15; P&lt;0.05)</td>
<td>(F_{(1,16)}=0.27; P&gt;0.05)</td>
</tr>
<tr>
<td>% of (\alpha_2) area</td>
<td>(F_{(1,16)}=0.00; P&gt;0.05)</td>
<td>(F_{(1,16)}=9.35; P&lt;0.01)</td>
<td>(F_{(1,16)}=0.17; P&gt;0.05)</td>
</tr>
<tr>
<td>% gephyrin area</td>
<td>(F_{(1,16)}=1.24; P&gt;0.05)</td>
<td>(F_{(1,16)}=6.52; P&lt;0.05)</td>
<td>(F_{(1,16)}=0.02; P&gt;0.05)</td>
</tr>
</tbody>
</table>
Figure 5.4: Effects of sex and early-life condition on colocalisation of the $\alpha_2$ subunit with gephyrin in the granule cell layer of the dentate gyrus. Data points represent average (n=5)±SEM (a) number of colocalised clusters / 1000 $\mu$m$^2$; b) size of colocalised clusters ($\mu$m$^2$); c) area covered by clusters ($\mu$m$^2$)/1000 $\mu$m$^2$. Bars represent significant main effects at *p<0.05 or ** p<0.01 for early-life condition.

Figure 5.5: Representative z-stack projections showing $\alpha_2$ subunit colocalisation with the presumed synaptic marker gephyrin in the granule cell layer of the dentate gyrus of the hippocampus. Images are taken from male (a, b,) and female (c, d) mice exposed to EH (a,c) and NH (b, d) early-life conditions. The GABA$\alpha_2$ receptor $\alpha_2$ subunit protein is stained in red, gephyrin is stained in green. Yellow depicts sites of protein colocalisation. Scale 10$\mu$m.
5.4. Discussion

5.4.1. Effects of early-life stress on protein clustering

5.4.1.1. Effects of early-life stress on GABA_A receptor α_2 subunit protein clusters

Findings of the present study indicate that regardless of sex there was a reduction in the mean size of α_2 subunit clusters in NH relative to EH mice in the adult DG granule cell layer. The mean number of α_2 subunit clusters per unit area was not affected by early-life condition. Nonetheless, the reduction in the size of α_2 subunit clusters was consistent with a reduction in the mean surface area covered by these clusters per unit area of the granule cell layer. As this reduction in size was observed in the absence of altered expression of the α_2 subunit protein (see section 4.3.2.4), it cannot be explained by a lack of available α_2 protein for incorporation into receptors. Therefore, this finding suggests that early-life condition had a specific effect on the process of GABA_A receptor clustering in the hippocampal dentate gyrus of both male and female mice.

A reduction in cluster size in NH relative to EH mice is consistent with the hypothesis that early-life stress can affect developmental processes in the GABAergic system. In most brain regions cluster size is greater in adulthood relative to early-life, suggesting developmental processes determine GABA_A receptor cluster size (Hutcheon et al., 2004). However, it is observed that more mature rats show a decrease in the size of α_2 clusters in the DG of the hippocampus relative to younger rats (Hutcheon et al., 2004). In the present study, NH mice showed a reduction in α_2 subunit cluster size relative to EH mice suggesting that early-life stress (NH group) may enhance the developmental processes governing GABA_A receptor clustering.
5.4.1.2. Effects of early-life stress on gephyrin protein clusters

This study also showed that gephyrin clustering properties were not affected by either sex or early-life condition. Whilst no previous studies have examined the effects of early-life stress on gephyrin clustering, a lack of sex differences in this protein is consistent with recent work showing that variations in sex-hormone levels during pregnancy or administration of the contraceptive pill do not affect gephyrin expression or clustering (Sassoe-Pognetto et al., 2007). As gephyrin cluster properties were unaffected by early-life stress, it is predicted that alterations in $\alpha_2$ subunit clustering or colocalisation with gephyrin (see below) would not be caused via a disruption of gephyrin, which is proposed to act as an anchoring ‘scaffold’ for receptors in the synapse (Fritschy et al., 2008).

5.4.2. Effects of early-life stress on $\alpha_2$-gephyrin colocalisation

Another major finding of the present study was that, regardless of sex, there was a reduction in the number of overlapping clusters for the $\alpha_2$ and gephyrin proteins (colocalisation) in the NH compared with EH group. Whilst the size of these $\alpha_2$-gephyrin colocalised clusters did not vary between groups, the surface area covered by them was altered in accordance with the reduction in the number. Under the presumption that gephyrin is a marker for $\alpha_2$ subunits present in the synapse, this finding then shows that the $\alpha_2$-subunit containing $\text{GABA}_A$ receptor synaptic clusters were equivalent in size, but less frequent in the adult DG of the NH compared with the EH condition. To our knowledge, these findings are the first to indicate that early-life stress has long lasting effects on $\alpha_2$ subunit-containing $\text{GABA}_A$ receptor synaptic
clusters. Future work should examine whether this loss in GABA$_A$ receptor synaptic clustering is also observed in other brain regions.

It is possible that these variations in synaptic clustering occur via a loss of the $\gamma_2$ subunit, which is required for synaptic clustering of GABA$_A$ receptors (Essrich et al., 1998). In support of this idea, a reduction in the $\gamma_2$-dependent benzodiazepine binding sites has been observed in forebrain homogenates of NH relative to EH mice (Bodnoff et al., 1987). Furthermore, significant reductions in $\gamma_2$ subunit mRNA expression have been observed in the brainstem and amygdalar nuclei of NH relative to EH rats (Caldji et al., 2000b). However, loss of the $\gamma_2$ subunit would be expected to result in a loss of clustering of both GABA$_A$ receptor subunits and gephyrin (Alldred et al., 2005; Essrich et al., 1998; Li et al., 2005; Schweizer et al., 2003). The present study did not find any changes in gephyrin clustering properties, suggesting that the $\gamma_2$ subunit may not have been affected. Thus, an investigation of $\gamma_2$ subunit protein expression in the DG is required to determine if the loss of GABA$_A$ receptor synaptic clusters may be mediated via a loss of the $\gamma_2$ subunit.

5.4.3. GABA$_A$ receptor synaptic clustering, brain function and behaviour

Whilst further work is required to determine whether the observed disruptions to GABA$_A$ receptor clustering extend to other subunits or brain regions, the present study provides a basis to suggest alterations in brain function and behaviour as a result of reduced synaptic clustering. Following a loss of synaptic clusters GABAergic synaptic strength appears to be reduced (Crestani et al., 1999; Essrich et al., 1998; Levi et al., 2004) with studies indicating that a reduced surface area covered by synaptic clusters is associated with reductions in the mean amplitude of miniature inhibitory post-synaptic
currents (mIPSCs) (Levi et al., 2004; Nusser et al., 1997). Furthermore, alterations in the frequencies of single channel conductance times (Crestani et al., 1999) have suggested that loss of $\text{GABA}_A$ receptor synaptic clustering may result in an increase in extrasynaptic receptors. Thus NH mice would be expected to have alterations in inhibitory transmission as a result of the lost synaptic clusters.

Consistent with functional alterations in inhibitory transmission, previous studies suggest behavioural changes in mice with altered synaptic clustering. For example, reductions in clustering of $\alpha_2$-subunit containing $\text{GABA}_A$ receptors in the dentate gyrus of the mouse hippocampus are associated with a more anxious and behaviourally reactive phenotype (Crestani et al., 1999). Given the findings of Crestani et al. (1999), reductions in $\text{GABA}_A$ receptor synaptic clustering observed in NH mice relative to EH mice in the present study are consistent with the enhanced anxiety observed in chapter 3 for NH animals. Thus, the present study expands on work of others suggesting that early-life stress-induced behavioural changes may be mediated by alterations in $\text{GABA}_A$ receptors in terms not only of their expression and regional distributions (See chapter 4; Bodnoff et al., 1987; Bolden et al., 1990; Caldji et al., 2000a; Caldji et al., 2000b), but also their subcellular distributions.

It is also of interest that the study by Crestani et al. (1999) showed that the reduction in $\text{GABA}_A$ receptor synaptic clustering is associated with specific attentional biases towards threatening cues and an inability to ignore irrelevant information in the environment. These types of attentional biases are characteristic of depression and anxiety disorders and may also be relevant to symptoms observed in schizophrenia. In schizophrenia and anxiety disorders, a loss of $\gamma_2$ subunit protein expression or benzodiazepine binding sites, which require the $\gamma_2$ subunit, has been observed.
(Huntsman et al., 1998; Malizia et al., 1998; Nutt and Malizia 2001; Squires et al., 1993; Tiihonen et al., 1997; Tokunaga et al., 1997). Given that \( \gamma_2 \) subunit-containing receptors are usually found in synaptic clusters (Kneussel et al., 1999b), it is possible that a deficiency in the expression of this subunit causes or reflects a loss of GABA\(_A\) receptor synaptic clusters in such illnesses. Reduced GABA\(_A\) receptor synaptic clustering may thus be relevant to our understanding of these diseases and so it is important for future work to investigate synaptic clustering of GABA\(_A\) receptors in the brains of people suffering from illnesses such as schizophrenia, depression and anxiety disorders.

Interestingly, it has recently been observed that enhanced activity at \( \alpha_2 \)-containing GABA\(_A\) receptors improves cognitive symptoms that are observed in people with schizophrenia (Lewis et al., 2008). This finding provided support for the hypothesis that deficient GABAergic signalling via this GABA\(_A\) receptor subtype in a subset of GABAergic neurons may underlie the negative symptoms in schizophrenia (Lewis et al., 2008). Deficient signalling via the \( \alpha_2 \) receptor subtype in disorders such as schizophrenia may be related to deficiencies in the synaptic clustering of such receptors in the PFC. Such deficient synaptic clustering may potentially even arise from the deficiencies observed in the presynaptic components of GABAergic transmission that are observed in subsets of GABAergic neurons in schizophrenia (Akbarian et al., 1995; Volk et al., 2000; Guidotti et al., 2000; Straub et al., 2007). Thus, future studies should specifically look at GABA\(_A\) receptor clustering in the DLPFC of the schizophrenic brain.
5.4.4. Conclusions

Findings from the present study indicated that GABA\(_A\) subcellular distribution is affected by early-life stress. Regardless of sex, mice exposed to the NH early-life condition showed a reduction in the size and surface area covered by \(\alpha_2\) subunit-containing GABA\(_A\) receptor clusters. This alteration in \(\alpha_2\) subunit clusters did not arise from a loss of \(\alpha_2\) subunit protein, which showed equivalent IR between groups in this area (chapter 4). Therefore, early-life stress specifically affected cluster formation in the granule cell layer of the DG and this is consistent with the previous hypothesis that early-life condition affects GABA\(_A\) receptor developmental processes in the NH relative to the EH group. The number of synaptic \(\alpha_2\) subunit containing GABA\(_A\) receptors was also reduced in NH relative to EH mice suggesting reduced inhibitory synaptic strength that is relevant to explaining the increased anxiety and behavioural reactivity of NH relative to EH mice. This is the first study to show that early-life stress can affect the cellular distributions of GABA\(_A\) receptors. Further work is required to examine the potential role of the \(\gamma_2\) subunit in mediating altered GABA\(_A\) receptor synaptic clustering, as well as to determine how other brain regions and GABA\(_A\) receptor subunit clusters may be affected by early-life stress. Furthermore, given the relevance of early-life paradigms for research into psychiatric disorders such as schizophrenia and depression, this study highlights the importance of examining how GABA\(_A\) receptor cellular distributions may be altered in such diseases.
PART D:

EARLY-LIFE STRESS AND ACUTE ADULTHOOD STRESS
CHAPTER 6:
Effects of Early-Life Stress on GABA<sub>A</sub> Receptor Responses to Adulthood Stress

6.1. Introduction

6.1.1 Background

Variations in stress reactivity have been associated with several illnesses including mood disorders, diabetes, autoimmune disorders and coronary heart disease (Chrousos and Gold, 1992; Higley <i>et al.</i>, 1991; McEwan and Stellar, 1993; Seckl and Meaney, 2004). In particular, prior stress is associated with the onset of symptoms in psychiatric disorders such as depression (Heim and Nemerhoff, 2001) and schizophrenia (McGrath <i>et al.</i>, 2003). The association of stress exposure with symptom onset has resulted in the “diathesis-stress” or ‘two-hit’ hypotheses (McGrath <i>et al.</i>, 2003). These hypotheses suggest that an impairment in stress coping ability underlies the precipitation of disease symptoms in individuals predisposed to such illnesses due to genetic and/or early life environmental factors (McGrath <i>et al.</i>, 2003). Thus, in order to better understand the pathophysiology of these diseases, it is important to determine how neurochemical system responses to stress in adulthood may vary.

A large amount of research has indicated that early postnatal environment affects stress reactivity in adulthood. This makes animal models where early-life environment is manipulated highly relevant to the investigation of the physiological bases of altered stress coping in psychiatric illness (Ader <i>et al.</i>, 1970; Denenberg, 1964; Hess <i>et al.</i>, 1969; Meaney <i>et al.</i>, 1996). In the EH-NH model, stress responses have been shown to vary between groups on both behavioural and neuroendocrine levels. As explained previously, relative to the EH condition in adulthood, animals exposed to the NH
condition in early-life are behaviourally more reactive to stressful situations (Caldji et al., 2000b; Padoin et al., 2001; Pryce et al., 2001, Pryce et al., 2003). Enhanced behavioural reactivity of the NH group is correlated with long-lasting changes in neuroendocrine responses to stress. In adulthood, the NH group show enhanced and prolonged release of HPA axis hormones following exposure to a stressor compared with the EH group (Levine et al., 1967; Liu et al., 1997; Meaney et al., 1996; Meaney et al., 1989; Plotsky and Meaney, 1993). Enhanced HPA axis responses in the NH group are likely explained by enhanced neuronal activity, measured by cfos expression, following both physical and emotional stress in the brain regions signalling to the PVN of the hypothalamus, including the bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala, hippocampus, posterior cingulate cortex, piriform cortex (Abraham and Kovacs, 2000) and locus coeruleus (Pearson et al., 1997). Thus, the incoming stress signal appears to be increased in NH rodents and this results in enhanced neuroendocrine and behavioural responses to stress.

Given the evidence for altered adulthood HPA axis and behavioural stress responses as a result of early-life stress, it is likely that stress responses of neurotransmitter systems such as the GABAergic system may also be affected by early-life experience. As described in chapter 2, following a 3 minute swim stress, rapid changes in forebrain low-affinity GABA binding sites in females and group-stressed males are observed particularly in forebrain cortical regions (Skilbeck et al., 2008a; chapter 2). As electrophysiological studies indicate that micromolar concentrations of GABA are required for channel conductance, stress-induced alterations in low-affinity (1μM) [3H]GABA binding are indicative of alterations in GABA_A receptor function, (Baur and Siegel, 2003; Harris and Allan, 1985; Maconochie
et al., 1994). Thus, stress rapidly alters the availability of functional (low-affinity) GABA\textsubscript{A} receptor sites in a sex-dependent fashion (Skilbeck et al., 2008a), with females showing an increase and males showing a decrease or no change in functional GABA binding sites. As GABA\textsubscript{A} receptors are strongly implicated in the neuropathology of schizophrenia (Hinton and Johnston, 2008) and stress appears to precipitate the onset of psychotic episodes in people with schizophrenia (McGrath et al., 2003), variations in the effect of adulthood stress on GABA\textsubscript{A} receptors arising from stress in early-life may be relevant to understanding this disease.

Effects of early-life environment on stress-induced changes in \([^3]H\)GABA binding sites in adulthood would likely be sex-dependent. For example, in chapter 2 the effects of stress on \([^3]H\)GABA binding sites were observed to vary between males and females, with females showing increases and males showing decreases in the number of low-affinity \([^3]H\)GABA binding sites following a 3 minute swim stress (chapter 2; Skilbeck et al., 2008a). The magnitude of HPA axis stress responses are also sex-dependent (Akinci and Johnston, 1993) and previous work has suggested that early postnatal environment has sex-specific neuroendocrine effects following stress (Erskine et al., 1975; Higley et al., 1991; Liu et al., 2000; McCormick et al., 2005; Meaney et al., 2001; Sutanto et al., 1996; Weinberg et al., 1978; Weinberg and Levine, 1977).

Given that there appear to be sex differences in the onset and severity of symptoms in schizophrenia and stress is proposed to precipitate these symptoms, it is important to characterise how both males and females are affected by the combination of early-life and adulthood stress. Thus, effects of early-life stress on the adulthood stress-induced changes in \([^3]H\)GABA binding were examined in both males and females.
6.1.2. Aims

In accordance with the known effects of early-life environment on adulthood behavioural and neuroendocrine stress responses, the main purpose of the present study was to examine whether early-life stress affected the adulthood stress-induced changes in low-affinity (functional) GABA_A receptor binding sites, and whether such effects were sex-dependent. However, in order to examine this primary aim, it became important to examine the secondary aims: i) to determine the effects of early-life environment on GABA_A receptor binding sites and ii) to determine the effects of early-life environment on sex differences in GABA_A receptor binding sites. To examine these aims, low-affinity [^3]H]GABA binding was measured using quantitative receptor autoradiography in male and female mice that were exposed to either the NH or EH condition in early-life and either the stress or control condition in adulthood. We examined brain regions from the cortex and hippocampus where the most robust stress-induced changes were observed in chapter 2 (see publication Skilbeck et al., 2008a). This study is of relevance to understanding the biological mechanisms underlying the ‘two-hit’ hypotheses that are used to explain psychiatric disorders such as schizophrenia and depression which are associated with both early-life and adulthood stress.
6.2. Materials and methods

6.2.1. Materials

All materials for tissue preparation and autoradiography experiments have already been described in section 2.3.1.

6.2.2. Subjects

Subjects were those already described in section 3.2.2.1. Briefly, male (n=24) and female (n=24) Quackenbush Swiss (QS) albino mice born in the animal house were exposed to both early-life environmental manipulation (Males NH n=12, EH n=12; Females NH n=12, EH n=12) on PND 1-14 as described in section 3.2.2.4 combined with adulthood acute swim stress (control and stressed groups) as described in section 3.2.2.6. Thus brain sections from the following groups were examined in a 2 x 2 x 2 between-subjects design as shown in table 6.1.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EH</td>
<td>NH</td>
</tr>
<tr>
<td>Control</td>
<td>n=6</td>
<td>n=7</td>
</tr>
<tr>
<td>Swim stress</td>
<td>n=6</td>
<td>n=6</td>
</tr>
</tbody>
</table>

6.2.3. Tissue acquisition and preparation

Fresh frozen tissue was prepared and sectioned as described in section 3.2.2.7. Slides devoted to autoradiography were stored at -70°C prior to autoradiography experiments, which were carried out within 14 days of sectioning.
6.2.4. Receptor binding assays

Low (1000nM [^3^H]GABA) affinity GABA binding sites were examined using the radioligand binding assay procedure described in section 2.3.4.

6.2.5. Generation of autoradiograms

Autoradiograms were generated as described in section 2.3.5.

6.2.6. Brain regions examined

The cortical and hippocampal brain regions examined are described in section 2.3.6.

6.2.7. Analysis of binding

Analysis of binding site density by region was performed as described in section 2.3.7.

6.2.8. Statistical analysis

Graphs for each brain region were compiled in Prism 4.0 and groups were compared by a three-way between-subjects ANOVA (sex x early-life environment x adulthood stress) using SPSS 15.0. The source of significant two-way interactions (sex x early-life environment; sex x adulthood stress, and early-life environment x adulthood stress) and significant three-way interactions (sex x adulthood stress x early-life environment) were determined using means comparison contrasts.
6.3. Results

6.3.1. Cortical regions

Figure 6.1 shows 1000nM \[^{3}\text{H}]\text{GABA}\) binding in various cortical regions of male and female mice exposed to either NH or EH conditions in early life, and either acute swim stress or no stress in adulthood. Table 6.2 shows the results of a three-way ANOVA (sex x adulthood stress condition x early-life manipulation) of this data for each brain region examined. There were no significant three-way interactions in any of the cortical regions examined, meaning that differences between any two of the three factors (sex, stress, early-life condition) did not depend on the third factor. Table 6.2 shows that there were significant two-way interactions between early-life and adulthood stress in the cingulate and frontal cortices and the upper (layer I-III) and lower (layer IV-VI) cortical layers, indicating that the effects of adulthood stress on \[^{3}\text{H}]\text{GABA}\) binding depend on early-life condition, regardless of sex. Table 6.2 also shows there were significant sex x stress 2-way interactions in the cingulate and whole frontal cortices, particularly in the upper (layer I-III) cortical layers, indicating that the effects of adulthood stress on GABA binding varied between sexes regardless of early-life condition. It was decided that an examination of simple contrast effects would best explain the source of these interactions.

Sex differences

Table 6.3 shows results from the simple contrast effects analysis. Sex differences were observed for unstressed mice only and were similar in EH and NH mice. In both EH and NH groups, males had more 1000nM \[^{3}\text{H}]\text{GABA}\) binding sites than females in the cingulate (EH \(p=0.013\); NH \(p=0.011\)) and whole frontal cortex (EH \(p=0.011\); NH
p=0.017), the lower cortical layers (EH p=0.045 NH p=0.040) and upper cortical layers (EH=0.040; NH p=0.042). As can be seen in table 6.3, no sex differences were found between stressed groups in either early-life condition. Thus, regardless of early-life condition, sex differences in low-affinity GABA binding sites are removed by adulthood swim stress.

Effects of adulthood stress

Adulthood stress affected 1000nM $[^3]$H]GABA binding in EH males and NH females but had no effect on NH males or EH females. As shown in table 6.3, NH males were not affected by stress in any cortical regions whilst EH males experienced stress-induced reductions in GABA binding in the cingulate cortex (p=0.001), frontal cortex (p=0.009) and both the upper (p=0.019) and lower (p=0.006) cortical layers. In contrast, for females it was the EH mice that were unaffected by adulthood stress whilst the NH females showed adulthood stress-induced increases in GABA binding in the cingulate cortex (p=0.044), frontal cortex (p=0.032) and both the upper (p=0.021) and lower (p=0.047) cortical layers. Thus EH males and NH females experience adulthood stress-induced alterations in low-affinity $[^3]$H]GABA binding in opposite directions to one another whilst NH males and EH females appear insensitive to the effects of stress on low-affinity $[^3]$H]GABA binding.

Effects of early-life condition

1000nM $[^3]$H]GABA binding was affected by early-life condition in mice that were not exposed to acute adulthood swim stress. In the unstressed female group NH mice showed reduced $[^3]$H]GABA binding sites relative to EH mice in the cingulate
cortex (p=0.030), frontal cortex (p=0.025) and both the upper (p=0.040) and lower (p=0.038) cortical layers. In the unstressed male group, NH mice showed reduced [$^3$H]GABA binding sites relative to EH mice in the cingulate cortex (p=0.041) and frontal cortex (p=0.038), specifically in the lower (p=0.039) cortical layers. As shown in table 6.3, early-life condition did not affect low-affinity [$^3$H]GABA binding in males or females that were exposed to acute swim stress in adulthood.

Summary for cortical regions

As shown in figure 6.2 on representative autoradiographs, in unstressed mice early-life condition affected the number of low-affinity [$^3$H]GABA binding sites in both males and females from the NH and EH groups. Males had more low-affinity GABA binding sites than females in certain forebrain cortical regions which was observed for each of the EH and NH groups. Early-life condition also determined how animals responded to adulthood stress with only EH males and NH females showing adulthood stress-induced changes in [$^3$H]GABA binding, which involved decreases and increases, respectively.
Figure 6.1 Effects of sex, early-life condition and adulthood stress on 1000nM [3H]GABA binding sites in cortical regions. Data are expressed as mean±SEM for the a) cingulate b) frontal c) temporal cortical regions and d) upper e) lower cortical layers. *p<0.05, **p<0.01 for significant stress-induced differences from control mice of the same sex and early-life handling condition. ^p<0.05 for significant sex differences relative to male controls of the same early-life and adulthood stress condition. #p<0.05 for significant effects of NH relative to EH controls of the same sex.
Table 6.2: Results of 3-way ANOVA tests for 1000nM $[^3]$H]GABA binding in cortical regions. Tests reaching significance with $p<0.05$ are highlighted.

<table>
<thead>
<tr>
<th>Main Effects</th>
<th>Cingulate</th>
<th>Frontal</th>
<th>Temporal</th>
<th>Upper</th>
<th>Lower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>$F_{(1,40)}=3.25$, $p=0.081$</td>
<td>$F_{(1,40)}=2.37$, $p=0.131$</td>
<td>$F_{(1,40)}=0.07$, $p=0.790$</td>
<td>$F_{(1,40)}=3.04$, $p=0.089$</td>
<td>$F_{(1,40)}=1.59$, $p=0.215$</td>
</tr>
<tr>
<td>Early-life</td>
<td>$F_{(1,40)}=0.44$, $p=0.511$</td>
<td>$F_{(1,40)}=0.10$, $p=0.754$</td>
<td>$F_{(1,40)}=3.44$, $p=0.071$</td>
<td>$F_{(1,40)}=1.70$, $p=0.200$</td>
<td>$F_{(1,40)}=0.00$, $p=0.952$</td>
</tr>
<tr>
<td>Stress</td>
<td>$F_{(1,40)}=4.75$, $p=0.037$</td>
<td>$F_{(1,40)}=3.45$, $p=0.070$</td>
<td>$F_{(1,40)}=0.20$, $p=0.661$</td>
<td>$F_{(1,40)}=1.60$, $p=0.213$</td>
<td>$F_{(1,40)}=3.40$, $p=0.073$</td>
</tr>
</tbody>
</table>

Two Way Interactions

| Sex x Early-life | $F_{(1,40)}=0.16$, $p=0.688$ | $F_{(1,40)}=0.03$, $p=0.861$ | $F_{(1,40)}=0.03$, $p=0.862$ | $F_{(1,40)}=0.00$, $p=0.953$ | $F_{(1,40)}=0.40$, $p=0.530$ |
| Sex x Stress   | $F_{(1,40)}=12.68$, $p=0.001$ | $F_{(1,40)}=4.82$, $p=0.048$ | $F_{(1,40)}=0.01$, $p=0.942$ | $F_{(1,40)}=4.90$, $p=0.047$ | $F_{(1,40)}=2.11$, $p=0.161$ |
| Early-Life x Stress | $F_{(1,40)}=5.54$, $p=0.025$ | $F_{(1,40)}=4.50$, $p=0.040$ | $F_{(1,40)}=0.22$, $p=0.641$ | $F_{(1,40)}=4.40$, $p=0.042$ | $F_{(1,40)}=4.65$, $p=0.037$ |

Three-Way Interaction

| Sex x Early-Life x Stress | $F_{(1,40)}=0.24$, $p=0.627$ | $F_{(1,40)}=0.03$, $p=0.874$ | $F_{(1,40)}=3.51$, $p=0.068$ | $F_{(1,40)}=0.04$, $p=0.850$ | $F_{(1,40)}=0.10$, $p=0.760$ |

Figure 6.2: Representative autoradiographs showing effects of sex, early-life condition and adulthood stress on forebrain 1000nM $[^3]$H]GABA binding sites. Images are from male (a, b, e, f) and female (c, d, g, h) mice exposed to early-life conditions of NH (a-d) or EH (e-h) and adulthood conditions of no stress (a, c, e, g) or 3 minute swim stress (b, d, f, h). Scale bar represents 0.5cm.
Table 6.3: Simple contrast effect comparisons for 1000nM GABA binding in cortical regions.

Highlighted boxes show significant effects at p<0.05

<table>
<thead>
<tr>
<th></th>
<th>Male vs. Female</th>
<th>Control vs. Stress</th>
<th>EH vs. NH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Adulthood Stress</td>
<td>EH</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>NH</td>
<td>EH</td>
</tr>
<tr>
<td></td>
<td>NH</td>
<td></td>
<td>NH</td>
</tr>
<tr>
<td></td>
<td>P=0.013</td>
<td>P=0.011</td>
<td>P=0.673</td>
</tr>
<tr>
<td></td>
<td>P=0.011</td>
<td>P=0.017</td>
<td>P=0.910</td>
</tr>
<tr>
<td></td>
<td>P=0.040</td>
<td>P=0.042</td>
<td>P=0.884</td>
</tr>
<tr>
<td></td>
<td>P=0.045</td>
<td>P=0.040</td>
<td>P=0.938</td>
</tr>
<tr>
<td></td>
<td>P=0.011</td>
<td>P=0.017</td>
<td>P=0.817</td>
</tr>
<tr>
<td></td>
<td>P=0.017</td>
<td></td>
<td>P=0.915</td>
</tr>
<tr>
<td></td>
<td>P=0.042</td>
<td></td>
<td>P=0.798</td>
</tr>
<tr>
<td></td>
<td>P=0.040</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                  | Control         | EH                  |
|                  | Male            | NH                  |
|                  | Female          |                    |
|                  | P=0.001         | P=0.060             | P=0.474   |
|                  | P=0.009         | P=0.426             | P=0.218   |
|                  | P=0.019         | P=0.585             | P=0.358   |
|                  | P=0.006         | P=0.676             | P=0.263   |
|                  | P=0.044         |                    |           |
|                  | P=0.032         |                    |           |
|                  | P=0.021         |                    |           |
|                  | P=0.047         |                    |           |
|                  | P=0.025         |                    |           |
|                  | P=0.288         |                    |           |
|                  | P=0.061         |                    |           |
|                  | P=0.604         |                    |           |
|                  | P=0.232         |                    |           |
6.3.2. Hippocampal regions

Figure 6.3 and 6.4 show 1000nM $[^3]$H]GABA binding in various hippocampal regions of male and female mice exposed to either NH or EH handling conditions in early life, and either acute swim stress or no stress in adulthood. Table 6.4 shows there were no significant interaction effects in any of the hippocampal regions examined. However, there was a significant main effect of sex, meaning males have a greater number of 1000nM $[^3]$H]GABA binding sites than females in both the dentate gyrus and whole hippocampus, regardless of stress and early-life condition. Analysis of simple main effects given in table 6.5 show this effect occurred due to a significant reduction in $[^3]$H]GABA binding of unstressed NH females relative to unstressed EH females and unstressed NH males in each of the hippocampal subregions that were examined (see table 6.5).
Figure 6.3 Effects of sex, early-life condition and adulthood stress on 1000nM $[^3]$H$\text{GABA}$ binding sites in hippocampal regions. Data are expressed as mean±SEM for the a) whole hippocampus and b) CA1-CA2 c) CA3 and d) dentate gyrus subregions of hippocampus. ^^=p<0.01 for significant sex differences relative to male controls of the same early-life and adulthood stress condition. ##=p<0.01 for significant effects of NH relative to EH controls of the same sex.
Table 6.4: Results of 3-way ANOVA tests for 1000nM [³H]GABA binding in the hippocampus. Tests reaching significance with p<0.05 are highlighted.

<table>
<thead>
<tr>
<th>Main Effects</th>
<th>CA1</th>
<th>CA3</th>
<th>DG</th>
<th>HIPP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>$F_{(1,41)}=1.21, p=0.278$</td>
<td>$F_{(1,41)}=1.41, p=0.242$</td>
<td>$F_{(1,40)}=8.50, p=0.006$</td>
<td>$F_{(1,40)}=4.13, p=0.044$</td>
</tr>
<tr>
<td><strong>Early-life</strong></td>
<td>$F_{(1,41)}=1.90, p=0.176$</td>
<td>$F_{(1,41)}=2.31, p=0.136$</td>
<td>$F_{(1,40)}=0.70, p=0.410$</td>
<td>$F_{(1,40)}=0.71, p=0.403$</td>
</tr>
<tr>
<td><strong>Stress</strong></td>
<td>$F_{(1,41)}=0.06, p=0.813$</td>
<td>$F_{(1,41)}=0.01, p=0.936$</td>
<td>$F_{(1,40)}=0.38, p=0.544$</td>
<td>$F_{(1,40)}=0.07, p=0.799$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Two Way Interactions</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex x Early-life</strong></td>
<td>$F_{(1,41)}=1.30, p=0.261$</td>
<td>$F_{(1,41)}=1.69, p=0.201$</td>
<td>$F_{(1,40)}=2.33, p=0.136$</td>
<td>$F_{(1,40)}=2.67, p=0.110$</td>
</tr>
<tr>
<td><strong>Sex x Stress</strong></td>
<td>$F_{(1,41)}=0.01, p=0.918$</td>
<td>$F_{(1,41)}=0.00, p=0.966$</td>
<td>$F_{(1,40)}=2.06, p=0.161$</td>
<td>$F_{(1,40)}=0.11, p=0.742$</td>
</tr>
<tr>
<td><strong>Early-Life x Stress</strong></td>
<td>$F_{(1,41)}=2.10, p=0.155$</td>
<td>$F_{(1,41)}=2.50, p=0.122$</td>
<td>$F_{(1,40)}=0.01, p=0.924$</td>
<td>$F_{(1,40)}=0.87, p=0.355$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Three-Way Interaction</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex x Early-Life x Stress</strong></td>
<td>$F_{(1,41)}=2.30, p=0.138$</td>
<td>$F_{(1,41)}=2.30, p=0.137$</td>
<td>$F_{(1,40)}=2.16, p=0.151$</td>
<td>$F_{(1,40)}=3.86, p=0.057$</td>
</tr>
</tbody>
</table>

Figure 6.4: Representative autoradiographs showing effects of sex, early-life condition and adulthood stress on 1000nM [³H]GABA binding sites at the level of the hippocampus. Images are from male (a, b, e, f) and female (c, d, g, h) mice exposed to early-life conditions of NH (a-d) or EH (e-h) and adulthood conditions of no stress (a, c, e, g) or 3 minute swim stress (b, d, f, h). Scale bar represents 0.5cm.
Table 6.5: Simple contrast effect comparisons for 1000nM GABA binding in hippocampal regions. Highlighted boxes show significant effects at p<0.05.

<table>
<thead>
<tr>
<th></th>
<th>Male vs. Female</th>
<th>Control vs. Stress</th>
<th>EH vs. NH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Adulthood Stress</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>NH</td>
<td>EH</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>P=0.454</td>
<td>P=0.012</td>
<td>P=0.378</td>
</tr>
<tr>
<td>CA1-CA2</td>
<td>P=0.923</td>
<td>P=0.045</td>
<td>P=0.466</td>
</tr>
<tr>
<td>CA3</td>
<td>P=0.410</td>
<td>P=0.033</td>
<td>P=0.474</td>
</tr>
<tr>
<td>Dentate Gyrus</td>
<td>P=0.505</td>
<td>P=0.002</td>
<td>P=0.482</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Stress</td>
<td>Control</td>
</tr>
<tr>
<td></td>
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<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>EH</td>
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<td>EH</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>P=0.825</td>
<td>P=0.414</td>
<td>P=0.162</td>
</tr>
<tr>
<td>CA1-CA2</td>
<td>P=0.881</td>
<td>P=0.817</td>
<td>P=0.459</td>
</tr>
<tr>
<td>CA3</td>
<td>P=0.960</td>
<td>P=0.989</td>
<td>P=0.116</td>
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<tr>
<td>Dentate Gyrus</td>
<td>P=0.739</td>
<td>P=0.074</td>
<td>P=0.712</td>
</tr>
</tbody>
</table>
6.4. Discussion


Table 6.6 summarises findings regarding sex differences in the present study according to early-life and adulthood stress conditions. As shown in table 6.6, in the cortex, males exposed to both the EH and NH conditions had increased $[^3]H$GABA binding sites relative to females. Similarly, in the hippocampus, males also had an increased number of $[^3]H$GABA binding sites but this sex-difference was only observed in the NH group. Whilst no previous studies have examined the effects of early-life stress on both male and female $[^3]H$GABA binding, these findings are consistent with previous studies showing sex differences in cortical and hippocampal low-affinity GABA binding (see chapter 2, Skilbeck et al., 2008a). As indicated in chapter 2, increased low-affinity GABA binding sites in males relative to females suggests that the number of functional GABA$_A$ receptors are higher in the male cortex. This may be relevant to explaining the sex differences in behavioural sensitivities to GABA$_A$ receptor compounds that have been observed in many studies (Bujas et al., 1997; Crippens et al., 1999; Fernandez-Gausti and Picazo, 1997; Fernandez-Gausti and Picazo, 1999; Guillet and Dunham, 1995; Gulinello and Smith, 2003; Kokka et al., 1992; Manev et al., 1987; Pericic and Bujas, 1997; Pericic et al., 1999; Tayyabkhan et al., 2002; Webb et al., 2002; Wilson 1992; Wilson et al., 2004). Furthermore, as the present study indicates that sex differences in GABA binding are affected by early-life stress at least in the hippocampus, the varied early-life environments of different animal rearing facilities may help explain some of the discrepancies in the literature regarding such sex differences (see literature review). Thus, the present study replicates findings...
of chapter 2 but extends upon them to show that the effects of early-life condition on sex differences in GABA binding are regionally dependent.

### Table 6.6. Summary of results indicating regional sex differences in $[^3]H$GABA binding according to early-life and adulthood stress condition

<table>
<thead>
<tr>
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</thead>
<tbody>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cingulate</td>
<td>M&gt;F</td>
<td>M&gt;F</td>
</tr>
<tr>
<td>Whole frontal</td>
<td>M&gt;F</td>
<td>M&gt;F</td>
</tr>
<tr>
<td>Upper</td>
<td>M&gt;F</td>
<td>M&gt;F</td>
</tr>
<tr>
<td>Lower</td>
<td>M&gt;F</td>
<td>M&gt;F</td>
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<tr>
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<td>-</td>
</tr>
<tr>
<td>Stress</td>
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<tr>
<td>Cingulate</td>
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<tr>
<td>Whole frontal</td>
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<tr>
<td>Upper</td>
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<td>Temporal</td>
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</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole Hippocampus</td>
<td>-</td>
<td>M&gt;F</td>
</tr>
<tr>
<td>CA1-CA2</td>
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<td>M&gt;F</td>
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<tr>
<td>CA3</td>
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<td>M&gt;F</td>
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<tr>
<td>DG</td>
<td>-</td>
<td>M&gt;F</td>
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<tr>
<td>Stress</td>
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</tr>
<tr>
<td>Whole Hippocampus</td>
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<tr>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DG</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Abbreviations:** Mice exposed to 3 minute swim stress in adulthood (S), mice were not exposed to adulthood swim stress and thus represent the unstressed control group (C).

### 6.4.2. Effects of early-life stress on $[^3]H$GABA binding in unstressed mice

#### 6.4.2.1. Males

Table 6.7 summarises the observed effects of early-life stress on $[^3]H$GABA binding in males and females according to adulthood stress condition. As shown in table 6.7, early-life condition only affected $[^3]H$GABA binding in mice that were not exposed to adulthood stress. In males, the NH group showed a reduced number of low-affinity $[^3]H$GABA binding sites relative to the EH group in forebrain cortical regions such as the cingulate and frontal cortices. This was particularly evident in the lower cortical layers (IV-VI). These findings are consistent with previous studies showing a small but significant reduction in low-affinity sites (Bolden et al., 1990) in forebrain cortical regions of NH males relative to EH males. The present study expands on the
findings of Bolden et al. (1990) whose study was conducted in forebrain homogenates, by demonstrating that specific cortical regions are deficient in low-affinity $[^3\text{H}]$GABA binding sites in adulthood as a result of early-life condition. Furthermore, given the findings of chapter 4 where NH males showed significant reductions in $\alpha_2$ subunit expression across each of the cortical laminae IV, V, VI, it is possible that the loss of low-affinity sites in the lower cortical layers of NH mice is at least partially explained by a loss of receptors containing the $\alpha_2$ subunit.

### 6.4.2.2. Females

Also shown in table 6.7, similarly to NH males, NH females also showed a reduction in the number of low-affinity $[^3\text{H}]$GABA binding sites relative to EH females. However, early-life condition appears to affect the number of $[^3\text{H}]$GABA binding sites to a greater extent in females than it does in males as all forebrain cortical and hippocampal regions examined showed a reduction in the number of low-affinity sites for NH relative to EH females. To our knowledge, this is the first study to report a widespread and long-lasting deficit in GABA binding in females exposed to a stressful early-life condition (NH). Interestingly, previous studies have also observed sex differences in the effects of early-life environment on the long-term behavioural and neuroendocrine effects. Females are more sensitive than males to the adulthood effects of pre-natal stress (Richardson et al., 2006), brief periods of isolation (Kosten et al., 2005), and prolonged maternal separation (Mesquita et al., 2007; Slotten et al., 2006). Such findings indicate that the events of early-life may be more important for the long-term development of females.
The loss of binding sites for $[^3 \text{H}]$GABA in NH females suggests a loss of GABA$_A$ receptors in regions of the frontal and cingulate cortex as well as the hippocampus. Results from the immunohistochemistry experiments in chapter 4 support a loss of $\alpha_1$ and $\alpha_2$ subunit containing receptors from regions of the hippocampus in NH females (see chapter 4). However, whilst $\alpha_2$ subunit protein was reduced in the cortex, $\alpha_1$ subunit protein was increased, suggesting that perhaps other GABA$_A$ receptor subunits are affected to a greater extent by early-life condition. Alternatively, as chapter 5 found deficits in the formation of membrane clusters, it may be that the effects of early-life environment on the number of GABA$_A$ receptor binding sites arise not from impaired protein expression but rather from alterations in receptor formation, membrane insertion or recycling. Nonetheless, this study shows a long-lasting, widespread effect of early-life stress on GABA binding in females.

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortex</strong></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>EH&gt;NH</td>
<td>EH&gt;NH</td>
</tr>
<tr>
<td>Cingulate</td>
<td>EH&gt;NH</td>
<td>EH&gt;NH</td>
</tr>
<tr>
<td>Whole frontal</td>
<td>EH&gt;NH</td>
<td>EH&gt;NH</td>
</tr>
<tr>
<td>Upper</td>
<td>-</td>
<td>EH&gt;NH</td>
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<td>EH&gt;NH</td>
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<tr>
<td>Temporal</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Stress</strong></td>
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<td></td>
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<tr>
<td>Cingulate</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Whole frontal</td>
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<tr>
<td><strong>Hippocampus</strong></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>EH&gt;NH</td>
<td>EH&gt;NH</td>
</tr>
<tr>
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<td>EH&gt;NH</td>
</tr>
<tr>
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<td>EH&gt;NH</td>
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<td>EH&gt;NH</td>
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<tr>
<td><strong>Stress</strong></td>
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<td></td>
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<tr>
<td>Whole Hippocampus</td>
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<tr>
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<td>CA3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DG</td>
<td>-</td>
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</tr>
</tbody>
</table>

**Abbreviations:** Mice exposed to 3 minute swim stress in adulthood (S), mice were not exposed to adulthood swim stress and thus represent the unstressed control group (C).
6.4.3. Relevance of the effects of early-life stress on [³H]GABA binding

The effects of early-life stress on adulthood [³H]GABA binding in mice that were not exposed to swim stress extend on previous observations of this thesis (see part C: chapter 3-5) and the work of others (Bodnoff et al., 1987; Bolden et al., 1990; Caldji et al., 2000a; Caldji et al., 2000b) suggesting that the effects of early-life stress on adulthood behaviour may be mediated by alterations in GABA receptors. As the number of low-affinity sites labelled by [³H]GABA provides a measurement of the total number of functional binding sites available, the observed loss of GABA binding sites in the hippocampus of NH females and the cortex of both NH males and females is likely indicative of an impairment of GABAergic function in adulthood. Impaired function in this major inhibitory neurotransmitter system may be relevant to the enhanced behavioural reactivity and anxiety displayed by NH mice relative to EH mice (see chapter 3). Furthermore, the finding that females are more sensitive than males to the effects of early-life stress on [³H]GABA binding is relevant to understanding illnesses such as depression and anxiety that are more prevalent in women and are strongly associated with early-life experience (Becker et al., 2007; Simonds and Whiffen, 2003; Young et al., 1990).

6.4.4. Effects of early-life on adulthood stress-induced changes in GABA binding

As can be seen in table 6.8, results of the present study indicate that early-life condition affects adulthood stress-induced changes in [³H]GABA binding in males and females. In males [³H]GABA binding in the NH group was not affected by stress, but in the EH group there was a stress-induced decrease in [³H]GABA binding in all forebrain cortical regions examined. In contrast, in females the EH group were
unaffected by adulthood stress, whilst the NH group showed a stress-induced increase in \[^{3}\text{H}]\text{GABA}\) binding in all forebrain cortical regions examined. Thus, in both males and females early-life condition affects adulthood stress-induced changes in GABA binding.

<table>
<thead>
<tr>
<th>Table 6.8. Summary of the effects of early-life condition on adulthood stress-induced changes in [^{3}\text{H}]\text{GABA}) binding according to sex.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortex</strong></td>
</tr>
<tr>
<td><strong>Male</strong></td>
</tr>
<tr>
<td>Cingulate</td>
</tr>
<tr>
<td>Whole frontal</td>
</tr>
<tr>
<td>Upper</td>
</tr>
<tr>
<td>Lower</td>
</tr>
<tr>
<td>Temporal</td>
</tr>
<tr>
<td><strong>Female</strong></td>
</tr>
<tr>
<td>Cingulate</td>
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<tr>
<td>Whole frontal</td>
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<tr>
<td>Upper</td>
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<td>Lower</td>
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<tr>
<td>Temporal</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
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<td><strong>Male</strong></td>
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<tr>
<td>Whole Hippocampus</td>
</tr>
<tr>
<td>CA1-Ca2</td>
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<tr>
<td>DG</td>
</tr>
<tr>
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</tr>
<tr>
<td>Whole Hippocampus</td>
</tr>
<tr>
<td>CA1-Ca2</td>
</tr>
<tr>
<td>CA3</td>
</tr>
<tr>
<td>DG</td>
</tr>
</tbody>
</table>

**Abbreviations:** Mice exposed to 3 minute swim stress in adulthood (S), mice were not exposed to adulthood swim stress and thus represent the unstressed control group (C).

These effects of early-life condition on adulthood stress-induced changes in GABA binding are relevant to discrepancies in the literature regarding the effects of stress. For example, in males, adulthood stress is observed to decrease (Biggio et al., 1981; Concas et al., 1985; Corda et al., 1985; Cuadra and Molina, 1992), increase (Wilson and Biscardi, 1992), or have no effect (Akinci and Johnston, 1993; Skerritt et al., 1981) on cortical low-affinity GABA\_A receptor binding sites. As the present study shows GABA binding in EH and NH males is affected differently by stress in adulthood, discrepancies in the literature may be at least partially explained by
variations in the early-life environments of different animal rearing facilities from which animals are obtained.

Following on from this line of reasoning, as the early-life environment of mice obtained for the study in chapter 2 is unknown, comparisons between the present study and that in chapter 2 require caution. Previous work has indicated that NH mice are more stress responsive in terms of behaviour, HPA axis secretions and neuronal activity following stress, relative to the EH group (Caldji et al., 2000b; Levine et al., 1967; Liu et al., 1997; Meaney et al., 1996; Meaney et al., 1989; Padoin et al., 2001; Plotsky and Meaney, 1993; Pryce et al., 2001, Pryce et al., 2003). Thus, in the present study, it was expected that the EH group would show similar effects of stress on GABA binding as the mice in chapter 2, whilst the NH group would show an exaggeration of these effects. This hypothesis assumes that the early-life environment of mice used in chapter 2, which were obtained from an animal rearing facility and underwent no systematic early-life intervention, was similar to that of the EH group in the present study. However, the findings of the present study question this assumption as the effects of adulthood stress on EH mice are not consistent with those reported in chapter 2. For example, in chapter 2 adulthood acute swim stress increased low-affinity GABA binding sites in females but did not affect these sites in males. In contrast, in the present study EH females did not show adulthood stress-induced changes and EH males showed stress-induced reductions in GABA binding. Therefore, despite our expectations, it is predicted that the early-life environment of the mice in chapter 2 was more like that of the NH group than the EH group as the changes in the number of cortical GABA binding sites observed for NH mice are consistent with those of the mice in chapter 2. Furthermore, as NH females showed 30-40% increases whilst the females in chapter 2 showed only 10-25%
increases in GABA binding following stress, NH females did show an exaggerated effect of stress on GABA\textsubscript{A} receptor binding relative to mice in chapter 2, consistent with enhanced stress reactivity in the NH relative to the EH group.

The present finding that GABA\textsubscript{A} receptor adulthood stress-responses are affected by early-life environment contributes to our understanding of the neurochemical changes underlying impaired stress reactivity and coping. As already mentioned, NH-reared groups show increased HPA axis responses and increased behavioural reactivity following stress relative to EH-reared groups (Caldji \textit{et al.}, 2000a; Caldji \textit{et al.}, 2000b; Levine \textit{et al.}, 1967; Liu \textit{et al.}, 1997; Meaney \textit{et al.}, 1996; Meaney \textit{et al.}, 1989; Padoin \textit{et al.}, 2001; Plotsky and Meaney, 1993; Pryce \textit{et al.}, 2001, Pryce \textit{et al.}, 2003). Altered stress-responsivity arising from early-life stress appears to result in differences in stress coping, with NH mice showing prolonged HPA axis stress responses (Levine \textit{et al.}, 1967; Liu \textit{et al.}, 1997; Meaney \textit{et al.}, 1996; Meaney \textit{et al.}, 1989; Plotsky and Meaney, 1993) and ‘helpless’ behaviours during stress as opposed to the active coping behaviours displayed in the EH mice (Hsu \textit{et al.}, 2003). As the present findings demonstrated that the NH group have a different neurochemical response to stress in the GABAergic system relative to the EH group, the effects of early-life stress on adulthood stress-induced changes in GABA\textsubscript{A} receptors may be relevant to differential adulthood stress-coping between EH and NH groups. Thus, from the findings of the present study it may be proposed that stress-induced increases in GABA\textsubscript{A} receptors observed in NH females may contribute to impaired recovery from stress, whilst stress-induced decreases observed in EH males may be advantageous to stress coping. Furthermore, the present observation that neurochemical stress responses are altered in an animal model of impaired stress reactivity is of importance to diseases
such as schizophrenia and depression where stress is associated with the onset of
disease symptoms but only in certain individuals (McGrath et al., 2003).

6.4.5. Sex-dependent effects of early-life stress on adulthood GABA_A receptors

Findings of the present study indicated that the early-life condition affected the
adulthood stress-induced changes in [3H]GABA binding differently in males and
females. Such sex differences in stress reactivity are relevant to diseases in which sex
and stress are associated with the onset and severity of disease symptoms such as
schizophrenia and depression. Only EH males and NH females showed stress-induced
changes in GABA binding and these changes were in opposite directions. Consistent
with this finding, sex differences in the effects of early-life environment on stress
responsivity both in terms of behaviour and serum corticosterone have been observed
previously (Mitev et al., 2003). Sex differences in the effects of early-life environment
on stress-induced changes in GABA_A receptors may be related to the effects of
early-life environment on corticosteroid responses to stress. For example, whilst both
males and females show a prolonged corticosteroid response to stress (Meaney et al.,
1985; Meaney et al., 1991), only in males does the NH group show increased levels of
corticosteroids following exposure to a stressor in adulthood relative to the EH group
(Ader, 1975; Meaney et al., 1989; Meaney et al., 1996; Plotsky and Meaney, 1993).
Corticosteroids act directly on GABA_A receptors as bi-directional modulators (Ong et
al., 1987; Ong et al., 1990), but are also necessary for the stress-induced synthesis of
the potent GABA_A receptor neurosteroid modulators (Mitev et al., 2003) and either of
these endogenous GABA_A receptor modulators may underlie the effects of stress on
GABA_A receptors. Alternatively, given that the present thesis has indicated that
GABA_A receptor expression (see chapter 4) and binding is affected in a sex-dependent fashion by early-life condition, changes in GABA_A receptors may be upstream of the sex-dependent effects on corticosteroids. In order to determine if GABA_A receptor stress responses may be a cause or effect of the changes in corticosteroid release, future studies are required to determine how early-life environment and stress affect GABA_A receptors on the neuronal projections to the PVN of the hypothalamus and how this varies between males and females.

### 6.4.6. Absence of stress-induced sex differences

The present study also showed that no sex differences occurred between stressed animals in [^3]H GABA binding sites regardless of early-life condition. This finding is consistent with the findings of chapter 2 which showed that stress eliminated baseline sex differences. As mentioned in chapter 2 this finding suggests that following adulthood stress, sex differences in GABAergic signalling and behaviours mediated via this neurotransmitter system would be removed. Thus the present study replicates the findings of chapter 2 but extends upon them by suggesting that the removal of baseline sex differences in [^3]H GABA binding following stress appears to be unaffected by early-life condition.

### 6.4.7. Conclusions

The present observations highlight the sensitivity of the GABAergic system to environmental stress in both early-life and adulthood. Early-life environment affected the number of functional [^3]H GABA binding sites in unstressed male and female mice. Baseline sex differences in the number low-affinity [^3]H GABA binding sites that were
reported in chapter 2 were present regardless of differences in $[^1H]$GABA binding resulting from early-life environment. Importantly, it was also observed that adulthood stress-induced changes in GABA$_A$ receptors are dependent on early-life condition suggesting neurochemical correlates for the altered adulthood stress-responsivity and coping that differentiates the early-life environmental groups used in the present study. The interaction of early-life and adulthood stress varied between males and females and it was suggested that this may occur via a mechanism involving endogenous GABA$_A$ receptor modulators such as corticosteroids and/or neurosteroids. Such sex-dependent changes in neurochemical stress responses that are related to different early-life conditions may contribute to our future understanding of the sex differences observed in diseases such as depression and schizophrenia.
PART E:

GENERAL DISCUSSION AND CONCLUSIONS
CHAPTER 7:
General Discussion

7.1 Overview of findings

The main aims of this thesis were to examine the effects of adulthood and early-life stress on GABA_\textsubscript{A} receptors, to aid understanding of the neurophysiology of stress, of possible sex differences in stress responses, and of the potential role of stress in the GABA_\textsubscript{A} receptor abnormalities that are observed in psychiatric illnesses. The major findings of this thesis were that the rapid and sex-dependent effects of adulthood stress on GABA binding occur in a regionally-dependent manner, that early-life stress has long-term effects on GABA_\textsubscript{A} receptor protein subunit regional densities and receptor synaptic clustering, and that early-life stress can affect adulthood stress-induced changes in GABA binding. In the course of this work, sex differences in GABA binding, subunit protein expression and the effects of stress on GABA_\textsubscript{A} receptors were also identified. To our knowledge no previous studies have reported such findings. The potential implications and future directions that arise from this work are discussed below.

7.2 Stress and GABA_\textsubscript{A} receptors

Results reported in this thesis indicated that GABA_\textsubscript{A} receptors are affected in the short and long-term by environmental stressors. A brief 3 minute swim stress in adulthood induced rapid changes in forebrain GABA binding sites in females and group stressed males (chapter 2). As well, stress over the first two weeks of post-natal life produced long-term effects on GABA_\textsubscript{A} receptors in terms of both regional and laminar protein subunit expression (chapter 4) and cellular protein distribution (chapter 5). These effects highlight the sensitivity of the GABAergic system to changes in the
environment and exemplify how prior experience may affect neurochemical signalling over both the short and long term.

These short and long-term changes in GABA<sub>A</sub> receptors that are incurred by stress have potential clinical implications. For example, many drugs which act on GABA<sub>A</sub> receptors including anaesthetics and anxiolytic agents are used clinically, thus it is likely the effects of such drugs are altered in individuals who experience prior stressful events in early-life or in adulthood. Along the same lines, behaviours such as anxiolysis, sedation and myorelaxation that are associated with signalling via GABA<sub>A</sub> receptors may also be altered by prior experience. Furthermore, behavioural abnormalities, such as those observed in psychiatric disorders, may arise due to prior stressful experiences in early-life or adulthood.

7.3 Sex differences and GABA<sub>A</sub> receptors

7.3.1 Baseline sex differences

In this thesis GABA<sub>A</sub> receptor sex differences were observed in control mice that had not been exposed to any stressful experiences. For example, low affinity \[^{3}H\]GABA binding was observed to be greater in forebrain cortical regions of male mice relative to female mice in two separate studies (see chapter 2 and chapter 6). That males have an increased number of low-affinity ‘functional’ \[^{3}H\]GABA binding sites in certain forebrain cortical regions may help explain studies showing that, compared with females, males show behavioural responses to lower doses of GABA<sub>A</sub> receptor compounds such as ethanol (Crippens et al., 1999; Tayyabkhan et al., 2002; Webb et al., 2002; Wilson et al., 2004), allopregnanalane (Fernandez-Gausti and Picazo, 1997;
1999; Guinello and Smith, 2003) and diazepam (Fernandez-Gausti and Picazzo, 1990; 1997; Fernandez-Gausti and Picazzo, 1999; Wilson et al., 2004).

\(\text{GABA}_A\) receptor subunits possibly involved in the sex-difference in the number of GABA binding sites observed in the frontal cortex were also examined in this thesis. Immunohistochemistry studies (chapter 4) showed that \(\alpha_1\) and \(\alpha_2\) subunit expression was similar in control males and females across a number of brain regions with the exception of the outer layers of the somatosensory cortex where males were observed to have increased IR for the \(\alpha_1\) subunit expression compared with females. This increase in \(\alpha_1\) subunit expression in male mice, suggests that the increased number of cortical GABA binding sites in males (reported in chapters 2 and 6) may be at least partially due to the increased number of cortical \(\alpha_1\)-subunit containing \(\text{GABA}_A\) receptors also observed in males. Given that \(\alpha_1\)-subunit selective compounds such as zolpidem and zopiclone are used clinically for the short-term treatment of insomnia, it is important for future work to examine whether there are sex differences in the required doses of these drugs, or in the recent reports of adverse side-effects from these drugs. Furthermore, sex differences in the expression of other \(\alpha\) subunits that were not examined in this thesis may also contribute to sex differences in the number of \(^{1}H\text{GABA}\) binding sites and sensitivity to GABAergic compounds. In particular, the \(\alpha_3\) subunit which is transcribed from a gene on the X chromosome, is strongly expressed on neurons receiving monoaminergic projections (Gao et al., 1993) and has been associated with sensorimotor deficits in subunit knockout studies (Hauser et al., 2005; Yee et al., 2005), suggesting this subunit may be particularly important in sex differences in the symptoms and treatment of diseases such as schizophrenia.
7.3.2 Sex differences in stress responsivity

As a number of diseases have both stress and sex as predisposing factors, another aim of this thesis was to examine sex differences in the effects of stress on GABA<sub>A</sub> receptors. Effects of early-life stress on GABA<sub>A</sub> receptors were largely consistent across sexes. For example both males and females showed similar reductions in low affinity [<sup>3</sup>H]GABA binding, α<sub>2</sub> subunit expression in the lower layers of the SS cortex and synaptic clustering of GABA<sub>A</sub> receptors in the dentate gyrus (chapter 4). In contrast, stress in adulthood affected [<sup>3</sup>H]GABA binding differently in males and females. Whilst the effects of adulthood stress on GABA binding were dependent on the early-life stress condition animals were exposed to, adulthood stress altered GABA binding in opposite directions in males and females such that baseline sex differences were removed (chapter 2 and chapter 6). These findings suggest that following stress, sex differences in GABAergic signalling and behaviours mediated via this neurotransmitter system would be reduced, highlighting the potential for stress to affect sex differences in responses to GABAergic agents in a clinical setting.

7.4 Stress, sex differences and psychiatric disorders

The involvement of stress in psychiatric disorders such as depression, anxiety disorders (generalised anxiety disorder, panic disorder, social phobia, PTSD) and schizophrenia is well documented, however the neurophysiological basis for how stress may be associated with these disorders is unclear. As GABA<sub>A</sub> receptors are affected in each of these disorders (see section 1.2), and the present thesis shows they are affected by stress in the short and long term (chapters 2-6), GABA<sub>A</sub> receptors present a potential site by which stress may affect neurochemical signalling resulting in behavioural
abnormalities that present as disease symptoms. However, given that such disorders occur on a genetic background, it is difficult to reproduce the GABA_A receptor abnormalities observed in psychiatric disorders via purely exposing mice to early-life stress. As was the case in this thesis, the regional selectivity of changes in α subunit expression was not consistent with the changes in α subunit expression that are observed in the brains of people with schizophrenia or depression (chapter 4). However, in depression GABA_A receptor abnormalities are by no means conclusive. Furthermore, the regional dependence of the changes in α subunit expression that arose in mice exposed to early-life stress are consistent with a disruption of the developmental α subunit switch. Whilst future studies are required to ensure changes in the expression of the α subunits are a result of developmental abnormalities, the fact that early-life stress potentially alters brain development is of relevance to disorders such as schizophrenia for which abnormal brain development is a primary feature. As well, the alterations in adulthood stress reactivity that were observed in mice exposed to early-life stress (chapter 6) is of interest for disorders such as schizophrenia and depression where stress-vulnerability or two-hit hypotheses are proposed as an explanation for the association of symptom onset, severity and disease outcome with stressful life events. Therefore, an animal model involving a combination of early-life stress with the genetic abnormalities proposed for such psychiatric disorders would likely be informative regarding how GABA_A receptor pathologies are acquired in such diseases. Whilst these diseases appear to be polygenetic in origin, investigation of the involvement of different genes using knockout mouse models, as is currently the fashion, could be done in combination with studies of variations in early-life environment. Thus, future studies
should seek to examine the effects of early-life stress in animal models of the genetic abnormalities proposed to play a role in disorders such as schizophrenia and depression.

In anxiety disorders, little investigation into GABA_A receptor pathologies in subjects with such disorders has been performed aside from in vivo imaging of GABA_A receptor binding sites. Interestingly, animal models of anxiety disorders have suggested that reductions in synaptic clustering are associated with specific attentional deficits that are observed in a number of psychiatric disorders. In the present thesis early-life stress was observed to reduce GABA_A receptor synaptic clustering in the dentate gyrus suggesting that early-life stress may be sufficient to produce the attentional deficits that are associated with several psychiatric illnesses such as depression, schizophrenia and anxiety disorders. However, no previous studies have examined how GABA_A receptor subcellular distribution is affected in the brains of patients affected by such diseases. Thus in addition to current studies examining regional and subregional distributions of proteins and mRNA that are proposed to be associated with certain psychiatric illnesses, future work should seek to determine if abnormal GABA_A receptor synaptic clustering occurs in the brains of subjects with psychiatric disorders such as depression, schizophrenia and anxiety disorders.

7.4 General Conclusions

In conclusion, this thesis shows that GABA_A receptors are sensitive to subtle changes in the environment in both early-life and adulthood. The stress sensitivity of GABA_A receptors both in the short and long-term suggests that both behaviours and clinically relevant drugs that are mediated via this system may be affected by prior stressful experiences throughout the lifespan. This thesis also sheds light on the
proposition of sexual differentiation of GABA_{A} receptors, with evidence suggesting that baseline sex differences exist which likely affect how males and females respond behaviourally and pharmacologically. The short and long-term stress-sensitivity of the GABAergic system also implicates GABA_{A} receptors in the non-genetic aetiology of psychiatric illnesses that are epidemiologically associated with sex and stress such as schizophrenia, depression and anxiety disorders. Further investigation into the role of neurosteroids in mediating stress-induced changes in GABA_{A} receptors and potential sex differences in the sensitivity to such effects may help our understanding of the mechanism by which GABA_{A} receptors are affected by stress in the short and long-term.
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