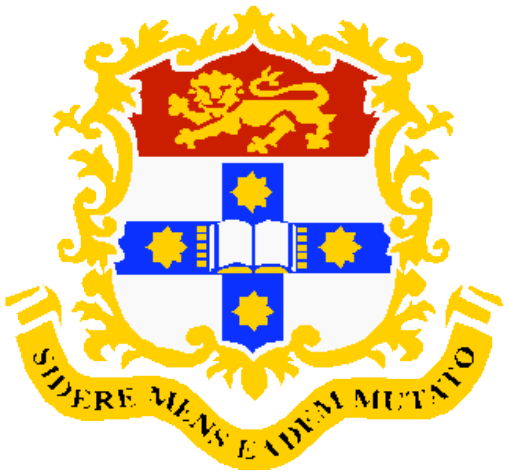
STRESS AND $GABA_A$ RECEPTOR REGULATION

A thesis submitted in fufilment of the requirement for the degree of

Doctor of Philosophy

by

Kelly Johanne Skilbeck
BSc(Advanced) (Hons)



Department of Pharmacology
Faculty of Medicine

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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 (7^{th} edition)". Australian Government Publishing Service, Canberra, 2004.

SUMMARY

1. $GABA_A$ 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 $GABA_A$ receptors may be relevant to understanding the association between stress and psychiatric disorders. Thus, this thesis examined how $GABA_A$ 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 $GABA_A$ receptor binding in the brains of male and female mice were examined using quantitative receptor autoradiography. The total number of $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ receptors were then examined.
4. Regional densities of $GABA_A$ receptor α_1 and α_2 subunit proteins were observed in the adult brain of male and female mice using immunoperoxidase histochemistry. NH males showed a loss of the α_2 subunit from the thalamus and the lower layers

(IV-VI) of the primary somatosensory cortex, whilst NH females showed a reduction of α_2 but an increase in α_1 protein in the lower layers of the primary somatosensory cortex only. These regionally specific alterations in the α_1:α_2 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 α_2 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 [^{3}H]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

†**K.J. Skilbeck,** T. Hinton & G.A.R. Johnston. 2008. Sex-differences and stress: Effects on regional high and low affinity [^{3}H]GABA binding. *Neurochem. Int.*, **52**, 1212-1219.

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K.J. Skilbeck, J.N O'Reilly, G.A.R. Johnston & T. Hinton. 2007. The effects of antipsychotic drugs on $GABA_A$ receptors depend on receptor subtype and treatment period. *Schizophr. Res.* **90**, 76-80.

Presentations

†**K.J. Skilbeck,** Stress and $GABA_A$ receptor regulation. Bosch Institute Distinguished Seminars Program, Sydney, 21st September, 2008.

†**K.J. Skilbeck,** G.A.R. Johnston & T. Hinton. Early-life environment produces long-lasting effects on GABA-A receptors. 27th Annual Meeting of the Australian Neuroscience Society, Hobart, 27-30 January, 2008.

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I.S. McLennan, **K.J. Skilbeck**, T. Hinton, G.A.R. Johnston. $MIS^{-/-}$ mice have cryptic neurological deficiencies. Australasian Winter Brain Research Organisation, Christchurch, August, 2007.

†**K.J. Skilbeck,** T. Hinton, G.A.R. Johnston. Sex and stress-induced differences in high and low affinity GABA binding by region. 7th International Brain Research Organisation (IBRO) World Congress of Neuroscience, Melbourne, 12-17th July 2007.

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K.J. Skilbeck, G.A.R. Johnston & T. Hinton. The effects of antipsychotic drug treatment on orthosteric and allosteric binding sites of the $GABA_A$ receptor. Institute for Biomedical Research Vth Annual Young Investigators Symposium, Sydney, 15 December, 2005.

K.J. Skilbeck, J.N O'Reilly, G.A.R. Johnston & T. Hinton. Antipsychotic drug effects on $GABA_A$ receptors. The Australasian Schizophrenia Conference, Fremantle, August, 2006.

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† Represents Publications and Communications arising from this thesis

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ABBREVIATIONS

5-HT	Serotonin
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
AFR	Animal facility reared early life manipulation condition
AMYG	Amygdala
ANOVA	Analysis of Variance
AP	Allopregnanalone / 3α-hydroxy-5α-pregnane-20-one
APMPA	3-Aminopropyl-(methyl)phosphinic acid
BDNF	Brain Derived Neurotrophic Factor
BLa	Basolateral nucleus of the amygdala
B_{MAX}	Saturation binding maximum constant
BNST	Bed nucleus of the stria terminalis
BSA	Bovine serum albumin
CA	Cornu Ammonis
CeA	Central nucleus of the amygdala
CING	Cingulate cortex
CNS	Central nervous system
CRH / CRF	Corticotropin-releasing hormone / Corticotropin-releasing factor
DAB	Diaminobenzidene
DG	Dentate gyrus of the hippocampus
DPSS	Diode-pumped solid-state laser
ED	Early-life deprivation early-life manipulation condition
EH	Early-life handled early-life manipulation condition
EPM	Elevated plus maze
GABA	gamma-Aminobutyric acid
GABARAP	GABA receptor associated protein
GABA-T	GABA-transaminase
GAD	Glutamate decarboxylase
GAT-1 / GAT-3	GABA transporter type 1 /GABA transporter type 3
GR	Glucocorticoid receptor
HIPP	Hippocampus
HPA	Hypothalamic-pituitary-adrenal
HRP	Horse-radish peroxidase
IC50	Concentration of drug that inhibits 50% of response
IgG	Immunoglobulin G
IHC	Immunohistochemistry
ip	Intraperitoneal
IR	Immunoreactivity
Isopentane	2-Methylbutane
iv	Intravenous
K_D	Dissociation constant
La	Lateral nucleus of the amygdala
Laser	Light Amplification by Stimulated Emission of Radiation
LC	Locus Coerelus
LD	Lateral dorsal nucleus of the thalamus
LS	Lateral septum

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	Sympatheric-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	Tetrahydrodeoxycorticosterone
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:
$GABA_A$ Receptors, Sex-Differences and Stress

1.1 The GABAergic system

1.1.1. GABA as a neurotransmitter

γ-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 α-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.

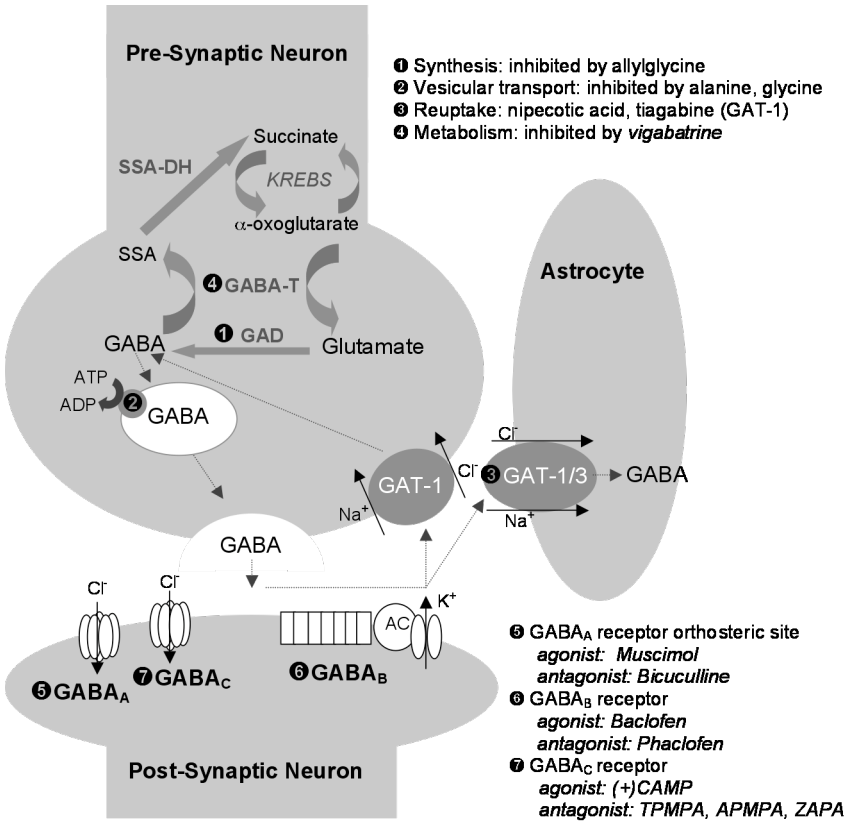


Figure 1.1: Physiology and pharmacology of GABA$_A$ receptor transmission

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. $GABA_A$ receptor structure

$GABA_A$ 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). $GABA_A$ 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 $GABA_C$ receptors are homomeric because functional receptors form from a single subunit protein.

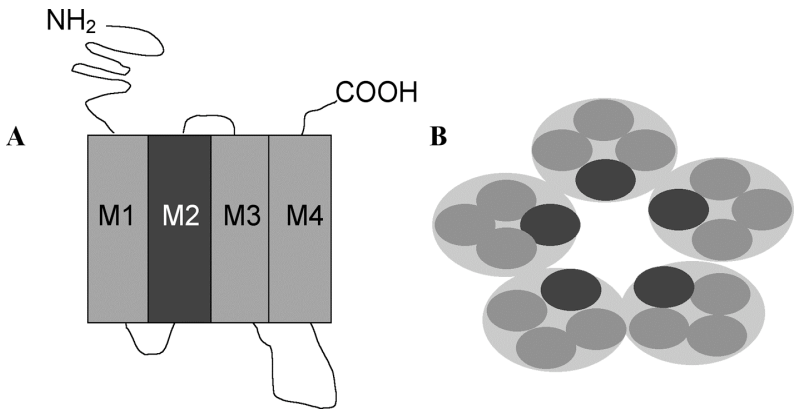


Figure 1.2: $GABA_A$ receptor subunit structure and arrangement. A. Schematic structure of a subunit of a $GABA_A$ receptor showing the agonist-binding extracellular domain and the four transmembrane domains. **B**. Pentameric structure of a $GABA_A$ receptor showing M2 domains facing the ion-conducting pore. *Adapted from Whiting, 2003*

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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ 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, $GABA_A$ 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. $GABA_A$ receptor subtypes

1.2.4.1. Subunit diversity

$GABA_A$ 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 $GABA_A$ 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 α (α_1-α_6), four β (β_1-β_4), three γ (γ_1-γ_3, 2 splice variants; γ_{2short}, γ_{2long}), one δ, one ε, and one θ subunit (Whiting, 2003). Splice variants also exist for the α_5, α_6, β_2, β_3 and γ_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 $GABA_A$ 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 α, β and γ 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 γβαβα, substantially reducing the number of possible configurations (Baumann *et al.,* 2002). However, ∂ and ε subunits may be able to replace γ, and θ may replace β 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 $GABA_A$ receptor subtypes

Immunohistochemical studies indicate that $GABA_A$ receptor subtypes are differentially distributed in the CNS (Pirker *et al.,* 2000). The most common subtype (~43%) contains α_1, $\beta_{2/3}$ and γ_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 α_1 and β_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 β_3 and γ_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 $\alpha_2\beta_{2/3}\gamma_2$ and $\alpha_3\beta_n\gamma_{2/3}$ subunit combinations are also common and are expressed mainly in regions where α_1 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 α_3 subunits predominate in the inner layers of the cortex, α_2 subunits predominate in the outer layers (Pirker *et al*., 2000; Zimprich *et al*., 1991). All three β subunits are widely distributed in the brain with complementary expression in subcortical and cerebellar regions and a pattern of β_2 predominance on interneurons (Miralles *et al*., 1999; Pirker *et al*., 2000). In contrast to the widely distributed $\alpha_{1/2}$ β and γ_2 subunits, the $\alpha_{3\text{-}6}$, γ_1 and ∂ subunits are largely confined to particular regions. For example, the α_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 α_5 is largely confined to the hippocampus (Fritschy and Mohler, 1995). Finally, immunohistochemical staining patterns show an overlap of α_1 and β_2, α_2 and β_3, $\alpha_{4/6}$ and ∂ 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. $GABA_A$ receptor pharmacology

1.2.5.1. The orthosteric site

The orthosteric site of $GABA_A$ 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 $GABA_A$ receptor agonist exists that does not act on $GABA_B$ or $GABA_C$ receptors (Johnston, 2005). For example muscimol acts as an agonist at the $GABA_A$ receptor orthosteric site but also acts as a potent agonist on the $GABA_C$ receptor (Johnston, 2005). Partial agonists that have reduced maximal efficacy compared with GABA also exist such as THIP, which acts as an antagonist at $GABA_C$ receptors (Johnston, 2005).

The $GABA_A$ receptor orthosteric site is thought to exist at the interface of the α and β subunits of $GABA_A$ receptors (Baur and Siegel, 2003). The orthosteric binding site has been extensively studied using radiolabelled agonists such as [^{3}H]GABA and [^{3}H]muscimol and antagonists such as [^{3}H]bicuculline and [^{3}H]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. allopregnanalone, 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 ($\alpha_5>\alpha_1>\alpha_6>\alpha_3$) (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 γ_2 subunits to increase the frequency of ion-channel opening. Benzodiazepines produce high affinity (nM) modulation of GABA at subtypes containing γ_2 subunits, with only low affinity (mM) enhancement if γ_1 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 α_4 and α_6 containing subtypes (Luddens *et al.,* 1991). Similarly, ethanol enhancement of GABA chloride currents depends on ∂ 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 γ_{2L} subunit show slightly greater sleep times in response to benzodiazepines and the α_1 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). β_3 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 ∂ 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 γ_2 or β_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 ∂ subunit deficient mice (Mihalek *et al.,* 1999). Studies with knockout mice have suggested that different α subunit isoforms may be involved in different behavioural effects of drugs, with α_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 α_5 subunits mediating spatial memory (Johnston, 2005). Compounds developed with preferential affinities for α_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 γ_2 subunit has also been implicated in anxiety as mice heterozygous for the γ_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 α_2 receptors are common in extrasynaptic regions and γ_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 [^{3}H]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 α_1, α_2, α_3, α_4 and α_5 subunit mRNAs (Impagnatiello *et al.,* 1998; Onhuma *et al.,* 1999; Pesold *et al.,* 1998; Volk *et al.,* 2002) and increased α_1 and $\beta_{2/3}$ subunit protein in the PFC of the schizophrenic brain (Ishikawa *et al.,* 2004). However, whilst the total population of $GABA_A$ receptors, labelled by [^{3}H]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 γ_2 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 $GABA_A$ 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}H]muscimol binding that are observed post-mortem in schizophrenia but rather, result in no change, or reductions in [^{3}H]muscimol 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}H]muscimol 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}H]flunitrazepam binding in the PFC following prolonged administration of antipsychotic drugs (Skilbeck *et al.,* 2007; Skilbeck *et al.,* 2008b). Thus, $GABA_A$ 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 $GABA_A$ receptors in schizophrenia holds. For example, alterations in $GABA_A$ receptors may result from an adaptation to impaired presynaptic GABAergic function, or an adaptation to changes in other neurotransmitter systems. However, in support of a role for $GABA_A$ receptors in the disease symptoms, certain studies have shown a correlation between symptom severity and reduced *in vivo* binding at the benzodiazepine site of $GABA_A$ receptors (Asai *et al.,* 2008; Ball *et al.,* 1998; Busatto *et al.,* 1997). Furthermore, recent studies showing that $GABA_A$ receptor α_3 and α_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: α_3 and α_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}C]flumazenil and [^{123}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}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 α_1, α_3, α_4 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 β_2, α_2 and γ_2 but decrease α_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 $GABA_A$ receptor regulation

1.3.1. $GABA_A$ receptor trafficking and membrane expression

The mechanisms involved in $GABA_A$ 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).

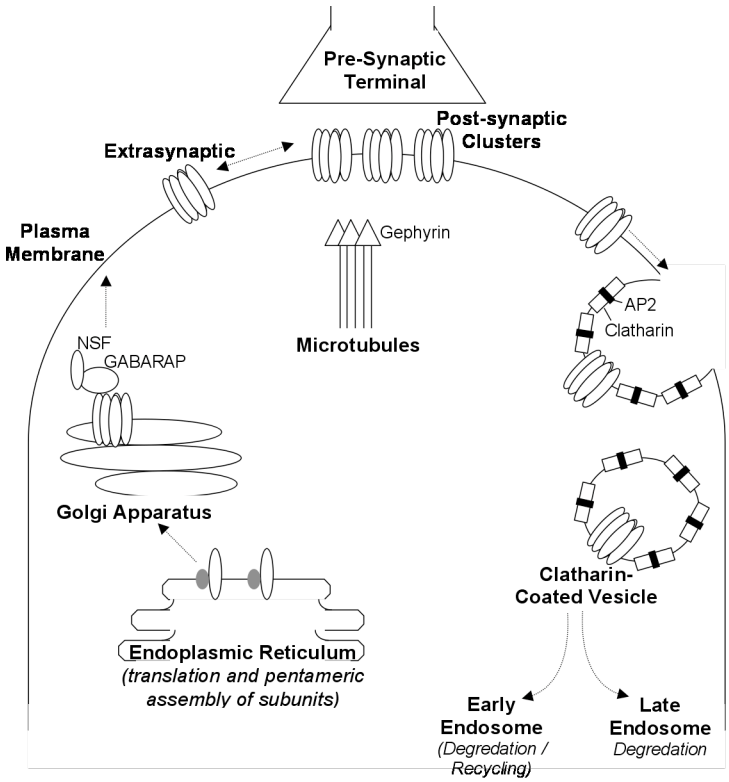


Figure 1.3: $GABA_A$ receptor trafficking. *Adapted from Lusher and Keller, 2001.*

1.3.2. Receptor assembly

$GABA_A$ receptors are synthesised in the endoplasmic reticulum of the neuronal cell body. Following translation of $GABA_A$ receptor subunit mRNA to protein in the endoplasmic reticulum, $GABA_A$ 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 γ_{2S}, β_1, β_3 and the chick β_4 subunits can reach the plasma membrane (Barnes, 2000; Kittler *et al.,* 2002) and in mammals only β_1 and β_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 α and β_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 γ_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 αβ, αβγ/∂, may actually be expressed on the membrane as functioning receptors.

1.3.3. Distribution of $GABA_A$ receptor subtypes on the membrane

$GABA_A$ receptors are usually found on post-synaptic densities, dendrites and cell bodies but studies suggest variations in the membrane locations between different $GABA_A$ 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 α_5 subunits are found almost exclusively on soma and dendrites, whilst α_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 $GABA_A$ 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 γ_2 (Essrich *et al.,* 1998) and certain subunits such as the α4 and δ 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 ∂-subunit containing $GABA_A$ 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 ∂-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 α subunit variants (1-3) as well as the γ_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 α_2 and γ_2 subunit clusters (Kneussel *et al.,* 1999b), and in experiments showing reduced α_2 and γ_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 $GABA_A$ receptors (Jacob *et al.,* 2005). Thus, when gephyrin auto-oligimerises it is thought to provide a scaffold that facilitates $GABA_A$ receptor clustering beneath the synapse (Jacob *et al.,* 2005; Levi *et al.,* 2004; Studler *et al.,* 2005; Yu *et al.,* 2007).

Interestingly, different $GABA_A$ receptors appear to vary in terms of the extent to which they colocalise with gephyrin. For example, in contrast to α_{1-3}, β and γ_2 subunits, the extrasynaptically located α_4, α_5 and δ 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 α_2 subunit intracellular loop to regulate the synaptic localisation of α_2 containing $GABA_A$ receptors in cultured cortical neurons (Tretter *et al.,* 2008). Despite this, as the α_{2-3} subunits show diffuse IR in addition to the clustered punctate staining that colocalises with gephyrin, it appears that α 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 γ_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 γ_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 γ_2 subunit-deficient mice lose both gephyrin and γ_2 receptor clusters (Alldred *et al.,* 2005; Essrich *et al.,* 1998; Yu *et al.,* 2007).

Whilst the role of gephyrin in $GABA_A$ receptor synaptic clustering is still under investigation, studies indicate that reductions in the colocalisation of gephyrin with $GABA_A$ receptor subunit proteins affects GABAergic function. For example, reduced $GABA_A$ receptor colocalisation with gephyrin results in alterations in single channel conductance times (Crestani *et al.,* 1999) and in the mean amplitude, but not the frequency of whole cell mIPSCs (Levi *et al.,* 2004; Kneussel *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 *et al.,* 1999; Levi *et al.,* 2004).

Functional alterations in GABAergic synaptic function appear to translate into behavioural differences. For example, a loss of α_1 and α_2 receptor clusters in hippocampus without alteration in gephyrin clusters is observed in mice lacking dystrophin in which animals show severe cognitive deficits (Kneussel *et al.,* 1999b). Furthermore, a loss of $GABA_A$ receptor synaptic clusters in the hippocampus and cortex of heterozygous γ_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 *et al.,* 1999). Thus, deficits in $GABA_A$ receptor synaptic clustering appear to translate into a more anxious, behaviourally reactive phenotype reminiscent of anxiety disorders and depression in humans (Crestani *et al.,* 1999).

1.3.5. Endocytosis, degradation and recycling

$GABA_A$ receptors undergo constitutive endocytosis under basal conditions to facilitate receptor turnover. $GABA_A$ 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 α and β 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 $GABA_A$ receptor regulation

$GABA_A$ receptor phosphorylation is a complex topic. A number of residues within the β and γ_2 subunits are capable of binding known kinases. The β_{1-3} subunits may be phosphorylated by protein kinase A, C, G (PKA; PKC, PKG) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (McDonald and Moss, 1997; Moss *et al.,* 1992). The γ_2 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 $GABA_A$ receptor phosphorylation state via the β or γ_2 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_2 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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ 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 α_1 subunit (Churn *et al.,* 2002). Phosphorylation has also been implicated in behavioural effects of allosteric modulators with PKCε (Hodge *et al.,* 1999) but not PKCγ (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 $GABA_A$ receptor expression

1.4.1. $GABA_A$ receptor onset and maturation

The developmental onset and maturational changes in brain $GABA_A$ 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 $GABA_A$ receptor binding sites (Schlumpf *et al.,* 1983; Shaw *et al.,* 1991), subunit mRNA expression (Laurie *et al.,* 1992; MacLennan *et al.,* 1991; Poulter *et al.,* 1992; Poulter *et al.,* 1993; Zhang *et al.,* 1991) and electrophysiological responses (Kellogg and Pleger, 1989) all show that $GABA_A$ receptors are abundant and functional in early brain development appearing by 15-18 weeks gestation in human cortex (Aaltonen *et al.,* 1983; Brooksbank *et al.,* 1982), foetal day 60 in the developing macaque cortex (Hendrickson *et al.,* 1994; Shaw *et al.,* 1991) and around gestational day 14 in rat brainstem (Poulter *et al.,* 1992; Schlumpf *et al.,* 1983). Radioligand binding studies suggest that the total population of $GABA_A$ receptors (both high and low affinity) measured by [^{3}H]GABA or [^{3}H]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 *et al.,* 1991) brain increases dramatically after birth. The subset of $GABA_A$ receptors containing the γ_2 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 *et al.,* 1991) and by birth in primates (Shaw *et al.,* 1991), consistent with the loss of γ_2 mRNA from PND 14 in rats (Gambarana *et al.,* 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 ($\alpha_{1/5}$ subunit-containing) and type II ($\alpha_{2/3}$ 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;

Hornung and Fritschy, 1996; Lopez-Tellez *et al.,* 2004; McKernan *et al.,* 1991). $\alpha_{2/3}$ Subunit mRNAs are first detected in rat brain at E15 with α_5 appearing at E17. α_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 α_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 α_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 α_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 α_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 α_1 immunoreactivity (Fritschy *et al.,* 1994; Hornung and Fritschy, 1996; Lopez-Tellez *et al.,* 2004; McKernan *et al.,* 1991). The adult α 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 α_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 α subunit 'switch' during brain maturation (Davis *et al.,* 2000; Kanaumi *et al.,* 2006) and certain nuclei of the brainstem show constant α_2 expression but a 'switch' from α_3 to α_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 α_1 immunoreactivity at any age and maintain intense α_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 α_1 or α_2 subunits (Fritschy *et al.,* 1994).

The pattern of the developmental 'switch' in $GABA_A$ receptor α subunits shows an area and lamina specific pattern however, it is unknown what signals trigger its onset. The onset of α_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 α_1 immunoreactivity (Fritschy *et al.,* 1994; Hendrickson *et al.,* 1994; Hornung and Fritschy, 1996; Paysan *et al.,* 1994) and displacement of [^{3}H]flunitrazepam binding by α_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, α_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 α_2 staining in the adult brain.

Table 1.1: Maturational changes in α_1 and α_2 subunit protein expression by region

Region		α_1	α_2	Species	Reference
Cortex	Whole	↑	↓	Rat	McKernan *et al.,* 1991
	Primary sensory (BA17, S1)	↑	↓	Marmoset Rat Macaque	Hornung and Fritschy, 1996 Fritschy *et al.,* 1994 *Paysan *et al.,* 1994 Hendrickson *et al.,* 1994
	Temporal	^↑	0	Human	Kanaumi *et al.,* 2006
	Motor, association areas	↑	↓	Marmoset Rat Macaque	Hornung and Fritschy, 1996 Fritschy *et al.,* 1994 Paysan *et al.,* 1994 Hendrickson *et al.,* 1994
	Infragranular layers	^↑	0	Marmoset Rat Macaque	Hornung and Fritschy, 1996 Fritschy *et al.,* 1994 Paysan *et al.,* 1994 Hendrickson *et al.,* 1994
Hippocampus	Whole	↑	0	Human Marmoset Rat	Kanaumi *et al.,*2006 Hornung and Fritschy, 1996 Fritschy *et al.,* 1994 Lopez-Tellez *et al.,* 2004
	Dentate Gyrus	^↑	↓	Human Rat	Kanaumi *et al.,* 2006 Lopez-Tellez *et al.,* 2004
	CA1	↑	↑	Human Rat	Kanaumi *et al.,* 2006 Davis *et al.,* 2000 Lopez-Tellez *et al.,* 2004
	CA3	0 (Human) ↑ (Rat)	0	Human Rat	Kanaumi *et al.,* 2006 Davis *et al.,* 2000 Lopez-Tellez *et al.,* 2004
Thalamus	Whole	↑	↓	Marmoset Rat	Hornung and Fritschy, 1996 Fritschy *et al.,* 1994
	Ventrolateral nucleus	↑	↓	Rat	Davis *et al.,* 2000
	Laterodorsal nucleus	↑	↓	Rat	Okada *et al.,* 2000
Hypothalamus	POA VMN	0 ↓	↑ ↑	Rat	Davis *et al.,* 2000
Amygdala	Whole	↓	↓	Rat	Davis *et al.,* 2000
Basal Forebrain	Globus pallidus	↑	↓	Marmoset Rat	Hornung and Fritschy, 1996 Fritschy *et al.,* 1994
	Substantia nigra	↑	↓		
	Medial septum	↑	↓		
	Pallidum	↑	↓		
Cerebellum	Whole	↑	↓	Rat	McKernan *et al.,* 1991 Fritschy *et al.,* 1994
Brainstem	Pre-botzinger complex	↑	0	Rat	Liu and Wong-Riley, 2004
	NTS Cuneate	↑ ↑		Rat	Liu and Wong-Riley, 2006

*Denotes references that only apply to α_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 α_2 subunits with α_1, with all neurons in a given region being affected by the subunit 'switch' (Fritschy *et al.,* 1994; Hornung and Fritschy, 1996). Disappearance of α_2 occurs first from dendrites then cell bodies and neuropil whilst α_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 α_2 subunit-containing neurons is unlikely as it has been shown that the increase in α_1 precedes the loss of α_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 α_1 and α_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 α 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 β subunit may be prominent in very early development where immunoreactive colocalisation is not as evident as the adult pattern of α_1-$\beta_{2/3}$ and α_2-$\beta_{2/3}$ colocalisation (Hendrickson *et al.,* 1994; Hornung and Fritschy, 1996). However, studies of β subunit mRNA expression suggest limited β_1 expression throughout development while reports on the β_2 and β_3 subunit genes are conflicting. Some reports suggest both β_2 and β_3 mRNAs are highly expressed at birth

(Gambarana *et al.,* 1991; Poulter *et al.,* 1993), whilst other studies observed that β_3 predominates in earlier development and remains constant, whilst β_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 α subunit expression alters $GABA_A$ 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 α_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, α_1 subunit containing receptors have reduced sensitivity to diazepam and clonazepam compared with the α_2 and α_3 containing subtypes (Puia *et al.*, 1991). Furthermore, in α_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 $GABA_A$ receptor sites that are measured by [^{3}H]muscimol, [^{3}H]flumazenil, [^{35}S]TBPS (Sur *et al.,* 2001), and the reduced muscimol-stimulated chloride uptake (Blednov *et al.,* 2003) observed in these mice. Thus, the α subunit 'switch' appears to alter adulthood receptor expression and pharmacology.

The 'switch' from α_2 to α_1 also leads to alterations in channel gating properties and such changes are associated with certain behaviours. α_2 and α_3 subunit-containing receptors show slower decay times and greater current amplitudes of mIPSPs than α_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 α_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 α_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 α 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 α 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 $GABA_A$ 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 α subunit 'switch' has also been implicated in the onset of synaptogenesis. The onset of α_1 and disappearance of α_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, α_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 α subunit maturational changes in brain development.

1.4.7. Summary

In summary, there are well known variations in the expression of $GABA_A$ receptor α subunits in the developing brain with the α_2 subunit predominating in the immature brain and the α_1 subunit in the adult brain. This subunit 'switch' is likely important given the different pharmacological and electrophysiological profiles for the α_1 and α_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 $GABA_A$ 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 $GABA_A$ receptor allosteric modulators

Drug	Behavioural Effect of drug (test)	Species	More sensitive sex
Ro 15-4513 FG7142	Learning Impairment (operant conditioning)	Mice	**Males** (Bao *et al.,* 1992)
	Reduced Activity (Open Field)	Rats	**Males (**Meng and Drugan, 1993)
Diazepam	Anxiolysis (Light-dark transitions)	Mice	**Males (**depends on female oestrous cycle) (Carey *et al.,* 1992)
	Protection from PTZ-induced seizure	Rats	**No difference** (Kokka *et al.,* 1992; Wilson and Biscardi, 1992)
	Protection from bicuculline-induced seizure	Rats	**No difference** (Wilson, 1992)
	Anxiolysis (plus-maze)	Rats	**Males** - Low dose (Wilson *et al.,* 2004)
	Anxiolysis (defensive prod burying)	Rats	**Males - Low dose** (Fernandez-Guasti and Picazzo, 1990; 1997; 1999) **No difference – High dose** (Wilson *et al.,* 2004; Boehm *et al.,* 2002)
	Reduced conflict behaviour (punished vs. unpunished drinking)	Rats	**No Difference** (Pericic and Pivac, 1995)
Allopregnanalone	Anxiolysis (plus-maze)	Rats	**No difference** (Fernandez-Guasti and Picazo, 1999)
	Anxiolysis (defensive prod burying)	Rats	**Males** (Fernandez-Guasti and Picazo, 1997; 1999)
	Anxiolysis (acoustic startle)	Rats	**Males** (Guinello and Smith, 2003)
	Anxiolysis (grooming)	Rats	**Females** (Zimmerberg *et al.,* 1999)
Ethanol	Protection from PTZ-induced seizure	Rats	**No difference** (Kokka *et al.,* 1992; Wilson and Biscardi, 1992)
	Anxiolysis (plus maze)	Rats	**No difference – low / high dose** (Wilson *et al.,* 2004; 1992; Stock *et al.,* 2000)
	Defensive prod-burying	Rats	**No difference – low dose** (Wilson *et al.,* 2004; Boehm *et al.,* 2004)
	Operant Conditioning	Mice	**No difference** (Bao *et al.,* 1992)
	Sedation (high dose)	Rats	**Males** (Webb *et al.,* 2002; Tayyabkhan *et al.,* 2002; Crippens *et al.,* 1999; Wilson *et al.,* 2004)

Table 1.3: Sex-differences in convulsant activity of $GABA_A$ receptor channel blockers and orthosteric site antagonists

Drug	Species	More sensitive sex
PTZ (i.v)	Rats	**Males** (Kokka *et al.,* 1992 ; Pericic and Bujas, 1997a)
Bicuculline (i.v)	Rats	**Males** (Pericic *et al.,* 1999; Guillet and Dunham, 1995; Pericic and Bujas, 1997a & b; Wilson 1992; Bujas *et al.*,, 1997; Manev *et al.,* 1987) **No difference** (Wilson and Biscardi, 1992; Devaud *et al.,* 1995) **Females** (Finn and Gee, 1994)
Bicuculline (i.p)	Rats	**No difference** (Pericic *et al.,* 1986)
Picrotoxin (i.v)	Rats	**Males** (Pericic and Bujas, 1997a)
Picrotoxin (i.v)	Mice	**No difference** (Pericic and Bujas, 1997a)
Picrotoxin (i.p)	Rats	**Females** (Pericic *et al.,* 1985)
Picrotoxin (i.p)	Cats	**Females** (Pericic *et al.,* 1986)
Picrotoxin (i.p)	Mice	**Males** (Pericic *et al.,* 1986)

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 $GABA_A$ 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 $GABA_A$ receptors.

1.5.4. Effects of gonadal hormones on drug sensitivity

Sex-differences in responses to drugs acting on $GABA_A$ 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 $GABA_A$ 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 ovariectimised 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 $GABA_A$ receptors

Whilst it is unclear if sex-differences exist in $GABA_A$ 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 *et al.,* 1997; Wilson, 1992; Wilson and Biscardi, 1992) of whole cortical and cerebellar [^{3}H]bicuculline sites (Kokka *et al.,* 1992), however, regional information is not available. Interestingly, intact females do show a lower GABA IC_{50} than males for displacement of [^{3}H]bicuculline binding, suggesting that overall female $GABA_A$ receptors are more sensitive to the effects of GABA. This difference was eliminated by gonadectomy (Bitran *et al.,* 1991; Wilson, 1992) and both testosterone (Bitran *et al.,* 1993) and oestrogen (Perez *et al.,* 1988) administration have been observed to affect chloride influx to cortical synaptosomes (Bitran *et al.,* 1993) and cortical TBPS / TBOB binding (Perez *et al.,* 1988). Thus, the presence of gonadal hormones in adulthood likely affects the functional state of the $GABA_A$ 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. [^{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 *et al.,* 1996). However, these sex differences depend on the brain region as males appear to have higher [^{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}H]muscimol 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}H]muscimol 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}H]muscimol binding sites have been observed in females compared with males. Thus, [^{3}H]muscimol 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}H]flunitrazepam 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 $GABA_A$ (α_2- and α_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 (α_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 α_4 (Gallo and Smith, 1993; Guinello *et al.,* 2003; Lovick *et al.,* 2005; Smith *et al.,* 1998; Sundstrom-Poromaa *et al.,* 2003) and ∂ 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 ∂ 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 $GABA_A$ receptors, this is by no means a robust finding. *Ex vivo* investigations provide more convincing results regarding sex-differences in $GABA_A$ receptors, with evidence to suggest that $GABA_A$ 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 $GABA_A$ receptor sensitivity, without information on sex differences in $GABA_A$ receptor expression, it is difficult to predict how GABAergic function varies between the sexes.

1.6. Acute stress and $GABA_A$ 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).

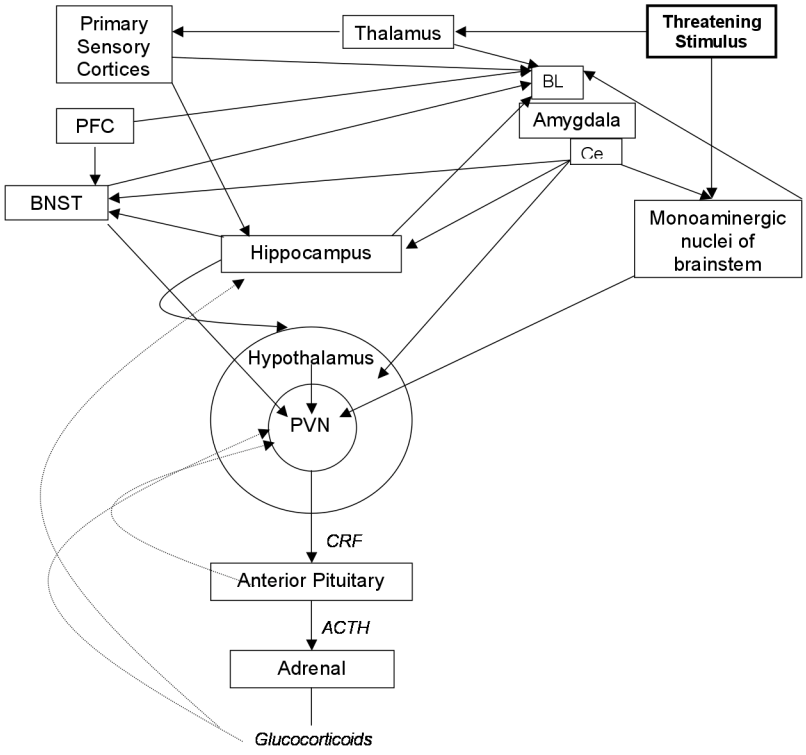


Figure 1.4: Diagrammatic representation of brain regions involved in stress responses. Adapted from Herman *et al.,* 2005.

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 ovarectomised 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 $GABA_A$ receptors

1.6.3.1. Overview

Not surprisingly, studies of the effects of acute adulthood stress on $GABA_A$ 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 $GABA_A$ receptors.

1.6.3.2. Orthosteric binding sites

Studies measuring [^{3}H]GABA binding suggest that the availability of low-affinity binding sites (B_{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}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 $GABA_A$ receptor binding even in the same stress paradigm.

There appear to be sex differences in the effects of stress on $GABA_A$ receptors. Studies have shown rapid increases in female but no change in male low-affinity (B_{MAX}) [^{3}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}H]GABA binding show that female mice appear to contain higher concentrations of endogenous inhibitors of [^{3}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}S]TBPS binds within the channel domain of the $GABA_A$ receptor. Reduced binding of [^{35}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}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}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}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.

Table 1.4: Stress-induced changes in $GABA_A$ receptor orthosteric site binding maximum

Stress	Animal	Radioligand	Change	Reference
Swim (3 minutes, 32°C)	Male mice Female mice	[^{3}H]GABA, low affinity	No change Increase	Akinci and Johnston, 1993
Swim (3 minutes, 32°C)	Male mice Female mice	[^{3}H]GABA, low affinity	No change Increase	Skerritt *et al.,* 1981
Swim (15 minutes, 25°C)	Male rats	[^{3}H]Muscimol	No change	Motohashi *et al.,* 1993
Swim (10 minutes 17°C)	Male Rats	Muscimol-stimulated Cl^- uptake	Increase	Schwartz *et al.,* 1987
Footshock	Male Rats	[^{3}H]GABA, low affinity	Decrease	Biggio *et al.,* 1981
	Male Rats Male Rat Pairs	[^{3}H]GABA, low affinity [^{3}H]GABA, low affinity	Decrease No Change	Cuadra and Molina, 1993
	Male rats	[^{3}H]Muscimol	Increase	Drugan *et al.,* 1993
	Male rats	Muscimol-stimulated Cl^- uptake	No Change	Drugan *et al.,* 1989
	Male rats	Muscimol-stimulated Cl^- uptake	No Change	Drugan *et al.,* 1989
Handling naïve exposure to death by guillotine	Male Rats	[^{3}H]GABA, low affinity	Decrease	Biggio *et al.,* 1981

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 [^{3}H]flunitrazepam and [^{3}H]βCCE binding in the cortex of male rats following swim stress (Medina *et al.,* 1983a; Medina *et al.,*

1983b). 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 ([^{3}H]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 $GABA_A$ 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 [^{3}H]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 γ_2-containing $GABA_A$ receptor subtypes.

Table 1.5: Stress-induced changes in $GABA_A$ receptor benzodiazepine site binding maximum

Stress	Animal	Radioligand	Change	Region	Reference
Swim (25°C, 15 min)	Male rats	[^{3}H]Flunitrazepam	Increase No change No change	Cortex Hippocampus Cerebellum	Motohashi *et al.,* 1993
Swim (6°C, 3 min)	Male rats	[^{3}H]Flunitrazepam	Increase No change	Cortex Cerebellum	Soubrie *et al.,* 1980
Swim	Male rats	[^{3}H]Flunitrazepam	Increase	Cortex	Rago *et al.,* 1989
Swim (18°C, 15 min)	Rats	[^{3}H]Flunitrazepam [^{3}H]βCCE	Decrease	Cortex	Medina *et al.,* 1983a; 1983b
Swim (6°C, 10 min)	Male mice	[^{3}H]Flunitrazepam [^{3}H]Ro 15-1788	No change No change No change Decrease No change Increased	Cortex Hippocampus Cerebellum Cortex Hippocampus Cerebellum	Park *et al.,* 1993
Swim (32°C, 3 min)	Male mice Female mice	[^{3}H]Diazepam	No change No change	Forebrain	Skerritt *et al.,* 1981
Swim (25°C) Footshock Immobilisation Isolation	Male mice	[3H]Diazepam	No change Decrease Increase No change	Forebrain	Braestrup *et al.,* 1979
Handling	Male rats	[^{3}H]Flunitrazepam	Decrease	Cortex	Mennini *et al.,* 1989; Andrews *et al.,* 1992
Social Defeat	Male mice	[^{3}H]Flunitrazepam [^{3}H]Ro 15-1788	No change Increase	Cortex Hypothalamus Cerebellum Cortex Hypothalamus Cerebellum	Miller *et al.,* 1987
Conflict Footshock	Male rats	[^{3}H]Diazepam	Decrease	Cortex	Lippa *et al.,* 1981
Swim (38°C, 15 min)	Male Chicks Female Chicks	[^{3}H]Flunitrazepam	Increase	Forebrain	Salvatierra *et al.,* 1994; Benavidez and Arce, 2002; Martijena *et al.,* 1992; Primus and Kellogg, 1991
Learned helplessness	Male rats	[^{3}H]Ro15-1788	Decrease Decrease Decrease No Change No Change	Cortex Hippocampus Striatum Cerebellum Hypothalamus	Drugan *et al.,* 1989
Footshock	Male rats	[^{3}H]Ro15-1788	No Change	Cortex Hippocampus Striatum Cerebellum Hypothalamus	Drugan *et al.,* 1989

1.6.4. Endogenous mediators of $GABA_A$ 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 $GABA_A$ 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 (μM) negative modulators of $GABA_A$ receptors (Majewska *et al.,* 1990; Majewska and Schwartz, 1987). As mentioned above, steroid action is affected by phosphorylation state and subunit composition, with the ∂ 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 $GABA_A$ receptor function may contribute to observations of rapid changes in $GABA_A$ receptor binding following stress, they are not sufficient to explain them, as altered [^{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. $GABA_A$ receptors and behavioural changes following acute stress

Consistent with stress-induced changes in $GABA_A$ receptors, acute stress alters behavioural sensitivities to $GABA_A$ 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 $GABA_A$ receptors, consistent with a loss of functional GABA binding sites in stressed males. Consistent with sex differences in the effects of stress on $GABA_A$ 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 $GABA_A$ receptor antagonists is blocked by finasteride inhibition of THDOC synthesis

(Barbaccia *et al.,* 1998) implicating neurosteroids in the effects of stress on $GABA_A$ 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 $GABA_A$ 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)

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

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 coerulus 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 γ_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 γ_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 α_1 subunit contribution. These findings were confirmed by observations of decreased α_1 and increased α_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 α_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 α_1 and α_2 subunit expression are consistent with disruptions in the development of the GABAergic system, in that there appears to be an impairment in the α_1/α_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 α 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:

$GABA_A$ RECEPTORS AND ACUTE ADULTHOOD STRESS

CHAPTER 2:
Effects of Adulthood Stress on $GABA_A$ 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 $GABA_A$ 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_{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 $GABA_A$ receptor function (Baur and Siegel, 2003; Harris and Allan, 1985; Maconochie *et al.*, 1994). Thus, stress rapidly alters the availability of functional (low-affinity) $GABA_A$ 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; 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), 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:

$$[L] + [R] \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} [LR]$$

where k_{+1} and k_{-1} 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_{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_{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}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 $GABA_A$ receptor binding, and to provide a better understanding of the mechanism of rapid $GABA_A$ 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 = $e^{-0.056.t}$. 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 Finetechnical (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 β emitter, binding would only be expected to occur in the top 5μm of tissue exposed to [^{3}H]GABA (Kuhar and Unnerstall, 1985). Sections were cut from three blocks at levels of bregma; 1.1-0.62mm, -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°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/mmol) was used as this is the experimentally derived concentration of GABA at which B_{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}H]GABA site 1000nM [^{3}H]GABA was used as this is the experimentally derived concentration of GABA at which B_{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}H]GABA stock (87 Ci/mmol) was diluted 1/10 with unlabelled GABA such that the final specific activity of [^{3}H]GABA in experiments with 1000nM [^{3}H]GABA was 8.7 Ci/mmol. Non-specific binding of [^{3}H]GABA was determined on additional sections at each concentration by adding 100μM GABA to the radioligand incubation medium.

As [^{3}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_{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}H]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}H]GABA

	High-affinity	Low-affinity
K_d (nM)	5-20nM	100-400nM
[GABA] for B_{MAX} (nM)	30nM	1000nM
References	Olsen *et al.*, 1981 Guidotti *et al.*, 1979 Enna and Snyder, 1975	Olsen *et al.*, 1981 Guidotti *et al.*, 1979 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.

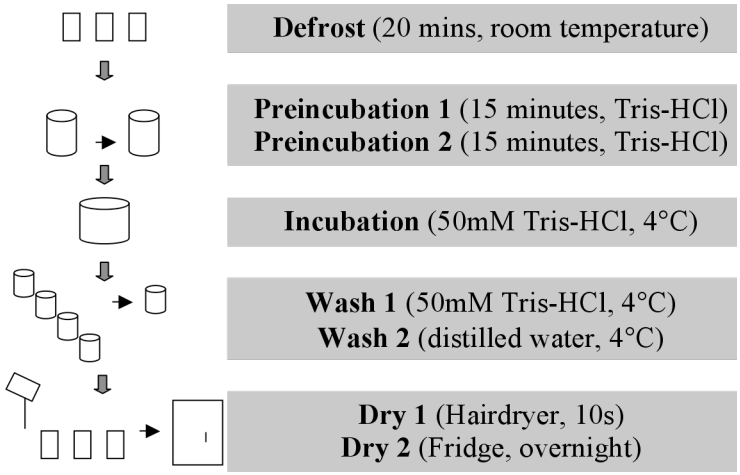


Figure 2.1: Schematic of the radioligand binding procedure

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.

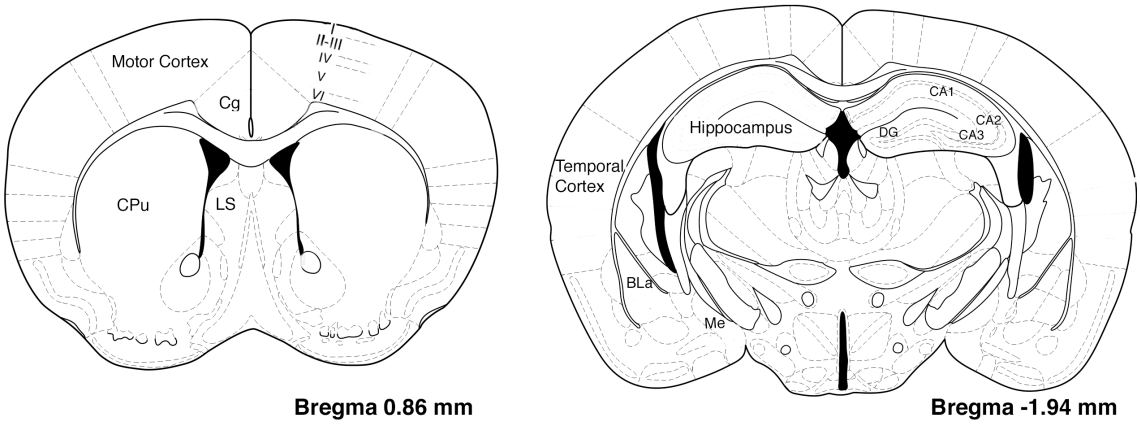


Figure 2.2: Brain regions examined in acute stress autoradiography. Images are reproduced with permission from "The Mouse Brain Atlas in Stereotaxic Coordinates" (Paxinos & Franklin, 2001). Regions examined in this experiment are labelled with the following abbreviations: cingulate cortex (Cg); caudate 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 [^{3}H]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 [^{3}H]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 $GABA_A$ 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}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}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}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}H]GABA binding (male>female) were removed by stress (male=female).

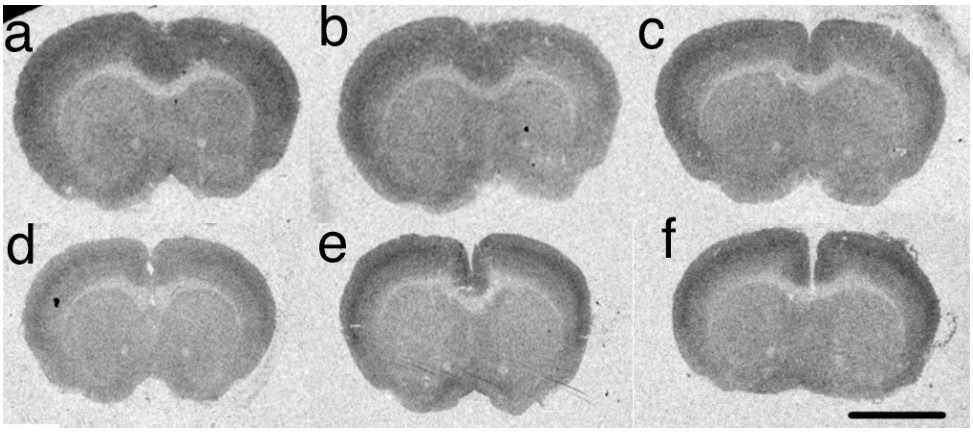


Figure 2.3: Representative autoradiographs of forebrain 1000nM [^{3}H]GABA binding sites. Pictures are from male (a, b, c) and female (d, e, f) mice exposed to no adulthood stress (a, d), individual 3 minute adulthood swim stress (b, e) and group stress in adulthood (c, f). Scale bar represents 0.5cm.

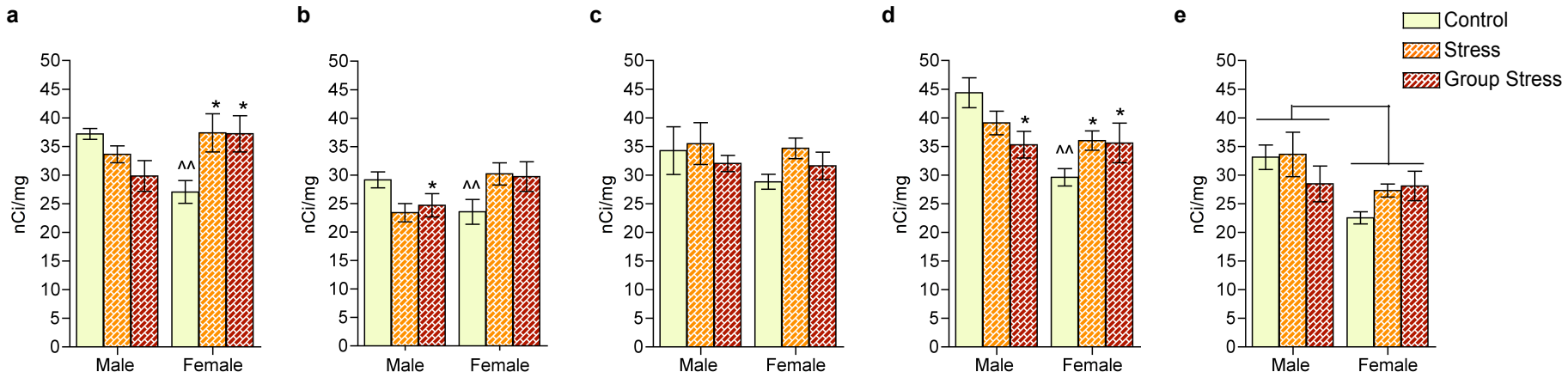


Figure 2.4: Effects of sex and adulthood stress on 1000nM [^{3}H]GABA binding sites in cortical regions. Data are expressed as mean ± SEM for a) cingulate b) frontal c) temporal cortical regions and d) upper e) lower cortical layers. *=p<0.05, **=p<0.01 for significant stress induced differences relative 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 [^{3}H]GABA binding in cortical regions. *Tests reaching significance with p<0.05 are highlighted.*

	Cingulate		Frontal		Temporal		Upper		Lower	
Main Effects										
Sex	$F_{(1,27)}$=3.03,	p=0.087	$F_{(1,27)}$=8.91,	p=0.006	$F_{(1,27)}$=0.26,	p=0.613	$F_{(1,27)}$=8.94,	p=0.006	$F_{(1,27)}$=7.88,	p=0.009
Stress	$F_{(2,27)}$=0.89,	p=0.424	$F_{(2,27)}$=0.33,	p=0.720	$F_{(2,27)}$=0.84,	p=0.442	$F_{(2,27)}$=0.41,	p=0.666	$F_{(2,27)}$=0.29,	p=0.752
Interaction										
Sex x Stress	$F_{(2,27)}$=6.56,	p=0.005	$F_{(2,27)}$=4.33,	p=0.023	$F_{(2,27)}$=0.08,	p=0.927	$F_{(2,27)}$=8.30,	p=0.002	$F_{(2,27)}$=2.68,	p=0.087

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.

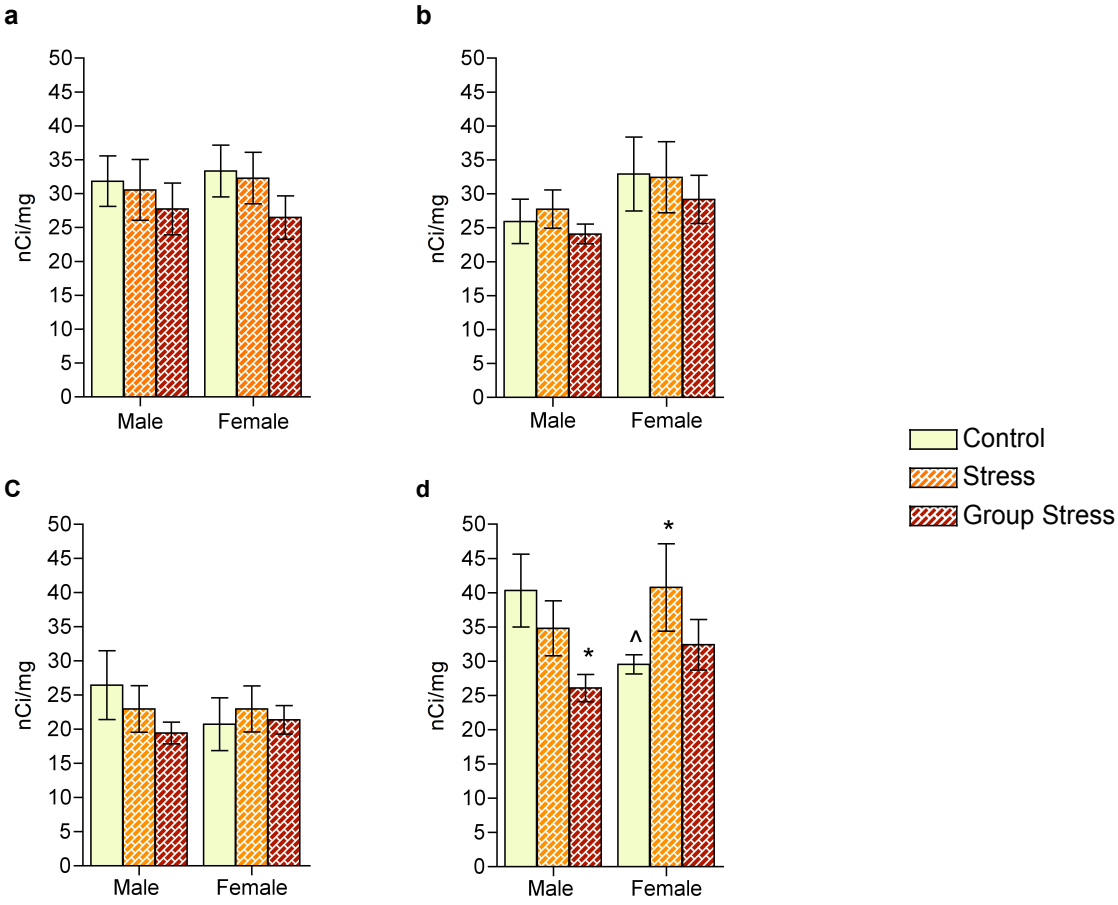


Figure 2.5: Effects of sex and adulthood stress on 1000nM [^{3}H]GABA binding sites in the hippocampus. Data are expressed as mean ± SEM for a) whole hippocampus and b) CA1-CA2 c) CA3 d) dentate gyrus subregions of the hippocampus. *=p<0.05 for significant stress induced differences relative to control 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 [^{3}H]GABA binding in hippocampal regions.
Tests reaching significance with p<0.05 are highlighted

	Hippocampus	CA1-CA2	CA3	Dentate Gyrus
Sex	$F_{(1,27)}$=0.05, p>0.05	$F_{(1,27)}$=0.77, p>0.05	$F_{(1,27)}$=1.02, p>0.05	$F_{(1,27)}$=0.34, p>0.05
Stress	$F_{(2,27)}$=1.14, p>0.05	$F_{(2,27)}$=2.51, p>0.05	$F_{(2,27)}$=0.58, p>0.05	$F_{(2,27)}$=1.96, p>0.05
Sex x Stress	$F_{(2,27)}$=0.10, p>0.05	$F_{(2,27)}$=0.36, p>0.05	$F_{(2,27)}$=1.23, p>0.05	$F_{(2,27)}$=3.49, p<0.05

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.

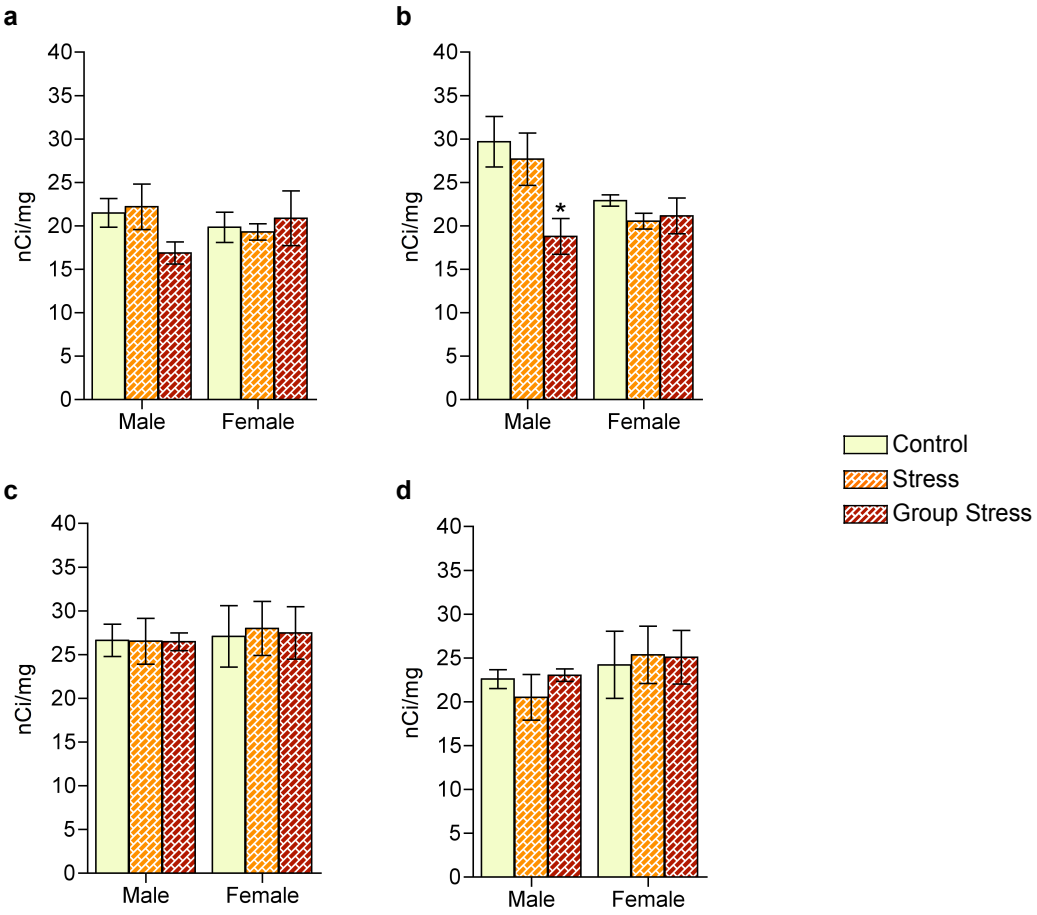


Figure 2.6: Effects of sex and adulthood stress on 1000nM [^{3}H]GABA binding sites in various subcortical regions. Data are expressed as mean ± SEM for the a) caudate putamen b) lateral septum c) baolateral amygdala and d) medial amygdala. *=p<0.05, for significant stress induced differences relative 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

	Caudate-Putamen	Lateral Septum	Basolateral Amygdala	Medial Amygdala
Sex	$F_{(1,27)}$=3.01, p=0.914	$F_{(1,27)}$=1.57, p=0.221	$F_{(1,27)}$=0.00, p=0.963	$F_{(1,27)}$=1.48, p=0.235
Stress	$F_{(2,27)}$=0.52, p=0.603	$F_{(2,27)}$=2.85, p=0.075	$F_{(2,27)}$=0.22, p=0.805	$F_{(2,27)}$=0.08, p=0.925
Sex x Stress	$F_{(2,27)}$=1.55, p=0.230	$F_{(2,27)}$=3.40, p=0.038	$F_{(2,27)}$=0.20, p=0.823	$F_{(2,27)}$=0.19, p=0.830

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.

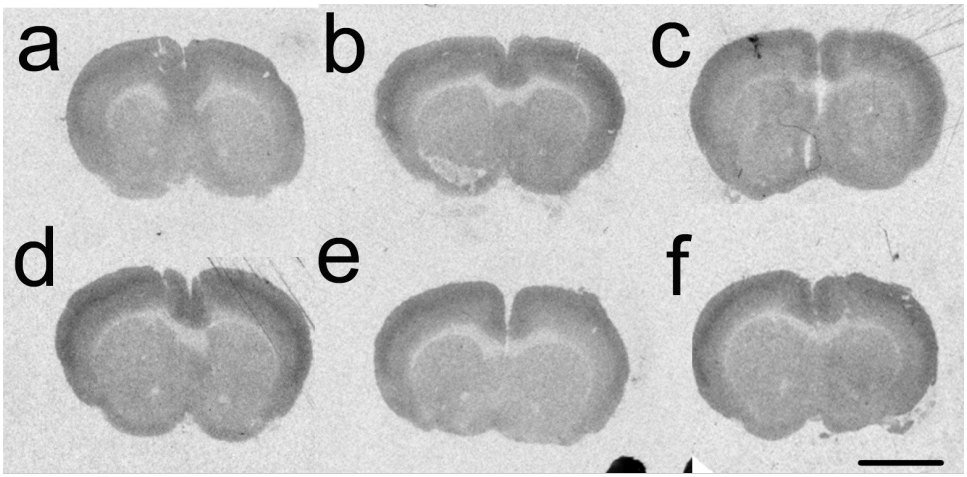


Figure 2.7: Representative autoradiographs of forebrain 30nM [^{3}H]GABA binding sites. Images are from male (a, b) and female (c, d) mice exposed to no adulthood stress (a, c) and individual 3 minute adulthood swim stress (b, d). Scale bar represents 0.5cm.

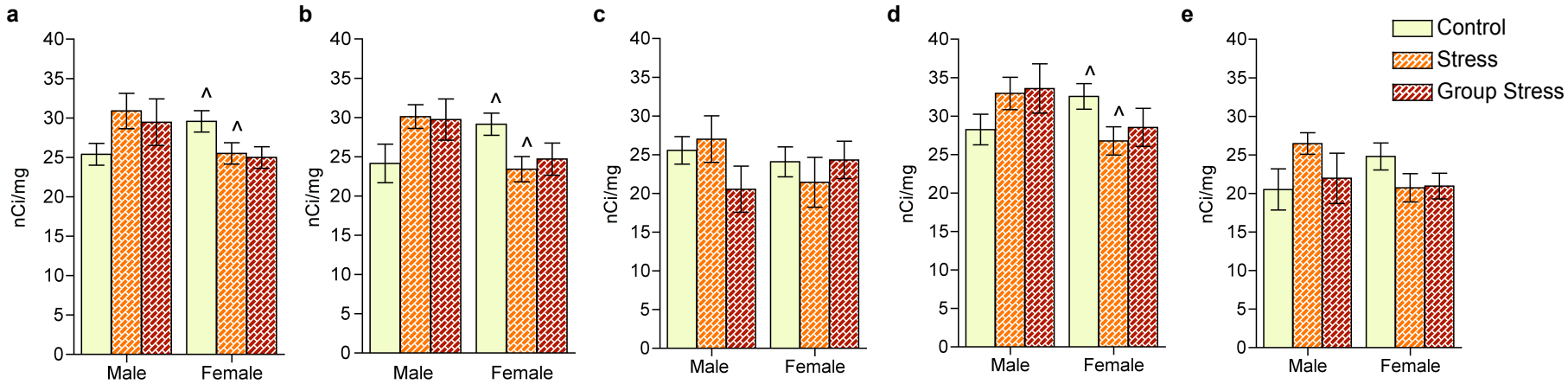


Figure 2.8: Effects of sex and adulthood stress on 30nM [^{3}H]GABA binding sites in cortical regions. Data are expressed as mean ± SEM for the a) cingulate b) frontal c) temporal cortical regions and d) upper e) lower cortical layers. ^=p<0.05 for significant sex differences relative to male controls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

Table 2.5: Results of 2-way ANOVA test for 30nM [^{3}H]GABA binding in cortical regions. *Tests reaching significance with p<0.05 are highlighted.*

	Cingulate		Frontal		Temporal		Upper		Lower	
Main Effects										
Sex	$F_{(1,27)}$=3.27,	p>0.05	$F_{(1,27)}$=1.35,	p>0.05	$F_{(1,27)}$=0.26,	p>0.05	$F_{(1,27)}$=1.19,	p>0.05	$F_{(1,27)}$=0.22,	p>0.05
Stress	$F_{(2,27)}$=0.20,	p>0.05	$F_{(2,27)}$=0.09,	p>0.05	$F_{(2,27)}$=0.46,	p>0.05	$F_{(2,27)}$=0.32,	p>0.05	$F_{(2,27)}$=0.50,	p>0.05
Interaction										
Sex x Stress	$F_{(2,27)}$=4.30,	P<0.05	$F_{(2,27)}$=4.93,	p>0.05	$F_{(2,27)}$=1.56,	p>0.05	$F_{(2,27)}$=4.09,	P<0.05	$F_{(2,27)}$=2.70,	p>0.05

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.

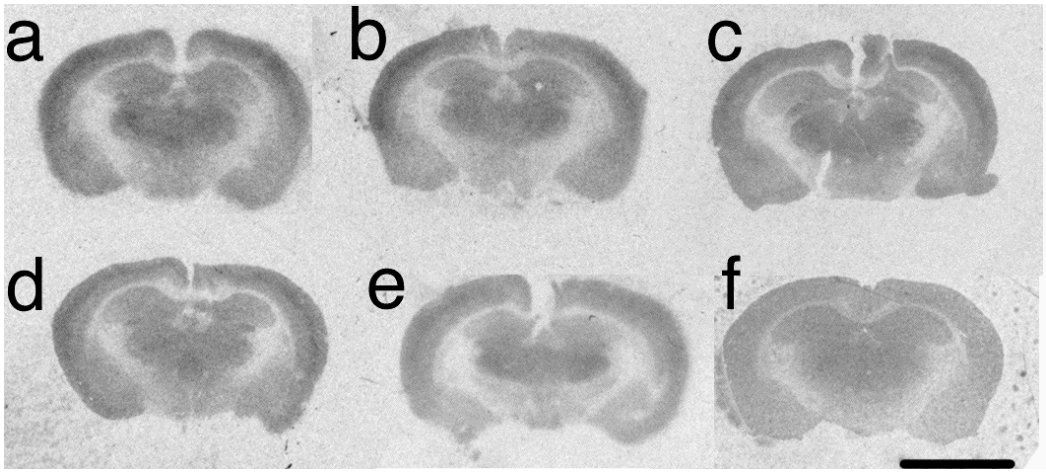


Figure 2.9: Representative autoradiographs of hippocampal 30nM [^{3}H]GABA binding sites. Images are from male (a, b) and female (c, d) mice exposed to no adulthood stress (a, c) and individual 3 minute adulthood swim stress (b, d). Scale bar represents 0.5cm.

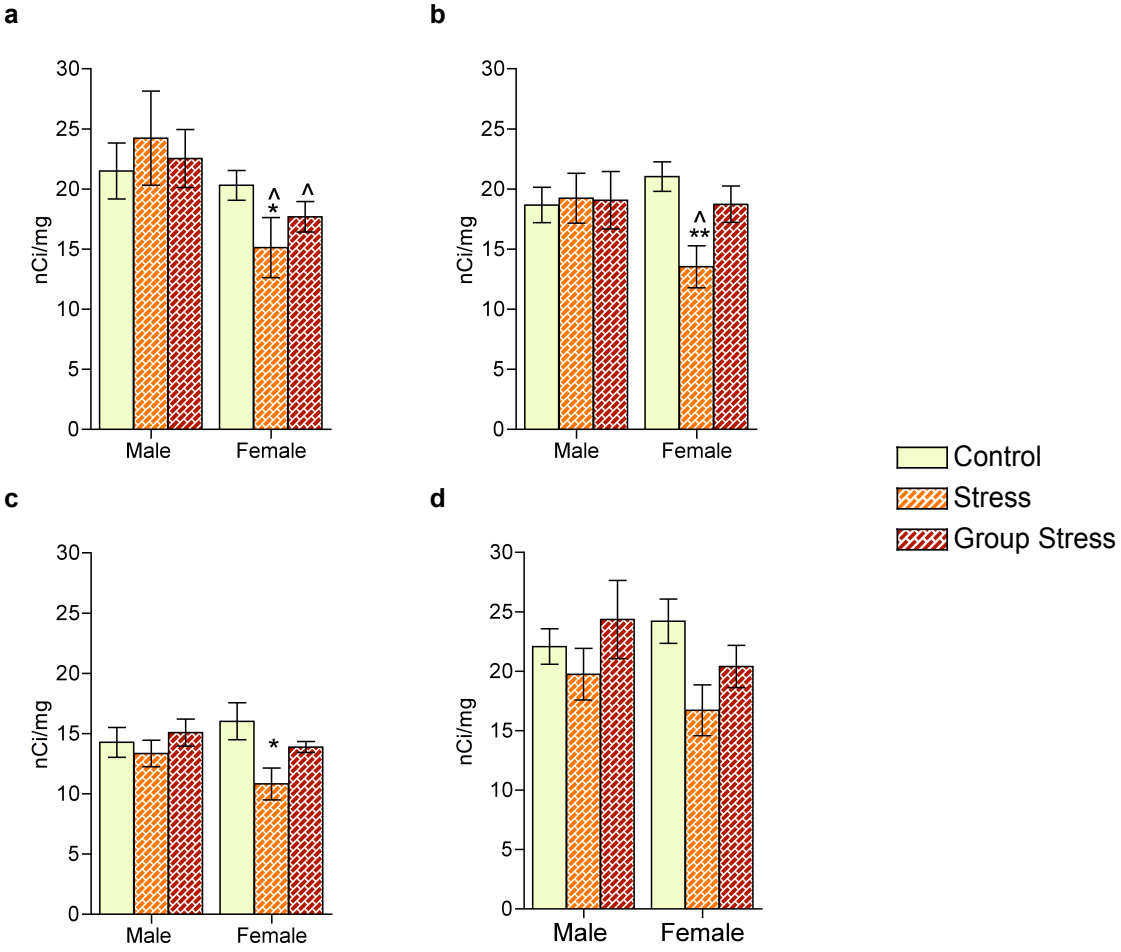


Figure 2.10: Effects of sex and adulthood stress on 30nM [^{3}H]GABA binding sites in hippocampal regions. Data are expressed as mean ± SEM for the a) whole hippocampus and b) CA1-CA2 c) CA3 d) dentate gyrus subregions of the hippocampus. *=p<0.05, **=p<0.01 for significant stress-induced differences relative to control mice of the same sex. ^=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*

	Hippocampus	CA1-CA2	CA3	Dentate Gyrus
Sex	$F_{(1,27)}$=3.88, p<0.05	$F_{(1,27)}$=1.91, p>0.05	$F_{(1,27)}$=0.13, p>0.05	$F_{(1,27)}$=0.13, p>0.05
Stress	$F_{(2,27)}$=0.62, p>0.05	$F_{(2,27)}$=2.12, p>0.05	$F_{(2,27)}$=3.01, p>0.05	$F_{(2,27)}$=1.11, p>0.05
Sex x Stress	$F_{(2,27)}$=3.12, p<0.05	$F_{(2,27)}$=3.41, p<0.05	$F_{(2,27)}$=3.47, p<0.05	$F_{(2,27)}$=0.80, p>0.05

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.

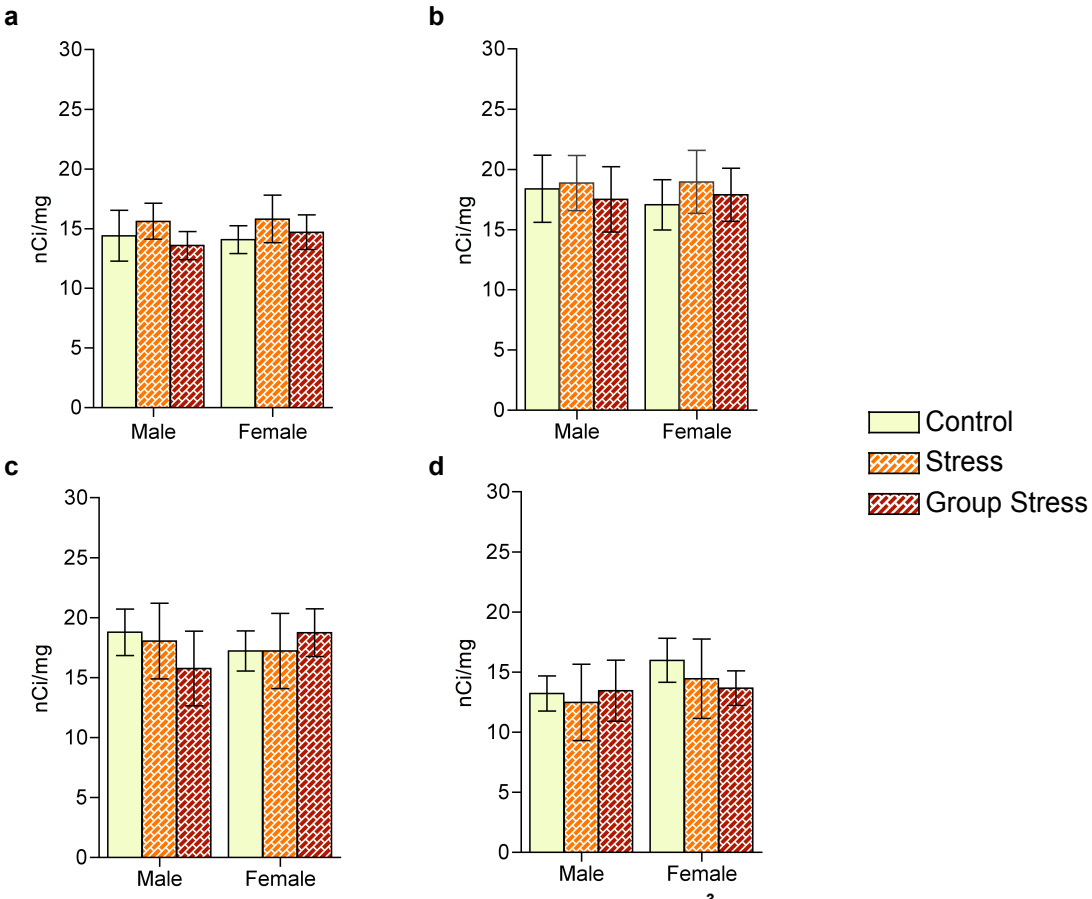


Figure 2.11: Effects of sex and adulthood stress on 30nM [^{3}H]GABA binding sites in subcortical regions. Data are expressed as mean ± SEM for the a) caudate putamen b) lateral septum c) basolateral amygdala and d) medial amygdala.

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*

	Caudate-Putamen	Lateral Septum	Basolateral Amygdala	Medial Amygdala
Sex	$F_{(1,27)}$=0.06, p>0.05	$F_{(1,27)}$=0.01, p>0.05	$F_{(1,27)}$=0.00, p>0.05	$F_{(1,27)}$=0.91, p>0.05
Stress	$F_{(2,27)}$=0.59, p>0.05	$F_{(2,27)}$=0.13, p>0.05	$F_{(2,27)}$=0.05, p>0.05	$F_{(2,27)}$=0.12, p>0.05
Sex x Stress	$F_{(2,27)}$=0.10, p>0.05	$F_{(2,27)}$=0.06, p>0.05	$F_{(2,27)}$=0.47, p>0.05	$F_{(2,27)}$=0.19, p>0.05

2.5. Discussion

2.5.1. Baseline sex differences in regional [^{3}H]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 $GABA_A$ receptors responsible for mediating tonic non-densensitising GABAergic currents in the brain.

2.5.2. Stress-induced changes in [^{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 [^{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 $GABA_A$ 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 $GABA_A$ binding sites suggests a stress-induced loss of functional $GABA_A$ receptor sites in male frontal cortical regions. Interestingly, forced swim stress has been observed to remove anxiolytic effects of diazepam (Briones-Aranda *et al.*, 2005), reduce the anti-seizure efficacy of benzodiazepines (Deutsch *et al.*, 1990) and reduce the convulsive activity of $GABA_A$ receptor antagonists (Drugan *et al.*, 1985; Pericic *et al.*, 2000; Pericic *et al.*, 2001; Soubrie *et al.*, 1980), suggesting impaired sensitivity of $GABA_A$ 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 *et al.*, 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}H]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}H]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}H]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 α_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 $GABA_A$ 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 $GABA_A$ 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 3.1: Timeline of animal model.

Time Period	*Stage of animal model*
ED 1-21	Gestation period
PND 0-14	EH-NH model
PND 14-21	Routine monitoring
PND 21	Weaning
PND 22-57	Routine Monitoring
PND 58-77	Routine monitoring & experimenter handling
PND 78-79	Elevated Plus Maze
PND 90-91	Adulthood stress protocol and euthanasia

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 immunohistochemsitry (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.

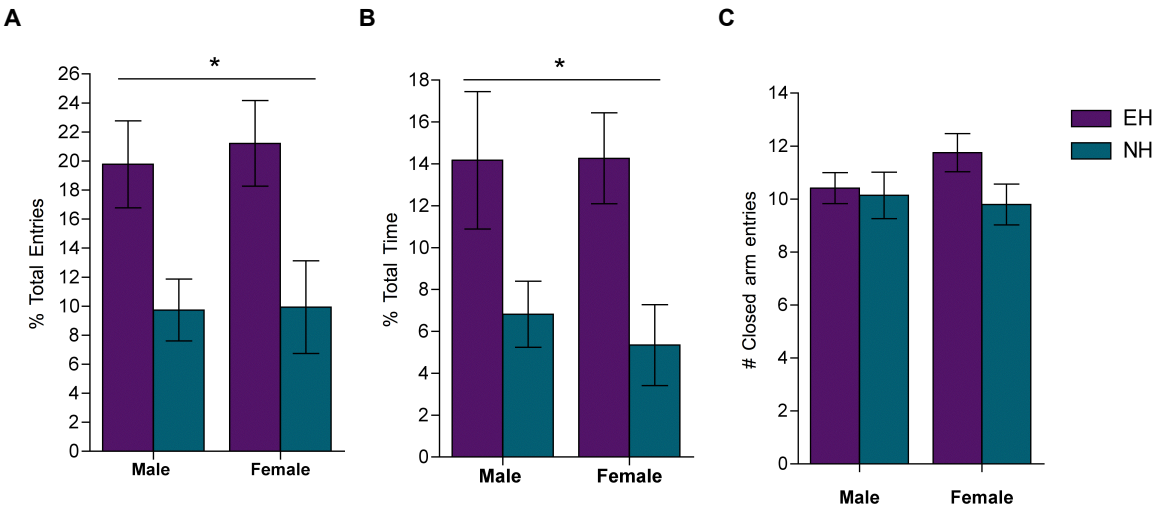


Figure 3.1: The effects of sex and early-life condition on elevated plus-maze behaviours. Figures represent % of entries into the open arms **(A),** the time spent on the open arms **(B),** and the total number of closed arm entries (**C**) over 5 minutes of free exploration of the elevated plus maze. 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 $GABA_A$ Receptor α 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.

$GABA_A$ receptors undergo marked changes in their subunit composition during development, involving the gradual replacement of the α_2 subunit with the α_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 α_2 to α_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 α_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 α_2 subunits with the α_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 α_1 and α_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 α_1 and α_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 γ_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 α_1 subunit in the dentate gyrus and amygdala (Caldji *et al.*, 2000b; Hsu *et al.*, 2003), and the α_2 subunit in the dentate gyrus (DG) (Hsu *et al.*, 2003) of male rats. Surprisingly, no previous studies have examined changes in α subunit expression in regions such as the primary sensory cortices and the thalamus, where the developmental subunit switch in α subunits is most prominent. Thus, the present study investigated the effects of early-life stress on α_1 and α_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 $GABA_A$ 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 α_1 staining procedure), or in immune (PAP method) or non-immune avidin- or streptavidin-biotin complexes (see section 4.2.5 - α_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 $GABA_A$ receptor α_1 and α_2 subunit expression in a variety of brain regions. Immunoperoxidase histochemistry was used to examine the relative density of each of the α_1 and α_2 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.

4.2.2.2. 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 $GABA_A$ 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 α_1 primary antibody but did not affect the α_2 subunit staining. Thus, 0.1 M TBS^+ pH 7.4 was used for washing slides and reagent dilution throughout the α_1 staining procedure whilst standard 0.1 M TBS pH 7.4 was used for the α_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 µL 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 α_1 subunit staining, negative isotype controls were used where the primary antibody was replaced with rabbit IgG (Dako, Carpenteria, CA, USA) at the same protein concentration. For α_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. α_1 Immunoperoxidase staining

4.2.4.1. Antibodies

i) Primary antibody

A polyclonal rabbit IgG directed against the $GABA_A$ receptor α_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 α_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 α_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 α_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. α_1 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% H_20_2 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 H_20_2 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-H_2O_2 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. α_2 Immunoperoxidase staining

4.2.5.1. Antibodies

i) Primary Antibody

A polyclonal guinea-pig antibody directed against the α_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 α_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 α_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. α_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 α_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 α_2 subunit staining. Of note, α_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 α_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 α_2 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 α_2 subunit staining. Thus no blocking step was performed for the α_2 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) Coverslipping slides

Slide coverslipping was performed as described in section 4.2.4.2 (see part vi).

4.2.6. Image acquisition

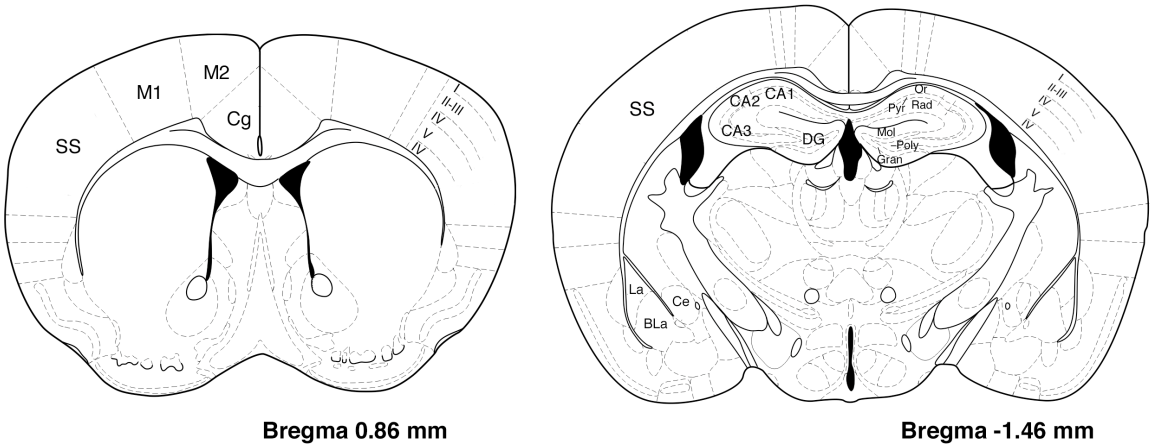
4.2.6.1. Brightfield microscopy image capture

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.



Bregma 0.86 mm **Bregma -1.46 mm**

Figure 4.1: Brain regions examined in immunohistochemistry experiments. 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); motor cortices (M1, M2); somatosensory cortex (SS); Cortical layers (I-VI); basolateral amygdala (BLa); central amygdala (Ce); lateral amygdala (La); dentate gyrus (DG); CA1 stratum oriens (Or); CA1 pyramidal cell layer (Pyr); CA1 stratum radiatum (Rad); DG molecular cell layer (Mol); DG granule cell layer (Gran); DG polymorphic cell layer (Poly).

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 $GABA_A$ 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 $GABA_A$ 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 α_1 or α_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 $GABA_A$ receptors

4.3.1. α_1 Subunit immunoreactivity

4.3.1.1. α_1 Subunit distribution

α_1 Subunit immunohistochemistry (IHC) revealed a selective pattern of distribution (see table 4.1). In general, the α_1 subunit expression was greatest in the cortex with abundant expression throughout the frontal (motor), somatosensory and cingulate cortices of all animals. α_1 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_1 IR was greatest in the CA1 region with only weak to moderate staining in the DG. Furthermore, the α_1 subunit was not expressed in the layers with the greatest α_2 IR - the cell body layers (pyramidal layers of CA1-CA3 and the granule cell layer of the dentate gyrus). α_1 staining was moderate in the amygdala, being particularly weak in the central nucleus where α_2 staining was strong. In the thalamus, the α_1 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_1 expression was moderate.

4.3.1.2. α_1 Subunit regional IR

Table 4.1 shows the optical density of α_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 α_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).

	Brain Region	Male		Female	
		EH	**NH**	**EH**	**NH**
CORTEX	CING	88.92±7.4 (5)	87.43±7.8 (6)	78.50±3.3 (5)	89.35±8.6 (5)
	M1	84.20±11.3 (5)	84.95±6.1 (6)	74.67±4.2 (5)	94.01±7.2 (5)
	M2	82.46±14.5 (5)	80.57±7.0 (6)	79.08±5.6 (5)	94.75±8.0 (5)
	SS	85.49±7.7 (5)	72.74±8.0 (6)	75.97±6.0 (5)	84.43±5.4 (5)
HIPP	CA1	105.70±8.9 (5)	100.67±11.9 (6)	108.45±5.7 (5)	105.36±8.3 (5)
	CA3	48.25±3.8 (5)	46.32±5.3 (6)	48.83±3.3 (5)	50.68±6.6 (5)
	DG	68.96±5.4 (5)	61.59±6.1 (6)	62.05±5.1 (5)	57.56±4.4 (5)
THAL	VL	87.06±9.1 (5)	83.74±8.2 (6)	81.11±6.8 (5)	80.30±8.8 (5)
	LD	96.32±6.5 (5)	94.00±7.7 (6)	93.05±7.6 (5)	88.64±7.1 (5)
AMYG	Lateral	77.28±10.7 (4)	86.2±3.4 (5)	96.0±10.6 (4)	77.91±10.7 (4)
	BLa	53.28±11.8 (4)	57.93±13.0 (5)	63.25±8.4 (4)	59.56±8.5 (4)
	CeA	17.13±2.2 (4)	12.46±6.4 (4)	15.09±5.5 (4)	16.69±1.8 (4)

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. α_1 Subunit IR in cortical laminae

Figures 4.2 and 4.3 show the effects of sex and early-life environment on α_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 α_1 subunit IR is significantly different between sexes in layers II-III. This means that regardless of early-life condition, males showed greater α_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 α_1 subunit IR. As shown in table 4.2, significant sex x early-life interactions were observed for α_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 α_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 α_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 4.2: Results of 2-way ANOVA tests for α_1 subunit immunoreactivity in cortical laminae.
Tests reaching significance with p<0.05 are highlighted.

Cortical Layer	**Sex**	**Early-life environment**	**Sex x Early-life environment**
I	$F_{(1,17)}$=0.02, p>0.05	$F_{(1,17)}$=0.86, p>0.05	$F_{(1,17)}$=0.26, p>0.05
II-III	$F_{(1,17)}$=4.70, p<0.05	$F_{(1,17)}$=4.14, p>0.05	$F_{(1,17)}$=0.71, p>0.05
IV	$F_{(1,17)}$=1.56, p>0.05	$F_{(1,17)}$=0.27, p>0.05	$F_{(1,17)}$=4.50, p<0.05
V	$F_{(1,17)}$=1.79, p>0.05	$F_{(1,17)}$=0.42, p>0.05	$F_{(1,17)}$=4.44, p<0.05
VI	$F_{(1,17)}$=0.56, p>0.05	$F_{(1,17)}$=3.36, p>0.05	$F_{(1,17)}$=4.56, p<0.05

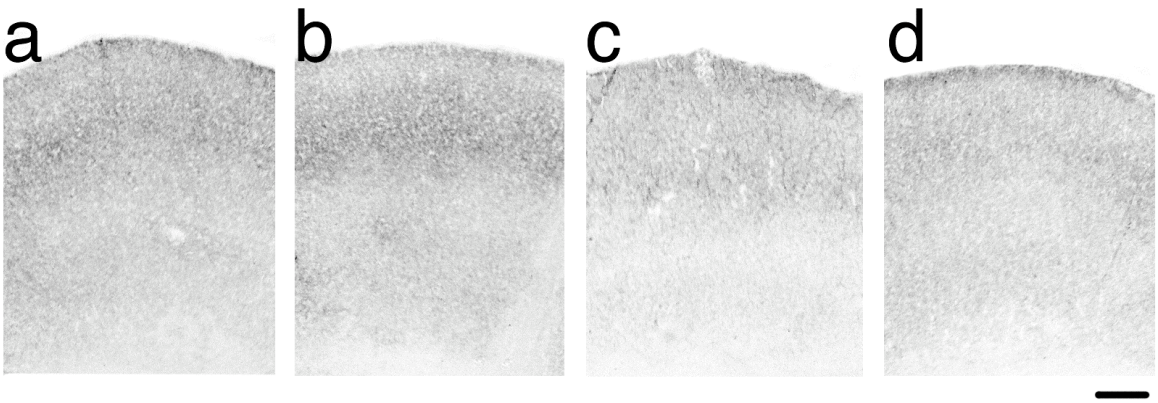


Figure 4.2: Representative images of α_1 subunit immunoreactivity in the somatosensory cortex. Images are taken from male (a,b) and female (c,d) mice exposed to EH (a,c) and NH (b, d) early-life environmental conditions. Scale 1 mm.

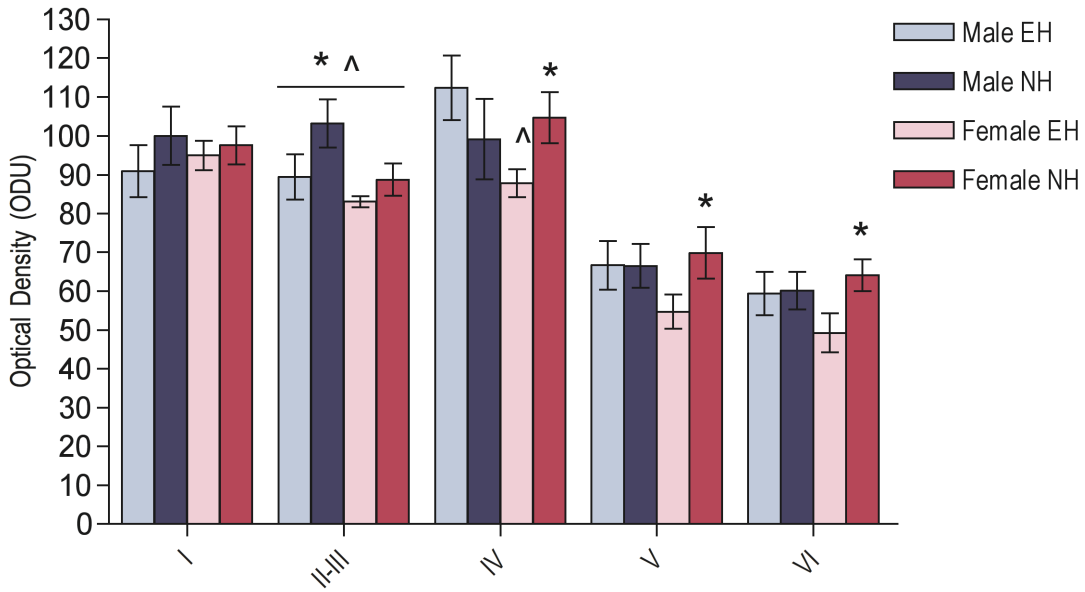


Figure 4.3: The effects of sex and early-life condition on α_1 subunit expression by cortical layer. Data represent mean relative optical density±SEM in optical density units (ODU). *p<0.05 denotes significant effects of early-life relative to EH group of same sex following a significant interaction. ^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. α_1 Subunit IR in hippocampal laminae

Figures 4.4 and 4.5 show the effects of sex and early-life condition on α_1 subunit IR in hippocampal layers. Results of the two-way ANOVA shown in table 4.3 indicate a significant reduction in α_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 4.3: Results of 2-way ANOVA tests for α_1 subunit immunoreactivity in layers of the hippocampus CA1 and DG subregions. *Tests reaching significance with p<0.05 are highlighted.*

Hippocampal Layer	**Sex**	**Early-life environment**	**Sex x Early-life environment**
Stratum oriens	$F_{(1,19)}$=1.24, p>0.05	$F_{(1,19)}$=1.12, p>0.05	$F_{(1,19)}$=0.45, p>0.05
Pyramidal cell	$F_{(1,19)}$=0.01, p>0.05	$F_{(1,19)}$=0.00, p>0.05	$F_{(1,19)}$=1.02, p>0.05
Stratum radiatum	$F_{(1,19)}$=1.59, p>0.05	$F_{(1,19)}$=0.38, p>0.05	$F_{(1,19)}$=0.80, p>0.05
Molecular cell	$F_{(1,19)}$=0.00, p>0.05	$F_{(1,19)}$=4.22, p<0.05	$F_{(1,19)}$=0.55, p>0.05
Granule cell	$F_{(1,19)}$=0.40, p>0.05	$F_{(1,19)}$=0.31, p>0.05	$F_{(1,19)}$=0.24, p>0.05
Polymorphic	$F_{(1,19)}$=0.04, p>0.05	$F_{(1,19)}$=4.12, p<0.05	$F_{(1,19)}$=0.97, p>0.05

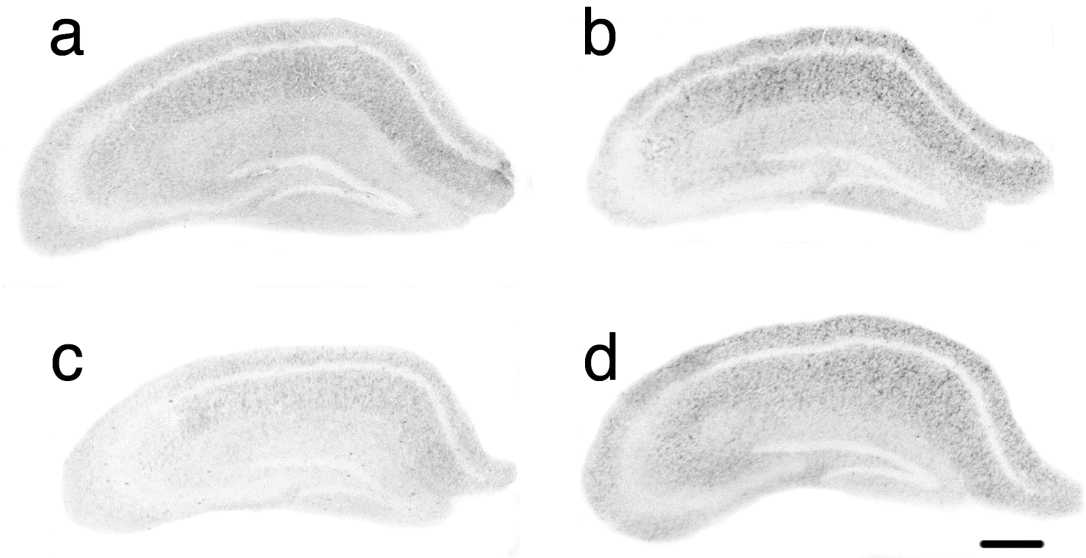


Figure 4.4: Representative images of α_1 subunit IR in the hippocampus. Images are taken from male (a,c) and female (b, d) mice exposed to EH (a,b) and NH (c,d) early-life environmental conditions. Scale 1 mm.

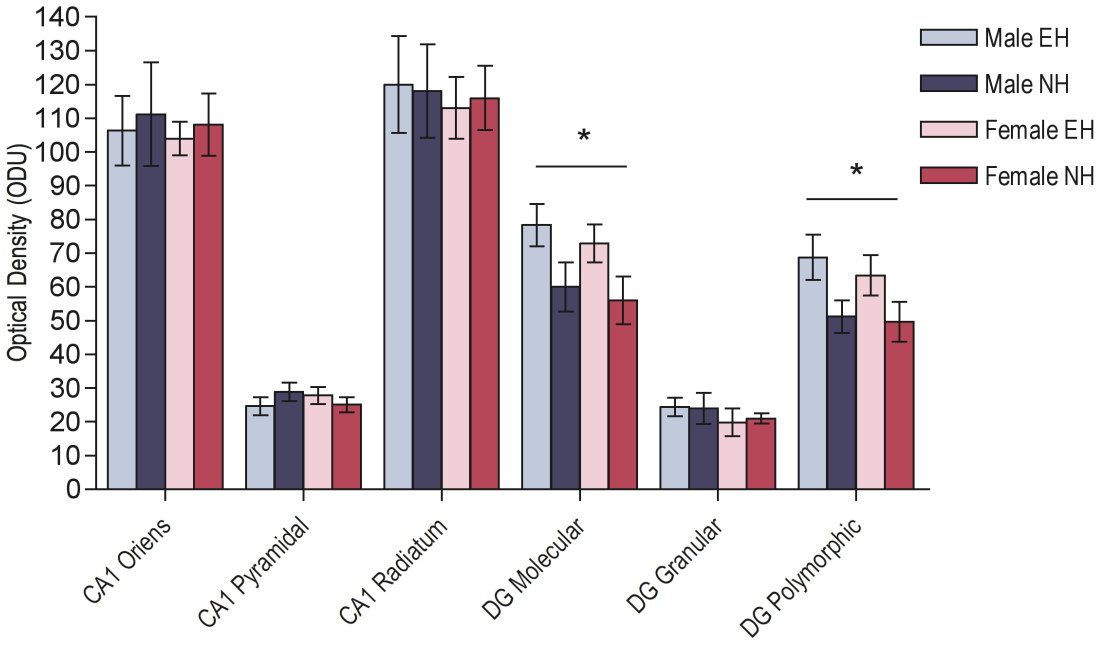


Figure 4.5: The effects of sex and early-life condition on α_1 subunit expression by layer in the CA1 and dentate gyrus hippocampal subregions. Data represent mean relative optical density±SEM in optical density units (ODU). Bars represent significant main effects at $p<0.05$ for * early-life condition.

4.3.2. α_2 Subunit immunoreactivity

4.3.2.1. α_2 Subunit distribution

Similar to α_1 subunit expression, α_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 α_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 α_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 α_2 subunit (see figure 4.8) with strong bands of staining distinguishing the cell body layers where α_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 α_2 immunostaining in the hippocampus was the polymorphic cell layer of the dentate gyrus. In the amygdala, α_2 subunit IR was also more abundant than α_1 with strong staining in nuclei where α_1 was weak such as the central nucleus of the amygdala. In the thalamus, the α_2 subunit was only weakly expressed with the exception of nuclei such as the reticular and the periventricular nuclei where the α_1 expression was low.

4.3.2.2. α_2 Subunit regional IR

Table 4.4 shows the effects of early-life condition and sex on the mean α_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 α_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 α_2 subunit IR compared with EH males (mean difference 25.2±9.4; p=0.016), early-life environment did not affect α_2 IR in females (p=0.763). EH females also showed reduced α_2 IR relative to EH males (mean difference 21.3±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 α_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 α_2 subunit IR in this region compared with males regardless of early-life environment (mean difference: EH 13.6±5.5; NH 11.0±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 α_2 subunit IR relative to EH males (mean difference 14.2±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 $GABA_A$ receptor α_2 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).

	Brain Region	Male		Female	
		EH	**NH**	**EH**	**NH**
CORTEX	CING	90.06±7.4 (6)	83.94 ±4.6 (6)	102.48±8.3 (6)	92.50±5.5 (5)
	M1	97.02±7.3 (6)	81.79±6.9 (6)	84.79±8.4 (6)	77.91±8.3 (5)
	M2	85.42±6.6 (6)	80.97±4.1 (6)	85.17±9.5 (6)	90.62±9.4 (5)
	S♠	96.98±7.8 (6)*	71.79±2.4 (6)	75.71±7.5 (6)	72.63±8.0 (5)
HIPP	CA1	77.25±3.7 (6)	84.22±3.5 (5)	84.88±1.6 (6)	88.99±5.2 (5)
	CA3	89.12±3.6 (6)	92.18±8.8 (5)	105.27±4.8 (6)	98.65±7.8 (5)
	DG✝	98.01±3.8 (6)	95.11±8.1 (5)	111.65±2.7 (6)	106.11±6.7 (5)
THAL	VL	55.87±2.4 (6)	56.01±1.9 (5)	50.28±4.2 (6)	47.06±5.9 (5)
	LD	58.97±2.0 (6)	45.81±5.1 (5)*	51.91±4.0 (6)	53.62±6.9 (5)
AMYG	Lateral	82.18±3.1 (6)	86.57±9.8 (6)	90.12±5.4 (6)	88.81±10.2 (5)
	BLa	82.54±2.4 (6)	89.22±10.0 (6)	90.86±2.5 (6)	90.89±8.7 (5)
	CeA	101.83±6.0 (6)	100.34±10.3 (6)	102.51±3.8 (6)	98.73±10.1 (5)

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_2 subunit immunoreactivity in various brain regions. *Tests reaching significance with p<0.05 are highlighted.*

Brain Region	**Sex**	**Early-life environment**	**Sex x Early-life environment**
CING	$F_{(1,19)}=2.49$, p>0.05	$F_{(1,19)}=1.47$, p>0.05	$F_{(1,19)}=0.08$, p>0.05
M1	$F_{(1,19)}=1.00$, p>0.05	$F_{(1,19)}=1.88$, p>0.05	$F_{(1,19)}=0.27$, p>0.05
M2	$F_{(1,19)}=0.41$, p>0.05	$F_{(1,19)}=0.01$, p>0.05	$F_{(1,19)}=0.45$, p>0.05
SS	$F_{(1,19)}=2.20$, p>0.05	$F_{(1,19)}=4.52$, p<0.05	$F_{(1,19)}=5.58$, p<0.05
CA1	$F_{(1,17)}=2.86$, p>0.05	$F_{(1,17)}=2.28$, p>0.05	$F_{(1,17)}=0.15$, p>0.05
CA3	$F_{(1,17)}=3.27$, p>0.05	$F_{(1,17)}=0.08$, p>0.05	$F_{(1,17)}=0.60$, p>0.05
DG	$F_{(1,17)}=4.62$, p<0.05	$F_{(1,17)}=0.54$, p>0.05	$F_{(1,17)}=0.05$, p>0.05
VL	$F_{(1,17)}=3.19$, p>0.05	$F_{(1,17)}=0.14$, p>0.05	$F_{(1,17)}=0.18$, p>0.05
LD	$F_{(1,17)}=0.06$, p>0.05	$F_{(1,17)}=0.88$, p>0.05	$F_{(1,17)}=4.42$, p<0.05
Lat	$F_{(1,19)}=0.57$, p>0.05	$F_{(1,19)}=0.00$, p>0.05	$F_{(1,19)}=0.23$, p>0.05
BLa	$F_{(1,19)}=0.46$, p>0.05	$F_{(1,19)}=0.21$, p>0.05	$F_{(1,19)}=0.20$, p>0.05
CeA	$F_{(1,19)}=0.00$, p>0.05	$F_{(1,19)}=0.11$, p>0.05	$F_{(1,19)}=0.02$, p>0.05

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), lateral amygdaloid nucleus (lat), basolateral amygdaloid nucleus (BLa), central amygdaloid nucleus (CeA).

4.3.2.3. α_2 Subunit IR in cortical laminae

Figures 4.6 and 4.7 show the effects of sex and early-life condition on α_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 α_2 subunit IR in layer IV, V and VI, indicating that regardless of sex, NH mice have reduced α_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 α_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 α_2 subunit immunoreactivity in cortical laminae. *Tests reaching significance with p<0.05 are highlighted.*

Cortical Layer	**Sex**	**Early-life environment**	**Sex x Early-life environment**
I	$F_{(1,17)}$=1.97, p>0.05	$F_{(1,17)}$=4.08, p>0.05	$F_{(1,17)}$=4.17, p>0.05
II-III	$F_{(1,17)}$=0.66, p>0.05	$F_{(1,17)}$=3.89, p>0.05	$F_{(1,17)}$=2.53, p>0.05
IV	$F_{(1,17)}$=3.23, p>0.05	$F_{(1,17)}$=7.68, p<0.05	$F_{(1,17)}$=0.47, p>0.05
V	$F_{(1,17)}$=3.49, p>0.05	$F_{(1,17)}$=5.80, p<0.05	$F_{(1,17)}$=0.04, p>0.05
VI	$F_{(1,17)}$=2.61, p>0.05	$F_{(1,17)}$=4.43, p<0.05	$F_{(1,17)}$=0.04, p>0.05

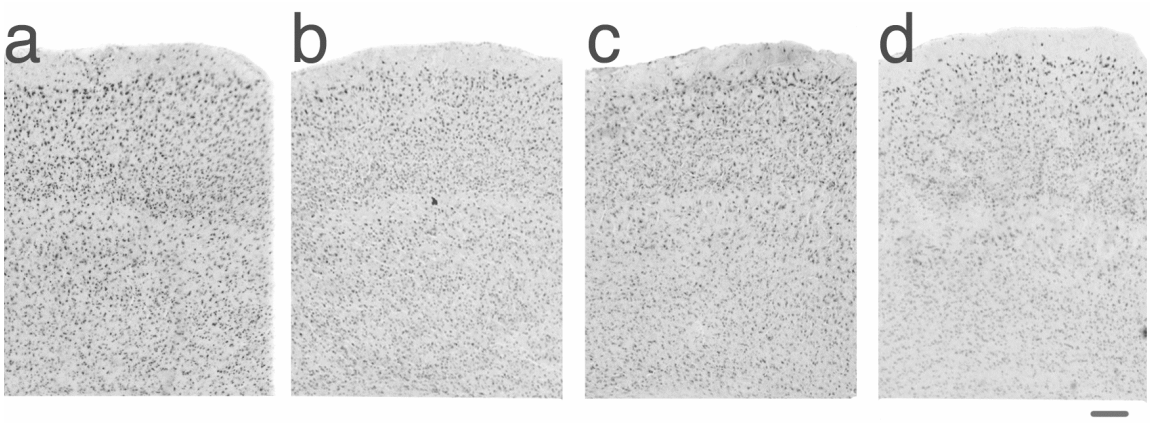


Figure 4.6: Representative images of α_2 subunit IR in the somatosensory cortex. Images are taken from male (a,b) and female (c,d) mice exposed to EH (a,c) and NH (b, d) early-life environmental conditions. Scale 1mm.

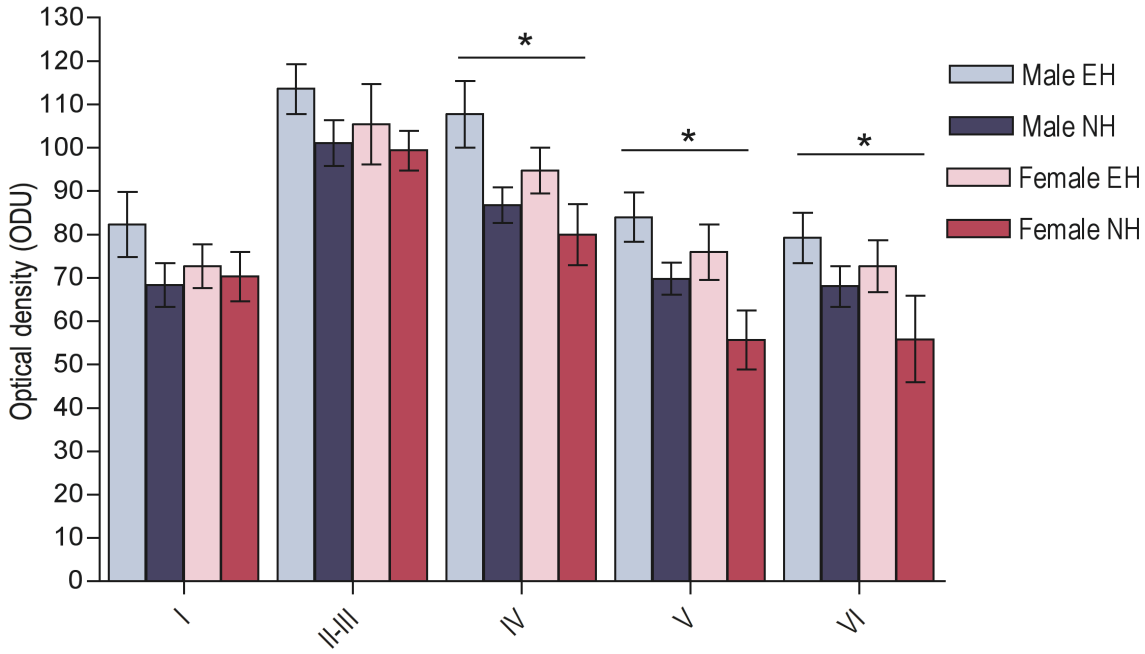


Figure 4.7: The effects of sex and early-life condition on α_2 subunit expression by cortical layer. Data represent mean relative optical density±SEM in optical density units (ODU). *p<0.05, **p<0.01 denote significant effects of early-life relative to EH group of same sex following a significant interaction. ^ 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. α_2 Subunit IR in hippocampal laminae

Figures 4.8 and 4.9 show the effects of sex and early-life condition on α_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 α_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 4.7: Results of 2-way ANOVA tests for α_1 subunit immunoreactivity in layers of the hippocampus CA1 and DG subregions. *Tests reaching significance with p<0.05 are highlighted.*

Hippocampal Layer	**Sex**	**Early-life environment**	**Sex x Early-life environment**
Stratum oriens	$F_{(1,19)}$=5.35, p<0.05	$F_{(1,19)}$=0.30, p>0.05	$F_{(1,19)}$=0.19, p>0.05
Pyramidal cell	$F_{(1,19)}$=2.26, p>0.05	$F_{(1,19)}$=0.00, p>0.05	$F_{(1,19)}$=0.03, p>0.05
Stratum radiatum	$F_{(1,19)}$=10.27, p<0.05	$F_{(1,19)}$=0.54, p>0.05	$F_{(1,19)}$=0.00, p>0.05
Molecular cell	$F_{(1,19)}$=7.10, p<0.05	$F_{(1,19)}$=0.65, p>0.05	$F_{(1,19)}$=0.76, p>0.05
Granule cell	$F_{(1,19)}$=0.79, p>0.05	$F_{(1,19)}$=0.02, p>0.05	$F_{(1,19)}$=0.06, p>0.05
Polymorphic	$F_{(1,19)}$=1.67, p>0.05	$F_{(1,19)}$=0.03, p>0.05	$F_{(1,19)}$=0.09, p>0.05

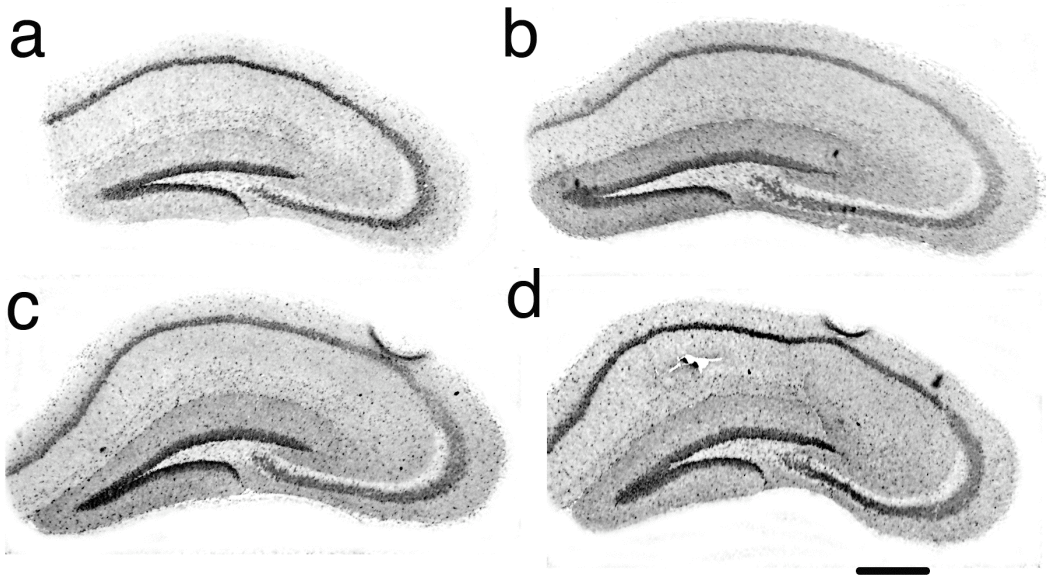


Figure 4.8: Representative images of α_2 subunit IR in the hippocampus. Images are taken from male (a, c) and female (b, d) mice exposed to EH (a,b) and NH (c, d) early-life environmental conditions. Scale 1mm.

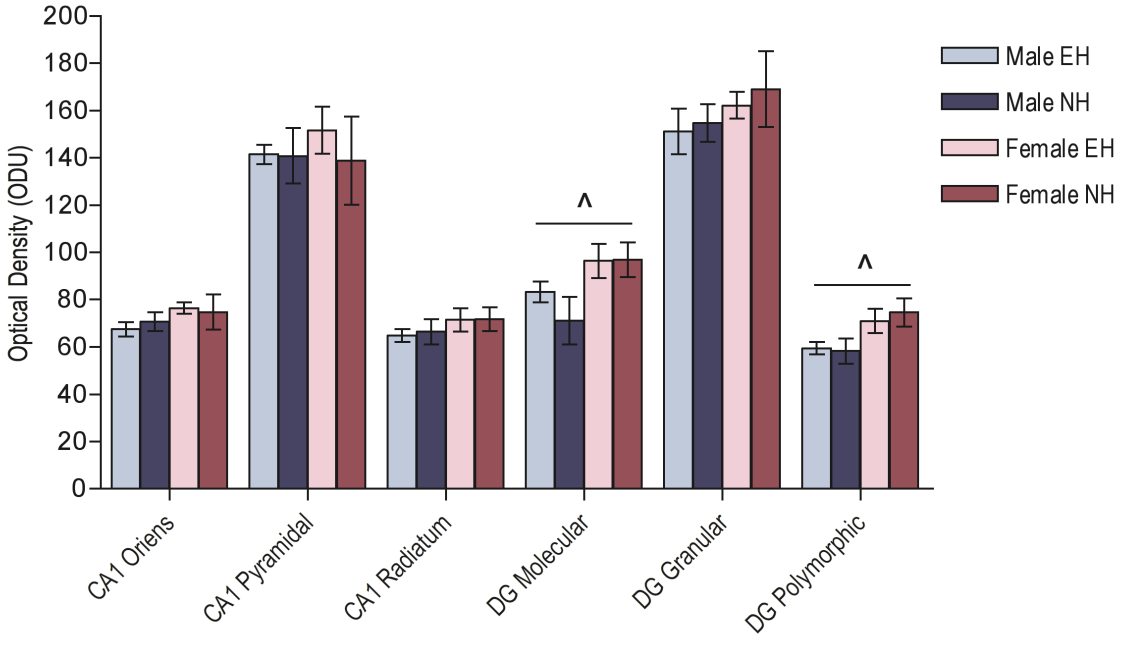


Figure 4.9: The effects of sex and early-life condition α_2 subunit expression in the CA1 and DG hippocampal subregions. Data represent mean relative optical density±SEM in optical density units (ODU). Grouped bars represent significant main effects of sex for individual layers of the hippocampus at ^ $p<0.05$.

4.4. Discussion

4.4.1. Sex differences in $GABA_A$ receptor α subunit expression

Results from the present study showed sex differences in $GABA_A$ receptor α_1 and α_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 α_1 subunit compared with females. Consistent with this finding, previous work has observed increased α_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 α_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 α_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 α_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 α_1 or α_2 $GABA_A$ receptor subunits. There are limited studies examining α_1 and α_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 α_4 and δ 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 α_1 or α_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 α_1 and α_2 subunits appear to be largely conserved across most brain regions.

Whilst the sex differences in α_1 and α_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 α_1 and α_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 α_1 and α_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 α_1-selective compounds such as zolpidem.

4.4.2. Effects of early-life stress on $GABA_A$ receptor α subunit IR

4.4.2.1. Effects of early-life stress on adult male $GABA_A$ receptor α subunit IR

Findings of the present study indicate long-term effects of early life stress on $GABA_A$ receptor α subunit protein regional densities in males. In adulthood, NH males showed reduced α_2 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 α_1 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 α_1 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 α_2 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 α_2 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 α_2 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 α_2 subunit protein expression is not affected in the DG.

No early-life environment-induced changes were observed in the α_1 or α_2 subunit protein density or distribution in any other brain regions. The regionally limited effects of early-life stress observed for the α_1 subunit in the present study are consistent with a previous study showing that zolpidem binding at α_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 α_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 α_2 subunit are regionally dependent.

4.4.2.2. Effects of early-life stress on adult female $GABA_A$ receptor α subunit IR

Findings of the present study also indicated long-term effects of early-life stress on $GABA_A$ receptor α subunit protein densities in females. Similarly to males, adult NH females showed a reduction in α_2 subunit density in the lower SS cortical layers as well as a reduction in α_1 subunit density in the polymorphic and molecular cell layers of the DG relative to EH females. NH females also showed an increase in α_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 α 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 $GABA_A$ receptor α 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 $GABA_A$ receptor subunit expression at different developmental time-points, and so cannot causally relate adulthood changes in α subunit expression to a disruption of the developmental changes in $GABA_A$ receptors, the regional dependence of the effects observed in the present study are consistent with disruptions of the α subunit developmental reshuffling that occurs over the first few postnatal weeks. The α_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 α_2 expression in the supragranular layers of the SS cortex, which undergo only small reductions in α_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 α 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 α subunit expression with both the α_1 and α_2 subunits being affected but in opposite directions. This suggests that both the developmental increase in the α_1 subunit and decrease in the α_2 subunit may be disrupted in females.

Interestingly, if early-life effects on adulthood α 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 α_2:α_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 α_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 α_1:α_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 α subunit switch.

4.4.4. Relevance of altered $GABA_A$ receptor α 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*

al., 1987; Bolden *et al.*, 1990; Caldji *et al.*, 2000a; Caldji *et al.*, 2000b). Unfortunately, it is difficult to differentiate between differences in $GABA_A$ receptor subunit expression and a loss in the number of GABAergic cells within a region using $GABA_A$ 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 α_1 and α_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 α_1:α_2 ratio in a regionally specific fashion whilst EH group showed a reduced ratio of α_1:α_2 subunits. It is well documented that different $GABA_A$ 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 $GABA_A$ receptor subunits are associated with different behavioural effects of benzodiazepines, with the α_1 subunit being associated with the sedative, amnesic, and anticonvulsant actions, whilst α_2, α_3, and α_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, α_2 subunit-containing receptors show slower decay times and greater mIPSP current amplitudes than α_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 α_2:α_1 subunit ratios (Franks and Lieb, 1994). Thus, the increased α_1:α_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 α_1:α_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 α_1 and α_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 α_1 and α_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 α_1, α_3, α_4 and δ 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 α 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 α_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 α_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 α_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 α subunit expression. The α_1 and α_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 α_1:α_2 subunit ratios of the lower cortical layers where the NH group showed an increased α_1:α_2 subunit ratio as a

result of reduced α_2 expression in males and a combined reduction of α_2 and increase of α_1 expression in females. The increased α_1:α_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 α_1:α_2 subunit ratio that was observed in the present study is consistent with the region-dependent variations in α_1:α_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 α 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 α_1:α_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 (Bolthalter *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 $GABA_A$ receptor α_2 and γ_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 α_2 and γ_2 subunit-containing $GABA_A$ 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 $GABA_A$ receptor synaptic clustering.

Previous studies have observed that alterations in $GABA_A$ receptor synaptic clustering result in variations in brain function and behaviour. In particular, a reduction in colocalisation of the α_2 subunit with gephyrin in the hippocampus (CA1 and DG) of mice heterozygous for the $GABA_A$ receptor γ_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 $GABA_A$ receptor synaptic clustering.

$GABA_A$ receptor synaptic clustering is particularly likely to be sensitive to early-life environmental manipulations. Recruitment of $GABA_A$ 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 α_2 to α_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 $GABA_A$ receptors and their cellular location. To examine $GABA_A$ receptor clustering the α_2 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 $GABA_A$ 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_2 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 $GABA_A$ 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) α_2 primary antibody

At the time of experimentation no guinea pig raised anti-$GABA_A$ receptor α_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 α_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 α_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 α_2 subunit immunoreactivity that was identical with previous descriptions for the α_2 subunit (Fritschy and Mohler, 1995).

ii) Gephyrin primary antibody

The monoclonal mouse antibody (clone mAB7a; 1µg/µL) 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 α_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 α_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 α_2 antibody, or mouse IgG_1 (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 α_2 subunit antibodies) was replaced with a negative isotype control solution, containing equal rabbit and mouse IgG concentrations as the α_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 α_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 α_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 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 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.

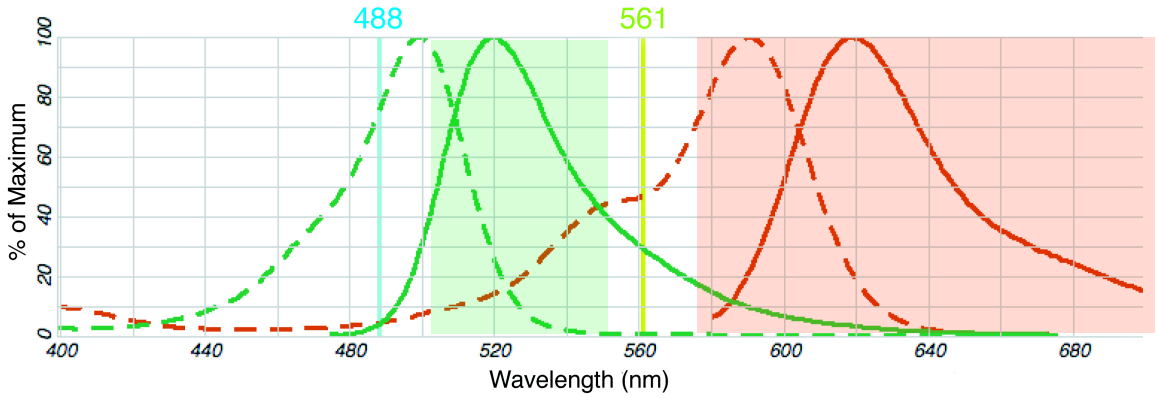


Figure 5.1: Excitation and emission spectra for fluorescent dyes Alexa Fluor 488 and Alexa Fluor 594. Absorption spectra are represented as dashed lines, whilst emission spectra are represented by filled lines. Green lines show the Alexa 488 spectra, whilst red lines show the Alexa 594 spectra. Laser 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 μm^2) was selected based on previous investigations investigating the size range of clusters for a similar experiment (Christie *et al.*, 2002; Marty *et al.*, 2004; Sassoe-Pognetto *et al.*, 2000) and the Nyquist criterion, which requires 2.3 pixels to digitally sample a minimum resolved distance.

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 *et al.*, 1999; Koksma *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 *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 nm^2 (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 α_2, gephyrin and colocalised gephyrin clusters (number, size, area covered) and the % area colocalised for each of the α_2 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. α_2 Subunit-containing $GABA_A$ receptor clusters

Figure 5.2 shows the effects of sex and early-life condition on α_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 μm^2 was not significantly different between EH and NH conditions, there was a significant main effect of early-life stress on the size of α_2 subunit clusters ($F_{(1,16)}=9.59$, $p<0.01$) and the area covered by α_2 subunit clusters ($F_{(1,16)}=11.30$, $p<0.01$). Thus, regardless of sex, α_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 μm^2; and females: 0.19±0.09 μm^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 α_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).

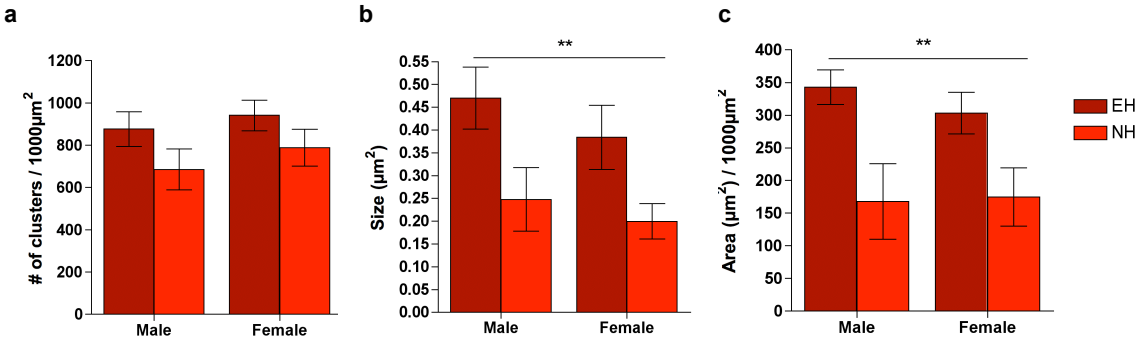


Figure 5.2: Effects of sex and early-life condition on α_2 receptor subunit clusters in the granule cell layer of the dentate gyrus. Data points represent average(n=5)±SEM (a) number of clusters / 1000 µm^2; b) size of clusters (µm^2); and c) area covered by clusters (µm^2)/ 1000 µm^2. Bars represent 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 α_2 subunit protein clusters in the hippocampal granule cell layer. *Tests reaching significance with p<0.05 are highlighted.*

Feature	Sex	Early-life condition	Sex x Early-life condition
Count / 1000 µm^2	$F_{(1,16)}$=1.05; P>0.05	$F_{(1,16)}$=4.22; P>0.05	$F_{(1,16)}$=0.04; P>0.05
Size (µm^2)	$F_{(1,16)}$=0.32; P>0.05	$F_{(1,16)}$=9.59; P<0.01	$F_{(1,16)}$=0.08; P>0.05
Area (µm^2) / 1000 µm^2	$F_{(1,16)}$=0.13; P>0.05	$F_{(1,16)}$=11.30; P<0.01	$F_{(1,16)}$=0.26; P>0.05

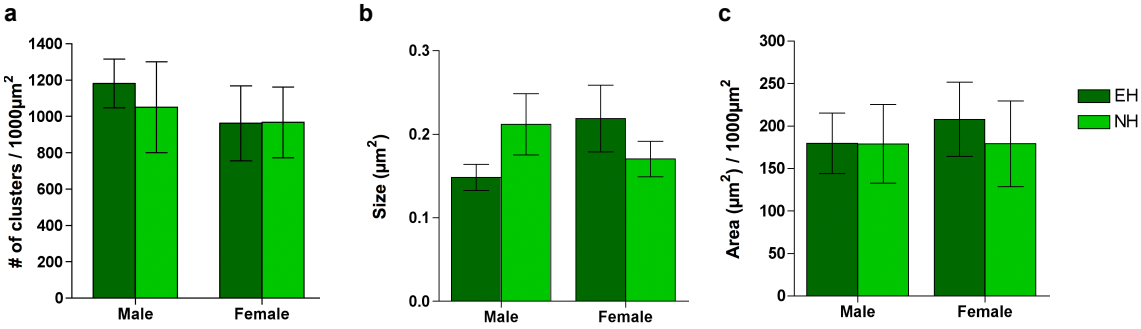


Figure 5.3: Effects of sex and early-life condition on gephyrin protein clusters in the granule cell layer of the dentate gyrus. Data points represent average (n=5) ±SEM (a) number of clusters / 1000 µm^2; b) size of clusters (µm^2); and c) area covered by clusters µm^2 / 1000 µm^2.

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.

Feature	Sex	Early-life condition	Sex x Early-life condition
Count / 1000 µm^2	$F_{(1,16)}$=0.57; P>0.05	$F_{(1,16)}$=0.10; P>0.05	$F_{(1,16)}$=0.12; P>0.05
Size (µm^2)	$F_{(1,16)}$=0.23; P>0.05	$F_{(1,16)}$=0.06; P>0.05	$F_{(1,16)}$=3.47; P>0.05
Area (µm^2) / 1000 µm^2	$F_{(1,16)}$=0.10; P>0.05	$F_{(1,16)}$=0.11; P>0.05	$F_{(1,16)}$=0.10; P>0.05

5.3.3. α_2 Subunit and gephyrin colocalisation

Figure 5.4 and 5.5 show the effects of sex and early-life condition on α_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 μm^2; and females: 332.28±149.73 /1000 μm^2) and occupy a smaller area (mean difference for males: 43.47±18.24 μm^2/1000 μm^2; and females: 30.14±12.24 μm^2/1000 μm^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 α_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 α_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 5.3: Results of two way-ANOVA tests examining between-group differences in features of α_2 and gephyrin overlap in the hippocampal granule cell layer. *Tests with p<0.05 are highlighted.*

Feature	Sex	Early-life condition	Sex x Early-life condition
Count / 1000 μm^2	$F_{(1,16)}$=0.03; P>0.05	$F_{(1,16)}$=5.60; P<0.05	$F_{(1,16)}$=0.06; P>0.05
Size (μm^2)	$F_{(1,16)}$=0.04; P>0.05	$F_{(1,16)}$=0.00; P>0.05	$F_{(1,16)}$=0.09; P>0.05
Area (μm^2) / 1000 μm^2	$F_{(1,16)}$=1.01; P>0.05	$F_{(1,16)}$=8.15; P<0.05	$F_{(1,16)}$=0.27; P>0.05
% of α_2 area	$F_{(1,16)}$=0.00; P>0.05	$F_{(1,16)}$=9.35; P<0.01	$F_{(1,16)}$=0.17; P>0.05
% gephyrin area	$F_{(1,16)}$=1.24; P>0.05	$F_{(1,16)}$=6.52; P<0.05	$F_{(1,16)}$=0.02; P>0.05

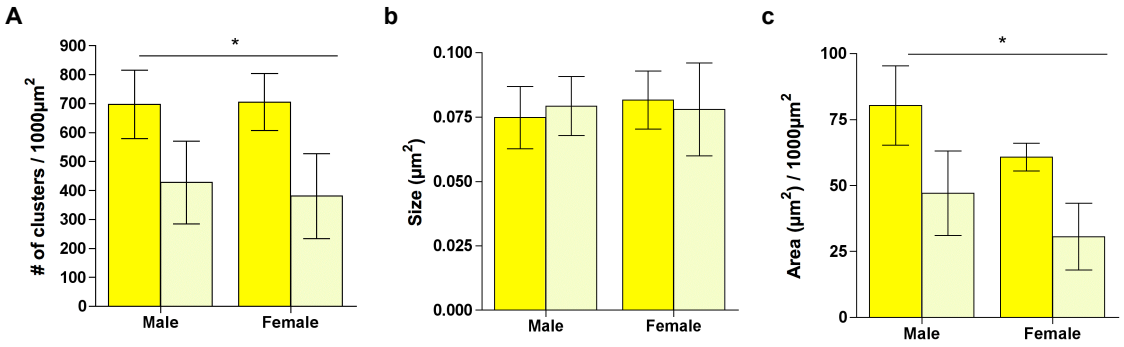


Figure 5.4: Effects of sex and early-life condition on colocalisation of the α_2 subunit with gephyrin in the granule cell layer of the dentate gyrus. Data points represent average(n=5)±SEM (a) number of colocalised clusters / 1000 µm^2; b) size of colocalised clusters (µm^2); c) area covered by clusters (µm^2)/ 1000 µm^2. Bars represent significant main effects at *p<0.05 or ** p<0.01 for early-life condition.

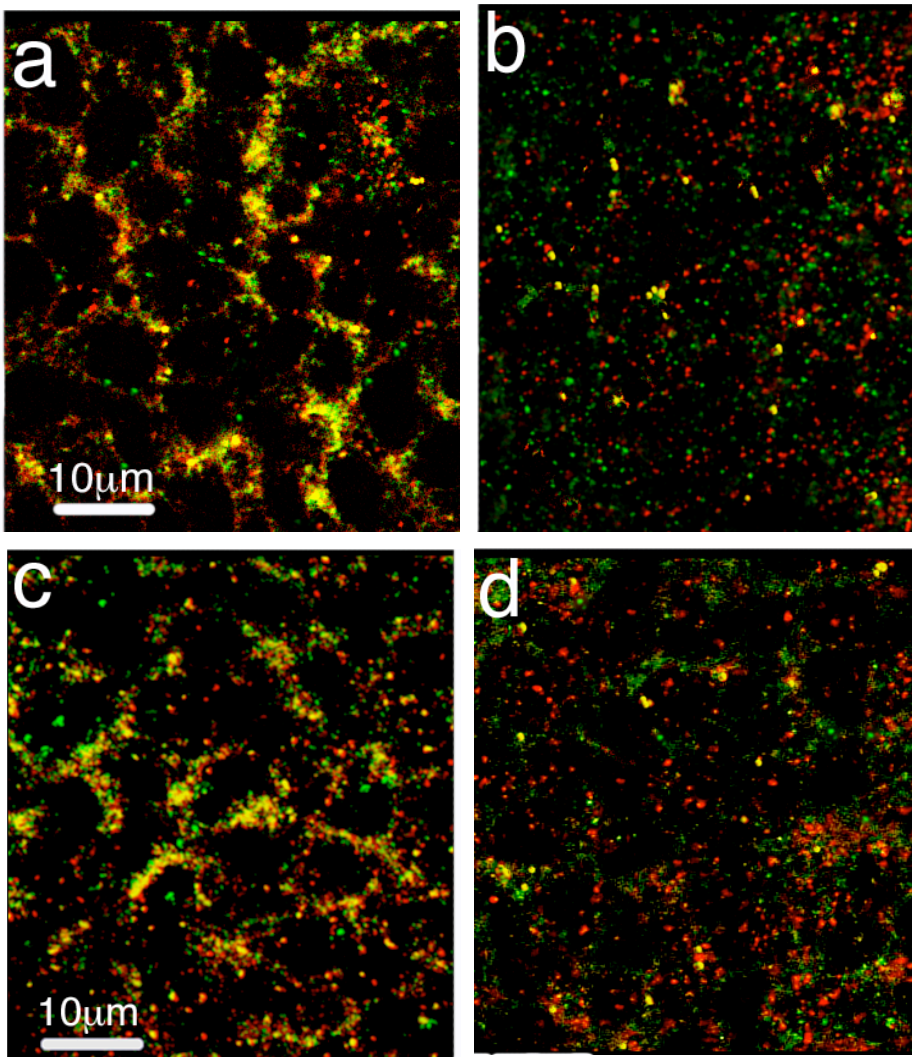


Figure 5.5: Representative z-stack projections showing α_2 subunit colocalisation with the presumed synaptic marker gephyrin in the granule cell layer of the dentate gyrus of the hippocampus. Images are taken from male (a, b,) and female (c, d) mice exposed to EH (a,c) and NH (b, d) early-life conditions. The $GABA_A$ receptor α_2 subunit protein is stained in red, gephyrin is stained in green. Yellow depicts sites of protein colocalisation. Scale 10µm.

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 α_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 α_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 α_2 and gephyrin proteins (colocalisation) in the NH compared with EH group. Whilst the size of these α_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 α_2 subunits present in the synapse, this finding then shows that the α_2-subunit containing $GABA_A$ 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 α_2 subunit-containing $GABA_A$ 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 γ_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 γ_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 γ_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 γ_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 γ_2 subunit may not have been affected. Thus, an investigation of γ_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 γ_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 $GABA_A$ 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 α_2-subunit containing $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ 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 γ_2 subunit protein expression or benzodiazepine binding sites, which require the γ_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 γ_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 α_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 α_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 α_2 subunit-containing $GABA_A$ receptor clusters. This alteration in α_2 subunit clusters did not arise from a loss of α_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 α_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 γ_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 $GABA_A$ 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 *et al.*, 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 *et al.*, 2003). The association of stress exposure with symptom onset has resulted in the "diathesis-stress" or 'two-hit' hypotheses (McGrath *et al.*, 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 *et al.*, 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 *et al.*, 1970; Denenberg, 1964; Hess *et al.*, 1969; Meaney *et al.*, 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) [^{3}H]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) $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ 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 6.1: Study design for examination of combined early-life environmental manipulation and adulthood stress

	Males		Females	
	EH	NH	EH	NH
Control	n=6	n=7	n=6	n=5
Swim stress	n=6	n=6	n=6	n=6

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 -70ºC 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}H]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}H]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}H]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.

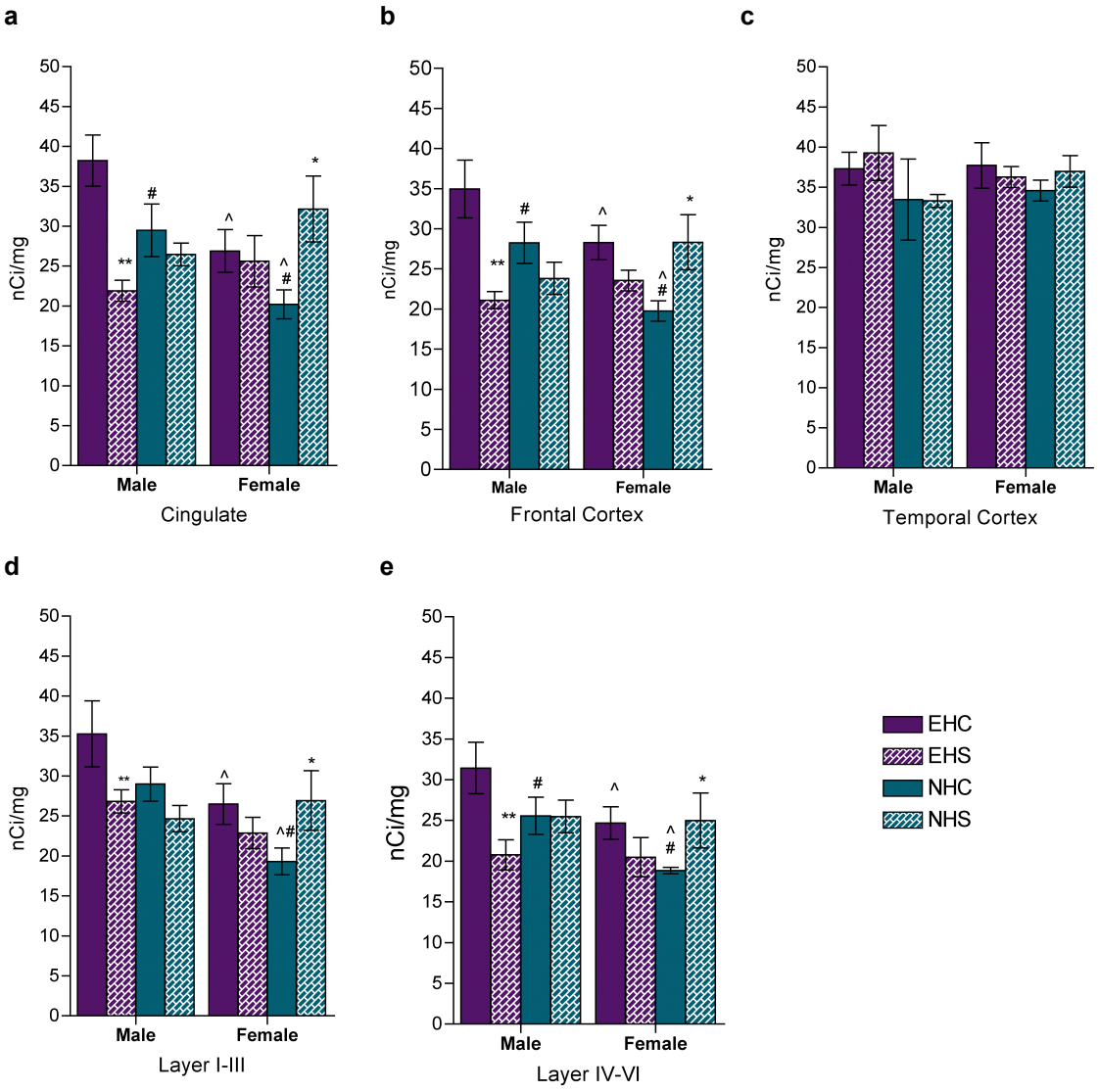


Figure 6.1 Effects of sex, early-life condition and adulthood stress on 1000nM [^{3}H]GABA binding sites in cortical regions. Data are expressed as mean±SEM for the a) cingulate b) frontal c) temporal cortical regions and d) upper e) lower cortical layers. *=p<0.05, **=p<0.01 for significant stress-induced 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.*

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

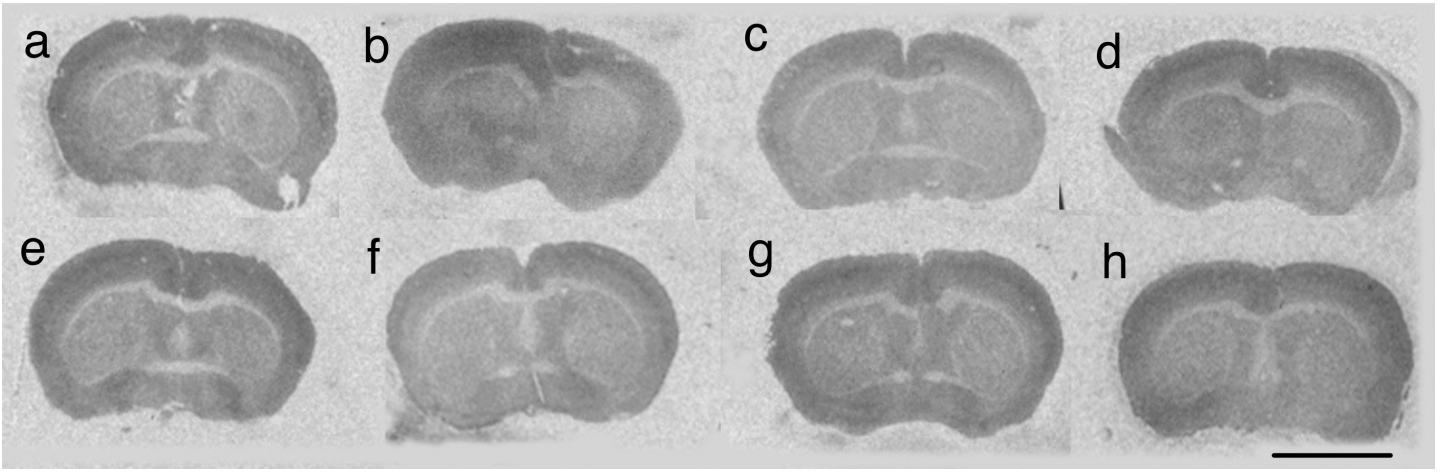


Figure 6.2: Representative autoradiographs showing effects of sex, early-life condition and adulthood stress on forebrain 1000nM [^{3}H]GABA binding sites. Images are from male (a, b, e, f) and female (c, d, g, h) mice exposed to early-life conditions of NH (a-d) or EH (e-h) and adulthood conditions 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

Male vs. Female

	Control		Adulthood Stress	
	EH	NH	EH	NH
Cingulate	P=0.013	P=0.011	P=0.673	P=0.179
Frontal	P=0.011	P=0.017	P=0.910	P=0.817
Upper Layers	P=0.040	P=0.042	P=0.884	P=0.915
Lower Layers	P=0.045	P=0.040	P=0.938	P=0.798

Control vs. Stress

	Male		Female	
	EH	NH	EH	NH
Cingulate	P=0.001	P=0.060	P=0.474	P=0.044
Frontal	P=0.009	P=0.426	P=0.218	P=0.032
Upper Layers	P=0.019	P=0.585	P=0.358	P=0.021
Lower Layers	P=0.006	P=0.676	P=0.263	P=0.047

EH vs. NH

	Male		Female	
	Control	Stress	Control	Stress
Cingulate	P=0.041	P=0.281	P=0.030	P=0.061
Frontal	P=0.038	P=0.448	P=0.025	P=0.288
Upper Layers	P=0.109	P=0.778	P=0.040	P=0.604
Lower Layers	P=0.039	P=0.362	P=0.038	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).

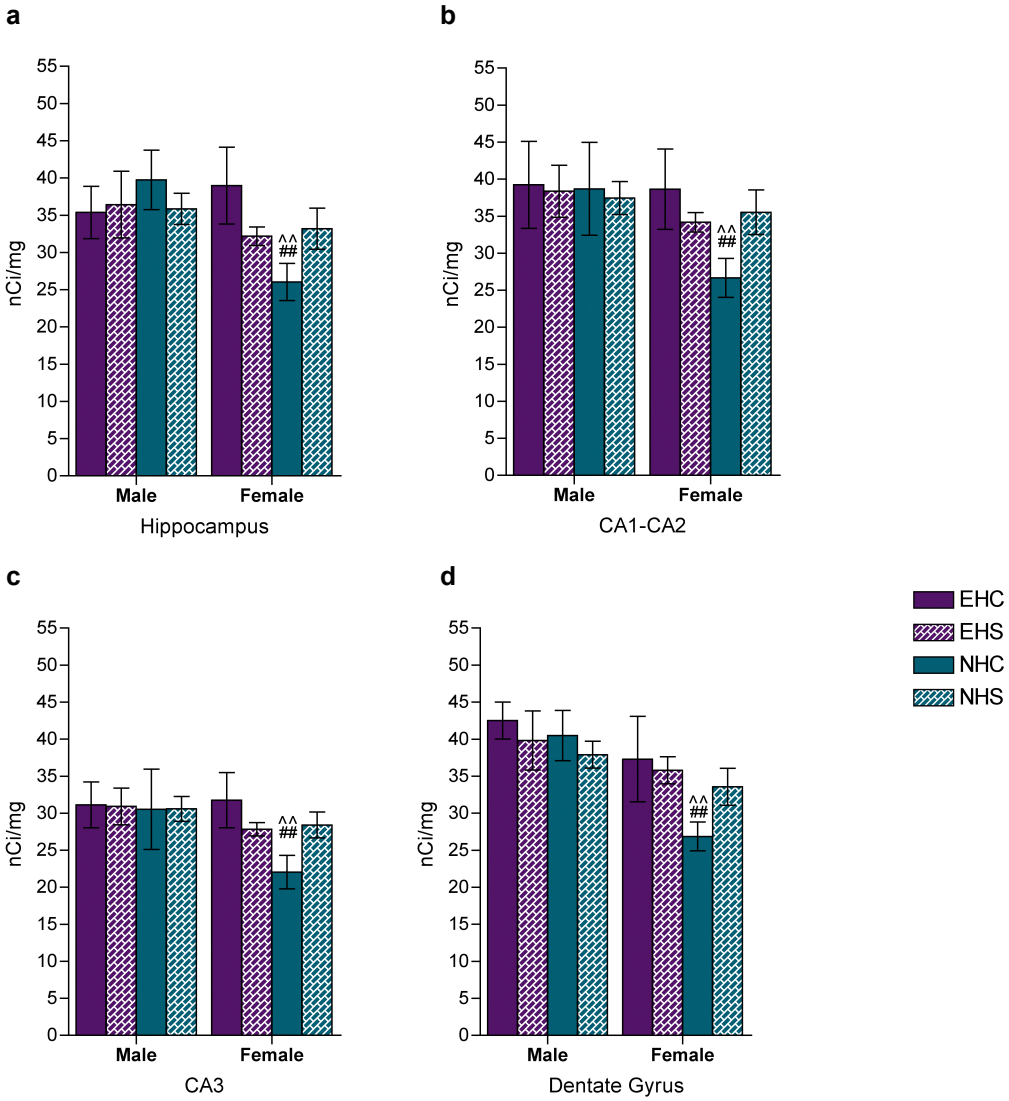


Figure 6.3 Effects of sex, early-life condition and adulthood stress on 1000nM [^{3}H]GABA binding sites in hippocampal regions. Data are expressed as mean±SEM for the a) whole hippocampus and b) CA1-CA2 c) CA3 and d) dentate gyrus subregions of hippocampus. ^^=p<0.01 for significant sex differences 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 [^{3}H]GABA binding in the hippocampus. *Tests reaching significance with p<0.05 are highlighted.*

	CA1		CA3		DG		HIPP	
Main Effects								
Sex	$F_{(1,41)}$=1.21,	p=0.278	$F_{(1,41)}$=1.41,	p=0.242	$F_{(1,40)}$=8.50,	p=0.006	$F_{(1,40)}$=4.13,	p=0.044
Early-life	$F_{(1,41)}$=1.90,	p=0.176	$F_{(1,41)}$=2.31,	p=0.136	$F_{(1,40)}$=0.70,	p=0.410	$F_{(1,40)}$=0.71,	p=0.403
Stress	$F_{(1,41)}$=0.06,	p=0.813	$F_{(1,41)}$=0.01,	p=0.936	$F_{(1,40)}$=0.38,	p=0.544	$F_{(1,40)}$=0.07,	p=0.799
Two Way Interactions								
Sex x Early-life	$F_{(1,41)}$=1.30,	p=0.261	$F_{(1,41)}$=1.69,	p=0.201	$F_{(1,40)}$=2.33,	p=0.136	$F_{(1,40)}$=2.67,	p=0.110
Sex x Stress	$F_{(1,41)}$=0.01,	p=0.918	$F_{(1,41)}$=0.00,	p=0.966	$F_{(1,40)}$=2.06,	p=0.161	$F_{(1,40)}$=0.11,	p=0.742
Early-Life x Stress	$F_{(1,41)}$=2.10,	p=0.155	$F_{(1,41)}$=2.50,	p=0.122	$F_{(1,40)}$=0.01,	p=0.924	$F_{(1,40)}$=0.87,	p=0.355
Three-Way Interaction								
Sex x Early-Life x Stress	$F_{(1,41)}$=2.30,	p=0.138	$F_{(1,41)}$=2.30,	p=0.137	$F_{(1,40)}$=2.16,	p=0.151	$F_{(1,40)}$=3.86,	p=0.057

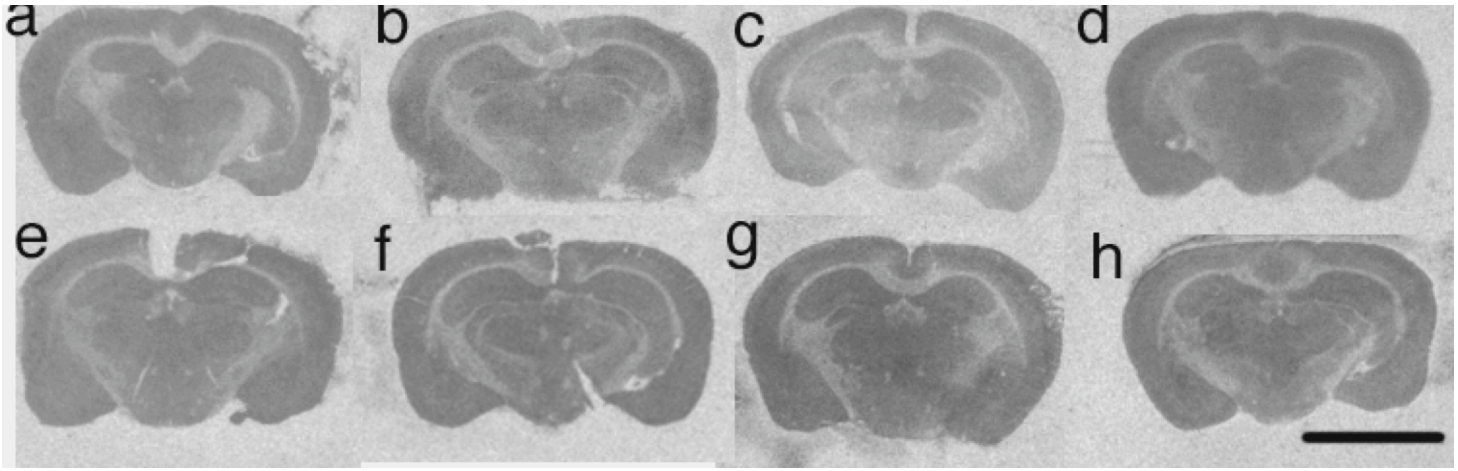


Figure 6.4: Representative autoradiographs showing effects of sex, early-life condition and adulthood stress on 1000nM [^{3}H]GABA binding sites at the level of the hippocampus. Images are from male (a, b, e, f) and female (c, d, g, h) mice exposed to early-life conditions of NH (a-d) or EH (e-h) and 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.

	Male vs. Female			
	Control		Adulthood Stress	
	EH	NH	EH	NH
Hippocampus	P=0.454	P=0.012	P=0.378	P=0.553
CA1-CA2	P=0.923	P=0.045	P=0.466	P=0.721
CA3	P=0.410	P=0.033	P=0.474	P=0.592
Dentate Gyrus	P=0.505	P=0.002	P=0.482	P=0.413

	Control vs. Stress			
	Male		Female	
	EH	NH	EH	NH
Hippocampus	P=0.825	P=0.414	P=0.162	P=0.160
CA1-CA2	P=0.881	P=0.817	P=0.459	P=0.155
CA3	P=0.960	P=0.989	P=0.116	P=0.165
Dentate Gyrus	P=0.739	P=0.074	P=0.712	P=0.270

	EH vs. NH			
	Male		Female	
	Control	Stress	Control	Stress
Hippocampus	P=0.387	P=0.898	P=0.013	P=0.834
CA1-CA2	P=0.925	P=0.864	P=0.033	P=0.814
CA3	P=0.888	P=0.933	P=0.008	P=0.891
Dentate Gyrus	P=0.310	P=0.715	P=0.032	P=0.694

6.4. Discussion

6.4.1. Baseline sex differences in [^{3}H]GABA binding

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.

			EH	NH
Cortex	Control	Cingulate	M>F	M>F
		Whole frontal	M>F	M>F
		Upper	M>F	M>F
		Lower	M>F	M>F
		Temporal	-	-
	Stress	Cingulate	-	-
		Whole frontal	-	-
		Upper	-	-
		Lower	-	-
		Temporal	-	-
Hippocampus	Control	Whole Hippocampus	-	M>F
		CA1-CA2	-	M>F
		CA3	-	M>F
		DG	-	M>F
	Stress	Whole Hippocampus	-	-
		CA1-CA2	-	-
		CA3	-	-
		DG	-	-

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}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 α_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 α_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}H]GABA binding sites relative to EH females. However, early-life condition appears to affect the number of [^{3}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}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 α_1 and α_2 subunit containing receptors from regions of the hippocampus in NH females (see chapter 4). However, whilst α_2 subunit protein was reduced in the cortex, α_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 6.7. Summary of the effects of early-life condition on [^{3}H]GABA binding according to sex and adulthood stress condition.

			Male	Female
Cortex	**Control**	Cingulate	EH>NH	EH>NH
		Whole frontal	EH>NH	EH>NH
		Upper	-	EH>NH
		Lower	EH>NH	EH>NH
		Temporal	-	-
	Stress	Cingulate	-	-
		Whole frontal	-	-
		Upper	-	-
		Lower	-	-
		Temporal	-	-
Hippocampus	**Control**	Whole Hippocampus	-	EH>NH
		CA1-CA2	-	EH>NH
		CA3	-	EH>NH
		DG	-	EH>NH
	Stress	Whole Hippocampus	-	-
		CA1-CA2	-	-
		CA3	-	-
		DG	-	-

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 [^{3}H]GABA binding

The effects of early-life stress on adulthood [^{3}H]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 $GABA_A$ receptors. As the number of low-affinity sites labelled by [^{3}H]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 [^{3}H]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 [^{3}H]GABA binding in males and females. In males [^{3}H]GABA binding in the NH group was not affected by stress, but in the EH group there was a stress-induced decrease in [^{3}H]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}H]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 6.8. Summary of the effects of early-life condition on adulthood stress-induced changes in [^{3}H]GABA binding according to sex.

			EH	NH
Cortex	**Male**	Cingulate	S<C	-
		Whole frontal	S<C	-
		Upper	S<C	-
		Lower	S<C	-
		Temporal	-	-
	Female	Cingulate	-	S>C
		Whole frontal	-	S>C
		Upper	-	S>C
		Lower	-	S>C
		Temporal	-	-
Hippocampus	**Male**	Whole Hippocampus	-	-
		CA1-CA2	-	-
		CA3	-	-
		DG	-	-
	Female	Whole Hippocampus	-	-
		CA1-CA2	-	-
		CA3	-	-
		DG	-	-

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 $GABA_A$ 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 $GABA_A$ 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 *et al.*, 2000a; 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). 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 *et al.*, 1967; Liu *et al.*, 1997; Meaney *et al.*, 1996; Meaney *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 *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 $GABA_A$ 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 $GABA_A$ 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 [^{3}H]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 [^{3}H]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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ receptors

Results reported in this thesis indicated that $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ receptors that are incurred by stress have potential clinical implications. For example, many drugs which act on $GABA_A$ 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 $GABA_A$ 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 $GABA_A$ receptors

7.3.1 Baseline sex differences

In this thesis $GABA_A$ 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 $GABA_A$ 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).

$GABA_A$ 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 α_1 and α_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 α_1 subunit expression compared with females. This increase in α_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 α_1-subunit containing $GABA_A$ receptors also observed in males. Given that α_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 α subunits that were not examined in this thesis may also contribute to sex differences in the number of [^{3}H]GABA binding sites and sensitivity to GABAergic compounds. In particular, the α_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 $GABA_A$ receptors. Effects of early-life stress on $GABA_A$ receptors were largely consistent across sexes. For example both males and females showed similar reductions in low affinity [^{3}H]GABA binding, α_2 subunit expression in the lower layers of the SS cortex and synaptic clustering of $GABA_A$ receptors in the dentate gyrus (chapter 4). In contrast, stress in adulthood affected [^{3}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 $GABA_A$ 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), $GABA_A$ 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.

PART F:

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