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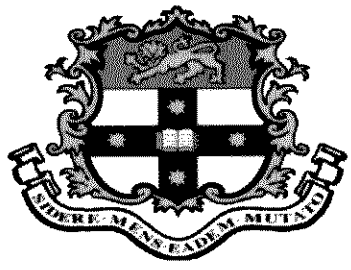
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IONOTROPIC GABA RECEPTORS IN THE CENTRAL NERVOUS SYSTEM AND IN SCHIZOPHRENIA

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

Department of Pharmacology

The University of Sydney, Australia

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STATEMENT

"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 acknowledgment 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." Australian Government Publishing Service, Canberra, 1990.

ABSTRACT

GABA is the principle transmitter mediating inhibition in the mammalian CNS, and acts to balance neural excitation, which is mediated predominantly by glutamate. Three known classes of GABA receptors exist, the ionotropic $GABA_A$ and $GABA_C$ receptors, and the metabotropic $GABA_B$ receptors. The $GABA_C$ receptors are the least studied of the three. These receptors are composed of ρ subunits, which have been cloned from retina. Thus, most of what is known about the $GABA_C$ receptors is derived from studies in the retina, but not in other areas of the CNS. The aims of this study were to map $GABA_C$ receptor subunits in rat and human brain in an attempt to understand their molecular composition in the CNS. Concurrent investigation of $GABA_A$ receptor subunits was undertaken, since their presence in the CNS is prolific and well documented.

In situ hybridisation and immunohistochemistry techniques were employed, and aspects of the procedures were varied to optimise detection of the receptor subunits in question. $GABA_C$ receptor ρ1 and ρ2 subunit mRNAs were detected in rat retina and cerebellum, and the ρ2 subunit mRNA was encountered in some regions of the rat forebrain and midbrain using *in situ* hybridisation histochemistry. ρ3 Subunit mRNA, on the other hand, could not be detected in retina or CNS. The $GABA_C$ receptor protein was observed in the retina and weakly in the cerebellum, yet it could not be detected in rat forebrain using immunohistochemistry. Demonstration of ρ subunit mRNAs in human brain was difficult owing to their very low expression levels, however the ρ1 and ρ2 transcripts could be detected in the cerebellum.

The $GABA_A$ receptor α1 and β3 subunit mRNA and β2/β3 subunit protein were widely distributed throughout the rat brain, in agreement with the literature. The transcripts were further detected in all regions of the human brain investigated; the middle frontal cortex, superior temporal gyrus, polar temporal cortex, hippocampus, amygdala, primary visual cortex and cerebellum.

An imbalance between neural excitation and inhibition is thought to be involved in symptoms of schizophrenia. For this reason, the ionotropic GABA receptor subunit

mRNAs were investigated using *in situ* hybridisation in two regions of heteromodal association cortex of schizophrenic and matched control brains. All tissue was obtained from the New South Wales Tissue Resource Centre following rigorous assessment and diagnosis. Post-mortem middle frontal cortex and superior temporal gyrus sections were examined, corresponding to the dorsolateral prefrontal cortex and auditory association cortex, respectively. Trends were observed for altered expression patterns of the $GABA_A$ receptor $\alpha 1$ and $\beta 3$ subunit mRNAs in schizophrenic brain. Increased expression of $GABA_A$ receptor $\alpha 1$ subunit mRNA was observed in the middle frontal cortex and $\beta 3$ subunit mRNA in the superior temporal gyrus. A converse decrease in expression of $\alpha 1$ subunit mRNA in the superior temporal gyrus, and $\beta 3$ subunit mRNA in the middle frontal cortex was encountered. These findings point to regional variations in subunit expression in schizophrenic subjects.

Finally, two-electrode voltage clamp electrophysiology was used to assess the activity of neuroleptic drugs on human recombinant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. The typical neuroleptics, chlorpromazine and haloperidol, and the atypical neuroleptic, clozapine, were tested. Drugs were applied in increasing concentrations to the oocytes alone and in the presence of GABA or the selective $GABA_A$ receptor antagonist, bicuculline. Chlorpromazine at concentrations between 10 and 600μM was found to weakly activate human recombinant $\alpha 1\beta 2\gamma 2_L$ $GABA_A$ receptors in a dose-dependent manner, an effect which was not significantly blocked by co-administration of bicuculline. A maximal response could not be ascertained owing to the high concentrations of chlorpromazine required, thus a valid EC_{50} could not be determined. Haloperidol at concentrations between 10 and 600μM did not exhibit any activity at the $\alpha 1\beta 2\gamma 2_L$ subclass of $GABA_A$ receptors. Clozapine, on the other hand, was an antagonist at these receptors, inhibiting the response of 10μM GABA with an IC_{50} of $314.77 \pm 2.27\mu$M (95% CI 138.45-715.65). This activity may be important for both the side effects and the therapeutic efficacy of clozapine in patients with schizophrenia.

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3-APA	3-aminopropylphosphonic acid
3-APS	3-aminopropanesulfonic acid
3-APPA	3-aminopropylphosphinic acid
3-APMPA	3-aminopropyl(methyl)phosphinic acid
4-ABPA	4-aminobutylphosphonic acid
ANOVA	Analysis Of Variance
BSA	bovine serum albumin
CA1	cornu Ammonis area 1
CA2	cornu Ammonis area 2
CA3	cornu Ammonis area 3
CACA	*cis*-4-aminocrotonic acid
cDNA	complimentary deoxyribonucleic acid
CNS	central nervous system
cRNA	complimentary ribonucleic acid
dATP	deoxyadenosine triphosphate
DAVA	δ-aminovaleric acid
DEPC	diethylpyrocarbonate
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E. Coli	*Escherichia coli*
GABA	γ-aminobutyric acid
GABA-T	GABA-transaminase/aminotransferase
GAD	glutamic acid decarboxylase
GAT-1	GABA transporter type 1
GAT-3	GABA transporter type 3
I4AA	imidazole-4-acetic acid
IgG	immunoglobulin G
MFC	middle frontal cortex
MFG	middle frontal gyrus
mRNA	messenger ribonucleic acid
NMDA	*N*-methyl-D-aspartate
ODN	oligodeoxyribonucleotide
P4MPA	(piperidin-4-yl)methylphosphinic acid
P4S	piperidine-4-sulfonic acid
PFC	prefrontal cortex
PKC	protein kinase C
RNases	ribonucleases
SR-95531	2-(3-carboxypropyl)-3-amino-6-*p*-methoxyphenylpyridazinium bromide
STG	superior temporal gyrus
TACA	*trans*-4-aminocrotonic acid
(+)-TACP	(+)-*trans*-(1*S*,3*S*)-3-aminocyclopentane-1-carboxylic acid)
TAMP	*trans*-2-(aminomethyl)cyclopropanecarboxylic acid
TBOB	*t*-butylbicycloorthobenzoate
TBPS	*t*-butylbicyclophosphorothionate
TDT	terminal deoxytransferase
TEA	triethanolamine
THIP	4,5,6,7-tetrahydroisoxazole[4,5-*c*]pyridin-3-ol
TPMPA	1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid
UV	ultraviolet
ZAPA	Z-3-[(aminoiminomethyl)thio]prop-2-enonic acid

PART 1

MAPPING IONOTROPIC GABA RECEPTORS IN RAT AND HUMAN BRAIN

MAPPING IONOTROPIC GABA RECEPTORS IN RAT AND HUMAN BRAIN

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION TO THE GABAERGIC SYSTEM

1.1.1 GABA as a Neurotransmitter

γ-Aminobutyric acid (GABA) is a non-protein amino acid, which was first discovered in the central nervous system (CNS) by Roberts and Frankel (1950). GABA is distributed predominantly in grey matter of the mammalian brain and spinal cord, with the highest concentrations in the substangtia nigra and areas of the basal ganglia followed by the hypothalamus (DiFiglia & Aronin, 1990; Fahn, 1976). GABA is in fact distributed throughout the entire CNS, with varying concentrations in different regions.

GABA is synthesised from the amino acid L-glutamate via α-decarboxylation by glutamate decarboxylase (GAD) (Martin & Rimvall, 1993; Petroff & Rothman, 1998; Roberts & Frankel, 1950). As the rate limiting enzyme for GABA synthesis, GAD regulates GABA-mediated neural transmission (Chase & Walters, 1976; Varju *et al.*, 2001). Two isoforms of GAD have been identified, GAD_{65} and GAD_{67} (Erlander *et al.*, 1991; Kaufman *et al.*, 1991; Martin & Rimvall, 1993; Varju *et al.*, 2001). Two processes terminate the action of GABA in the synapse. GABA is taken up via the Na^+-dependent, high affinity GABA transporters (GAT-1 and GAT-3) into the presynaptic terminal for re-release or into post-synaptic terminals and neighbouring glial cells (Chase & Walters, 1976; Kanner & Bendehan, 1990; Miller *et al.*, 1997; Storm-Mathisen *et al.*, 1976). GABA is also metabolised via the tricarboxylic acid cycle in mitochondria by GABA-aminotransferase (GABA-T) to succinic semialdehyde then oxidised by succinic semialdehyde dehydrogenase to succinic acid (Chase & Walters, 1976; Martin & Rimvall, 1993; Petroff & Rothman, 1998; Roberts & Breghoff, 1953).

GABA is now thought to be the principle mediator of neuronal inhibition in the CNS (Roberts & Kuriyama, 1968; Curtis & Johnston, 1970; Iversen, 1984; Olsen, 1991; Waagepetersen *et al.*, 1999). GABA is synthesised in 20-30% of all CNS neurons (Varju *et al.*, 2001) and is the primary transmitter at 25-50% of synapses in mammalian

brain (Petroff & Rothman, 1998). Its ubiquitous presence means that almost all neurons either release GABA, express GABA receptors, or are innervated by neurons which do (Koella, 1981; Olsen, 1991). For these reasons it is expected that most brain functions involve GABAergic transmission (Koella, 1981). Consequently, the role of GABA in normal information processing is crucial, as GABA acts to keep processes of the CNS under control (Koella, 1981; Roberts, 1984; Roberts, 1991).

1.1.2 Inhibitory Interneurons

Approximately 70-85% of neurons in the cortex are excitatory spiny neurons (pyramidal cells), which are likely to use glutamate as a neurotransmitter and are identified by the presence of many dendritic spines (Deakin *et al.*, 1997; Kandel *et al.*, 1991; Waldvogel *et al.*, 2000). 15-30% of neurons in the cortex are non-spiny local circuit neurons (interneurons), which mainly use GABA and neuropeptides as their transmitters and are identified by the absence of dendritic spines (Cauli *et al.*, 1997; Curtis & Johnston, 1974; Fagg & Foster, 1983; Iversen, 1984; Kandel *et al.*, 1991; Kubota *et al.*, 1994; Waldvogel *et al.*, 2000). While the excitatory pyramidal cells are projection neurons, their axons projecting to other regions of the brain, interneurons tend to have shorter axons, not extending beyond the cortex (Kandel *et al.*, 1991). The spiny stellate cells are excitatory interneurons which utilise glutamate (De Lima & Voigt, 1997), also referred to as granule cells.

The small interneurons of the CNS play a fundamental role in regulating communication between the larger afferent and efferent neurons. These inhibitory interneurons typically synapse on excitatory spiny (pyramidal) neurons as well as other inhibitory interneurons, and are also innervated by pyramidal cells and other interneurons (Kawaguchi & Kubota, 1997). Thus, GABA modulates neuronal activity locally, through release from interneurons (Fagg & Foster, 1983). There exists roughly one inhibitory synapse for every five to six excitatory synapses (Waldvogel *et al.*, 2000). The smaller number of inhibitory interneurons and inhibitory synapses implies that fewer neurons are involved in inhibition, therefore little inhibition is required to be functionally relevant (Waldvogel *et al.*, 2000).

Inhibitory synapses are located on or near the soma of pyramidal cells, and on dendritic shafts, while excitatory synapses are predominantly localised on dendritic spines (Kandel *et al.*, 1991). The proximity of inhibitory interneurons to the soma suggests that they influence the generation of action potentials both directly and strongly (Condé *et al.*, 1994; DeFelipe *et al.*, 1989; Kandel *et al.*, 1991; Kawaguchi & Kubota, 1998). In this way, GABA can "modulate and sculpture the activity generated by the direct excitatory input" (Roberts, 1984). GABA is therefore essential for the overall balance of excitation and inhibition in the CNS.

Inhibitory GABAergic interneurons have been distinguished morphologically by the pattern of dendritic arborisation, electrophysiologically by firing patterns, and through expression of calcium-binding proteins. These calcium-binding proteins are commonly used as neuronal markers, to assist investigators in the examination of heterogeneous GABAergic interneurons. The most commonly described interneurons are basket cells, chandelier cells, arcade cells, neurogliaform cells, double bouquet cells and cells of Martinotti (Kandel *et al.*, 1991; Kawaguchi & Kubota, 1997). The presence of many types of GABAergic interneurons indicates that GABAergic interactions in the cortex are various and complex (DiFiglia & Aronin, 1990).

1.1.3 Non-isocortical GABAergic Neurons

In addition to neocortical interneurons, GABA is located in different cell types in many brain regions (Fagg & Foster, 1983). For example, GABAergic interneurons exist in the hippocampal formation (eg: basket cells), thalamus, hypothalamus and olfactory bulb, striatum, substantia nigra, pons and cerebellar cortex (eg: Golgi and stellate cells) (DiFiglia & Aronin, 1990; Fagg & Foster, 1983; Waldvogel *et al.*, 1999).

Some GABAergic projection neurons also exist in non-isocortical regions (Fagg & Foster, 1983). GABAergic neurons project from the posterior striatum, globus pallidus and nucleus accumbens to the substantia nigra (DiFiglia & Aronin, 1990; Fagg & Foster, 1983; Waldvogel *et al.*, 1999). The striopallidal pathway from the striatum to the globus pallidus is known to be GABAergic (DiFiglia & Aronin, 1990; Fagg & Foster, 1983). Neurons projecting from the substantia nigra to the ventromedial thalamus and

globus pallidus to the subthalamic nucleus and thalamus are also GABAergic (Fagg & Foster, 1983; Waldvogel *et al.*, 1999), as are neurons projecting from the reticular thalamic nucleus to other thalamic nuclei (DiFiglia & Aronin, 1990). The Purkinje cells of the cerebellum, likewise, are GABAergic projection neurons (Fagg & Foster, 1983).

1.1.4 Disinhibition

Normal activity of the CNS is mediated by the "coordinated dynamic interplay of excitation and inhibition", both within and between neuronal systems (Roberts, 1976). Neurons releasing GABA may synapse on excitatory neurons so that activation of the receptors decreases the release of the excitatory transmitter. GABAergic neurons may also synapse on inhibitory neurons. Activation of the receptors on inhibitory neurons leads to decreased release of the inhibitory transmitter. Inhibiting the inhibitory synapse leads to a decreased inhibitory tone, or "disinhibition", which potentiates excitation (Olsen & Avoli, 1997; Roberts, 1976).

GABA-mediated tonic inhibition acts to constrain transmission of information (Roberts, 1991). This is fundamental to the normal function of the CNS. Roberts (1991) points out that disinhibition is also a necessary process for normal CNS function, as it permits variability in function, essential for adaptation to the complexity of internal and external stimuli. However, impaired connections involving inhibitory neurons may generate aberrations in information processing, hence impaired behavioural versatility (Roberts, 1991). "Disease in the CNS is believed to be a consequence of incoordination between inhibition and excitation" (Roberts, 1984).

1.2 GABA Receptors

GABA is a flexible molecule that adopts many conformations in order to bind to receptors, through which its inhibitory effects are mediated (Johnston, 1996a). GABA is the endogenous neurotransmitter for three known classes of receptors: $GABA_A$, $GABA_B$ and $GABA_C$ receptors. The GABA receptor classes are delineated on the basis of structural, physiological and pharmacological differences. $GABA_A$ and $GABA_C$ receptors are ionotropic, while the $GABA_B$ receptors are metabotropic (for review of $GABA_B$ receptors see Blein *et al.*, 2000).

1.2.1 Ionotropic GABA Receptors

$GABA_A$ and $GABA_C$ receptors are ionotropic receptors belonging to a superfamily of ligand gated ion channels (LGICs), or transmitter-gated ion channels. LGICs differ from the other major classes of receptors, which include the metabotropic (G protein-coupled) receptors, the enzyme-associated receptors, and the ligand-dependent transcriptional regulators, based on structure and method of transduction (Barnard, 1996). LGICs are subdivided into nine superfamilies (for more extensive review see Barnard, 1996). The ionotropic GABA receptors belong to the cys-loop superfamily, also referred to as the nicotinicoid superfamily (Barnard, 1996; Chebib & Johnston, 2000; Karlin, 2002; Le Novère & Changeux, 1999; Ortells & Lunt, 1995). Of the cys-loop LGIC family, $GABA_A$ receptors appear to be the most complex, while $GABA_C$ receptors are the simplest (Le Novère & Changeux, 1999).

The structure of nicotinicoid LGICs has been deduced mainly from work by Unwin (1989) on the nicotinic acetylcholine (nACh) receptor isolated from *Torpedo electroplax*. Other receptors in the nicotinicoid LGIC family are assumed to resemble the nACh receptors in topology and molecular structure (Karlin, 2002; Le Novère & Changeux, 1999; Taylor & Lummis, 1993; Unwin, 1989; Unwin, 1993), based on sequence homology and analysis of single channel behaviour (Unwin, 1989). This structure has been established for the $GABA_A$ receptors by studying rotational symmetry of receptors isolated from porcine brain cortex visualised using electron microscopy (Nayeem *et al.*, 1994). Figure 1.1a schematically depicts the nicotinicoid LGIC receptor model based on nACh receptors. In general, these receptors are glycoproteins composed of five membrane-spanning subunits which form a central pore, the ion channel (Karlin, 2002; Macdonald & Olsen, 1994; Unwin, 1989). Figure 1.1b schematically represents the structure of a typical LGIC subunit. Each subunit contains four putative hydrophobic transmembrane regions, the second one (M2) of which lines the channel pore (Ortells & Lunt, 1995; Schofield *et al.*, 1987; Unwin, 1989, 1993).

A hydrophilic amine terminal (N terminal region) extends from the first transmembrane (M1) domain into the extracellular space (Hackam *et al.*, 1997; Le Novère & Changeux, 1999; Unwin, 1993) and contains putative glycosylation sites (Kushner *et al.*, 1989;

Schofield *et al.*, 1987; Tyndale *et al.*, 1995). The cys-loop family is so named for the pair of cysteines at 15-residue spacing located in the N terminal (Barnard *et al.*, 1987; Karlin, 2002). Amino acid residues within the N terminal and the highly conserved cysteine-cysteine bridge are thought to be responsible for the binding of ligands to the receptor (Ortells & Lunt, 1995).

The carboxyl terminal (C terminal) extends from the fourth transmembrane domain, also into the extracellular space (Le Novère & Changeux, 1999; Unwin, 1993). A small extracellular loop exists between transmembrane domains M2 and M3 (Unwin, 1993). The cytoplasmic loop between the third and fourth transmembrane domains contains consensus sequences for cAMP and protein kinase activity for phosphorylation and subsequent desensitisation (Enz & Cutting, 1998; Kushner *et al.*, 1989; Tyndale *et al.*, 1995; Unwin, 1993). The cytoplasmic loop is also involved in receptor clustering (Macdonald & Olsen, 1994), and anchors the receptor via specific anchoring proteins to the cytoskeleton (Enz & Cutting, 1998).

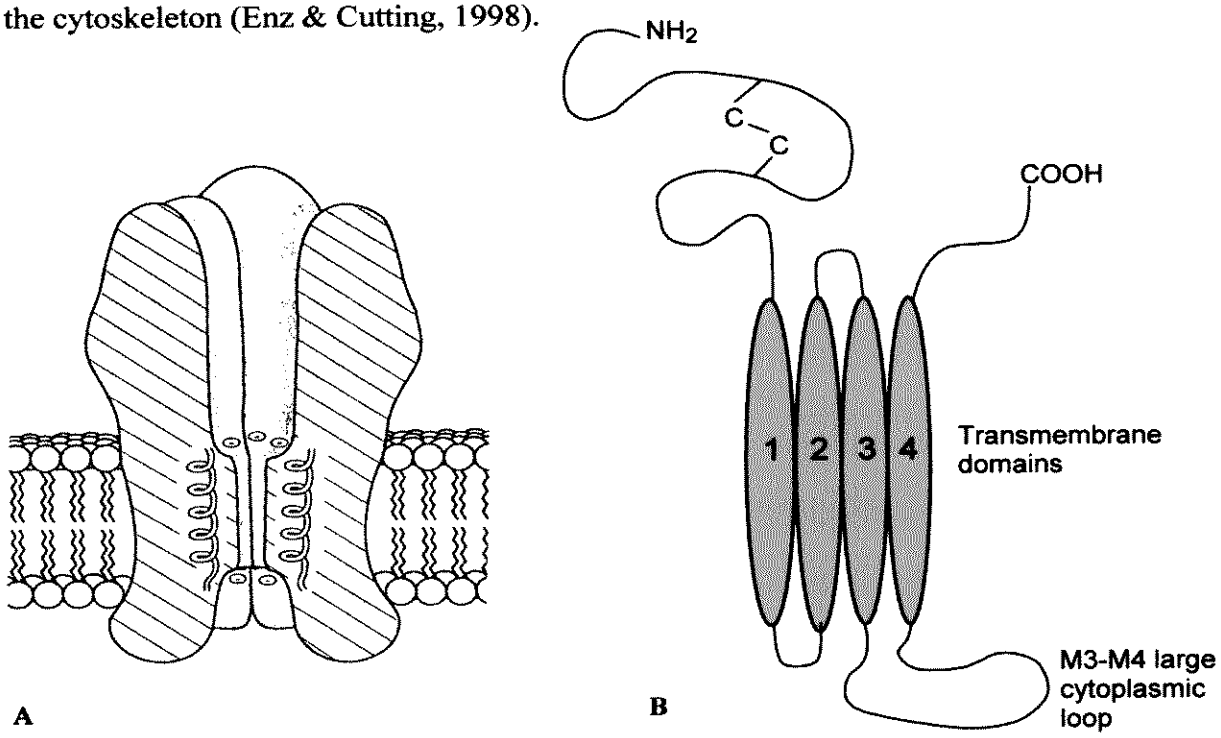


Figure 1.1: Structure of nicotinicoid LGIC. **A.** Putative arrangement of subunits in membrane (adapted from Unwin, 1989). Most anterior subunit removed to provide cross section displaying alpha helical arrangement of transmembrane domain 2 (M2) pore lining segment. **B.** Schematic diagram of typical LGIC subunit topology showing major features.

Activation of ionotropic GABA receptors causes an early (fast) inhibitory post-synaptic potential (IPSP). $GABA_A$ and $GABA_C$ receptors conduct anions, specifically chloride ions (Iversen, 1984), demonstrated by the fact that their reversal potential occurs near the chloride equilibrium potential predicted by the Nernst equation (Cutting *et al.*, 1991; Cutting *et al.*, 1992; Levitan *et al.*, 1988; Martina *et al.*, 1995). When GABA or its structural analogues bind to the ionotropic receptor, the ligand gated chloride channel changes conformation, allowing increased conductance of chloride ions through the channel. The equilibrium potential for chloride is close to resting membrane potential, therefore increased chloride conductance stabilises membrane potential, thereby reducing excitatory depolarisation of the post-synaptic membrane (Costa, 1998; Iversen, 1984; Olsen, 1991; Olsen & Avoli, 1997).

1.2.2 $GABA_A$ Receptors

$GABA_A$ receptors in the CNS were discovered when the alkaloid, bicuculline, was shown to specifically antagonise the inhibitory effect of GABA in the cat spinal cord (Curtis *et al.*, 1970; Curtis & Johnston, 1974). The $GABA_A$ receptors are the most extensively studied and well characterised of the GABA receptors, particularly due to their pharmacological relevance to specific behaviours such as anxiety and sleep.

1.2.2.1 Molecular cloning of $GABA_A$ receptor subunits

The first $GABA_A$ receptor subunits were affinity purified from bovine cerebral cortex and subsequent probes were used to screen bovine brain cDNA libraries, yielding two clones (Schofield *et al.*, 1987). These were designated α and β and were found to possess 35% sequence similarity (Schofield *et al.*, 1987). Injection of the subunit mRNAs in *Xenopus* oocytes demonstrated that both subunits were required for correct functional assembly of receptors with similar pharmacology to that observed *in vivo*, except that no benzodiazepine activity was observed (Lolait *et al.*, 1989a; Lolait *et al.*, 1989b; Schofield *et al.*, 1987; Ymer *et al.*, 1989a).

To date, fifteen $GABA_A$ receptor subunits have been cloned from the mammalian CNS. These are the α subunits (6), β subunits (3), γ subunits (3, with two splice variants, $\gamma 2_{Long}$ and $\gamma 2_{Short}$), δ subunit (1), ε subunit (1) and the most recently described θ

subunit (Barnard *et al.*, 1998; Bonnert *et al.*, 1999; Chebib & Johnston, 2000; Davies *et al.*, 1997; Moragues *et al.*, 2000; Whiting *et al.*, 1990). Splice variants further exist for the α5, α6, β2 and β3 subunits (Barnard *et al.*, 1998; Kirkness *et al.*, 1993; Korpi *et al.*, 1994). The different families of receptor subunits are structurally distinct and genetically different (Costa, 1998). Approximately 30% amino acid sequence homology exists across the subunit classes, and 60-80% homology within the subunit classes (Chebib & Johnston, 2000; Costa, 1998).

1.2.2.2 $GABA_A$ receptor composition

$GABA_A$ receptors are heterooligomeric, in that a combination of subunits is required to form fully functional receptors *in vivo* and in cell expression systems (Levitan *et al.*, 1988; Schofield *et al.*, 1987; Sieghart *et al.*, 1999). A number of attempts have been made to ascertain the subunit composition of $GABA_A$ receptors. More recently, subtractive purification methods combined with subunit stoichiometry studies established that the majority of $GABA_A$ receptors are assembled from two α, two β and one γ subunit (Bormann, 1988; Macdonald & Olsen, 1994; Pritchett *et al.*, 1989; Sieghart *et al.*, 1999). Immunohistochemical and *in situ* hybridisation studies assessing the colocalisation of subunits on neuronal membranes have also shown that most $GABA_A$ receptors in the brain are a combination of α, β and γ subunits (Benke *et al.*, 1991b; Laurie *et al.*, 1992; Pirker *et al.*, 2000; Sieghart *et al.*, 1999; Wisden *et al.*, 1992). β subunits contain a consensus sequence for cAMP-dependent phosphorylation (Lolait *et al.*, 1989a; Ymer *et al.*, 1989b; Schofield *et al.*, 1987), which appears to be important in regulation of receptor activity and cellular location (Costa, 1998; Tyndale *et al.*, 1995). Thus the β subunits are a conceivably crucial component of the $GABA_A$ receptor, evidenced by the fact that that most $GABA_A$ receptors contain β subunits (Tyndale *et al.*, 1995). It is likely that the γ subunit may be replaced by δ or ε subunits in minor receptors (Bormann, 1988; Macdonald & Olsen, 1994; Sieghart *et al.*, 1999) and the θ subunit might replace a β subunit (Bonnert *et al.*, 1999; Sieghart *et al.*, 1999). This allows for a very large number of possible subunit combinations, however a large number is not anticipated to exist *in vivo* (Chebib & Johnston, 2000; Johnston, 1996a). The necessity for the presence of particular subunits may limit the diversity of $GABA_A$ receptors expressed in cell membranes (Costa, 1998).

Immunoprecipitation studies using antisera directed against specific $GABA_A$ receptor subunits in solubilised preparations of mammalian brain have allowed researchers to determine the subunit composition of $GABA_A$ receptors (Costa, 1998; McKernan & Whiting, 1996). The most commonly occurring $GABA_A$ receptor subunit composition is the $\alpha1\beta2\gamma2$ combination, which is sensitive to benzodiazepines (Sieghart, 2000) and thought to account for around 43% of $GABA_A$ receptors in the CNS (McKernan & Whiting, 1996). Other researchers have also demonstrated that the $\alpha1$, $\beta2/3$ and $\gamma2$ subunits co-precipitate, and that these receptors are ubiquitous throughout the brain, accounting for 40-50% of rat cerebral $GABA_A$ receptors (Benke *et al.*, 1990; Benke *et al.*, 1991b; De Blas, 1996). Around 18% of $GABA_A$ receptors *in vivo* are formed from $\alpha2\beta2/3\gamma2$ subunits, and around 17% of the $GABA_A$ receptors are comprised of $\alpha3\beta n\gamma2/3$ subunits (McKernan & Whiting, 1996). Other less-abundant subtypes of $GABA_A$ receptors are present throughout the brain with more discrete distributions, such as the $\alpha6\beta n\gamma2/\delta$ subunit combination which has been localised in the cerebellum and accounts for a further 4% of the $GABA_A$ receptors (McKernan & Whiting, 1996).

Immunohistochemical detection of $GABA_A$ receptor subunits in rat brain additionally showed that the $\alpha1$, $\beta1$-3 and $\gamma2$ subunits were the most widespread (Fritschy & Möhler, 1995; Pirker *et al.*, 2000), confirming their predominance throughout the brain. Results from *in situ* hybridisation studies assessing the distribution of subunit mRNAs throughout the brain have revealed individual subunits display distinct but overlapping cellular and regional distribution (Laurie *et al.*, 1992; Persohn *et al.*, 1992; Wisden *et al.*, 1992). Colocalisations that appear most common are the $\alpha1\beta2\gamma2$, $\alpha2\beta3$, $\alpha5\beta1$ and $\alpha4\delta$ mRNAs, with the $\alpha1$, $\beta2$ and $\gamma2$ subunit mRNAs being most widely distributed (Laurie *et al.*, 1992; Persohn *et al.*, 1992; Wisden *et al.*, 1992). Neurons express from two to many different $GABA_A$ receptor subunit mRNAs (Sieghart *et al.*, 1999).

1.2.2.3 Diversity of $GABA_A$ receptors

Due to the diversity of the $GABA_A$ receptor subunits, many subtypes of $GABA_A$ receptors exist which are differentially distributed throughout the CNS and which demonstrate differing pharmacological sensitivity. This large heterogeneity in receptor subtypes suggests that discrete $GABA_A$ receptor subtypes are responsible for distinct neural processes and perhaps disease states.

The type of α subunit influences the sensitivity of the receptor to GABA. Levitan and co-workers (1988) expressed recombinant bovine $GABA_A$ receptors composed of either the α1, α2 or α3 subunit with the β subunit in *Xenopus* oocytes, and observed that exchanging α subunits produced a 30 fold change in sensitivity to GABA. The α subunit isoform also dramatically changes the affinity and efficacy of allosteric modulators acting at the benzodiazepine binding site (Barnard *et al.*, 1998; Puia *et al.*, 1991). Exchanging α subunits did not affect qualitative properties of the expressed $GABA_A$ receptor, such as bicuculline and barbiturate sensitivity, and Hill coefficients (Levitan *et al.*, 1988). Subsequent expression of the α subunits using Northern blot analysis of mRNA from bovine cerebellum, cortex and hippocampus showed that the three α subunits differed in their abundance and distribution (Levitan *et al.*, 1988). Such findings affirm that neuronal responsiveness to GABA is influenced by diversified subunit expression (Levitan *et al.*, 1988).

The type of γ subunit appears to be important in the activities of benzodiazepine binding site ligands (Pritchett *et al.*, 1989). High sensitivity to benzodiazepines requires the presence of the γ2 subunit (Pritchett *et al.*, 1989), since replacement of this subunit with γ1 subunits in recombinant $GABA_A$ receptors expressed in HEK293 cells resulted in a marked reduction in benzodiazepine facilitation of GABA-induced currents (Puia *et al.*, 1991). The type of γ subunit is also critical to neurosteroid sensitivity. The presence of γ1 subunits enhances sensitivity to neurosteroids (Puia *et al.*, 1993). These findings confirm that drug sensitivity is dictated by the subunit composition of the $GABA_A$ receptor complex (Wafford *et al.*, 1991).

Ethanol sensitivity is conferred by the presence of the $\gamma2_{Long}$ splice variant of the γ2 subunit (Wafford *et al.*, 1991). Recombinant receptors comprised of $\alpha1\beta1\gamma2_{Short}$ and α1β1 subunits displayed no sensitivity to ethanol (Wafford *et al.*, 1991; Wafford & Whiting, 1992). Moreover, antisense oligonucleotides complementary to the $\gamma2_{Long}$ subunit which had been hybridised with mouse brain mRNA then injected into oocytes, prevented ethanol enhancement of GABA (Wafford *et al.*, 1991). Since the $\gamma2_{Long}$ subunit accommodates 8 amino acids in the large intracellular loop which contain a consensus site for protein kinase C (Whiting *et al.*, 1990), the potentiation of $GABA_A$

receptors by ethanol obviously requires phosphorylation by protein kinase C (Wafford *et al.*, 1991; Wafford & Whiting, 1992).

The multigenic nature of the $GABA_A$ receptor family of subunits permits regulation of the amounts and distribution of functional receptors (Tyndale *et al.*, 1995). This molecular heterogeneity, coupled with differences in pharmacological sensitivities to a wide variety of ligands as well as receptor subtype distributions, results in remarkable receptor diversity. Receptor diversity may be an evolutionary phenomenon to allow a variety of neuronal responses to GABA, with a consequent increase in plasticity (Persohn *et al.*, 1992; Tyndale *et al.*, 1995).

1.2.2.4 Pharmacology of $GABA_A$ receptors

To date, there is no known compound that selectively activates the $GABA_A$ receptor, which also binds to the $GABA_C$ receptor. The convulsant alkaloid, bicuculline, is a selective, competitive antagonist for $GABA_A$ receptors (Curtis & Johnston, 1974; Johnston, 1996a).

In addition to the GABA binding site, many sites are present for allosteric modulation of the $GABA_A$ receptors, as indicated by the diversity of chemicals acting at these receptors (Johnston, 1996a; Chebib & Johnston, 2000). The clinical effects of such compounds are partly due to modulation of the allosteric binding sites on $GABA_A$ receptors (Sieghart *et al.*, 1999). Johnston (1996a) proposed an additional 10 binding sites on the $GABA_A$ receptor. These include: a site for picrotoxinin, closely associated with the ion channel (Johnston, 1996a; Macdonald & Olsen, 1994); a site for barbiturates; a site for benzodiazepines and β-carbolines; a site for neuro-active steroids (potentially two sites, one for facilitation of the GABA response and one for direct action (Puia *et al.*, 1990)); a site for ethanol; a site for inhalation anaesthetics; and sites for zinc ions and other divalent cations. The various agonists, antagonists and modulators of the $GABA_A$ receptors are reviewed extensively by Johnston (1996a) and Chebib and Johnston (2000).

1.2.2.5 Functional importance of $GABA_A$ receptors

$GABA_A$ receptors are relevant to many CNS functions. $GABA_A$ receptors modulate anxiety, feeding and drinking behaviour, circadian rhythms, sleep, cognition, vigilance, muscle tension, epileptiform activity, memory and learning (Benke *et al.*, 1990; Puia *et al.*, 1990; Sieghart *et al.*, 1999). Clinically, $GABA_A$ receptor ligands are used in general anaesthesia, anxiolysis, sedation, epilepsy and muscle relaxation (Johnston, 1996a).

$GABA_A$ receptors are also functionally important in development of the CNS. Picrotoxinin-sensitive, bicuculline-sensitive $GABA_A$ receptors were shown to inhibit migration of postmitotic cells from the cortical plate to their target destinations of the embryonic rat cortex during cortical development (Behar *et al.*, 2000). Furthermore, activation of $GABA_A$ receptors depolarises immature hippocampal neurons during development, through the outward flow of chloride ions (Martina *et al.*, 1995).

1.2.3 $GABA_C$ Receptors

1.2.3.1 Discovery of GABA receptors with novel pharmacology

The notion of another subtype of GABA receptors emerged when *cis*-4-aminocrotonic acid (CACA), an analogue of GABA restricted in the folded conformation, was found to depress firing of cat spinal interneurons in a manner that what not inhibited by bicuculline or strychnine (Johnston *et al.*, 1975). CACA also failed to influence GABA uptake or the GABA-catabolising enzyme, 2-oxoglutarate aminotransferase (Johnston *et al.*, 1975). The actions of CACA lead to the premise that GABA may activate a previously uncharacterised class of receptors in the mammalian CNS (Johnston *et al.*, 1975).

Consequently, a series of GABA analogues of restricted conformation were assayed on synaptic membranes prepared from rat cerebellum (Drew *et al.*, 1984). These analogues were found to have inhibitory activity in the CNS in the presence of bicuculline, and failed to inhibit the binding of labelled baclofen to synaptic membranes prepared from rat cerebellum. A third subtype of GABA receptor was thus defined on the basis of insensitivity to the $GABA_A$ receptor antagonist, bicuculline, and the $GABA_B$ receptor agonist, baclofen. These receptors were designated $GABA_C$ (Drew *et al.*, 1984).

GABA receptors with novel pharmacology were discovered in areas of the CNS across a range of species. Nistri and Sivilotti (1985) isolated bicuculline- and baclofen-insensitive responses to GABA in the frog optic tectum. The researchers demonstrated a dose-dependent enhancement by GABA of an optic nerve-evoked mono-synaptic excitatory amplitude, which was mimicked by muscimol, blocked by picrotoxin, and insensitive to bicuculline (Cherubini & Strata, 1997; Nistri & Sivilotti, 1985). Sivilotti & Nistri (1989) used a similar preparation to further characterise the novel response to GABA in frog optic tectum, and demonstrated that the GABA-evoked enhancement was chloride-dependent. The novel receptors also displayed unusual pharmacology. They were potently activated by 3-APS, muscimol and TACA, and to a lesser extent by CACA, and were not modulated by benzodiazepines or barbiturates (Sivilotti & Nistri, 1989). On the basis of these findings, the researchers concluded that the novel receptors they had characterised were similar to those reported by Drew *et al.* (1984).

Further work by Drew & Johnston (1992) using [^{3}H]GABA binding to rat cerebellar membranes supported earlier results of bicuculline- and baclofen-insensitive binding sites which were also displaced by muscimol and isoguvacine. Strata and Cherubini (1994) also isolated transient excitatory GABAergic responses in the developing rat hippocampus which were chloride-dependent and insensitive to bicuculline and baclofen. Intracellular recording from CA3 neurons in rat hippocampal slices revealed that bicuculline abolished the response evoked by isoguvacine, an agonist at $GABA_A$ receptors, however it merely reduced the GABA-evoked response. The GABA-evoked depolarisation was abolished by co-application of bicuculline and picrotoxin (Cherubini & Strata, 1997; Strata & Cherubini, 1994). The bicuculline-resistant response to GABA was also resistant to baclofen, the $GABA_B$ receptor antagonist (3-aminopropyl)(diethoxymethyl)phosphinic acid (CGP35348), and allosteric modulators of the $GABA_A$ receptors (Cherubini & Strata, 1997). These findings lead the authors to conclude that the novel response was through activation of $GABA_C$ receptors. Patch-clamp techniques were used by Martina and colleagues (1995) to examine whole cell and single channel properties of a novel response to GABA in acutely dissociated CA3 hippocampal neurons of the postnatal rat. Bicuculline blocked the response to isoguvacine but only partially reduced the response to GABA in these cells, in keeping with the results of Strata & Cherubini (1994). Furthermore, picrotoxin completely

abolished the bicuculline-insensitive response, while CACA reduced both bicuculline-sensitive and bicuculline-insensitive responses. The bicuculline-resistant response decreased with increasing age (Martina *et al.*, 1995).

Arakawa & Okada (1988) demonstrated novel GABAergic responses in the guinea pig superior colliculus. At low concentrations GABA enhanced the amplitude of post-synaptic potentials (PSPs) in slice preparations of superior colliculus superficial grey layers. At high concentrations, GABA depressed the post-synaptic potentials. This pattern was mimicked by muscimol, but not baclofen, and the excitatory response was not blocked by bicuculline. The authors suggested a situation where novel, high affinity $GABA_A$ receptors exist which are excitatory at low concentrations of GABA and are not blocked by bicuculline. More recently, Pasternack *et al.* (1999) and Boller & Schmidt (2001) measured electrically evoked field potentials in slices of superior colliculus from postnatal and adult rat brain that were modulated by drug application. The researchers showed that $GABA_C$ receptors are functionally expressed in the superior colliculus from relatively early in postnatal development, determined by a reduction in field potentials in the presence of TPMPA. CACA and low concentrations of muscimol potentiated the evoked responses, an effect that was blocked by TPMPA application (Pasternack *et al.*, 1999). This $GABA_C$ receptor response contrasted to the inhibitory effects of higher muscimol concentrations, presumably acting at the $GABA_A$ receptors (Pasternack *et al.*, 1999). These results were confirmed by whole cell patch clamping of superior colliculus neurons (Boller & Schmidt, 2001).

Additionally, Boue-Grabot and co-workers (2000) using whole-cell patch clamp recording from single thyrotropin-secreting cells of the rat pituitary gland revealed bicuculline-insensitive Cl^- currents that readily desensitised.

1.2.3.2 $GABA_C$ receptors in retina

Polenzani *et al.* (1991) first demonstrated that the retina contains GABA receptors which are insensitive to bicuculline and baclofen by injecting $poly(A)^+$ RNA from bovine retina into *Xenopus* oocytes to generate two pharmacologically distinct GABA receptors. The researchers used two electrode voltage clamp techniques to assess the pharmacology of receptors formed on the oocyte surface. The $GABA_A$ receptor

component was blocked by bicuculline, however the other expressed receptors were sensitive to GABA, insensitive to bicuculline and baclofen, and were not modulated by benzodiazepines and barbiturates. At the same time, poly(A)$^+$ RNA from bovine and rat cortex were also expressed in *Xenopus* oocytes. Subsequently expressed receptors displayed none of the novel pharmacological and physiological responses, suggesting that they were entirely $GABA_A$ receptors.

Woodward *et al.* (1993) also expressed bovine retinal mRNA in *Xenopus* oocytes to further characterise the novel pharmacological responses of the receptors. These responses were compared directly to receptors translated from rat cortical mRNA. As was the case for Polenzani and colleagues (1991), the cortical receptors displayed none of the novel responses, demonstrating only responses similar to those of native $GABA_A$ receptors. The retinal bicuculline- and baclofen-insensitive GABA receptors had a greater affinity for GABA and muscimol than the corresponding retinal or cortical bicuculline-sensitive $GABA_A$ receptors. Furthermore, the novel retinal receptors were shown to be sensitive to many $GABA_A$ receptor agonists and antagonists, as well as to some $GABA_B$ receptor antagonists.

Native $GABA_C$ receptors were observed *in situ* in retinal preparations. The GABA-responsive receptors were demonstrated to be selective for chloride ions, but insensitive to baclofen and bicuculline (Dong *et al.*, 1994; Qian & Dowling, 1993; Wang *et al.*, 1994). Electrophysiological recordings provided evidence of baclofen- and bicuculline-insensitive GABA gated chloride channels in cultured cone-driven horizontal cells isolated from the catfish retina (Dong *et al.*, 1994), in cultured rod-driven horizontal cells isolated from the white perch retina (Qian & Dowling, 1993; Qian & Dowling, 1994), in isolated hybrid bass retinal bipolar cells (Qian & Dowling, 1995), in isolated bipolar cells of the tiger salamander retina (Lukasiewicz & Werblin, 1994; Lukasiewicz *et al.*, 1994), and in isolated rat retinal bipolar cells (Feigenspan *et al.*, 1993; Feigenspan & Bormann, 1994). The physiological and pharmacological responses of native $GABA_C$ receptors differed considerably from the $GABA_A$ receptor responses in retina, as outlined in ensuing paragraphs.

1.2.3.3 Molecular cloning of ρ subunits and expression in Xenopus *oocytes*

The $GABA_C$ receptor subunit cDNA, termed ρ1, was first isolated from a human retinal cDNA library using polymerase chain reaction and mixed oligonucleotides for conserved regions (Cutting *et al.*, 1991). This cDNA exhibited features which suggested it was related to the $GABA_A$ and glycine receptor subunits, but represented another class of these proteins, most closely related to the $GABA_A$ receptor β subunits (Cutting *et al.*, 1991). Screening of human fœtal brain, 1 day old human cortex and adult rat cortex cDNA libraries failed to identify a cDNA clone containing the ρ1 sequence (Cutting *et al.*, 1991). However, Northern blot analysis showed ρ1 cDNA hybridised to mRNA in poly(A)-selected RNA extracted from bovine retina, confirming the results of Polenzani *et al* (1991) that a novel GABA receptor type exists in bovine retina, and further, establishing that it is in fact comprised of ρ1 subunits.

ρ2 cDNA was identified shortly thereafter by screening of a genomic DNA library using a portion of the ρ1 cDNA, and subsequent screening of a human retinal cDNA library (Cutting *et al.*, 1992). The novel amino acid sequence displayed 30-38% homology with $GABA_A$ receptor subunits, but displayed 74% sequence homology with the human ρ1 cDNA, indicating that it belonged to the same family as the ρ1 subunits (Wang *et al.*, 1994). Polymerase chain reaction (PCR) amplification of ρ1 and ρ2 cDNA revealed mapping to human chromosome 6, bands q14-q21 (Cutting *et al.*, 1992), further consolidating their designation to a novel subclass of ionotropic GABA receptor subunits.

The expression of recombinant ρ1 mRNA in *Xenopus* oocytes and mammalian cell lines demonstrated the formation of robust homo-oligomeric ion channel receptors displaying GABA-responsive chloride conductance (Cutting *et al.*, 1991; Kusama *et al.*, 1993a; Kusama *et al.*, 1993b; Shimada *et al.*, 1992; Wang *et al.*, 1994). These recombinant receptors exhibited physiological and pharmacological properties similar to $GABA_C$ receptors formed from retinal mRNA (Cutting *et al.*, 1991), showing benzodiazepine-, barbiturate- and bicuculline-insensitivity (Shimada *et al.*, 1992). Expression of *in vitro* transcribed RNA from ρ2 cDNA in *Xenopus* oocytes similarly produced GABA-gated chloride-selective ion channels, with substantially lower currents than those produced by oocytes injected with ρ1 RNA (Cutting *et al.*, 1992; Wang *et al.*, 1994). This

suggests that ρ2 subunits possibly require coexpression with other subunits to form fully functional receptors (Cutting *et al.*, 1992).

Following the cloning of human ρ1 and ρ2 subunit cDNAs, ρ1 and ρ2 subunit cDNAs were isolated from rat retinal cDNA libraries (Enz *et al.*, 1995; Ogurusu *et al.*, 1995; Zhang *et al.*, 1995). The rat retinal ρ subunits displayed 74% and 91% homology to human ρ1 and ρ2 amino acid sequences respectively (Ogurusu *et al.*, 1995). Ogurusu and Shingai (1996) cloned a third ρ subunit from a rat retina cDNA library, which exhibited 63% homology to the human ρ1 amino acid sequence and 62% similarity to the rat ρ2 amino acid sequence. ρ3 Expression in oocytes also produced GABA-gated chloride currents different to those formed by $GABA_A$ receptor subunits (Ogurusu & Shingai, 1996), but with similar pharmacology and functional properties to that reported for rat ρ1 and ρ2 subunit receptors (Shingai *et al.*, 1996). The partial sequence for a putative ρ3 subunit has also been cloned from human bacteriophage λ genomic libraries and localised to chromosome 3q11-q13.3 (Bailey *et al.*, 1999). Furthermore, splice variants of the ρ1 receptor have also been identified (Martínez-Torres *et al.*, 1998).

$GABA_C$ receptor subunits were subsequently cloned from the retina of chick (ρ1 and ρ2) (Albrecht & Darlison, 1995), mouse (ρ1 and ρ2) (Greka *et al.*, 1998) and white perch (ρ1-ρ5) (Qian & Dowling, 1993).

Since homooligomeric receptors formed from recombinant ρ1, ρ2 and ρ3 subunits expressed in *Xenopus* oocytes displayed many of the functional and pharmacological properties of $GABA_C$ receptors formed from retinal mRNA and native $GABA_C$ receptors identified in the bovine, fish and rat retina, it was concluded that $GABA_C$ receptors *in vivo* are composed of ρ1, ρ2 or ρ3 subunits (Bormann & Feigenspan, 1995; Wang *et al.*, 1994; Zhang *et al.*, 2001).

1.2.3.4 $GABA_C$ receptor composition

In retina and regions of the rat brain, ρ subunits are expressed in similar cells and regions, albeit in varying levels. This suggests that, *in vivo*, $GABA_C$ receptors may be composed of single subunits (homooligomers) (Cutting *et al.*, 1991; Kusama *et al.*,

1993a; Kusama *et al.*, 1993b; Shimada *et al.*, 1992) or a combination of ρ subunits (pseudo-heterooligomers) (Enz & Cutting, 1998; Enz & Cutting, 1999a). This contrasts to the $GABA_A$ receptors, which are heterooligomeric, composed of a number of subunits. Recombinant $GABA_C$ receptors can be formed from ρ1, ρ2 (not rat) or ρ3 subunits in a homooligomeric fashion (Chebib *et al.*, 1998; Chebib *et al.*, 2001; Chebib & Johnston, 2000; Cutting *et al.*, 1991; Cutting *et al.*, 1992; Enz & Cutting, 1999a; Kusama *et al.*, 1993a; Ogurusu & Shingai, 1996; Ogurusu *et al.*, 1999; Shingai *et al.*, 1996; Vien *et al.*, 2002). Robust recombinant pseudo-heterooligomeric receptors can also be formed from ρ1 and ρ2 subunits or ρ2 and ρ3 subunits, displaying distinct biophysical properties and differing pharmacological properties to homooligomeric receptors (Enz & Cutting, 1999a; Ogurusu *et al.*, 1999; Zhang *et al.*, 1995). Hackam and colleagues (1997) also demonstrated the assembly of ρ1 with ρ2 subunits *in vitro*, which implies that they are able to form pseudo-heterooligomeric receptors *in vivo*.

Native $GABA_C$ receptors in rat retina display characteristics similar to those of receptors in *Xenopus* oocytes formed from coexpression of ρ1 and ρ2 subunits (Chebib & Johnston, 2000). This suggests that rat retinal $GABA_C$ receptors are pseudo-homooligomeric (ρ1ρ2) with differing properties to homooligomeric receptors comprised of either ρ1 or ρ2 subunits (Enz & Cutting, 1998; Enz & Cutting, 1999a). Rat ρ2 subunits do not form receptors when expressed in *Xenopus* oocytes (Zhang *et al.*, 1995). While human ρ2 subunits form efficient receptors in *Xenopus* oocytes, their whole cell currents are an order of magnitude lower than receptors comprised of ρ1 subunits (Chebib & Johnston, 2000; Enz & Cutting, 1998; Wang *et al.*, 1994). However, the pharmacological characteristics of the ρ2 receptors, for the most part, are similar to the ρ1 receptors (Chebib & Johnston, 2000; Kusama *et al.*, 1993b).

The core structure of the ρ subunits, including the transmembrane spanning domains and the proximal N terminal region, is conserved across species (Zhang *et al.*, 2001). The largest deviations in sequence homology for the three subunits occur at the distal N terminal region and the cytoplasmic loop between transmembrane domains 3 and 4 (Zhang *et al.*, 2001). Amino acids in the distal N terminal region are thought to be important for the different binding characteristics of the three ρ subunits. Two agonist binding domains have in fact been localised to the N terminus between the cys-loop and

TM1 of the ρ1 subunit using site-directed mutagenesis (Amin & Weiss, 1994). Hackam *et al.* (1997) showed that the N terminal directs subunit interaction into homo- or pseudohetero-oligomeric receptors by employing *in vitro* translated proteins and immunoprecipitation with subunit-specific antibodies. To this end, the ρ1 subunits contain assembly signals which allow them to form more efficient homooligomeric receptors than the ρ2 subunits (Hackam *et al.*, 1998). Enz & Cutting (1999b) identified a 70 amino acid sequence in the human ρ1 subunit N terminal domain that was important for assembly of the ρ subunits and receptor formation. $GABA_A$ receptor β1 subunit chimeras containing the 70 amino acids identified in the ρ1 subunit were shown to interfere with assembly of receptors from ρ1 subunits, but not ρ2 subunits, suggesting separate regions for homo- and pseudohetero-oligomeric receptor assembly (Enz & Cutting, 1999b).

Amino acids in the M3 – M4 cytoplasmic loop are regarded as important to subunit-specific intracellular functions, such as phosphorylation and binding to microtubule associating proteins (MAPs) (Zhang *et al.*, 2001). The cytoplasmic loop of the rat ρ2 subunit has been shown to contain two such sites (Johnston, 1996b). It also cannot be excluded that the cytoplasmic loop contains sequences for subunit assembly which are active in combination with the N terminal (Enz & Cutting, 1998; Hackam *et al.*, 1997), since deletions of amino acids in the ρ1 subunit C terminal interfered with receptor assembly (Enz & Cutting, 1999b).

1.2.3.5 Differences between $GABA_A$ and $GABA_C$ receptors

Much contention has rested over the reputation of the ρ subunits as constituting a separate class of GABA receptor or merely belonging to the $GABA_A$ receptor family of subunits (Bormann, 2000; Chebib & Johnston, 2000). However, there is a great deal of evidence to suggest that the $GABA_C$ receptors are physiologically and pharmacologically distinct from both the $GABA_A$ and $GABA_B$ receptors.

Homology between subunits of different members of the LGICs is 10-40% (Olsen & Tobin, 1990; Unwin, 1993), while homology between subunits within a specific receptor family is greater than 40% (Unwin, 1993). ρ Subunits within a species display 60-74% homology, and 95% homology exists between ρ subunits of rat and human.

Only 30-38% sequence homology exists between $GABA_C$ and $GABA_A$ receptor subunits (Cutting *et al.*, 1991; Bormann & Feigenspan, 1995; Cherubini & Strata, 1997). Thus, on the basis of Unwin's (1993) characterisation, ρ subunits belong to a different class of receptors to $GABA_A$ receptor subunits.

Activation, desensitisation, conductance and gating properties demarcate $GABA_C$ from the $GABA_A$ receptors (Amin & Weiss, 1994; Bormann & Feigenspan, 1995). Native and recombinant $GABA_C$ receptors are more sensitive to GABA than $GABA_A$ receptors (Amin & Weiss, 1994; Bormann & Feigenspan, 1995; Polenzani *et al.*, 1991). Channel opening and closing times of the $GABA_C$ receptor are longer than for $GABA_A$ receptors (Amin & Weiss, 1994; Feigenspan *et al.*, 1994; Feigenspan & Bormann, 1998; Martina *et al.*, 1995; Polenzani *et al.*, 1991). Thus, $GABA_C$ receptor-mediated inhibition is thought to occur at lower concentrations of GABA and to last longer (Bormann & Feigenspan, 1995). Hill slopes are steeper for $GABA_C$ receptors, representing a greater number of binding sites required for activation (Amin & Weiss, 1994; Feigenspan & Bormann, 1998; Kusama *et al.*, 1993a). $GABA_C$ receptors formed from recombinant ρ subunits expressed in *Xenopus* oocytes do not readily desensitise compared to the $GABA_A$ receptors (Amin & Weiss, 1994; Cherubini & Strata, 1997; Feigenspan & Bormann, 1998; Polenzani *et al.*, 1991; Qian & Dowling, 1993). Furthermore, retinal $GABA_C$ receptors have a much smaller chloride conductance compared to retinal $GABA_A$ receptors (Bormann & Feigenspan, 1995; Feigenspan & Bormann, 1994).

No evidence exists for *in vivo* co-localisation of $GABA_C$ receptor subunits with $GABA_A$ receptor subunits in the retina (Enz *et al.*, 1996; Koulen *et al.*, 1998) or co-assembly when expressed in *Xenopus* oocytes (Kusama *et al.*, 1993a; Shimada *et al.*, 1992). Moreover, human ρ1 and ρ2 subunits do not co-immunoprecipitate with $GABA_A$ receptor α1, α5 and β1 subunits, demonstrating a lack of physical interaction between the $GABA_C$ and $GABA_A$ receptor subunits (Hackam *et al.*, 1998). In fact, Hackam and colleagues (1998) found that the assembly properties for ρ subunits differed from those for $GABA_A$ receptor subunits, suggesting that the $GABA_C$ receptors are likely to be formed from ρ subunits only.

Co-assembly of perch and rat ρ1 subunits with γ2 subunits in *Xenopus* oocytes has been achieved. Qian & Ripps (1999) maintained that the receptors formed from perch ρ1 and human γ2 subunits resembled the responses of native $GABA_C$ receptors in perch retina moreso than homooligomeric receptors comprised of ρ1 subunits alone. Pan and colleagues (2000) reported the co-assembly of mutant or wild type rat ρ1 subunits with the human $\gamma2_{Short}$ subunit and rat glycine α subunit in *Xenopus* oocytes. The co-assembly and formation of functional receptors was postulated on the basis of changed receptor pharmacology and physiology for the wild type or mutant ρ1 subunits when expressed with the $GABA_A$ receptor γ2 or glycine receptor α subunit. No changes were observed when the human $GABA_A$ receptor α1 and β2 or glycine receptor β subunits were co-injected with the wild type or mutant ρ1 subunits. However the ability for these subunits to co-assemble in *Xenopus* oocytes does not mean that such an event would occur *in vivo*. Subunits from different species were co-expressed in the studies described. Moreover, the results do not necessarily reflect the *in vivo* situation, since the ρ1 subunit was mutated in TM2, the pore lining segment of the subunit (Enz & Cutting, 1998). Further, subcellular localisation of ρ1 with γ2 subunits *in vivo* remains to be demonstrated (Zhang *et al.*, 2001).

1.2.3.6 Status of $GABA_C$ receptors in the CNS

What we know of $GABA_C$ receptors to date has been elucidated predominantly from retinal preparations or recombinant receptors composed of subunits cloned from retina.

Patch-clamp recordings combined with single cell reverse transcription - polymerase chain reaction (RT-PCR) in acutely dissociated adult rat retinal bipolar cells were performed in order to correlate $GABA_C$ receptor responses with expression of ρ transcripts. ρ1 Subunit mRNA was localised exclusively to the retinal bipolar cells and could be convincingly correlated with the familiar bicuculline- and diazepam-insensitive $GABA_C$ receptor responses (Yeh *et al.*, 1996; Zhang *et al.*, 2001). ρ2 Subunit mRNA was expressed in both bipolar and ganglion retinal cells, and no bicuculline-resistant responses to GABA were found in the ganglion cells (Yeh *et al.*, 1996), suggesting the ρ2 subunits may combine to form bicuculline-insensitive receptors on bipolar cells, and bicuculline-sensitive receptors on ganglion cells. The elimination of the ρ1 subunit gene

in ρ1-knockout mice resulted in a lack of $GABA_C$ receptor expression in the retina (McCall *et al.*, 2002), suggesting again these subunits are necessary for the formation of $GABA_C$ receptors in the retina.

$GABA_C$-like responses observed in the hippocampus, amygdala and pituitary gland are different to those detected in the retina, indicating slightly different receptor subtypes are responsible (Boue-Grabot *et al.*, 2000; Cherubini & Strata, 1997; Delaney & Sah, 1999). The fact that ρ1 subunits are found predominantly in the retina means most of the described properties of $GABA_C$ receptors are mediated by ρ1 receptors. The fact that ρ2 receptors are found predominantly outside the retina, notably in the hippocampus, means that the different responses observed in the hippocampus and other regions may be mediated by ρ2 receptors. In the hippocampus, for example, $GABA_C$ receptors are less sensitive to GABA and cannot be distinguished by CACA (Cherubini & Strata, 1997).

While total RNA extracted from rat and bovine brain did not produce any responses resembling those of $GABA_C$ ρ-like receptors isolated from rat and bovine retina (Polenzani *et al.*, 1991; Woodward *et al.*, 1993), abundant evidence still exists for the presence of ρ subunits in the mammalian CNS. As mentioned previously, the first evidence of GABA receptors with novel pharmacology was discovered in the cat spinal cord (Johnston *et al.*, 1975). Moreover, GABA receptors with novel pharmacology and responses different to those of $GABA_A$ or $GABA_B$ receptors, were characterised in several brain regions, namely rat cerebellum, frog optic tectum, postnatal rat hippocampal cells, rat pituitary gland cells and rat and guinea pig superior colliculus (Arakawa & Okada, 1988; Boller & Schmidt, 2001; Boue-Grabot *et al.*, 2000; Drew *et al.*, 1984; Drew & Johnston, 1992; Martina *et al.*, 1995; Nistri & Sivilotti, 1985; Pasternack *et al.*, 1999; Sivilotti & Nistri, 1989; Strata & Cherubini, 1994).

ρ Subunits have been mapped to various regions of the CNS (refer to sections 1.4 and 1.6). However, the molecular composition of the $GABA_C$ receptors in areas of the CNS outside the retina in not entirely clear, and whether $GABA_C$ receptors in other areas of the CNS resemble the retinal receptors requires further study (Zhang *et al.*, 2001). This could be achieved using functional *in vivo* assays with selective pharmacological agents

and single-channel patch-clamp techniques such as those employed by Yeh and co-workers (1996).

Delaney and Sah (1999) discovered a novel receptor in the central nucleus of the rat amygdala which was resistant to bicuculline, sensitive to the selective $GABA_C$ receptor antagonist (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid (TPMPA), yet was inhibited by the benzodiazepines flurazepam and diazepam, an effect which was antagonized by the benzodiazepine antagonist Ro 15-1788. When the $GABA_C$ receptor component was blocked in the central nucleus, using TPMPA, the benzodiazepines produced a typical facilitatory response in the remaining receptors, which were assumed to be the $GABA_A$ receptors. These results may demonstrate the presence of $GABA_C$-like receptors in the central nucleus of the amygdala, which are modulated by benzodiazepines in a novel way. It is possible that the $GABA_C$ receptor subunits may assemble with $GABA_A$ receptor subunits to produce these novel responses. Alternatively, splice variants of the $GABA_C$ receptor subunits may account for the responses.

1.2.3.7 Splice variation in subunits

Alternative splicing of receptor subunit pre-mRNA increases the coding potential of the isoforms, thereby increasing the diversity of receptor responses (Maniatis & Tasic, 2002; Martínez-Torres *et al.*, 1998). Martínez-Torres and colleagues (1998) discovered alternative forms of the ρ1 subunit in a human retinal cDNA library. Subsequent cloning and sequencing of these transcripts demonstrated subunits with alternative splicing of 51 deleted nucleotide bases and 450 deleted nucleotide bases in the N terminal region of the ρ1 subunit, termed ρ1Δ51 and ρ1Δ450 respectively. The deletion was shown to begin at the same splice site. The ρ1Δ450 cRNA did not form functional receptors when expressed in *Xenopus* oocytes, however, when co-expressed with ρ1 subunit mRNA, decreased the GABA-evoked current to half of its maximum response (Martínez-Torres *et al.*, 1998). The ρ1Δ51 cRNA expressed functional GABA-sensitive receptors in *Xenopus* oocytes with similar pharmacological and physiological characteristics to those of the recombinant ρ1 subunits; it was insensitive to bicuculline, not desensitised by prolonged application of GABA, and was negatively modulated by Zn^{2+} (Demuro *et al.*, 2000; Martínez-Torres *et al.*, 1998). The ρ1Δ51 subunit

receptors, however, were more sensitive to GABA, TPMPA and Zn^{2+} compared to the ρ1 subunit receptors, and showed a higher level of cooperativity (Demuro *et al.*, 2000). The questions of whether the alternative splicing is tissue specific and what impact it has upon the function of $GABA_C$ receptors formed from ρ1 subunits remain to be elucidated (Martínez-Torres *et al.*, 1998).

It would be interesting to discover if similar alternative splice variants exist for the $GABA_C$ receptor subunits outside of the retina, in the CNS. Some evidence for variants of the ρ1 and ρ2 subunits was reported recently by Boue-Grabot *et al.* (2000), who demonstrated, using Northern blot experiments, that the ρ1 and ρ2 subunit mRNAs from the rat pituitary gland were shorter in size than those expressed in the retina. Wegelius and co-workers (1998) also discovered three different sized transcripts of ρ2 mRNA using Northern analysis. 2.3 and 2kb fragments were found in retina, hippocampus and superior colliculus, and only the 2kb fragment in the cortex. Results from Northern blots must be interpreted cautiously since shorter fragments may merely represent degradation products (Barton *et al.*, 1993).

1.2.3.8 Agonists

Many compounds acting at the $GABA_A$ receptors also act at $GABA_C$ receptors but with differing affinities and efficacies. To date, there is no known compound that selectively activates the $GABA_A$ receptor, which does also bind to the $GABA_C$ receptor. However, there are selective ligands for $GABA_C$ receptors. *cis*-4-Aminocrotonic acid (CACA), which acts as a very weak agonist at $GABA_A$ receptors (EC_{50} 5mM), acts as a partial agonist at the ρ-like receptors (Woodward *et al.*, 1993). (±)-*cis*-2-(Aminomethyl)cyclopropanecarboxylic acid ((±)-CAMP) is also a selective agonist of $GABA_C$ receptors (Duke *et al.*, 1998; Duke *et al.*, 2000). Since it is now known that CACA has a weak affinity for a GABA uptake system (Chebib & Johnston, 1997), and activates α6 subunit-containing $GABA_A$ receptors in the cerebellar granule cell layer (Wall, 2001), it appears (±)-CAMP is the most selective agonist for $GABA_C$ receptors (Chebib & Johnston, 2000; Duke *et al.*, 2000). Separation of the racemic mixture into its enantiomers (+)-CAMP and (-)-CAMP further demonstrated that (+)-CAMP is an agonist while (-)-CAMP is an antagonist at ρ1 receptors expressed in *Xenopus* oocytes (Duke *et al.*, 2000).

The $GABA_A$ receptor agonists, *trans*-4-aminocrotonic acid (TACA) and (±)*trans*-2-(aminomethyl)cyclopropanecarboxylic acid (TAMP) ((±)-TAMP), act as potent agonists at the ρ-like receptors and recombinant ρ1 receptors, with greater affinity for the ρ-like receptors than GABA (Chang *et al.*, 2000; Kusama *et al.*, 1993a; Kusama *et al.*, 1993b; Woodward *et al.*, 1993). TACA also acts as a potent agonist at $GABA_C$ receptors in native preparations (Bormann & Feigenspan, 1995; Feigenspan *et al.*, 1993; Lukasiewicz *et al.*, 1994; Qian & Dowling, 1993; Sivilotti & Nistri, 1989), and (±)-TAMP is a moderately potent partial agonist at human recombinant ρ2 receptors expressed in *Xenopus* oocytes (Kusama *et al.*, 1993b). Furthermore, the enantiomers (+)-TAMP and (-)-TAMP are potent partial agonists at human recombinant ρ1 receptors expressed in *Xenopus* oocytes (Duke *et al.*, 2000).

Muscimol, an agonist at $GABA_A$ receptors, is a potent partial agonist at ρ-like receptors expressed from retinal poly(A)$^+$ RNA injected into *Xenopus* oocytes, at native $GABA_C$ receptors and at human recombinant ρ1 and ρ2 receptors expressed in *Xenopus* oocytes (Bormann & Feigenspan, 1995; Chang *et al.*, 2000; Kusama *et al.*, 1993a; Kusama *et al.*, 1993b; Qian & Dowling, 1994; Shimada *et al.*, 1992; Woodward *et al.*, 1993).

1,2,3,6-Tetrahydropyridine-4-carboxylic acid (isoguvacine), a weak partial agonist at $GABA_A$ receptors, is a partial agonist at the ρ-like receptors expressed from bovine retinal mRNA (Woodward *et al.*, 1993) and human recombinant $GABA_C$ receptors formed from ρ subunits (Chang *et al.*, 2000). However, at white perch retinal $GABA_C$ receptors, isoguvacine is a weak agonist (Qian & Dowling, 1994).

1.2.3.9 Antagonists

4,5,6,7-Tetrahydroisoxazole[4,5-*c*]pyridin-3-ol (THIP), a partial agonist at $GABA_A$ receptors, is a moderately potent competitive antagonist at ρ-like receptors expressed from retinal poly(A)$^+$ RNA injected into *Xenopus* oocytes (Woodward *et al.*, 1993) and in rat and white perch retina (Bormann & Feigenspan, 1995; Qian & Dowling, 1994). Piperidine-4-sulfonic acid (P4S), isonipecotic acid and 3-aminopropanesulfonic acid (3-APS), also partial agonists at $GABA_A$ receptors, are competitive antagonists at the

$GABA_C$ receptors (Bormann & Feigenspan, 1995; Qian & Dowling, 1994; Woodward *et al.*, 1993). Furthermore, Z-3-[(aminoiminomethyl)thio]prop-2-enonic acid (ZAPA), which is a potent agonist at $GABA_A$ receptors, acts as a competitive inhibitor of the ρ-like receptors (Woodward *et al.*, 1993).

Imidazole-4-acetic acid (I4AA) is a partial agonist at native salamander and recombinant human and fish $GABA_C$ receptors (Chang *et al.*, 2000; Chebib *et al.*, 1998; Kusama *et al.*, 1993a; Kusama *et al.*, 1993b; Lukasiewicz & Werblin, 1994; Zhang *et al.*, 2001), but antagonises native fish, porcine and rat $GABA_C$ receptors (Bormann & Feigenspan, 1995; Qian & Dowling, 1994; Qian & Dowling, 1995; Zhang *et al.*, 2001). Isoguvacine and I4AA are more potent at recombinant human ρ2 receptors compared to human ρ1 receptors (Chebib *et al.*, 1998; Kusama *et al.*, 1993b). *trans*-4-Amino-2-methylbut-2-enoic acid (2-MeTACA) is a partial agonist at human ρ2 receptors expressed in *Xenopus* oocytes, but displays antagonist properties at the recombinant ρ1 receptors (Chebib *et al.*, 1998).

2-(3-Carboxypropyl)-3-amino-6-*p*-methoxyphenylpyridazinium bromide (SR-95531) is a potent antagonist at cortical $GABA_A$ receptors expressed in *Xenopus* oocytes, with weak antagonist effects at the ρ-like receptors expressed from retinal mRNA in *Xenopus* oocytes (Woodward *et al.*, 1993) and in rat and perch retina (Bormann & Feigenspan, 1995; Feigenspan & Bormann, 1994). However, SR-95531 displayed no effect at recombinant receptors formed from ρ1 subunits (Kusama *et al.*, 1993a) or $GABA_C$ receptors in native fish and tiger salamander retinal preparations (Lukasiewicz *et al.*, 1994; Qian & Dowling, 1994).

The $GABA_B$ receptor agonists 3-aminopropylphosphonic acid (3-APA), 3-aminopropylphosphinic acid (3-APPA or CGP27492) and 3-aminopropyl(methyl)phosphinic acid (3-APMPA) are potent antagonists at the ρ-like receptors expressed from retinal mRNA in *Xenopus* oocytes (Woodward *et al.*, 1993; Zhang *et al.*, 2001) and in native retinal preparations (Qian & Dowling, 1994). δ-Aminovaleric acid (DAVA) and 4-aminobutylphosphonic acid (4-ABPA) are antagonists at $GABA_B$ receptors, which also display potent and weak antagonist effects at the ρ-receptors respectively (Woodward *et al.*, 1993).

Alkyl phosphinic acid analogues of GABA developed by Ciba Geigy (now Novartis), which were shown to have antagonist effects on $GABA_B$ receptors (Froestl *et al.*, 1995b), have also demonstrated antagonist effects at human recombinant $GABA_C$ receptors expressed in *Xenopus* oocytes (Chebib *et al.*, 1998 and unpublished observations).

Ragozzino *et al.* (1996) and Murata *et al.* (1996) synthesised the first selective $GABA_C$ receptor antagonist, (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid (TPMPA), from structure activities of 3-APMPA and isoguvacine. TPMPA acted as an antagonist at recombinant $GABA_C$ receptors expressed in *Xenopus* oocytes (Ragozzino *et al.*, 1996) and attenuated the inhibitory action of GABA on spinal reflexes in the neonatal rat (Rozzo *et al.*, 1999). The action of TPMPA is selective for the $GABA_C$ receptors, with 100 times greater potency at $GABA_C$ compared to $GABA_A$ receptors expressed in *Xenopus* oocytes (Ragozzino *et al.*, 1996). TPMPA is 8 fold less sensitive at recombinant receptors formed from ρ2 subunits than ρ1 subunits (Chebib *et al.*, 1998).

A number of derivatives of TPMPA were developed by Johnston and co-workers, including (piperidin-4-yl)methylphosphinic acid (P4MPA) (Hanrahan *et al.*, 2001). P4MPA is a selective antagonist at $GABA_C$ receptors, with equipotent effects at receptors formed from ρ1 and ρ2 subunits (Johnston *et al.*, 1998, registered patent).

1.2.3.10 Channel blockers

Picrotoxin, the non-competitive blocker of the $GABA_A$ receptor channel (Iversen, 1984), has a similar action at novel $GABA_C$-like receptors in native preparations (Lukasiewicz *et al.*, 1994; Qian & Dowling, 1993; Qian & Dowling, 1994; Sivilotti & Nistri, 1994; Strata & Cherubini, 1994), as well as at $GABA_C$-like receptors expressed in *Xenopus* oocytes (Shimada *et al.*, 1992; Woodward *et al.*, 1993). However, picrotoxinin, the toxic component of picrotoxin, exerts little effect on rat bipolar cells (Feigenspan *et al.*, 1993; Feigenspan & Bormann, 1994). Species-specific variation in subunit composition is believed to account for the variable sensitivity to picrotoxin (Yeh *et al.*, 1996). Picrotoxin was found to antagonise homooligomeric $GABA_C$ receptors comprised of ρ1 subunits, not homooligomeric ρ2 or pseudo-heterooligomeric native

ρ1ρ2 receptors (Bormann & Feigenspan, 1995; Cutting *et al.*, 1992; Enz & Cutting, 1999a). Moreover, human ρ1 and ρ2 subunits are sensitive to picrotoxin, while rat subunits are not, possibly due to single amino acid residue in rat ρ2 subunit (Zhang *et al.*, 1995; Zhang *et al.*, 2001).

Other channel blockers such as *t*-butylbicyclophosphorothionate (TBPS) antagonise the $GABA_C$ receptors (Bormann & Feigenspan, 1995 Feigenspan & Bormann, 1994; Qian & Dowling, 1994; Shimada *et al.*, 1992).

1.2.3.11 Allosteric modulators

$GABA_C$ receptors appear to be insensitive to $GABA_A$ receptor allosteric modulators such as benzodiazepines, barbiturates and neurosteroids (Johnston, 1996b; Shimada *et al.*, 1992), however they are modulated by protons, multivalent cations, dopamine and protein kinase C (PKC) (Wegelius *et al.*, 1996; Zhang *et al.*, 2001). Zn^{2+} inhibits GABAergic currents, and a site of interaction for Zn^{2+} modulation has been identified on the $GABA_C$ receptor (Enz & Cutting, 1998; Johnston, 1996b; Qian & Dowling, 1995; Wang *et al.*, 1995). PKC and PKC activators modulate $GABA_C$ receptors by inhibiting responses in rat retinal bipolar cells and recombinant receptors formed from human ρ subunits expressed in *Xenopus* oocytes and HEK293 cells (Feigenspan & Bormann, 1994; Zhang *et al.*, 2001).

A recent study has reported the modulation of recombinant $GABA_C$ receptors formed from ρ1 subunits expressed in *Xenopus* oocytes by neuro-active steroids such as alphaxalone and allopregnanolone. At high concentrations, these compounds potentiated GABA-evoked currents and prolonged the decay time (Kendall *et al.*, 1999). Delaney & Sah (1999) discovered a novel $GABA_C$ receptor in rat amygdala that was insensitive to bicuculline, barbiturates, general anaesthetics and $GABA_B$ receptor antagonists, but was inhibited by benzodiazepines. Thus, contrary to original perceptions, $GABA_C$ receptors may be allosterically modulated by selective compounds that also modulate $GABA_A$ receptors.

1.2.3.12 Functional importance of $GABA_C$ receptors

The presence of $GABA_C$ receptors in the retina is well established, and their role in this structure has been surmised. Owing to their localisation on bipolar and horizontal retinal cells, $GABA_C$ receptors are thought to mediate lateral inhibition of light responses (Bormann & Feigenspan, 1995; Feigenspan & Bormann, 1998). Lukasiewicz and Werblin (1994) suggest that presynaptic $GABA_C$ receptors at bipolar cell terminals modulate release of excitatory neurotransmitters. The location of $GABA_C$ receptors on the axon terminals of rod bipolar cells means they receive feedback from GABAergic amacrine cells and may modulate transmitter release from the bipolar cells in an autoreceptor fashion (Zhang *et al.*, 2001).

The physiological role of functional $GABA_C$ receptors outside the retina, in the CNS, remains to be established (Johnston, 1996b). However, their distribution through areas of the brain involved in vision (see section 1.4) suggests an important role for $GABA_C$ receptors in the visual system (Boue-Grabot *et al.*, 1998). A role for $GABA_C$ receptors in the superior colliculus has been postulated. $GABA_C$ receptors show limited localisation to interneurons of the rat superior colliculus, activation of which increases activity of projection neurons through disinhibition (Pasternack *et al.*, 1999; Boller & Schmidt, 2001). $GABA_C$ receptors may therefore modulate feedforward inhibition to superior colliculus projection neurons (Pasternack *et al.*, 1999; Boller & Schmidt, 2001). Moreover, the selective elimination of the ρ1 subunit in ρ1-knockout mice lead to altered visual processing in these animals (McCall *et al.*, 2002).

$GABA_C$ receptors appear to play a role in cortical development. Picrotoxin-sensitive, bicuculline-insensitive $GABA_C$-like receptors regulate migration of postmitotic cells from the telencephalic ventricular zone to the intermediate zone of the embryonic rat cortex during cortical development (Behar *et al.*, 2000).

The presence of $GABA_C$-like receptors in the hippocampus of postnatal rats further suggests a role for these receptors in hippocampal development and activity. Strata & Cherubini (1994) surmise that the novel, transient $GABA_C$ receptor response in rat hippocampal neurons for the first two postnatal weeks plays a role in neurite growth and strengthening of synaptic connections in the critical period of postnatal

development. Martina *et al.* (1995) speculate that the novel bicuculline- and baclofen-resistant response to GABA found in acutely dissociated CA3 hippocampal neurons of postnatal rats potentiates the depolarising activity of GABA during a critical period of development, playing an essential role in synaptogenesis.

1.3 Review of $GABA_A$ Receptor Distribution in Rat Brain

As mentioned previously, $GABA_A$ receptors are ubiquitous throughout the mammalian CNS, however the various subunits are present in differing concentrations in different regions (Fritschy & Möhler, 1995). The rat brain has proved to be a useful model of mammalian brain, thus $GABA_A$ receptor subunits are most commonly mapped in this species. Mapping of $GABA_A$ receptor subunit distributions in the rat brain has been accomplished using a variety of techniques, namely *in situ* hybridisation, immunohistochemistry and immunoprecipitation.

1.3.1 *In Situ* Hybridisation Studies

A number of *in situ* hybridisation experiments exploring the distribution and relative density of $GABA_A$ receptor subunit mRNAs in rat brain have been carried out (Laurie *et al.*, 1992; Lolait *et al.*, 1989a; Persohn *et al.* 1992; Pritchett *et al.*, 1989; Wisden *et al.*, 1992), the results of which are summarised in table 1.1 (page 32). The subunit mRNAs indicated in the table are reported as showing a moderate to strong positive signal.

1.3.2 Immunoprecipitation and Immunohistochemical Studies

Immunoprecipitation and immunohistochemistry experiments using antibodies directed against specific $GABA_A$ receptor subunit proteins have been performed (Benke *et al.*, 1991a; Benke *et al.*, 1991b; Bonnert *et al.*, 1999; Fritschy & Möhler, 1995; Pirker *et al.*, 2000; Schwartzer *et al.*, 2001; Wainwright *et al.*, 2000). Owing to the fact that different research groups may use different antisera for the receptor subunits and different strains of rats are used, some studies observe subunit immunoreactivity in brain areas where others may not. In general, however, most studies report on a similar regional distribution of subunits. Table 1.2 (page 33) summarises the majority of subunits for which immunoreactivity and immunolabelling was observed in various brain regions.

There exists a good correlation between immunohistochemistry and *in situ* hybridisation distributions of the $GABA_A$ receptor subunits, with some exceptions, which may be due to the differing localisation of mRNA, in cell soma, and protein expression, in cell membranes and often at synapses (De Blas, 1996). Differing results between the two techniques may also arise from the fact that mRNAs are not necessarily translated into proteins, and due to the differing sensitivities of the techniques or antibodies used.

1.4. Review of $GABA_C$ Receptor Distribution in Rat Brain

The molecular composition of the $GABA_C$ receptors in areas of the CNS outside the retina is not entirely clear (Zhang *et al.*, 2001). Whether $GABA_C$ receptors in other areas of the CNS resemble the retinal receptors requires further study (Zhang *et al.*, 2001). Mapping techniques can be used in order to ascertain the localisation of subunits comprising the $GABA_C$ receptors in order to determine the putative subunit composition. $GABA_C$ receptor subunits have been detected in a number of CNS regions using RT-PCR, Northern and Southern blots, *in situ* hybridisation and immunohistochemistry. In most studies, the greatest density of subunits has been found in the retina. The ρ2 subunits have been found to predominate in the CNS (Enz & Cutting, 1998; Enz & Cutting, 1999a; Wegelius *et al.*, 1998). Some studies have also failed to detect ρ1 subunits outside of the retina. The fact that in some studies subunits could only be detected by RT-PCR suggests that these subunits are expressed at very low levels in the CNS (Enz *et al.*, 1995; Zhang *et al.*, 2001).

1.4.1 Localisation of mRNA

Owing to the sensitivity of the techniques and the fact that specific subunit mRNAs may be investigated, localisation of ρ subunit mRNAs have been ascertained using Northern blotting, RT-PCR and *in situ* hybridisation histochemistry (Albrecht *et al.*, 1997; Boue-Grabot *et al.*, 1998; Boue-Grabot *et al.*, 2000; Enz & Cutting, 1999a; Enz *et al.*, 1995; Enz & Cutting, 1998; Ogurusu *et al.*, 1995; Ogurusu & Shingai, 1996; Wegelius *et al.*, 1998). The results from these studies are summarised in table 1.3 (page 34).

Table 1.1: Distribution of $GABA_A$ receptor subunit mRNAs in rat brain determined by *in situ* hybridisation histochemistry

Brain region	Subunits present
Olfactory bulb	α1, α2, α3, α4, α5, β1, β2, β3, γ2, δ
Tenia tecta	α1, α2, α4, α5, β1, β3
Cerebral cortex	α1, α2, α3, α4, α5, β1, β2, β3, γ1, γ2, γ3, δ
Piriform cortex	α1, α2, α3, β2, β3, γ2
Septum	α1, α2, α3, β2, β3, γ1, γ2
Diagonal band	α1, α2, α3, β2, β3, γ2, γ3
Nucleus accumbens	α2, α4, β3
Globus pallidus	α1, β2, γ1, γ2
Ventral pallidum	α1, β2, γ1, γ2
Islands of Calleja	α1, β2, γ1
Entopeduncular nucleus	α1, β2
Striatum	α2, α4, β3, γ2, δ
Bed nucleus of stria terminalis	α1, α2, α3, α4, β1, β2, β3, γ1, γ2
Claustrum	α1, α2, β1, β2, β3, γ3
Subthalamic nucleus	α1, β2, γ2
Hippocampal formation	
CA1	α1, α2, α4, α5, β1, β3, γ1, γ2
CA2	α1, a2, α5, β1, β2, β3, γ2
CA3	α1, α2, α4, α5, β1, β2, β3, γ2
Dentate gyrus	α1, α2, α4, α5, β1, β2, β3, γ2, δ
Subiculum	α1, α5, β3, γ2, δ
Amygdaloid complex	
Basolateral nuclei	α1, α2, β3, γ2
Central nucleus	α2, γ1
Medial nucleus	α2, β2, β3, γ1
Thalamus	α1, α3, α4, β1, β2, β3, γ2, δ (LGN)
Hypothalamus	α2, β3, γ2
Medial habenular nucleus	α2, β3, γ2
Zona incerta	α1, β2, γ2
Superior colliculus	α1, α3, α5, β1, β2, β3, γ2
Inferior colliculus	α1, β2, γ2
Red nucleus	α1, β2, γ2
Subtantia nigra	α1, α3, β2, γ1, γ2
Inferior olivary nucleus	α1, α2, α4, β3, γ1
Cerebellar cortex	
Granule cell layer	α1, α4, α6, β1, β2, β3, γ2, δ
Purkinje cell layer	α1, β2, β3, γ1 (Bergmann glia), γ2
Molecular layer	α1, γ2
Deep cerebellar nuclei	α1, β2, γ2
Brainstem nuclei	
Nucleus interpositus	α1, β2, γ2
Vestibular nucleus	α1, α2, β2, β3, γ2
Facial nucleus	α1, α2, β2, β3
Motor trigeminal nucleus	α2, β1, β2, γ2
Pontine nuclei	α1, γ2, β3
Spinal cord	α1, α2, α3, β2, β3, γ2

Table 1.2: Distribution of $GABA_A$ receptor subunits in rat brain determined by immunohistochemistry and immunoprecipitation methods.

Brain region	Subunits present
Olfactory bulb	α1, α2, α3, α5, β1, β2, β3, γ2, δ
Cerebral cortex	α1, α2, α3, α4, α5, β1, β2, β3, γ2, γ3, δ
Piriform cortex	α1, α2, α3, β2/3, γ2
Insular cortex	α1, α2, α3, β2/3, γ2
Septum	α1, α2, α3, α4, β1, β3, γ1, γ2
Nucleus accumbens	α1, α2, α3, α4, α5, β1, β2, β3, γ2, γ3, δ
Globus pallidus	α1, α3, α5, β2, β3, γ1, γ2
Islands of Calleja	α2, α3, β2/3, γ2
Entopeduncular nucleus	α1, α3, α5, β1, β2, β3, γ1, γ2
Striatum	α2, α3, α4, α5, β1, β2, β3, γ2, δ, θ
Bed nucleus of stria terminalis	α1, α2, α3, β2/3, γ1, γ2
Claustrum	α1, α3, β2/3, γ2
Subthalamic nucleus	α1, α2, α5, β2, β3, γ2, γ3, δ
Endopiriform nucleus	α1, α3, α5, β2/3, γ2
Hippocampal formation	
CA1	α1, α2, α3, α4, α5, β2, β3, γ2, θ
CA2	α5, β1, β2, γ2
CA3	α1, α2, α3, α5, β2, β3, γ2, θ
Dentate gyrus	α1, α2, α4, α5, β2/3, γ2, δ
Subiculum	α1, α5, β1, β2, β3, γ2, δ
Amygdaloid complex	
Basolateral nuclei	α1, α2, α3, α5, β1, β2, β3, γ2
Central nuclei	α1, α2, α3, β1, β2, β3, γ1, γ2
Medial nuclei	α1, α2, α3, β1, β2, β3, γ1, γ2
Thalamus	α1, α2, α3, α4, β2, γ2, γ3 (MGN), δ
Hypothalamus	α1, α2, α3, α5, β1, β2, γ2, θ
Medial habenular nucleus	γ2
Superior colliculus	α2, α3, α4, α5, β2/3, γ2
Inferior colliculus	α1, α3, α5, β2/3, γ2
Red nucleus	α1, α5, β2/3, γ2
Substantia nigra	α1, α3, α4, α5, β1, β2, β3, γ1, γ2, γ3, δ
Ventral tegmental area	α1, α3, α5, β1, β2, β3, γ2, γ3, δ
Inferior olivary nucleus	α2, α3, α5, γ2
Cerebellar cortex	
Granule cell layer	α1, α6, β2, β3, δ
Purkinje cell layer	α1, γ2
Molecular layer	α1, β1, β2/3, γ2
Deep cerebellar nuclei	α1, β2/3, γ2
Brainstem	α1, α4, γ2, γ3
Vestibular nucleus	α1, α3, β2/3, γ2
Facial nucleus	α1, α2, γ2
Motor trigeminal nucleus	α2, γ2
Pontine nuclei	α2, α3, α5, β2/3, γ2

Table 1.3: Distribution of $GABA_C$ receptor subunit mRNAs in rat brain using Northern blotting, RT-PCR and *in situ* hybridisation.

Brain region	Subunits present
Olfactory bulb	ρ2
Cerebral cortex	ρ2, ρ3
Basal ganglia	ρ2, ρ3
Hippocampus	ρ1, ρ2 (also specifically in CA1 region), ρ3
Pituitary gland	ρ1, ρ2
Thalamus	ρ1, ρ2 (also specifically in the LGN), ρ3
Midbrain	ρ2, ρ3
Superior colliculus	ρ1, ρ2
Substantia nigra	ρ2
Cerebellum	ρ1, ρ2 (also specifically in the Purkinje cell layer), ρ3
Brainstem	ρ1
Spinal cord	ρ1 (also dorsal root ganglia), ρ2, ρ3 (dorsal root ganglia)
Retina	ρ1, ρ2, ρ3

1.4.2 Immunohistochemical Studies

A number of studies to date have investigated the distribution of $GABA_C$ receptor subunits in the mouse and rat retina using immunohistochemistry, with strongest immunoreactivity occurring in the inner plexiform layer (Enz *et al.*, 1996; Koulen *et al.*, 1998; Wässle *et al.*, 1998). Few studies, however, have examined the distribution of $GABA_C$ receptors outside of the retina, in the CNS, using immunohistochemistry. One study has confined $GABA_C$ receptor immunoreactivity to the somata and dendrites of the cerebellar Purkinje cells (Boue-Grabot *et al.*, 1998). Other studies localised $GABA_C$ receptor immunoreactivity in the superficial grey layer of the superior colliculus of postnatal rats (Clark *et al.*, 2001; Pasternack *et al.*, 1999). Since the antiserum used in these studies was raised against amino acids possessed by both ρ1 and ρ2 subunits, the $GABA_C$ receptors immunolabelled in the cerebellar Purkinje cells and superficial grey of the superior colliculus may be composed of either subunits

1.5. Review of $GABA_A$ Receptor Distribution in Human Brain

While the rat brain provides an acceptable model of the mammalian CNS, in order to fully understand the nature of $GABA_A$ receptors relevant to human function, investigation of the receptor subunits in human brain is necessary. Such knowledge is especially useful for comprehending the therapeutic effects of compounds acting at these receptors. A limited number of studies have been performed to assess the distribution and density of $GABA_A$ receptor subunits in human brain. Human tissue is generally difficult to obtain and the variance between subjects is quite large, making

analyses of many brain regions in many subjects difficult. Nevertheless, immunohistochemistry to evaluate protein localisation and quantitative receptor autoradiography techniques to gauge binding sites have been utilised to ascertain the $GABA_A$ receptor subunits in various brain regions, the results of which have been summarised in table 1.4 (page 36) (Houser *et al.*, 1988); Loup *et al.*, 1998; Vogt *et al.*, 1990; Wainwright *et al.*, 2000; Waldvogel *et al.*, 1999). mRNA distribution of the $GABA_A$ receptor subunits has also been assessed using *in situ* hybridisation and RT-PCR in human brain, predominantly in disease states, however these studies also provide information on subunit distribution in normal brain, the results of which are also summarised in table 1.4 (Akbarian *et al.*, 1995b; Dodd & Lewohl, 1998; Hadigham *et al.*, 1996; Hawkins & Dodd, 2000; Lewohl *et al.*, 1997; Mitsuyama *et al.*, 1998; Mizukami *et al.*, 1998; Montpied *et al.*, 1989; Ohnumna *et al.*, 1999). It is important to note that in this instance, the lack of reported subunits in a given brain region does not preclude their presence in this region, it merely indicates a lack of information.

1.6. Review of $GABA_C$ Receptor Distribution in Human Brain

Only two published studies have examined the distribution of ρ subunits in the human brain, both of which measured mRNA levels by total RNA extraction from brain regions. RT-PCR and Northern blotting localised $GABA_C$ receptor ρ1 and ρ2 subunits to the following areas of the human CNS; the frontal and temporal cortices, striatum, thalamus, midbrain and cerebellum (Cutting *et al.*, 1991; Enz & Cutting, 1999a). ρ1 Subunit mRNA expression was much lower in most brain regions than ρ2 subunit mRNA, and ρ2 subunit mRNA alone was also detected in the parietal and occipital cortices (Enz & Cutting, 1999a). Furthermore, both subunits were detected in the retina (Cutting *et al.*, 1991; Enz & Cutting, 1999a). No studies have been published to date examining the protein distribution of $GABA_C$ receptors in human brain using immunohistochemistry or quantitative receptor autoradiography.

Table 1.4: Distribution of $GABA_A$ receptor subunits in human brain assessed by *in situ* hybridisation, immunohistochemistry and quantitative receptor autoradiography.

Brain region	Subunits/radioligand	Reference
Cerebral cortex	α1, α2, a3	Loup *et al.* (1998); Montpied *et al.* (1989)
Frontal cortex	α1, α4, β3	Mitsuyama *et al.* (1998); Ohnumna *et al.* (1999)
Superior frontal cortex	α1, α2, α3	Dodd & Lewohl, (1998); Lewohl *et al.* (1997)
Middle frontal gyrus	α1, α2, α5, β1, β2, γ2	Akbarian *et al.* (1995b)
Motor cortex	α1, α2, α3	Lewohl *et al.* (1998)
Basal Ganglia		
Striatum	α1, α2, α3, β2/3, γ2	Waldvogel *et al.* (1999)
Globus pallidus	α1, α3, β2/3, γ2	Waldvogel *et al.* (1999)
Posterior cingulate cortex	[^{3}H]Muscimol binding	Vogt *et al.* (1990)
Hippocampal formation		
CA1	α1, α2, α3, α5, γ2, [^{3}H]Ro 15-1788 binding, β2, β3	Houser *et al.* (1988); Loup *et al.* (1998); Wainwright *et al.* (2000); Mizukami *et al.* (1998)
CA2	α1, α2, α5, γ2, [^{3}H]Ro 15-1788 binding, β2, β3	Houser *et al.* (1988); Loup *et al.* (1998); Wainwright *et al.* (2000); Mizukami *et al.* (1998)
CA3	α1, α2, α5, γ2, [^{3}H]Ro 15-1788 binding, β2, β3	Houser *et al.* (1988); Loup *et al.* (1998); Wainwright *et al.* (2000); Mizukami *et al.* (1998)
Dentate gyrus	α1, α2, α5, γ2, [^{3}H]Ro 15-1788 binding	Houser *et al.* (1988); Loup *et al.* (1998); Wainwright *et al.* (2000)
Subiculum	α1, [^{3}H]Ro 15-1788 binding	Houser *et al.* (1988)
Entorhinal cortex	α1, α3, β2, γ2	Houser *et al.* (1988); Longson *et al.* (1997)
Cerebellum	α1, α6	Hadigham *et al.* (1996); Montpied *et al.* (1989)
Brainstem	α1, α2, α3, β1	Hawkins & Dodd (2000)

[^{3}H]Muscimol binds to all $GABA_A$ receptors and [^{3}H]Ro 15-1788 binds to high affinity benzodiazepine binding sites on the $GABA_A$ receptors.

1.7 RECEPTOR MAPPING

The motives for anatomical and biochemical mapping of receptors in the central nervous system are numerous. Localisation of receptors in neuronal networks provides a view of the biochemical organisation of the brain (Kuhar, 1987), and allows us to interpret the physiological roles of receptors on the basis of their regional distribution. Mapping of receptors in conjunction with pharmacological studies further facilitates our comprehension of the mechanism by which drugs exert their physiological effects (Kuhar, 1987). The most important aspect of receptor mapping is the fact that it provides information on receptors *in situ*, and not in isolated systems.

1.7.1 Mapping Techniques

A number of techniques have been developed which provide information on receptor localisation in the CNS, each relying on the formation of a stable complex which can be

detected (Watson & Cullinan, 2000). Choice of which technique to use is dependent on the information required and the level of quantification one wishes to achieve.

Immunohistochemistry, developed by Coons and co-workers (1941), exploits the relationship between antibodies and antigens to label receptor proteins and assess their localisation and densities in specific regions of the brain. This technique can be applied to *in vitro* tissue sections mounted on slides. *In vitro* radioligand binding utilises ligands radiolabelled with ^{3}H or ^{125}I, to visualise receptor binding sites autoradiographically. When applied to tissue sections mounted on slides, this technique is commonly referred to as quantitative receptor autoradiography (Young and Kuhar, 1979). Information can be obtained not only on receptor binding site localisation, but on binding characteristics of the ligand, such as kinetics and pharmacological specificity (Hudson, 1993). Finally, the more recently developed technique of *in situ* hybridisation histochemistry (Gall & Pardue, 1969) can be applied to examine the distribution and density of messenger RNA of specific receptor subunits.

Depending on the technique used, mapping experiments can be extended to investigate new pharmacological agents, localisation of novel receptor subtypes, as well as genetic variants of receptor subtypes and their expression in the CNS. Furthermore, while it is important to investigate the normal neurochemical organisation of the brain, receptor mapping is also useful for studying changes in neuropathological states, especially neuropsychiatric illnesses (Kuhar, 1987; Royston, 1995).

The opportunity to study receptors at the levels of both mRNA and protein allows the determination of a number of properties of the receptors in question as well as substantiation of findings for each methodology. mRNA Localisation provides genetic information on the receptor, relative density, and on cells of origin. Immunohistochemistry permits visualisation of the location and relative density of the receptor protein.

1.8 Aims

A review of the literature makes it evident that localisation in the CNS and subunit composition of $GABA_C$ receptors in the CNS is questionable. What we know of

$GABA_C$ receptors to date has been elucidated predominantly from retinal preparations or recombinant receptors composed of subunits cloned from retina.

The primary aims were, therefore, to map the distribution of $GABA_C$ receptor subunit mRNA and protein in rat brain using *in situ* hybridisation and immunohistochemistry. A further aim was to determine the presence of these subunits in human brain. Two $GABA_A$ receptor subunits were also explored using these techniques, since their presence in the CNS is well documented. Mapping techniques enable researchers to speculate on the putative subunit composition of ionotropic GABA receptors through their colocalisation, as well as to infer a role for the receptors according to regions of the CNS to which they are mapped.

MAPPING IONOTROPIC GABA RECEPTORS IN RAT AND HUMAN BRAIN
CHAPTER 2: IN SITU HYBRIDIZATION HISTOCHEMISTRY

2.1 INTRODUCTION AND AIMS

2.1.1 Introduction to the Methods

In situ hybridisation histochemistry is a combined anatomical and molecular biological technique used to study the spatial and temporal patterns of gene expression, specifically messenger RNA (mRNA) (Harrison & Pearson, 1995; Henderson, 1996; Mladanic *et al.*, 2000; Stahl & Baskin, 1993). *In situ* hybridisation allows anatomical localisation of mRNA in cell bodies (Harrison & Pearson, 1995; Watson & Cullinan, 2000), and is particularly useful for the examination of the distribution and density of particular mRNAs in small anatomic regions or small sections of heterogeneous and anatomically complicated tissue such as the brain (Henderson, 1996; LeMoine *et al.*, 1994; Uhl, 1988). Furthermore, *in situ* hybridisation is suitable for examination of changes in gene expression, distribution and density, in pathological states (Henderson, 1996; Higgins & Mah, 1989).

Both non-radioactive and radioactive markers can be used for *in situ* hybridisation (Stahl *et al.*, 1993). For the current thesis, only radioactive *in situ* hybridisation was used. Radioactive *in situ* hybridisation is more sensitive than non-radioactive methods, is readily quantifiable, and provides the most consistent results (Heath *et al.*, 1996b; Henderson, 1996; Penschow & Coghlan, 1994; Stahl *et al.*, 1993; Wilcox, 1993).

2.1.1.1 Principles of hybridisation

Binding occurs between mRNA in the cytoplasm and strands of labelled DNA or RNA by complementary base pairing to form a stable hybrid (Watson & Cullinan, 2000). When the DNA or RNA strand is labelled, the location and density of mRNA can be assessed (Heath *et al.*, 1996b; Henderson, 1996). The hybrid forms through hydrogen bonding of complementary nucleic acids inherent in the DNA and RNA structure (Henderson, 1996).

2.1.1.2 Choice of probes

Successful hybridisation is highly dependent on good probes (Uhl, 1988). A number of probe types are available for *in situ* hybridisation, however oligodeoxyribonucleotide (ODN) probes, first implemented by Lewis *et al.* (1986) for hybridisation, were selected for experiments in the current thesis. ODN probes are short, single stranded DNA probes, and were chosen for a number of reasons. They are manufactured commercially and are easy to obtain, can be labelled with a number of markers, and do not require extensive molecular biological expertise for their use (Harrison & Pearson, 1995; Higgins & Mah, 1989; Lewis *et al.*, 1986; Penschow & Coghlan, 1994; Stahl *et al.*, 1993). Furthermore, ODN probes may be designed to select for particular isoforms or splice variants of the isoforms of mRNA in question (Heath *et al.*, 1996b; Lewis *et al.*, 1986; Penschow & Coghlan, 1994). ODN probes are less vulnerable to degradation by ribonucleases (RNases) and can penetrate tissue more readily due to their small size compared to RNA probes. However, ODN probes are not as sensitive as RNA probes due to their comparatively short length, form less stable hybrids, and require longer exposure time (Henderson, 1996; Lewis *et al.*, 1986; Watson & Cullinan, 2000; Wilcox, 1993).

2.1.1.3 Design of oligodeoxyribonucleotide probes

ODN probes are designed from the coding strand of a published cDNA sequence (Henderson, 1996), which is identical to the mRNA sequence except thymine is replaced by uracil in the mRNA. Two probes are designed, a "sense" probe and an "antisense" probe. The sense probe corresponds to the mRNA sequence, and is therefore used as a background control as it should not hybridise to mRNA in the tissue. The antisense probe is the complementary sequence of the mRNA, and should therefore hybridise to mRNA in the tissue if it is present. In preparation, the mRNA sequence is copied as a single stranded cDNA sequence (the oligonucleotide) using reverse transcription (Stahl *et al.*, 1993). Figure 2.1 below illustrates the process of probe design from a cDNA sequence obtained from the literature:

cDNA	5′	GGC CGA AAA TAC	3′
mRNA	5′	GGC CGA AAA UAC	3′
		‖‖ ‖‖ ‖‖ ‖‖	
'Antisense probe	3′	CCG GCT TTT ATC	5′
Sense probe	5′	GGC CGA AAA TAC	3′

Figure 2.1: Demonstration of oligodeoxyribonucleotide probe design for *in situ* hybridisation using a cDNA sequence obtained from the literature. The cDNA corresponds to the mRNA. The sense probe is identical to this sequence. The antisense probe is the complement of this sequence. Adapted from Stahl *et al.* (1993).

A "good" ODN probe obeys a number of rules which optimise its capacity to hybridise *in situ*. These probes are between 30 and 50 nucleotide bases long (Heath *et al.*, 1996b; Henderson, 1996) which is a good size for maximal specificity but small enough to readily penetrate the tissue (Stahl *et al.*, 1993). The greater the length of the probe, the better the signal to noise ratio (Heath *et al.*, 1996b). The GC content of probes should be between 45-60% to ensure stability during hybridisation (Stahl *et al.*, 1993). This is because guanine forms three hydrogen bonds with cytosine, compared with two hydrogen bonds between adenine and thymine. This GC content is also suitable for incubation and washing at temperatures below 60°C. Temperatures above 60°C can destroy the tissue integrity (Penschow & Coghlan, 1994). ODN probes which target specific regions of known mRNAs also maximise the selectivity of the probe (Heath *et al.*, 1996b; Uhl, 1988). When designing probes for isoforms of gene families, sequences with the lowest possible homology between the isoforms must be chosen to remove the possibility of hybridisation to related genes of high nucleotide sequence homology (Stahl *et al.*, 1993). Generally, the 5' terminal region, 3' terminal region, and 5' and 3' untranslated regions are the least conserved between isoforms (Heath *et al.*, 1996b; Mengod & Vilaró, 1993). Since efficiency of hybridisation may differ between mRNA species, probe length, GC content and specific activity must be similar to facilitate reliable comparison between different mRNAs (Higgins & Mah, 1989).

Additional factors must be considered in probe design to yield the best possible probe for maximal hybridisation. Sequences should not contain more than three Gs in a row due to formation of probe tetramers (Stahl *et al.*, 1993). Computer programs such as Net Primer online (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html) are now available to assess the quality of a chosen probe. These programs can

evaluate the ability of the probe to form hair pin loops, dimers or cross-dimers and palindromes, all of which create self-hybridisation, thus reducing the ability of the probe to hybridise to mRNA *in situ*, hence reducing hybridisation signal (Uhl, 1988).

2.1.1.4 Choice of isotope

For the use of oligodeoxyribonucleic acid probes, isotopic markers are essential (Henderson, 1996), presumably because the sensitivity of the marker offsets the lower sensitivity of the probe. β emitting isotopes are used for radioactive *in situ* hybridisation. High activity isotopic labelling is required as the number of molecules to be labelled is generally small (Gall & Pardue, 1969). ^{35}S is the isotope of common choice as it permits quite accurate localisation of radioactive signal after a relatively short exposure time (Harrison & Pearson, 1995; Penschow & Coghlan, 1994; Wilcox, 1993). The drawbacks of ^{35}S are its tendency to produce high background due to its reactivity and strong affinity for proteins (Penschow & Coghlan, 1994), and its relatively short half life which limits the amount of time in which to use it.

2.1.1.5 Tissue preparation

Tissue preparation for *in situ* hybridisation constitutes a major component of the experimental procedure, therefore tissue handling and conscientious preparation are of ultimate importance to obtain highest possible anatomical and histological quality (Herkenham, 1988; Royston, 1995; Wilcox, 1993).

Tissue is prepared fresh frozen as this increases sensitivity, facilitates probe penetration, and prevents loss of mRNA content (Henderson, 1996; Penschow & Coghlan, 1994). Tissue is rapidly isolated and frozen in liquid nitrogen or isopentane to preserve morphology and prevent freezing artefacts which occur through formation of ice crystals from water in the tissue (Grzanna, 1982; Higgins & Mah, 1989). Tissue must not be thawed prior to sectioning, and must not dessicate with long term storage at low temperatures (Penschow & Coghlan, 1994). -20°C is an adequate storage temperature over long periods (years) without loss of mRNA, however the tissue can deteriorate (Penschow & Coghlan, 1994). Tissue may be stored for several years at -70°C without loss of mRNA (Harrison & Pearson, 1995).

Tissue is sectioned at 5-20μm in a cryostat and thaw mounted onto slides coated with an adherent (Henderson, 1996; Higgins & Mah, 1989). 3-Aminopropyltriethoxysilane (silane) is generally used for this purpose. A 2% solution of silane in acetone coats the glass slide with aminoalkyl groups which allow covalent bonds to form with aldehyde and ketone groups in the frozen tissue (Rentrop *et al.*, 1986). While silane coating slides is the preferred method of the author, alternative such as poly-L-lysine or chrome alum and gelatine-coated slides can also be used (Heath *et al.*, 1996b; Henderson, 1996; Wilcox, 1993).

Sections should be fixed immediately after cutting, or stored no longer than 10 days prior to fixation, to prevent liberation of RNases which will degrade RNA (Higgins & Mah, 1989) and to avoid deterioration of tissue integrity. Once sectioned and fixed, tissue can be stored for 2 years, before it begins to lose signal (Harrison & Pearson, 1995). Some researchers have proven that storage longer than 2 years has no discernible effect on mRNA, since fixed sections are robust (Heath *et al.*, 1996a; Wilcox, 1993), and experience suggests that sections stored up to 3 years demonstrate no appreciable loss of mRNA signal. Sections should be stored in airtight conditions below -15°C (Herkenham, 1988).

2.1.1.6 Pretreatment

Pretreatment of tissue is carried out with the aims of reducing background signal, preserving tissue morphology, retaining RNA and increasing probe penetration into the tissue (Heath *et al.*, 1996b; Henderson, 1996; Penschow & Coghlan, 1994). All tissue for experimentation must be processed in the same manner to reduce the possibility of variations in hybridisation signal (Harrison & Pearson, 1995).

Fixation allows the preservation of morphology and mRNA throughout subsequent experimentation (Gall & Pardue, 1969). Fixatives do not fix the mRNA molecules, however the mRNA molecules become immobilised through fixation of surrounding proteins (Watson & Cullinan, 2000). This prevents degradation of mRNA by RNases or diffusion away from the tissue section (Watson & Cullinan, 2000). Tissue is generally fixed with fresh 4% paraformaldehyde (Heath *et al.*, 1996b; Henderson, 1996; Wilcox, 1993). Fresh paraformaldehyde is used to reduce background and prevent re-

polymerisation (Heath *et al.*, 1996b; Henderson, 1996; Wilcox, 1993). Fixative should not be of basic pH either, as this may cause mRNA hydrolysis (Michael & Priestley, 1996).

Basic proteins must be removed from the tissue as they may interfere with hybridisation and cause non-specific binding (Gall & Pardue, 1969). Acetic anhydride in triethanolamine hydrochloride is used to acetylate basic amino groups in the tissue and on the slides, decreasing electrostatic binding of the probe to these basic groups (Heath *et al.*, 1996b; Higgins & Mah, 1989; Watson & Cullinan, 2000). Ethanol and chloroform disrupt or remove the lipid component of the tissue to enhance penetration of the probe (Henderson, 1996) and decrease non-specific binding of the probe to tissue lipids (Heath *et al.*, 1996b). Chloroform also functions to inactivate RNases (Henderson, 1996).

2.1.1.7 Controls

Rigorous use of controls permits the determination of specific hybrids (Uhl, 1988) and reduces the chance of detecting false positive results (Lewis *et al.*, 1986). Controls must be interpreted cautiously, therefore use of more than one type of control in experiments is favoured (Chesselet *et al.*, 1996; Stahl & Baskin, 1993).

2.1.1.7.1 Sense probe control

As mentioned previously, hybridisation with the sense probe should not yield a specific signal as it is identical to part of the mRNA sequence, not complementary to it (Chesselet *et al.*, 1996; Watson & Cullinan, 2000). Sense probes contain the same GC content as antisense probes. When the sense probe is added to hybridisation buffer and incubated with adjacent sections to the antisense probe, resulting signal can be determined as artefact, hybridisation between GC-rich portions of a probe and unrelated sequences (Chesselet *et al.*, 1996; Henderson, 1996), therefore non-specific hybridisation (Harrison & Pearson, 1995; Penschow & Coghlan, 1994). For quantitation, the background sense signal is subtracted from the antisense signal (Harrison & Pearson, 1995; Heath *et al.*, 1996b). This is especially useful when comparing pathological states to control groups (Harrison & Pearson, 1995).

2.1.1.7.2 Nonsense probes

Non-specific probes which are not complementary to any known transcript are another negative control which tests for artefact (Harrison & Pearson, 1995; Heath *et al.*, 1996b; Penschow & Coghlan, 1994).

2.1.1.7.3 Excess unlabelled probe

Addition of excess (around 50 times) unlabelled antisense probe to tissue during hybridisation is useful for determining specificity of signal (Harrison & Pearson, 1995; Heath *et al.*, 1996b; Henderson, 1996; Penschow & Coghlan, 1994; Stahl *et al.*, 1993). The concentration of mRNA of interest is small relative to the amount of mRNA in tissue, therefore the number of potential non-specific sites is large (Heath *et al.*, 1996b). Excess unlabelled probe should compete with the labelled probe for specific hybridisation sites, hence reduce the resultant signal (Chesselet *et al.*, 1996; Heath *et al.*, 1996b; Lewis *et al.*, 1986; Stahl *et al.*, 1993). The specific signal should not diminish if excess unlabelled probe for another mRNA is added to the hybridisation mixture (Mengod & Vilaró, 1993; Penschow & Coghlan, 1994).

2.1.1.7.4 Positive control

A positive control probe with known cellular distribution may be employed in experiments to control for positive hybridisation and for efficiency of the experimental procedure (Wilcox, 1993).

2.1.1.7.5 Multiple antisense probes

An additional control is the use of multiple antisense probes for the same mRNA, which ensures specificity as the results from each probe should produce the same distribution and strength of signal (Heath *et al.*, 1996b; Stahl & Baskin, 1993; Wilcox, 1993).

2.1.1.8 In situ *hybridisation*

In situ hybridisation involves, first, the labelling of an ODN probe at the 3' end with ^{35}S molecules. This labelling is facilitated by the enzyme terminal deoxytransferase (TDT) (Roychoudry & Wu, 1980; Stahl *et al.*, 1993). TDT catalyses the repetitive addition of mononucleotides from a dNTP, such as [^{35}S]-dATP, to the terminal 3'-OH of a DNA initiator, such as an ODN probe (Henderson, 1996; Promega product information). 1mM Co^{2+} (as $CoCl_2$) in the buffer allows the tailing of any type of 3'- end with varying efficiencies (Promega product information). Duration of incubation period with

TDT, concentration of enzyme and amounts of dNTPs relative to probe contribute to the resulting length of tail (Higgins & Mah, 1989; Penschow & Coghlan, 1994).

After labelling, probes are purified through Sephadex columns to remove the unincorporated label (Blum & Roberts, 1989; Rentrop *et al.*, 1986). Each ODN probe is then prepared in a hybridisation buffer with dithiothreitol and applied in excess to the tissue sections (Watson & Cullinan, 2000). Dithiothreitol is a strong reducing agent, preventing formation of disulphide bridges between ^{35}S molecules and cysteine residues on tissue proteins to aid elimination of non-specific background observed with the use of ^{35}S-labelled probes (Heath *et al.*, 1996b; Higgins & Mah, 1989). Between 3% and 7% DTT is used in the hybridisation solution. Overnight incubation (16-18 hours) allows hybridisation between the labelled probe and target mRNA in the tissue (Heath *et al.*, 1996b). Wilcox (1993) maintains that hybridisation is complete after 4 hours of incubation, however Heath *et al.* (1996b) have found that less than 12 hours incubation produces remarkably less signal than 16 hours incubation.

The *in situ* hybridisation buffer includes a number of components which are critical for the hybridisation process. Formamide disrupts hydrogen bonds to destabilise hybrids formed between G-C and A-U (Stahl *et al.*, 1993), hence decreases the temperature required for hybridisation (Heath *et al.*, 1996b; Henderson, 1996). Dextran sulfate in the buffer raises the surface tension to stop large sections from drying out (Gentleman & Roberts, 1995), and decreases the amount of probe required to be effective (Henderson, 1996). Heparin is an anionic competitive inhibitor of RNase (Gozes, 1988). Additional nucleotides in the form of herring sperm DNA and Polyadenylic acid (Poly(A)) competitively inhibit probe binding to non-specific nucleotides in the tissue (Heath *et al.*, 1996b; Henderson, 1996). Denhardt's solution effectively reduces non-specific binding to macromolecules in the sections (Denhardt, 1966; Heath *et al.*, 1996b).

Hybridisation is carried out at 15°C below melt temperature (T_m) (Davis *et al.*, 1986; Heath *et al.*, 1996b). T_m is the temperature at which 50% of hybrids dissociate to single strands (Harrison & Pearson, 1995; Heath *et al.*, 1996b; Henderson, 1996). 15°C below T_m is thought to favour formation of specific hybrids while precluding formation of non-specific hybrids. Many researchers hybridise at a standard 37°C owing to the fact

that the T_m is relevant to filter hybridisation (Henderson, 1996), however it has also been applied to *in situ* hybridisation. The equation for determination of T_m (Davis *et al.*, 1986) is given in section 2.3.7. This equation calculates the stability of the hybrid, accounting for Na^+ concentration in the buffer, GC content, length of the probe and formamide concentration in the buffer.

Hybridisation is contingent predominantly upon three parameters: a) concentration of Na^+, b) temperature, and c) concentration of formamide (Henderson, 1996). Increasing temperature decreases hybridisation (Harrison & Pearson, 1995) presumably by melting apart mismatched hybrids. Increased salt (Na^+) concentration stabilises the hybrids by neutralising electrostatic repulsive forces between negatively charged phosphate groups on opposite strands and by reducing base solubility and strengthening hydrophobic interactions (Henderson, 1996; Stahl *et al.*, 1993). Formation of specific hybrids is maximised under conditions of high stringency, when: a) concentration of Na^+ is decreased, b) temperature is increased, and c) concentration of formamide is increased, according to the equation for T_m (Henderson, 1996). These parameters may be altered empirically to determine the conditions for optimal hybridisation.

2.1.1.9 Washing

After hybridisation, sections are washed to remove excess unhybridised or weakly hybridised probe, thus leaving specifically bound probe (Henderson, 1996; Watson & Cullinan, 2000), and to permit dispersion of material remaining in the section (Uhl, 1988).

Washes are carried out under both high and low stringency conditions in saline sodium citrate (SSC) buffer. High stringency conditions incorporate washes at T_w, 15°C below T_m without formamide (Heath *et al.*, 1996b) (for equation see section 2.3.8) at low salt concentrations (Henderson, 1996; Stahl *et al.*, 1993). Increasing wash temperature increases the stringency of the wash as it facilitates melting of non-specific hybrids, thereby eliminating them (Stahl *et al.*, 1993). Low stringency conditions require washes at room temperature or in higher salt concentrations in the buffer (Henderson, 1996).

2.1.1.10 Potential sources of background

In situ hybridisation with ^{35}S-labelled ODN probes presents a number of possible sources of background, therefore it is of great importance to optimise techniques to maximise the signal to noise ratio.

Background may be produced by probes which are poorly prepared, impure or partially degraded, too concentrated, or have dried out during hybridisation (Penschow & Coghlan, 1994). Further caution is required during probe preparation, as the cobalt ions in the TDT buffer can precipitate (Penschow & Coghlan, 1994), resulting in background.

Repeated freezing and re-thawing of the isotope before use contributes high background (Wilcox, 1993). It is therefore recommended that ^{35}S be used immediately after thawing, and remaining label is discarded rather than frozen for re-use (Wilcox, 1993). However, owing to the expense of *in situ* hybridisation experiments, especially the [^{35}S]-dATP, it is often not economical to do this.

As mentioned previously, non-specific binding between the probe and non-specific tissue components may contribute to background. Availability of unpaired bases leads to potential interaction with proteins and lipids (Lewis *et al.*, 1986). Furthermore, ^{35}S has an affinity for proteins, which is exacerbated by its radioactive decay products (Penschow & Coghlan, 1994). Availability of unpaired bases also leads to potential formation of non-complementary base pairs, or mismatched sequences (Lewis *et al.*, 1986), under conditions of low stringency (Henderson, 1996). Probes are capable of cross hybridisation, analogous to cross reactivity with antibodies (Watson & Cullinan, 2000). Use of high stringency conditions and incorporation of pretreatment steps and buffer components should largely remove the occurrence of non-specific binding and hybridisation.

Other sources of background include inadequately frozen or fixed sections so that mRNA disseminates through the tissue (Penschow & Coghlan, 1994), excess formamide in the tray during incubation, drying out of sections during incubation or wash steps (Heath *et al.*, 1996b), washing in inadequate volumes of SSC (Penschow & Coghlan, 1994), and high temperatures for hybridisation and washing (Heath *et al.*, 1996b).

Structures in the brain containing high levels of rRNA may display background signal which is commonly perceived as specific (Higgins & Mah, 1989). Such areas include the dentate gyrus and pyramidal layers of the hippocampus, granule cell layer of the cerebellum, primary olfactory and piriform cortices and medial habenular nucleus (Higgins & Mah, 1989). Thus, hybridisation signals in these regions must be interpreted cautiously and confirmed using other methods (Higgins & Mah, 1989).

On occasion, the sense probe produces a strong signal, which may be explained by a number of factors. The strong signal may occur through hybridisation to rRNA (Harrison & Pearson, 1995), which is abundant in all tissues. The sense probe may be hybridising specifically to some unidentified mRNA (Heath *et al.*, 1996b). Alternatively, sense probes may recognise endogenous "antisense" mRNA in the tissue, created by transcription of part of both DNA strands (symmetrical gene transcription) (Chesselet *et al.*, 1996; Higgins & Mah, 1989). The sense probe will hybridise to the complementary antisense mRNA, producing a misleading non-specific signal (Chesselet *et al.*, 1996; Higgins & Mah, 1989). This phenomenon of symmetrical gene transcription is potentially more common than was previously thought (Higgins & Mah, 1989). Where the sense probe produces an uncharacteristic strong signal, the experiment is usually repeated until a negative signal with the sense probe is obtained (Wilcox, 1993), or new probes must be designed and purchased (Harrison & Pearson, 1995).

2.1.2 Autoradiography

Autoradiography involves the localisation and recording of a radiolabel within a solid specimen (Old & Primrose, 1994). Spatial information must be preserved for accurate determination of location and extent of radiolabelled binding (Hudson, 1993; Rogers, 1975).

2.1.2.1 Macroscopic autoradiography

Macroscopic autoradiography is suitable for studying structures greater than 0.5mm diameter (Rogers, 1975). It is therefore useful for mapping receptors and variations in mRNA at the regional level in heterogeneous tissues such as brain (Heath *et al.*, 1996b;

Hudson, 1993; LeMoine *et al.*, 1994). This form of autoradiography employs autoradiographic film, nuclear emulsion coated on plastic, which permits ease of exposure and quantification of optical density (Uhl, 1988). Once the film is developed, it is digitised for quantification through image analysis (Royston, 1995). Macroscopic autoradiography is a favourable step before microscopic autoradiography to establish the success of the experiment, and to determine exposure time for microscopic autoradiography (Harrison & Pearson, 1995).

2.1.2.2 Microscopic autoradiography

Microscopic autoradiography is useful for whenever radioactivity must be resolved to sources of cellular dimensions (Harrison & Pearson, 1995; Rogers, 1975). Because of its capacity for greater resolution (Hudson, 1993), microscopic autoradiography is advantageous in situations where macroscopic autoradiography is not sensitive enough to detect low abundance mRNAs (LeMoine *et al.*, 1994). Microscopic autoradiography is also employed to determine whether signal above background in the autoradiogram is specific to cell bodies (Mladanic *et al.*, 2000). Microscopic autoradiography provides information on mRNA abundance in different neuronal populations, deviation in number of cells expressing mRNA, and differences in levels of mRNA for individual cells (Higgins & Mah, 1989; LeMoine *et al.*, 1994).

Microscopic autoradiography utilises liquid photographic emulsion into which tissue sections are dipped to coat them. Radioactivity exposes silver grains in the emulsion over the site of hybridisation (Heath *et al.*, 1996b). Following exposure and development, the pattern of radioactivity can be analysed at the cellular level within distinct structures of the tissue, discernible by light microscopy (Heath *et al.*, 1996b; Royston, 1995).

2.1.2.3 Analysis of macroscopic autoradiography

Results are usually expressed as the concentration of radioactivity in radioactivity bound/tissue section, or radioactive concentration (Ci)/mg tissue (Palacios *et al.*, 1988). For *in situ* hybridisation the optical density reflects distribution and density of mRNA in the tissue. Progressive darkening of the emulsion, or increase in optical density, is

directly proportional to the amount of radioactivity in the section over a limited range (Baskin & Stahl, 1993; Rogers, 1975).

Optical density is not linearly related to radioactive content of the tissue (Baskin & Stahl, 1993; Kuhar, 1987; Kuhar & Unnerstall, 1985). Radioactivity increases more rapidly than optical density in the film, therefore co-exposure of radioactive standards of known activity alongside treated sections is crucial to adequate quantitation of autoradiographic images (Kuhar & Unnerstall, 1985; Uhl, 1988).

Calibration is achieved using radioactive standards exposed with the tissue. For isotopic *in situ* hybridisation utilising ^{35}S-labelled probes, ^{14}C plastic standards are suitable, owing to the long half life and similar emission energy (Baskin & Stahl, 1993; Miller, 1991). Optical density values obtained from the film for each standard are measured and fit to the known concentration of polymer radioactivity (nCi/g) (Palacios *et al.*, 1988). A non-linear regression fitting the curve to a first order polynomial (straight line) or second order polynomial (non-linear) curve is carried out to determine the relationship between optical density and concentration of radioactivity (Palacios *et al.*, 1988). Optical density measurements of the section are then standardised to the radioactive standards to indicate the approximate radioactivity (nCi/g tissue equivalent) of the section (Rogers, 1975).

Examination of optical density in different regions of the brain is accomplished by outlining parts of the section on the digitised image to obtain the average optical density within the defined region (Royston, 1995). Multiple outlining and a consistent set of outlining criteria are necessary to reduce measurement error (Royston, 1995). Labelled regions can be correlated to anatomical structures in a brain atlas by staining the same or adjacent sections with a cell marker such as cresyl violet (Hudson, 1993; Palacios *et al.*, 1988).

2.1.2.4 Counterstaining

Sections are counterstained to provide identification of structures for quantification (Hudson, 1993). Cresyl violet is often used as a histological counterstain (Heath *et al.*, 1996b; Hudson, 1993) as it stains phosphate groups of nucleic acids, hence reveals cell

nuclei (Paulsen, 1993) and therefore cell bodies. Histological counterstaining of emulsion dipped sections, however, should be light so that microscopic investigation of grains is not impeded (Hudson, 1993). It is of utmost importance for the purpose of quantification that degree of shrinkage of the specimen on fixation and dehydration is carefully controlled (Rogers, 1975). Reagents used must also be free of contaminants to prevent chemical interactions and anomalous results (Rogers, 1975).

2.1.2.5 Interpreting the results

Optical density signal obtained from film or grain count density obtained from emulsion reflects the density and distribution of radiolabelled probe (Royston, 1995), which effectively allows the determination of density and localisation of mRNA in the tissue. As the signal or grain count increases, one can infer that there is a greater density of the molecules of interest (Heath *et al.*, 1996b).

Intrasection, intersection and interexperiment variability exists (Heath *et al.*, 1996b). Intersection variability may be surmounted by measuring from two or more sections. Interexperiment variability can be overcome by performing single complete experiments with common probes and exposure time, such that groups within a single experiment can be compared (Heath *et al.*, 1996b). Replication of experiments also decreases variability.

The technique of *in situ* hybridisation is semi-quantitative for a number of reasons, especially since the results give the degree of relative change in mRNA, and do not allow inference or measurement of the actual number of mRNA molecules per region per cell (Baskin & Stahl, 1993; Harrison & Pearson, 1995).

Measuring mRNA allows one to infer the capacity of the cell to synthesise protein (Watson & Cullinan, 2000). It is accepted that mRNA is generally present if both transcription and translation are occurring, therefore one can assume that if mRNA is present, the encoded protein is also being synthesised (Harrison & Pearson, 1995). Changes in the rate of mRNA synthesis, however, are not necessarily coupled to changes in the rate of protein synthesis, and alterations may occur in mRNA without subsequent changes in protein synthesis (Harrison & Pearson, 1995). These factors

emphasise the need to investigate transmitter receptors at both the mRNA and the protein levels *in situ* (Harrison & Pearson, 1995; Heath *et al.*, 1996b).

2.1.3 Advantages and Limitations of ISHH

2.1.3.1 Advantages

In situ hybridisation allows measurement of mRNA *in situ*, without having to be extracted from homogenised tissue, as in RT-PCR and Northern blotting (Harrison & Pearson, 1995). Greater anatomical sensitivity is therefore obtained, for example, mRNA levels in specific layers of brain regions (Harrison & Pearson, 1995).

In situ hybridisation is also a highly specific and sensitive technique which provides high resolution (Uhl, 1988). Specificity is obtained from the high degree of control over probe design (Watson & Cullinan, 2000). Signal can be obtained even from very low abundance mRNAs (Watson & Cullinan, 2000). Furthermore, information for gene expression can be visualised at the cellular level (Stahl & Baskin, 1993; Uhl, 1988) and cell bodies of origin for molecules of interest can be identified (Harrison & Pearson, 1995; Watson & Cullinan, 2000).

2.1.3.2 Limitations

One of the limitations of *in situ* hybridisation is that the biochemical mechanisms underlying changes in mRNA levels cannot be elucidated, as one cannot determine whether changes in mRNA expression are due to changed transcription rate or changed mRNA stability (Uhl, 1988).

Another limitation of *in situ* hybridisation is the chance of detecting unrelated gene products that are not of interest. Mladanic *et al.* (2000) found that ODN probes thought to be specific for the mRNA in question in fact detected unrelated gene products whose sequence homology was only 55% of the target mRNA, even under conditions of high stringency. This result emphasises the necessity to use probes directed against different regions of the same target mRNA. Mladanic and colleagues did, however, suggest that the same problem may not occur for other researchers using probes from different regions of the mRNA of interest.

2.1.4 Post-mortem Tissue and Factors Affecting Tissue Preparation

One of the major problems involved with the use of post-mortem human brain tissue is the brain-to-brain variability (Harrison, 1996), especially in mRNA yield and integrity (Higgins & Mah, 1989). This variability may be amplified by pre-, peri- and post-mortem factors, as well as experimental procedures. For this reason, optimisation of techniques, for example, with rat brain, is required before use of post-mortem human tissue (Higgins & Mah, 1989).

mRNA may be susceptible to degradation during the post-mortem interval, or the interval between time of death and time of tissue freezing and fixation (Barton *et al.*, 1993; Higgins & Mah, 1989). It is now known that post-mortem interval exerts a relatively minor effect on mRNA detection in human brain (Barton *et al.*, 1993; Heath *et al.*, 1996a). Studies have shown that mRNA in human brain appears to remain intact up to 48 hours post-mortem (Harrison, 1996; Harrison & Pearson, 1995; Heath *et al.*, 1996a; Higgins & Mah, 1989). This may be due to inactivation of ribonucleases (RNases) which usually degrade the RNA (Barton *et al.*, 1993; Harrison & Pearson, 1995). It is important, however, to recognise that mRNA degradation is dependent on the factor being assessed (Barton *et al.*, 1993; Harrison & Pearson, 1995). Different mRNAs may degrade at different rates. Individual differences in post-mortem delay may be a factor contributing to variability (Higgins & Mah, 1989).

Agonal state, the state of the person before death, has the greatest impact on tissue quality post-mortem. mRNA is susceptible to hypoxia, acidosis, pyrexia, dehydration, seizure and other factors which may occur before death depending on the cause of death (Barton *et al.*, 1993; Harrison & Pearson, 1995; Heath *et al.*, 1996a). Individual differences in agonal state may be a factor contributing towards variability (Barton *et al.*, 1993; Higgins & Mah, 1989). Furthermore, different agonal states affect different mRNAs (Barton *et al.*, 1993). Measurement of brain pH is known to reasonably represent mRNA quality, such that decreasing pH indicates worsening agonal state, resulting in decreased mRNA preservation (Harrison, 1996; Heath *et al.*, 1996a; Kingsbury *et al.*, 1995).

mRNA may be susceptible to degradation during fixation or freezing techniques (Higgins & Mah, 1989). Dissection, storage and histological procedures may also contribute to liberation of RNases which degrade RNA (for more information see Higgins & Mah, 1989). Rigorous control of histological procedures may alleviate these problems (Rogers, 1975).

As a result of the inherent variability in brain tissue, coupled with peri-mortem factors and experimental procedures, standardised protocols for brain collection and processing are essential to meaningful, quantifiable results.

2.1.5 Choice of Subunits

$GABA_C$ receptors are the most recently described GABA receptors, and little is understood of their distribution and physiological role in the brain (Chebib & Johnston, 2000). All $GABA_C$ receptor ρ subunits (ρ1, ρ2 and ρ3) were therefore chosen for investigation in the present thesis.

Well-characterised subunits from the $GABA_A$ receptor family were chosen for these studies in order to compare with the relative densities and distributions of the ρ subunits. In this way, the $GABA_A$ receptor subunits were used as positive controls. Two $GABA_A$ receptor subunits were chosen, the α1 subunit and the β3 subunit.

The most commonly occurring $GABA_A$ receptor subunit composition is the α1β2γ2 combination, which is sensitive to benzodiazepines (Sieghart, 2000) and thought to account for around 43% of $GABA_A$ receptors in the CNS (McKernan & Whiting, 1996). Immunoprecipitation studies with an α1 subunit antibody in purified receptor preparations from rat brain have shown that approximately 90% of $GABA_A$ receptors contain the α1 subunit (Benke *et al.*, 1991a). Moreover, immunohistochemical studies investigating the distribution of 13 $GABA_A$ receptor subunits in rat brain showed that the α1 subunit was the most widely distributed, present in almost all brain regions (Pirker *et al.*, 2000). Therefore the α1 subunit was chosen for these studies not only for its pharmacological importance, but also for its ubiquitous distribution in the CNS which would enable easy detection.

Around 18% of $GABA_A$ receptors *in vivo* are formed from $\alpha2\beta2/3\gamma2$ subunits, and around 17% of the $GABA_A$ receptors are comprised of $\alpha3\beta n\gamma2/3$ subunits, as *determined by immunoprecipitation studies (McKernan & Whiting, 1996).* $GABA_A$ receptors containing β3 subunits are therefore well characterised. The β3 subunit was additionally chosen on the basis of its molecular similarity to ρ subunits and their similar patterns of known distribution. $GABA_C$ receptor ρ subunits are more like β subunits than any other $GABA_A$ receptor isoform, branching from a common ancestral arm in the evolutionary tree of the nicotinicoid LGIC superfamily (Ortells & Lunt, 1995; Tyndale *et al.*, 1995).

2.1.6 Aims

$GABA_C$-like responses have been reported in various regions of the mammalian brain including the cat spinal cord (Johnston *et al.*, 1975), superior colliculus (Arakawa & Okada, 1988; Pasternack *et al.*, 1999), postnatal rat hippocampus (Martina *et al.*, 1995), and rat amygdala (Delaney & Sah, 1999). The nature of the receptors in these areas, however, is not known. The fact that three subunits exist suggests a small degree of heterogeneity for the $GABA_C$ receptors, but whether they exist as pseudo-heterooligomers or merely homooligomers in the CNS is not known. Assessing the mRNA distribution of all three subunits in adjacent sections may point to potential colocalisation of subunits to form receptors, and to determine which subunits may be responsible for the reported $GABA_C$-like responses.

$GABA_C$ receptors have been localised using a variety of techniques in areas of rat brain (refer to section 1.4). The first aim of this chapter was to determine the optimal experimental conditions for *in situ* hybridisation using rat brain, and to confirm the presence of $GABA_C$ receptor subunit mRNA in the rat brain. The α1 and β3 subunit probes were used as positive controls for this purpose. The current work diverges from previously published work in that the probes for the ρ subunits are directed against mRNA corresponding to the N terminal region of the resultant protein, while previous studies have utilised probes directed against mRNA corresponding to the large cytoplasmic loop. Where problems were encountered with sense probes in the present study, different probes were designed against the cytoplasmic loop.

Many studies convincingly demonstrate the presence of ρ subunits in rat retina (Enz *et al.*, 1995; Koulen *et al.*, 1998; Wegelius *et al.*, 1998). Furthermore, $GABA_C$-like responses in the retina have been correlated to the presence of ρ1 subunits (McCall *et al.*, 2002; Yeh *et al.*, 1996). With this in mind, rat retina was employed in the current thesis with the purpose of ensuring the $GABA_C$ receptor subunit probes could be used to identify the subunits in question, as well as to confirm previous findings.

The distribution of $GABA_C$ receptor subunit mRNA in human brain has been investigated by total RNA extraction and blotting, however no study has examined the subunit distribution *in situ*. Another aim of the present chapter was therefore to apply the optimised *in situ* hybridisation techniques to ascertain the distribution of $GABA_C$ receptor subunit mRNA *in situ* in human brain. In all studies, the distribution and density of $GABA_C$ receptor subunit mRNA was compared to the α1 and β3 $GABA_A$ receptor subunits, which are well characterised and known to exhibit prolific expression in the CNS. This work thus further extends our knowledge of the distribution and relative density of $GABA_A$ receptor subunit mRNA in the human brain as well.

Tissue from the middle frontal cortex, hippocampus, central nucleus of the amygdala, pole of the temporal cortex, anterior superior temporal gyrus, primary visual cortex and cerebellum was used. These regions were chosen on the basis of their availability, and for the reason that ρ subunit mRNA had been localised in some of the regions by RT-PCR and Northern blots (Enz & Cutting, 1999a; Cutting *et al.*, 1991). The central nucleus of the amygdala was also chosen for study on the basis of $GABA_C$-like responses reported in the rat amygdala. It is important to determine the distribution of subunits in anatomical regions of the brain in order to infer a role for these receptors based on the function of the brain region it is mapped to.

2.2. MATERIALS

2.2.1 Subjects

2.2.1.1 Rat tissue

Male Sprague-Dawley rats aged 6 weeks and weighing 200 to 250gm were purchased from Australian Laboratory Services (Perth, Australia). Animals were housed in groups

of two to five in plastic cages for a minimum of 1 week. Animals were housed under a 12 hour/12 hour light/dark cycle at constant temperature (20-21°C) and humidity (45-65%) with access to standard rat chow and water *ad libitum*. Light intensity in the housing area was held at 60 lux, and 15 room air changes occurred daily for ventilation. Animal ethics approval was granted for experimentation (ACEC number K21/1-99/3/2803).

2.2.1.2 Human tissue

Human tissue was obtained from the New South Wales Tissue Resource Centre located in the Department of Pathology at the University of Sydney. The Tissue Resource Centre carried out all processing of the tissue from collection to sectioning. Information on tissue collection and processing was obtained from the New South Wales Tissue Resource Centre Guidelines for Researchers. Tissue was collected from the New South Wales Institute of Forensic Medicine, Glebe. The Police Report of Death was reviewed for potential cases that met initial inclusion criteria. Potentially infectious tissue was excluded from the collection. If the case met inclusion criteria for further studies (see Appendix 1), the Consultant Neuropathologist, Professor Clive Harper, was contacted for review and approval of the case (Central Sydney Area Health Service ethics approval, protocol X98-0216). The brain was weighed and placed in a labelled container for transport to the Tissue Resource Centre. Each brain was entered onto the Tissue Resource Centre Network Database and assigned a pathology identification number. All subsequent referral to the brain was made using the identification number. The brain volume was estimated and the brain was dissected hemispherically. One hemisphere was processed for fixation and the other for fresh frozen material (refer to section 2.3.2.2). Fixed tissue first underwent neuropathological examination to remove the possibility of any other neuropathological state which may have posed a hazard to laboratory personnel or confounded results obtained from tissue delineated for investigation of a single disease state.

Inclusion criteria for collection of both schizophrenia and control cases are listed in Appendix 1. These criteria are reproduced verbatim from the New South Wales Tissue Resource Centre Guidelines for Researchers. In addition to the inclusion/exclusion criteria, psychiatric cases were rigorously and systematically characterised. Psychiatric

diagnosis was confirmed retrospectively and a detailed review of all available medical records undertaken by a clinician. Information was additionally obtained for family, drug and alcohol, and treatment history. Postmortem serology and toxicology information was obtained from the post-mortem report. Diagnostic criteria; the Feighner criteria, Research Diagnostic Criteria, and the Item Group Checklist of the Schedule for Clinical Assessment in Neuropsychiatry; were applied for DSM-IIIR and ICD10 diagnoses. Fulfillment of two out of the three criteria was required for diagnosis of schizophrenia.

Area Health hospitals and information supplied to the coroner were researched extensively to ascertain whether control cases indicated any history of known psychiatric illness. Control cases were excluded if a history of psychiatric illness was apparent.

Tissue was allocated on the basis of availability after discussion with the Tissue Resource Centre Coordinator. Formal requests were made (see Appendix 2 for request form and disclaimer) and reviewed by Professor Clive Harper, Professor of Neuropathology, on the basis of ethics approval (approval number 99/01/15) for the project for which the tissue was to be used.

For mapping experiments, control tissue was predominantly used that did not have a history of neuropsychiatric illness. Tissue rendered unsuitable for matched cases was also obtained for two brain areas (amygdala and polar temporal cortex). One schizophrenic and one alcoholic subject were also used in these studies. While subunit expression may be altered in these pathological states, for the purposes of the current study, to determine whether the subunits are present or not, the pathological state was not expected to confound results. Table 2.1 (page 60) provides subject information for regions of the brain used in experimentation. A sample size of two at minimum was used for mapping experiments. Tissue from the middle frontal cortex, hippocampus, central nucleus of the amygdala, pole of the temporal cortex, anterior superior temporal gyrus, primary visual cortex and cerebellum was used.

Table 2.1: Subject information for human brain regions used in mapping experiments

Subject identification	Tissue type	Region	Cause of Death
Unsuitable	unsuitable	CeA, TCx	Information not available
13994	control	CeA, TCx, Cer	Ischaemic heart disease
13031	alcoholic	VCx	Information not available
12861	control	VCx, Hpp	Atherosclerotic cardiovascular disease
12876	schizophrenia	Hpp	Clozapine toxicity
13283	control	Hpp, Cer	Coronary arteries disease
12886	control	STG, MFC	Carbon monoxide poisoning
12858	control	STG, MFC, VCx	Cardiac arrest
12862	control	STG, MFC, VCx	Ischaemic heart disease
12889	control	STG, MFC, VCx	Ischaemic heart disease
12870	control	STG, MFC, VCx	Acute myocardial infarction
13082	control	STG, MFC	Suicide by hanging

Key: CeA = central nucleus of the amygdala, TCx = polar temporal cortex, Cer = cerebellum, VCx = primary visual cortex, Hpp = hippocampus, STG = superior temporal gyrus, MFC = middle frontal cortex

2.2.2 Oligodeoxyribonucleotide Probes

Oligodeoxyribonucleotide (ODN) probes were designed (see section 2.3.3) and purchased online from Gibco BRL Life Technologies (http://www.lifetech.com), desalted at 50nmol. Table 2.2 (section 2.3.3) provides the sequence information for each ODN probe.

2.2.3 Radioisotopes

Deoxyadenosine 5'-[α-^{35}S]thiophosphate, triethylammonium salt ([^{35}S]-dATP) for *in situ* hybridisation was purchased from Amersham Pharmacia Biotech (Amersham International/Pharmacia Biotech, Amersham, U.K). All [^{35}S]-dATP had a specific activity of 1000Ci/mmol.

2.2.4 Reagents and Chemicals

Polyadenylic acid (Poly(A)) and 50x Denhardt's solution were purchased from Sigma-Aldrich (St Louis, MO, USA), mesh ion exchange resin AG501-X8 (molecular biology grade) was purchased from Bio-Rad Laboratories (Hercules, CA, USA) and herring sperm DNA was purchased from Promega Corporation (Madison, WI, USA).' Terminal deoxytransferase (TDT) with 5x terminal deoxytransferase labelling buffer were purchased from Promega Corporation (Madison, WI, USA)

3-Aminopropyltriethoxysilane (silane), diethylpyrocarbonate (DEPC), dithiothreitol (DTT), triethanolamine HCl (TEA-HCl), formamide, acetic anhydride, 2-methylbutane (isopentane), dextran sulfate, sodium citrate, ethylenediaminetetraacetic acid (EDTA), sodium hydroxide pellets, heparin, sodium phosphate monobasic (anhydrous), sodium phosphate dibasic (anhydrous) and paraformaldehyde powder were purchased from Sigma-Aldrich (St Louis, MO, USA). Cresyl violet acetate was obtained from BDH Laboratory Supplies (Poole, England).

Polyvinylpyrrolidone, ficoll, tris-HCl, sodium chloride and phosphate buffered saline (PBS) tablets were purchased from ICN Biochemicals (Costa Mesa, CA, USA). All products were molecular biology grade.

Acetone, chloroform and absolute ethanol were purchased from APS Finechem (Seven Hills, NSW, Australia). Xylene and histolene were obtained from Fronine (Riverstone, NSW, Australia). All solvents were analytical reagent (AR) grade.

2.2.5 Autoradiography and Photography

Hypercassettes, Hyperfilm βmax, Hypercoat dipping vessels, autoradiographic ^{14}C microscales and Emulsion LM-1 were purchased from Amersham Pharmacia Biotech (Amersham International/Pharmacia Biotech, Amersham, U.K).

Phenisol developer, Hypam Rapid Fixer LCS and In-1 Indicator Stop Bath were purchased from Ilford Imaging Australia Pty. Ltd. (St Leonards, NSW, Australia).

2.3. METHODS

2.3.1 Slide Preparation

Starfrost microscope slides (Livingstone International Pty Ltd, Rosebery, NSW, Australia) were loaded into Lipshaw metal staining racks (Shandon Lipshaw, Inc., Pittsburgh, PA, USA) and rinsed in Decon-90 then distilled water to clean them prior to coating. Slides were coated by immersion in a 2% solution of 3-aminopropyltriethoxysilane (silane) in acetone (see Appendix 3 for solution). Slides

were immersed for 5 minutes in acetone, then for 30 minutes in 2% silane solution, and rinsed briefly in milliQ water. All solutions were contained in Lipshaw glass staining jars covered with aluminium foil. Slides were dried under a stream of air overnight then baked for 8 hours at 160°C or 4-6 hours at 200°C to kill RNases.

2.3.2 Processing of Tissue

2.3.2.1 Rat tissue

Animals were culled using CO_2 gas in a chamber and decapitated using a guillotine. The brains were dissected rapidly on ice and plunged into 2-methylbutane on dry ice to freeze immediately.

Retina were obtained by removal of the intact eye cup and immersed in 4% paraformaldehyde in 0.1M PBS, as suggested by Penschow & Coghlan (1994) for fixation of small specimens.

2.3.2.2 Human tissue

Human tissue was obtained from autopsy and frozen as tissue blocks no more than 48 hours post-mortem, as described in section 2.2.1.2. Processing of human tissue was carried out by the New South Wales Tissue Resource Centre, as outlined in the Tissue Resource Centre Guidelines for Researchers. For fresh frozen tissue, the hemisphere was placed on its medial surface and 1-1.5cm coronal slices were made. Specific structures including caudate putamen, amygdala, basal forebrain, primary visual cortex, cerebellum and brainstem were dissected and placed caudal surface down. Brain slices and dissected blocks were frozen rapidly at -80°C, then stored in labelled bags at -80°C.

2.3.2.3 Tissue sectioning

Rat brains were cut transversely with a scalpel blade and mounted rostral-caudal from the forebrain to midbrain, or caudal-rostral from the spinal cord or cerebellum to the midbrain, onto microtome stages, surrounded by OCT embedding compound (Tissuetek, Miles Inc., USA). Rat retina were either dissected and mounted onto microtome stages, surrounded by OCT embedding compound, or the entire eyecup was embedded in OCT lens down in plastic moulds. Embedded rat retina was mounted sideways on the

cryostat stage for sectioning on the sagittal axis. Unused portions of tissue were stored, surrounded by OCT embedding compound, at -20°C.

Frozen tissue blocks from requested regions of human brain were mounted caudal-rostral onto microtome stages, and surrounded by OCT embedding compound.

Coronal rat brain sections were cut in a cryostat (Cryostat-E, Reichert-Jung, Vienna, Austria) maintained at -20°C, and thaw mounted onto silane coated slides. Rat retina sections were cut in the sagittal plane in the same manner as rat brain. All human tissue was cut coronally in a cryostat (Shandon Cryotome E, Shandon Lipshaw, Inc., Pittsburgh, PA, USA) maintained at -18°C, by the New South Wales Tissue Resource Centre Coordinator, and thaw mounted onto silane coated slides.

Brain tissue was sectioned at 12μm and 1-2 sections (for human brain) or 2-3 sections (for rat brain) were arranged on a single slide. Retinal tissue was sectioned at 20μm with 3-4 sections per slide. Sections were stored in slide boxes for a maximum of 12 days at -20°C, however most tissue was pretreated for *in situ* hybridisation within 3 days of sectioning.

2.3.3 Design and Ordering of Oligodeoxyribonucleotide Probes

Nucleotide sequences from cloned cDNA for the target receptor subunit were obtained from the published literature. Entire sequences were entered into a spreadsheet for comparison and areas of conservation were highlighted. Areas of high conservation were avoided to prevent cross hybridisation between differing subunits, since the focus of the present study was to ascertain the differential distribution of the differing subunits. In non-conserved regions, sequences of 42 nucleotide bases in length were identified with a GC content of between 48 and 57% (refer to Table 2.2). Using an internet-based cDNA library called BLAST (http://www.ncbi.nlm.nih.gov/BLAST), selected oligodeoxyribonucleotide (ODN) sequences were entered and cross-checked for significant homology with all other nucleotide sequences in the BLAST database. This step precludes hybridisation to mRNAs of similar sequence that are not of interest. The selected ODN sequences were ordered through Gibco BRL Life Technologies over the

internet (http://www.lifetech.com). Both sense and antisense ODN probes were ordered. Antisense strands were reversed to give 5' to 3' strands as requested by the company, in order to simplify the manufacturing process.

Probes were designed to target translated regions of mRNA coding for both rat and human ionotropic GABA receptor subtypes; ρ1 and ρ2 subunits of the $GABA_C$ receptor, and α1 and β3 subunits of the $GABA_A$ receptor. The ρ3 subunit for rat brain was also designed. cDNA Sequences were acquired for human ρ1 (Cutting *et al.*, 1991), ρ2 (Cutting *et al.*, 1992), α1 (Schofield *et al.*, 1989) and β3 (Wagstaff *et al.*, 1991) subunits. Further sequences were obtained for rat ρ1 (Zhang *et al.*, 1995), ρ2 (Ogurusu *et al.*, 1995), ρ3 (Ogurusu & Shingai, 1996), α1 (Seeburg *et al.*, 1990) and β3 (Ymer *et al.*, 1989b) subunits. All probes were designed from regions corresponding to the translated N terminus of the receptor, except for the β3 subunit and the human ρ2 subunit probes, which corresponded to the cytoplasmic loop between transmembrane domains 3 and 4 of the receptor subunit. The N terminal and cytoplasmic loop demonstrate the least conservation among the ionotropic GABA receptor genes (Mladanic *et al.*, 2000). In addition, a nonsense strand was designed which corresponded to no known DNA sequence.

Table 2.2: Sequences (antisense strand) of oligodeoxyribonucleotide probes designed for *in situ* hybridisation. All probes are 42 nucleotide bases in length with a GC content of between the recommended 45 and 60 %. Sequences are written 5' to 3'.

Probe	Sequence (5' to 3')	GC content
Rat ρ1	254 cagaattgggctgccttgcttgtgggcatcgtcatgtgcacc 213	57%
Rat ρ2	252 ccatctcttcctcctgggttttctgctctccacaagagccat 211	52%
Rat ρ3	306 ttgtttcggtgaagagaggcattgctggtggggttccttgac 265	52%
Rat α1	156 ttcatcttgggagggctgtccatagcttcttcccgagagagt 115	52%
Rat β3	1429 cctggaccatctgtctatggcattcacatcggttagatcagg 1388	50%
Nonsense	42 aacgtgagggtgcacatcgggtcgtccgtattcttgtaattg 1	50%
Human ρ1	172 ttctcgtctttgtctttggggcctgcctttcttagacatctc 131	48%
Human ρ2	1322 actcaggcccaggtggaccactattttgtcttgcctttcttc 1281	50%
Human α1	307 taatgacggctgtccatagcttcttccagtcagtgtgctcag 266	50%
Human β3	1412 cctggaccatctgtctatggcattcacatcggttagatcagg 1371	50%

Sense and antisense ODN probes were centrifuged briefly to collect the DNA at the bottom of the tube, then reconstituted with 1mL of autoclaved DEPC-treated milliQ water and allowed to stand for 2 minutes. Tubes were briefly vortexed and 30μL of each tube were removed to sterilised microfuge tubes. The 30μL were diluted to 2pmol/μL with the addition of autoclaved DEPC-treated milliQ water (Heath *et al.*,

1996b) and aliquoted in 40µL aliquots into sterilised microfuge tubes. ODN probes were stored indefinitely at -20°C.

2.3.4 Sterilisation of Glass and Plastic Ware

All procedures for *in situ* hybridisation histochemistry were carried out in RNase-free conditions. This required all glass and plastic ware to be rinsed with 0.1M diethylpyrocarbonate (DEPC) in milliQ water (see Appendix 3) and left for 24 hours at room temperature, then autoclaved to render them sterile (Heath *et al.*, 1996b). DEPC inhibits ribonucleases (RNases) by reacting with the imidazole nitrogens of histidine and free amino groups (Gozes, 1988). All solutions were similarly made up in 0.1M DEPC-treated milliQ water and autoclaved before use. All eppendorf tubes and pipette tips were autoclaved before use.

2.3.5 Pretreatment of Sections

No longer than 12 days after sectioning, slides were loaded into sterile polyacetal slide racks (HD Scientific, Blacktown, NSW, Australia) and pretreated according to the procedure of Heath *et al.* (1996b) and Le Corre *et al.* (2000). All solutions for pretreatment were made up in sterile glassware and poured into sterile polyacetal staining boxes (HD Scientific, Blacktown, NSW, Australia). Details for solution preparation are outlined in Appendix 3. Tissue was fixed for 5 minutes in 4% paraformaldehyde then rinsed briefly through 0.01M phosphate buffered saline solution twice. Tissue was placed in a solution of 0.1M triethanolamine (TEA) containing acetic anhydride (0.25% v/v) for 10 minutes. Tissue was then taken through a series of ethanol solutions; 70% for 1 minute, 80% for 1 minute, 95% for 2 minutes and 100% for 1 minute. Tissue was placed in chloroform (AR grade) for 10 minutes, then placed in 100% ethanol for 1 minute and finally 95% ethanol for 1 minute. Tissue was dried overnight under a stream of air and stored for up to 3 years in slide boxes at -70°C.

2.3.6 Labelling and Purification of ODN Probes

ODN probes were labelled in sterilised microfuge tubes at 37°C through the addition of [^{35}S]-dATP to the 3' end of the probe, catalysed by terminal deoxytransferase as first

described by Roychoudhury & Wu (1980). Briefly, 3μL (6pmol) of probe were added to 15μL of 5x terminal deoxytransferase labelling buffer (containing 215mM cacodylate buffer, 2.15mM $CoCl_2$, 0.215mM DTT in 35μL reaction volume), 9μL of autoclaved DEPC-treated milliQ water, 6μL (60pmol) of [^{35}S]-dATP, and 2μL (30u) of terminal deoxytransferase, to make a final reaction volume of 35μL. The procedure followed that described by Le Corre *et al.* (2000). Tubes were stored on ice throughout the procedure. Microfuge tubes containing the reaction volume were incubated in a water bath at 37°C for 30 minutes and the reaction was terminated by the addition of 15μL Reagent A (containing 0.01M triethanolamine, 0.1M tris-HCl, 10μM ethylenediaminetetraacetic, pH 7.7), making a final volume of 50μL for purification.

Labelled probes were purified using Microspin G-50 sephadex purification columns (Amersham International/Pharmacia Biotech, Amersham, UK). Microspin G-50 columns were vortexed briefly to resuspend the gel filtration resin and the bottom enclosure was snapped off. Microspin columns were centrifuged at 735x g for 1.5 to 2 minutes over sterile microfuge tubes to remove the liquid component of the filtration gel. 50μL of labelled ODN probe were added carefully to the centre of the compacted gel filtration resin bed and the Microspin columns were centrifuged over new sterile microfuge tubes at 735x g for 3 to 4 minutes. Optimal centrifugation times were determined as 2 minutes for the pre-spin and 3 minutes for the purification spin. The resulting purified ODN probes were collected in the bottom of the microfuge tubes. 55μL of autoclaved DEPC-treated milliQ water were added to each tube and the tubes were vortexed briefly then placed on ice. 1μL of purified product was added to 5mL Emulsifier Safe or Ultima Gold scintillant (Packard Bioscience, Mount Waverley, VIC, Australia) in Beckman 5mL Snap-Cap Bio-Vials (Beckman Coulter, Fullerton, CA, USA) and the level of radioactivity was counted in CPM (counts per minute) on a Beckman β scintillation counter (Beckman Coulter, Fullerton, CA, USA).

2.3.7 *In Situ* Hybridization

Incubation temperature (T_i) was calculated by subtracting 15°C from the melt temperature (T_m) (Davis *et al.*, 1986; Heath *et al.*, 1996b). T_m and is derived from the following equation:

$T_m = 16.6\log[Na^+] + 0.41(\%GC) + 81.5 - \%mismatches - 675/ODN\ length - 0.65(\%formamide)$

where $[Na^+]$ is the concentration of sodium in sodium citrate buffer used in the hybridisation buffer; %GC is the GC content of the ODN probe (expressed as an integer, not a decimal); %mismatches is the percentage of mismatches between the probe and the original sequence; ODN length is number of nucleotide bases in the ODN probe; and %formamide is the percentage of formamide used in the hybridisation buffer and in the incubation tray.

Hybridisation buffer containing 4x sodium citrate buffer (600mM NaCl, 60mM sodium citrate, pH 7.0), deionised formamide (50% v/v), dextran sulfate (10% w/v), 50x Denhardt's solution (1% v/v bovine serum albumin, ficoll and polyvinylpyrrolidone), 10mg/mL Herring sperm DNA, 10mg/mL Poly(A), 20mM sodium phosphate buffer (see Appendix 3) and 185units/mg heparin was heated in a water bath at T_i for 30 minutes prior to hybridisation. In this time, perspex Nunc bioassay dishes (Nalge Nunc International, Naperville, IL, USA) were lined with 2 sheets of 24cm Whatman's filter paper #1 (Lab Supply, Milperra, NSW, Australia) soaked in 50% formamide (see Appendix 3). Slides were removed from the -20°C freezer and allowed to thaw to room temperature for a minimum of 20 minutes, then placed on the filter paper at least 5mm apart.

The amounts of ODN probe, hybridisation buffer and dithiothreitol required were calculated for each probe using 1 x 10^6 CPM per section in a volume of 75µL per section for rat brain and 75-100µL per section for human brain (Heath *et al.*, 1996b; Le Corre *et al.*, 2000). Sections were hybridised with a total of 75µL or 100µL solution containing labelled probe, hybridisation buffer and 3% or 5% 1mM dithiothreitol, and coverslipped. Sections were incubated in the bioassay dishes humidified with 50% formamide for 16-20 hours at T_i. Where the T_i differed between probes, an average T_i was used. All hybridisation was performed under sterile conditions using autoclaved pipette tips and autoclaved microfuge tubes or sterile conical tubes. The procedure followed that of Le Corre *et al.* (2000).

2.3.8 Washing Hybridized Sections

Wash temperature (T_w) was calculated by subtracting 15°C from the following equation (Davis *et al.*, 1986; Heath *et al.*, 1996b):

$$T_w = 16.6\log[Na^+] + 0.41(\%GC) + 81.5 - \%mismatches - 675/ODN\ length$$

where $[Na^+]$ is the concentration of sodium in sodium citrate buffer for washing; %GC is the GC content of the ODN probe (expressed as an integer, not a decimal); %mismatches is the percentage of mismatches between the probe and the original sequence; and ODN length is number of nucleotide bases in the ODN probe.

Procedure followed that of Heath *et al.* (1996b) and Le Corre *et al.* (2000) with minor modifications. Coverslips were removed by dipping slides in a beaker of 1x SSC (20x sodium citrate buffer diluted in milliQ water) at room temperature. Sections were then washed under high stringency conditions in 1x SSC three times for 20 minutes at T_w, then twice under low stringency conditions in 1x SSC for one hour at room temperature. At no time during the wash steps were sections allowed to dry out. Slides were rinsed briefly in milliQ water then dried overnight under a stream of air or on the bench top.

2.3.9 Autoradiography

Slides were arranged in an autoradiography cassette and taped down to avoid movement. ^{14}C microscales were prepared according to the procedure recommended by the manufacturer. Briefly, microscales were placed for 15 minutes in milliQ water heated to 55°C, then mounted onto silane coated slides and allowed to dry. One high (30 to 862nCi/g) and one low (0.1 to 98.26nCi/g) activity ^{14}C microscale were placed in the autoradiography cassette with the sections. Hyperfilm β-max was exposed to sections in a darkroom under Kodak red safe light. The cassette was locked and covered in aluminium foil then stored for 3 to 4 weeks at room temperature. This period of exposure was determined empirically as that which produced optimal signal for the positive control probes (α1 and β3 antisense).

Film was developed for 4-5 minutes in a 1 in 5 dilution of Phenisol. The developing reaction was stopped by immersing the film for 30 seconds in a 1 in 40 dilution of In-1

Indicator Stop Solution. Film was fixed for 7 minutes in a 1 in 5 dilution of Hypam Rapid Fixer, then washed under tap water for 5 minutes and dried in a drying cupboard.

2.3.10 Emulsion Dipping Sections

Selected sections were isolated and placed in black polyacetal staining racks and boxes. Emulsion dipping was carried out in a darkroom under Kodak red safe light which was at least two feet from the workspace (Uhl, 1988) and covered with paper towelling to reduce light. LM-1 emulsion was warmed in a water bath at 42°C for 30-45 minutes then transferred to a Hypercoat dipping cylinder using a syringe. The dipping cylinder was maintained in the water bath and sections were dipped by slowly lowering the slide into the emulsion then evenly removing the slide, wiping the back of the slide against the dipping cylinder to remove excess emulsion. All sections were dipped in a similar way to avoid uneven emulsion coating which can lead to problems in analysis (Rogers, 1975). All sections from a single experiment were dipped during the same session. After being dipped, the back of each slide was wiped to remove excess emulsion and the slide was placed in a Nunc bioassay dish. Bioassay dishes were covered with foil and placed overnight in a dark cupboard. Slides were transferred to black polyacetal staining racks and boxes and stored with a sachet of silica gel for 6-8 weeks at 4°C, covered with foil. This period of exposure was determined empirically as that which produced optimal signal for the positive control probes (α1 and β3 antisense)

Solutions for developing emulsion dipped sections were made up and allowed to equilibrate to room temperature before use. Volumes of Phenisol (1 in 5 dilution), In-1 Indicator Stop Solution (1 in 40 dilution), and Hypam Rapid Fixer (1 in 5 dilution) were made according to the number of boxes of slides required for developing (250mL per box). 250mL of each solution were poured into black polyacetal staining boxes and sections were taken through the solutions thus; 5 minutes in Phenisol, 2 minutes in stop solution and 10 minutes in Fixer. The process was repeated in fresh solutions for each subsequent rack of slides. Sections were washed for 30 minutes under tap water and distilled water and allowed to dry overnight under a stream of air. Sections were finally stained with 0.5% cresyl violet to visualise cell bodies.

2.3.11 Cresyl Violet Staining

Sections were loaded into staining racks and rinsed through water briefly, then placed in 0.5% cresyl violet solution (refer to Appendix 3) for 1 minute. Sections were removed and again rinsed in water, then rinsed 5-20 times in differentiating solution until cell nuclei were purple-blue and background was mainly colourless (ascertained by microscopic inspection). Sections were once again rinsed briefly through water then dehydrated through a series of ethanol solutions by 5-10 rinses in 70% ethanol, 95% ethanol (twice) and 100% ethanol (twice). Slides were then placed in xylene or histolene for 1 minute, then transferred to another container of xylene or histolene for 1 minute, then coverslipped with 24x50mm coverslips using DPX mountant (Crown Scientific, Moorebank, NSW, Australia). Slides were placed on a flat surface overnight for mountant to dry, then stored at room temperature.

2.3.12 Quantitation of Autoradiograms

Autoradiograms were scanned by a laser beam (1mV, HeNe, wavelength 632.8nm) on a Molecular Dynamics Personal Densitometer (Molecular Dynamics/Pharmacia Biotech, Palo Alto, CA, USA) into Image Quant version 1.1 software (Molecular Dynamics/Pharmacia Biotech, Palo Alto, CA, USA). Alternatively, autoradiograms were scanned with a fluorescent lamp on a Bio-Rad GS-690 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) into Molecular Analyst 2.1 software (Molecular Biosciences Group, Bio-Rad Laboratories, Hercules, CA, USA) then exported as tiff files. For rat brain, optical density was measured in anterior coronal sections at the level of the olfactory nuclei and the caudate/putamen (bregma 3.7 to 1mm), in more medial coronal sections at the level of the hippocampus (bregma -1.8 to -5.3mm), in more posterior coronal sections at the level of the superior colliculus (bregma -5.6 to -6.3mm), in coronal sections at the level of the cerebellum (bregma -10.04 to -13.8mm) and in spinal cord at the cervical level. All sections were analysed with reference to a rat brain atlas (Paxinos & Watson, 1986). For human brain, optical density was measured from whole sections and, where applicable, grey matter. Optical density measurements were obtained in triplicate to reduce error. Average optical density values were obtained from two or three consecutive sections. Magnification of the image allowed optical density measurements to be obtained from individual structures or layers. These structures in

the rat brain included varying regions of the cortex, hypothalamus, thalamus, septum, amygdala, hippocampal formation, caudate-putamen, midbrain, granule cell and molecular layers of the cerebellar cortex, brainstem and spinal cord grey matter. Adjacent sections were cresyl violet stained to give the comparable structural detail.

2.3.13 Analysis

Optical density measurements were standardised to concentration of radioactivity per gram of tissue (nCi/g) using ^{14}C microscales with the GraphPad Prism 3.0 program (GraphPad Inc., San Diego, CA, USA) on an Apple Macintosh computer. Density of grey in the autoradiogram was used as an indicator of the presence and level of mRNA in the tissue (Harrison & Pearson, 1995). Non-linear regression analysis was applied to the optical density measurements obtained for the microscales (Worthington *et al.*, 1998). Two fits were compared, a polynomial first order curve (linear curve) ($Y = A + BX$) and a polynomial second order curve ($Y = A + BX + CX^2$). The curve which best fit the equation was applied to the data. Most often the polynomial first order curve was the most appropriate, indicating a linear relationship between the optical density measurements obtained from the film and the level of radioactivity. Where the polynomial second order curve best fit (non-linear relationship), two linear curves were constructed and all optical density values below the point of inflexion were transformed according to the lower curve, while all optical density values above the point of inflexion were transformed according to the upper curve. In this way, all optical density measurements were transformed in a linear fashion such that the relationship between optical density and radioactivity was linear.

Once the standard curves were constructed, optical density measurements for sections were transformed using a straight line transformation to corresponding radioactivity measurements (nCi/g). The equation for the straight line transformation is as follows:

$$Y = (Y-A)/B$$

where Y is the optical density measurement, A is the y intercept, and B is the slope of the curve.

In this way, arbitrary optical density measurements obtained from the film were standardised to specific levels of radioactivity which then corresponded to the level of

mRNA labelled in the tissue. Where necessary, background signal was subtracted from the specific signal using the transform Y=Y-K in GraphPad Prism 3.0. Final mRNA densities were expressed in nCi/g tissue equivalent after sense was subtracted from the antisense signal.

2.3.14 Statistics

For sample sizes greater than four, the Kolmogorov-Smirnov (KS) test of Normality was performed using GraphPad Prism 3.0 to ensure data fit a Guassian distribution for subsequent statistical analyses. Where sample size was four or less, it was assumed that the data obtained from the population followed an approximate Normal distribution. To determine whether significant levels of mRNA occurred in each brain region, dependent samples two tailed Student's *t*-tests were performed between density measurements for sense or antisense plus excess unlabelled probes (background controls) and antisense probes using GraphPad Prism 3.0 on an Apple Macintosh or SPSS 10.0 for Windows on a PC. Statistical significance was taken at the $p<0.05$ level.

2.3.15 Microscopic Autoradiographic Analysis

Emulsion dipped sections were examined under brightfield microscopy to visualise the silver grains corresponding to labelled mRNA. Sections were examined under a Nikon Eclipse E800 (Nikon Corporation, Kanagawa, Japan) microscope using a flat-field apochromatic 20x objective lens. Digital images were captured using a Fujix HC-1000 3CCD Image Capturing Camera (Fuji Xerox Co., Ltd., Tokyo, Japan) into Adobe Photoshop 5.0 on an Apple Macintosh computer.

2.3.16 Controls

Positive controls were employed in the present study, namely the use of $GABA_A$ receptor $\alpha 1$ and $\beta 3$ subunit probes.

A number of negative controls were applied in experiments, including a sense probe, competition of labelled antisense probe with excess (50x) unlabelled probe, as well as the use of a nonsense probe. An additional negative control was also assessed using rat

brain: the introduction of unlabelled sense probe to corresponding labelled antisense probe to form a double hybrid in solution which should preclude antisense hybridisation with mRNA in tissue.

2.3.17 Variations in Experimental Procedure

A number of variations were incorporated into the experimental procedure to ascertain sources of background and to optimise the techniques. Variations in experimental procedure were tested using rat tissue owing to its ease of accessibility. Differing concentrations of dithiothreitol were added to the hybridisation solution (3%, 5% and 7%) to determine the most appropriate concentration. Since DTT is a reducing agent, the concentration which reduces background maximally without interfering with the strength of specific hybridisation signal must be resolved. Dithiothreitol was added to the wash buffer to determine whether this reduced non-specific binding of ^{35}S-labelled probes to tissue components as suggested (Higgins & Mah, 1989; Nicholson *et al.*, 1996). Stringency of washes was altered to determine whether this had any effect on signal to noise ratio. 1x and 0.5x SSC as wash buffers were compared, and the addition of a wash in cold SSC before hot washes was tested. This cold, low stringency wash is used by some researchers, thus its effect on background signal was assessed. An extra step was incorporated in the washing procedure where alternating boxes were used and transferral of slide racks between boxes was rapid to avoid tissue drying out at any time during and between washes. Some authors suggest the use of parafilm for incubation of sections instead of coverslips since parafilm is sterile, and this was tested. Furthermore, a prehybridisation step where sections were incubated for several hours in hybridisation buffer without labelled probe was tested to determine whether this step reduced background signal. Prehybridisation is thought to act essentially as a blocking step to saturate sites which may bind the probe non-specifically (Henderson, 1996; Higgins & Mah, 1989; Gozes, 1988).

2.4 RESULTS

2.4.1 Specificity of Signal

Specificity of hybridisation signal was evidenced by the use of negative controls. Hybridisation with sense probes generally demonstrated weak homogeneous labelling, compared to the corresponding antisense probe, which displayed a discrete and heterogeneous pattern of labelling. In some cases, however, the sense probe produced a strong homogeneous signal, particularly for the ρ2, ρ3 and β3 subunit sense probes. New human ρ2 sense probes were designed as suggested by Harrison & Pearson (1995) to counteract this problem, nevertheless the same difficulty was encountered with the new probes, with sense probe hybridisation producing a generally high signal in some tissue. It was also noted that in some experiments, this high sense signal was specific to some subjects but not others, suggesting the presence of symmetrical gene transcription. Other factors which confirmed specificity of signal included white matter showing no hybridisation signal above background, and the nonsense probe producing no observable signal above background. Furthermore, signal was competitively reduced by the addition of excess unlabelled probe to the hybridisation solution. Since the use of competition with unlabelled antisense probe is a more relevant means of ascertaining background signal, this negative control was used in situations where the sense probe produced a strong signal, to confirm specificity of the antisense probe signal. Finally, introducing unlabelled sense probe to corresponding labelled antisense probe to form a double hybrid in solution was shown to preclude antisense hybridisation with mRNA in tissue (data not shown).

2.4.2 Factors Affecting mRNA Signal Intensity

Hybridisation buffer was found to affect mRNA signal intensity. In experiments where the only variable was differing batches of hybridisation buffer, it was found that the use of some buffers yielded a higher mRNA signal intensity than others. This is depicted graphically in figure 2.2, where NMDA receptor NR1a and NR1b splice variants (obtained from Dr Sylvie Le Corre, Department of Pathology, University of Sydney), as well as $GABA_C$ receptor ρ2 subunit antisense probes have been used. Length of

exposure of sections to film also affected signal intensity, with increasing time of exposure increasing signal intensity (data not shown).

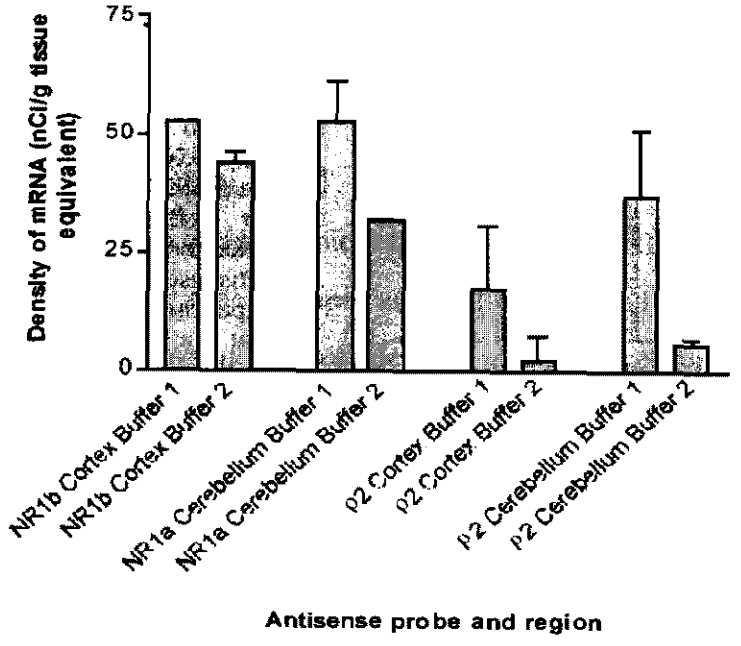


Figure 2.2: Bar chart comparing mRNA density for different subunit antisense probes hybridised in different batches of hybridisation buffer (buffer 1 or 2). Results were obtained from different regions of whole rat brain sections. Data are expressed as mean±S.D. A lack of error bars indicates n=1. It is apparent that the batch of hybridisation buffer affected signal intensity.

Increasing concentration of DTT in the hybridisation buffer led to decreased signal, as depicted in figure 2.3. Here, $GABA_A$ receptor $\alpha 1$ and $\beta 3$ subunit antisense probes have been used to exemplify the fact. A concentration of either 3% or 5% DTT was thus used in all experiments.

Incorporating a cold wash before the hot washes made no difference to hybridisation signal. The exchange of 1x SSC for 0.5x SSC also made little difference to signal intensity. This is depicted graphically in figure 2.4, where $GABA_A$ receptor $\alpha 1$ and $\beta 3$ subunit antisense probes have been used to exemplify the fact. As a result, 1x SSC was used for most experiments.

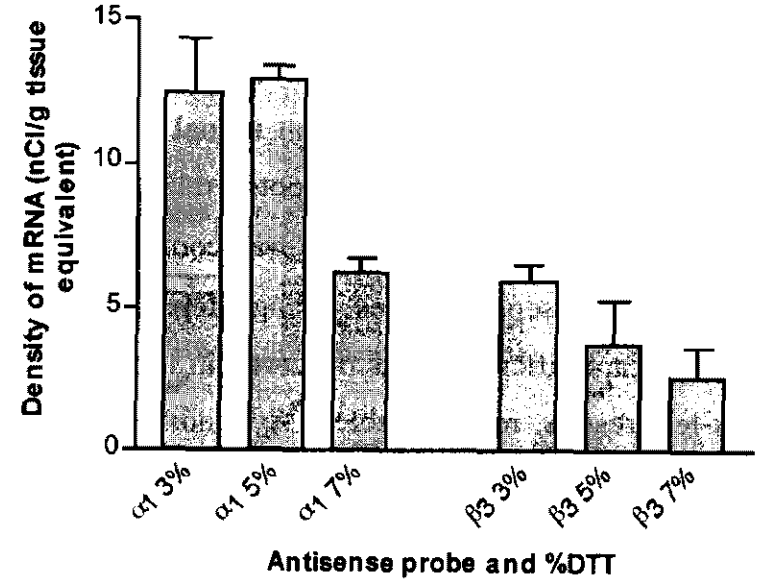


Figure 2.3: Bar graph comparing the effects of %DTT in the hybridisation solution on mRNA signal intensity in rat brain sections. It appears that for the $\alpha 1$ subunit antisense probe, 3% or 5% DTT had the same effect on mRNA signal, while 7% DTT markedly diminished signal. For the $\beta 3$ subunit antisense probe, as %DTT increased, signal decreased. Data are expressed as mean±S.D.

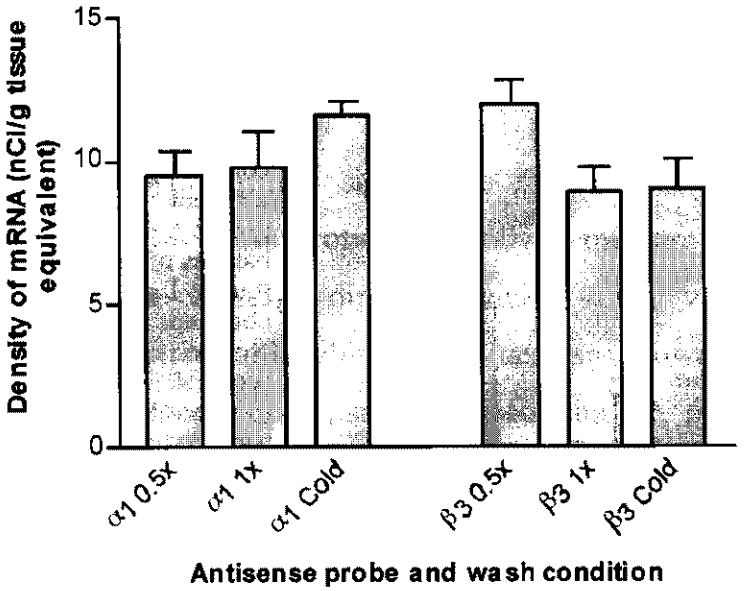


Figure 2.4: Comparison of wash conditions on mRNA signal detection in rat whole brain sections. 0.5x and 1x refers to the concentration of SSC in the wash buffer, while Cold refers to the use of a cold wash in 1x SSC prior to normal washing in 1x SSC. It appears concentration of SSC had no effect on mRNA detection for the α1 subunit antisense probe. In this experiment, it is apparent that washing in 0.5x SSC marginally increased β3 subunit signal intensity. Data are expressed as mean±S.D.

2.4.3 Factors Affecting Signal to Noise Ratio

In many experiments problems were encountered with high background, generally with the appearance of black speckles on the film, either on the tissue or on the slide, or both. In some experiments this was so marked that determination of specific signal was impossible. It was discovered that storage of labelled probes for re-use increased background dramatically, as did excess formamide in the incubation tray.

Briefly, storage time, exchange of coverslips for parafilm, addition of an incubation step with hybridisation buffer prior to hybridisation with the labelled probe, changes in wash techniques to avoid tissue drying out between solutions, and introduction of 10% DTT to the wash buffer made no difference to level of background signal (data not shown).

2.4.4 Rat Retina

Optical density was used as a measure of mRNA density in rat retinal sections. When signal intensity for sections hybridised with the $GABA_A$ receptor α1 and β3 subunit and $GABA_C$ receptor ρ1, ρ2 and ρ3 subunit antisense probes was compared to that of sections hybridised with corresponding sense probes using dependent samples Student's *t*-tests, a statistically significant difference was observed in the rat retina for the ρ1 and ρ2 subunits (for full *t*-test results, refer to table i, Appendix 4). Figure 2.5 portrays this graphically. This verifies the presence of ρ1 and ρ2 subunit mRNAs

above background in the rat retina. From figure 2.5 it is also apparent that the α1 and β3 subunit mRNAs were detected in the retina, however these findings were marginally non-significant (for full *t*-test results, refer to table i, Appendix 4). The ρ3 subunit antisense probe failed to produce any signal above background levels (data not shown).

The relative density of α1, β3, ρ1 and ρ2 subunit mRNAs in rat retina is shown in figure 2.6. This graph depicts the density of mRNA in nCi/g tissue equivalent in retina, quantified using macroscopic autoradiography. It is apparent that the $GABA_C$ receptor ρ2 subunit occurs at greatest density in the rat retina, followed by the ρ1 subunit. The $GABA_A$ receptor α1 and β3 subunits were present at lower levels than the $GABA_C$ receptor subunits.

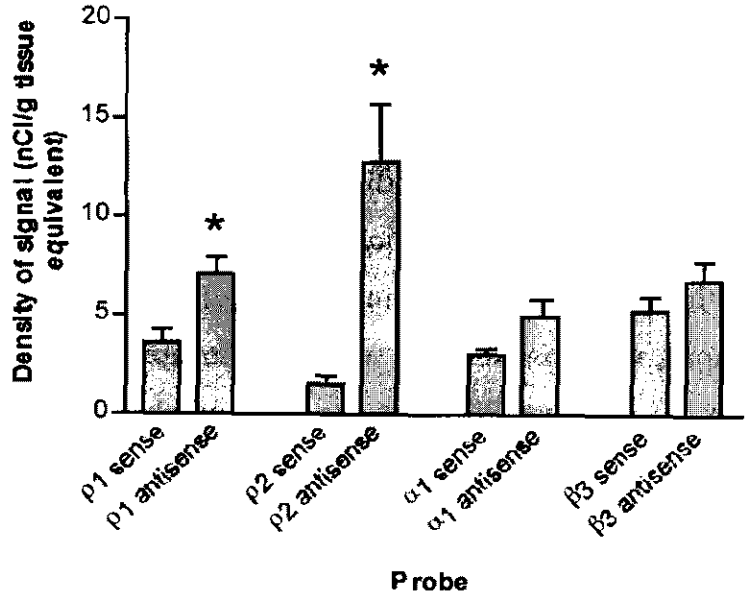


Figure 2.5: Bar graph comparing sense to antisense signal for $GABA_C$ receptor ρ1 and ρ2 subunits and $GABA_A$ receptor α1 and β3 subunits in rat retina. Sense probe gives background signal while antisense probe gives specific signal. Data are expressed as mean±s.e.mean. Paired samples Student's *t*-tests were performed to determine whether a significant difference existed between sense and antisense probes. Statistical significance between signals is denoted by *. For *t*-test results, refer to table i, Appendix 4.

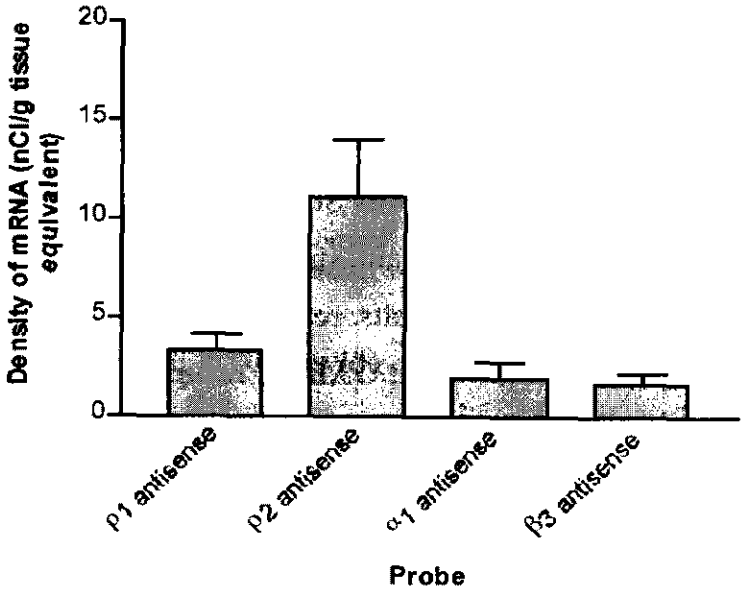


Figure 2.6: Bar graph showing relative density of $GABA_C$ receptor ρ1 and ρ2 subunit and $GABA_A$ receptor α1 and β3 subunit mRNAs in rat retina (n=4). Data are expressed as mean±s.e.mean.

2.4.5 Rat Brain

2.4.5.1 $GABA_A$ receptor subunit distribution

Optical density was used as a measure of mRNA expression in rat brain sections. The α1 subunit sense probe produced very low signal at background levels only. Furthermore, addition of excess unlabelled antisense probe to the hybridisation buffer containing labelled α1 subunit antisense probe dose-dependently abolished specific signal (data not shown). The β3 subunit sense probe for the most part produced low signal at background levels only, and addition of excess unlabelled antisense probe to the hybridisation buffer containing labelled β3 subunit antisense probe abolished specific signal (refer to figure 2.10). In many experiments, the β3 subunit sense probe did produce a strong homogeneous signal, as mentioned in section 2.4.1.

When signal intensity for sections hybridised with the $GABA_A$ receptor α1 subunit antisense probe was compared to that of sections hybridised with sense probe using dependent samples Student's *t*-tests, a statistically significant difference was observed in the rat central nervous system at the level of the forebrain, specifically at the levels of the caudate-putamen and hippocampus, when assessed as whole sections (for full *t*-test results, refer to table ii, Appendix 4). Figure 2.7 portrays this graphically. This indicates that $GABA_A$ receptor α1 subunit mRNA was detected above background in the rat central nervous system when assessed as whole sections. From figure 2.7 it is also apparent that the subunit mRNA was detected in cerebellar sections, however this finding was marginally non-significant due to larger variability. α1 Subunit mRNA could not be detected above background in the spinal cord.

A comparison of signal intensity for sections hybridised with the $GABA_A$ receptor β3 subunit sense and antisense probes using dependent samples Student's *t*-tests showed a statistically significant difference at the level of the forebrain, specifically at the levels of the caudate-putamen and hippocampus (for full *t*-test results, refer to table ii, Appendix 4). Figure 2.8 depicts this graphically. This indicates that $GABA_A$ receptor β3 subunit mRNA was detected above background in the rat central nervous system when assessed as whole sections. From figure 2.8 it is also apparent that the subunit mRNA was

detected in cerebellar and spinal cord sections, however these finding were marginally non-significant due to lower sample size and larger variability.

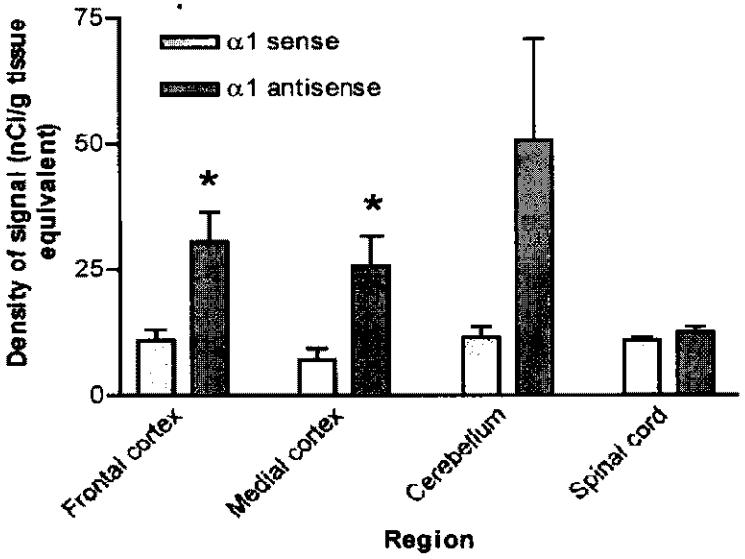


Figure 2.7: Bar graph comparing signal obtained for $GABA_A$ receptor α1 subunit probes in rat brain sections. Sense probe gives background signal while antisense probe gives specific signal. Data are expressed mean±s.e.mean. Paired samples Student's *t*-tests were performed to determine whether a significant difference existed between sense and antisense probe signals. A significant difference is designated by the *. Frontal cortex (n=5) refers to coronal sections at the level of the olfactory nuclei and caudate-putamen (bregma 3.70 to 0.48mm). Medial cortex (n=5) refers to coronal sections at the level of hippocampus and superior colliculus (bregma -2.30 to -6.80). Cerebellum (n=4) refers to coronal sections at the level of the cerebellum (bregma -9.80 to -13.8mm). Spinal cord (n=3) refers to coronal sections at the level of the cervical spinal cord.

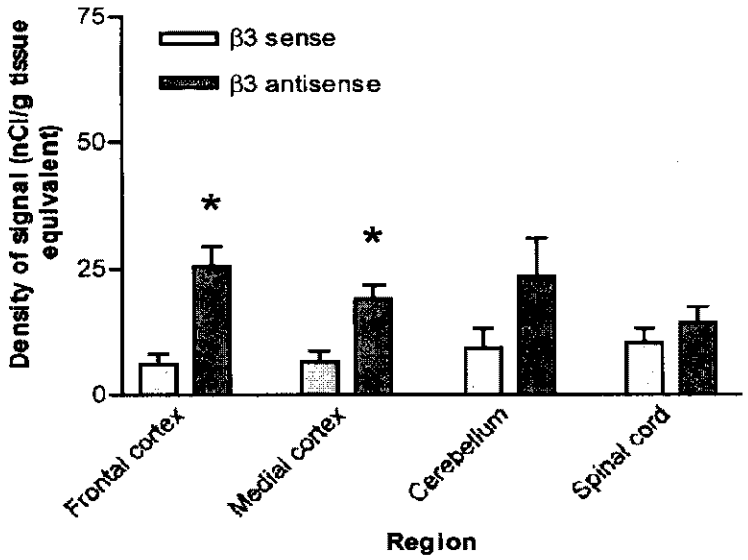


Figure 2.8: Bar graph comparing signal obtained for $GABA_A$ receptor β3 subunit probes in rat brain sections. Sense probe gives background signal while antisense probe gives specific signal. Data are expressed mean±s.e.mean. Paired samples Student's *t*-tests were performed to determine whether a significant difference existed between sense and antisense probe signals. A significant difference is designated by the *. Frontal cortex (n=5) refers to coronal sections at the level of the olfactory nuclei and caudate-putamen (bregma 3.70 to 0.48mm). Medial cortex (n=6) refers to coronal sections at the level of hippocampus and superior colliculus (bregma -2.30 to -6.80). Cerebellum (n=3) refers to coronal sections at the level of the cerebellum (bregma -9.80 to -13.8mm). Spinal cord (n=3) refers to coronal sections at the level of the cervical spinal cord.

Figures 2.9 and 2.10 provide images of rat brain sections hybridised with α1 and β3 subunit probes, respectively, from autoradiograms, showing sense probe, antisense probe and competition labelling at various levels of the central nervous system.

Figures 2.11 and 2.12 present images of rat brain sections hybridised with α1 and β3 subunit probes, respectively, after emulsion dipping to reveal microscopic silver grain deposition indicative of mRNA distribution. At the microscopic level of investigation, clustering of silver grains around cell soma, where mRNA is located, is characteristic of specific mRNA labelling. Microscopic examination of emulsion dipped sections hybridised with α1 or β3 subunit antisense probe revealed a large degree of heterogeneity in silver grain distribution, with dense clustering of silver grains around cells and discrete labelling in most brain regions, denoting the presence of specific α1 or β3 subunit mRNA labelling. Density of mRNA varied in different regions, consistent with varying densities of signal observed at the macroscopic level.

At the microscopic level the α1 subunit sense probe yielded very low levels of silver grains evenly distributed across cellular and non-cellular components of the tissue, which signifies background signal only. In some sections hybridised with β3 subunit sense probe, density of silver grains was moderate but distribution was even across regions without clustering around cell soma, in keeping with the strong, generalised signal sometimes observed at the macroscopic level. In other sections hybridised with the β3 subunit sense probe, very low levels of silver grains with homogenous distribution were observed, characteristic of background signal.

Examination of autoradiograms and microscopic examination of emulsion dipped sections demonstrated the presence of α1 and β3 subunit mRNA signal in regions of the rat brain outlined in table 2.3 (page 96). In the thalamus, β3 subunit mRNA was more noticeable in the paraventricular nuclei. β3 subunit mRNA expression was also more prominent in the lateral septum, than in the medial septum. The relative densities of α1 and β3 subunit mRNA in differing regions of the rat brain are shown in figures 2.13 and 2.14 respectively. The graphs depict the density of mRNA in nCi/g tissue equivalent for the main regions of the rat brain which could be quantified using macroscopic autoradiography.

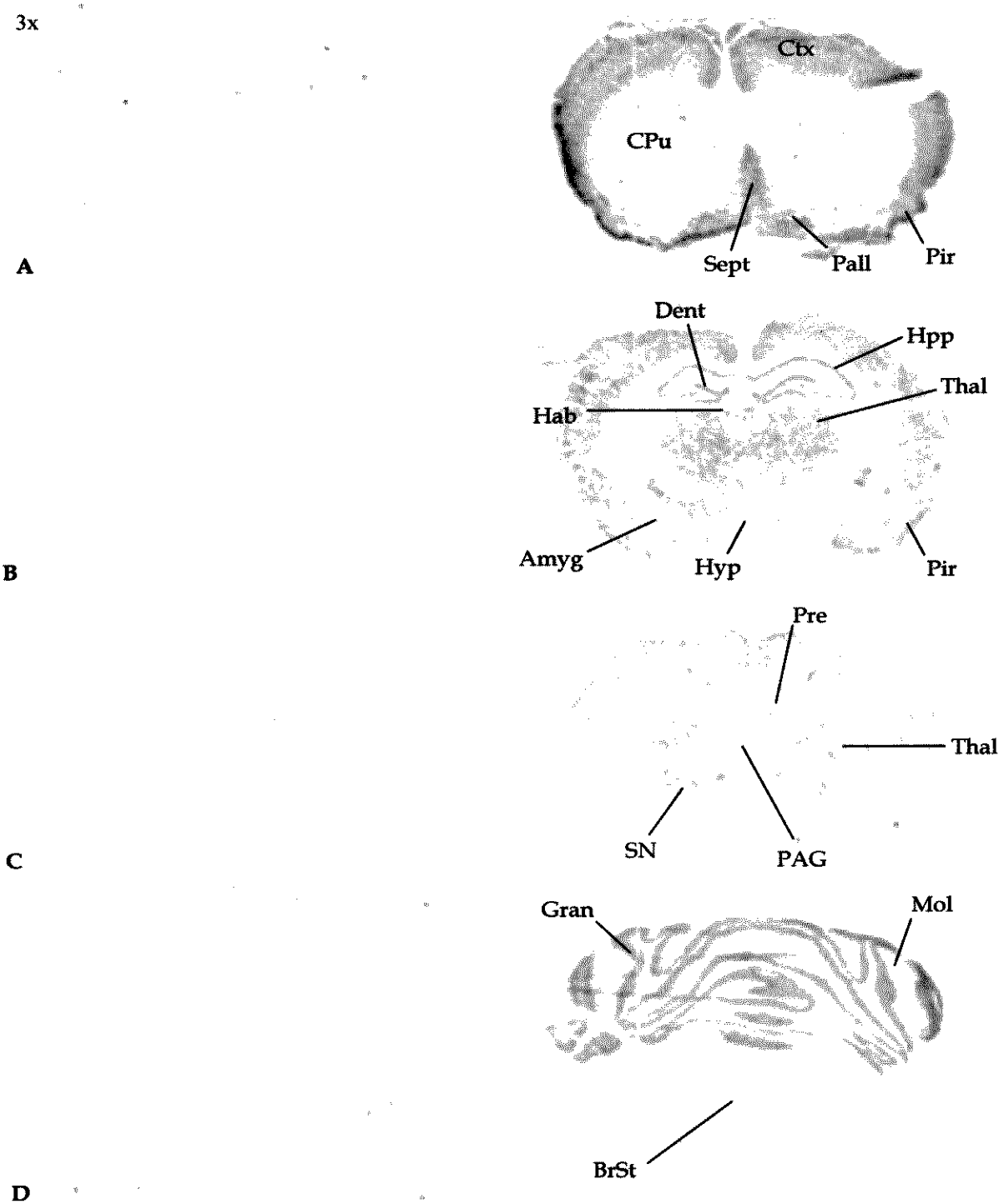


Figure 2.9: Autoradiograph images of α1 subunit mRNA distribution in coronal rat brain sections. Sections on the left were hybridised with sense probe, showing background signal only. Sections on the right were hybridised with antisense probe. Increased density of grey depicts labelling of mRNA by the antisense probe, specifically in the regions shown. **A.** In anterior forebrain sections at the level of the caudate-putamen (bregma 1mm). **B.** In medial forebrain sections at the level of the hippocampus (bregma -3.3mm). **C.** In posterior forebrain/midbrain sections at the level of the PAG (bregma -5.3mm). **D.** In cerebellar sections (bregma -10.3mm). Ctx = cortex, CPu = caudate-putamen, Pir = piriform cortex, Sept = septum, Pall = pallidum. Hpp = hippocampus, Dent = dentate gyrus, Hab = habenular nucleus, Amyg = amygdala, Thal = thalamus, Hyp = hypothalamus, Pre = pretectal nuclei, SN = substantia nigra, PAG = periaqueductal grey, Gran = granule cell layer, Mol = molecular layer, BrSt = brainstem. Magnification 3 times original macroscopic images.

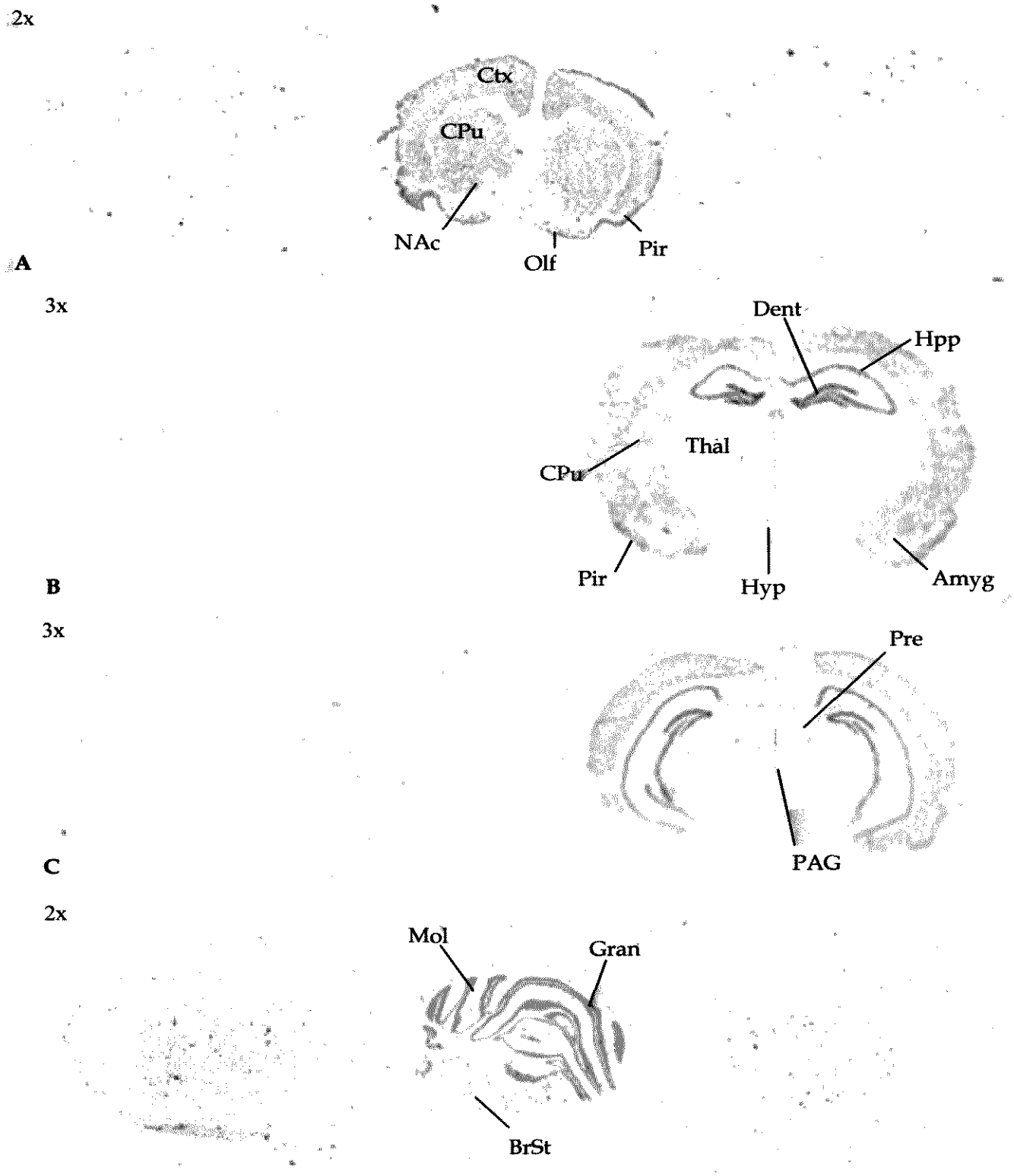


Figure 2.10: Autoradiograph images of β3 subunit mRNA distribution in coronal rat brain sections. Sections on the left were hybridised with sense probe. Sections on the right were hybridised with antisense probe. Where present, sections on the far right were hybridised with antisense probe in the presence of unlabelled excess probe. Sense and antisense plus excess unlabelled antisense probes give background signal only. Increased density of grey in sections hybridised with antisense probe depicts labelling of mRNA by the antisense probe, specifically in the regions shown. **A.** In anterior forebrain sections at the level of the caudate-putamen (bregma 1mm). **B.** In medial forebrain sections at the level of the hippocampus (bregma -3.6mm). **C.** In posterior forebrain/midbrain sections at the level of the PAG (bregma -5.2mm). **D.** In cerebellar sections (bregma -10.52mm). **E.** In cervical spinal cord. Ctx = cortex, CPu = caudate-putamen, NAc = nucleus accumbens, Pir = piriform cortex, Olf = olfactory tubercle, Hpp = hippocampus, Dent = dentate gyrus, Amyg = amygdala, Thal = thalamus, Hyp = hypothalamus, Gran = granule cell layer, Mol = molecular layer, BrSt = brainstem. *Magnification 2 to 3 times original macroscopic images.*

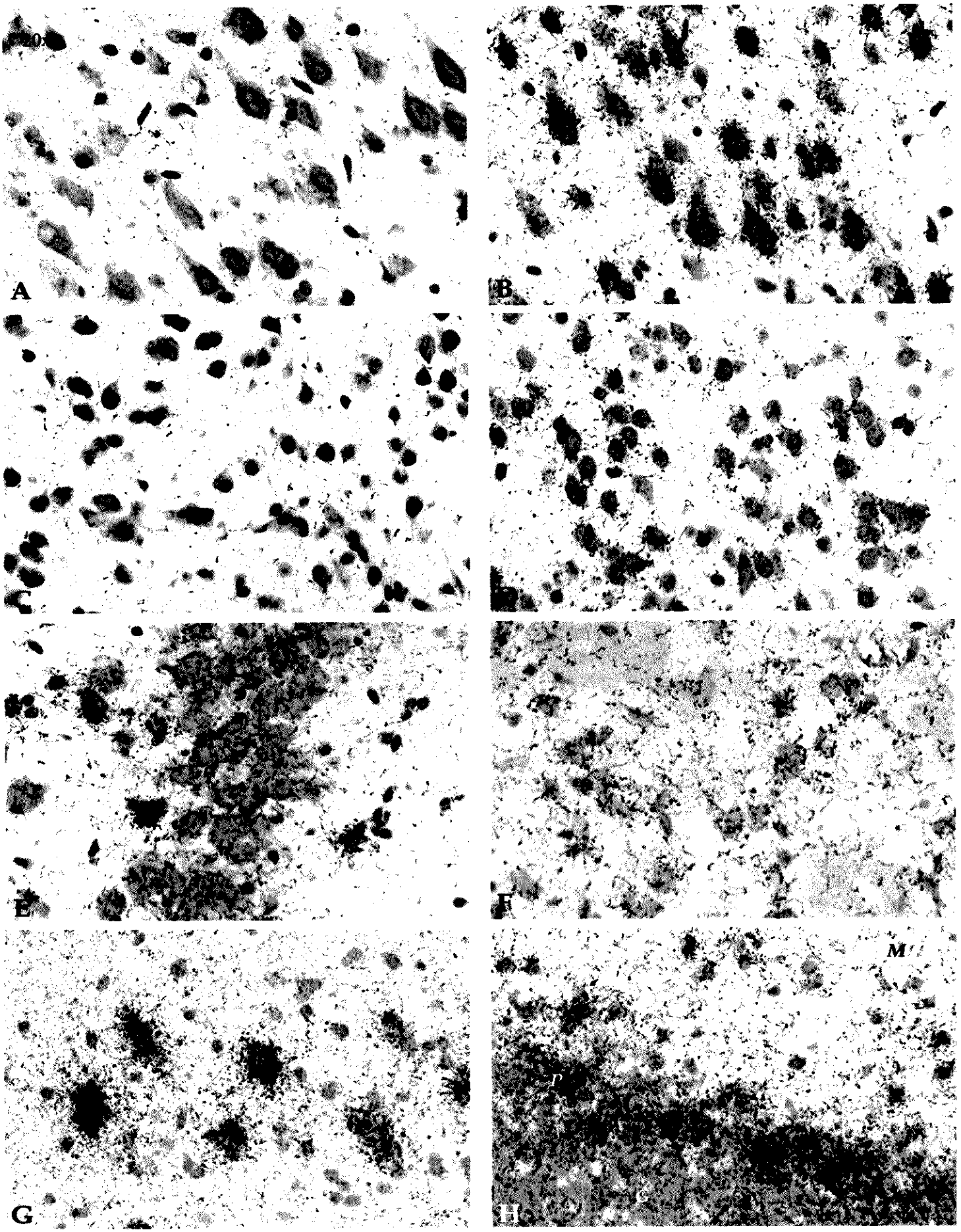


Figure 2.11: Micrographs of sections hybridised with $GABA_A$ receptor $\alpha 1$ subunit probes after emulsion dipping. A and C depict background signal obtained from sense or antisense with excess unlabelled probe hybridisation. B, D, E, F, G and H depict labelling of mRNA by antisense probe, evident by clustering of silver grains around cell bodies. All sections have been counterstained with cresyl violet to show cell soma. **A** and **B**. Cortex. **C** and **D**. Medial amygdaloid nucleus. **E**. Hippocampus. **F**. Caudate-putamen. **G**. Substantia nigra. **H**. Cerebellar cortex. Key in H. *G* = granule cell layer. *M* = molecular layer. *P* = Purkinje cell layer. Images captured at 20x magnification.

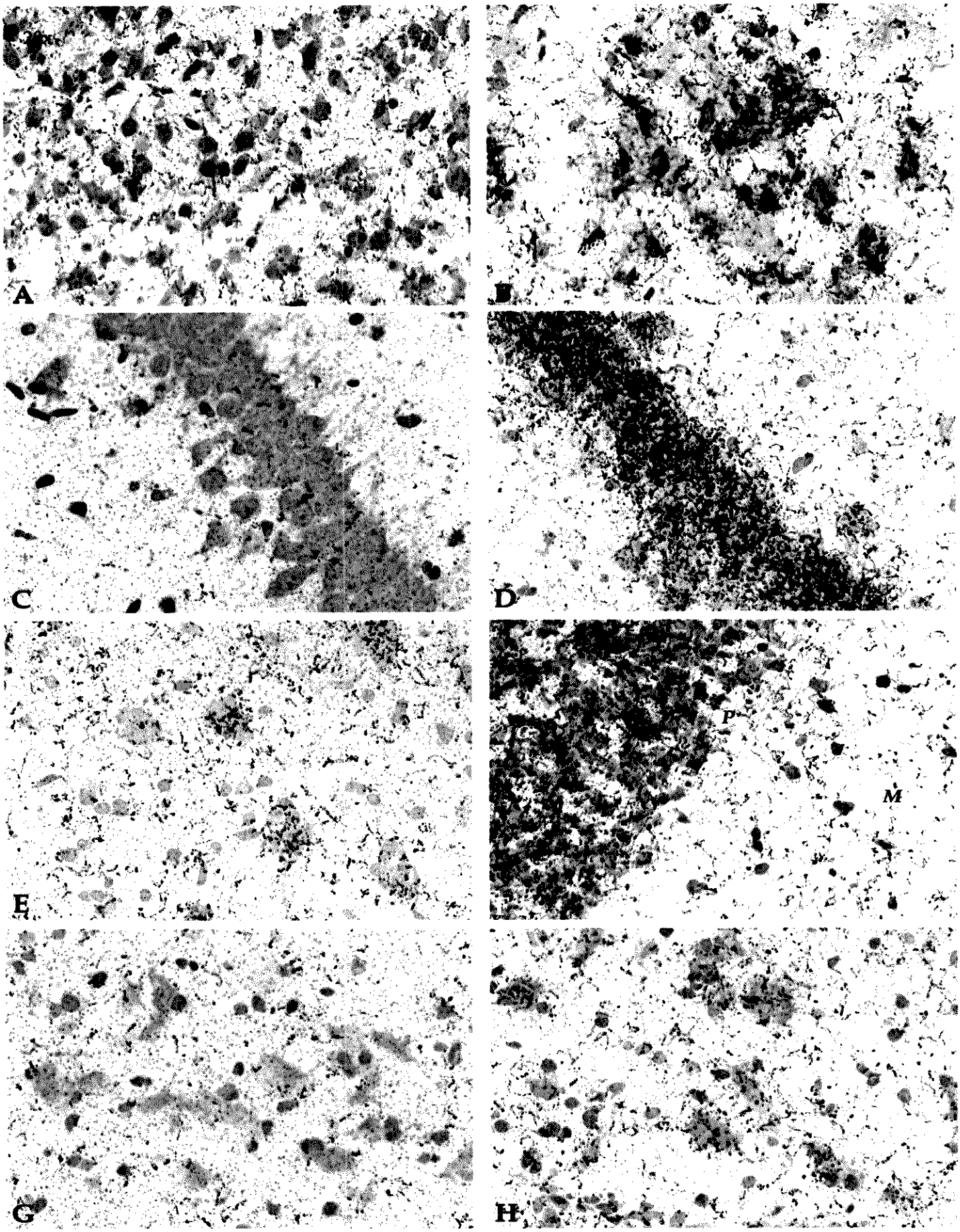


Figure 2.12: Micrographs of sections hybridised with $GABA_A$ receptor β3 subunit probes after emulsion dipping. C and G depict background signal obtained from sense or antisense with excess unlabelled probe hybridisation. A, B, D, E, F and H depict labelling of mRNA by antisense probe, evident by clustering of silver grains around cell bodies. All sections have been counterstained with cresyl violet to show cell soma. **A**. Cortex. **B**. Caudate-putamen. **C** and **D**. Hippocampus. **E**. Substantia nigra. **F**. Cerebellar cortex. **G** and **H**. Spinal cord. Key in F. *G* = granule cell layer. *M* = molecular layer. *P* = Purkinje cell layer. Images captured at 20x magnification.

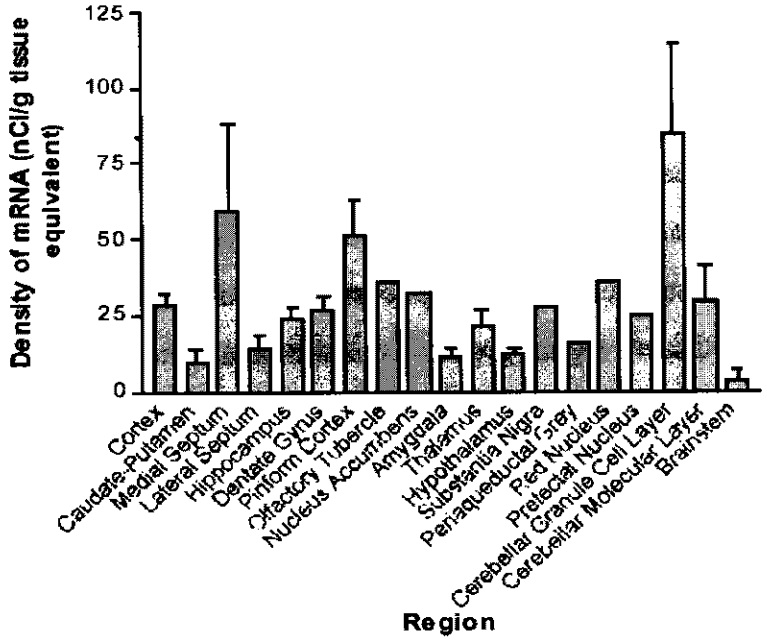


Figure 2.13: Bar graph representing density of $GABA_A$ receptor $\alpha 1$ subunit mRNA in varying regions of rat brain, quantified macroscopically. Data are expressed as mean±s.e.mean. For cortex, n=7; hippocampus, dentate gyrus, caudate-putamen and thalamus, n=5; amygdala, hypothalamus, piriform and cerebellar cortices, n=4; olfactory tubercle, nucleus accumbens, substantia nigra, periaqueductal grey, red nucleus and pretectal nuclei, n=1.

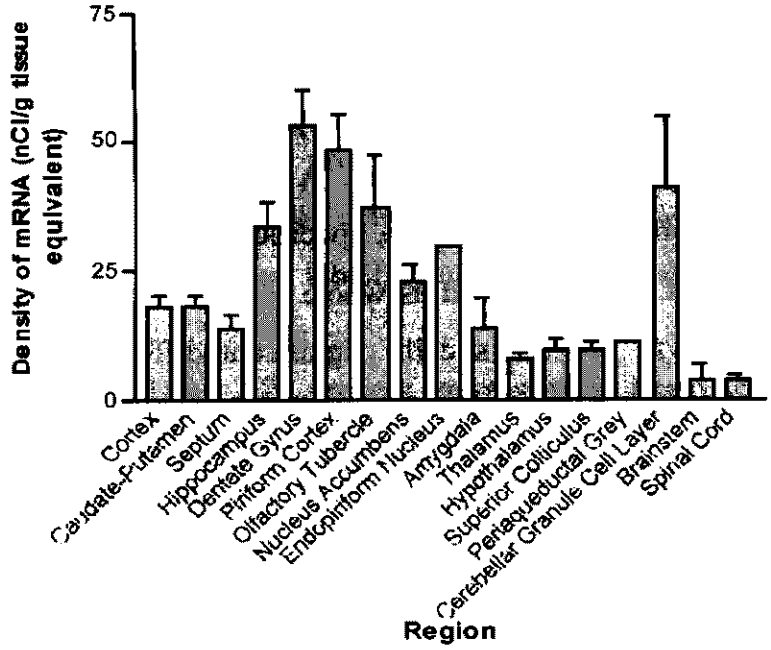


Figure 2.14: Bar graph representing density of $GABA_A$ receptor $\beta 3$ subunit mRNA in varying regions of rat brain, quantified macroscopically. Data are expressed as mean±s.e.mean. For cortex, n=8; hippocampus and dentate gyrus, n=6; nucleus accumbens, caudate-putamen and piriform cortex, n=5; thalamus and septum, n=4; hypothalamus olfactory tubercle cerebellum and spinal cord, n=3; amygdala, superior colliculus and brainstem, n=2; endopiriform nucleus and periaqueductal grey, n=1.

2.4.5.2 $GABA_C$ receptor subunit distribution

The ρ1 subunit sense probe produced very low signal at background levels only. Addition of excess unlabelled antisense probe to the hybridisation buffer containing labelled ρ1 subunit antisense probe did not dose-dependently abolish specific signal in all experiments, demonstrating that the antisense signal obtained was not necessarily specific (data not shown). In some instances when this occurred, it was usually the case that the antisense probe did not produce a signal greater than the sense probe. The ρ2 and ρ3 subunit sense probes often produced low signal at background levels only, and addition of excess unlabelled antisense probe to the hybridisation buffer containing labelled ρ2 subunit antisense probe abolished specific signal (refer to figure 2.19). In many experiments, however, the ρ2 and ρ3 sense probes did produce a strong, homogeneous signal, as mentioned in section 2.4.1.

When signal intensity for sections hybridised with $GABA_C$ receptor subunit antisense probes was compared to that of sections hybridised with sense probes using dependent samples Student's *t*-tests, the following results were obtained. A statistically significant difference in ρ1 subunit probes was observed in forebrain, specifically at the levels of the caudate-putamen and hippocampus, when assessed as whole sections (for full *t*-test results, refer to table ii, Appendix 4). No statistically significant differences were observed for ρ2 or ρ3 subunit probes (for full *t*-test results, refer to table ii, Appendix 4). Figures 2.15, 2.16 and 2.17 graphically represent sense and antisense signals for the ρ1, ρ2 and ρ3 subunit probes respectively.

The data indicate that $GABA_C$ receptor ρ1 subunit mRNA was detected above background at the levels of the rat central nervous system mentioned above, when assessed as whole sections. From figure 2.15 it is also apparent that the subunit mRNA was detected in cerebellar sections, however this finding was marginally non-significant due to larger variability. ρ1 Subunit mRNA could not be detected above background in the spinal cord. While the whole-section optical density for ρ1 antisense probe is significantly different to the sense probe (background), shown in figure 2.15, closer examination of macroscopic images reveals that signal in discrete brain regions was only discernible in one subject, suggesting that the mRNA is detectable only at very low levels.

Although no statistically significant differences between ρ2 sense and antisense probes were encountered, these findings were only marginally non-significant and may represent the fact that between subject variability was great, and that in many experiments the ρ2 sense probe produced a strong, homogenous signal. Specific signal for ρ2 subunit antisense probe was in fact apparent at all levels of the rat brain, with the exception of the spinal cord, as shown in figure 2.23. Since many regions of the rat brain could be quantified macroscopically and positive signal was produced by the antisense probe after sense probe signal had been subtracted, one may affirm that ρ2 subunit mRNA was detectable above background in most subjects.

The results suggest that $GABA_C$ receptor ρ3 subunit mRNA was not detectable above background in the rat central nervous system. The large variability seen in cerebellar sections accounts for the fact that in one subject, signal was observed for the antisense probe above background.

Figures 2.18 and 2.19 provide images of rat brain sections hybridised with ρ1 and ρ2 subunit probes, respectively, from autoradiograms, showing sense probe, antisense probe and competition labelling at various levels of the central nervous system.

Figures 2.20 and 2.21 present images of rat brain sections hybridised with ρ1 and ρ2 subunit probes, respectively, after emulsion dipping to reveal microscopic silver grain deposition. Microscopic examination of emulsion dipped sections hybridised with ρ1 subunit antisense probe revealed no heterogeneity in the distribution of silver grains, denoting an absence of specific ρ1 subunit mRNA labelling. Silver grains were distributed evenly across all regions with no clustering around cell soma to indicate specific mRNA labelling, as seen in figure 2.20B. This concurs with macroscopic images showing an homogenous signal without discrete labelling, even though the antisense signal was greater than the sense probe signal. The exception to this finding is in the cerebellum, where density of silver grains appeared greater over the granule cell layer. In three subjects, density of silver grains was also increased over the hippocampus and dentate gyrus at this level of resolution, which was not apparent in macroscopic examination. Sections hybridised with ρ1 subunit sense probe

demonstrated low levels of silver grains with an even distribution, characteristic of background labelling.

Microscopic examination of emulsion dipped sections hybridised with ρ2 subunit antisense probe revealed heterogeneity in silver grain distribution, indicating the presence of specific ρ2 subunit mRNA labelling. A relatively high level of background was evidenced by silver grain distribution across non-perikarya components of the tissue. However, specific labelling was apparent from clustering of silver grains, albeit at low levels, around cell bodies. These findings reinforce macroscopic images which depict low specific signal. In some sections hybridised with ρ2 subunit sense probe, density of silver grains was moderate but distribution was even across regions without clustering around cell soma, consistent with the strong, generalised signal sometimes observed at the macroscopic level. In other sections hybridised with the ρ2 subunit sense probe, very low levels of silver grains with homogenous distribution were observed, indicative of background signal.

Examination of autoradiograms and microscopic examination of emulsion dipped sections demonstrated the presence of ρ1 and ρ2 subunit mRNA signal in regions of the rat brain outlined in table 2.3 (page 97), and the presence of ρ3 subunit mRNA signal in the cerebellar cortex of only one subject. The relative densities of ρ1 and ρ2 subunit mRNAs in differing areas of the rat brain are shown in figures 2.22 and 2.23 respectively. These graphs depict the density of mRNA in nCi/g tissue equivalent for the main regions of the rat brain which could be quantified using macroscopic autoradiography.

Specific signal in the brain regions outlined for ρ1 subunit mRNA was detected macroscopically in one subject, except for the cerebellar cortex (n=6). Microscopic examination of ρ2 mRNA labelling revealed the increased density of signal in the amygdala was not due to specific labelling, yet specific labelling of the mRNA in spinal cord was evident at this higher level of resolution in one subject. In addition, silver grain density was increased in the lateral septum and Islands of Calleja, which could not be determined macroscopically.

No heterogeneity in the distribution of silver grains was observed with ρ3 subunit mRNA labelling, denoting an absence of specific labelling. Silver grains were distributed evenly across all regions with no clustering around cell nuclei to indicated specific mRNA expression (data not shown). This is concordant with macroscopic images exhibiting a generalised signal without discrete labelling. In many instances, sections hybridised with ρ3 subunit sense probe displayed a moderate density of silver grains with an even distribution across cell soma and non-perikarya components of the tissue, consistent with the generalised, moderate signal observed at the macroscopic level.

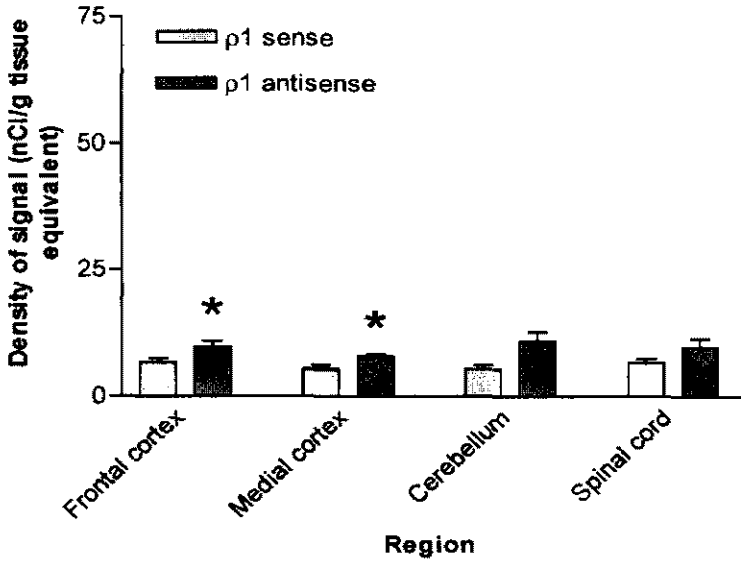


Figure 2.15: Bar graph comparing signal obtained for $GABA_C$ receptor ρ1 subunit probes in rat brain sections. Sense probe gives background signal while antisense probe gives specific signal. Data are expressed as mean±s.e.mean. Paired samples Student's *t*-tests were performed to determine whether a significant difference existed between sense and antisense probes. A significant difference is designated by the *. Frontal cortex (n=4) refers to coronal sections at the level of the olfactory nuclei and caudate-putamen (bregma 3.70 to 0.48mm). Medial cortex (n=5) refers to coronal sections at the level of hippocampus and superior colliculus (bregma -2.30 to -6.80mm). Cerebellum (n=5) refers to coronal sections at the level of the cerebellum (bregma -9.80 to -13.8mm). Spinal cord (n=2) refers to coronal sections at the level of the cervical spinal cord.

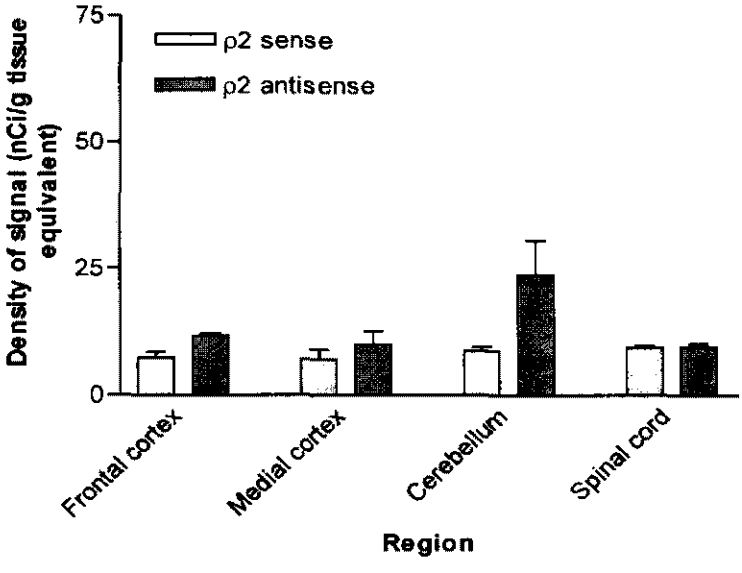


Figure 2.16: Bar graph comparing signal obtained for $GABA_C$ receptor ρ2 subunit probes in rat brain sections. Sense probe gives background signal while antisense probe gives specific signal. Data are expressed as mean±s.e.mean. Paired samples Student's *t*-tests were performed to determine whether a significant difference existed between sense and antisense probes. A significant difference is designated by the *. Frontal cortex (n=3) refers to coronal sections at the level of the olfactory nuclei and caudate-putamen (bregma 3.70-0.48mm). Medial cortex (n=6) refers to

coronal sections at the level of hippocampus and superior colliculus (bregma -2.30 to -6.80mm). Cerebellum (n=6) refers to coronal sections at the level of the cerebellum (bregma -9.80 to -13.8mm). Spinal cord (n=3) refers to coronal sections at the level of the cervical spinal cord.

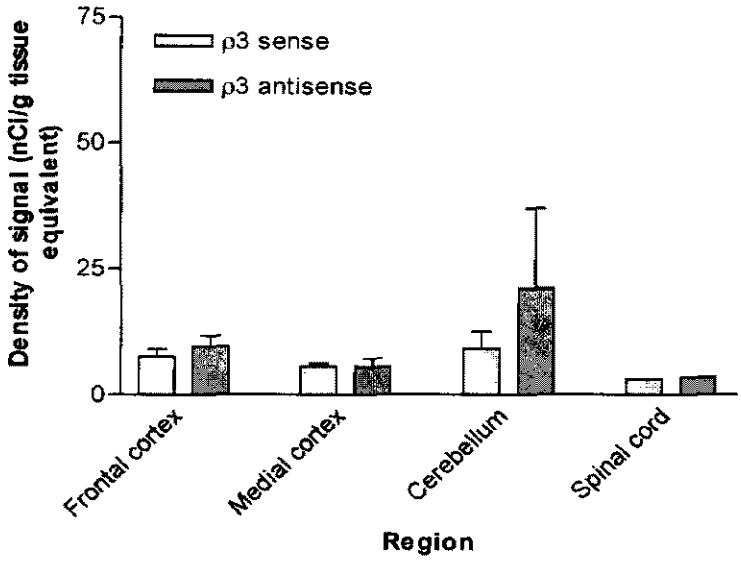


Figure 2.17: Bar graph comparing signal obtained for $GABA_C$ receptor ρ3 subunit probes in rat brain sections. Sense probe gives background signal while antisense probe gives specific signal. Data are expressed as mean±s.e.mean. Paired samples Student's *t*-tests were performed to determine whether a significant difference existed between sense and antisense probes. A significant difference is designated by the *. Frontal cortex (n=5) refers to coronal sections at the level of the olfactory nuclei and caudate-putamen (bregma 3.70 to 0.48mm). Medial cortex (n=4) refers to coronal sections at the level of hippocampus and superior colliculus (bregma -2.30 to -6.80mm). Cerebellum (n=3) refers to coronal sections at the level of the cerebellum (bregma -9.80 to -13.8mm). Spinal cord (n=1) refers to coronal sections at the level of the cervical spinal cord.

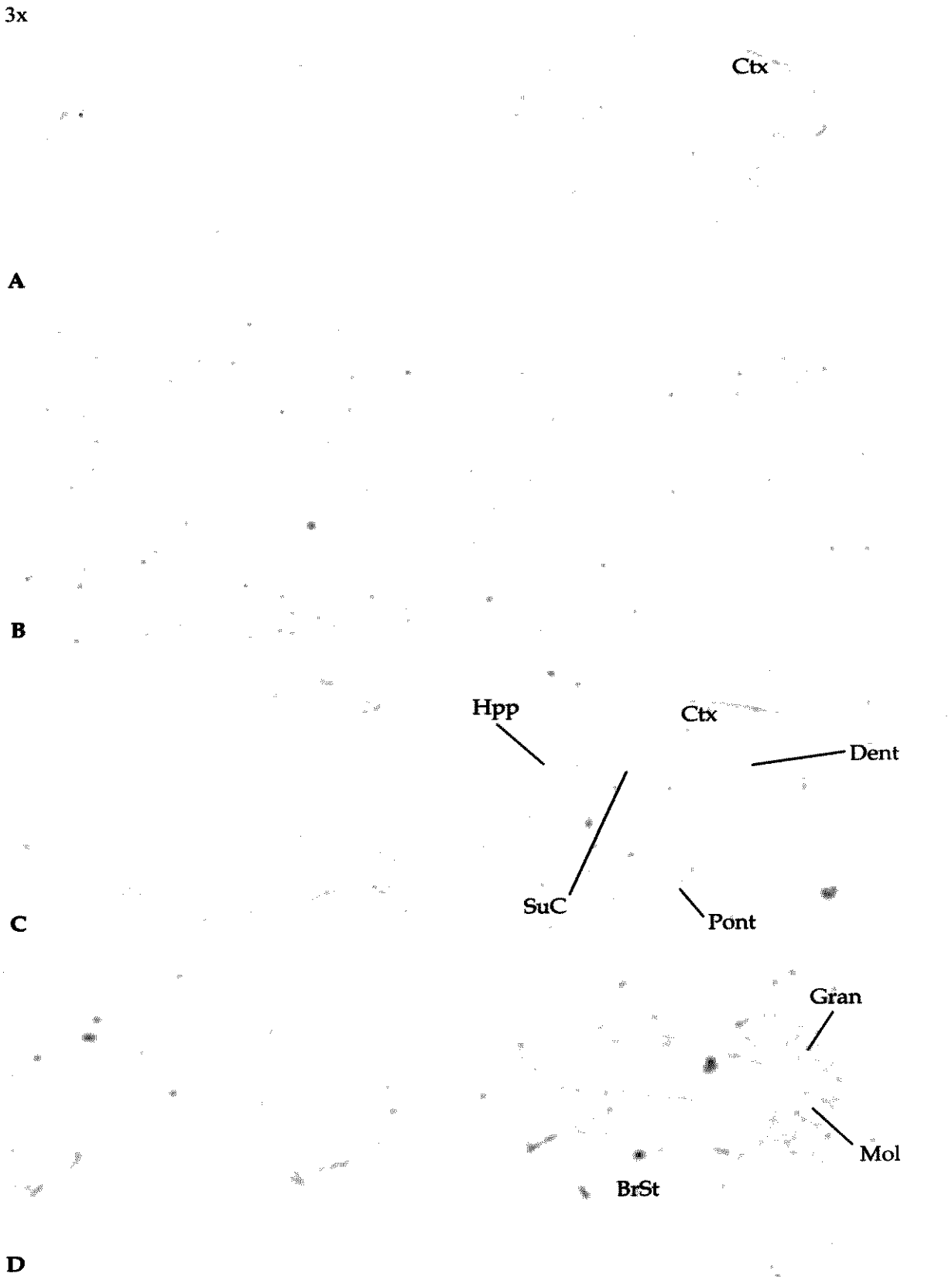


Figure 2.18: Autoradiographic images of ρ1 subunit mRNA distribution in coronal rat brain sections. Sections on the left were hybridised with sense probe, giving background signal only. Sections on the right were hybridised with antisense probe. Darker grey observed in discrete brain regions of sections hybridised with antisense probe depicts labelling of mRNA by the antisense probe, specifically in the regions shown. **A.** In anterior forebrain sections at the level of the caudate-putamen (bregma 1mm). **B.** In medial forebrain sections at the level of the hippocampus (bregma –3.6mm). **C.** In posterior forebrain/midbrain sections at the level of the superior colliculus (bregma -5.6mm). **D.** In cerebellar sections (bregma -12.8mm). Ctx = cortex, Hpp = hippocampus, Dent = dentate gyrus, SuC = superior colliculus, Pont = pontine nuclei, Gran = granule cell layer, Mol = molecular layer, BrSt = brainstem.

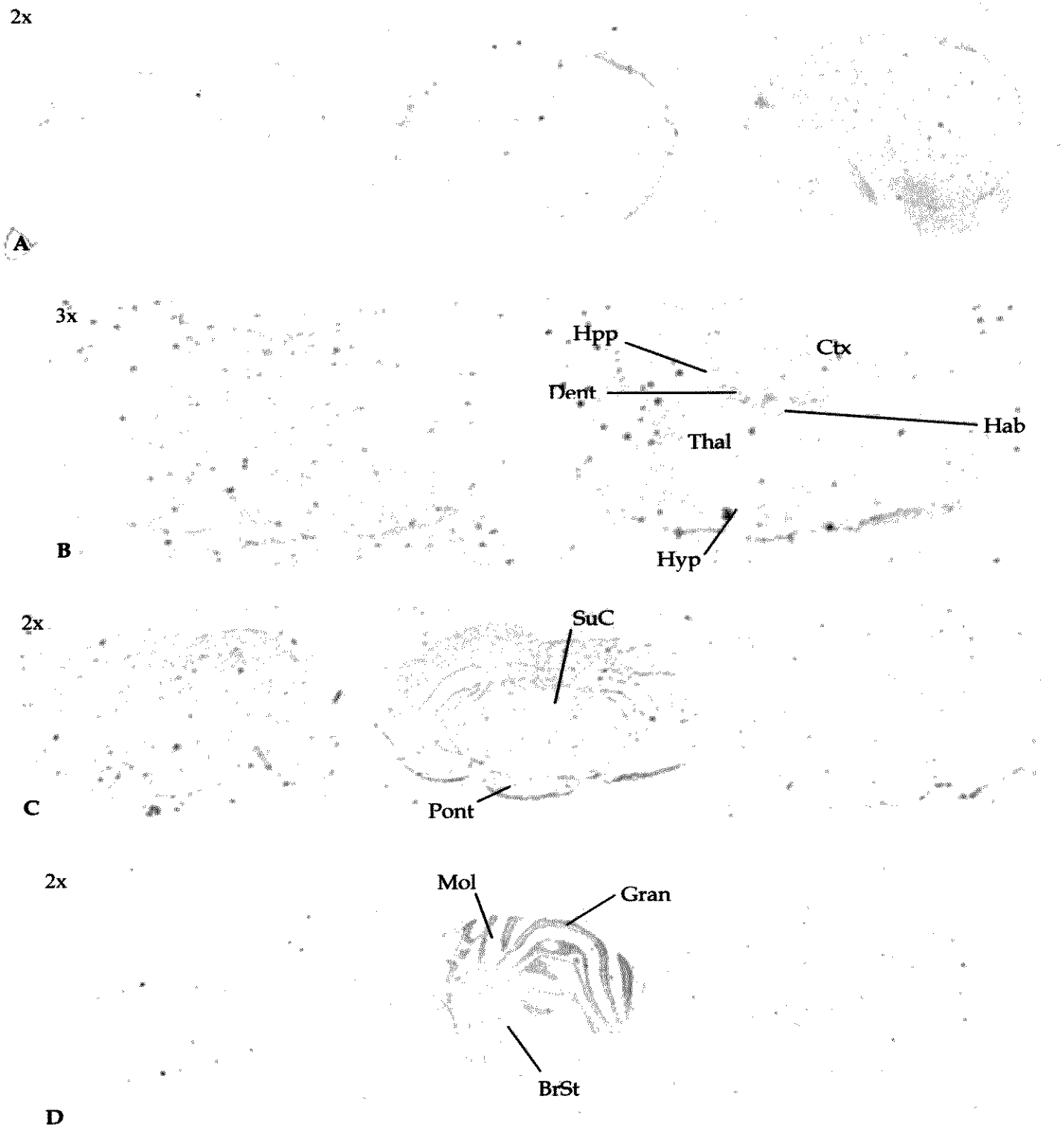


Figure 2.19: Autoradiographic images of ρ2 subunit mRNA distribution in coronal rat brain sections. Sections on the left were hybridised with sense probe. Sections on the right were hybridised with antisense probe. Where present, sections on the far right were hybridised with antisense probe in the presence of unlabelled excess probe. Sense and antisense plus excess unlabelled antisense probes give background signal only. Darker grey observed in discrete brain regions of sections hybridised with antisense probe depicts labelling of mRNA by the antisense probe, specifically in the regions shown. **A.** In anterior forebrain sections at the level of the caudate-putamen (bregma 1mm). **B.** In medial forebrain sections at the level of the hippocampus (bregma -3.6mm). **C.** In posterior forebrain/midbrain sections at the level of the superior colliculus (bregma -5.8mm). **D.** In cerebellar sections (bregma -10.52mm). Ctx = cortex, Hpp = hippocampus, Dent = dentate gyrus, Amyg = amygdala, Hab = habenular nucleus, Hyp = hypothalamus, SuC = superior colliculus, Pont = pontine nuclei, Gran = granule cell layer, Mol = molecular layer, BrSt = brainstem. Magnification 2-3 times original macroscopic images.

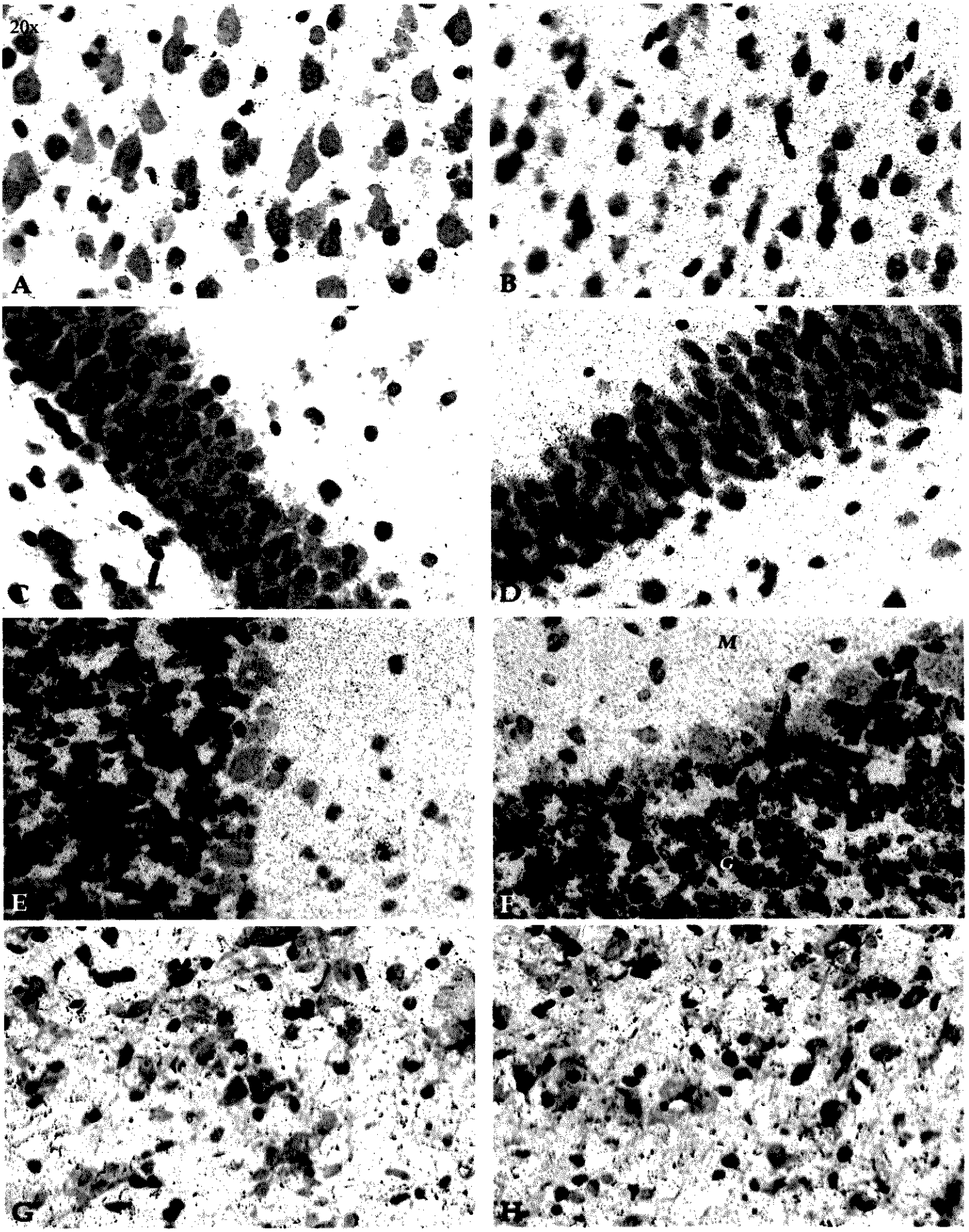


Figure 2.20: Micrographs of sections hybridised with $GABA_C$ receptor ρ1 subunit probes after emulsion dipping. A, C, E and G depict background signal obtained from sense or antisense with excess unlabelled probe hybridisation. B, D, F and H depict labelling of mRNA by antisense probe, evident by clustering of silver grains around cell bodies. All sections have been counterstained with cresyl violet to show cell soma. **A** and **B**. Cortex. **C** and **D**. Dentate gyrus. **E** and **F**. Cerebellar cortex. **G**. and **H**. Spinal cord. Key in F. *G* = granule cell layer. *M* = molecular layer. *P* = Purkinje cell layer. Images captured at 20x magnification.

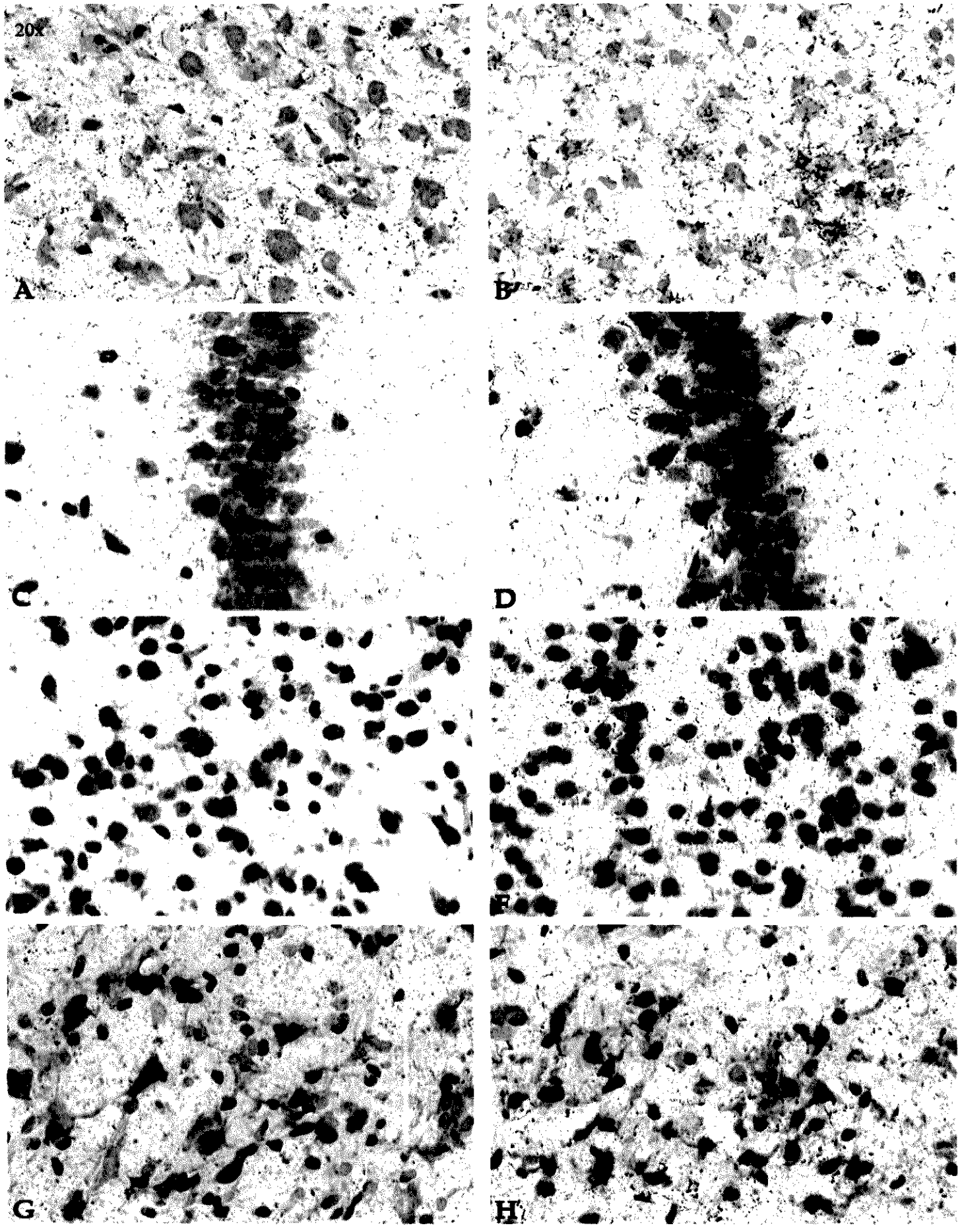


Figure 2.21: Micrographs of sections hybridised with $GABA_C$ receptor ρ2 subunit probes after emulsion dipping. A, C, E and G depict background signal obtained from sense or antisense with excess unlabelled probe hybridisation. B, D, F and H depict labelling by antisense probe, evident by clustering of silver grains around cell bodies. All sections have been counterstained with cresyl violet to show cell soma. **A** and **B**. Cortex. **C** and **D**. Hippocampus. **E** and **F**. Ventromedial hypothalamus. **G**. and **H**. Spinal cord. Images captured at 20x magnification.

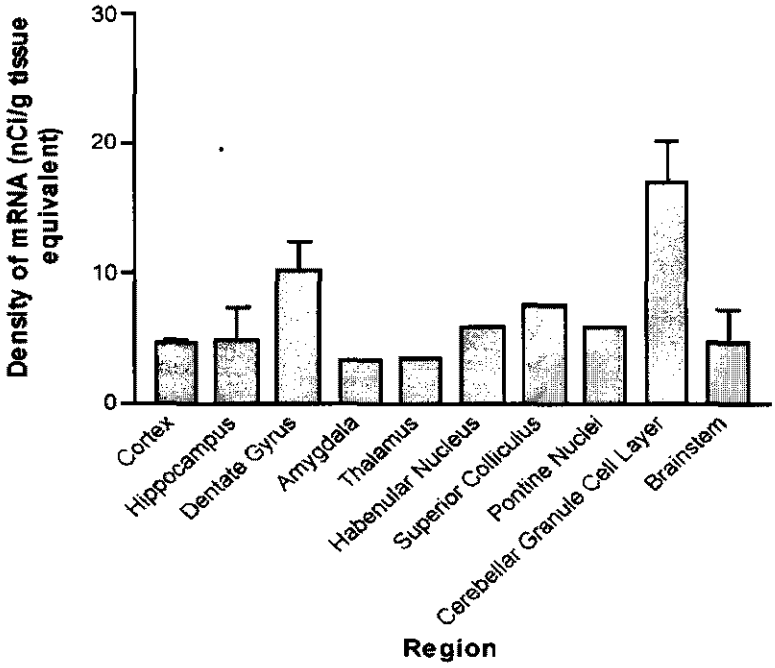


Figure 2.22: Bar graph representing density of $GABA_C$ receptor ρ1 subunit mRNA in varying regions of rat brain, quantified macroscopically. Data are expressed as mean±s.e.mean. For all regions, n=1, apart from cerebellar and brainstem regions, where n=6. An omission of error bars indicates results obtained from one experiment only.

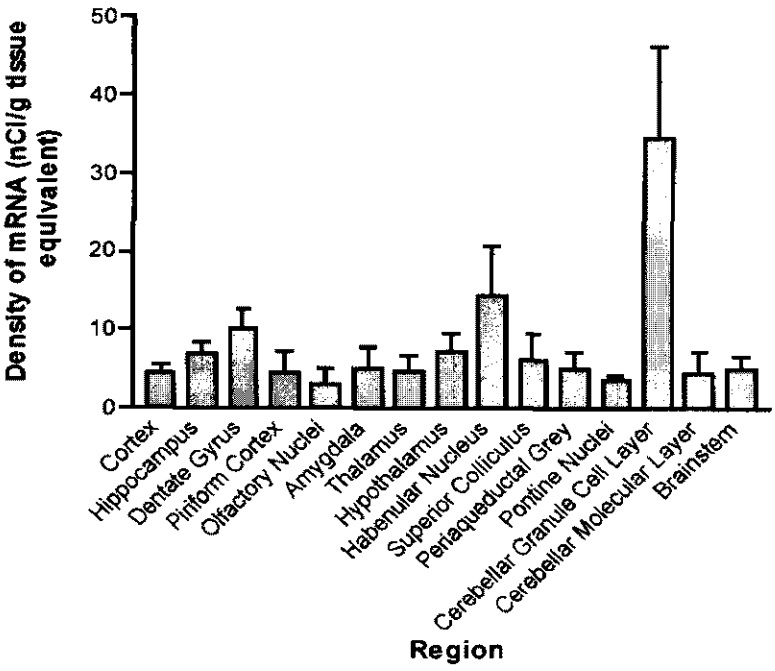


Figure 2.23: Bar graph representing density of $GABA_C$ receptor ρ2 subunit mRNA in varying regions of rat brain, quantified macroscopically. Data are expressed as mean±s.e.mean. For cortex and cerebellar granule cell layer, n=6; hippocampus, dentate gyrus and brainstem, n=5; amygdala, thalamus and hypothalamus, n=4; cerebellar molecular layer, n=3; piriform cortex, olfactory nuclei, superior colliculus, habenular nucleus and periaqueductal grey, n=2; pontine nuclei, n=1.

Table 2.3: Distribution of $GABA_A$ receptor α1 and β3 subunit mRNAs and $GABA_C$ receptor ρ1, ρ2 and ρ3 subunit mRNAs in rat brain, ascertained from macroscopic and microscopic autoradiography. ✓ indicates the presence of the subunit, with a weak to strong signal.

Brain region	α1	β3	ρ1	ρ2	ρ3
Olfactory nucleus	✓	✓		✓	
Cerebral cortex					
cingulate	✓	✓	✓	✓	
frontal	✓	✓	✓	✓	
parietal	✓	✓			
visual	✓	✓		✓	
Piriform cortex	✓	✓		✓	
Olfactory tubercle	✓	✓			
Septum					
medial	✓				
lateral	✓	✓		✓	
Claustrum	✓	✓			
Endopiriform nucleus	✓	✓			
Islands of Calleja		✓		✓	
Nucleus accumbens	✓	✓			
Globus pallidus	✓				
Ventral pallidum	✓				
Striatum		✓		✓	
Bed nucleus of stria terminalis	✓				
Entopeduncular nucleus	✓				
Diagonal band					
of vertical limb	✓	✓			
of horizontal limb	✓				
Habenular nuclei		✓		✓	
Hippocampal formation					
CA1	✓	✓	✓	✓	
CA2	✓	✓		✓	
CA3	✓	✓		✓	
Dentate gyrus	✓	✓	✓	✓	
Amygdaloid complex					
Basolateral nuclei	✓	✓			
Central nuclei					
Medial nuclei	✓	✓			
Thalamus	✓	✓		✓	
Hypothalamus	✓	✓		✓	
Arcuate nucleus	✓				
Zona incerta	✓				
Pretectal nuclei	✓	✓			
Superior colliculus	✓	✓		✓	
Red nucleus	✓	✓			
Periaqueductal grey	✓	✓			
Subtantia nigra	✓	✓			
Interpeduncular nuclei	✓				
Cerebellar cortex					
Granule cell layer	✓	✓	✓	✓	✓
Purkinje cell layer	✓				
Molecular layer	✓				
Brainstem	✓	✓		✓	
Spinal cord		✓		✓	

2.4.6 Human Brain

Optical density was used as a measure of mRNA density in human brain sections. Figure 2.24 presents bar graphs showing the density of $GABA_C$ receptor ρ1 and ρ2 subunit, and $GABA_A$ receptor α1 and β3 subunit mRNAs in various regions of the human brain. Statistical significance is designated by the *, its position indicating statistical significance between the probe it lies above and the probe to the left of it.

2.4.6.1 $GABA_A$ receptor subunit distribution

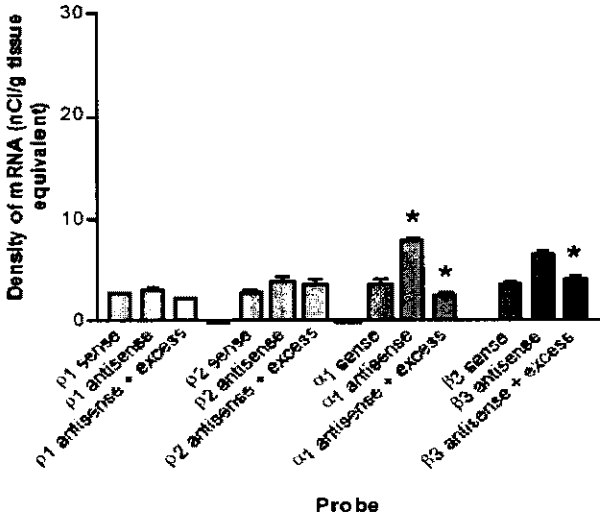
The α1 subunit sense probe produced low signal at background levels only. The β3 subunit sense probe mainly produced low signal at background levels only, however in some cases it was seen to produce a strong homogenous signal as described in section 2.4.1. Addition of excess unlabelled antisense probe to the hybridisation buffer containing labelled α1 or β3 subunit antisense probes abolished specific signal (see figure 2.24).

When signal intensity for sections hybridised with the $GABA_A$ receptor α1 subunit antisense probe was compared to that of sections hybridised with sense probe using dependent samples Student's *t*-tests, a statistically significant difference was observed in the middle frontal cortex and hippocampus, when assessed as whole sections (for full *t*-test results, refer to table iii, Appendix 4). A comparison of signal intensity for sections hybridised with the antisense probe with sections hybridised with excess unlabelled antisense probe produced a statistically significant difference the middle frontal cortex and superior temporal gyrus (for full *t*-test results, refer to table iii, Appendix 4). Figure 2.24 portrays this graphically. This indicates that $GABA_A$ receptor α1 subunit mRNA was detected significantly above background in the middle frontal cortex (n=6), hippocampus (n=2) and superior temporal gyrus (n=6) of the human brain.

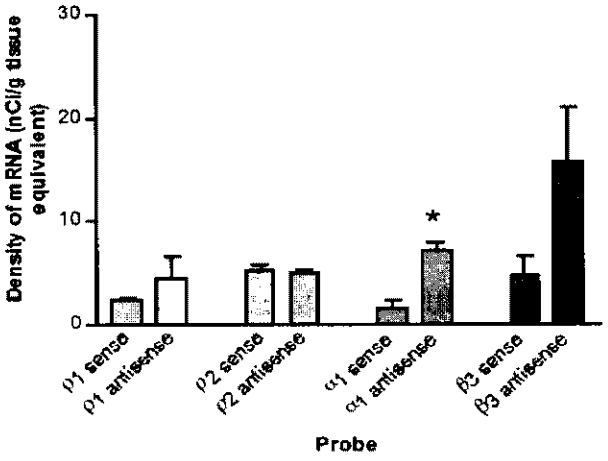
Comparison of signal intensity for sections hybridised with the $GABA_A$ receptor β3 subunit antisense probe to that of sections hybridised with sense probe using dependent samples Student's *t*-tests, failed to produce a statistically significant difference, when assessed as whole sections (for full *t*-test results, refer to table iii, Appendix 4). However, when signal intensity for sections hybridised with the antisense probe was

compared to that of sections hybridised with excess unlabelled antisense probe, a statistically significant difference was observed in the middle frontal cortex (n=6) (for full *t*-test results, refer to table iii, Appendix 4). This verifies that $GABA_A$ receptor β3 subunit mRNA was detected significantly above background in the middle frontal cortex (n=6). Figure 2.24 displays this graphically.

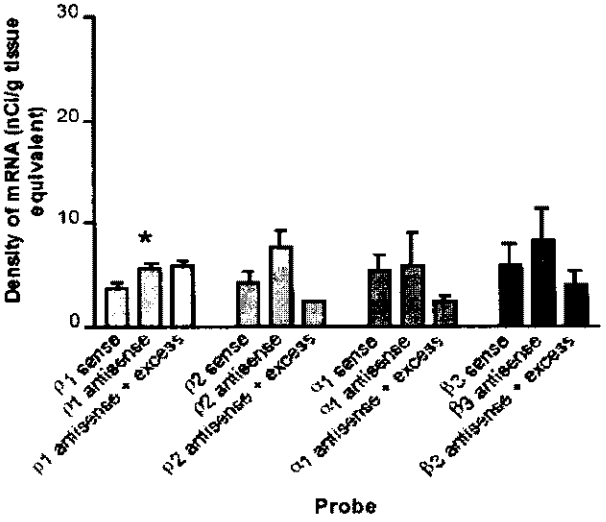
The relative density of α1 and β3 subunit mRNAs in differing regions of the human brain is shown in figure 2.25 and 2.26 respectively, once sense or excess unlabelled antisense probe signal has been subtracted to leave specific antisense probe signal. These graphs depict the density of mRNA in nCi/g tissue equivalent for the main regions of the human brain which could be quantified using macroscopic autoradiography. From figures 2.25 and 2.26 it is apparent that the α1 and β3 subunit transcripts were detectable above background, although not statistically significantly, in other regions of the human brain tested. For the α1 subunit mRNA, these areas included the central nucleus of the amygdala, polar temporal cortex, primary visual cortex and cerebellum. In particular, the comparison of sense to antisense probes in the primary visual cortex is only marginally non-significant (t=2.678, df=4, p=0.055). For the β3 subunit transcript, regions of detection included the hippocampus, central nucleus of the amygdala, polar temporal cortex, primary visual cortex and cerebellum. Density of β3 mRNA appears to be very low in the superior temporal gyrus and cerebellar molecular layer. In particular, the comparison of sense to antisense probes in the middle frontal cortex is only marginally non-significant (t=2.533, df=5, p=0.052). Furthermore, the difference between β3 subunit antisense and antisense plus excess unlabelled probe signal in the cerebellum is also rather large (t=7.791, df=1, p=0.081). Lack of statistical significance is due to small sample size and large inter-subject variability.



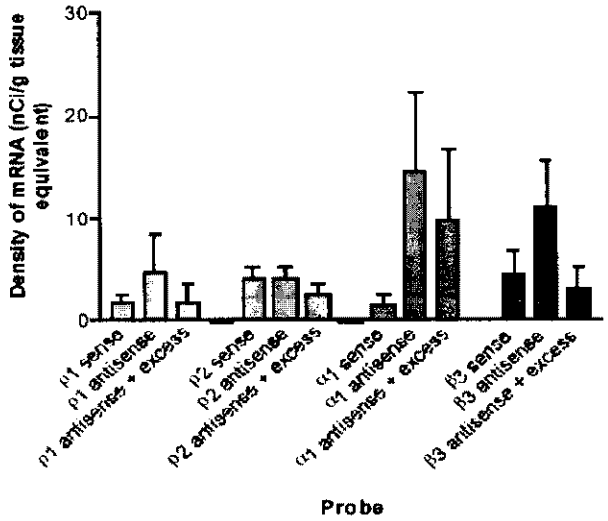
A. Bar graph showing sense, antisense and antisense with excess unlabelled probe signal for $GABA_C$ receptor ρ1 and ρ2 subunits (n=5), and $GABA_A$ receptor α1 and β3 subunits (n=6) in the middle frontal cortex. Statistical significance is denoted by *.



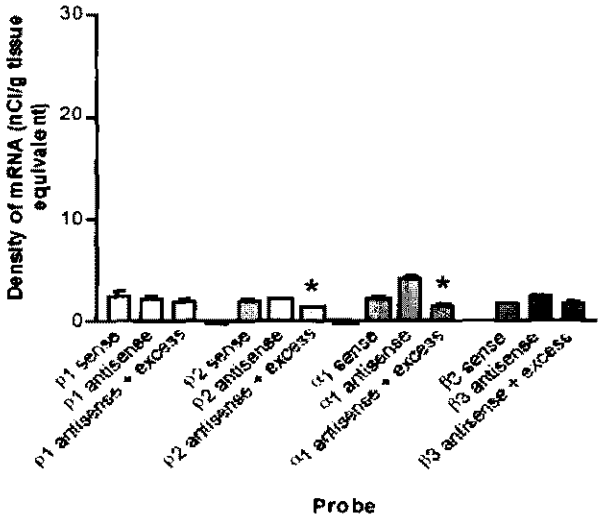
B. Bar graph showing sense and antisense probe signal for $GABA_C$ receptor ρ1 and ρ2 subunits, and $GABA_A$ receptor α1 and β3 subunits in the hippocampus. n=2 for ρ1 and α1 subunits. n=3 for ρ2 and β3 subunits. Statistical significance is denoted by *.



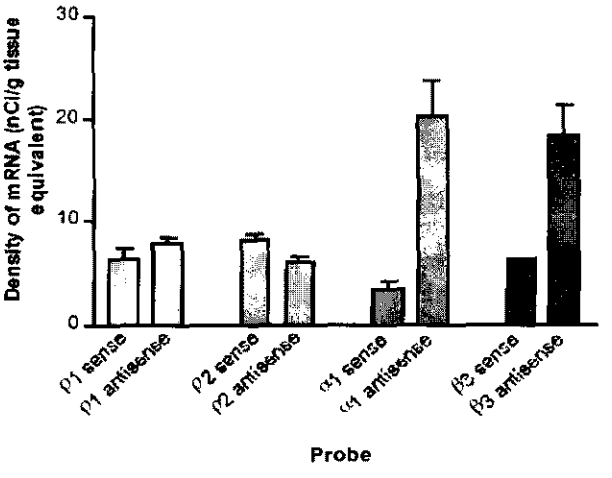
C. Bar graph showing sense, antisense and antisense with excess unlabelled probe signal for $GABA_C$ receptor ρ1 and ρ2 subunits, and $GABA_A$ receptor α1 and β3 subunits in the central nucleus of the amygdala (n=2). Statistical significance is denoted by *.



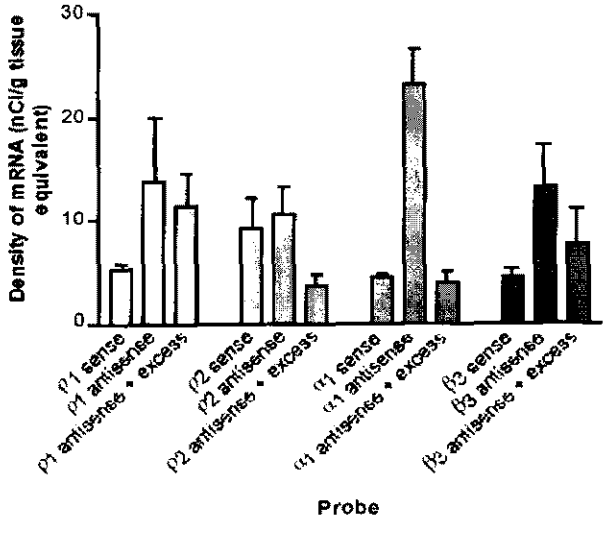
D. Bar graph showing sense, antisense and antisense with excess unlabelled probe signal for $GABA_C$ receptor ρ1 and ρ2 subunits, and $GABA_A$ receptor α1 and β3 subunits in the polar temporal cortex (n=2).



E. Bar graph showing sense, antisense and antisense with excess unlabelled probe signal for $GABA_C$ receptor ρ1 and ρ2 subunits, and $GABA_A$ receptor α1 and β3 subunits in the superior temporal gyrus (n=6). Statistical significance is denoted by *.



F. Bar graph showing sense and antisense probe signal for $GABA_C$ receptor ρ1 and ρ2 subunits, and $GABA_A$ receptor α1 and β3 subunits in the primary visual cortex. n=6 for ρ1 subunits. n=5 for ρ2 and α1 subunits. n=3 for β3 subunits.



G. Bar graph showing sense, antisense and antisense with excess unlabelled probe signal for $GABA_C$ receptor ρ1 and ρ2 subunits, and $GABA_A$ receptor α1 and β3 subunits in the cerebellum (n=2).

Figure 2.24: Bar graphs comparing signal obtained from $GABA_C$ receptor ρ1 and ρ2 subunit, and $GABA_A$ receptor α1 and β3 subunit probes in regions of the human brain. **A.** Middle frontal cortex. **B.** Hippocampus. **C.** Central nucleus of the amygdala. **D.** Polar temporal cortex. **E.** Superior temporal gyrus. **F.** Primary visual cortex. **G.** Cerebellum. Sense and antisense + excess probes give background signal while antisense probe gives specific signal. Data are expressed as mean±s.e.mean. Paired samples Student's *t*-tests were performed to determine whether a significant difference existed between sense and antisense probes, and between antisense and antisense + excess unlabelled probes. Results from *t*-tests are given in Table iii, Appendix 4.

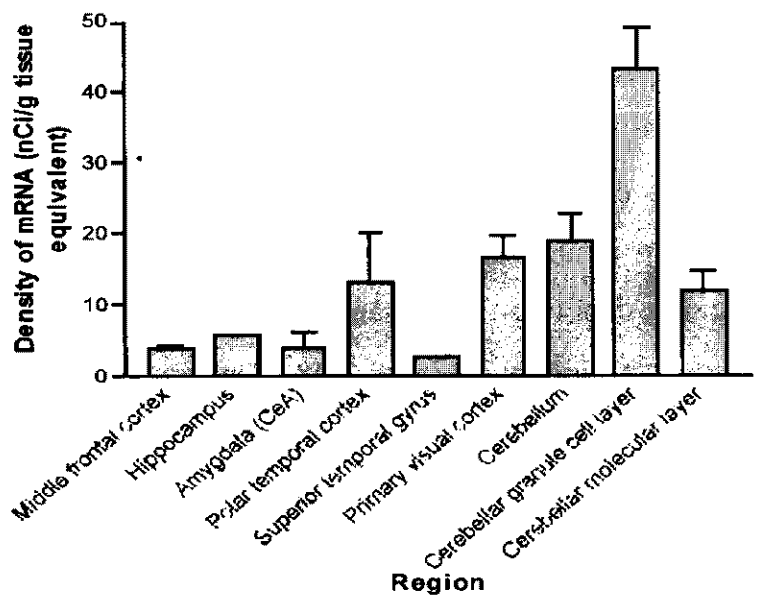


Figure 2.25: Bar graph representing density of $GABA_A$ receptor $\alpha 1$ subunit mRNA in regions of human brain, quantified macroscopically. Data are expressed as mean±s.e.mean. For middle frontal cortex and superior temporal gyrus, n=6; primary visual cortex, n=5; hippocampus, central nucleus of the amygdala, polar temporal cortex and cerebellum, n=2.

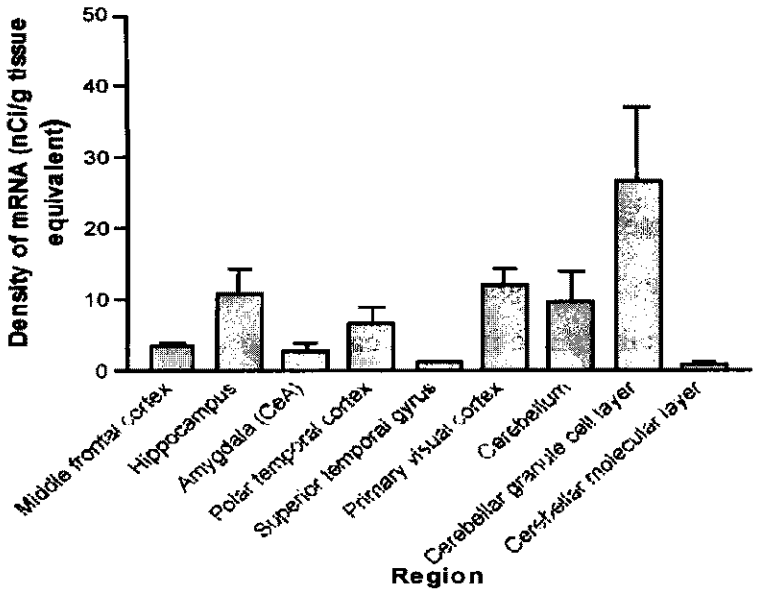


Figure 2.26: Bar graph representing density of $GABA_A$ receptor $\beta 3$ subunit mRNA in regions of human brain, quantified macroscopically. Data are expressed as mean±s.e.mean. For middle frontal cortex and superior temporal gyrus, n=6; hippocampus and primary visual cortex, n=3; central nucleus of the amygdala, polar temporal cortex and cerebellum, n=2.

Figures 2.27 and 2.28 provide images of human brain sections hybridised with $\alpha 1$ and $\beta 3$ subunit probes respectively from autoradiograms, showing sense and antisense probe. Figures 2.27 and 2.28 also present images of human brain sections hybridised with the antisense probes after emulsion dipping to reveal microscopic silver grain deposition indicative of mRNA distribution. At the microscopic level, the $\alpha 1$ subunit sense probe yielded very low levels of silver grains evenly distributed across cellular and non-cellular components of the tissue, which signifies background signal only. In some sections hybridised with $\beta 3$ subunit sense probe, density of silver grains was moderate but distribution was even across regions without clustering around cell soma, in keeping with the strong, generalised signal sometimes observed at the macroscopic level. In

other sections hybridised with the β3 subunit sense probe, very low levels of silver grains with homogenous distribution were observed, characteristic of background signal.

Examination of sections hybridised with either the α1 or β3 subunit antisense probe revealed a laminar pattern of distribution or discrete labelling, and clustering of silver grains around cells in most brain regions, denoting the presence of specific α1 and β3 subunit mRNA labelling. This is evident in figures 2.27 and 2.28. Density of mRNAs varied in different regions, consistent with varying densities of signal observed at the macroscopic level.

2.4.6.2 $GABA_C$ receptor mRNA distribution

The ρ1 subunit sense probe produced low signal at background levels only, and addition of excess unlabelled antisense probe to the hybridisation buffer containing labelled ρ1 subunit antisense probe for the most part abolished specific signal (see figure 2.24). The ρ1 subunit antisense probe also produced a low signal which in most cases appeared homogenous; for example, cortical laminae could not be distinguished even if whole section optical density was increased. This suggests perhaps that the signal obtained for ρ1 subunit probes was not specific, especially since in these cases, the excess unlabelled antisense probe did not abolish the signal. The bar graphs in figure 2.24 and the images in figure 2.31 depict these observations.

The ρ2 subunit sense probe commonly produced strong homogenous signal, as described in section 2.4.1. In many instances when this occurred experiments were repeated. Design and use of new probes corresponding to the large intracellular loop of the receptor subunit failed to alleviate the problem. Addition of excess unlabelled antisense probe to the hybridisation buffer containing labelled ρ2 subunit antisense probe, however, did abolish specific signal (refer to figure 2.24). The ρ2 subunit antisense probe also produced a low signal which, like the ρ1 subunit antisense probe, in many cases appeared homogenous. However, since excess unlabelled probe could abolish the signal, it was most likely to be specific to mRNA. The bar graphs in figure 2.24 and the images in figure 2.32 depict these observations.

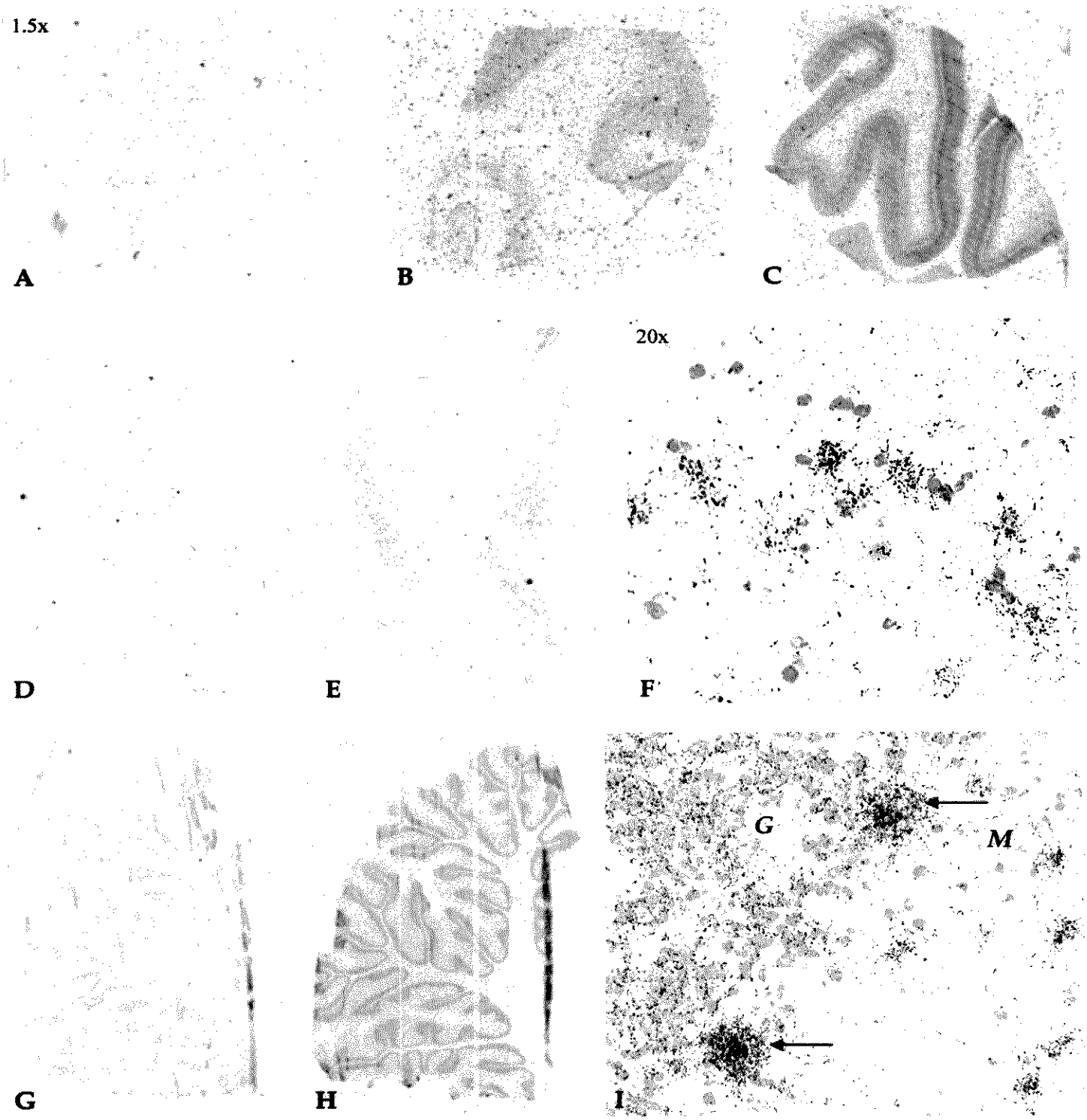


Figure 2.27: Autoradiogram and micrograph images of human brain sections hybridised with $GABA_A$ receptor $\alpha 1$ subunit sense and antisense probes. A, D & G were hybridised with sense probe, giving background signal only. B, C, E, F, H & I were hybridised with antisense probe. Increased density of grey in autoradiograms and clustering of silver grains around cell bodies in micrographs represent labelling of mRNA by the antisense probe. **A & B**. Hippocampus. **C**. Primary visual cortex. **D, E & F**. Polar temporal cortex. **G, H & I**. Cerebellum. Arrows point to Purkinje cells. Key in I: *M* = Molecular layer. *G* = Granule cell layer. Autoradiogram images 1.5x original macroscopic images. Micrographs taken at 20x magnification.

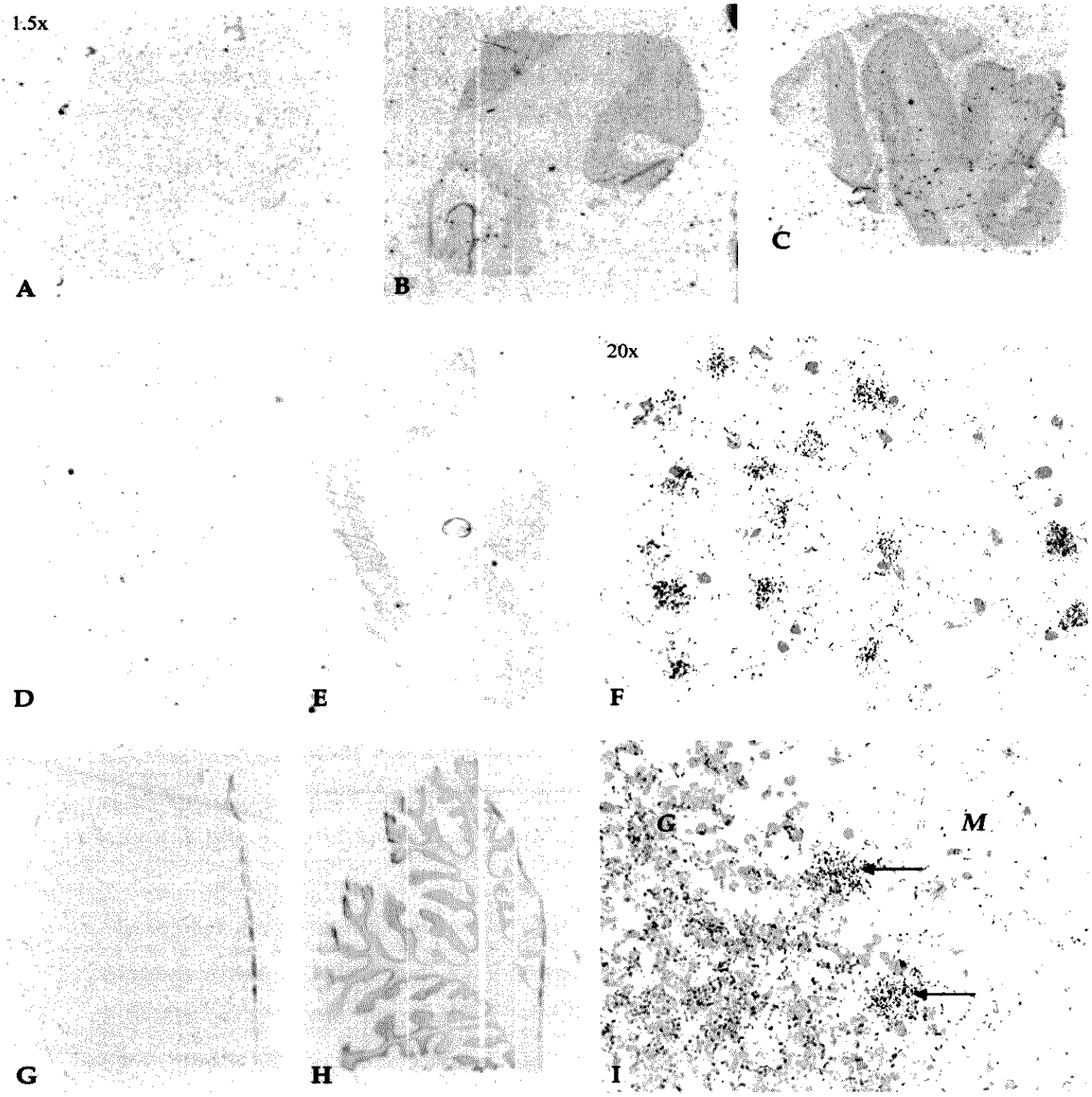


Figure 2.28: Autoradiogram and micrograph images of human brain sections hybridised with $GABA_A$ receptor β3 subunit sense and antisense probes. A, D & G were hybridised with sense probe, giving background signal only. B, C, E, F, H & I were hybridised with antisense probe. Increased density of grey in autoradiograms and clustering of silver grains around cell bodies in micrographs represent labelling of mRNA by the antisense probe. **A & B.** Hippocampus. **C.** Primary visual cortex. **D, E & F.** Polar temporal cortex. **G, H & I.** Cerebellum. Arrows on I point to Purkinje cells. Key in I: *M* = Molecular layer. *G* = Granule cell layer. Autoradiogram images 1.5x original macroscopic images. Micrographs taken at 20x magnification.

When signal intensity for sections hybridised with the $GABA_C$ receptor ρ1 subunit antisense probe was compared to that of sections hybridised with sense probe using dependent samples Student's *t*-tests, a statistically significant difference was observed in the amygdala, when assessed as whole sections (for full *t*-test results, refer to table iii, Appendix 4). This indicates that $GABA_C$ receptor ρ1 subunit mRNA was detected significantly above background in the amygdala (n=2). However, as shown in figure 2.24C, the antisense signal could not be abolished by the addition of excess unlabelled antisense probe, implying the observed increase in antisense signal is not specific to the mRNA present. When signal intensity for sections hybridised with the antisense probe was compared to that of sections hybridised with excess unlabelled antisense probe, no statistically significant differences were observed, when assessed as whole sections (for full *t*-test results, refer to table iii, Appendix 4).

A comparison of signal intensity for sections hybridised with the $GABA_C$ receptor ρ2 subunit antisense probe and sections hybridised with sense probe revealed no statistically significant difference, when assessed as whole sections (for full *t*-test results, refer to table iii, Appendix 4). When signal intensity for sections hybridised with the antisense probe was compared to that of sections hybridised with excess unlabelled antisense probe, a statistically significant difference was observed in the superior temporal gyrus (for full *t*-test results, refer to table iii, Appendix 4). This indicates that the $GABA_C$ receptor ρ2 subunit mRNA was detected significantly above background in the superior temporal gyrus (n=6).

The relative densities of ρ1 and ρ2 subunit mRNAs in differing regions of the human brain are shown in figures 2.29 and 2.30 respectively, once sense or excess unlabelled antisense probe signal has been subtracted to leave specific antisense probe signal. These graphs depict the density of mRNA in nCi/g tissue equivalent for the main regions of the human brain which could be quantified using macroscopic autoradiography. From figures 2.29 and 2.30 it is apparent that the ρ1 and ρ2 subunit transcripts were detectable above background, although not statistically significantly, and only at very low levels, in other regions of the human brain tested. For ρ1 subunit mRNAs, these included the central nucleus of the amygdala and primary visual cortex. Cerebellar sections demonstrated a higher level of ρ1 subunit mRNA, specifically in the

granule cell layer, as shown in figure 2.31. From figure 2.30 it is apparent that the ρ2 subunit mRNA was detectable above background in the central nucleus of the amygdala, middle frontal cortex and polar temporal cortex. In particular, the difference between antisense and antisense plus excess unlabelled probe signals was rather large for the polar temporal cortex (t=7.694, df=1, p=0.082). Cerebellar sections demonstrated a higher level of ρ2 subunit mRNA, specifically in the granule cell layer, as shown in figure 2.32.

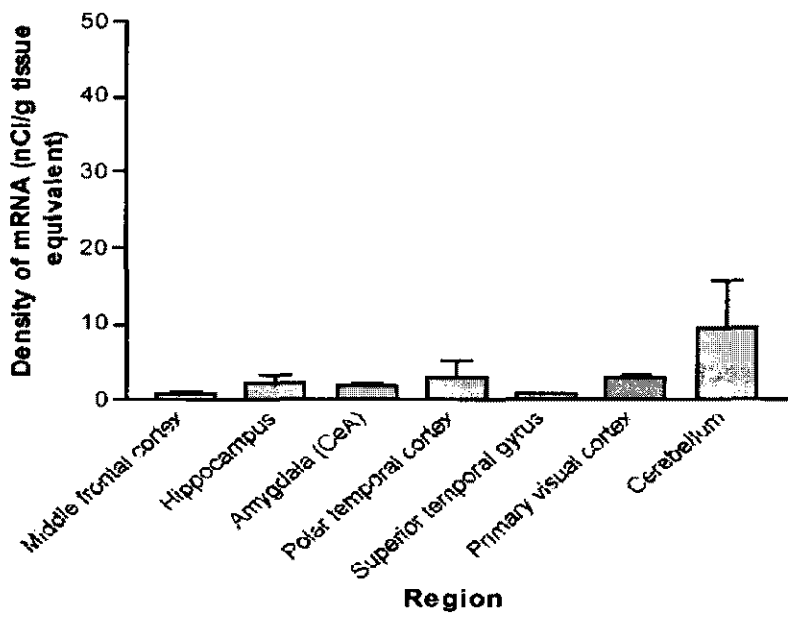


Figure 2.29: Bar graph representing density of $GABA_C$ receptor ρ1 subunit mRNA in regions of human brain, quantified macroscopically. Data are expressed as mean±s.e.mean. For superior temporal gyrus, n=6; middle frontal cortex and primary visual cortex, n=5; hippocampus, central nucleus of the amygdala, polar temporal cortex and cerebellum, n=2.

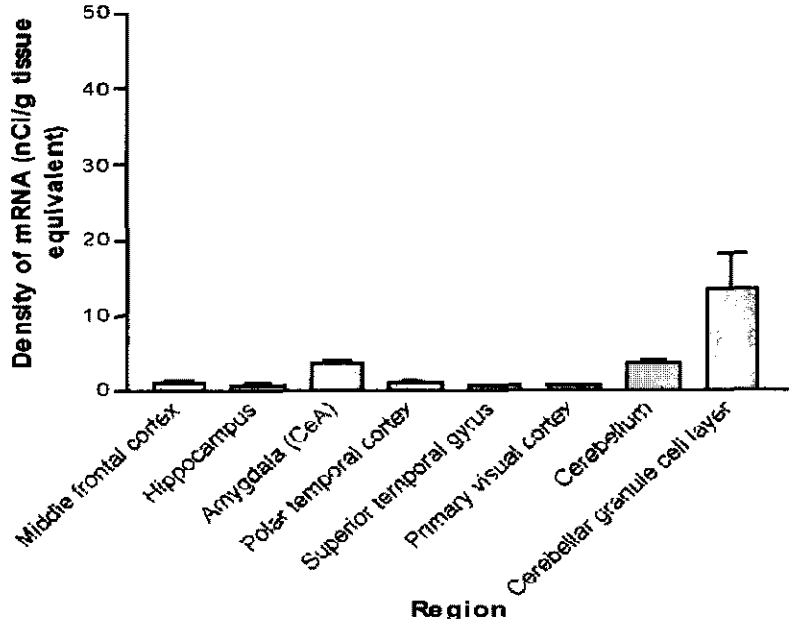


Figure 2.30: Bar graph representing density of $GABA_C$ receptor ρ2 subunit mRNA in regions of human brain, quantified macroscopically. Data are expressed as mean±s.e.mean. For superior temporal gyrus, n=6; middle frontal cortex and primary visual cortex, n=5; hippocampus, n=3; central nucleus of the amygdala, polar temporal cortex and cerebellum, n=2.

Figures 2.31 and 2.32 provide images of human brain sections hybridised with ρ1 and ρ2 subunit probes, respectively, from autoradiograms, showing sense probe, antisense

probe and antisense with excess unlabelled probe. Figures 2.31 and 2.32 also show images of human brain sections hybridised with ρ1 or ρ2 subunit probes after emulsion dipping to reveal microscopic silver grain deposition indicative of mRNA distribution. ρ1 Subunit sense probe produced low levels of silver grains evenly distributed across tissue components, signifying background labelling only. In some sections hybridised with ρ2 subunit sense probe, density of silver grains was moderate but distribution was even across regions without clustering around cell soma, consistent with the strong, generalised signal sometimes observed at the macroscopic level. In other sections hybridised with the ρ2 subunit sense probe, very low levels of silver grains with homogenous distribution were observed, indicative of background signal.

Microscopic examination of emulsion dipped sections hybridised with ρ1 or ρ2 subunit antisense probe often failed to reveal heterogeneity in the distribution of silver grains, without discrete distribution, denoting the absence of specific mRNA labelling. Silver grains were observed at low density, however not clustered around cell nuclei, evident in figure 2.31 and 2.32 F and I. These finding reinforce macroscopic images which depict low homogenous signal, even though antisense signal was greater than sense probe signal. Moreover, a relatively high level of background was evidenced by silver grain distribution across non-perikarya components of the tissue. However, specific labelling was apparent in the cerebellum from clustering of silver grains in the granule cell layer, albeit at low levels, around cell bodies.

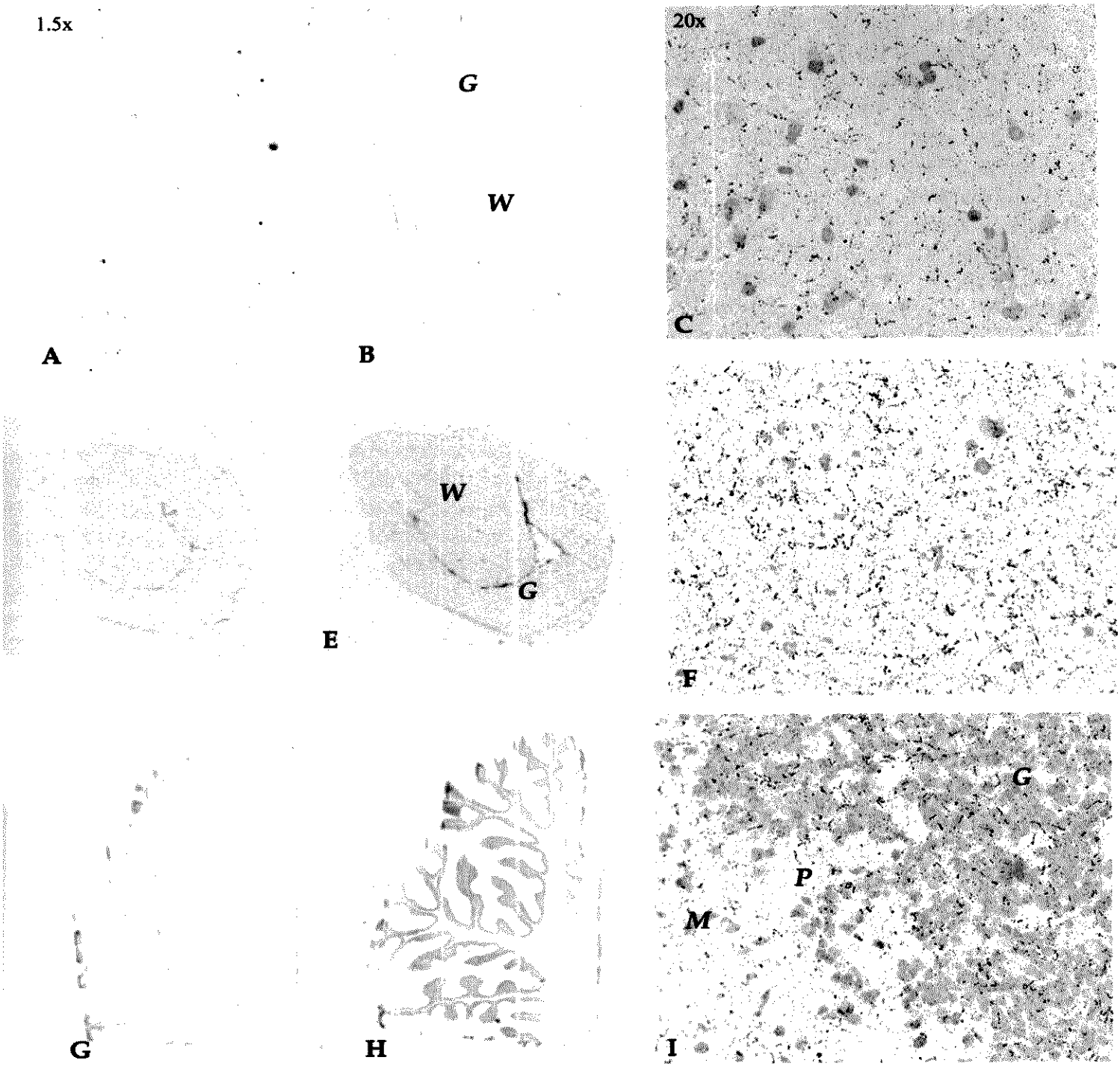


Figure 2.31: Autoradiogram and micrograph images of human brain sections hybridised with $GABA_C$ receptor ρ1 subunit sense and antisense probes. A, D & G were hybridised with sense probe, giving background signal only. B, C, E, F, H & I were hybridised with antisense probe. Increased density of grey in autoradiograms and clustering of silver grains around cell bodies in micrographs represent labelling of mRNA by the antisense probe. **A, B & C.** Polar temporal cortex. **D, E & F.** Primary visual cortex. **G, H & I.** Cerebellum. Key in B: *W* = White matter. *G* = Grey matter. Key in I: *M* = Molecular layer. *P* = Purkinje cell layer. *G* = Granule cell layer. Autoradiogram images 1.5x original macroscopic images. Micrographs taken at 20x magnification.

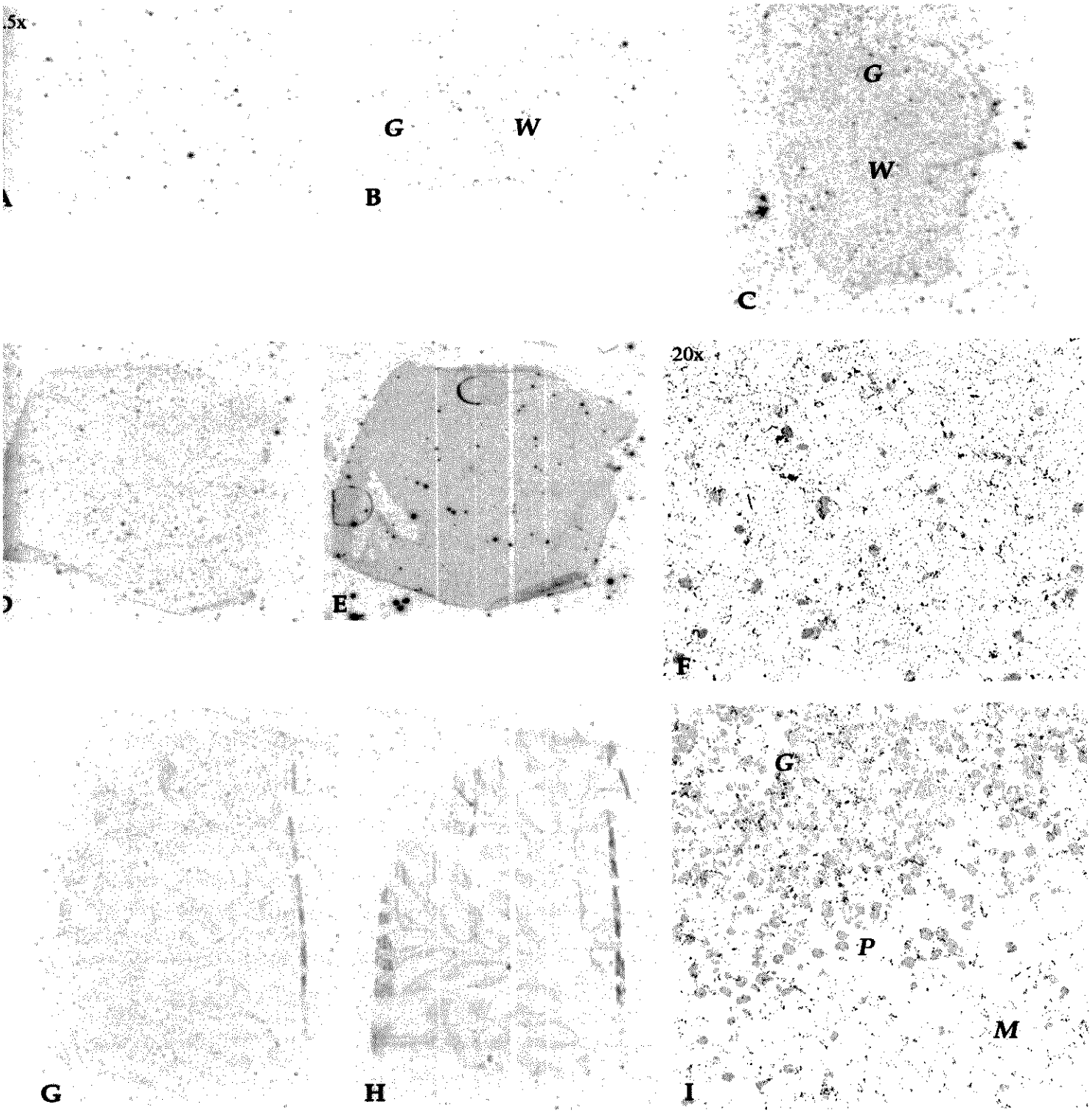


Figure 2.32: Autoradiogram and micrograph images of human brain sections hybridised with $GABA_C$ receptor ρ2 subunit sense and antisense probes. A, D & G were hybridised with sense probe, giving background signal only. B, C, E, F, H & I were hybridised with antisense probe. Increased density of grey in autoradiograms and clustering of silver grains around cell bodies in micrographs represent labelling of mRNA by the antisense probe. **A & B.** Middle frontal cortex. **C.** Superior temporal gyrus. **D, E & F.** Central nucleus of amygdala. **G, H & I.** Cerebellum. Key in B: *W* = White matter. *G* = Grey matter. Key in I: *M* = Molecular layer. *P* = Purkinje cell layer. *G* = Granule cell layer. Autoradiogram images 1.5x original macroscopic images. Micrographs taken at 20x magnification.

2.5 Discussion

The main findings of the current study were replication of published data on the distribution of ionotropic GABA receptor mRNAs in rat brain, and localisation of $GABA_A$ receptor mRNAs in human brain. To this end, ρ1 and ρ2 subunit mRNAs were confirmed in rat retina and α1, β3, ρ1 and ρ2 subunit mRNAs were detected in rat brain, supporting previous research. Furthermore, while it was difficult to localise $GABA_C$ receptor mRNAs in human brain, the present study demonstrated the presence of $GABA_A$ receptor mRNAs in regions of the human brain that have not previously been investigated.

2.5.1 Specificity of Signal and Factors Affecting Signal to Noise Ratio

One aim of this chapter was to establish an optimal *in situ* hybridisation protocol using rat brain. Optimisation of techniques entails producing a reliable protocol rendering high signal to noise ratio, that is, high specific signal with low background, and consistent results. Given the nature of *in situ* hybridisation, the length of the procedure, the number of factors involved in each step of the procedure, and the intrinsic problems of working with RNA, this task proved to be somewhat difficult. While all measures were taken to ensure highest laboratory standards, *in situ* hybridisation provides a wealth of possibilities for opportunistic contaminants. Consequently, some experiments had to be repeated in order to obtain reliable results.

A number of procedures were varied in order to test factors which may have contributed to the problems enumerated in section 2.4.3. Stringency of washes was altered to assess the effects on signal to noise ratio, with little effect, as seen in figure 2.4. As mentioned in section 2.4.3, tissue storage time, exchange of coverslips for parafilm, addition of an incubation step with hybridisation buffer prior to hybridisation with the labelled probe, and introduction of 10% DTT to the wash buffer also made no difference to level of background signal. It was thought that background may have been caused by tissue drying out between washes. Use of alternating solutions and rapid transferral of slide racks to avoid drying out of tissue during and between washes, though, made no difference to the background signal. Since the problems of high background arose with

particular probes in particular tissues, it seemed unlikely that it was due to contamination of the hybridisation buffer or its components.

The most likely factor contributing towards the high background encountered in some experiments was storage of labelled probes for re-use. While unused labelled probes were stored for no longer that 1 week at -20°C and were not used on more than one other occasion, freezing and re-thawing of the isotope is still thought to contribute to background (Wilcox, 1993). ^{35}S has an affinity for proteins, which is exacerbated by its radioactive decay products (Penschow & Coghlan, 1994). Furthermore, storage of labelled probe may have lead to partial degradation of the probes (Penschow & Coghlan, 1994). Another factor contributing to high background on the slide was excess formamide in the incubation tray. It was discovered that decreasing the amount of formamide in the incubation trays and using probes only once, immediately after they were labelled, reduced the problem of background remarkably.

It was also surmised that the terminal deoxytransferase was a contributing factor to the high background observed in some experiments. Duration of incubation period with TDT, concentration of enzyme and amounts of dNTPs relative to probe contribute to the resulting length of tail (Higgins & Mah, 1989; Penschow & Coghlan, 1994). While it was certainly the case that older TDT stocks markedly diminished tail labelling, hence radioactive counts, there was no consistent evidence to suggest that prolonged incubation with TDT contributed toward background. Calculations also showed that the amounts of enzyme and dNTPs (derived from [^{35}S]-dATP) relative to the probe concentration in the protocol used for these experiments, were in fact less than those used in many *in situ* hybridisation protocols in the literature, thus, this was not thought to be a contributing factor. The manufactured solution containing [^{35}S]-dATP did not contain cobalt ions, however one cannot exclude the possibility that cobalt ions in the TDT buffer may have precipitated during probe preparation, resulting in background. A further possibility is that the high background, stemming from the black speckles in particular, may have been caused by 'dirty' alcohol (Dr Louise Nicholson, personal communication). Although all steps were taken to minimise contamination of any solutions used, one cannot rule out this possibility, and filtering of ethanol should be carried out in any subsequent experiments.

Specificity of hybridisation signal was evidenced by the use of negative controls. Hybridisation with sense probes generally demonstrated weak, homogeneous labelling, compared to the corresponding antisense probe, which in most instances displayed a discrete and heterogeneous signal pattern. As described in section 2.4.1, in some cases the sense probe produced a strong homogeneous signal, which may be accounted for by a number of factors. The sense probe may have been hybridising to rRNA (Harrison & Pearson, 1995), which is abundant in all tissues, or to some unidentified mRNA (Heath *et al.*, 1996b). The latter suggestion is unlikely, however, since the observed signal was not confined to discrete structures or laminae. Since this high sense signal was specific to certain subjects but not others in some experiments, the probes may have been recognising endogenous "antisense" mRNA in the tissue, created by transcription of part of both DNA strands (symmetrical gene transcription) (Chesselet *et al.*, 1996; Higgins & Mah, 1989) to produce a misleading non-specific signal. Where possible, the experiment was repeated to obtain a negative signal with the sense probe. Where this failed consistently for the human ρ2 subunit, new probes were designed and purchased, however the sense probe continued to produce a strong signal on occasion.

Other factors which confirmed specificity of signal included white matter showing no hybridisation signal above background. While this was observed at all times for the $GABA_A$ receptor subunit α1 and β3 subunit antisense probes, this was not the situation in some instances for the $GABA_C$ receptor ρ1 subunit in rat forebrain, and the ρ1 and ρ2 subunits in human brain, evidenced in figures 2.31 and 2.32 respectively. This makes questionable the specific nature of the signal obtained. The use of a nonsense probe consistently produced signal at background levels only (data not shown). Furthermore, antisense signal was, for the most part, competitively reduced by the addition of excess unlabelled probe to the hybridisation solution. Unfortunately, again, this was not necessarily the situation for the ρ1 antisense with excess unlabelled probe, as it did not always reduce antisense signal. This was apparent in the superior temporal gyrus and central nucleus of the amygdala, and in some instances with rat brain (data not shown). Since the use of competition with unlabelled antisense probe is a more relevant means of ascertaining background signal, where this control did not reduce antisense signal, the antisense signal was not deemed specific. Specificity of signal was also evidenced at the microscopic level by clustering of silver grains, indicative of

mRNA labelling, over cell soma, and the heterogeneous distribution of silver grains over different brain regions.

2.5.2 Ionotropic GABA Receptor mRNAs in Rat Retina

Functional $GABA_C$ receptors are known to exist in retina (Feigenspan & Bormann, 1998), and the ρ subunits were isolated and cloned from retina. $GABA_C$-like responses in the retina have been correlated to the presence of ρ1 subunits (McCall *et al.*, 2002; Yeh *et al.*, 1996). One aim of the current study, therefore, was to assess whether the probes designed labelled the target ρ subunit mRNAs using rat retina as positive control tissue. The $GABA_A$ receptor α1 and β3 subunit mRNAs were also assessed, as they have previously been localised in retina using *in situ* hybridisation (Greferath *et al.*, 1993; Greferath *et al.*, 1995; Grünert, 1999, Kosaka *et al.*, 1994).

$GABA_C$ receptor ρ1 and ρ2 subunits were detected significantly above background in rat retina, evidenced by the statistically significant difference between signals elicited by sense (background) and antisense probes (refer to figure 2.5). These results concur with the literature on the prevalence of ρ subunits in rat retina and the correlation between the presence of ρ subunits and $GABA_C$-like responses consistently encountered from retina. Very importantly, since we expected to find the ρ subunit mRNA in rat retina, these results confirm that the probes designed do target the ρ subunits in rat retina. This was not the case, unfortunately, for the ρ3 subunit, which could not be detected above background levels. This may be because the sense probe produced a strong, homogenous signal in all tissues examined. The design of new probes may alleviate this problem, and is therefore an essential step for further investigation.

$GABA_A$ receptor α1 and β3 subunit antisense probe signals could not be detected significantly above background levels, although the lack of statistical significance was only marginal ($p=0.07$ and 0.09 for α1 and β3 subunits respectively, see table i, Appendix 4). Specific signal was apparent since subtraction of the sense probe left a specific component, that is, antisense signal above background levels, as seen in figure 2.6. The α1 and β3 subunit mRNAs were identified at lower levels than the ρ subunit mRNAs. Previous studies employing *in situ* hybridisation have localised α1 and β3

subunit mRNAs to the retinal inner nuclear and ganglion cell layers (Greferath *et al.*, 1993; Greferath *et al.*, 1995; Grünert, 1999; Kosaka *et al.*, 1994). The presence of the subunit mRNA in these regions was corroborated by immunohistochemistry in adjacent sections (Greferath *et al.*, 1993; Greferath *et al.*, 1995). Findings of the α1 and β3 subunit in rat retina in the current thesis are therefore consistent with the literature.

2.5.3 Ionotropic GABA Receptor mRNAs in Rat Brain

One aim of the current study was to assess the distribution and density of ionotropic GABA receptor mRNAs in rat brain prior to application to human brain. For this purpose, $GABA_A$ receptor α1 and β3 subunits were chosen for assessment as positive controls, given that they are well characterised. Investigation of these subunit transcripts further extends our knowledge of their distribution and relative density in rat brain. $GABA_C$ receptor ρ1, ρ2 and ρ3 subunits were chosen for investigation as they were only recently described and little is known of the composition or role of $GABA_C$ receptors in the CNS.

2.5.3.1 $GABA_A$ receptor subunit distribution

$GABA_A$ receptor α1 and β3 subunit mRNAs were observed in many regions of the rat brain, evidenced by a statistically significant difference between sense and antisense probe signals measured from whole rat brain sections, and at the microscopic level. Of all the subunit mRNAs measured in rat brain, the α1 subunit mRNA exhibited the most prolific distribution, in keeping with previous *in situ* hybridisation experiments (Laurie *et al.*, 1992; Persohn *et al.* 1992; Wisden *et al.*, 1992). Immunohistochemical experiments have supported this finding, showing that α1, β1-3 and γ2 subunits were the most widespread (Fritschy & Möhler, 1995; Pirker *et al.*, 2000). The α1β2γ2 subunit combination is known to be the most commonly occurring in rat brain, accounting for around 40-50% of all $GABA_A$ receptors (Benke *et al.*, 1991b; De Blas, 1996; McKernan & Whiting, 1996; Sieghart, 2000). Thus it appears consistent that in the present study, the α1 subunit should arise in most brain areas investigated.

In situ hybridisation studies have found that the β3 subunit co-localises with the α2 subunit most commonly (Laurie *et al.*, 1992; Persohn *et al.*, 1992; Wisden *et al.*, 1992).

McKernan & Whiting (1996) revealed approximately 18% of $GABA_A$ receptors *in vivo* are formed from $\alpha 2\beta 2/3\gamma 2$ subunits. Thus, the relatively extensive distribution of $\beta 3$ subunits observed in the current investigation is to be expected.

The distribution and relative density of $\alpha 1$ and $\beta 3$ subunit mRNAs in the present study correlated well with previous *in situ* hybridisation studies of the subunits in rat brain (Persohn *et al.*, 1992; Wisden *et al.*, 1992), as well as immunohistochemical studies (Fritschy & Möhler, 1995; Pirker *et al.*, 2000). A comparison of the distribution of these transcripts obtained from the current study, given in table 2.3, and results from previous *in situ* hybridisation studies, given in table 1.1, reveals many similarities.

The presence of $\alpha 1$ subunit mRNA was confirmed in the tenia tecta, neocortex, piriform cortex, globus pallidus, ventral pallidum, entopeduncular nucleus, hippocampal formation, medial and lateral septum, diagonal band of the vertical and hind limbs, thalamus, zona incerta, basolateral amygdaloid nuclei, superior colliculus, red nucleus, bed nucleus of the stria terminalis, substantia nigra, cerebellar cortical layers and brainstem. Moreover, in the current study, a stronger signal was observed in the medial thalamic nuclei, although mRNA was still abundant in the lateral thalamic nuclei. The medial septum also yielded far greater signal than the lateral septum, consonant with previous findings (Wisden *et al.*, 1992). In the present investigation, $\alpha 1$ subunit mRNA was also observed in the nucleus accumbens, endopiriform nuclei, hypothalamus and medial amygdaloid nuclei and weak labelling at the microscopic level in the caudate-putamen. Prior *in situ* hybridisation studies reported only weakly positive signal in the hypothalamus, nucleus accumbens, caudate-putamen and medial amygdala for the $\alpha 1$ subunit (Wisden *et al.*, 1992), yet previous immunohistochemical studies have confirmed the presence of $\alpha 1$ subunits in these regions, as indicated in table 1.2. The current study also reports the presence of $\alpha 1$ subunit mRNA in the olfactory tubercle, insular cortex, pretectal nuclei, interpeduncular nuclei and periaqueductal grey which has not been communicated in previous studies. Results from the present examination diverge from previous *in situ* hybridisation studies on the $\alpha 1$ subunit in that mRNA was not detected in the spinal cord.

The presence of the β3 subunit transcript was confirmed in the tenia tecta, neocortex, piriform cortex, nucleus accumbens, striatum, lateral septum, diagonal band of the vertical limb, hippocampal formation, basolateral and medial amygdaloid nuclei, thalamus, hypothalamus, medial habenular nuclei, superior colliculus, cerebellar granule cell layer, brainstem and spinal cord. The present examination also reports the appearance of β3 subunit mRNA in the olfactory tubercle, insular cortex, endopiriform nucleus and periaqueductal grey, which has not been communicated in previous studies. Immunohistochemical studies have confirmed the presence of β3 subunits in the insular cortex and endopiriform nucleus, as seen in table 1.2. Results from the current investigation diverge from previous *in situ* hybridisation studies on the β3 subunit in that the transcript was not detected in the cerebellar Purkinje cell layer, evident in figure 2.12F.

The relative densities of α1 and β3 subunit transcripts differed in regions of the brain quantified. α1 Subunit mRNA appeared to be most abundant in the cerebellum, specifically in the granule cell layer, followed by the medial septum and piriform cortex. The α1 subunit transcript was also abundant in the neocortex, dentate gyrus, hippocampus and molecular layer of the cerebellum. The thalamus showed marked density of mRNA, especially in the medial nuclei. Lower levels of α1 subunit mRNA were detected in the striatum, lateral septum, amygdala, hypothalamus and brainstem.

β3 subunit mRNA appeared in greatest density in the dentate gyrus, followed by the piriform cortex, granule cell layer of the cerebellum, olfactory tubercle and hippocampus. The transcript was also highly abundant in the nucleus accumbens, and showed a marked density in the neocortex and striatum, followed by the septum and amygdala. Low levels of β3 subunit mRNA were detected in the thalamus, hypothalamus and superior colliculus. Very low mRNA levels were found in the brainstem and spinal cord. It is difficult to comment on the relative density of α1 subunit transcripts in the olfactory tubercle, nucleus accumbens, substantia nigra, periaqueductal grey, red nucleus and pretectal nucleus, and β3 subunit mRNA in the periaqueductal grey and endopiriform nucleus, as these regions were quantified in only one subject.

A comparison of figures 2.13 and 2.14 displaying the relative density of α1 and β3 subunit mRNAs respectively reveals variation in their relative densities in differing brain regions. In the neocortex, α1 subunit mRNA appears to be more prolific than β3 subunit mRNA. This is also the case in the thalamus and granule cell layer of the cerebellum. On the other hand, β3 subunit mRNA occurs in greater abundance than the α1 subunit transcript in the striatum, hippocampus and dentate gyrus. In most other regions where both subunit mRNAs are expressed, they appear in similar densities. While the present study does not quantify the precise cellular distribution of α1 and β3 subunit mRNAs, their apparent co-distribution in many brain regions does not preclude co-localisation of the subunit mRNAs. Thus, it may be the case that in some brain regions, for example, in the cerebellar granule cell layer where both subunit mRNAs were localised, the two subunits combine to form $GABA_A$ receptors of the $\alpha1\beta3\gamma n$ composition.

As mRNA is the machinery for protein expression, the presence of mRNA generally predicts protein expression in the regions of the brain the transcript is mapped to. This may not always be the circumstance, however, where mRNA in the cell soma translates to protein which is transported down the axon of projection neurons to nerve terminals in other regions of the brain. Since GABAergic inputs are predominantly local, however, it is unlikely that the receptors would occur at sites remote from soma where translation is occurring (Dodd & Lewohl, 1998).

From the literature it appears that α1 and β3 subunit mRNAs are commonly observed in regions of the brain where their respective polypeptides have been localised, with immunohistochemical detection paralleling mRNA distribution for the most part. The extensive distribution of α1 and β3 subunit transcripts anticipates a role for these subunit in many brain functions. Owing to the heterogeneity of $GABA_A$ receptors, the composition of receptors containing the α1 or β3 subunits is likely to vary, thus the role of the subunit in differing regions may also vary. While the α1 subunit is most commonly found in conjunction with the β2 and γ2 subunits (Benke *et al.*, 1991b; De Blas, 1996; Laurie *et al.*, 1992; McKernan & Whiting, 1996; Persohn *et al.*, 1992; Wisden *et al.*, 1992), it may also be present in other subunit combinations. The role of

the receptor is also contingent upon the cell it is expressed in; interneuron or projection neuron; and whether it is expressed pre- or post-synaptically.

It is tempting to conclude a role for the receptor based on known functions of particular brain regions. In the case of the α1 and β3 subunits, one may hypothesise a role in most rat brain functions, including learning and memory (cortex, hippocampus, cerebellum), integration of sensory and motor functions (thalamus, cortex, basal ganglia, cerebellum), conditioned emotion and anxiety (amygdala), homeostatic mechanisms (hypothalamus), vision (retina, superior colliculus), pain mechanisms (periaqueductal grey) and control of lower order functions (brainstem).

2.5.3.2 $GABA_C$ receptor ρ1 subunit distribution

$GABA_C$ receptor ρ1 subunit mRNA was detected in rat brain, implied by a statistically significant difference between sense and antisense probe signals measured from whole rat brain sections. With exception to the cerebellar sections, the increase in antisense probe signal in comparison to sense did not, however, appear distinctive, in that the antisense signal was generalised without structural recognition. This suggests that either the antisense signal was not in fact specific for the ρ1 subunit mRNA, or that the mRNA was present at very low levels. Dissemination of mRNA can occur when tissue is inadequately frozen or fixed (Penschow and Coghlan, 1994), and this may explain the generalised signal. Nonetheless, the same tissue was used for all other probes, where antisense signal was definitive, hence incompetent tissue preparation cannot be the cause. Microscopic examination of emulsion dipped sections revealed no heterogeneity in the distribution of silver grains, denoting an absence of specific ρ1 subunit mRNA labelling. Emulsion dipping provides a more sensitive means of visualising the radioactively-labelled probe.

The distribution and relative density of ρ1 subunit mRNA in the current investigation correlated well with previous studies examining the distribution of ρ1 subunit mRNA using RT-PCR, Northern blotting and *in situ* hybridisation. Results obtained from the present study are given in table 2.3, and results from previous investigations are given in table 1.3. Most prior *in situ* hybridisation studies failed to detect ρ1 subunit mRNA outside of the retina (Albrecht *et al.*, 1997; Enz *et al.*, 1995; Wegelius *et al.*, 1998), and

results from the present examination are in keeping with this. The exception is the obvious presence of ρ1 subunit mRNA in the granule cell layer of the cerebellum. This result is confirmed by radioligand binding assays of the $GABA_C$ receptor (Drew *et al.*, 1984; Drew & Johnston, 1992). Prior *in situ* hybridisation studies have documented ρ1 subunit mRNA in the cerebellar cortex, in the Purkinje cell layer only (Boue-Grabot *et al.*, 1998), and immunohistochemistry against the $GABA_C$ receptor proved their existence in the Purkinje cell layer (Boue-Grabot *et al.*, 1998). Thus, findings in the current thesis diverge from previous studies. Probes used in the present examination were directed against the N terminal region of the ρ1 subunit, while those in previous *in situ* hybridisation studies were designed against the large intracellular loop of the subunit, however this made little difference to ρ1 subunit mRNA detection.

RT-PCR, which is a more sensitive method of mRNA detection, revealed ρ1 subunit mRNA expression in the hippocampus, thalamus, superior colliculus, cerebellum, brainstem and spinal cord (Boue-Grabot *et al.*, 1998; Wegelius *et al.*, 1998). In the current investigation, ρ1 subunit mRNA was quantified in the hippocampus, thalamus, superior colliculus and brainstem of only one subject. In the same subject, the mRNA was also observed in the cortex, dentate gyrus, pontine nuclei, and very low levels in the amygdala. In all other subjects, however, even at the cellular level in emulsion dipped sections, ρ1 subunit mRNA was not detected in most of these regions, although microscopic examination revealed increased density of silver grains over the hippocampus and dentate gyrus in two other subjects. An inability to detect ρ1 subunit mRNA in rat brain outside the retina and cerebellar granule cell layer for most subjects may imply that the transcript is not present in any other regions of the rat CNS. Alternatively, problems with the probes may have precluded optimal hybridisation to the tissue mRNA. An analysis of the ρ1 subunit probes in Net Primer gave an 83% rating, and revealed the potential for hairpin loops, dimerisation and palindromes which may have reduced the probe's ability to bind at full length to the tissue mRNA, hence decreasing hybridisation signal. An alternate and likely explanation is that the subunit mRNA is present at such low levels that it cannot be detected by *in situ* hybridisation, as was the case in previous studies. This possibility is highlighted by the finding of specific ρ1 subunit mRNA signal in one subject, as shown in figure 2.22. Investigation

of ρ1 subunit mRNA in rat brain using RT-PCR would help elucidate whether this was the case in the present examination.

One cannot disregard the important fact that while the α1 and β3 subunits have been cloned from rat brain, the ρ1 subunit has been cloned from rat retina only. Thus, while a strong positive signal could be obtained from rat retina using the ρ1 subunit probes designed for the current thesis, confirming the probes could recognise the ρ1 subunit mRNA, a similar signal was not consistently obtained from rat brain. One possible explanation is that the ρ1 subunit in rat brain is similar, but not identical in nucleotide sequence, to the retinal ρ1 subunit. The rat brain sequence may be of different length or may be a splice variant of the retinal equivalent. Evidence for splice variants comes from Northern blotting experiments where two different sized mRNAs for the ρ1 subunit were detected from rat retina and pituitary gland, a 5kb and 4.5kb fragment respectively (Boue-Grabot *et al.*, 2000). The discovery of alternative splicing in the N terminal of the human ρ1 subunit (Martínez-Torres *et al.*, 1998) alludes to a potentially similar situation with the rat retinal subunit. Since the probes used in the current study were designed against the N terminal region of the ρ1 subunit, an inability to detect mRNA may have resulted from probe design to a splice site in the nucleotide sequence. Investigation of alternative splicing of the ρ1 subunit in rat retina and brain using total RNA extraction and RT-PCR with primers designed to amplify the N-terminal region of the receptor subunit is necessary to address this point.

It is difficult to comment on the relative density of ρ1 subunit mRNA in regions of the rat brain since it was quantified in only one subject. Despite this, one may assert without hesitation that the greatest density of ρ1 subunit mRNA occurred in the cerebellar granule cell layer, evident in figure 2.22.

It is equally difficult to comment on the potential role for $GABA_C$ receptors comprised of ρ1 subunits in rat brain, given the subunit mRNA could be demonstrated repetitively in the retina and cerebellum only. While the precise cellular localisation of the ρ1 subunit mRNA has not been quantified in the current study, the distribution of the

transcript allows one to infer a role for $GABA_C$ receptors comprised of ρ1 subunits in vision (retina) and the coordination of vision and movement (cerebellum).

2.5.3.3 $GABA_C$ receptor ρ2 subunit distribution

$GABA_C$ receptor ρ2 subunit mRNA was detected in rat brain. While no statistically significant differences were observed between sense and antisense probe signals measured from whole rat brain sections, many regions of the rat brain could be quantified macroscopically and specific signal was produced by the antisense probe after sense probe signal had been subtracted. Microscopic examination of emulsion dipped sections revealed a degree of heterogeneity in silver grain distribution, denoting the presence of specific ρ2 subunit mRNA labelling. Thus, one may affirm that ρ2 subunit mRNA was detectable above background in most subjects. Large inter-subject variability accounts for the lack of statistical significance, as the density of ρ2 subunit mRNA differed between subjects such that in some subjects, a considerable density of mRNA was observed, while other subjects demonstrated a more conservative density. The lack of statistical significance between sense and antisense probes may also be accounted for by the strong, homogenous signal often produced by the ρ2 sense probe, discussed in section 2.5.1.

The distribution of ρ2 subunit mRNA in the current investigation correlated well with previous studies examining the distribution of ρ2 subunit mRNA using RT-PCR, Northern blotting and *in situ* hybridisation. A comparison of results from previous studies, given in table 1.3, and findings from the present examination, given in table 2.3, reveals many similarities. The presence of ρ2 subunit mRNA was confirmed in the neocortex, hippocampus, thalamus, superior colliculus, midbrain and cerebellum. In the present study, ρ2 subunit mRNA was also determined in the dentate gyrus, piriform cortex, habenular nuclei, hypothalamus and brainstem, as shown in figure 2.23. In one subject, density of silver grains was increased over cells in the islands of Calleja and the lateral septum. ρ2 subunit mRNA has not been reported in these brain areas in previous studies. Microscopic examination did not confirm the presence of ρ2 subunit mRNA in the amygdala as the silver grains did not appear to be clustered around neurons.

Higgins and Mah (1989) caution against interpretation of specific mRNA signal in brain structures containing high levels of rRNA, including the dentate gyrus and pyramidal layer of the hippocampus, granule cell layer of the cerebellum, piriform cortex and medial habenular nucleus. rRNA may exhibit high background in these regions which is commonly perceived as specific signal (Higgins & Mah, 1989). Nevertheless, since the antisense signal could be abolished by the addition of excess unlabelled probe, one may assume the antisense signal was specific. These findings should, however, be confirmed using other methods such as immunohistochemistry or radioligand binding.

Results from the current study diverge from previous investigations of ρ2 subunit mRNA in that the subunit was not consistently detected in the basal ganglia or spinal cord. Divergence in results may be accounted for by the fact that ρ2 subunit mRNA was previously discovered in these regions using RT-PCR and not *in situ* hybridisation (Enz *et al.*, 1995). The mRNA may occur in these areas at levels too low to be determined by *in situ* hybridisation. Probes used in the current thesis were directed against the N terminal region of the ρ2 subunit, while those in previous studies were designed against the large intracellular loop, however this is not anticipated to alter detection of the target mRNA. As for the ρ1 subunit, one cannot disregard the important fact that the ρ2 subunit has been cloned from rat retina only. A strong positive signal could be obtained from rat retina using the ρ2 subunit probes designed for the current investigation, confirming the probes could recognise the ρ2 subunit mRNA. Identification in rat brain, however, was not quite so robust, although specific signal was observed. Rat brain yielded much lower levels of ρ2 subunit mRNA than retina, concordant with the literature (Enz *et al.*, 1995; Wegelius *et al.*, 1998). Detection of target mRNA may be interrupted if the ρ2 subunit in rat brain is a variant of the retinal equivalent. Evidence for alternative splicing of the ρ2 subunit and a difference between retina and brain was documented by Boue-Grabot *et al.* (2000), who demonstrated, using Northern blot experiments, that ρ2 subunit mRNA from the rat pituitary gland was shorter in size than that from retina. Wegelius and coworkers (1998) also discovered three different sized transcripts of ρ2 mRNA using Northern analysis. 2.3kb and 2kb fragments were found in retina, hippocampus and superior colliculus, and only the 2kb fragment in the cortex.

In comparison to the $GABA_A$ receptor $\alpha 1$ and $\beta 3$ subunit mRNAs, it is apparent that the $\rho 2$ subunit mRNA is not very widespread in the rat CNS, which may belie rather specific functions of the $GABA_C$ receptors comprising this subunit. Moreover, it is expressed at much lower densities in the brain regions quantified compared to the $GABA_A$ receptor subunit mRNAs examined. The relative density of $\rho 2$ subunit mRNA varied between brain regions, apparent in figure 2.23. $\rho 2$ Subunit mRNA appeared most abundant in the cerebellar granule cell layer, followed by the habenular nuclei and dentate gyrus. The transcript was also prevalent in the hippocampus and hypothalamus. Rather low levels of the transcript occurred in the neocortex, piriform cortex, thalamus, superior colliculus, periaqueductal grey and brainstem. Very low levels of the mRNA were detected in the olfactory and pontine nuclei.

Based on current findings and those recorded in the literature, it is possible that cerebellar $GABA_C$ receptors reported by Drew and colleagues (1984) and Drew & Johnston (1992) are homooligomeric, formed from $\rho 1$ or $\rho 2$ subunits, or pseudo-heterooligomeric, formed from a combination of $\rho 1$ and $\rho 2$ subunits. Investigation of these subunits at the cellular level using immunohistochemistry is required before any further conclusions can be drawn. The possibility also remains that $\rho 2$ subunits combine with $GABA_A$ receptor subunits in the granule cell layer of the cerebellum (Wall, 2001). Given the localisation of $\rho 2$ subunit mRNA in the rat CNS, it seems likely that other $GABA_C$-like responses reported in mammalian brain, for example in the hippocampus and superior colliculus, may be mediated by receptors comprised of $\rho 2$ subunits. $GABA_C$-like responses observed in the hippocampus and pituitary gland are different to those detected in the retina, indicating slightly different receptor subtypes are responsible (Boue-Grabot *et al.*, 2000; Cherubini & Strata, 1997). The fact that $\rho 2$ receptors are found predominantly outside the retina, notably in the hippocampus, means that the different responses observed in the hippocampus and other regions may be mediated by $\rho 2$ receptors. In the hippocampus, for example, $GABA_C$ receptors are less sensitive to GABA and cannot be distinguished by CACA (Cherubini & Strata, 1997).

While human recombinant $\rho 2$ subunits expressed in *Xenopus* oocytes form functional receptors which are physiologically and pharmacologically characteristic of $GABA_C$

receptors, rat ρ2 subunits fail to form robust homooligomeric receptors in *Xenopus* oocytes (Zhang *et al.*, 1995). Although this may not be the case for ρ2 subunits in rat brain, one cannot confidently declare the existence of functional homooligomeric $GABA_C$ receptors formed from ρ2 subunits in rat CNS. Furthermore, ρ2 subunits have not been cloned from rat brain, therefore the presence of the subunit mRNA does not imply the formation of functional $GABA_C$ receptors. Moreover, at least in rat retina, $GABA_C$-like responses have been correlated to the presence of ρ1 subunits (McCall *et al.*, 2002; Yeh *et al.*, 1996) but not ρ2 subunits (Yeh *et al.*, 1996). Nonetheless, findings in the current study point to the existence of ρ2 subunit mRNA in rat brain, which may account for the $GABA_C$-like responses observed in mammalian CNS. The recent report of immunohistochemical localisation of the ρ subunit polypeptide in the superior colliculus of postnatal rats detected using immunohistochemistry (Clark *et al.*, 2001) affirms this possibility.

Results from the current thesis do not support the existence of $GABA_C$ receptors formed from ρ subunits in the spinal cord, where $GABA_C$-like responses were first discovered by Johnston and colleagues (1975). This may be due to a species difference - initial studies were performed in cats whereas the present study uses rats. Alternatively, the ρ subunits may have been present, but expressed at levels too low to be detected by *in situ* hybridisation. This is a likely explanation given previous examinations have isolated ρ1 subunit mRNA in the dorsal root ganglia and ρ2 subunit mRNA in the spinal cord using RT-PCR and Northern blotting (Enz *et al.*, 1995; Ogurusu *et al.*, 1995; Wegelius *et al.*, 1998). Although ρ2 subunit mRNA levels were too low to be detected in spinal cord at the regional level, at the microscopic level silver grains denoting labelled ρ2 subunit mRNA were apparent in one subject, seen in figure 2.21.

If one assumes the presence of ρ2 subunit mRNA generally predicts ρ2 subunit protein expression in the regions of the brain the mRNA was mapped to, one may infer a role for receptors comprising these subunits. Receptors containing the ρ2 subunit may therefore be involved in learning and memory (cortex, hippocampal formation, cerebellum), vision (retina, superior colliculus), coordination of vision and movement

(cerebellum), integration of sensory information (thalamus, cortex), homeostatic mechanisms (hypothalamus) and control of lower order functions (brainstem).

2.5.3.4 $GABA_C$ receptor ρ3 subunit distribution

$GABA_C$ receptor ρ3 subunit mRNA was not detected above background in rat brain except in the cerebellar granule cell layer of one subject. No statistically significant differences were observed between sense and antisense probe signals measured from whole rat brain sections. Macroscopic and microscopic examination further revealed no specific signal or labelling of discrete structures, and no heterogeneity in the distribution of silver grains, denoting an absence of specific ρ3 subunit mRNA labelling. These findings are surprising in light of previous findings of ρ3 subunit mRNA in regions of the rat brain (Boue-Grabot *et al.*, 1998; Wegelius *et al.*, 1998) outlined in table 1.3. Although one previous study failed to detect ρ3 subunit mRNA outside the retina using Northern blotting (Ogurusu & Shingai, 1996), the current examination failed to detect ρ3 subunit mRNA even in the retina. While the ρ3 sense probe commonly demonstrated a strong, homogenous signal, this factor alone does not account for the lack of statistical significance and inability to detect ρ3 subunit mRNA. Previous researchers have located ρ3 subunit mRNA in regions of the rat brain using RT-PCR, and it may be the circumstance that *in situ* hybridisation is not sensitive enough to detect the low levels of the ρ3 subunit transcript. Another possibility is that part or all of the mRNA may not have withstood post-mortem changes and tissue treatment. This seems unlikely, however it cannot be excluded. Again, as for the ρ1 and ρ2 subunits, it is necessary to bear in mind that the ρ3 subunit has been cloned from retina, therefore probes were designed against retinal subunit mRNA, not brain mRNA. This does not seem to have made a difference, nevertheless, considering ρ3 subunit could not be detected in rat retina using the probes designed for the present examination. Again, it is possible that splice variants exist of the ρ3 subunit, involving part of the nucleotide sequence the ρ3 subunit antisense probe was designed against, thus excluding hybridisation. Total RNA extraction and RT-PCR must be performed to address the possibility that mRNA was present at levels too low to be detected using *in situ* hybridisation in the current examination. This technique would also provide information on potential splice variants

of the ρ3 subunit in rat brain. Design of new probes for *in situ* hybridisation is also an essential step for further investigation.

2.5.4 Ionotropic GABA Receptors in Human Brain

Another aim of the current chapter was to apply the optimised *in situ* hybridisation techniques to ascertain the distribution of $GABA_C$ receptor subunit mRNA *in situ* in human brain. No study in the literature thus far has examined the ρ subunit distribution in human brain *in situ*. The distribution and density of $GABA_A$ receptor α1 and β3 subunit mRNAs was investigated in parallel to serve as positive controls and to further extend our knowledge of the expression of these subunits in human brain.

At this junction it is requisite to mention that although statistical analysis with human brain did not render significance for the most part, the qualitative information gained is still of great importance. Lack of statistical significance is rather meaningless owing to small sample size and large variability encountered between subjects.

2.5.4.1 $GABA_A$ receptor α1 subunit mRNA

$GABA_A$ receptor α1 subunit mRNA was detected significantly above background in the middle frontal cortex superior temporal gyrus and hippocampus of the human brain, as seen in figure 2.24. Examination of sections hybridised with α1 subunit antisense probe at the cellular level further supported the presence of α1 subunit mRNA labelling in these brain areas, and consolidated the presence the α1 subunit mRNA in other regions of the brain examined, including the central nucleus of the amygdala, polar temporal cortex, primary visual cortex and cerebellum. In the cerebellum, α1 subunit mRNA was localised to the granule cell layer, Purkinje cell layer and molecular layer, shown in figures 2.26 and 2.27. From these results it is reasonable to state that, as for the rat brain, α1 subunit mRNA appears to be rather widely distributed in human brain also.

Previous studies examining the presence of α1 subunit mRNA in human brain using *in situ* hybridisation and RT-PCR have localised the transcript to few regions of the brain, namely the frontal cortex, including prefrontal cortex, motor cortex, middle frontal gyrus and superior frontal cortex, the entorhinal cortex, and brainstem (Akbarian *et al.*, 1995b;

Dodd & Lewohl, 1998; Hawkins & Dodd, 2000; Lewohl *et al.*, 1997; Longson *et al.*, 1997; Mitsuyama *et al.*, 1998; Ohnuma *et al.*, 1999). Mapping of α1 subunit mRNA to the few brain regions mentioned here is contingent upon the limited number of studies assessing the distribution and density of $GABA_A$ receptor subunits in human brain, given that tissue is generally difficult to obtain and the variance between subjects is quite large. Many of the investigations have concentrated predominantly on disease states, however information on distribution in normal brain is also provided in such studies. Prior immunohistochemical analyses have localised α1 subunits to various human brain regions, including the cerebral cortex, striatum, globus pallidus, hippocampus, subiculum and entorhinal cortex (Houser *et al.*, 1988; Loup *et al.*, 1998; Wainwright *et al.*, 2000; Waldvogel *et al.*, 1999). Results from the present examination therefore add to the body of literature on the distribution of α1 subunit mRNA in human brain, reporting on its presence in the middle frontal cortex, superior temporal gyrus and hippocampus, and likely distribution in the central nucleus of the amygdala, polar temporal cortex, primary visual cortex and cerebellum. Findings in human brain parallel those of rat brain, since α1 subunit mRNA was localised to cerebral cortex, hippocampus, amygdala (although not central nucleus) and cerebellum of rat brain in the current study.

The relative density of α1 subunit mRNA differed in regions of the brain quantified, indicated in figure 2.25. α1 Subunit mRNA appeared to be most substantially expressed in the cerebellum, specifically in the granule and Purkinje cell layers, followed by the primary visual cortex then polar temporal cortex. mRNA was also abundant in the cerebellar molecular layer, followed by the middle frontal cortex and central nucleus of the amygdala. The lowest density of mRNA was discovered in the superior temporal gyrus.

2.5.4.2 $GABA_A$ receptor β3 subunit mRNA

$GABA_A$ receptor β3 subunit mRNA, like the α1 subunit transcript, was mapped to most areas of the brain examined in the current investigation. β3 Subunit mRNA was detected statistically significantly above background in the middle frontal cortex. Although the difference between sense and antisense signal in this region was not statistically significant, the difference between antisense and antisense with excess unlabelled probe signal was. The lack of statistical significance between sense and

antisense probe signals is best accounted for by the strong homogenous signal sometimes observed with the β3 sense probe. While statistical significance was not observed for other brain regions quantified, strong evidence for the presence of β3 subunit mRNA in these regions comes from the fact that signal was still observed when background was subtracted, and at the microscopic level where silver grain deposition was laminar and clustered around cell soma, as shown in figure 2.28. Thus, the β3 subunit mRNA was further encountered in the cerebellum, particularly the granule and Purkinje cell layers, primary visual cortex, hippocampus, temporal pole, middle frontal cortex, superior temporal gyrus and central nucleus of the amygdala.

Few published studies have reported on β3 subunit mRNA distribution in human brain. Mitsuyama and colleagues (1998) mapped β3 subunit mRNA to the frontal cortex, and the transcript has also been shown in the hippocampus CA1, CA2 and CA3 regions (Mizukami *et al.*, 1998). Immunohistochemical experiments utilising the β2/3 subunit antibody further demonstrated immunoreactivity in the striatum and globus pallidus (Waldvogel *et al.*, 1999). The current study thus supports previous findings, and contributes to the body of literature on the distribution of β3 subunit mRNA in human brain.

The relative density of β3 subunit mRNA differed in regions of the brain quantified, indicated in figure 2.26. β3 subunit mRNA appeared in greatest density in the cerebellar granule cell layer, followed by the primary visual cortex and hippocampus. β3 subunit mRNA was also abundant in the temporal pole, and showed a marked density in the middle frontal cortex, followed by the amygdala. Low levels of mRNA were expressed in the superior temporal gyrus. Negligible levels of mRNA were encountered in the cerebellar molecular layer. A comparison of figures 2.25 and 2.26 shows β3 subunit mRNA appeared in greater density than α1 subunit mRNA in the hippocampus. The α1 subunit mRNA was observed in greater abundance than β3 subunit mRNA in the temporal pole, superior temporal gyrus, primary visual cortex and granule and Purkinje cerebellar layers. Densities for both subunit mRNAs were equivalent in the middle frontal cortex and the amygdala.

2.5.4.3 Role of receptors formed from α1 and β3 subunits based on mRNA distribution

Putative subunit composition of $GABA_A$ receptors has been studied in rat brain, therefore it is difficult to comment on the likelihood for co-expression of α1 and β3 subunits in human brain. However, the apparent co-distribution of these two subunit mRNAs in human brain regions analysed in the present study imply their co-localisation, potentially forming $GABA_A$ receptors of the $\alpha1\beta3\gamma n$ combination. Additional analysis of the subunit mRNA distribution at the cellular level is required before any further conclusions of such nature can be drawn.

As mentioned in section 2.5.3.1, neural and synaptic complexities limit our capacity to infer receptor function, however on the gross level we can predict a role for receptors comprised of α1 and β3 subunits based on the function of brain regions to which the transcripts were mapped.

The middle frontal cortex is part of the prefrontal association cortex, essential for social behaviours, motor planning and cognitive processing, including analysis of visual and auditory information (Kupfermann, 1991; Zilles, 1990). The superior temporal gyrus is a component of the auditory association cortex, implicated in auditory processing (Barr & Kiernen, 1993; Craven, 2002). It is well established that the hippocampus plays an inherent role in learning and memory (Amaral & Insausti, 1990; Squire & Zola-Morgan, 1988). The temporal pole is limbic association cortex, important in integration of higher order sensory processes and emotional memory (Kupfermann, 1991). The primary visual cortex is essential for processing of visual information. The cerebellum coordinates planning, timing and patterning of skeletal muscle contractions during movement and plays a role in maintenance of posture and coordination of head and eye movements (Kelly & Dodd, 1991). Tissue used in the current thesis was acquired from the cerebrocerebellar region, which occupies the lateral part of the cerebellar hemisphere, and is involved in planning and initiation of movement, as well as learning of motor skills (Kandel, 1991; Ghez, 1991). Finally, the amygdala coordinates autonomic and endocrine responses (Kandel, 1991; Kelly & Dodd, 1991). It is also implicated in learned fears and emotions, and in social and emotional behaviour, principally via the basolateral amygdalar nuclei and central amygdala nucleus (Aggleton, 1993).

In summary, on the basis of current findings, the $GABA_A$ receptors consisting of α1 and β3 subunits are conceivably involved in cognitive processing, integration of sensory modalities and coordination of motor function, and social and emotional behaviours.

2.5.4.4 $GABA_C$ receptor ρ1 subunit mRNA

Sections hybridised with ρ1 subunit sense probe generally exhibited low background signal. Regardless, no statistically significant difference could be found between sense and antisense probe signals, apart from in the central nucleus of the amygdala. As mentioned previously, the lack of statistical significance is not entirely meaningful, in light of the small sample size used and the inherent inter-subject variability encountered with the use of human brain. Nonetheless, since the antisense signal observed in the amygdala could not be competitively reduced by the addition of excess unlabelled probe, the existence of the ρ1 subunit transcript in this region is questionable. While antisense signal generally occurred above background levels in most brain regions, microscopic examination in many areas failed to show silver grain clustering around cell soma, indicated in figure 2.31. Thus the ρ1 subunit transcript could not be easily demonstrated in the human brain regions analysed.

As with rat brain, strong evidence exists for ρ1 subunit mRNA in the human cerebellum, specifically in the granule cell layer, witnessed by the obvious laminar distribution of antisense signal at the regional level and specific labelling at the microscopic level, seen in figure 2.31. This finding is supported by the literature where ρ1 subunit mRNA was determined in human cerebellum using RT-PCR (Enz & Cutting, 1999a). There is some indication of ρ1 subunit mRNA in primary visual cortex, as a laminar distribution was observed in some subjects. However, at the microscopic level, no specific signal was observed, shown in figure 2.31. Investigation of ρ1 subunit distribution using RT-PCR in the occipital lobe did not demonstrate mRNA (Enz & Cutting, 1999a), however this study does not specify which part of the occipital lobe was sampled.

It is clear from figure 2.29 that where ρ1 subunit mRNA could be detected above background, it occurred at very low levels in comparison with the $GABA_A$ receptor subunit transcripts. This is in keeping with rat brain results in the current study, and reports in the literature of the very low levels of ρ1 subunit mRNA occurring in rat

brain. An inability to detect the ρ1 subunit transcript in some brain regions may be because the subunit simply does not exist in the areas examined. Alternatively, the ρ1 subunit mRNA may be present at levels too low to be detected using *in situ* hybridisation. In this instance, more sensitive methods such as RT-PCR must be employed to investigate the possibility. RT-PCR has in fact been used in one previous study examining the distribution of ρ1 subunit mRNA in human tissue, with the discovery of ρ1 subunit mRNA in the eye and cerebellum, and present at very low levels in the thalamus, midbrain, temporal cortex and frontal cortex (Enz & Cutting, 1999a). The subunit could not be detected in parietal and occipital cortices (Enz & Cutting, 1999a). Needless to say, the sample size for this study was one, and mRNA quantity and integrity varies greatly in human tissue (Higgins & Mah, 1989). Another study using Northern blotting with ρ1 subunit probes in human striatum yielded "very low or undetectable levels of hybridisation" (Cutting *et al.*, 1991). Both these studies affirm the presence of ρ1 subunit mRNA in human brain, albeit at very low levels.

It is possible that in the current study, the exposure time for ρ1 subunit probes could have been lengthened so that higher levels of mRNA could be detected, as is generally the practice for transcripts that are expressed at low levels. However, it was believed that such a practice may not have permitted the direct comparison of the relative densities of the ρ subunit mRNAs with those of the $GABA_A$ receptor subunit mRNAs, and the use of these subunits as positive controls. In fact, as the strength of signal also depends on the specific activity of the probe, the amount and concentration of the transcript in the tissue, and the affinity of the probe for the target, it would have been better practice to optimise the exposure time for all probes (Dr Peter Dodd, personal communication).

One must also bear in mind that, as with rat ρ subunits, the ρ subunits have been cloned from human retina and not brain. The nucleotide sequence for ρ1 subunits in human brain may be similar, but not identical to that for the retina. Tissue-specific variations in nucleotide sequence are not uncommon (Chabot, 1996; Cooper & Mattox, 1997; Maniatis & Tasic, 2002). The probability of differences in subunit sequence is increased by the fact that the probes were designed to regions of lowest conservation among subunits. Moreover, alternative splicing of the human ρ1 subunit was discovered

recently (Martínez-Torres *et al.*, 1998). Cloning and sequencing of these splice variants demonstrated subunits with alternative splicing of 51 deleted nucleotide bases and 450 deleted nucleotide bases in the N terminal region of the ρ1 subunit, termed ρ1Δ51 and ρ1Δ450 respectively. The deletion was shown to begin at the same splice site. The ρ1Δ51 cRNA expressed functional GABA-sensitive receptors in *Xenopus* oocytes with similar pharmacological and physiological characteristics to those of the recombinant ρ1 subunits (Demuro *et al.*, 2000; Martínez-Torres *et al.*, 1998). As it happens, the ρ1 subunit probes designed in the present investigation lie in the middle of the spliced region. It is thus crucial to investigate the possibility of similar splice variants of the ρ1 subunit in human brain using total RNA extraction and RT-PCR with primers designed to amplify the N-terminal region of the receptor subunit. Human brain may predominantly express the shorter variants, which would have precluded hybridisation using the ρ1 subunit probes designed for the current study, which targetted the region of known 51bp deletion.

Both rat and human recombinant ρ1 subunits form robust homooligomeric receptors with properties similar to those observed *in vivo* (Cutting *et al.*, 1991; Kusama *et al.*, 1993a; Kusama *et al.*, 1993b; Shimada *et al.*, 1992; Wang *et al.*, 1994). On this basis, it is possible to assert that ρ subunits expressed in human brain are likely to form homooligomeric $GABA_C$ receptors, although the possibility for interaction with other subunits remains. The apparent mapping of $GABA_C$ receptor ρ1 subunit mRNA to the primary visual cortex and cerebellar cortex in the present examination predicts a role for $GABA_C$ receptors comprised of these subunits in visual processing and in the coordination of vision and movement.

2.5.4.5 $GABA_A$ receptor ρ2 subunit mRNA

Experiments with ρ2 subunit probes were not always successful because of the problems encountered with the sense probe producing high, generalised signal. However, antisense signal was generally reduced by the addition of excess unlabelled probe, for example in the central nucleus of the amygdala, polar temporal cortex, cerebellum and superior temporal gyrus. Apparently specific signal also remained after subtraction of background signal in the amygdala, middle frontal cortex and polar temporal cortex, albeit very low levels, indicated in figure 2.30. Even so, the antisense

probe did not always produce heterogeneous signal, and sometimes failed to produce any signal above background, evident in figure 2.32. This may be because the subunit mRNA was present at very low levels such that laminar expression could not be deduced. An even distribution of signal across grey and white matter, however, suggests the signal observed was not always specific. Moreover, at the microscopic level, silver grain deposition was not always clustered around soma, denoting a lack of specific labelling, for example in the amygdala.

The cerebellum revealed an obvious discrete distribution of antisense signal at the regional level, specifically in the granule cell layer. Examination at the cellular level also disclosed specific labelling through clustering of silver grains around cell soma in some tissues, observed in figure 2.32. Findings of ρ2 subunit transcripts in cerebellum in the current study are supported by previous findings of ρ2 subunit mRNA in human cerebellum (Enz & Cutting, 1999a). The present study expands this knowledge to include the specific part of the cerebellum the transcripts were mapped to, namely the granule cell layer of the cerebellar cortex. Findings of ρ2 subunit mRNA in the human cerebellum are also consistent with similar findings in rat brain in the present investigation.

Some divergence in rat and human results is seen with ρ2 subunit mRNA localisation in the current investigation. For example, ρ2 subunit mRNA was mapped to the hippocampus in rat but not in human brain. The subunit may be present at much lower levels comparably in human brain, or the region of human hippocampus examined may not have contained the ρ2 subunits. ρ2 subunit mRNA was mapped to both rat and human frontal cortex, but the transcript could not be convincingly demonstrated in other regions of human brain examined. Quantification of transcript at the cellular level of analysis is required to ascertain whether the ρ2 subunit mRNA was present above background in human brain.

A previous study employing RT-PCR determined the presence of ρ2 subunit mRNA in human retina and in all brain regions tested, including the cerebellum, thalamus, midbrain, frontal, temporal, parietal and occipital cortices (Enz & Cutting, 1999a). Findings in the present examination could not confirm the presence of the transcripts in the temporal or

occipital cortices, although many factors could explain why. Different regions of the occipital and temporal cortices may have been sampled for RT-PCR. In addition, RT-PCR is a far more sensitive technique than *in situ* hybridisation, and the low density of mRNA may not have been estimable using *in situ* hybridisation alone. As with ρ1 subunits, it is possible that the exposure time for ρ2 subunit probes could have been lengthened so that higher levels of mRNA could be detected, as is generally the practice for transcripts with low expression levels. Since ρ2 subunits have been cloned from human retina, and not brain, tissue-specific variations in nucleotide sequence may prevail and alternate splicing which could preclude hybridisation using the probes designed for the current study.

It is obvious from figure 2.30 that where ρ2 subunit mRNA could be detected above background, it occurred at very low levels in comparison to the $GABA_A$ receptor subunit transcripts. This conforms to the pattern witnessed in rat brain in the current study, and reports in the literature of the low levels of ρ2 subunit mRNA occurring in the brain.

The ρ subunits expressed in human brain are likely to form homooligomeric $GABA_C$ receptors, although the possibility for interaction with other subunits remains. The lower currents produced by ρ2 subunits *in vitro* also imply that these subunits possibly require co-expression with other subunits to form fully functional receptors (Cutting *et al.*, 1992). Since the ρ1 subunits contain assembly signals which allow them to form more efficient homooligomeric receptors than the ρ2 subunits (Hackam *et al.*, 1998), pseudo-heterooligomeric receptor formation incorporating the ρ2 subunit seems likely. The co-distribution of ρ1 and ρ2 subunit mRNAs in the human cerebellar granule cell layer implies that the receptors may exist as pseudo-heterooligomers in this region of the brain. Little can be said for other brain regions, though.

Results from the current thesis provide some evidence for the existence of ρ subunit mRNAs in human brain. The advantages of this study over those previously conducted with the $GABA_C$ receptor subunits in human brain are that a larger sample size was used, and mRNA was investigated *in situ*, rather than by total RNA extraction and RT-PCR or blotting techniques. This allowed quantification of mRNA levels in specific

brain regions, and examination at the cellular level. A disadvantage of *in situ* hybridisation for these purposes, however, is that it is not as sensitive an assay as RT-PCR, even when examining mRNA at the microscopic level. This is because RT-PCR amplifies the transcript, while *in situ* hybridisation provides information only on its relative density in the natural state. Hence, RT-PCR should still be employed to investigate whether the transcripts are in fact existent, but at levels too low to be detected using *in situ* hybridisation. Immunohistochemical studies applying antibodies against the ρ subunit polypeptide, or quantitative autoradiographic experiments using tritiated ligands selective for the $GABA_C$ receptors, such as the recently available [^{3}H]P4MPA, must be performed with human brain to confirm the presence of ρ subunits in human brain.

2.5.5 Sources of Variability and Limitations with the Technique

Large inter-subject variability was encountered in the present investigation in both rat and human brain. While a sample size of 4 to 6 is generally sufficient for quantification of parameters in rat brain, this number may still not be large enough to obtain statistical significance, especially where large variability in mRNA levels occurs between subjects. Variability in mRNA levels for the transcripts investigated may be naturally large, therefore increasing sample size would be one way of overcoming this problem. The variability even between rat brains may be naturally large although the animals are of the same breed, as they were ordered at different times and were therefore not all from the same litter. Furthermore, although all efforts were made to ensure animals were of the same age, there may have been some variation in animal ages (by up to 2 weeks at most). It is well known that expression of $GABA_C$ receptors, for example, decreases over the course of post-natal development (Martina *et al.*, 1995; Strata & Cherubini, 1994), thus younger rats may have greater expression of the some subunits than older rats.

On the other hand, variability may have been introduced during tissue preparation. While consistent and rigorous tissue isolation and preparation techniques were adhered to, some tissues were nonetheless better preserved than others, meaning mRNA yields would have varied between subjects. Ideally, to reduce experimental variation, experiments would be performed on the same day, however the number of subjects and probes used in each experiment in the current study meant that numerous experiments

had to be performed over a long period. Replication of experiments, though, should have removed some of this experimental variability. Varying lengths of tissue storage may also have contributed to variability, as long term tissue storage is known to reduce mRNA yield (Harrison & Pearson, 1995).

One of the major problems involved with the use of post-mortem human brain tissue is the brain-to-brain variability (Harrison, 1996), especially in mRNA yield and integrity (Higgins & Mah, 1989). This variability may be amplified by pre-, peri- and post-mortem factors, as well as experimental procedures. Although the *in situ* hybridisation techniques were optimised using rat brain before use of post-mortem human tissue, large variability was still encountered in human brain, primarily because of the small sample size, as well as the sample used. It was stated that the disease state of some individuals used for the present examination should not affect the results in that all we were looking for was the presence of the subunit mRNA. However, it is known that, for example, $\alpha 1$ and $\beta 3$ subunit mRNAs are affected in the brain of alcoholics (Dodd & Lewohl, 1998; Lewohl *et al.*, 1997; Mitsuyama *et al.*, 1998; Thomas *et al.*, 1998) and schizophrenics (Ohnuma *et al.*, 1999). Thus, the decrease or increase in subunit mRNA as a result of these disease states may have complicated the mean density of mRNA detected, contributing to the lack of statistical significance. The sample size must be increased before definitive can be drawn from the results.

Use of post-mortem tissue is limited in that any results are based on the state of the brain at death, and no conclusions can be drawn beyond this (Harrison & Pearson, 1995). Post-mortem tissue may not maintain integrity of some mRNAs even if all possible measures are taken to ensure highest tissue quality and preparation. Post-mortem interval exerts a relatively minor effect on mRNA detection in human brain (Heath *et al.*, 1996b), however, variation in post-mortem interval may add to variability in mRNA detection nonetheless. Since predominantly control and tissue unsuitable for matched cases was used in the present study, post-mortem interval was not controlled for. Agonal state is more likely to affect mRNA levels and tissue quality post-mortem (Harrison & Pearson, 1995; Heath *et al.*, 1996a). Nevertheless, in the brain collection from which tissue was obtained, agonal state is incorporated into the inclusion criteria such that any compromised tissue is not processed. mRNA is also susceptible to

degradation during fixation and freezing (Higgins & Mah, 1989), and while rigorous control of histological procedures was employed, this may still have contributed to the observed variability in mRNA yields.

Finally, *in situ* hybridisation is a sensitive technique for assessing distribution and density of mRNA, however it does not give an indication of corresponding protein expression. While it is generally accepted that mRNA localisation dictates protein expression, mRNA is found in cell bodies, yet its translation products are regularly localised to axons and terminals, which may be located at some distance from the soma. The GABA receptors are commonly confined to local circuits, therefore they are not expected to be expressed at sites distant from the cell bodies transcribing them. In order to confirm the presence of the ionotropic GABA receptors mapped in rat and human brain in the current investigation, immunohistochemical analysis of their polypeptide distribution is necessary.

2.5.6 Conclusions

$GABA_A$ receptor $\alpha 1$ and $\beta 3$ subunit and $GABA_C$ receptor $\rho 1$ and $\rho 2$ subunit mRNAs were detected in rat retina, with the ρ subunit mRNAs present at greater density than $GABA_A$ receptor subunit transcripts, confirming the prevalence of the $GABA_C$ receptor subunits in rat retina. These findings are congruous with previous reports of $\alpha 1$, $\beta 3$, $\rho 1$ and $\rho 2$ subunits in rat retina, and justify the use of the probes designed for the present thesis in detecting the target mRNA. An inability to detect the $\rho 3$ subunit mRNA in rat retina may be explained by the consistently high, generalised signal produced by the sense probe in all tissues, making assessment of whether the antisense probe yielded specific or background signal impossible. Design of new probes to target $\rho 3$ subunit mRNA may overcome this dilemma.

$GABA_A$ receptor $\alpha 1$ and $\beta 3$ subunit probes provided a consistent signal in rat brain, and were therefore useful positive controls. These subunit transcripts were widely distributed throughout rat brain, in agreement with the literature, with $\alpha 1$ subunit mRNA exhibiting the most prolific expression. $GABA_C$ receptor subunits were far less prevalent in rat brain, and where they appeared to exist, they occurred at very low

densities. ρ2 subunit mRNA was convincingly demonstrated in a number of brain regions, consonant with previous reports. However, ρ1 and ρ3 subunit transcripts were not convincingly demonstrated in rat brain, apart from the cerebellar granule cell layer. Successful mapping of ionotropic GABA receptors in rat brain permitted optimisation of techniques and transferral to the human brain.

Large variability between subjects impeded wholly successful mapping of α1, β3, ρ1 and ρ2 subunits in human brain. Concordant with findings in rat brain, the $GABA_A$ receptor subunit transcripts were easier to detect and localised to most brain regions examined, while the $GABA_C$ receptor subunit mRNAs were present at very low levels in few regions investigated. The present examination does, nonetheless, extend the literature on the presence of α1 and β3 subunit mRNAs in regions of the human brain previously not reported. A number of factors can explain the large variability encountered in these experiments, the most important element being sample size, which must be increased before definitive conclusions can be drawn. The capacity to identify these transcripts at the regional and cellular levels can furnish valuable information on the ionotropic GABA receptors in human brain, both in the normal state as well as in pathophysiological processes.

MAPPING IONOTROPIC GABA RECEPTORS IN RAT AND HUMAN BRAIN
CHAPTER 3: IMMUNOHISTOCHEMISTRY

3.1 INTRODUCTION AND AIMS

3.1.1 Introduction to the Methods

Immunohistochemistry facilitates the identification of tissue components, usually proteins, *in situ* by taking advantage of the specific antigen-antibody reaction. The antigen is detected through tagging of the antibody using a fluorochrome or chromagen (Gentleman & Roberts, 1995; Grzanna, 1982; Van Noorden, 1986).

3.1.1.1 Antigens, antibodies and antibody-antigen interactions

The antigen, or immunogen, in immunohistochemistry is a peptide or protein component of the tissue against which a specific antibody is produced. The site on the surface of the antigen to which the specific antibody binds is the epitope, or antigenic site, and usually comprises one to six amino acid residues (Chemicon, 1998; Watson & Cullinan, 2000).

Antibodies are immunoglobulins that combine specifically with antigens. In immunohistochemistry, the antibody is directed against the antigenic site that caused its production in a susceptible animal (Chemicon, 1998). The most common antibodies produced for immunohistochemistry are the IgG class (Chemicon, 1998; Pearse, 1980). Antigen-antibody complexes form through hydrogen bonds, hydrophobic bonds, electrostatic interactions and Van der Waals forces (Chemicon, 1998). The rate of antibody-antigen binding is contingent upon temperature, pH and solvent, therefore the antibody-antigen interaction is amenable to control by variation of any of these factors (Chemicon, 1998). For immunohistochemical detection of an antigen, the epitope must be available for binding (Chemicon, 1998). If the epitope is altered through fixation or changes in pH, interaction with the antibody will be impaired (Chemicon, 1998; Pearse, 1980).

3.1.1.2 Types of antibodies

The primary antibody is the antibody raised specifically against the antigen of interest. Primary antibodies may be either polyclonal or monoclonal. Primary polyclonal antisera contain a mixture of antibodies that recognise and react specifically with a variety of different epitopes on an antigen (Chemicon, 1998; Gentleman & Roberts, 1995). Primary monoclonal antibodies recognise a single epitope, and are therefore homogeneous antibodies ideally with absolute specificity (Gentleman & Roberts, 1995). Monoclonal antisera are advantageous due to their specificity for a single epitope, resulting in lower background staining than the polyclonal antibodies (Chemicon, 1998). They may also be more robust, producing replicable staining across experiments (Chemicon, 1998).

The secondary antibody is an antibody raised against the IgG of the species in which the primary antibody was raised. For example, if the primary antibody is raised in rabbit and targets a receptor protein in rat, the secondary antibody is raised in another species other than rabbit or rat, such as goat, and targets all rabbit IgG.

3.1.1.3 Indirect immunohistochemistry methods

As opposed to the direct method where the primary antibody is labelled directly with a chromagen or fluorochrome and applied to the tissue (Boorsma, 1983; Grzanna, 1982; Pearse, 1980; Van Noorden, 1986), indirect immunohistochemical methods use an unlabelled primary antibody (Gentleman & Roberts, 1995; Van Noorden, 1986). The specifically bound primary antibody is then detected with a labelled secondary antibody (Grzanna, 1982; Pearse, 1980; Van Noorden, 1986). Several secondary antibody molecules can bind to the primary antibody, making the indirect method more sensitive than the direct method as smaller amounts of antigen can be detected and a greater signal can be obtained (Grzanna, 1982; Pearse, 1980; Van Noorden, 1986).

3.1.1.4 Immunofluorescence histochemistry

A common indirect method is fluorescence immunohistochemistry, developed by Coons and associates (1941). The basic idea of indirect immunofluoresence labelling is depicted in figure 3.1. Fluorescent labels such as fluorescein isothiocyanate (FITC), rhodamine isothiocyanate (RITC) and Texas Red are conjugated to the secondary

antibody without compromising the biological and immunogenic properties, and can be viewed using fluorescence microscopy (Coons *et al.*, 1941; Pryzwansky, 1982).

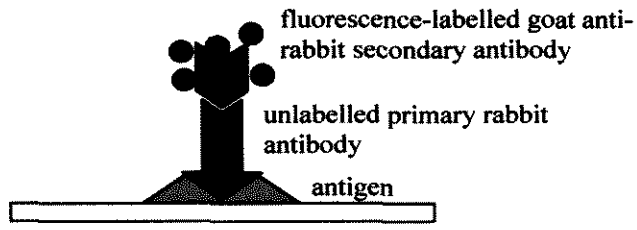


Figure 3.1: Schematic diagram of the principles of immunofluorescence histochemistry.

The immunofluorescence technique relies on the properties of fluorescing molecules for visualisation of the target antigen. The wavelength of light absorbed by the fluorescing molecules is shorter than that emitted (Sternberger, 1979). Under fluorescence microscopy, the light source is beyond the visible range and filters concentrate the light such that only the specifically stained fluorescent component of the tissue is viewed (Sternberger, 1979). The sensitivity of the technique lies in the fact that emitted light is viewed against a dark background (Sternberger, 1979). Nevertheless, even at full light transmission, intensity of signal is low, emphasising the need for a strong light source and excellent optics (Sternberger, 1979). Furthermore, fluorescence intensity fades upon exposure to light, so exposure times need to be minimised (Sternberger, 1979).

3.1.1.5 Immunoenzyme histochemistry

Avidin-biotin immunohistochemistry is a method of immunoenzyme histochemistry which involves conjugation of the secondary antibody to a biotin molecule. Biotin is a vitamin which interacts strongly with the glycoprotein avidin, resulting in an avidin-biotin complex (Boorsma, 1983). Avidin is added to the tissue in the form of streptavidin-peroxidase. The chromagen 3,3',5,5'-diaminobenzidine tetrachloride (DAB) is then combined with peroxide and added to the tissue, and reacts with the peroxidase to give an amorphous, insoluble brown precipitate (Gentleman & Roberts, 1995; Grzanna, 1982; Van Noorden, 1986). The basic concept of avidin-biotin immunoenzyme histochemistry is illustrated in figure 3.2.

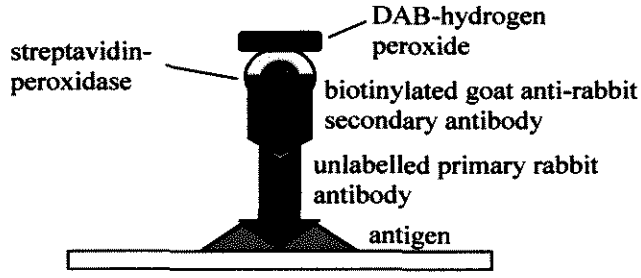


Figure 3.2: Schematic diagram of the principles of immunoenzyme immunohistochemistry, demonstrating reactive component to obtain final reaction product

Immunoenzyme histochemistry has a number of advantages, including the fact that staining does not fade if sections are mounted, creating, in effect, a permanent record. Furthermore, sections are analysed under brightfield light microscopy so that cell bodies and immunostained regions can be viewed together (Gentleman & Roberts, 1995; Grzanna, 1982; Pool *et al.*, 1983). Less primary antibody is required in this technique, and the final reaction product is also more easily quantified than immunofluorescence techniques. However, fluorophores are now available which are highly resistant to photobleaching and can be analysed repeatedly if tissue is stored correctly. Furthermore, while immunoenzyme methods produce a rather diffuse stain, immunofluorescence techniques produce very punctate labelling which can be viewed at high resolution and with confocal microscopy, allowing precise localisation of proteins to cellular compartments (Pool & Buijs, 1988).

3.1.1.6 Tissue preparation

Accurate detection of an antigen relies essentially upon preservation of tissue morphology and of the antigen in the tissue (Brandtzaeg, 1982; Pool & Buijs, 1988; Van Noorden, 1986). Tissue preparation must be optimised to ensure these factors are preserved.

For the detection of membrane-bound antigens such as ionotropic receptors, cryostat sectioned tissue that is fresh frozen or fixed after sectioning is optimal, as degradation of tissue and antigens is minimised (Grzanna, 1982; Van Noorden, 1986). Cryostat sectioning of fresh frozen tissue followed by fixation is also suitable for antigens which may lose their immunoreactivity easily (Pool *et al.*, 1983), although tissue prepared in this manner is more susceptible to non-specific staining than pre-fixed sections (Pearse,

1980). Cryostat sectioned tissue which was previously perfused with or immersed in fixative is also suitable (Gentleman & Roberts, 1995; Van Noorden, 1986), as diffusion of antigens is less likely to occur (Grzanna, 1982).

Preservation of tissue morphology is achieved by the use of fixatives, which render tissue components insoluble (Grzanna, 1982). Fixatives also suspend soluble antigens *in situ* (Sternberger, 1979; Van Noorden, 1986) preventing diffusion of the antigen within or out of the tissue (Brandtzaeg, 1982; Pool & Buijs, 1988; Sternberger, 1979). Furthermore, fixatives prevent autolysis through inactivation of lysosomal enzymes, and prevent bacterial and fungal growth (Brandtzaeg, 1982; Gentleman & Roberts, 1995). A number of fixatives can be used, depending on the way tissue is processed and the location and nature of the antigen of interest (Costa & Furness, 1983; Van Noorden, 1986). Ultimately, fixation should preserve anatomical detail without detrimentally affecting antibody-antigen binding (Gentleman & Roberts, 1995; Grzanna, 1982; Sternberger, 1979).

For cryostat sectioned tissue that will be analysed by light or fluorescence microscopy, fixation with formaldehyde is most common, particularly phosphate buffered paraformaldehyde (Gentleman & Roberts, 1995; Pool *et al.*, 1983; Van Noorden, 1986). Formaldehyde fixes tissue by cross-linking proteins (Grzanna, 1982; Sternberger, 1979; Van Noorden, 1986). The downfall of fixation with aldehydes is that cross-linking may occur to the extent that antigen sites become obscured from the antibody (Gentleman & Roberts, 1995; Grzanna, 1982; Sternberger, 1979; Van Noorden, 1986). In these circumstances, antigens which are present in low amounts may be masked completely (Grzanna, 1982). Fixation may also alter the antigen structure to interfere with antibody-antigen binding (Gentleman & Roberts, 1995). Peptides contain many sites for fixatives to conjugate, and changes to the tertiary structure of the antigen may occur as a result of conjugation with the fixative, compromising recognition of the antigen by the antibodies (Pool & Buijs, 1988). Antigen retrieval techniques involving heating of sections prior to experimentation have been introduced to break down some of the cross linkages and effectively render antigen sites available for subsequent antibody interaction. This does not necessarily alleviate the problem of changes in tertiary structure of the molecule to be identified, however. Fixation time and concentration can

also be carefully controlled to reduce obscuring of antigenic sites or structure (Chemicon, 1998; Costa & Furness, 1983; Pool & Buijs, 1988). Temperature and pH can additionally affect the fixation process (Brandtzaeg, 1982; Sternberger, 1979; Van Noorden, 1986), and must be carefully controlled. Higher pH increases cross linkage, hence fixation (Gentleman & Roberts, 1995).

Less permanent methods of fixation include pre-cooled ethanol and acetone. These solvents denature proteins by coagulation (Chemicon, 1998), precipitating proteins and carbohydrates in the tissue without incurring any change in their immunoreactivity (Brandtzaeg, 1982; Grzanna, 1982; Sternberger, 1979). Fixation with ethanol or acetone is appropriate for staining tightly bound tissue antigens, not diffusible antigens (Grzanna, 1982). Therefore, ethanol and acetone provide a gentler alternative for fixation when investigating membrane-bound receptors, but not, for example, neurotransmitter substances. Brandtzaeg (1982) in fact reported that ethanol fixation produced more sensitive and intense immunoreactive labelling than any of the cross-linking fixatives tested. Use of unfixed, frozen tissue sections is further recommended in situations where antigenic reactivity must be maximally preserved (Sternberger, 1979). However, soluble antigens are either lost from the tissue or diffuse within the tissue, therefore the suitability of frozen tissue is limited to insoluble, membrane-bound antigens (Sternberger, 1979).

Tissue which is not treated with fixative must be frozen rapidly in order to preserve tissue morphology and prevent freezing artefacts which occur through formation of ice crystals from water in the tissue (Grzanna, 1982). Reduction of crystal growth, or "cryo-protection" can be achieved by soaking tissue in sucrose solution for 24 hours (Gentleman & Roberts, 1995; Grzanna, 1982; Uhl, 1988).

Thorough washing of tissue after fixation using aldehydes is necessary to remove free aldehyde groups. Free aldehyde groups can attract immunoglobulins, which increases the opportunity for non-specific staining (Pool & Buijs, 1988; Pool *et al.*, 1983; Van Noorden 1986). Washing is generally carried out in the buffer in which the fixative was made up, for example, phosphate buffered saline (Costa & Furness, 1983).

3.1.1.7 Antigen retrieval

Antigen retrieval techniques involve heating of sections prior to experimentation in order to break down some of the fixative-induced cross linkages and effectively render antigen sites available for subsequent antibody interaction. This procedure is especially useful for tissue which has been perfused or drop-fixed, or in archival tissue which has been stored in fixative. Tissue which is not paraffin-embedded may be antigen retrieved through microwave irradiation or autoclaving (Chemicon, 1998). However, it is the experience of the author that these techniques can significantly disrupt the integrity of the tissue, and gentler retrieval techniques can be employed, such as heating tissue at 90°C in sodium citrate or phosphate buffers for 1-2 hours (Dixon & Harper, 2001 and unpublished observations). Another widely used method of antigen retrieval is sodium borohydride, which is an anti-fixative, acting to break cross-links created by aldehyde fixatives, effectively reducing the double bonds to single bonds (Eldred *et al.*, 1983; Stuart & Oorschot, 1995).

3.1.1.8 Potential sources of background

Background staining may be diffuse or structurally distinct (Van Noorden, 1986) and may be caused by a number of factors. Reagents used in the immunohistochemistry procedure may produce a background stain (Van Noorden, 1986). Concentrations of the primary and secondary antibodies may be too high and may bind electrostatically to basic proteins in the section, creating non-specific background staining through protein-protein interaction (Pearse, 1980; Pool & Buijs, 1988). Pretreatment with normal serum of the species from which the secondary antibody is derived may minimise non-specific adherence of the primary antibody (Sternberger, 1979). Dilution of the antibodies can ameliorate these circumstances (Pearse, 1980; Sternberger, 1979). Background staining may also result from non-specific binding of the antibodies to components of the tissue in addition to the target antigen (Grzanna, 1982).

Autofluorescence is another factor responsible for background staining and will mask specific staining if it occurs. Autofluorescence may arise for a number of reasons. Formaldehyde itself can produce autofluorescence (Van Noorden, 1986), therefore it is best to use the lowest possible concentration whilst maintaining tissue preservation. Intrinsically fluorescing components of the tissue, such as connective tissue or

pigmented molecules like lipofuscin, can also produce autofluorescence (Coons *et al.*, 1941; Pool *et al.*, 1983; Van Noorden, 1986). Furthermore, phosphate buffered saline solutions may promote bacterial growth, which can produce background autofluorescence, and these solutions should therefore be refreshed regularly (Pryzwansky, 1982).

Molecules in the tissue with related antigenic sites to the protein under question may attract the specific antiserum, resulting in a non-specific reaction (Pearse, 1980; Pool *et al.*, 1983). This problem, referred to as cross-reactivity, may occur with polyclonal antibodies, as they recognise a variety of epitopes, some of which may occur on related proteins (Pearse, 1980).

3.1.1.9 Controls

As mentioned above, the possibility exists that antisera raised against one peptide may cross-react with structurally similar peptides with homologous amino acid sequences (Gentleman & Roberts; Pearse, 1980; Sternberger, 1979). Controls are employed in immunohistochemistry to ensure that the tissue component under investigation is the only component producing immunoreactivity in the tissue, thus, that the antigen-antibody interaction is specific (Gentleman & Roberts, 1995; Pool *et al.*, 1983).

A positive control must first be carried out with tissue known to contain the target antigen, previously demonstrated through a reliable method (Chemicon, 1998; Pool & Buijs, 1988; Pool *et al.*, 1983; Van Noorden, 1986). Once it has been established that the optimal staining occurs in this tissue, then tissue which is not known to contain the antigen can be investigated (Pool *et al.*, 1983). Another form of positive control is the concurrent use of a primary antibody which has demonstrated robust expression in the tissue of interest.

Negative controls must be employed in immunohistochemical procedures to ensure specificity of the final observed immunoreactive signal. One type of negative control involves pre-incubation or incubation in the place of the primary antiserum with a pre-immune or non-immune serum, which possesses no immunoreactivity with any of the tissue components (Pearse, 1980; Pool & Buijs, 1988; Pool *et al.*, 1983; Sternberger,

1979). Comparison of tissue treated with non-immune serum and tissue incubated with primary antibody alone allows one to ascertain whether the specific primary antiserum alone is responsible for resultant immunostaining (Pearse, 1980; Pool *et al.*, 1983). Staining which persists after incubation with the non-immune serum implies the presence of contaminating antibodies arising from impurities in the immunogen, or contaminating antibodies in the serum present before immunisation commenced (Pearse, 1980; Pool & Buijs, 1988).

Alternate negative controls involve the omission of the primary or secondary antibody to determine the extent of non-specific background staining (Chemicon, 1998; Gentleman & Roberts, 1995; Pearse, 1980; Sternberger, 1979). No staining should occur in the absence of the primary antibody (Pearse, 1980), thus any observed immunoreactivity is deemed non-specific (Van Noorden, 1986).

3.1.1.10 Blocking and permeabilisation

Before addition of primary antibody to sections, the tissue must be treated with blocking agents in order to minimise non-specific staining, hence reducing background and increasing the signal to noise ratio. The blocking step incorporates normal serum, bovine serum albumin and Triton X-100.

Cryostat-sectioned tissue commonly contains endogenous receptors for the Fc portion of immunoglobulins, non-antigenic sites to which antibodies can attach non-specifically (Pool & Buijs, 1988; Sternberger, 1979; Van Noorden, 1986). In order to avoid this non-specific binding, tissue is initially blocked with normal serum of the species in which the secondary antibody was raised (Gentleman & Roberts, 1995; Sternberger, 1979; Van Noorden, 1986). In this way, Fc sites are saturated by immunoglobulin from the normal serum, rendering the sites unavailable for primary antibody attachment (Van Noorden, 1986). Blocking with normal serum additionally prevents the secondary antibody from binding to tissue immunoglobulin if cross-species reactivity is prone to occur (Gentleman & Roberts, 1995; Van Noorden, 1986).

Hydrophobic and electrostatic forces can create non-specific binding of immunoglobulin if tissue components are positively charged (Gentleman & Roberts, 1995; Pool *et al.*,

1983; Van Noorden, 1986). Blocking tissue with Triton X-100 can help decrease the hydrophobic and electrostatic forces, decreasing non-specific adherence of the antibodies, hence decreasing background staining (Gentleman & Roberts, 1995; Pool & Buijs, 1988; Pool *et al.*, 1983; Van Noorden, 1986).

Penetration of antibody in the tissue is often limited by the presence of intact membranes. Sectioning tissue permits access to interior cellular components, while freezing and thawing disrupt membranes, further increasing accessibility (Sternberger, 1979). However, tissue is commonly permeabilised with a detergent such as 0.1-2% Triton X-100 in the blocking step, as well as in the antibody incubation steps, to facilitate penetration of antibodies (Gentleman & Roberts, 1995; Pool & Buijs, 1988; Pool *et al.*, 1983; Sternberger, 1979).

3.1.1.11 Titration experiments

Intensity of signal for antigen detection is determined by the number of antibodies carrying the molecules for detection, such as the fluorochrome, that bind specifically to the target antigen and the intensity of background staining (Grzanna, 1982). The brightest signal may hinder maximal resolution (Pryzwansky, 1982). The concentration of antibody can be varied relative to the concentration of antigen to control the extent of antibody-antigen complex formation (Chemicon, 1998). This is achieved experimentally by titration of both primary and secondary antibodies to yield the optimal dilution which provides the highest signal with lowest background (high signal to noise ratio) (Van Noorden, 1986). Titration is also necessary as antibody concentrations may vary in different stocks of antisera (Chemicon, 1998). Dilutions in the range of 1 in 10 through to 1 in 10000 are typically used, depending on length of incubation, concentration of stock antibody solution and density of antigen sites.

3.1.2 Advantages and Limitations of Immunohistochemistry

3.1.2.1 Advantages

Some of the advantages of immunohistochemistry include the high contrast and high specificity staining which is readily repeatable (Gentleman & Roberts, 1995). Immunohistochemistry provides a powerful method for analysing specific biochemical

structures at the cellular level. Multiple cellular compartments can be visualised. Furthermore, very small levels of expression can be visualised (Watson & Cullinan, 2000).

3.1.2.2 Limitations

Immunohistochemical techniques present with some limitations. The specificity of immunolabelling is questionable in view of the potential for cross-reactivity. Fixation procedures may also immortalise the antigen in a way that does not necessarily reflect the antigen at the moment of death (Pool & Buijs, 1988). The most crucial limitation of immunofluorescence histochemistry is that it is not entirely quantitative (Watson & Cullinan, 2000). However, it is useful for qualitative evaluation of the presence of particular protein in tissue.

3.1.3 Aims

Expression of $GABA_C$ receptor subunit mRNA was confirmed in rat retina and cerebellum using *in situ* hybridisation, outlined in chapter 2 of the present thesis. As summarised in the literature review (see section 1.4.2), previous studies have demonstrated the presence of the $GABA_C$ receptor protein using immunohistochemistry in retina (Enz *et al.*, 1996; Koulen *et al.*, 1998; Wässle *et al.*, 1998), Purkinje cells of the cerebellum (Boue-Grabot *et al.*, 1998), and in the superior colliculus (Clark *et al.*, 2001; Pasternack *et al.*, 1999). At the time experiments in the present thesis were undertaken, $GABA_C$ receptors had only been localised to the Purkinje cells of the cerebellum and to the retina. Retinal and cerebellar tissue was therefore tested for the presence of $GABA_C$ receptor immunoreactivity in the current experiments to establish immunohistochemical techniques. A further aim was to investigate the presence of $GABA_C$ receptors in rat brain using available antisera and to consolidate results from *in situ* hybridisation experiments. Concurrently, the $GABA_A$ receptor $\beta2/\beta3$ subunit antibody was applied to the same tissue as a positive control, owing to its robust and prolific distribution, and to reinforce *in situ* hybridisation studies.

3.2 MATERIALS

3.2.1 Subjects

Male and female Sprague-Dawley rats aged 10-12 weeks were obtained from the Bosch Animal House. Animals were housed in same-sex groups of two to five in plastic cages. Animals were housed in Bosch Animal House under a 12 hour/12 hour light/dark cycle at constant temperature (20-21°C) and humidity (45-65%) with access to standard rat chow and water *ad libitum*. Light intensity in the housing area was held at 60 lux, and 15 room air changes occurred daily for ventilation. Animal ethics approval was granted for experimentation (ACEC number K21/1-99/3/2803).

3.2.2 Antibodies

3.2.2.1 Primary antibodies

$GABA_C$ receptor antisera were gifts from Dr Erica Fletcher (Department of Optometry and Vision Science, University of Melbourne, Parkville, Victoria, Australia) and Professor Heinz Wässle (Max-Planck-Institut fur Hirnforschung, Neuroanatomische Abteilung, Frankfurt, Germany). The antiserum produced by Wässle and colleagues (Enz *et al.*, 1996) was affinity-purified polyclonal IgG raised in rabbit (1:100) against residues 16-171 of the N terminal region of the rat ρ1 $GABA_C$ receptor subunit. This antibody was shown to cross react with ρ2 and ρ3 subunits using Western blots and transfected HEK293 cells (Enz *et al.*, 1996). The cross-reactivity is explained by the high sequence homology between the N terminal of ρ1 subunits and the ρ2 and ρ3 subunits (82% and 78% respectively) (Enz *et al.*, 1996). The antibody was also shown to produce strong immunoreactive labelling in rabbit, cat and macaque retina (Enz *et al.*, 1996). The second $GABA_C$ receptor antibody was developed by Maurice Garrett and colleagues (Boue-Grabot *et al.*, 1998). This antiserum was also affinity-purified and polyclonal IgG, raised in rabbit against residues 16-31 of the N terminal of the rat $GABA_C$ receptor ρ1 subunit. The antibody was shown to be specific to ρ1 subunits using Western blots and transfected HEK293 cells (Boue-Grabot *et al.*, 1998).

The $GABA_A$ receptor antiserum was purchased from Upstate Biotechnology through Auspep Pty. Ltd. (Parkville, VIC, Australia). The antiserum is protein G-purified

mouse monoclonal IgG, and is directed against the N terminal region of the bovine $GABA_A$ receptor β2 and β3 subunits at 55 and 57 kDa respectively (Upstate Biotechnology catalogue, 2001). This antibody cross reacts with bovine, human and rat brain (Upstate Biotechnology catalogue, 2001).

Rabbit IgG non-immune serum and MOPC-21 mouse IgG1kappa were obtained from Sigma-Aldrich (St Louis, MO, USA).

3.2.2.2 Secondary antibodies

Secondary antibodies for immunoperoxidase histochemistry were obtained from Vector Laboratories (Australian Laboratory Services, Paddington, QLD, Australia). Both biotinylated Anti-Rabbit IgG (H+L) made in goat and Anti-Mouse IgG (H+L) made in horse antibodies were obtained.

Secondary antibodies for immunofluorescence histochemistry were purchased from Molecular Probes (Bioscientific Pty. Ltd., Avondale, Arizona, USA). Both Alexa 594 goat anti-mouse IgG (H+L) conjugate (2mg/mL) and Alexa 594 goat anti-rabbit IgG (H+L) conjugate (2mg/mL) were purchased. Alexa 594 absorbs light energy at 594nm and fluoresces red at an excitation wavelength of 623nm, akin to the traditional Texas Red fluorochrome.

3.2.3 Reagents and Chemicals

Standard Vectastain Elite ABC Peroxidase kit and bovine serum albumin (immunohistochemical grade) were purchased from Vector Laboratories (Australian Laboratory Services, Paddington, QLD, Australia). 3,3',5,5'-diaminobenzidine (DAB) tetrachloride tablets were obtained from Sigma-Aldrich (St Louis, MO, USA). Normal goat serum and normal horse serum were purchased from Gibco BRL Life Technologies (Rockville, MD, USA). Halothane was obtained from CENVET Australia Pty/Ltd (Artamon, NSW, Australia).

Sodium phosphate monobasic (anhydrous), sodium phosphate dibasic (anhydrous), paraformaldehyde powder and sucrose were acquired from Sigma-Aldrich (St Louis,

MO, USA). Sodium chloride and phosphate buffered saline (PBS) tablets were purchased from ICN Biochemicals (Costa Mesa, CA, USA). 2-methylbutane was obtained from Sigma-Aldrich (St Louis, MO, USA). Cresyl violet acetate was obtained from BDH Laboratory Supplies (Poole, England).

3.3 METHODS

3.3.1 Processing of Tissue

3.3.1.1 Transcardial perfusion

Transcardial perfusion was performed by Des Richardson (Department of Pharmacology, University of Sydney). Animals were anaesthetised with halothane (1mg/mL v/v) in a chamber until general anaesthesia was evident. Anaesthesia was maintained using halothane in a nose cup as the animal was perfused transcardially. Perfusion was first performed with 0.9% saline solution (see Appendix 5) for 1-2 minutes to wash out blood, then for 5 to 10 minutes with 4% paraformaldehyde (see Appendix 5). Perfusion was deemed complete once tissue stiffening lessened and the liver turned from red to brown. Once perfusion was complete animals were decapitated with a guillotine and the brains rapidly dissected then stored overnight in 15% or 30% sucrose solution (see Appendix 5) for cryoprotection prior to sectioning.

3.3.1.2 Tissue preparation

Where rat brain was fixed post-mortem, rather than by perfusion, animals were first euthanased using CO_2 gas in a chamber and decapitated using a guillotine. The brains were dissected rapidly on ice and plunged into 2-methylbutane on dry ice to freeze immediately.

Rat brains were cut transversely with a scalpel blade and mounted rostral-caudal from the forebrain to anterior brain, or caudal-rostral from the spinal cord or cerebellum to the midbrain, onto microtome stages, surrounded by OCT embedding compound (Tissuetek, Miles Inc., USA). Unused portions of tissue were stored, surrounded by OCT embedding compound, at -20°C.

All rat tissue was cut in coronal sections in a cryostat (Cryostat-E, Reichert-Jung, Vienna, Austria) maintained at -20°C and thaw mounted onto silane coated slides, prepared in the manner outlined in section 2.3.1. Tissue was sectioned at 12μm and 2-3 sections were arranged on each slide, then stored in slide boxes at -20°C.

For tissue that was fixed post-mortem, fixation was carried out no more than 10 days following death. Sections were placed for 5 minutes in 4% paraformaldehyde then rinsed briefly through 0.01M phosphate buffered saline (PBS) solution (see Appendix 5 for solution). Otherwise, sections were immersed in 100% ethanol for 5 minutes. Alternately, sections processed for *in situ* hybridisation as described in section 2.3.5 were used.

Rat retina tissue was prepared by Dr Erica Fletcher (Department of Optometry and Vision Science, University of Melbourne, Parkville, Victoria, Australia) in the manner described by Fletcher *et al.* (1998).

3.3.2 Antigen Retrieval

A black polyacetal staining box (HD Scientific, Blacktown, NSW, Australia) containing 0.01M sodium citrate buffer (SCB) (1 in 10 dilution of 0.1M SCB - see Appendix 5) was suspended in a beaker of boiling water maintained on a hotplate. In this manner, 0.01M SCB was heated to approximately 90°C. Sections were placed in the SCB and allowed to heat for one to two hours, after which time the black polyacetal box was removed from the boiling water and allowed to cool to room temperature. This antigen retrieval technique was modified from that of Dixon & Harper (2001) for sections on slides.

3.3.3 Incubation with Blocking Solution

When using slides, incubation is performed in a humid chamber at room temperature (Costa & Furness, 1983) to ensure tissue does not dry out during incubation (Chemicon, 1998). All incubations were therefore performed at room temperature in Nunc bioassay dishes (Nalge Nunc International, Naperville, IL, USA) equipped with raised grids upon

which slides were supported horizontally. Dampened tissue was placed in the bottom of the tray for humidity.

Sections were thawed to room temperature then washed by immersion in Coplin staining jars containing roughly 60mL 0.1M Tris buffered saline (TBS) (see Appendix 5) or 0.01M phosphate buffered saline (PBS) (see Appendix 5). Washes were performed three times for ten minutes or twice for fifteen minutes, timed with a digital timer. Slides were then removed, excess moisture blotted off and sections were carefully circled with a Pap pen (ICN Biochemicals, Costa Mesa, CA, USA). Each slide was labelled according to the dilution and type of antibody to be used on it.

Blocking solution (10% normal serum v/v, 1% bovine serum albumin w/v, 1% Triton X v/v in 0.1M TBS or 0.01M PBS) was made (see Appendix 5) according to the number of sections, at 100μL per section. Sections were incubated for 1 hour with blocking solution.

3.3.4 Incubation with the Primary Antibody

Immunofluorescence procedure followed that of Fletcher *et al.* (1998). Immunoperoxidase procedure was similar to that of Dixon & Harper (2001), with modifications. Following incubation with blocking solution, slides were shaken, wiped and the Pap pen line reapplied where necessary. A base solution for the primary antibody was made (3% normal serum v/v, 1% Triton X v/v in 0.1M TBS or 0.01M PBS) according to the number of sections, at 100μL per section. Primary antibody was added to the required amount of solution to make the relevant dilution, determined empirically from titration experiments, as follows:

- For $GABA_C$ receptor antisera this was a dilution of 1 in 1000 for immunoperoxidase experiments and 1 in 100 for immunofluorescence experiments.
- For the $GABA_A$ receptor antiserum, this was 1 in 2000 for immunoperoxidase experiments and 1 in 1000 for immunofluorescence experiments.
- For primary antibody control, no primary antibody was added to the base solution.
- For rabbit non-immune antiserum, 1 in 100 dilution was applied.
- For MOPC-21 mouse IgG1kappa serum, 1 in 1000 dilution was used.

Optimal dilutions were determined as those which produced greatest signal with minimal background. Solutions were shaken gently before application. Sections were incubated with primary antibody overnight at room temperature.

3.3.5 Incubation with the Secondary Antibody

Following incubation with the primary antibody, sections were washed in 60mL 0.1M TBS or 0.01M PBS. Sections were separated for washes according to the primary antibody applied. Washes were performed three times for ten minutes or twice for fifteen minutes, timed with a digital timer. Slides were then removed and excess moisture wiped off.

A base solution for the secondary antibody was made (1% normal serum v/v, 1% Triton X v/v in 0.1M TBS or 0.01M PBS) according to the number of sections, at 100μL per section. Secondary antibody solutions were made and applied to sections in the same manner as for primary antibody solutions. Secondary antibody controls omitted any secondary antibody from the base solution. Sections were incubated for one hour at room temperature. Incubation with the secondary antibody for one hour is sufficient to visualise antigenic sites (Costa & Furness, 1983)

Following incubation with the secondary antibody, sections were washed in separate coplin jars according to the type of antibody used. Sections were washed in 0.1M TBS or 0.01M PBS, three times for ten minutes or twice for fifteen minutes.

3.3.5.1 Immunoperoxidase technique

Biotinylated goat anti-rabbit IgG for $GABA_C$ receptor antisera and horse anti-mouse IgG for $GABA_A$ receptor antiserum were diluted to 1 in 1000 in base solution according to manufacturer's instructions.

The amount of streptavidin-peroxidase required for the number of sections, at 100μL per section, was made up from the Standard Vectastain Elite ABC Peroxidase kit according to manufacturer's instructions. Briefly, 5μL of component A was mixed with

5μL of component B for each 1mL total solution in 0.1M TBS and allowed to stand for 20 to 30 minutes.

Following washes in 0.1M TBS, excess moisture was removed from slides and streptavidin-peroxidase was applied. Sections were incubated with streptavidin-peroxidase for 45 minutes at room temperature.

Sections were washed in 0.1M TBS in coplin staining jars, three times for ten minutes or twice for fifteen minutes. During this time, 0.02% DAB solution was made (see Appendix 5) and cooled on ice. The relevant quantity of DAB solution for application of 100μL per section was removed to a separate conical tube. Half the required DAB solution was pipetted onto slides and pre-incubated for 10 minutes. 3% hydrogen peroxide solution (see Appendix 5) was added to the remaining DAB solution immediately prior to use, giving a final concentration of 0.01% hydrogen peroxide (taking the entire quantity of DAB solution into account). This DAB-H_2O_2 solution was added in half quantity to each slide in turn and the reaction time gauged using a digital timer. Reaction time was determined as the time required for a black precipitate to appear. Once reaction product was visible, slides were washed immediately in 0.1M TBS. Sections were then washed in 0.1M TBS for five minutes and allowed to dry overnight under a fume hood. Sections were then coverslipped with 24x50mm coverslips (Lab Supply, Milperra, NSW, Australia) using DPX mountant (Crown Scientific, Moorebank, NSW, Australia). Slides were placed on a flat surface overnight for mountant to dry, then stored at room temperature until microscopic examination. Some sections were first counterstained with cresyl violet (see section 3.3.6) before examination.

3.3.5.2 Immunofluorescence technique

Fluorescence-conjugated Alexa 594 goat anti-rabbit IgG for $GABA_C$ receptor and non-immune rabbit antisera, and Alexa 594 goat anti-mouse IgG for $GABA_A$ receptor and MOPC-21 antisera were diluted to a 1 in 500 dilution in base solution according to manufacturer's instructions. This dilution proved to produce high background staining, and 1 in 1000 dilution was used for later experiments, as determined through titration

experiments performed by Hazel Hill (Department of Pharmacology, University of Sydney).

Solutions containing fluorescence-conjugated secondary antibodies were protected from light using aluminium foil. When sections were incubated with these secondary antibodies the bioassay dishes were covered with foil to reduce light exposure.

Following washes in 0.01M PBS, PBS/glycerol (1:1) mountant (see Appendix 5) was applied to each slide and sections coverslipped. Clear nail polish was applied to the edges of the coverslip to seal the mountant, as it does not dry. Fading of immunofluorescent label occurs if sections dry out (Michael & Priestley, 1996). Once nail polish was dry, sections were stored at 4°C in slide folders until microscopic examination.

3.3.6 Cresyl Violet Staining

Sections were loaded into staining racks and rinsed through water briefly, then placed in 0.5% cresyl violet solution (refer to Appendix 3) for 1 minute. Sections were removed and again rinsed in water, then rinsed 5-20 times in differentiating solution until cell nuclei were purple-blue and background was mainly colourless (ascertained by microscopic inspection). Sections were once again rinsed briefly through water then dehydrated through a series of ethanol solutions by 5-10 rinses in 70% ethanol, 95% ethanol (twice) and 100% ethanol (twice). Slides were then placed in xylene or histolene for 1 minute, then transferred to another container of xylene or histolene for 1 minute, then coverslipped with 24x50mm coverslips using DPX mountant. Slides were placed on a flat surface overnight for mountant to dry, then stored at room temperature.

3.3.7 Microscopy

Immunoreactivity was determined as presence of black precipitate in sections processed using immunoperoxidase histochemistry under brightfield microscopy. Immunoreactivity was determined as the presence of fluorescent puncta in sections processed using immunofluorescence techniques with fluorescence microscopy.

Sections processed using immunoperoxidase histochemistry were examined under a Nikon Eclipse E800 microscope (Nikon Corporation, Kanagawa, Japan) using flat-field apochromatic 10x, 20x, 40x and 60x objective lenses. Digital images were captured using a Fujix HC-1000 3CCD Image Capturing Camera (Fuji Xerox Co., Ltd., Tokyo, Japan) and Adobe Photoshop 5.0 on an Apple Macintosh computer.

Sections processed using immunofluorescence histochemistry were analysed under a Nikon Eclipse E800 microscope using flat-field apochromatic 10x, 20x, 40x and 60x objective lenses and fluorescence filters (FITC and Cy3/Texas Red). Digital images were captured using a SensiCam CCD Imaging system (The Cooke Corporation, Auburn Hills, MI, USA) into SensiCam 4.02 software on a PC computer.

3.3.8 Variations in Experimental Procedure

A number of parameters were tested in the experimental procedure to optimise immunoreactive staining. Fixation procedures were modified and different fixatives compared, since it was thought the fixation process may have altered the $GABA_C$ receptor epitope, preventing detection (Erica Fletcher, personal communication). Immunoperoxidase techniques were first implemented using primarily perfused rat cerebellum. In these experiments, perfusion time was tested at 10 minutes and 5 minutes. Rat cerebellar sections prepared for *in situ* hybridisation were also incorporated to assess whether perfusion or fixation post-mortem yielded best signal.

Immunofluorescence techniques were also applied to rat retinal, cerebellar and forebrain sections. In these experiments, pre-fixation by perfusion and post-fixation by immersion in paraformaldehyde were compared to assess optimal fixation procedures. Additionally, frozen tissue sections were used, as suggested by Ulrike Grünert (personal communication) to alleviate any loss of antigenic reactivity through fixation procedures. Lighter fixation procedures through ethanol immersion were also tested.

Antigen retrieval techniques were applied and compared to tissue that did not undergo antigen retrieval, as it was thought excessive fixation may have masked the antigen.

3.4 RESULTS

3.4.1 Antigen Retrieval

No difference in signal was encountered from sections antigen retrieved for one hour compared to two hours using the procedure described. Furthermore, little difference was observed between sections that were antigen retrieved and those that were not. At most, for some sections antigen retrieval yielded mildly brighter and more punctate labelling, however this was not widely encountered. Figure 3.3 depicts sections of rat cerebellum that were antigen retrieved compared with those that were not. These results were obtained from tissue processed for immunofluorescence histochemistry using the $GABA_C$ receptor antiserum produced by Wässle and colleagues (Enz *et al.*, 1996).

3.4.2 Controls

Omission of the primary antibody yielded only the expected level of background staining in tissue. This occurred with both the biotinylated and fluorescence-conjugated secondary antibodies. Control sections such as those in figures 3.5C and 3.6D showed some staining of white matter using the immunoperoxidase technique. Moreover, it is evident in figure 3.3C that the controls still demonstrated some immunoreactive staining using the immunofluorescence procedure. This background staining encountered with 1 in 500 dilution of the fluorescence-conjugated secondary antibodies did not occur when 1 in 1000 dilution of the secondary antibodies were used. A comparison of figures 3.3C and 3.4F illustrate this.

The use of Rabbit IgG and mouse MOPC-21 non-immune sera in place of the primary antibody failed to produce any staining, as seen in figures 3.4A and 3.4B, as did omission of secondary antibody (data not shown). Immunoreactive staining obtained with the $GABA_A$ receptor antiserum is included in figure 3.4 for comparison to the controls. These images are of rat forebrain sections using immunofluorescence histochemistry.

3.4.3 $GABA_A$ Receptor β2/β3 Subunit Antiserum

3.4.3.1 Immunoperoxidase histochemistry

Sections treated with the $GABA_A$ receptor β2/β3 subunit antiserum demonstrated strong immunoreactive staining in the rat cerebellum using immunoperoxidase techniques. Immunoreactive staining was qualified visually as that which appeared greater than in control sections. Figure 3.5 depicts images of rat cerebellar sections treated with the β2/β3 subunit antiserum in comparison to control and Nissl stained sections. Immunolabelling is clearly present in the granule cell and molecular layers of the cerebellum, and notably absent in the Purkinje cell layer. Staining appears to be localised to cell bodies in the granule cell and molecular layers. This tissue was fixed by perfusion for 10 minutes with 4% paraformaldehyde.

Figure 3.6 presents images of rat cerebellar sections treated with the β2/β3 subunit antiserum after different fixation procedures in comparison to control sections where primary antibody was omitted. These images demonstrate the effects of different fixation procedures on immunoreactivity. Fixation involving perfusion of tissue with 4% paraformaldehyde for 5 minutes yielded adequate immunoreactive staining (figure 3.6A), as did fixation in 4% paraformaldehyde for 5 minutes following fresh frozen cryostat sectioning (figure 3.6B). Tissue that was pretreated for *in situ* hybridisation, incorporating fixation for 5 minutes in 4% paraformaldehyde, on the other hand, did not produce favourable immunoreactive staining (figure 3.6C).

3.4.3.2 Immunofluorescence histochemistry

Tissue treated with the $GABA_A$ receptor β2/β3 subunit antiserum using immunofluorescence procedures yielded similar results to that obtained by immunoperoxidase techniques. Moreover, the pattern of immunoreactive labelling observed in cerebellar sections persisted regardless of fixation procedure. Tissue fixed by perfusion with 4% paraformaldehyde for 5 minutes, tissue that was pretreated for *in situ* hybridisation, and fresh frozen sections fixed in 4% paraformaldehyde or 100% ethanol for 5 minutes all showed robust, punctate immunolabelling in most brain regions investigated. Fresh frozen tissue, however, demonstrated reduced integrity and disrupted morphology following the immunohistochemical procedures (data not shown).

Figures 3.7A and 3.7B compare fresh frozen rat cerebellar sections fixed by immersion in either 4% paraformaldehyde or 100% ethanol for 5 minutes. It is evident that little difference exists between the intensity of labelling for either fixation procedure.

In rat cerebellar sections treated with β2/β3 subunit antibody, cell bodies in the granule cell and molecular layers yielded bright and punctate immunoreactive labelling compared to the control. There was an obvious lack of staining in the Purkinje cell layer. These results are presented in Figure 3.7. Extensive analysis of the localisation of β2/β3 subunit immunoreactivity in forebrain regions was not undertaken. However, in sections examined, immunoreactive labelling above background levels was clearly present in all cortical regions including the olfactory, piriform and insular cortices, as well as some subcortical structures, such as the amygdala and thalamus. Images of amygdala and cortex are displayed in Figure 3.7.

3.4.4 $GABA_C$ Receptor Antisera

3.4.4.1 Immunoperoxidase histochemistry

Sections treated with the $GABA_C$ receptor antisera demonstrated very weak and diffuse immunoreactive staining in the rat cerebellum. Immunoreactive staining was qualified as that which appeared greater than in control sections. Figure 3.3 depicts images of rat cerebellar sections applied with the $GABA_C$ receptor antisera in comparison to control tissue where the primary antibody was omitted. From these images it is apparent that the staining is weak and diffuse, however apparent in distinct brain regions. Therefore specific immunolabelling was observed in the granule cell and Purkinje cell layers of the cerebellum, but not in the molecular layer. As seen in Figure 3.3C, control sections where the primary antibody was omitted did not produce such staining. Control sections showed some background staining in the white matter. This staining was also evident in sections treated with $GABA_C$ receptor antisera, observed in figure 3.3B.

The weak staining was thought to be due to prolonged fixation time, therefore a shorter perfusion time of 5 minutes, as opposed to 10 minutes, was employed. Shorter perfusion time yielded comparable immunoreactive staining, although this was not quantified. Figure 3.5 compares immunoreactive staining in tissue perfused for 10

minutes (figure 3.5B) and 5 minutes (figure 3.5E). Tissue fixed after sectioning and prepared for *in situ* hybridisation, on the other hand, failed to demonstrate immunoreactivity (data not shown).

3.4.4.2 Immunofluorescence histochemistry

As it was difficult to obtain consistent and easily visualised results with the $GABA_C$ receptor antisera using immunoperoxidase histochemistry techniques, immunofluorescence histochemistry was employed. While immunoperoxidase histochemistry is more sensitive, in that lower dilutions of antibody are needed, immunofluorescence histochemistry produces punctate labelling that is easier to visualise.

Tissue fixed by perfusion with 4% paraformaldehyde for 5 minutes yielded immunoreactive labelling with the immunofluorescence technique. Tissue fixed after sectioning and prepared for *in situ* hybridisation, once again, failed to demonstrate immunoreactivity, as did fresh frozen tissue and sections fixed in 100% ethanol (data not shown). Fresh frozen tissue demonstrated reduced integrity and disrupted morphology following immunohistochemical procedures.

Both $GABA_C$ receptor antisera examined produced immunoreactive labelling that appeared greater than control tissue in the rat retina and cerebellum, but not forebrain. This is apparent in figures 3.3 and 3.8. Immunoreactive labelling in the retina was particularly strong and punctate in the outer and inner plexiform layers. Weak immunoreactivity was observed in the cerebellar Purkinje cell layer, and apparently in the granule cell layer. Figure 3.8D shows rat piriform cortex applied with the $GABA_C$ receptor antiserum. This image shows no immunolabelling above background, and is representative of that observed for other regions of the rat forebrain.

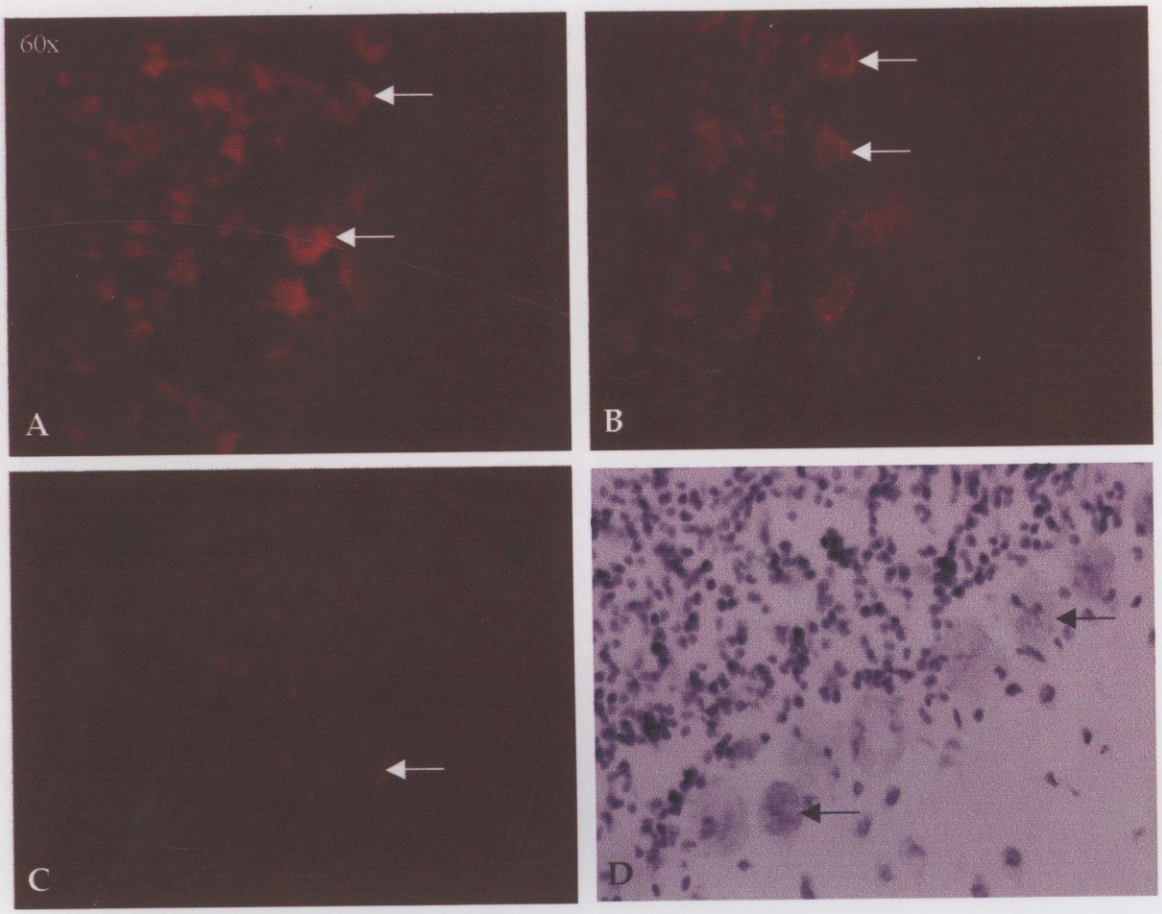


Figure 3.3: Photomicrographs of rat cerebellar sections following immunofluorescence histochemistry using the $GABA_C$ receptor antiserum manufactured by Heinz Wässle, supplied by Erica Fletcher. Tissue was perfused for 5 minutes with 4% paraformaldehyde. Secondary antibody dilution was 1 in 500. Images depict immunoreactive staining in **A**. sections that were antigen retrieved and **B**. sections that were not antigen retrieved. **C**. control tissue where the primary antibody was omitted. **D**. Nissl stained section. Arrows point to Purkinje cells. Images captured at 60x magnification.

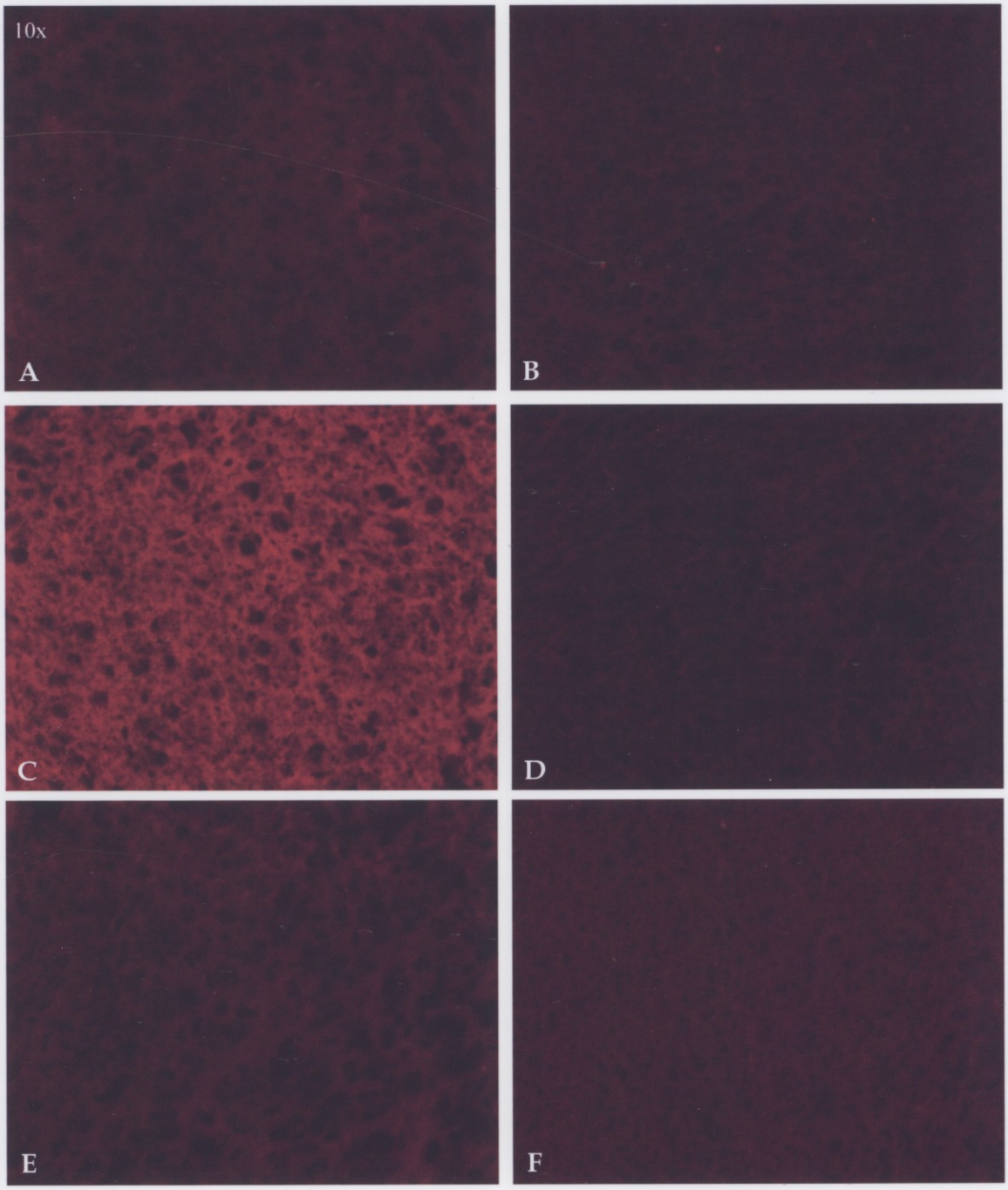


Figure 3.4: Photomicrographs of rat prelimbic cortex following application of differing antisera. Tissue was perfused for 5 minutes with 4% paraformaldehyde. Secondary antibody dilution was 1 in 1000. **A.** Mouse MOPC-21 non-immune serum. **B.** Rabbit IgG non-immune serum. **C.** $GABA_A$ receptor β2/β3 subunit antiserum. High level of immunofluorescent labelling is evident around cell soma as well as neuropil. **D.** $GABA_C$ receptor antiserum made by Heinz Wässle, supplied by Erica Fletcher. No immunofluorescent labelling above background is evident. **E.** Control where $GABA_A$ receptor primary antibody was omitted (goat anti-mouse secondary antibody). **F.** Control where $GABA_C$ receptor primary antibody was omitted (goat anti-rabbit secondary antibody). Images were captured at 10x magnification.

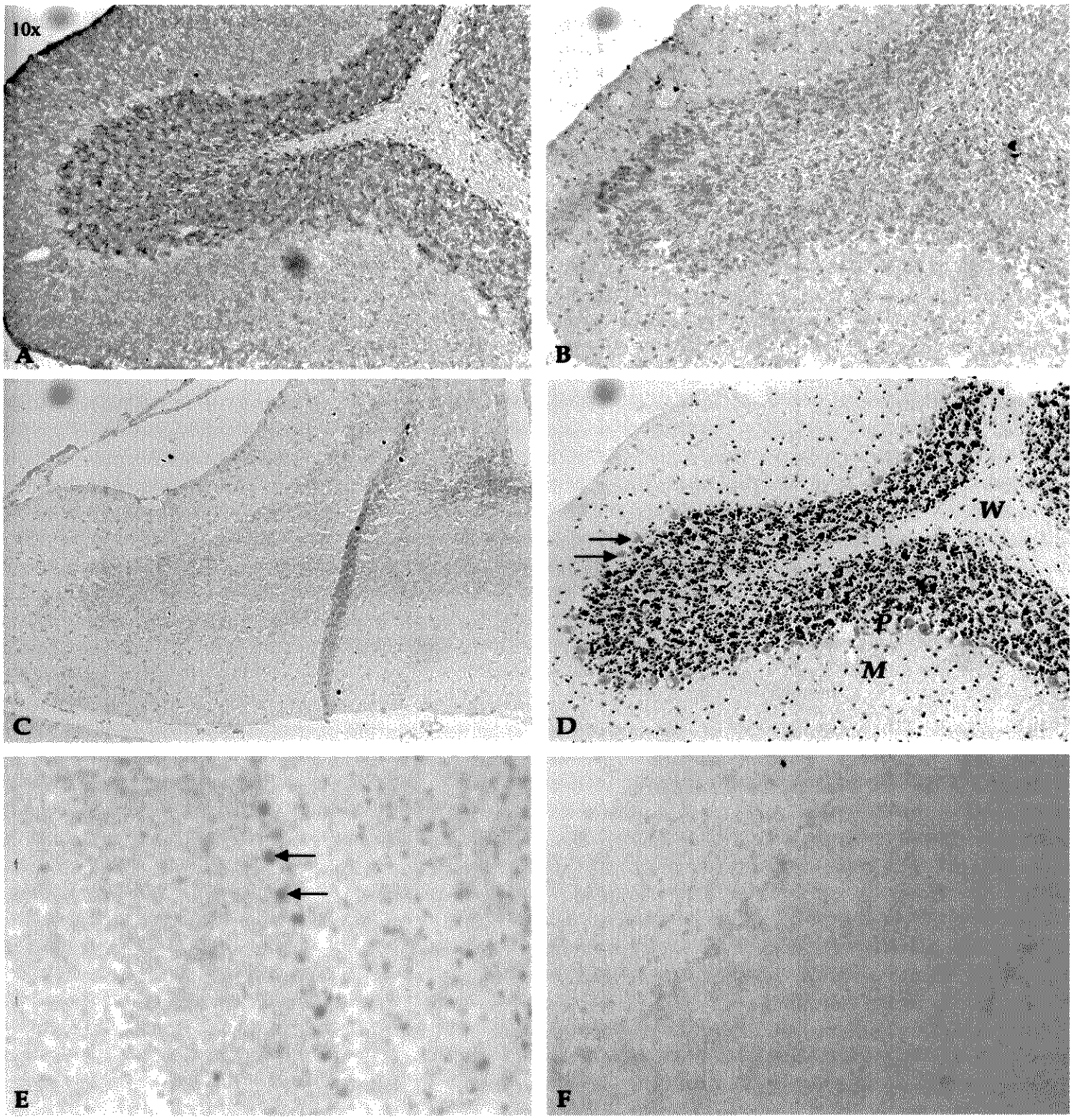


Figure 3.5: Photomicrographs of rat cerebellar sections following immunoperoxidase histochemistry. For A, B, C & D, tissue was perfused for 10 minutes with 4% paraformaldehyde. For E & F, tissue was perfused for 5 minutes with 4% paraformaldehyde. **A**. $GABA_A$ receptor β2/β3 subunit antiserum. **B**. $GABA_C$ receptor antiserum supplied by Erica Fletcher. **C**. Control where primary antibody was omitted. **D**. Adjacent nissl stained section. **E**. $GABA_C$ receptor antiserum supplied by Erica Fletcher. **F**. Control where primary antibody was omitted. Key in D: *W* = white matter. *G* = granule cell layer. *P* = Purkinje cell layer. *M* = molecular layer. Arrows point to large Purkinje cells. Images captured at 10x magnification.

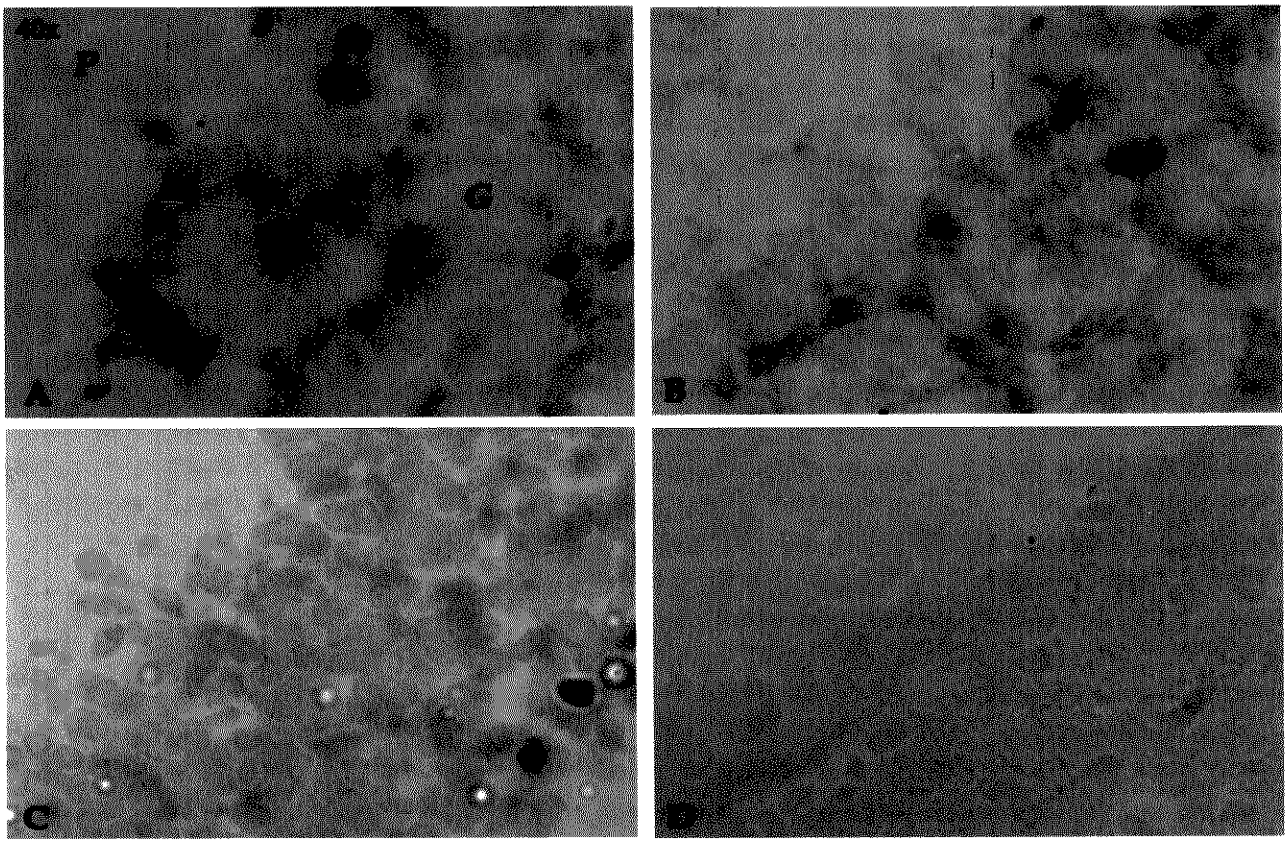


Figure 3.6: Photomicrographs of rat cerebellar sections following immunoperoxidase histochemistry using the $GABA_A$ receptor β2/β3 subunit antiserum. Images depict immunoreactive staining following differing fixation procedures. **A**. Tissue was perfused for 5 minutes with 4% paraformaldehyde. **B**. Tissue was fixed for 5 minutes with 4% paraformaldehyde following sectioning. **C**. Tissue was fixed for 5 minutes with 4% paraformaldehyde and pretreated for *in situ* hybridisation. **D**. Control with primary antibody omitted. Key in A: *G* = granule cell layer. *P* = Purkinje cell layer. Images captured at 40x magnification.

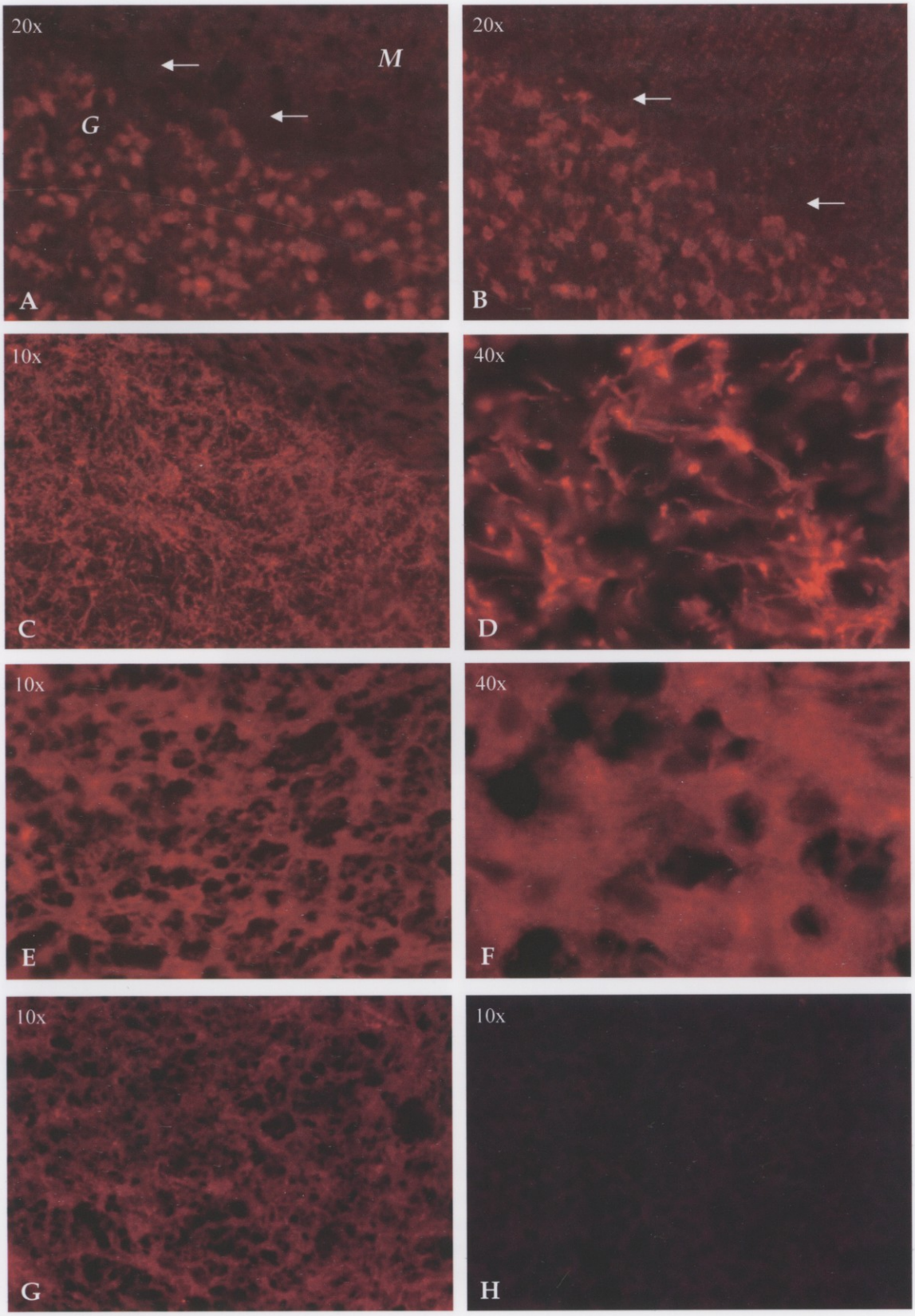


Figure 3.7: Photomicrographs of rat brain sections treated with $GABA_A$ receptor β2/β3 subunit antiserum (A-G) and control with primary antibody omitted (H). **A**. Cerebellum fixed by immersion in 4% paraformaldehyde. **B**. Cerebellum fixed by immersion in 100% ethanol. A & B were captured at 20x magnification. C, E, G & H were captured at 10x magnification. D & F were captured at 40x magnification. C-H from rat brain fixed by perfusion for 5 minutes with 4% paraformaldehyde. **C**. & **D**. Amygdala. **E**. & **F**. Cortex. **G**. & **H**. Olfactory nucleus. Key in A: *G* = granule cell layer. *M* = molecular layer. Arrows point to cerebellar Purkinje cells.

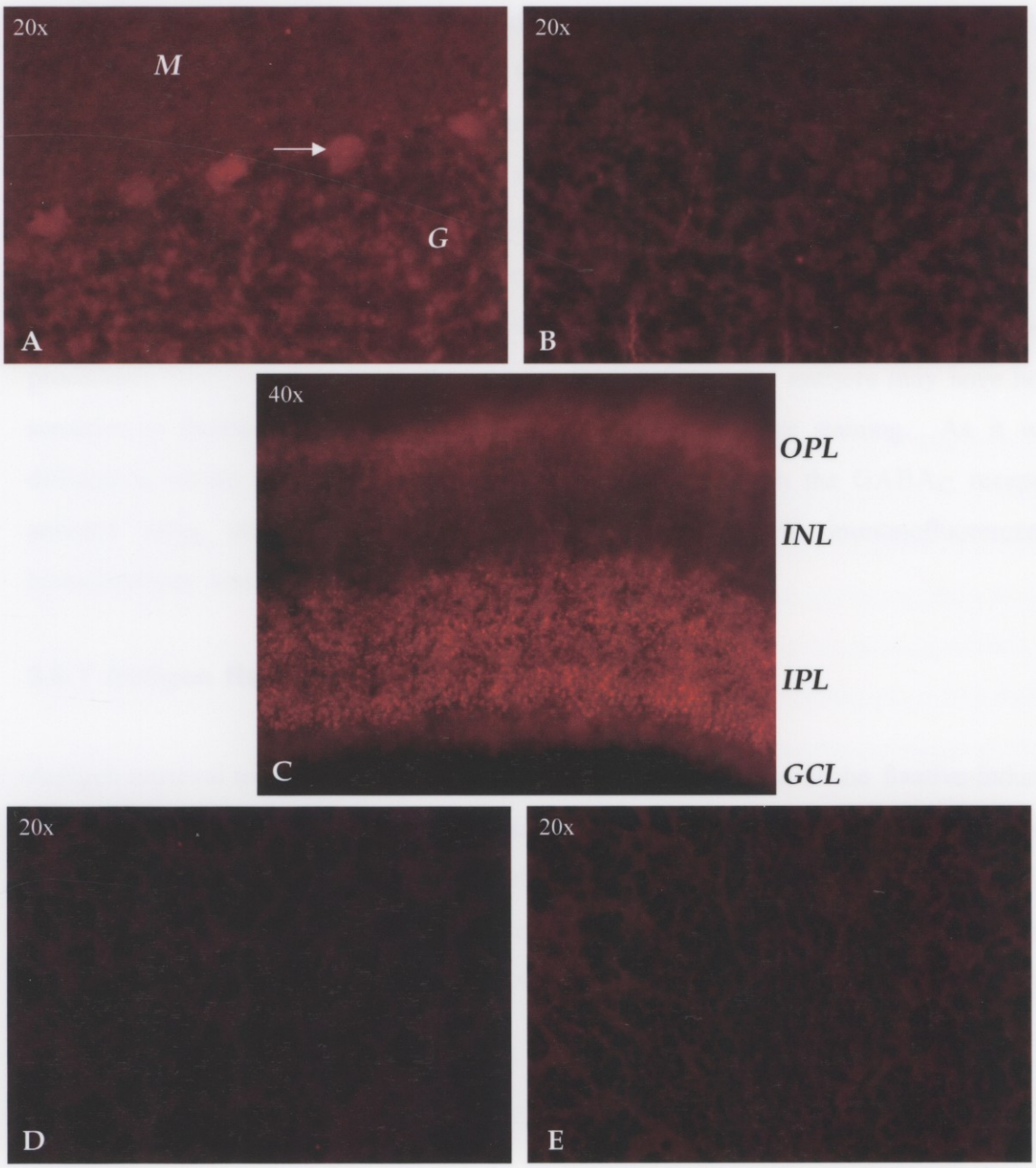


Figure 3.8: Photomicrographs of rat brain sections treated with $GABA_C$ receptor antisera and control where the primary antibody was omitted. Tissue was fixed by perfusion with 4% paraformaldehyde for 5 minutes, except retina (C), which was fixed by immersion in 4% paraformaldehyde (Fletcher *et al.*, 1998). **A**. Cerebellum treated with $GABA_C$ receptor antiserum manufactured by Maurice Garrett. **B**. Cerebellum control. **C**. Retina treated with $GABA_C$ receptor antiserum made by Heinz Wässle, supplied by Erica Fletcher. **D**. Piriform cortex treated with $GABA_C$ receptor antiserum obtained from Heinz Wässle. **E**. Piriform cortex control. A & B captured at 20x magnification. C captured at 40x magnification. D & E captured at 20x magnification. Key in A: *G* = granule cell layer. *M* = molecular layer. Arrows point to Purkinje cells. Key in C: *OPL* = outer plexiform layer. *INL* = inner nuclear layer. *IPL* = inner plexiform layer. *GCL* = ganglion cell layer.

3.5 DISCUSSION

The aims of the present study were to confirm the presence of $GABA_C$ receptors in the rat retina and cerebellum, and to extend this investigation to the rat forebrain. While determination of $GABA_C$ receptors in the retina was easily achieved, demonstration of immunoreactivity in the cerebellum was somewhat less straightforward. Furthermore, using the antisera provided, no immunoreactivity to $GABA_C$ receptors could be detected in the rat forebrain. Concurrently, immunoreactivity to $GABA_A$ receptor $\beta2/\beta3$ subunit was detected in all regions of the rat CNS investigated. Fixation procedures were varied, as it was thought the $GABA_C$ receptor antisera may have been sensitive to fixation, accounting for the lack of immunoreactive staining. As it was difficult to obtain consistent and easily visualised results with the $GABA_C$ receptor antisera using immunoperoxidase histochemistry techniques, immunofluorescence histochemistry was also employed.

3.5.1 Antigen Retrieval

Antigen retrieval techniques were employed to break down some of the fixative-induced cross linkages and effectively render antigen sites available for subsequent antibody interaction. Regardless, antigen retrieval made little difference, qualitatively, to the amount of immunoreactive staining observed. At most, for some sections antigen retrieval yielded mildly brighter and more punctate labelling, however this was not widely encountered.

3.5.2 Fixation Procedures

The $GABA_C$ receptor antisera are often sensitive to fixation (Fletcher *et al.*, 2001). Fixation procedures were varied to minimise the amount of fixation in order to obtain greater immunoreactive staining with the $GABA_C$ receptor antisera. Fixation time affects labelling of the antigen. For example, Greferath and coworkers (1995), in a study examining the localisation of $GABA_A$ receptor subunits in rat retina, showed that short fixation times (10-15 minutes) resulted in punctate labelling, while longer fixation times resulted in diffuse staining of dendrites and cell bodies. Fletcher and colleagues (2001) investigating $GABA_C$ receptors in the rat gastrointestinal tract, found fixation in

paraformaldehyde at 4°C produced optimal labelling. Fixation longer than 30 minutes under these conditions resulted in a loss of immunolabelling. In the present study, using immunoperoxidase histochemistry techniques, tissue perfused for 10 minutes was compared with that perfused for 5 minutes, however little difference in immunoreactive staining was observed. It may be worthwhile to investigate fixation of tissue post-mortem at reduced temperatures, as Fletcher and associates (2001) did for gastrointestinal tract, to see if $GABA_C$ receptor immunolabelling can be detected in the rat forebrain.

Using immunofluorescence histochemistry, tissue perfused for 5 minutes was compared to tissue fixed in 4% paraformaldehyde following sectioning. Again, little difference was observed. Tissue prepared for *in situ* hybridisation failed to demonstrate immunoreactivity to the $GABA_C$ receptor antisera. Since this tissue undergoes a series of treatments including dehydration and defatting, it is possible the weak antigenicity of the $GABA_C$ receptor could not survive such rigorous treatment. Nonetheless, the $GABA_A$ receptor $\beta 2/\beta 3$ subunit antibody produced robust and distinct immunoreactive labelling regardless of fixation procedure, attesting to its usefulness as a positive control.

Ethanol is a gentler means of fixation, and it was thought its use may attenuate the problem of antigen-site masking by aldehyde fixatives. While no difference in immunoreactive staining was observed between ethanol and paraformaldehyde fixed tissue using the $GABA_A$ receptor $\beta 2/\beta 3$ subunit antibody, no staining could be detected following application of the $GABA_C$ receptor antisera in ethanol-fixed tissue. The use of fresh frozen tissue also failed to produce any immunoreactive staining for both the $GABA_A$ and $GABA_C$ receptor antisera, and demonstrated a loss of integrity and disruption of morphology. It seems the fresh frozen tissue could not withstand extensive washing by immersion in buffers and prolonged incubation required of the immunohistochemical procedures. Solubilisation of the antigen or diffusion within the tissue may have occurred with the use of fresh frozen sections (Sternberger, 1979).

3.5.3 Controls

In order to demonstrate specificity of the primary antibody to the antigen site it was manufactured against, omission of the primary antibody should not produce immunoreactive labelling in the tissue under question (Pearse, 1980; Van Noorden, 1986). A small degree of background immunoreactive staining was witnessed when the primary antibody was omitted using both immunoperoxidase and immunofluorescence techniques. Background staining in the immunoperoxidase techniques may have occurred through prolonged reaction with DAB-H_2O_2. Additionally, the secondary antibody concentration may have been too high, creating electrostatic bonds with basic proteins in the section and protein-protein interactions (Pearse, 1980; Pool & Buijs, 1988). This appeared to be the case in immunofluorescence procedures. A 1 in 500 dilution of the secondary antibody, as suggested by the manufacturer, produced moderate background staining, which was ameliorated by dilution of the antibody to 1 in 1000. On the whole, omission of the primary antibody did not produce immunoreactive staining near the intensity observed when primary antibodies were applied to the tissue, indicating the primary antibodies were sufficiently specific to the antigens in question.

3.5.4 $GABA_A$ Receptor β2/β3 Subunit Antiserum

Immunoreactivity to the β2/β3 subunits of the $GABA_A$ receptor occurred predominantly in the granule cell layer of the cerebellum, and to a lesser extent in the molecular layer, denoting the presence of $GABA_A$ receptors containing β2 or β3 subunits in these areas of the cerebellum. No immunoreactivity was observed in the Purkinje cell layer. These findings are consistent with *in situ* hybridisation results outlined in chapter 2, where the β3 subunit mRNA was encountered primarily in the granule cell layer of rat cerebellum. This is also consistent with the literature, where β2/β3 subunit antibodies showed immunoreactive labelling in the cerebellar granule cell and molecular layers, but not the Purkinje cell layer (Fritschy & Möhler, 1995; Pirker *et al.*, 2000).

In the present experiments, immunoreactivity to the β2/β3 subunits also occurred in rat forebrain regions. Although extensive regional investigation was not undertaken, the

presence of immunoreactive labelling suggests $GABA_A$ receptors containing the β2 or β3 subunits were present in all regions of the cortex examined, including the piriform and insular cortices, the olfactory nucleus and the amygdala. Again this localisation is consistent with β3 subunit mRNA distribution derived from *in situ* hybridisation experiments outlined in chapter 2, as seen in table 2.3. Furthermore, the current results are supported by the literature, where β2/β3 subunit antibodies showed immunoreactive labelling in all cortical regions, including the piriform and insular cortices, as well as subcortical structures (Fritschy & Möhler, 1995; Pirker *et al.*, 2000; Schwartzer *et al.*, 2001; Wainwright *et al.*, 2000), outlined in table 1.2.

Not only was immunoreactivity to the β2/β3 subunits consistent with mRNA distribution, immunoreactivity could be reliably demonstrated in all experiments. The $GABA_A$ receptor β2/β3 subunit antibody therefore provided an excellent positive control.

3.5.5 $GABA_C$ Receptor Antisera

Functional $GABA_C$ receptors are known to exist in retina (Feigenspan & Bormann, 1998), and the ρ subunits, believed to be the molecular components of $GABA_C$ receptors, were isolated and cloned from retina. One aim of this study was to assess whether the $GABA_C$ receptor antisera provided could adequately detect $GABA_C$ receptors using rat retina as positive control tissue. The $GABA_C$ receptor antisera produced strong immunoreactive staining in the rat retina, particularly the outer and inner plexiform layers, indicating the presence of $GABA_C$ receptors in this structure, as well as the usefulness of the antisera. Detection of $GABA_C$ receptors in the retina using immunohistochemistry substantiates findings of $GABA_C$ receptor ρ1 and ρ2 subunit mRNAs in the rat retina using *in situ* hybridisation (chapter 2), and is consistent with the literature (Enz *et al.*, 1996; Koulen *et al.*, 1998).

$GABA_C$ receptor mRNA was convincingly demonstrated in the rat cerebellum using *in situ* hybridisation (chapter 2). Cerebellar tissue was therefore investigated for the presence of $GABA_C$ receptors using immunohistochemistry, and was used to assess experimental techniques for optimal immunoreactive staining. Immunolabelling of

$GABA_C$ receptors was observed predominantly in the Purkinje cell layer of the rat cerebellum, with some labelling detected in the granule cell layer. These observations support the localisation of $GABA_C$ receptor ρ1 and ρ2 subunit mRNA in the rat cerebellum using *in situ* hybridisation. mRNA was detected in the granule cell layer, but not the Purkinje cell layer. Quantification of grain density at the cellular level of analysis may confirm the presence of ρ subunit mRNAs above background levels in the Purkinje cell layer, however this remains to be determined. Results from the present study are partially consistent with the literature, where the $GABA_C$ receptor antiserum produced by Garrett and colleagues labelled Purkinje cells of the cerebellum (Boue-Grabot *et al.*, 1998). The authors did not report any labelling in the granule cell layer, however they used a 1 in 4000 dilution of the primary antibody with the immunoperoxidase technique. In the current experiments, a 1 in 1000 dilution of the primary antibody was used for immunoperoxidase experiments. A 1 in 100 dilution of the primary antibody was also applied using immunofluorescence procedures, which may have facilitated visualisation of immunoreactivity in the granule cell layer.

Given adequate localisation of $GABA_C$ receptors in the rat retina and cerebellum using the antisera provided and experimental protocol, another aim of this investigation was to probe rat forebrain for the presence of $GABA_C$ receptors. Subsequent to conduction of experiments in the current study, $GABA_C$ receptors were labelled in the rat superior colliculus using immunohistochemistry techniques (Clark *et al.*, 2001; Pasternack *et al.*, 1999). In the present examination, however, no immunoreactive labelling was observed in tissue applied with the $GABA_C$ receptor antisera in any region of the rat forebrain or midbrain. A small degree of immunofluorescence was observed, but this was no greater than that seen in sections treated with the secondary antibody only. It is possible that the background fluorescence exhibited by the secondary antibody at 1 in 500 dilution was masking weak primary signal from the $GABA_C$ receptor antibody. However, even when 1 in 1000 dilution of the secondary antibody was applied, no specific immunoreactive staining was encountered with the $GABA_C$ receptor antisera in rat forebrain. Since the $GABA_A$ receptor antibody produced strong staining in adjacent forebrain sections, experimental techniques are unlikely to be the reason for the lack of staining above background using the $GABA_C$ receptor antisera.

The antibodies against $GABA_C$ receptors used in the present study were directed against the ρ1 subunit. ρ1 Subunit mRNA was not well demonstrated outside the retina and cerebellum using *in situ* hybridisation, suggesting it is not present in rat forebrain regions. This may explain the lack of immunolabelling encountered. However, the antiserum developed by Wässle and colleagues is known to cross react with ρ2 and ρ3 subunits (Enz *et al.*, 1996) and ρ2 subunit mRNA could be convincingly demonstrated in rat forebrain (see chapter 2). As mentioned in section 2.5.3.2, the $GABA_C$ receptors have been cloned from rat retina only. Strong immunoreactive labelling could be obtained from rat retina using the $GABA_C$ receptor antisera provided, confirming the antisera could recognise the $GABA_C$ receptors. Identification in rat forebrain, however, was not possible. This may be because $GABA_C$ receptors in the forebrain are composed of different subunits, or splice variants of the known subunits, therefore detection was not possible. Alternately, the epitope may differ due to heterogeneity in post-translational modifications such as glycosylation (Chemicon, 1998).

Alternatively, the lack of fluorescence in forebrain sections treated with the $GABA_C$ receptor antisera may have resulted from a concentration of primary antibody that was not high enough to produce immunofluorescence in these structures. The optimal dilution of antisera was determined using rat cerebellar tissue, not forebrain sections. Titration experiments using rat forebrain sections must be conducted to investigate the possibility that higher concentrations of primary antibody are required.

The success of immunohistochemical procedures depends on the reaction of the antibody to the fixed tissue antigen (Pool & Buijs, 1988). Failure to detect robust immunoreactive staining of the $GABA_C$ receptors in the present examination may have been due to an alteration in the epitope to which the antisera were directed. Loss of soluble antigens may have occurred during tissue processing or incubation procedures (Pool & Buijs, 1988; Pool *et al.*, 1983). Antigenicity depends on conformational features of the protein that may be severely altered by the fixation process (Brandtzaeg, 1982). Folding of proteins may occur, making antigen sites inaccessible. Aldehyde fixation can denature the epitope (Chemicon, 1998; De Blas, 1996) rendering it undetectable. Thus, fixation with paraformaldehyde may have precluded the antibody-antigen interaction from occurring. 100% Ethanol was employed as an alternate means

of fixation, however this also failed to produce immunoreactive staining in the forebrain, as well as in the cerebellum.

The demonstration of an antigen in low concentrations is more difficult than where it is present in high concentrations (Pool & Buijs, 1988; Pool *et al.*, 1983). The ability to detect $GABA_C$ receptor subunit mRNAs using *in situ* hybridisation, but not the protein using immunohistochemistry, may be due to the greater sensitivity of the *in situ* hybridisation technique. Furthermore, the presence of mRNA does not ensure translation to protein. Nevertheless, "unsuccessful staining with a particular antiserum in a tissue investigated for the first time cannot be regarded as conclusive evidence that the tissue antigen under investigation is not present in the tissue" (Costa & Furness, 1983).

3.4.6 Conclusions

In summary, $GABA_A$ receptor β2/β3 subunits could be detected in all regions of the rat brain investigated regardless of fixation procedure. The $GABA_C$ receptors, on the other hand, were successfully demonstrated in the rat retina, at low densities in the rat cerebellum, but not in rat forebrain. These findings support the localisation of $GABA_C$ ρ1 and ρ2 subunit mRNAs in rat retina and cerebellum, outlined in chapter 2, but not in rat forebrain. The antisera used in these experiments have been used by other researchers to successfully label $GABA_C$ receptors in the rat pituitary gland (Boue-Grabot *et al.*, 2000), superior colliculus (Clark *et al.*, 2001; Pasternack *et al.*, 1999) and rat gastrointestinal tract (Fletcher *et al.*, 2001).

The status of functional $GABA_C$ receptors in the rat CNS is still questionable. While ρ2 subunit mRNA is clearly present in the rat CNS, ρ2 subunits fail to form robust homooligomeric receptors in *Xenopus* oocytes (Zhang *et al.*, 1995). Although this may not be the case for ρ2 subunits in rat brain, an inability to detect $GABA_C$ receptor immunoreactive labelling does little to verify the presence of functional $GABA_C$ receptors in rat brain. Indeed, an antibody directed against a different region of the ρ2 or ρ3 subunit may produce positive results in the CNS. Nevertheless, a lack of immunolabelling does not provide conclusive evidence that the antigen is not present in

the tissue. The antigenic site may have been altered by fixation, rendering it undetectable. Otherwise, receptors may be present in such low abundance that they could not be detected using immunohistochemistry without signal amplification.

The present study was qualitative, and specific immunoreactive staining was deemed as that which appeared greater than the background signal derived from controls where the primary antibody was omitted. In order to obtain conclusive evidence of $GABA_C$ receptors in rat brain using immunohistochemistry, quantitation must be performed, especially given the peptide's apparently low yield, for example, in the rat cerebellum.

Findings of $GABA_C$ receptors in the rat cerebellum in the current examination support binding studies of bicuculline- and baclofen-insensitive $GABA_C$ receptors in rat cerebellum (Drew *et al.*, 1984; Drew & Johnston, 1992). Binding studies with $GABA_C$ selective compounds, such as the recently available [^{3}H]P4MPA, will enable further evaluation of $GABA_C$ receptors in the CNS. Ultimately, functional analysis of $GABA_C$ receptors in the CNS must be undertaken to provide a clear idea of their location and functional role in the brain.

PART 2

IONOTROPIC GABA RECEPTORS IN SCHIZOPHRENIA

IONOTROPIC GABA RECEPTORS IN SCHIZOPHRENIA

CHAPTER 4: LITERATURE REVIEW

4.1 INTRODUCTION TO THE NEUROSCIENCE OF SCHIZOPHRENIA

"...that most characteristic plague of the twentieth century, schizophrenia. Is the mental disorder due to a chemical disorder?"

Aldous Huxley, The Doors of Perception

The ponderings of Aldous Huxley evolved from a comparison of the similarity between the effects of hallucinogenic drugs and symptoms of schizophrenia. Since drugs are known to affect neurochemistry, he contemplated whether schizophrenia might also involve changes in neurochemistry. Huxley's surmisings are accurate according to contemporary theory. Original neuroscientific theories of the pathophysiology of schizophrenia were based on the similarities between experiences and psychoses elicited by hallucinogenic drugs such as LSD and phencyclidine, and psychoses of patients with schizophrenia. The invention of drugs for the treatment of schizophrenia symptoms consolidated the connection between the symptoms and abnormalities in neural processes. Drugs manipulate neurotransmitter systems to alleviate the symptoms of the disorder.

The common biological view interprets all activities of the "mind" as a function of the brain. The hypothesis is that abnormal mental functioning has neuroanatomical and neurochemical substrates. Current research works to resolve the neuroanatomical and neurochemical dysfunctions responsible for the symptoms of schizophrenia. The difficulty of this task lies not only in the complexity of the disorder itself, but in the complexity of the central nervous system and the interplay of neurochemicals. There is no doubt that multiple transmitter systems and multiple structures are involved in the pathophysiology of schizophrenia.

4.2 HISTORY OF SCHIZOPHRENIA

Schizophrenia is a uniquely human disorder, which has been recognised in all cultures from antiquity. The disorder affects aspects of higher neural functions: cognition,

emotion (but not mood) and behaviour. Records reveal that schizophrenia was identified as early as 1400BC in ancient India (Summers *et al.*, 1989). Hippocrates wrote of the disorder in his manuscript *The Sacred Disease*, where he challenged the popular belief that mental disorders were of sacred origin, and instead arose from natural causes and an unhealthy brain (Costin & Draguns, 1989). What is now known as schizophrenia was initially referred to for some time as *dementia praecox*, a term coined by Bénédict Augustin Morel in his 1860 text *Traité des Maladies Mentale* (Costin & Draguns, 1989). "Dementia" pertained to the deterioration of intellectual capacity. "Praecox" related to the onset of the disorder primarily in young people (Summers *et al.*, 1989). Emil Kraepelin used the term dementia praecox to describe the disorder in his major text, *Psychiatrie*, published in 1896. In this text, Kraepelin eloquently and systematically described the symptoms of dementia praecox. The eminent psychiatrist, Eugene Bleuler, revolutionised the characterisation of schizophrenia in his 1908 volume *The Prognosis of Dementia Praecox: The Group of Schizophrenias* by introducing the term *schizophrenia* to describe the disorder. Bleuler believed that the disorder constituted, not a dementia *per se*, but a group of symptoms which involved the "tearing apart or splitting of psychic functions". Bleuler's observations were accurate in that schizophrenia is recognised as a syndrome, or group of symptoms, and is not neurodegenerative like the dementias, but neurodevelopmental. The word schizophrenia takes its roots in the Greek "schizein"; to split, and "phren"; the mind. For Bleuler, the "split" referred to the loss of capacity to connect thought processes with function (Andreasen, 2000; Costin & Draguns, 1989; Summers *et al.*, 1989).

4.3 Social Impact of Schizophrenia

Schizophrenia is a debilitating illness, which seriously affects not only those with the disorder, but families and friends and those in the wider community who may experience first hand the social consequences of the disorder. Current statistics depict an annual economic burden of $3 billion in Australia for treatment and loss of productivity (Mowry & Nancarrow, 2001).

Schizophrenia knows no socio-economic or cultural boundaries, nor does it show any obvious preference for gender. The prevalence of schizophrenia is estimated at 1% of the mean population, ranging from 0.3% in Taiwan to 3% in Finland (Andreasen, 2000;

Mowry & Nancarrow, 2001; Rowley *et al.*, 2001). Gender differences occur in age of onset (18-25 years of age for males and 25-35 years of age for females), but not in prevalence (Mowry & Nancarrow, 2001). Due to its high prevalence in the community, schizophrenia is an extremely important disorder to research.

4.4 SCHIZOPHRENIA SYNDROMES

Psychosis is a term used loosely to define a severe mental disorder characterised by disorganised thought processes, emotional disturbances, disorientation with time, space and person, and hallucinations and delusions (Chaplin, 1985). Psychosis is therefore inherent in schizophrenia, however it is not unique to schizophrenia. Other disorders involving psychosis include affective disorders, major depression and alcoholism. Since some of the symptoms are shared, the diagnosis of schizophrenia is reliant not only on the presentation of psychotic symptoms, but also on the combination of symptoms and length of time they are present.

Schizophrenia is characterised generally as a misinterpretation of reality - that is, a misinterpretation of information coming through an individual's sensory systems (Andreasen, 2000; Crowe, 1998). As mentioned earlier, the misinterpretation of reality is claimed to arise from aberrant neural processing. The manifestations of this reality distortion are commonly symptoms of psychomotor poverty and disorganised speech and behaviour, representing disorganised thought patterns.

The symptoms of schizophrenia are diverse, therefore persons with the disorder do not display all symptoms to the same extent. The term *schizophrenia* actually refers to a group of related disorders whose specific manifestations and aetiologies differ, but whose basic characteristics remain the same. It is technically more accurate to refer to schizophrenia as *schizophrenic disorders* or *disorders of the schizophrenic syndrome* (Costin & Draguns, 1989). However, diagnosis confines the term "schizophrenia" to a subgroup of persons reflecting some homogeneity, therefore the single term schizophrenia is commonly used throughout the literature and will be used in this thesis to refer to the complex set of syndromes that comprise the schizophrenic disorders.

4.4.1 Diagnostic Criteria

The Diagnostic and Statistical Manual of Mental Disorders, edition IV (DSM-IV), (American Psychiatric Association, 1994) is used as the benchmark for characterisation of schizophrenia across clinical, research and community health disciplines. According to DSM-IV (1994), diagnosis of schizophrenia requires a combination of representative symptoms; delusions, hallucinations, disorganised speech or behaviour, and negative symptoms (refer to table 1), existing for one month, with some signs of the disorder lasting six months or more. These symptoms cannot be better accounted for by another psychological disturbance, physiological explanation or medical condition (DSM-IV, 1994). In addition, symptoms should produce a marked impairment in work, interpersonal relationships, or self care (DSM-IV, 1994).

Schizophrenia must be distinguished from similar disorders such as schizotypal personality disorder and schizophreniform. Schizotypal personality disorder is a form of personality disorder which resembles schizophrenia, however the break from reality is not so extreme, and symptoms of delusions and hallucinations are not evident (Costin & Draguns, 1989). Schizophreniform is the classification used when the symptoms of schizophrenia are present for less than six months, and is therefore delineated as a separate disorder from schizophrenia (DSM-IV, 1994).

4.4.2 Problems with Diagnostic Criteria outlined in DSM-IV

There are a number of problems inherent in the DSM-IV diagnostic criteria for schizophrenia. Criterion-based diagnostic systems are purely descriptive, focusing on only a few of the symptoms that patients present with (Andreasen, 2000). Although it is known that the psychotic symptoms of schizophrenia are shared with a number of disorders, the syndromal nature of the disorder is not emphasised in the DSM-IV diagnostic criteria (Andreasen, 2000; Maj, 1998). Even with exclusion criteria in place, misdiagnosis can occur. The amount of weight given to each of the five groups of symptoms listed in the diagnostic criteria (see section 4.1) is equal, which makes questionable the diagnosis a clinician will make upon client presentation (Maj, 1998). Furthermore, psychotic symptoms are emphasised as the defining feature for diagnosis where they may not be in reality (Andreasen, 2000). Length of time symptoms have

been manifest is also dependent on the clinician's subjective judgement (Maj, 1998). The fact that exclusion criteria incorporate aetiology is also highly problematic. If the aetiology is known, for example the symptoms are due to a CNS infection, then schizophrenia is ruled out. However, schizophrenia may be caused in part by infection. It seems that if aetiology can be defined, then the diagnosis of schizophrenia cannot be made (Maj, 1998). Essentially, the final diagnosis of schizophrenia is a diagnosis by exclusion.

The totality of problems with diagnostic criteria impinges upon the reliability of diagnosis. Reliable diagnosis is critical to studies on schizophrenia using post-mortem tissue. Even if patient history is rigorous and conclusive, it amounts to little if diagnosis is not.

4.4.3 Positive and Negative Symptomatology of Schizophrenia

Symptoms of schizophrenia have been classified two-dimensionally into positive, reflecting an excess or distortion of normal function; and negative, reflecting a decrease or loss of normal function (Andreasen, 2000; DSM-IV, 1994) in accordance with the scheme put forth by Andreasen & Olsen (1982). Table 4.1 (page 183) details the positive/negative symptom division, adapted from Andreasen & Olsen (1982) and Davison & Neale (1996). An alternative three-dimensional scheme was proposed by Liddle (1987), comprising reality distortion syndrome, disorganisation syndrome and psychomotor poverty. Table 4.2 (page 184) depicts this alternate division of symptoms. Although this scheme provides an excellent point of departure for neurochemical and neuropathological studies into schizophrenia, the positive/negative symptomatology prevails in the recognised description and diagnosis of schizophrenia.

4.4.4 Problems with Positive and Negative Symptomatology

Much controversy has reigned over the simplistic division of the symptoms of schizophrenia into positive and negative. The distinction was made in an attempt to simplify in order to clarify (Andreasen, 1990). In the case of schizophrenia, however, the simplified conceptualisation of symptoms may not do justice to the diversity and complexity of observed behaviours.

Regardless of the refutations of the positive/negative symptoms division, this differentiation is inclusive of all symptoms and the usefulness of the distinction ensures the classification is still used in current clinical and research situations.

4.5 AETIOLOGIES OF SCHIZOPHRENIA

The aetiology of schizophrenia is questionable and a number of theories have been proffered. It is currently anticipated that schizophrenia is in fact a syndrome encompassing aetiologically diverse disorders sharing common clinical symptoms (Trojanowsky & Arnold, 1995). A genetic component is clearly indicated, perhaps increasing susceptibility to the disorder, however environmental factors are thought to play a major role.

4.5.1 Neurodevelopmental versus Neurodegenerative

While it was first accepted that schizophrenia was a neurodegenerative disorder, in that the disease progression was insidious and accompanied by increasing brain damage, it is now generally accepted that schizophrenia is in fact a neurodevelopmental disorder. Gliosis, or glial cell proliferation, is a marker for degeneration, and is not evident in post-mortem examination of brains from people with schizophrenia (Benes *et al.*, 1986; Egan & Weinberger, 1997; Falkai & Bogerts, 1986; Falkai *et al.*, 1988; Harrison, 1997; Kurumaji *et al.*, 1997; Rajkowska *et al.*, 1998; Roberts *et al.*, 1986; Roberts *et al.*, 1987; Selemon *et al.*, 1998).

A neurodevelopmental theory of schizophrenia proposes that schizophrenia is caused by problems in neural development and cortical maturation, and brain damage may be present but not progressive (Harrison, 1997). The problems in neural development may occur during the pre-, peri- or postnatal periods, from variations in nutritional factors, toxins, infectious agents, inflammation or obstetric complications (Andreasen, 2000; Carlson, 1991; Harrison, 1997; Holmes, 1997).

Table 4.1: Positive and Negative Symptoms of Schizophrenia. Adapted from Andreasen & Olsen (1982), DSM-IV (1994) and Davison & Neale (1996).

Positive symptoms	Negative symptoms
Hallucinations (distortion of perception) • auditory (voices conversing or voices commenting) • somatic/tactile • gustatory • olfactory • visual	**Flattened affect** • lack of facial expression • lack of spontaneous movement • poverty of gestures • poor eye contact • affective non-responsivity • inappropriate affect • lack of vocal inflections
Delusions (distortion of inferential thinking) • delusions of persecution • delusions of reference • delusions of jealousy • delusions of sin or guilt • delusions of grandiosity • religious delusions • somatic delusions • delusions of control • mind reading • made feelings • made volitional acts • made impulses • thought broadcasting • thought insertion • thought withdrawal	**Alogia (restrictions in fluidity and productivity of thought and speech)** • poverty of speech • poverty of content of speech • blocking • latency of response **Avolition/Apathy (restrictions in initiation of goal-directed behaviour)** • inattention to grooming/hygiene • impersistence • anergia (lack of energy) **Attention** • social (conversation) • under testing (serial 7's) • difficulty concentrating **Anhedonia (inability to experience pleasure)** • lack of recreational interests • lack of sexual interest • withdrawal from intimacy and closeness • withdrawal from friends/peers **Cognitive dysfunction** • confusion/disorientation • memory impairment • lack of insight
Disorganized thought and speech • loose associations/derailment • tangentaility • incoherence • illogicity • cognitive flooding/stimulus overload • circumstantiability • pressure of speech • clanging	
Disorganized/catatonic behaviour • clothing and appearance • inappropriate sexual behaviour • inappropriate social behaviour • aggressive/agitated • repetitive behaviour • catatonic posturing • catatonic rigidity • catatonic excitement • catatonic negativity • catatonic stupor	

Table 4.2: Three dimensional model of schizophrenia symptoms adapted from Liddle (1987).

	Macro level	Micro level
Reality distortion syndrome voices speak to patient delusions of persecution delusions of reference	temporal lobe abnormality; delusions, in particular, are associated with left temporal lobe lesions	good response to neuroleptics suggesting some neurochemical abnormality in dopamine system
Disorganisation syndrome inappropriate affect poverty of content of speech tangentiality derailment pressure of speech distractibility	frontal lobe abnormality; associated, in particular with mediobasal lesions; predominantly a right hemisphere dysfunction	mediobasal region
Psychomotor poverty syndrome poverty of speech decreased spontaneous movement unchanging facial expression paucity of expressive gesture	frontal lobe abnormality; associated primarily with the dorsolateral prefrontal cortex; predominantly a left hemisphere dysfunction; ventricular enlargement	dorsolateral prefrontal cortex

Factors interfering with neural development in these critical periods may trigger changes in the neural matrix, which subsequently manifest as the brain develops (Harrison, 1997; Trojanowsky & Arnold, 1995). Such changes in maturation include cell death, synaptogenesis, synaptic pruning, changes in neuronal phenotype and myelination, and schizophrenia may involve an aberration in any of these processes (Andreasen, 2000; Harrison, 1997). Brain development is not complete until late adolescence, which is the most common period of onset of schizophrenia (Andreasen, 2000; Egan & Weinberger, 1997; Weinberger, 1997). At this time, active myelination of corticolimbic areas is occurring (Benes, 1989). Interference with the developmental progression of neurons may result in structural aberrations and modified patterns of synaptic connectivity, contributing to impaired associative processing and behavioural changes observed in schizophrenia (Benes *et al.*, 1992a; Egan & Weinberger, 1997; Kovelman & Scheibel, 1984).

The neurodevelopmental theory depends upon environmental impacts leading to changes in neural development, and does not adequately accommodate the heritability of schizophrenia, unless one takes into account that environmental impacts upon susceptible individuals may eventually give rise to the disorder. Moreover, it may give us an idea of when the cause of schizophrenia emerges, but still fails to elucidate the specific aetiology.

4.5.2 Genetics of Schizophrenia

Schizophrenia clearly comprises a genetic aetiology, however attempts to ascertain the genetic loci of the disorder have emphasised the difficulties inherent in the investigation of complex psychiatric disorders.

4.5.2.1 Family, adoption and twin studies

Despite the inability for researchers to find the precise genetic loci of schizophrenia, there is much evidence to suggest that the disorder is a genetic one (Möhler, 1997). The genetic link can be traced through family, adoption, and twin studies, which demonstrate an increased lifetime risk of schizophrenia in adults related to persons with the disorder (Riley & Williamson, 2000). The prevalence of schizophrenia in offspring is 8% (Mowry & Nancarrow, 2001). Adopted children have the same risk as their biological families, and monozygotic twins show a 50% concordance (Kendler & Diehl, 1993; Mowry & Nancarrow, 2001; Sawa & Snyder, 2002).

These data demonstrate that the genetics of schizophrenia are complex and multiple genes may be involved (Hyman, 1999). It is currently believed that multiple genes of small to moderate effect confer risk, and non-genetic (environmental) factors also play a role (Möhler, 1997; Mowry & Nancarrow, 2001; Nestler, 1997).

4.5.2.2 Candidate gene association studies

Candidate gene association studies derive information from psychopharmacological studies and describe variations in genetic alleles that are associated with specific disease states. Alleles of the 5-HT_{2A} receptor gene and the dopamine D_3 receptor gene have been implicated from large sample studies (Mowry & Nancarrow, 2001; Sawa & Snyder, 2002; Weinberger, 1997). Weaker evidence for the NMDA receptor gene also exists (Mowry & Nancarrow, 2001).

4.5.2.3 Linkage studies

Co-localisation of cloned genes with a disease locus is critical to the identification of abnormal proteins in a disorder (Cutting *et al.*, 1992). Numerous linkage studies using DNA polymorphism technology have been applied to schizophrenia in an attempt to

discover a disease locus. Linkage studies use a map of DNA markers of known location evenly spaced across the genome in order to examine regions of chromosomes that appear to be linked to the disorder in immediate families (parents and offspring affected by the disorder) or pairs of siblings (Mowry & Nancarrow, 2001). Screens thus far have been unable to provide cogent evidence for linkage to any chromosomal regions, although some regions give multiple positive results to schizophrenia (Brzustowicz *et al.*, 2000; Riley & Williamson, 2000; Sawa & Snyder, 2002)

It is evident that a number of genomic regions may be implicated in schizophrenia, suggesting the disorder is multifactorial, with a complex mode of inheritance (Hyman, 1999; Sawa & Snyder, 2002; Weinberger, 1997). The fact that several genes may be involved indicates that individuals may be susceptible to the disorder, but environmental factors are necessary for precipitation of the symptoms (Carlson, 1991; Harrison, 1997; Hyman, 1999). This heterogeneity in genetic linkage is indicative of the heterogeneity of the disorder, and attests to the necessity for clear clinical delineation of aetiologically or genetically distinct subtypes. Adequate interpretation of current genetic information may lead to patterns of information, which subsequently point to definitive behavioural and symptomatic subtypes of schizophrenia. These subtypes, defined by modern technology, would surpass the traditional types of schizophrenia outlined in section 4.4.5, and may provide a new goal for research into schizophrenia.

4.6 Neuropathological Changes in Schizophrenia

Post-mortem brain studies and imaging techniques have been employed in the search for neuropathological alterations in the brain in schizophrenia and the anatomical substrates of the disorder. The pathology of schizophrenia is subtle, rarely exceeding two-fold changes, with an absence of specific neuropathological markers, and it is not focal to a single brain region, suggesting that the pathophysiology of schizophrenia involves many neural circuits and neurotransmitter systems (Andreasen, 2000; Jeste & Lohr, 1989; Mirnics *et al.*, 2000). Global neuropathological changes have been documented, as well as structural and chemical alterations in particular brain regions, most notably the frontal and temporal cortices. Other regions such as the thalamus and basal ganglia have also been implicated, but will not be discussed here.

4.6.1 Global Neuropathology

Generally, neuropathological findings in the brain in schizophrenia appear to be confined to the left hemisphere (Coffman *et al.*, 1984; Reynolds, 1991; Syvälahti, 1994). The most consistent finding from post-mortem studies and structural imaging techniques is lateral ventricular enlargement (Becker *et al.*, 1996; Brown *et al.*, 1986; Crow *et al.*, 1989; Heckers *et al.*, 1990; Johnstone *et al.*, 1976; Pakkenberg, 1987; Shelton *et al.*, 1988; Shenton *et al.*, 1992; Vita *et al.*, 1995; Weinberger *et al.*, 1979a; Young *et al.*, 1991) resulting from adjacent cortical loss (Brown *et al.*, 1986; Marsh *et al.*, 1994; Reynolds, 1991; Shenton *et al.*, 1992; Syvälahti, 1994). Other gross pathological changes encountered in schizophrenic brain are enlargement of cortical sulci and fissures, particularly the Sylvian fissure (Becker *et al.*, 1996; Holmes, 1997; Weinberger *et al.*, 1979b), reduced brain weight (Brown *et al.*, 1986; Pakkenberg, 1987; Rowley *et al.*, 2001), reduced cortical size and volume (Andreasen *et al.*, 1986; Pakkenberg, 1987), particularly in the temporal lobe (Becker *et al.*, 1996; Bogerts *et al.*, 1990a; Highley *et al.*, 1999; Marsh *et al.*, 1997; Sanfilipo *et al.*, 2000; Shenton *et al.*, 1992), and neocortical shrinkage with subsequent increased neuronal density (Pakkenberg, 1993; Selemon *et al.*, 1995; Selemon *et al.*, 1998). An absence of normal structural asymmetry or lateralisation is also commonly reported (Breier *et al.*, 1992; Coffman *et al.*, 1984; Luchins *et al.*, 1982; Maher *et al.*, 1998; Young *et al.*, 1991). Not all of these findings have been substantiated (Heckers *et al.*, 1990; Heckers *et al.*, 1991b; Young *et al.*, 1991) and some, such as ventricular enlargement, are not exclusive to schizophrenia, also being demonstrated in mood disorders (Roy *et al.*, 1998; Shelton *et al.*, 1988; Syvälahti, 1994).

4.6.2 Temporal Lobe Neuropathology

Neuropathological changes in temporolimbic structures of schizophrenic brain have been widely replicated. Both post-mortem studies and neuroimaging techniques have reported reduced volume, or atrophy, of the amygdala (Barta *et al.*, 1997; Bogerts *et al.*, 1985; Breier *et al.*, 1992; Marsh *et al.*, 1994; Rossi *et al.*, 1993; Shenton *et al.*, 1992), hippocampal formation and surrounding temporal cortex (Altshuler *et al.*, 1998; Becker *et al.*, 1996; Bilder *et al.*, 1995; Bogerts *et al.*, 1985; Bogerts *et al.*, 1990a; Bogerts *et al.*, 1990b; Breier *et al.*, 1992; Falkai & Bogerts, 1986; Gur *et al.*, 2000; Heckers *et al.*, 1991a; Jeste & Lohr, 1989; Marsh *et al.*, 1994; Rossi *et al.*, 1993; Shenton *et al.*, 1992;

Suddath *et al.*, 1989), parahippocampal gyrus and entorhinal cortex (Altshuler *et al.*, 1990; Barta *et al.*, 1997; Bogerts *et al.*, 1985; Brown *et al.*, 1986; Falkai *et al.*, 1988; Shenton *et al.*, 1992) and superior temporal gyrus (Barta *et al.*, 1990; Barta *et al.*, 1997; Gur *et al.*, 2000; Marsh *et al.*, 1997; Highley *et al.*, 1999; Sanfilipo *et al.*, 2000; Shenton *et al.*, 1992) in schizophrenia. Not all these findings, however, have been replicated (Altshuler *et al.*, 1990; Heckers *et al.*, 1990; Heckers *et al.*, 1991a; Heckers *et al.*, 1991b; Rajarenthinam *et al.*, 2001; Roy *et al.*, 1998; Vita *et al.*, 1995). It is generally accepted that reduced structural volume reflects reduced function (Bogerts *et al.*, 1990b; Heckers *et al.*, 1991a). As mentioned above, enlargement of the lateral ventricles, especially in the temporal horn, has also been documented (Becker *et al.*, 1996; Brown *et al.*, 1986; Crow *et al.*, 1989; Heckers *et al.*, 1990; Marsh *et al.*, 1997; Roy *et al.*, 1998; Shenton *et al.*, 1992).

As well as gross pathological changes, changes in cell size and number have been observed in temporal lobe structures, although not in all studies conducted. Reduced neuronal size was encountered in the hippocampus, parahippocampal gyrus and entorhinal cortex (Falkai *et al.*, 1988; Zhang & Reynolds, 2002). Decreased cell numbers were also detected in the hippocampus, parahippocampal gyrus and entorhinal cortex (Falkai *et al.*, 1988; Falkai & Bogerts, 1986), although not in the amygdala (Pakkenberg, 1990). Additionally, changes in particular neuronal populations are evident (Heckers & Konradi, 2002). A reduced density of glutamatergic pyramidal cells was found in regions CA1 to CA4 of the hippocampal formation (Falkai & Bogerts, 1986; Heckers & Konradi, 2002; Jeste & Lohr, 1989), and in the anterior cingulate cortex, albeit non-significantly (Benes *et al.*, 1991). Findings of reduced pyramidal cell number or density have not been replicated in all studies (Benes *et al.*, 1998). Furthermore, deficits in small GABAergic interneurons have been reported in the anterior cingulate cortex and hippocampus (Benes *et al.*, 1986; Benes *et al.*, 1991; Benes *et al.*, 1998; Heckers & Konradi, 2002), with no change in neuronal size or glial cell number. Increased numbers of vertical associative or commissural axons were also encountered in the anterior cingulate and entorhinal cortices (Benes *et al.*, 1987; Longson *et al.*, 1996). These fibres were subsequently shown to be associative (ipsilateral) glutamatergic afferents (Benes *et al.*, 1992a), and their increased number is thought to indicate changes in afferent input to the cingulate and entorhinal cortices in schizophrenia (Benes *et al.*, 1987; Longson *et al.*,

1996). Altered orientation and disorganisation of left hippocampal pyramidal cells and dendrites were also observed in schizophrenic brains, without changes in cell density (Kovelman & Scheibel, 1984; Scheibel & Kovelman, 1981). While another study failed to replicate these findings, the level of cellular disarray observed in schizophrenic tissue in this study was correlated with severity of symptoms (Altshuler *et al.*, 1987).

4.6.3 Prefrontal Cortex Neuropathology

Prevailing prefrontal cortical pathology encountered in schizophrenic brain is enlarged ventricles and reduced grey matter (Pakkenberg, 1987; Selemon *et al.*, 1995; Shelton *et al.*, 1988; Weickert & Kleinman, 1998). Reduced volume and thickness of the prefrontal cortex has been observed (Andreasen *et al.*, 1986; Andreasen *et al.*, 1994; Sanfilipo *et al.*, 2000; Selemon *et al.*, 1995; Selemon *et al.*, 1998; Shelton *et al.*, 1988; Woo *et al.*, 1997; Woodruff *et al.*, 1997), although not all studies have confirmed this finding (Beasley & Reynolds, 1997; Pierri *et al.*, 1999; Reynolds & Beasley, 2001; Reynolds *et al.*, 2002). The reduction in volume and cortical atrophy is thought to be due to a loss of neuropil with consequent shrinkage of the intraneuronal space (Selemon *et al.*, 1995).

Reduced neuronal densities have also been found in the prefrontal cortex, including reduced density of small, presumably GABAergic interneurons, with no change in neuronal size or glial cell number (Benes *et al.*, 1986; Benes *et al.*, 1991), and reduced pyramidal cell somal size in Brodmann's area 9 (Pierri *et al.*, 2001; Rajkowska *et al.*, 1998), indicative of pyramidal cell atrophy (Selemon & Goldman-Rakic, 1999). Rajkowska and colleagues (1998) also encountered reduced pyramidal cell density in the dorsolateral prefrontal cortex. Conversely, increased glutamatergic pyramidal cell density in layer V (Benes *et al.*, 1991), and increased density of neuronal and glial cell in Brodmann's areas 9 and 46 (Selemon *et al.*, 1995; Selemon *et al.*, 1998), particularly of the small to medium sized neurons (Rajkowska *et al.*, 1998) have also been reported. However, apparent increases in cell densities may reflect decreased neuropil as a result of cortical atrophy (Selemon *et al.*, 1995; Selemon & Goldman-Rakic, 1999).

4.6.3.1 Hypofrontality and cognitive deficits in schizophrenia

Hypofrontality, or decreased glucose utilisation and blood flow to the prefrontal cortex, is a common finding in neuroimaging studies of schizophrenic brains (Andreasen *et al.*, 1992; Andreasen *et al.*, 1996; Lewis, 1995; Spence *et al.*, 1998; Syvälahti, 1994; Weickert & Kleinman, 1998; Weinberger *et al.*, 1992; Wolkin *et al.*, 1992). Hypofrontality occurs both at rest and during performance of cognitive tasks (Franzén & Ingvar, 1975a; Franzén & Ingvar, 1975b; Spence *et al.*, 1998; Weinberger *et al.*, 1986). Decreased task performance corresponds to reduced activation of the prefrontal cortex, or greater hypofrontality, which reverses as symptoms improve (Franzén & Ingvar, 1975b; Spence *et al.*, 1998; Weinberger *et al.*, 1986).

In particular, schizophrenic subjects display deficits in attention, working memory and tasks involving the temporal integration of information (Cadenhead & Braff, 1995; Lewis & Anderson, 1995). Performance declines more readily in situations requiring multiple tasking, high processing load or other variables that place greater demand on efficient information processing (Cadenhead & Braff, 1995). These irregularities are similar to those observed in experimental lesion and trauma-induced lesion studies. Prefrontal cortex lesions in non-schizophrenic individuals have led to avolition, apathy, anhedonia, perseverative behaviours, impoverished speech and flattening of emotions, all of which are present in schizophrenia (Andreasen *et al.*, 1986; Wolkin *et al.*, 1992). This evidence has given rise to the theory that the negative symptoms of schizophrenia arise from prefrontal cortex dysfunction (Andreasen *et al.*, 1986; Sanfilipo *et al.*, 2000; Weinberger *et al.*, 1986; Wolkin *et al.*, 1992), which is manifest in hypofrontality (Lewis, 1995; Lewis & Anderson, 1995; Trojanowsky & Arnold, 1995; Weinberger *et al.*, 1986; Weinberger *et al.*, 1994; Wolkin *et al.*, 1992). Some studies, though, have correlated reduced activation of the dorsolateral prefrontal cortex with positive symptoms of schizophrenia (Menon *et al.*, 2001). Such dysfunctions may result from subtle alterations in the intrinsic neural circuitry of the prefrontal cortex (Lewis & Anderson, 1995).

The prefrontal cortex has afferent and efferent connections to all other neocortical regions, the cingulate cortex, the limbic systems and the basal ganglia (Andreasen *et al.*, 1992), all of which are implicated in the pathology of schizophrenia. It is quite feasible

that schizophrenia involves a breakdown, not only in these structures, but in pathways between the prefrontal cortex and the cingulate cortex, limbic system and temporal cortex. It has also been speculated that prefrontal cortex deficits result from primary deficits in other brain regions that impinge upon the prefrontal cortex (Lewis *et al.*, 2001; Suddath *et al.*, 1989; Weinberger *et al.*, 1986; Weinberger *et al.*, 1992).

4.7 NEUROTRANSMITTERS IN SCHIZOPHRENIA

It is anticipated that anatomical abnormalities in schizophrenia are accompanied by disturbances in neurochemistry (Deakin *et al.*, 1997; Reynolds, 1991; Simpson *et al.*, 1992). Current research dictates the involvement of four predominant neurotransmitter systems in schizophrenia: dopamine, serotonin (5-HT), glutamate and GABA. An imbalance in neurochemistry is highly plausible in schizophrenia, in light of the overwhelming evidence of exacerbation or amelioration of symptoms by drugs.

4.7.1 Dopamine Hypothesis of Schizophrenia

The involvement of dopamine in schizophrenia was first postulated on the basis of the pharmacological activity of neuroleptic drugs. The antihistamine drug, chlorpromazine, was discovered to have a sedative effect in human subjects, calming and creating a sense of "disinterest", without augmenting mental capacity (Carlson, 1991). When tested on patients with schizophrenia, affective disorders and neuroses, chlorpromazine had little effect on the symptoms of neurosis or affective psychosis, but produced a profound effect on symptoms of schizophrenia (Delay & Deniker, 1952, cited in Carlson, 1991). In these patients, chlorpromazine reduced the symptoms, not merely through sedative masking, but in a manner dependent upon the individual's symptoms (Carlson, 1991).

It was not until neuroleptics were shown to selectively antagonise the dopamine D_2 receptors (Creese *et al.*, 1976), that the "dopamine hypothesis" of schizophrenia was born. The dopamine hypothesis states that overactivity of dopamine neurotransmission is the underlying cause of schizophrenia (Owen & Simpson, 1995; Stahl & Wets, 1988). Abnormal neurotransmission in the dopaminergic mesolimbic/mesocortical pathways extending from the ventral tegmental area to the nucleus accumbens (mesolimbic) and limbic and prefrontal cortices (mesocortical) is implicated in the symptomatology of

schizophrenia (Stahl & Wets, 1988; Syvälahti, 1994). Initially it was thought that overactivity of dopamine was due to an excess of dopamine, which increases neurological activity via the dopamine D_2 receptors, leading to the symptoms of psychosis (Halberstadt, 1995). This hypothesis was reinforced by observations that neuroleptics reversed many psychotic symptoms by antagonising the D_2 receptors, and that drugs like amphetamines which act to increase levels of dopamine in the synapse, caused psychosis similar to the positive symptoms observed in schizophrenia (Halberstadt, 1995; Owen & Simpson, 1995; Reynolds, 1991; Sawa & Snyder, 2002; Stahl & Wets, 1988; Syvälahti, 1994). This theory, however, was flawed in that few researchers could substantiate findings of increased levels of dopamine or its metabolites in the brain or cerebrospinal fluid of patients with schizophrenia (Owen & Simpson, 1995; Reynolds, 1988; Rowley *et al.*, 2001).

A second theory was proposed, that schizophrenia is associated with increased dopamine receptor number or sensitivity (Halberstadt, 1995; Owen & Simpson, 1995). This hypothesis was based on findings of increased dopamine receptors in the CNS and increased binding and affinity of dopamine in the CNS of individuals with schizophrenia (Halberstadt, 1995; Owen & Simpson, 1995; Reynolds, 1988). However, most studies were conducted on individuals receiving neuroleptic treatment, and it is well established that neuroleptics up-regulate dopamine D_2 receptors (Burt *et al.*, 1977; Halberstadt, 1995; Owen & Simpson, 1995; Reynolds, 1988). Neuroimaging studies of neuroleptic-naïve patients have also failed to demonstrate increases in D_2 receptors (Weickert & Kleinman, 1998).

It is now widely accepted that the dopamine hypothesis does not adequately explain the disturbances in schizophrenia (Reynolds *at al.*, 1990; Rowley *et al.*, 2001). While selective D_2 receptor antagonists ameliorate the positive symptoms of schizophrenia, they fail to reduce the negative symptoms. Furthermore, neuroleptics decrease dopaminergic activity immediately, but take several weeks to become therapeutically effective (Halberstadt, 1995; Syvälahti, 1994). Nonetheless, given the obvious crucial role for dopamine receptors in schizophrenia, contemporary theories must take a dysfunction in dopaminergic transmission in schizophrenia into account. An alternate dopamine hypothesis of schizophrenia proposes that dopamine overactivity in

schizophrenia may result from deficiencies in one or more other neurotransmitter systems which ordinarily oppose or modulate the effects of dopamine (McCarthy *et al.*, 1981; Perry *et al.*, 1979b; Roberts, 1972; van Kammen, 1977). Activities that are usually suppressed are not, resulting in inappropriate behaviours (McCarthy *et al.*, 1981). Thus, candidate transmitter systems have subsequently been examined.

4.7.2 Serotonin Involvement in Schizophrenia

The serotonin hypothesis of schizophrenia advocates that the symptoms of schizophrenia extend from an underactivity of the serotoninergic system. Low serotonin levels lead to reduced inhibition of dopamine, resulting in increased neurological arousal, hence psychosis (Holmes, 1997). The hypothesis was first proposed on the basis of the hallucinatory effects of serotoninergic drugs such as lysergic acid diethylamide (LSD) (Rang *et al.*, 1999; Rowley *et al.*, 2001; Syvälahti, 1994). LSD was thought to antagonise serotonin receptors, however it was subsequently demonstrated to be a partial agonist. Reinforcement for the role of serotonin in the pathogenesis of schizophrenia arose from the work of Meltzer (1989) who discovered the clinical efficacy of atypical neuroleptics such as clozapine was in part due to their strong affinity for the serotonin receptors, specifically the 5-HT_1 and 5-HT_2 receptors. 5-HT_2 receptor occupancy was observed with atypical but not typical neuroleptics (Nyberg *et al.*, 1996), which may account for the fact that atypical neuroleptics are more efficacious. Atypical neuroleptics are more effective than typical neuroleptics in reducing the negative symptoms of schizophrenia and in treatment-resistant patients (Dean, 2001; Holmes, 1997; Meltzer, 1999; Sawa & Snyder, 2002) even though they show low dopamine D_2 receptor occupancy at therapeutic doses (Meltzer, 1999; Rowley *et al.*, 2001).

Nevertheless, drugs selective for the 5-HT receptors have no clinical efficacy in schizophrenic patients (Owen & Simpson, 1995; Syvälahti, 1994), highlighting the important therapeutic role of the dopaminergic system in schizophrenia. Moreover, there do not appear to be any changes in brain concentrations of serotonin or its metabolites in schizophrenia, nor any consistent alterations in serotonin receptor binding (Owen & Simpson, 1995; Reynolds, 1988). More selective ligands have

permitted the evaluation of specific serotonin receptors (Dean & Hayes, 1996; Dean *et al.*, 1999). Ligands selective for 5-HT_{2A} receptors, such as [^{3}H]ketanserin, have demonstrated a reduced density of binding sites in the prefrontal cortex (Brodmann's areas 8, 9 and 10) in schizophrenic subjects, attesting to a role for the 5-HT_{2A} receptors in the pathophysiology of schizophrenia (Dean, 2001; Dean & Hayes, 1996; Dean *et al.*, 1999).

4.7.3 Glutamatergic Hypothesis of Schizophrenia

As mentioned in section 1.1.2, pyramidal cells constitute the major projection neurons of the cerebellar cortex, utilising glutamate as their transmitter. It stands to reason, thus, that glutamatergic transmission may be altered in schizophrenia, accounting for aberrant cortico-cortical integration.

Schizophrenia was thought to involve hypoactivity of glutamatergic neurotransmission. This hypothesis was propounded on the basis of findings that phencyclidine (PCP), a non-competitive NMDA receptor antagonist precipitates schizophrenia-like symptoms, both positive and negative, and exacerbates these symptoms in patients with schizophrenia (Abe *et al.*, 2000; Deakin *et al.*, 1997; Halberstadt, 1995; Itil *et al.*, 1967; Kaneko *et al.*, 1996; Owen & Simpson, 1995; Thornberg & Saklad, 1996). Findings of decreased glutamate innervation in the hippocampus and temporal lobe structures substantiated the hypothesis of reduced glutamate transmission. Studies into brain glutamate concentrations, however, have proved inconclusive in light of inconsistent results (Owen & Simpson, 1995).

On the basis of PCP action, an hypothesis of NMDA receptor hypofunction was proposed (Olney & Farber, 1995). As NMDA receptor antagonism elicits psychotic symptoms, schizophrenia may involve a reduction in function or number of NMDA receptors. This is substantiated by animal studies of reduced NMDA receptor expression. Mice with an NMDA receptor NR1 subunit gene knockdown display behavioural abnormalities associated with symptoms of schizophrenia (Mohn *et al.*, 1999) and reduced 2-deoxyglucose uptake in selective brain regions consistent with that

observed in schizophrenic patients (Duncan *et al.*, 2002). However not all studies have demonstrated alterations in NMDA receptors (Kerwin *et al.*, 1990).

Investigations of glutamate receptors and uptake sites in schizophrenic brain have subsequently yielded disparate findings. Binding to glutamate receptors and uptake sites has generally been found to be reduced in the basal ganglia and temporal lobe structures (Deakin *et al.*, 1997; Halberstadt, 1995; Humphries *et al.*, 1996; Kerwin *et al.*, 1990; Owen & Simpson, 1995; Reynolds, 1991; Simpson *et al.*, 1992). Receptors and uptake sites, on the other hand, are found to be increased in the frontal cortex (Deakin, 1994; Deakin *et al.*, 1989; Deakin *et al.*, 1997; Owen & Simpson, 1995; Simpson *et al.*, 1992; Toru *et al.*, 1988). Upregulation of glutamate receptors is thought to occur as compensation for reduced innervation (Akbarian *et al.*, 1996; Ohnuma *et al.*, 1998; Tsai *et al.*, 1995), and is consistent with findings of reduced glutamate content in schizophrenic prefrontal cortex relative to controls (Ohnuma *et al.*, 1998; Tsai *et al.*, 1995). In addition, metabotropic glutamate receptor subunit mRNAs were reported to be decreased in temporal lobe structures (Eastwood *et al.*, 1995; Halberstadt, 1995; Humphries *et al.*, 1996) and NMDA receptor subunit mRNAs were increased in the prefrontal cortex (Akbarian *et al.*, 1996; Ohnuma *et al.*, 1998). However, reports of increased NMDA receptor binding and subunit mRNA expression in the superior temporal cortex (Grimwood *et al.*, 1999; Le Corre *et al.*, 2000; Nudmamud & Reynolds, 2001) do not comply with the above patterns of glutamate receptor alterations in schizophrenia.

While regional differences and inconsistent evidence preclude a simple theory of glutamatergic abnormality, two general hypotheses do exist for glutamate dysfunction in schizophrenia. Glutamatergic hypoactivity in the temporal lobes is associated with positive symptoms of schizophrenia, while hypoinnervation in the frontal cortex is assumed in the negative symptoms (Deakin, 1994; Owen & Simpson, 1995). The documented reduction in synaptic markers (Glantz & Lewis, 1997) and dendritic spines (Garey *et al.*, 1998; Glantz & Lewis, 2000) also suggests a loss of glutamatergic synapses, which may result in receptor up-regulation. NMDA receptors are located on pyramidal cell dendrites and dendritic spines (Garey *et al.*, 1998). Thus, altered

patterns of glutamatergic cell connectivity evidently contribute to the pathophysiology of schizophrenia (Benes *et al.*, 1992a; Deakin *et al.*, 1997; Owen & Simpson, 1995).

4.7.4 GABAergic Theory of Schizophrenia

Much evidence exists to suggest that the GABA neurotransmitter system is involved in the pathophysiology of schizophrenia, with GABA transporters, GABA receptors, and inhibitory GABAergic interneurons all being implicated.

4.7.4.1 GABA hypothesis of deficient inhibition

GABA is the principle neurotransmitter mediating neuronal inhibition in the mammalian CNS, and is involved in a diversity of functions and disease states, including anxiety, sleep, memory and epilepsy. Therefore an abnormality in GABA transmission may contribute considerably towards the symptoms of schizophrenia. The possibility that a failure in inhibitory mechanisms could lead to some of the symptoms of schizophrenia was first postulated by Bleuler in his distinguished work, *Die Prognose der Dementia Praecox – Schizophreniegruppe* (1908). Bleuler states that in the schizophrenias "it is as if the physiological inhibitions and pathways have lost their significance". Maher (1966) suggested that the general deficiency of attention in schizophrenia is related to a failure of inhibitory mechanisms. The proposition that the defect in inhibitory mechanisms involves GABAergic systems was first put forth by Roberts (1972), and encompasses the GABA deficiency hypothesis. Roberts proposed that reduced GABAergic function with a consequent imbalance between GABAergic and dopaminergic transmission could play a primary pathogenetic role in schizophrenia. The reduced inhibitory control of dopaminergic neurons may lead to overactivity of these neurons, which is compensated for by the introduction of dopamine D_2 receptor antagonists (Carlson, 1991). Subsequently, the idea was proffered that schizophrenia involves a deficit in inhibitory processes controlling affect and cognition (Aggleton, 1993; Ball *et al.*, 1998). The lack of inhibition leads to an increased awareness of preconscious material that interferes with the individual's ability to interact appropriately with the world (Cadenhead & Braff, 1995).

To this end, deficits in cortical inhibition have been observed in unmedicated schizophrenic subjects using transcranial magnetic stimulation (Daskalakis *et al.*, 2002).

Since normal activity of the CNS is mediated by the "coordinated dynamic interplay of excitation and inhibition", both within and between neuronal systems (Roberts, 1976), perturbations in inhibitory transmission may result in an incoordination between inhibition and excitation (Roberts, 1984), resulting in aberrant information processing.

Reduced GABAergic neurotransmission is a possible neurochemical correlate of anatomical alterations in schizophrenia (Reynolds *et al.*, 1990). Under activity of GABAergic transmission may be characterised by decreased GABA synthesis, decreased GABA release, decreased receptor responsiveness and GABA binding, decreased receptor number, reduced inhibitory interneurons, or a combination of these. An increase in receptor number may compensate for the decreased responsiveness or functional reduction in GABA output. In fact, the experimental evidence attesting to a deficit in GABA transmission is abundant.

4.7.4.2 Changes in GAD activity in schizophrenia

GAD is the synthesising enzyme for GABA. Its concentration thus represents the level of synthetic activity occurring in the brain. Bird *et al* (1977) measured GAD concentration in post-mortem brains of schizophrenic patients and found decreased activity compared to controls in the nucleus accumbens, putamen, amygdala and hippocampus. Sherman and colleagues (1991) also demonstrated decreased GAD activity in prefrontal and temporal cortical tissue homogenates of schizophrenic brain compared to controls. Further, McGeer & McGeer (1977) found decreased GAD activity in sensory association cortex, the temporal pole, the calcarine fissure and insular cortex in subjects with schizophrenia. Some studies, however, have not replicated these findings, failing to demonstrate changes in GAD activity in post-mortem schizophrenic brain (Bennett *et al.*, 1979; Bird *et al.*, 1979; Cross and Owen, 1979; Hanada *et al.*, 1987).

While GAD concentration is stable post-mortem, prolonged terminal illness is correlated with decreased GAD concentration in post-mortem tissue (Akbarian *et al.*, 1995b; Bird *et al.*, 1978; Bird *et al.*, 1979; Cross and Owen, 1979; Curzon & Kantamaneni, 1978; Czudek & Reynolds, 1990; Perry *et al.*, 1979a; Perry *et al.*, 1979b; Spokes *et al.*, 1979; Spokes *et al.*, 1980). Thus, alternate methods to measure GAD activity have been

sought, particularly mRNA expression. Akbarian and colleagues (1995a) showed decreased GAD_{67} mRNA density in the dorsolateral prefrontal cortex of schizophrenic brains compared to controls, significantly in the superficial cortical layers, without a corresponding loss of neurons. This study was replicated by Volk *et al.* (2000), who suggested the reduction occurs in a subset of neurons. Mirnics and colleagues (2000) report decreased transcript levels for GAD_{67} in prefrontal cortex of schizophrenic subjects assessed using microarrays. Bunney *et al.* (1993) and Guidotti and colleagues (2000) also demonstrated decreased expression of GAD_{67} mRNA in the prefrontal cortex in schizophrenia, particularly in the superficial laminae. However, Todtenkopf & Benes (1998) found no significant decrease in GAD_{65} density in the hippocampus in schizophrenia using immunohistochemical techniques. The authors did show a positive correlation between density of GAD_{65} immunoreactive puncta and neuroleptic dose. In other words, as the neuroleptic dose increased, density of GAD_{65} increased. Furthermore, two neuroleptic-free subjects demonstrated the lowest GAD_{65} density. This was also the case in the studies of Akbarian *et al.* (1995a) and Volk *et al.* (2000), where neuroleptic-free subjects had lower GAD_{67} mRNA levels than the neuroleptic-treated subjects, suggesting decreased GAD expression is not the consequence of neuroleptic treatment.

Decreased GAD expression in post-mortem brain may represent reduced presynaptic turnover of GABA which is associated with defective inhibitory transmission (Akbarian *et al.*, 1995a). On the other hand, decreased GAD concentration might signify a loss of GABAergic neurons (Czudek & Reynolds, 1990), however this theory is not supported by the data (Akbarian *et al.*, 1995a; Bunney *et al.*, 1993; Volk *et al.*, 2000). Volk and colleagues (2000) predict that GABA uptake is impaired because of decreased synaptic connections, which result, for example, from reduced chandelier cell axon terminals (Woo *et al.*, 1998). This may produce an increase in GABA activity at post-synaptic sites, with a corresponding down-regulation of GAD.

4.7.4.3 Changes in GABA levels in blood, CSF and brain in schizophrenia

Changes in GABA levels have been measured to ascertain whether the symptoms of schizophrenia are caused or exacerbated by abnormal GABA anabolism, release or catabolism. GABA has been assayed in cerebrospinal fluid (CSF) and blood as the

presence of neurotransmitters in these fluids is thought to predict levels in the brain (Gerner & Hare, 1981; McCarthy *et al.*, 1981; Perry & Hansen, 1976; Petty & Sherman, 1984). Most studies have reported no changes in plasma or CSF levels of GABA in schizophrenic individuals compared to neurological controls or normal subjects (Gerner & Hare, 1981; Gerner *et al.*, 1984; Gold *et al.*, 1980; Lichtshtein *et al.*, 1978; Perry *et al.*, 1989; Petty & Sherman, 1984; van Kammen *et al.*, 1998; Zimmer *et al.*, 1981). On the other hand, van Kammen *et al.* (1982) detected significantly lower levels of GABA in the CSF of young female patients with schizophrenia compared to age and gender matched controls, but not in chronic schizophrenics. In contrast, McCarthy and coworkers (1981) reported high CSF GABA levels in chronic but not acute schizophrenic patients. Plasma GABA levels were also decreased in patients who displayed sulcal widening of the prefrontal cortex, taken as a measure of cortical atrophy (van Kammen *et al.*, 1998). It is clear that the results from studies of the CSF and blood concentrations of GABA in schizophrenic subjects compared to normal controls are inconsistent. These inconsistencies may be related to the methods used to assay GABA (McCarthy *et al.*, 1981; Perry & Hansen, 1976), or the samples from which patients were selected (Gerner & Hare, 1981; McCarthy *et al.*, 1981).

Brain GABA content has been investigated extensively in schizophrenic compared to control tissue. Ohnuma and colleagues (1999) observed a decrease in GABA content measured using HPLC in the prefrontal cortex of schizophrenic subjects relative to controls. This was positively correlated to a decrease in GAT-1 mRNA density. Sherman *et al.* (1991) also found reduced GABA release from synaptosomes of temporal cortex in schizophrenic compared to control tissue. Findings of reduced GABA content in the nucleus accumbens, thalamus and amygdala (Perry *et al.*, 1979b; Sherman *et al.*, 1991; Spokes *et al.*, 1980) could not be replicated by others (Cross *et al.*, 1979; Korpi *et al.*, 1987; Spokes *et al.*, 1980; Perry *et al.*, 1989). Nor were decreased GABA levels encountered in other brain areas measured (Korpi *et al.*, 1987; Toru *et al.*, 1988).

Measurement of GABA levels in biopsy or autopsy material is difficult to interpret owing to the fact that synthesis of GABA continues under hypoxic conditions while GABA catabolism does not (Petroff & Rothman, 1998). In animals, brain GABA levels rise within two minutes of decapitation unless the animals or brain are kept frozen

(Czudek & Reynolds, 1990; Fahn, 1976; Perry *et al.*, 1979a). Freezing of the brain before quantitation may ameliorate this circumstance (Petroff & Rothman, 1998), however diverse tissue preparation methods create inconsistencies between studies, hence the large range of results. Thus, measuring the amount of transmitter is not a reliable indicator of GABAergic function (Simpson *et al.*, 1989), although findings of decreased GABA in association with schizophrenia negate the post-mortem increase in GABA concentration. Decreased GABA concentration in the brain in schizophrenia is not thought to reflect neuroleptic activity, since animal studies have shown that neuroleptic treatment has no effect or in fact increases GABA concentrations (Kornhuber *et al.*, 1984; Lloyd & Hornykiewicz, 1977; Perry *et al.*, 1979a; Sherman *et al.*, 1991). Moreover, levels of GABA in the CSF of schizophrenic patients not treated with neuroleptics were not significantly different to those of controls, while levels were increased in patients treated with neuroleptics (Zimmer *et al.*, 1981).

4.7.4.4 Changes in GABA uptake sites in schizophrenia

Changes in GABA uptake sites are thought to correlate with changes in GABAergic neurons and terminals. [^{3}H]Nipecotic acid and [^{3}H]tiagabine are selective inhibitors of GABA uptake by the GAT-1 transporter into the presynaptic neuron (Czudek & Reynolds, 1990; Sundman-Eriksson *et al.*, 2002). [^{3}H]Nipecotic acid binding, in particular, has commonly been employed in studies assessing changes in GABA uptake sites in schizophrenia. Alterations in [^{3}H]nipecotic acid and [^{3}H]tiagabine binding suggest changes in GABA uptake sites, hence aberrations in GABA uptake in schizophrenia.

Simpson *et al.* (1989), using whole brain homogenates, observed a symmetrical decrease in [^{3}H]nipecotic acid binding to amygdala and hippocampus of schizophrenic brain relative to matched controls. This study further detected a reduction in [^{3}H]nipecotic acid binding in left polar temporal cortex of schizophrenic brain. Because saturation binding experiments were not performed, the authors could not comment upon whether the reduction in [^{3}H]nipecotic acid binding was due to a loss of uptake sites or altered affinity of [^{3}H]nipecotic acid for the uptake sites. Simpson *et al.* (1992) demonstrated decreased binding of [^{3}H]nipecotic acid to neuronal GABA uptake sites in putamen retrieved from whole tissue homogenates of schizophrenic brain compared to matched

controls. A non-statistically significant decrease in binding in the caudate nucleus and lateral pallidum of post mortem schizophrenic brains was also observed. These changes were thought to explain the mild motor deficits seen in schizophrenia.

Reynolds *et al.* (1990) partially confirmed the results of Simpson *et al.* (1989) with the finding of a bilateral loss of [^{3}H]nipecotic acid binding to GABA uptake sites in hippocampal slices prepared from schizophrenic brains. However, Simpson and colleagues (1998) failed to confirm these and their original findings. Simpson *et al.* (1998) found no overall change in [^{3}H]nipecotic acid binding to membranes prepared from tissue homogenates from frontal and temporal lobe structures, including cingulate gyrus, polar temporal cortex, amygdala, hippocampus and parahippocampal gyrus. A tendency toward increased [^{3}H]nipecotic acid binding in the cingulate gyrus, hippocampus and superior temporal gyrus was conclusively not disease related. Furthermore, the number of [^{3}H]tiagabine binding sites were found to be elevated in tissue homogenates of cingulate cortex from schizophrenic brains relative to controls, and unchanged in the caudate nucleus (Sundman-Eriksson *et al.*, 2002). The authors suggest the increase in GAT-1 binding compensates for reduced numbers of GABA neurons. Post-mortem interval, agonal state, neuroleptic treatment and age were all controlled for in these studies. Inconsistent findings may have resulted from differences in sample size, statistical analysis or tissue preparations used. Results must also be interpreted cautiously in light of the fact that prolonged illness can decrease binding to GAT-1 transporters post-mortem (Czudek & Reynolds, 1990), emphasising the necessity for matched controls in experimental design.

Ohnuma *et al.* (1999) found decreased GAT-1 mRNA density measured by *in situ* hybridisation in the prefrontal cortex of schizophrenic tissue compared to controls. This was positively correlated with decreased GABA content in the same region, suggesting a compensatory decrease in GAT-1 to ensure sustained GABA concentration in the synaptic cleft. These findings extend binding studies to demonstrate that not only GAT-1 binding, but mRNA is also decreased in schizophrenia.

The axon cartridges of chandelier cells, a subclass of GABAergic interneurons, preferentially express GAT-1 (Lewis, 1995). Decreased GAT-1 mRNA expression and

GAT-1 immunoreactive axon cartridges have been found, particularly in the superficial layers, of the dorsolateral prefrontal cortex of schizophrenic subjects compared to normal and psychiatric control subjects (Lewis *et al.*, 1999; Pierri *et al.*, 1999; Volk *et al.*, 2001; Woo *et al.*, 1998). Such a decrease in GAT-1 immunoreactivity and mRNA expression may arise from a reduction in chandelier cell number or degree of arborisation, or may represent a decrease in expression of the transporter itself, indicative of deficient GABAergic transmission in schizophrenia (Lewis *et al.*, 1999; Pierri *et al.*, 1999; Woo *et al.*, 1998). Psychiatric control subjects treated with neuroleptic drugs and monkeys administered haloperidol demonstrated either no change or an increase in GAT-1 mRNA expression and immunoreactivity, thus the observed decreases in GAT-1 in schizophrenic subjects cannot be attributed to neuroleptic treatment. (Pierri *et al.*, 1999; Volk *et al.*, 2001; Woo *et al.*, 1998).

Neuronal transporters are necessary for the maintenance of normal inhibition (Petroff & Rothman, 1998), functioning to remove excess synaptic transmitter. If GABA synthesis or release is deficient in schizophrenia, however, it is reasonable that GAT-1 density decreases to compensate, such that GABA activity in the synapse is prolonged. Alternatively, decreased GAT-1 density may be a primary deficit, contributing to decreased turnover of GABA in schizophrenia. GAD may therefore be downregulated to compensate for the potential excess of GABA (Pierri *et al.*, 1999). The decrease in uptake has also been interpreted as an indicator of loss of integrity of GABAergic neurons in schizophrenia (Reynolds *et al.*, 1990).

4.7.4.5 Changes in GABA receptors in schizophrenia

A number of studies investigating changes in GABA receptors in schizophrenia have relied on quantitative receptor autoradiography techniques. Increased [^{3}H]muscimol binding to tissue homogenates isolated from dorsolateral prefrontal cortex and caudate nucleus was observed in schizophrenic compared to control brain (Hanada *et al.*, 1987). Increased bicuculline-sensitive [^{3}H]muscimol binding to sections of anterior cingulate and prefrontal cortex, chiefly in layer II, was also shown in schizophrenic compared to control tissue (Benes *et al.*, 1992b; Benes *et al.*, 1996b; Dean *et al.*, 1998; Dean *et al.*, 1999). This increase in [^{3}H]muscimol binding was further apparent in the hippocampal formation (Benes *et al.*, 1996a). However, Bennett *et al.* (1979) failed to reveal a change

in Na^{2+}-independent [^{3}H]GABA binding to frontal cortex homogenates of schizophrenic brain. Increased binding of [^{3}H]GABA to the caudate nucleus of schizophrenic brains was observed, with no change in [^{3}H]diazepam binding (Owen *et al.*, 1981), suggesting the increased binding of [^{3}H]GABA was to GABA receptors other than those sensitive to benzodiazepines. Farnbach-Pralong and coworkers (1998) failed to demonstrate an increase in [^{3}H]muscimol binding to sections obtained from the auditory-linguistic association cortex of schizophrenic relative to control brain, implying region-specific alterations in GABAergic transmission in the brain in schizophrenia.

An increase in [^{3}H]muscimol and [^{3}H]GABA binding represents an increase in $GABA_A$ receptor binding sites in schizophrenia, signifying elevated receptor number. The increase in binding in the prefrontal cortex and hippocampal CA1 region occurred primarily to receptors located on glutamatergic pyramidal cells (Benes *et al.*, 1996b). Perhaps this reflects a compensatory up-regulation of GABA receptors in response to defective inhibitory modulation, caused by a reduced number of inhibitory interneurons, deficient transport or release of GABA (Benes *et al.*, 1992b; Benes *et al.*, 1996b; Benes *et al.*, 1997; Weickert & Kleinman, 1998). The increase in GABA receptor number was associated with non-pyramidal neurons in the hippocampal CA3 region, suggesting disinhibitory modulation (Benes *et al.*, 1996a; Benes *et al.*, 1997). An alternative hypothesis is that up-regulation conceivably results in hypersensitivity of the post-synaptic GABA receptors to GABA, which may actually lead to schizophrenic symptoms (Meldrum, 1982).

Squires and colleagues (1993) encountered decreased [^{3}H]flunitrazepam binding to tissue homogenates from anterior cingulate cortex, somatomotor cortex, hippocampus, cerebellar cortex and globus pallidus of schizophrenic subjects compared to controls. Decreased binding to peripheral-type benzodiazepine receptors by [^{3}H]PK 11195 was observed in superior parietal and primary visual cortices and putamen of schizophrenic compared to control brains (Kurumaji *et al.*, 1997). No change was evident in other regions investigated, including the prefrontal and temporal cortices. Reduced [^{123}I]iomazenil binding was correlated with poorer cognitive function in schizophrenic patients during neuropsychological tasks measured using single photon emission tomography (SPECT) (Ball *et al.*, 1998). The authors suggested that reduced

benzodiazepine receptor density, implied by decreased binding, results in impaired cortical efficiency. Busatto and colleagues (1997) also demonstrated reduced *in vivo* [^{123}I]iomazenil binding in relation to the severity of negative symptoms in the medial frontal lobe and positive symptoms in the left temporal lobe in schizophrenia.

Researchers predict that the loss of benzodiazepine binding sites is contingent upon atrophy or reduced neuronal density, specifically a loss of glutamatergic pyramidal cells (Busatto *et al.*, 1997; Squires & Saederup, 1991; Squires *et al.*, 1993; Verhoeff, 1997). GABAergic interneurons synapse on pyramidal cells, which are reported as reduced in schizophrenia (Benes *et al.*, 1991; Falkai & Bogerts, 1986), although not in all studies (Benes *et al.*, 1991; Rajkowska *et al.*, 1998; Selemon *et al.*, 1995; Selemon *et al.*, 1998). Moreover, $GABA_A$ receptors sensitive to benzodiazepines are highly localised to these neurons (Wisden *et al.*, 1992). This is supported by findings in schizophrenic brain of decreased γ2 subunit mRNA expression (Akbarian *et al.*, 1995b; Huntsman *et al.*, 1998), the subunit required for high-affinity benzodiazepine binding (Pritchett *et al.*, 1989).

In quantitative autoradiographic post-mortem studies of schizophrenic brain, a dissociation in binding to $GABA_A$ receptors is apparent, depending on the ligand used. Ligands such as [^{3}H]muscimol which bind to all $GABA_A$ receptors, generally demonstrate increased binding, implying increased receptor numbers, while tritiated benzodiazepines, binding only to a subset of the $GABA_A$ receptors, tend to yield decreased binding or no change (Benes *et al.*, 1997). When two different high affinity binding sites located on a single macromolecular complex display contrasting alterations in their density, uncoupling in their regulation is said to take place (Benes, 1999). This may occur with the benzodiazepine-sensitive $GABA_A$ receptors, which bind both muscimol and high affinity benzodiazepines (Benes, 1999). Few researchers account for the fact that muscimol is equipotent at $GABA_C$ receptors, hence [^{3}H]muscimol may also be binding to $GABA_C$ receptors in the CNS, partly explaining the differences between [^{3}H]muscimol and benzodiazepine binding.

Findings of decreased benzodiazepine receptors in the brain is schizophrenia have not been substantiated in all studies, however. Kiuchi *et al.* (1989) observed an increase in benzodiazepine receptors measured by [^{3}H]flunitrazepam binding to tissue

homogenates of schizophrenic brain compared to controls. The increased binding was observed in many regions of the brain, including the dorsolateral and orbital frontal cortices, superior, medial and inferior temporal gyri, hippocampus and putamen. Increased binding was not correlated to neuroleptic or benzodiazepine treatment, but was positively correlated with GABA concentration in some regions. However, only a single concentration of ligand was used, hence the researchers were unable to determine receptor density (Reynolds & Stroud, 1993). Abidargham *et al.* (1994) also showed a higher benzodiazepine receptor distribution volume in temporal and occipital cortices of schizophrenics relative to controls measured using [^{123}I]iomazenil SPECT. On the other hand, Pandey *et al.* (1994; 1997) using [^{3}H]RO15-1788 (flumazenil) binding to homogenates of prefrontal cortex (Brodmann's area 10) from schizophrenic and control subjects found no significant difference in binding between the two groups. When schizophrenic patients were analysed as neuroleptic-medicated or neuroleptic-free, those that were neuroleptic-medicated showed significantly lower [^{3}H]RO15-1788 binding compared to neuroleptic-free patients and normal controls. Such a finding indicates that decreased benzodiazepine binding in schizophrenia may be the consequence of a down-regulation of receptors by neuroleptic medication (Pandey *et al.*, 1994; Pandey *et al.*, 1997). Busatto and colleagues (1997) also acknowledge that the reduced patterns of [^{123}I]iomazenil binding in schizophrenic brain *in vivo* were influenced by neuroleptic treatment. Animal studies have further demonstrated neuroleptic-induced changes in benzodiazepine receptor binding. For example, the number of [^{3}H]flunitrazepam binding sites was decreased in rat cerebellar membrane preparations after 12 months administration of haloperidol, sulpiride or clozapine, although no change was seen in the striatum (Rupniak *et al.*, 1987).

Reynolds and Stroud (1993) failed to demonstrate changes in [^{3}H]flunitrazepam binding to tissue homogenates of hippocampus from schizophrenic subjects compared to controls. Using positron emission tomography (PET), [^{11}C]flumazenil binding was not found to be significantly altered in drug-naïve schizophrenic patients (Sedvall *et al.*, 1995). Moreover, no change in [^{3}H]diazepam binding was observed in caudate or putamen of schizophrenic compared to control brain (Owen *et al.*, 1981). Increased binding to hippocampal $GABA_A$ receptors (Benes *et al.*, 1996a) was not accompanied by an increase in benzodiazepine receptor binding, as measured by [^{3}H]flunitrazepam

autoradiography (Benes *et al.*, 1997). In totality, then, the evidence for changes in benzodiazepine receptors measured with radioactive ligand binding in schizophrenia is inconsistent.

Changes in GABA receptors in schizophrenia have also been measured using *in situ* hybridisation histochemistry and immunohistochemistry. Akbarian and coworkers (1995b) found a decrease in mRNA levels for the $GABA_A$ receptor $\alpha1$, $\alpha2$, $\alpha5$, $\beta1$, $\beta2$ and $\gamma2$ subunits across all layers of the middle frontal gyrus (prefrontal cortex) in schizophrenic compared to control tissue. None of the decreases, however, were statistically significant, indicating gene expression was not substantially influenced by prefrontal cortex dysfunction in schizophrenia (Akbarian *et al.*, 1995b). No change in laminar pattern of gene expression was evident. Huntsman and coworkers (1998), using *in situ* hybridisation and semi-quantitative RT-PCR, demonstrated a decrease in $GABA_A$ receptor $\gamma2$ subunit expression, specifically a 51.7% decrease in the $\gamma2_{Short}$ variant and 17% decrease in the $\gamma2_{Long}$ variant, in the dorsolateral prefrontal cortex of schizophrenic brains compared to matched controls. The $\gamma2$ subunits are critical for high affinity benzodiazepine binding (Pritchett *et al.*, 1989). These findings therefore lend weight to the reports of decreased benzodiazepine binding sites in the dorsolateral prefrontal cortex in schizophrenia.

Ohnuma *et al.* (1999) found a selective and discrete increase in $\alpha1$ subunit mRNA measured by *in situ* hybridisation in the dorsolateral prefrontal cortex (Brodmann's area 9) of post-mortem schizophrenic brain compared to controls. No change in laminar pattern of gene expression was evident. This increase was coupled to a decrease in GAT-1 mRNA and a decrease in GABA content in this region. The change in mRNA expression extends prior ligand binding studies which showed increased [^{3}H]muscimol binding in the prefrontal cortex of schizophrenic brain compared to controls (Benes *et al.*, 1996b; Hanada *et al.*, 1987). The authors speculated that $GABA_A$ receptor mRNA was up-regulated in the prefrontal cortex to compensate for decreased GABA release. However, their findings are in disagreement with those of Akbarian *et al.* (1995b). Impagnatiello *et al.* (1998) also showed an increase in $\alpha1$ as well as $\alpha5$ subunit mRNA expression in prefrontal cortex of post-mortem schizophrenic brain. Further, Pesold and colleagues (1998) demonstrated increased immunolabelling of α and $\beta2/3$ subunit

proteins, with a corresponding decrease in γ2 subunit peptide labelling in the prefrontal cortex of schizophrenic subjects. Using RT-PCR, they further revealed that α1 and α3 subunit mRNAs are increased in this region in schizophrenia. These results are compatible with previous studies showing increased [^{3}H]muscimol but decreased or unaltered tritiated benzodiazepine binding to $GABA_A$ receptors in the prefrontal cortex in schizophrenia, and the authors conclude that schizophrenia involves an increase in $GABA_A$ receptor subtypes devoid of the γ2 subunit, that is, receptors that do not bind high affinity benzodiazepines.

An animal model of schizophrenia using phencyclidine (PCP) to induce a psychotic-like state in rats, demonstrated a down-regulation of $GABA_A$ receptor α1 subunit mRNA after acute PCP administration, and a decrease in β2 and β3 subunit mRNA expression after chronic PCP administration (Abe *et al.*, 2000). The mRNAs were measured in 17 brain regions, and significant decreases in expression were found in the hippocampus, cingulate and temporal cortices for the α1 subunit, the cerebellum for the β2 subunit, and the caudate-putamen, cerebellum, cingulate, parietal and temporal cortices for the β3 subunit. A corresponding decrease in [^{35}S]TBPS binding was observed in the cerebral cortices and cerebellum, suggesting reduced $GABA_A$ receptor density. This result contrasts to that of Kaneko and colleagues (1996), who describe increased [^{125}I]iomazenil binding in the parietal cortex after acute and chronic PCP administration, and in the hippocampal CA1 region after repeated administration. However, binding was decreased in the olfactory and piriform cortices after chronic administration. Discrepancies in findings may be due to the ligands, since TBPS is a non-competitive $GABA_A$ receptor antagonist acting on the ion channel, while iomazenil binds to the benzodiazepine site to allosterically modulate $GABA_A$ receptors.

The reported changes in GABA receptor binding and subunit mRNA and protein expression point to an aberration in these receptors in schizophrenia. Altered receptor binding and expression may merely represent a compensatory mechanism for structural or chemical anomalies. Nonetheless, alterations of GABA receptor subunit assembly and/or structure may contribute to the symptoms of schizophrenia (Costa, 1992). Notably, only the $GABA_A$ receptors have been studied in schizophrenia, and it would be interesting to investigate the $GABA_B$ and $GABA_C$ receptors in the disorder.

4.7.4.6 Changes in GABAergic neurons in schizophrenia

GABAergic neurons may be visualised by a number of methods, including the presence of GAD, by size and shape, or using calcium-binding proteins and neuropeptides. GAD has been found to be decreased in schizophrenic brains compared to controls (Akbarian *et al.*, 1995a; Volk *et al.*, 2000). However, in these studies, neuronal density was not decreased, thus reduced GAD expression does not imply reduced neuronal densities. Utilising GAD to visualise GABAergic cells may incur problems in light of more recent evidence that small, glutamatergic granule cells of the hippocampal formation express GAD following temporal lobe seizures (Schwartzer & Sperk, 1999).

Some changes to GABAergic cells assessed by size and shape have been documented in schizophrenia. A preferential loss of interneurons in the prefrontal and anterior cingulate cortices, most remarkably in layer II, were reported (Benes *et al.*, 1986; Benes *et al.*, 1991). These cells were thought to be basket cells, owing to their wide distribution through cortical laminae (Benes *et al.*, 1992b). The loss of basket neurons is potentially accompanied by an up-regulation of post-synaptic $GABA_A$ receptors in schizophrenic brain (Benes *et al.*, 1992b). Reductions in non-pyramidal cells were also encountered using a stereological counting technique in the hippocampal CA1-CA4 regions of schizophrenic brain compared to controls, with no significant difference in pyramidal cell numbers (Benes, 1999; Benes *et al.*, 1998).

Bunney *et al.* (1993) failed to replicate decreased interneuron density in the prefrontal cortex. Akbarian and colleagues (1995b) and Volk *et al.* (2000) also found no change in cell numbers in the dorsolateral prefrontal cortex. Selemon and colleagues (1995, 1998) in fact demonstrated increased densities of pyramidal and non-pyramidal cells without cell loss, coupled with decreased cortical volume in the dorsolateral prefrontal (Brodmann's areas 9 and 46) and primary visual cortices of schizophrenic brains compared to control subjects. Rajkowska *et al.* (1998) also found increases in the density of small neurons, presumably interneurons, in the dorsolateral prefrontal cortex. Measurement of neuronal densities, however, may not accurately represent cell number (Selemon & Goldman-Rakic, 1999). Increased neuronal density may in fact reflect unaltered cell number if cortical volume were reduced (Selemon & Goldman-Rakic, 1999). Thus, evidence to suggest that in schizophrenic cortex, total neural number is not

necessarily decreased (Pakkenberg, 1993; Akbarian *et al.*, 1995a) and cell packing density is increased (Pakkenberg, 1993; Selemon *et al.*, 1995; Selemon *et al.*, 1998), is indicative of reduced cortical neuropil (Lewis & Anderson, 1995; Selemon *et al.*, 1995; Selemon *et al.*, 1998; Selemon & Goldman-Rakic, 1999). Neuropil refers to the axon terminals, distal dendrites and dendritic spines, the synaptic elements (Lewis *et al.*, 1999; Selemon & Goldman-Rakic, 1999).

Discrepancies in the above findings may be explained by differing criteria for identification of interneurons in the different studies and the methods by which the neurons were distinguished. Thickness of tissue sections and sample size may also have contributed to inconsistencies in cell measurements (Reynolds *et al.*, 2001; Selemon & Goldman-Rakic, 1999), as well as cell counting methodologies used (stereological versus two-dimensional) (Benes & Berretta, 2001; Selemon *et al.*, 1998). Incongruity in results could have arisen from differences in brain areas analysed. Deficits in interneurons were reported in Brodmann's areas 10 (orbital prefrontal cortex) and 24 (cingulate cortex) (Benes *et al.*, 1986; Benes *et al.*, 1991). No changes were observed when measured in Brodmann's areas 9 and 46 (dorsolateral prefrontal cortex) (Akbarian *et al.*, 1995b; Selemon *et al.*, 1995; Selemon *et al.*, 1998; Volk *et al.*, 2000). Furthermore, it is entirely likely that subclasses of interneurons are preferentially altered in schizophrenia, perhaps as a consequence of aberrations during cortical development (Benes, 1999; Lewis *et al.*, 1999). Measures of total neuronal density lack the resolution to identify losses in neuronal subclasses (Beasley & Reynolds, 1997; Pakkenberg, 1993). Refining analysis on the basis of neuronal subsets may provide a more accurate indication of changes in interneuron densities in schizophrenia.

4.7.4.7 Changes in GAT-1-containing chandelier cells

The chandelier class of GABAergic interneurons has been investigated in the prefrontal cortex in schizophrenia. Chandelier cells are found predominantly in the superficial cortical layers, where their axon terminals synapse in characteristic cartridge arrays on the axon initial segment of pyramidal neurons (Lewis, 1995; Lewis *et al.*, 1999). This synaptic organisation permits the inhibitory cells to exert inhibitory control over action potential propagation in the pyramidal cells, thus regulating cortical output (Lewis, 1995). The axon cartridges of chandelier cells preferentially express GAT-1, and

decreased GAT-1 mRNA and peptide densities have been shown in the prefrontal cortex of schizophrenic subjects compared to controls (Lewis, 1995; Lewis *et al.*, 1999; Volk *et al.*, 2001; Woo *et al.*, 1998). A decrease in GAT-1 immunoreactive axon cartridges is conceivably the result of decreased chandelier cell number in the prefrontal cortex, a reduction in the extent of axonal arborisation of chandelier cells, consistent with the reduced neuropil hypothesis, or the consequence of a decrease in the number of axon terminals (Lewis *et al.*, 1999; Woo *et al.*, 1998).

4.7.4.8 Changes in calcium-binding protein-containing interneurons

Inhibitory GABAergic interneurons have been distinguished morphologically by the pattern of dendritic arborisation, and through expression of calcium-binding proteins. Visualising GABAergic interneurons by calcium-binding protein expression is useful as subclasses of neurons may be selectively identified, however cell counting methods are also required in these studies. In the rat and human temporal neocortex, the calcium-binding protein, calretinin, is found in bipolar cells (Del Río & DeFelipe, 1996; Kubota *et al.*, 1994; Rogers, 1992), and is localised to double bouquet cells of the monkey neocortex (Condé *et al.*, 1994). Parvalbumin, another calcium-binding protein, is contained in both the chandelier and basket cells of rat and monkey neocortex and human temporal cortex (Cauli *et al.*, 1997; Celio & Heizmann, 1981; Condé *et al.*, 1994; DeFelipe *et al.*, 1989b; Del Río & DeFelipe, 1994; Kawaguchi & Kubota, 1997; Kawaguchi & Kubota, 1998). In the monkey prefrontal neocortex, calcium-binding protein, calbindin D_{28k}, was localised to cells of Martinotti and neurogliaform cells (Condé *et al.*, 1994) as well as double bouquet and arcade cells (DeFelipe *et al.*, 1989a). Calbindin D_{28k} was also found in double bouquet cells of the human temporal neocortex (Del Río & DeFelipe, 1996).

A decrease in the density of parvalbumin-immunoreactive neurons was observed in Brodmann's areas 9, 10 and 46 of the prefrontal cortex, significantly in layers III and IV, in the entorhinal cortex and in the hippocampus of schizophrenic brain compared to controls (Beasley & Reynolds, 1997; Beasley *et al.*, 2002; Lewis *et al.*, 2001; Reynolds & Beasley, 2001; Reynolds *et al.*, 2002; Zhang & Reynolds 2002). A non-significant reduction in parvalbumin-immunoreactive neuron density in the anterior cingulate cortex of schizophrenic subjects relative to controls has also been found (Cotter *et al.*, 2002).

Another study found no change in calbindin-positive cell density in Brodmann's area 9 in schizophrenic subjects, however morphological abnormalities and cellular disarray were prominent (Iritani *et al.*, 1999). A significant reduction in calbindin-immunoreactive neurons in layer II of Brodmann's area 9 from schizophrenic brain was also evident (Beasley *et al.*, 2002). Further, a non-significant reduction in calbindin-immunoreactive neuronal density was demonstrated in Brodmann's area 46 (Reynolds *et al.*, 2002) and the anterior cingulate cortex (Cotter *et al.*, 2002) of schizophrenic subjects relative to controls. No change in calretinin-containing interneurons in Brodmann's areas 9, 10 and 46 of the prefrontal cortex, the anterior cingulate cortex, the entorhinal cortex or the hippocampus have been observed in schizophrenic tissue (Beasley *et al.*, 2002; Cotter *et al.*, 2002; Reynolds & Beasley, 2001; Reynolds *et al.*, 2002; Woo *et al.*, 1998; Zhang & Reynolds, 2002).

The reduction in parvalbumin- and calbindin-containing cell densities has not been replicated in all studies. Woo and colleagues (1997) did not encounter a change in parvalbumin-containing interneurons in the dorsolateral prefrontal cortex (Brodmann's areas 9 and 46) or primary visual cortex (Brodmann's area 17) of schizophrenic subjects compared to controls. Daviss and Lewis (1993) observed an increase in the density of calbindin-positive interneurons in the superficial layers of the dorsolateral prefrontal cortex of schizophrenic patients. Kalus and coworkers (1997) also showed an increase in parvalbumin-immunoreactive neuron density in the anterior cingulate region of schizophrenic cortex, without a change in total neuronal density. The parvalbumin-stained neurons were thought to be chandelier cells, due to the characteristic labelling of axon cartridges (Kalus *et al.*, 1997).

Calcium-binding proteins are thought to be neuroprotective against toxic calcium influx following, for example, NMDA receptor activation and excitotoxicity (Beasley & Reynolds, 1997; Reynolds & Beasley, 2001; Reynolds *et al.*, 2001). Parvalbumin- and calbindin-containing neurons appear late in post-natal development, around 3 months of age, which is much later than glutamate receptor development at 18 to 20 weeks gestation (Beasley & Reynolds, 1997; Reynolds & Beasley, 2001; Reynolds *et al.*, 2002; Thornberg & Saklad, 1996). Researchers have therefore speculated that a "window of vulnerability" exists during which the neurons are sensitive to excitatory damage

(Beasley & Reynolds, 1997; Olney & Farber, 1995; Reynolds & Beasley, 2001; Reynolds *et al.*, 2002). Deficits in interneurons containing calcium-binding proteins may therefore result from some trauma inducing excitotoxicity, such as neonatal hypoxia or inflammatory responses to infection, which would have developmental consequences (Beasley & Reynolds, 1997; Reynolds *et al.*, 2001; Reynolds *et al.*, 2002). Parvalbumin-dense staining in layer III of Brodmann's area 9 has been attributed to staining of vertically oriented rows of puncta, corresponding to chandelier cell axon terminals (Reynolds *et al.*, 2001). The observed reduction in width of the parvalbumin-dense layer provides further evidence for a deficit in the chandelier cells in schizophrenia (Reynolds *et al.*, 2001). Reduced parvalbumin staining also supports findings by Benes and coworkers (1986; 1991) of a selective loss of presumably basket cells in the schizophrenic cortex, since parvalbumin is also localised to basket cells. Loss of immunoreactivity most likely reflects a reduction in cell number, but may also represent decreased expression of parvalbumin and calbindin (Beasley *et al.*, 2002). Decreased expression may result from reduced cellular activity, perhaps through reduced innervation of GABAergic interneurons (Beasley & Reynolds, 1997), which is consistent with GABAergic hypofunction in any case.

4.7.4.9 Changes in neuropeptide-containing interneurons

GABAergic cells may also be identified on the basis of co-localised neuropeptides, including cholecystokinin (CCK), somatostatin, neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP) (Reynolds *et al.*, 2001). CCK content, immunoreactivity and mRNA were found to be reduced in the orbitofrontal cortex, inferior frontal gyrus, temporal cortex, superior temporal gyrus, parahippocampal gyrus, hippocampus, amygdala, entorhinal cortex and subiculum of schizophrenic subjects relative to controls (Bachus *et al.*, 1997; Gabriel *et al.*, 1996; Roberts *et al*, 1983; Virgo *et al.*, 1995). Bachus and colleagues (1997), however, failed to demonstrate a change in CCK mRNA levels in the schizophrenic dorsolateral prefrontal cortex. Somatostatin content and immunoreactivity were decreased in the orbitofrontal cortex, inferior frontal gyrus, cingulate cortex, superior temporal gyrus, parahippocampal gyrus and lateral thalamus in schizophrenic brain (Gabriel *et al.*, 1996; Nemeroff *et al.*, 1983; Roberts *et al.*, 1983). No changes in somatostatin were detected in the amygdala, nucleus accumbens and caudate nucleus (Nemeroff *et al.*, 1983). NPY content was also reduced in the cingulate

cortex and superior temporal gyrus (Gabriel *et al.*, 1996). Since these neuropeptides are found almost exclusively in GABAergic neurons, their losses may reflect a loss of the particular cells (Bachus *et al.*, 1997; Gabriel *et al.*, 1996; Reynolds *et al.*, 1990).

4.7.4.10 Reelin expression and GABA interneurons

Reelin is an extracellular matrix protein that regulates neuronal migration and positioning, and its mRNA is expressed almost exclusively in GABAergic cells, especially in the superficial cortical layers (Costa *et al.*, 2001; Impagnatiello *et al.*, 1998; Sawa & Snyder, 2002). Reelin mRNA expression and protein density have been found to be reduced by up to 50% in the prefrontal cortex, hippocampus, striatum and cerebellum of post-mortem schizophrenic brain (Impagnatiello *et al.*, 1998; Sawa & Snyder, 2002). Guidotti and colleagues (2000) further demonstrated a downregulation of reelin and GAD_{67} mRNA in Brodmann's area 9 of the dorsolateral prefrontal cortex in patients with schizophrenia. A decrease in reelin-immunoreactive cells was also encountered in the prefrontal cortex of schizophrenic subjects (Costa *et al.*, 2001). Together, the data signify a possible reduction in reelin-expressing GABAergic cells or cell function in schizophrenic brain. Additionally, reelin downregulation lends further support to the neurodevelopmental hypothesis of schizophrenia owing to its role in neuronal migration during corticogenesis (Costa *et al.*, 2001).

4.7.4.11 Consequences of changes in GABAergic neurons and synapses

In total, the evidence points to a selective deficit in a subpopulation of GABAergic neurons in the schizophrenic brain (Cotter *et al.*, 2002; Zhang & Reynolds, 2002). However, even if GABAergic interneurons are not consistently altered in schizophrenia, synaptic irregularities may occur, especially in the dorsolateral prefrontal cortex, where most research has been focussed. Within the cortex, pyramidal cells synapse upon the dendritic spines of other pyramidal cells, (Lewis & Anderson, 1995), and are also innervated by interneurons at the soma, axon initial segment, dendrites and dendritic spines. Synaptic irregularities in schizophrenia are manifest in decreased synaptic markers like synaptophysin in Brodmann's areas 9 and 46, indicative of reduced synaptic density, hence integrity of synaptic function (Glantz & Lewis, 1997). Reduced pyramidal cell somal size is also evident, suggestive of decreased axonal and dendritic arborisation (Pierri *et al.*, 2001; Rajkowska *et al.*, 1998). Deficient synaptic

activity is apparent with a reduction in pyramidal cell dendritic spines, a marker for pyramidal cell input, in Brodmann's areas 46 of the dorsolateral prefrontal cortex and 22 and 38 of the temporal cortex (Garey *et al.*, 1998; Glantz & Lewis, 2000; Lewis & Anderson, 1995). Additionally, the reduced neuropil observed in schizophrenic brain is a marker of diminished synaptic activity (Selemon *et al.*, 1995; Selemon *et al.*, 1998).

Whether the primary pathogenetic factor is structural or molecular, it appears that a dissociation of cortical activity may exist in schizophrenia due to impaired local intrinsic cortical connections, hence cortical output (Selemon & Goldman-Rakic, 1999). GABAergic interneurons provide stability to pyramidal cells through feedback and feedforward inhibition (Benes & Berretta, 2001; Keverne, 1999). Thus, interneuron connectivity may be compromised, impinging on inhibitory output onto both inhibitory and excitatory cells in the cortex in schizophrenia (Glantz & Lewis, 1997; Lewis, 1998). This is consistent with Robert's (1972) notion of reduced inhibition in schizophrenia, the consequences of which are anticipated to have a significant impact on the activity of local circuits in the cortex (Benes *et al.*, 1992b).

Findings of changes in GABAergic interneurons in schizophrenia, coupled with changes in glutamatergic pyramidal cells, particularly in the superficial laminae of the cortex, in fact support the notion that schizophrenia is a disorder involving aberrant cortico-cortical connections, since the supragranular layers project predominantly to other cortical regions. Supragranular cortical layers are important for associative, integrative processing owing to predominant cortico-cortical connections (Lee & Tobin, 1995; Zilles, 1991), disruptions of which may lead to the disruptions of higher cognition observed in schizophrenia (Lee & Tobin, 1995).

GABAergic cells and receptors maintain a critical role in brain development, facilitating neuronal migration and mediating differentiation and synaptogenesis of pyramidal cells (Behar *et al.*, 2000; Keverne, 1999). Alterations in GABAergic cell function or number during cortical development may thus have enduring effects, lending vulnerability to schizophrenia.

4.7.4.12 Genetic studies with the GABA receptor subunits

Linkage analysis and genotyping failed to demonstrate a linkage between schizophrenia and $GABA_A$ receptor $\alpha1$, $\alpha2$, $\alpha4$, $\alpha6$, $\beta1$ and $\beta3$ subunit genes in sibling pairs (Byerley *et al.*, 1995). However, failure to demonstrate linkage may be influenced by small sample size and substantial genetic heterogeneity (Coon *et al.*, 1994). Candidate gene studies have provided some evidence of a polymorphism in the $\beta1$ subunit in schizophrenic patients, which may have a pathogenetic effect (Coon *et al.*, 1994).

4.7.4.13 Pharmacological evidence for a role of GABA in schizophrenia

If schizophrenia is due, in part, to a deficiency in GABAergic transmission, increasing GABA activity in the CNS might exert a therapeutic effect (Fuxe *et al.*, 1975; van Kammen, 1977). This rather simple hypothesis has not withstood investigation, as GABAergic drugs have shown little therapeutic efficacy in the treatment of schizophrenia (van Kammen *et al.*, 1982; Weinberger, 1997).

Activation of $GABA_A$ receptors by agonists such as muscimol and THIP produce psychotomimetic responses in humans (Costa, 1992; Weinberger, 1997) and exacerbate the symptoms of schizophrenia (Tamminga *et al.*, 1978). THIP did not ameliorate dyskinetic symptoms in schizophrenic patients either (Korsgaard *et al.*, 1982). The $GABA_B$ receptor agonist, baclofen, was also shown to augment schizophrenic symptoms in 9 of 12 schizophrenic patients trialled (Simpson *et al.*, 1976; van Kammen *et al.*, 1982). Moreover, baclofen failed to attenuate tardive dyskinesias in psychiatric outpatients (Glazer *et al.*, 1985). Blockers of GABA catabolism also produce psychotomimetic effects (Costa, 1992; Lautin *et al.*, 1980), as do GABA uptake inhibitors (Sedman *et al.*, 1990). A human safety and tolerance study of GABA uptake inhibitor, CI-966, produced no effects in 11 of 16 subjects, but elicited psychotic symptoms in two subjects with no history of prior psychosis (Sedman *et al.*, 1990).

From the literature it is apparent that elevating the concentration or activity of GABA through administration of GABA agonists, GABA uptake inhibitors and GABA-transaminase inhibitors produces schizophrenia-like symptoms or exacerbates existing symptoms. This overactivity refutes the proposition that schizophrenia involves deficient GABA transmission. However it is possible that agents are acting at

presynaptic sites, altering GABA release, resulting in deficient GABA transmission. Furthermore, a pre-existing up-regulation of $GABA_A$ receptors (Benes *et al.*, 1992b; Benes *et al.*, 1996b) may lead to hypersensitivity of post-synaptic GABA receptors to GABA, leading to symptoms of schizophrenia (Meldrum, 1982).

Benzodiazepines can exacerbate psychosis in schizophrenia (Meldrum, 1982). Nonetheless, there is some evidence for a therapeutic effect of benzodiazepines in schizophrenia, with favourable outcomes and a reversal of negative symptoms in some studies (see Wassef *et al.*, 1999, for review). The benzodiazepine partial agonist, bretazenil, was trialled for antipsychotic efficacy. Most patients responded to bretazenil treatment with a regression of symptoms and reversal of dyskinesias (Delini-Stula & Berdah-Tordjman, 1996). When used as adjunct therapy to neuroleptic treatment, benzodiazepines also demonstrated some efficacy (Wassef *et al.*, 1999). Smaller doses of benzodiazepines were required when used in conjunction with neuroleptics, suggestive of a synergistic relationship between the drug classes (Wassef *et al.*, 1999).

The literature suggests a therapeutic role in schizophrenia for GABA-transaminase inhibitors, which increase synaptic GABA concentrations. Although some studies found sodium valproate enhanced psychotic symptoms in schizophrenic patients (Costa, 1992; Lautin *et al.*, 1980; Wassef *et al.*, 1999), a number of researchers have demonstrated therapeutic effects (see Wassef *et al.*, 1999, for review). Again, encouraging results were obtained when sodium valproate was administered in combination with neuroleptics (Wassef *et al.*, 1999). γ-Vinyl GABA, another GABA-transaminase inhibitor, decreases symptoms of tardive dyskinesia in patients with schizophrenia by effectively increasing the concentration of GABA in the CNS (Thaker *et al.*, 1987). Thus, modification of GABAergic activity through allosteric modulation or inhibition of its breakdown, rather than direct agonist action, may be a preferable means of attenuating symptoms of schizophrenia, or adverse effects of neuroleptic compounds. GABAergic agents may also provide additional antipsychotic activity in combination with neuroleptics (Wassef *et al.*, 1999).

4.7.4.14 Effects of neuroleptics on GABAergic transmission

Neuroleptic treatment has produced varied effects on GAD expression in animal studies. Prolonged haloperidol administration increased GAD mRNA and activity in the globus pallidus, striatum and entopeduncular nucleus of cataleptic rats (Delfs *et al.*, 1995; Rupniak *et al.*, 1987). Prolonged treatment with fluphenazine, a phenothiazine, increased GAD mRNA levels in the caudate nucleus, nucleus accumbens and entopeduncular nucleus in dyskinetic rats (Johnson *et al.*, 1994). Clozapine, on the other hand, was shown to marginally decrease GAD mRNA expression in the rat globus pallidus (Delfs *et al.*, 1995) and had no effect on GAD activity in the striatum (Rupniak *et al.*, 1987). Neither acute nor chronic treatment with haloperidol or clozapine was shown to significantly alter GAD concentrations in the rat substantia nigra (Lloyd & Hornikiewicz, 1977; Rupniak *et al.*, 1987) or GAD_{67} mRNA densities in the monkey prefrontal cortex (Volk *et al.*, 2000).

Animal studies have shown that neuroleptic treatment has no effect or in fact increases GABA concentrations (Kornhuber *et al.*, 1984; Lloyd & Hornykiewicz, 1977; Perry *et al.*, 1979a; Sherman *et al.*, 1991). Kornhuber *et al.* (1984) showed a significant increase in GABA in rat striatum with prolonged haloperidol exposure. Chronic haloperidol administration also increased GABA-immunoreactivity in the rat prefrontal cortex, implying that haloperidol increases the concentration of GABA, probably by inhibiting the inhibitory effect of dopamine on GABA release (Vincent *et al.*, 1994). Moreover, levels of GABA in the CSF of schizophrenic patients not treated with neuroleptics were not significantly different to those of controls, while levels were elevated in patients treated with neuroleptics (Zimmer *et al.*, 1981). Not all these findings have been substantiated. While acute administration of clozapine and haloperidol decreased GABA levels in the rat substantia nigra, chronic administration produced no significant effect (Kornhuber *et al.*, 1984; Lloyd & Hornykiewicz, 1977). Prolonged haloperidol or chlorpromazine administration also failed to alter GABA or GAD content in rat mesolimbic regions or striatum (Perry *et al.*, 1979b).

Neuroleptics increase GABA release from synaptosomal preparations of schizophrenic cerebral cortex and rat brain (Sherman *et al.*, 1991). Chronic haloperidol treatment potentiated K^+-induced GABA release in the rat caudate-putamen but not the prefrontal

cortex, measured using intracranial microdialysis (See & Lynch, 1995). This effect may be mediated via neuroleptic antagonism of dopamine receptors (See & Lynch, 1995). Clozapine and haloperidol were shown to elevate GABA release in the striatum, and haloperidol increased GABA release in the globus pallidus (Drew *et al.*, 1990). Another study employing *in vivo* microdialysis, however, showed that haloperidol and clozapine decreased extracellular GABA levels in the rat prefrontal cortex, with no effect on striatal GABA levels (Bourdelais & Deutch, 1994). This effect was thought to occur through dopaminergic mechanisms, as haloperidol would act to inhibit the dopaminergic facilitation of GABA release in the prefrontal cortex (Bourdelais & Deutch, 1994). Discrepancies in results may arise from differences in brain regions where neuroleptics were administered and where GABA release was measured. Generally, however, it follows that D_2 receptor blockade, such as that which occurs with neuroleptic drugs, increases GABA synthesis and release, which may form part of the therapeutic effect of neuroleptics (Lindefors, 1993). Dopamine receptors have been localised to GABAergic interneurons, lending further evidence to this hypothesis.

Neuroleptic treatment affects GABA receptor expression measured by radioligand binding. [^{3}H]Muscimol binding was reduced in the hippocampus and temporal cortex after chronic treatment with chlorpromazine, haloperidol, clozapine or olanzapine in rats, the most notable decreases occurring following clozapine and olanzapine administration (Farnbach-Pralong *et al.*, 1998). Haloperidol, however, had no effect on [^{3}H]muscimol binding in the rat striatum, while clozapine and olanzapine decreased binding in this region (Dean *et al.*, 2001). On the other hand, prolonged haloperidol treatment is known to increase [^{3}H]muscimol binding to $GABA_A$ receptors in the rat substantia nigra (See *et al.*, 1989; See *et al.*, 1990), although this is not coupled with a decrease in GABA levels, since chronic treatment with haloperidol was not shown to significantly alter GABA in the rat substantia nigra (Kornhuber *et al.*, 1984; Lloyd & Hornikiewicz, 1977). Chronic administration of haloperidol decreased [^{35}S]TBPS binding in the globus pallidus and increased binding in the ventrolateral striatum of rats exhibiting dyskinetic symptoms. Owing to the different patterns of binding observed in different brain regions, [^{35}S]TBPS binding was further speculated to involve the $GABA_C$ receptors, which are sensitive to TBPS (Sasaki *et al.*, 1997).

Neuroleptic compounds are known to displace binding to $GABA_A$ receptors, demonstrating affinity for the receptors. Fluphenazine decreased [^{3}H]muscimol binding in the caudate-putamen, nucleus accumbens and dentate gyrus of dyskinetic rats (Johnson *et al.*, 1994). Chlorpromazine inhibited [^{3}H]muscimol binding to $GABA_A$ receptors in rat cerebral cortical synaptoneurosomes (Schwartz & Mindlin, 1988). Clozapine (100μM) was also shown to decrease [^{3}H]muscimol and [^{3}H]SR 95531 binding to $GABA_A$ receptors in rat cortical and cerebellar membrane preparations (Korpi *et al.*, 1995). Finally, chlorpromazine (500μM) and haloperidol (200μM) were found to act as non-competitive inhibitors of [^{3}H]GABA uptake in rat brain cortical slices (Iversen & Johnston, 1971).

Chlorpromazine inhibited [^{3}H]TBOB binding to the picrotoxin-binding site on the $GABA_A$ receptor channel pore in a non-competitive fashion in rat cerebral cortical synaptoneurosomes (Schwartz & Mindlin, 1988). Haloperidol alters [^{35}S]TBPS binding to the ion channel in rat basal ganglia (Sasaki *et al.*, 1997). Haloperidol (100μM) was also shown to decrease [^{35}S]TBPS binding in rat cortical, hippocampal and cerebellar membrane preparations, and potentiate the GABA-induced inhibition of [^{35}S]TBPS binding (Korpi *et al.*, 1995). The inhibitory action of 1μM GABA on [^{35}S]TBPS binding in rat forebrain preparations was partially reversed by 30μM clozapine (Squires and Saederup, 1997). This effect was thought to be regulated by a subset of the $GABA_A$ receptors (Squires & Saederup, 1997). Clozapine inhibited [^{35}S]TBPS binding and reversed the inhibition of binding by 5μM GABA in rat hippocampus and cortical membrane preparations and whole sections (Korpi *et al.*, 1995). Regional differences in the extent of clozapine's effects suggested subtype selectivity of the drug's action.

Neuroleptic effects on GABA currents recorded from cells *in vitro* have been reported. Some studies failed to demonstrate an effect of chlorpromazine on GABAergic transmission. GABA-induced depolarisation of cat primary afferent neurons was unaltered at concentrations of chlorpromazine up to 100μM (Higashi *et al.*, 1981). Chlorpromazine also had no effect on spontaneously firing Purkinje cells in the cat cerebellum (Maruyama & Kawasaki, 1976). On the other hand, chlorpromazine inhibited miniature GABAergic currents in cultured rat hippocampal neurons through a

reduction in binding and an increase in the unbinding rate of GABA at the $GABA_A$ receptor (Mozrzymas *et al.*, 1999). Chlorpromazine blocked GABA currents in cultured chick spinal cord neurons in a non-competitive manner (Zorumski & Yang, 1988). Haloperidol (100μM), on the other hand, failed to modify GABA currents in cultured chick spinal cord neurons (Zorumski & Yang, 1988). Furthermore, haloperidol had no effect on current elicited by 3μM GABA in isolated rat dorsal root ganglion neurons, but did reduce the clozapine-induced inhibition of GABA, possibly by acting as an antagonist at the picrotoxin binding site (Yokota *et al.*, 2002). This is consistent with evidence that haloperidol reverses picrotoxin-induced impairment of sensorimotor gating in rats (Japha & Koch, 1999) and alters [^{35}S]TBPS binding to the picrotoxin-binding site in the $GABA_A$ receptor channel pore in rat basal ganglia (Sasaki *et al.*, 1997). Maruyama (1974) and Maruyama and Kawasaki (1976) observed a GABA-mimetic effect of haloperidol and droperidol on spontaneously firing Purkinje cells in the cat cerebellum. These drugs were found to depress firing, and the effects of GABA and droperidol were blocked by the $GABA_A$ receptor antagonist, bicuculline (Maruyama & Kawasaki, 1976). Clozapine inhibited the current induced by 3μM GABA in isolated rat dorsal root ganglion neurons (Yokota *et al.*, 2002). Clozapine also reduced picrotoxin-mediated inhibition in this study. Clozapine has been shown to inhibit IPSCs elicited by 500μM GABA in cultured rat ventral tegmental area neurons at concentrations of 10μM upwards (Michel & Trudeau, 2000).

Muscimol-induced $^{36}Cl^-$ uptake into rat cerebral cortical synaptoneurosomes was inhibited by chlorpromazine in a Ca^{2+}-dependent manner (Schwartz & Mindlin, 1988). Clozapine (100μM) was further shown to inhibit picrotoxin-sensitive GABA-induced ^{36}Cl flux into brain vesicles, indicating that it is an antagonist at $GABA_A$ receptors (Korpi *et al.*, 1995).

A few studies involving cell expression systems have been carried out with neuroleptics. Clozapine was found to inhibit GABA-evoked Cl^- currents at recombinant $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes at concentrations between 10 and 300μM (Asproni *et al.*, 2002). Haloperidol at low concentrations inhibited GABA-induced depolarisation of cat primary afferent neurons, while at concentrations above 10μM, GABA-induced depolarisation was enhanced (Higashi *et al.*, 1981). Droperidol, another

butyrophenone compound used for neuroleptanaesthesia, was shown to inhibit GABA at $\alpha1\beta1\gamma2$ receptors expressed in *Xenopus* oocytes with an IC_{50} of 12.6 nM (Flood & Coates, 2002). This inhibitory effect reversed at 5μM, and at high concentrations (100μM), droperidol activated the receptors in the absence of GABA (Flood & Coates, 2002). Using HEK293 cells expressing different combinations of $GABA_A$ receptor subunits, Korpi *et al.* (1995) demonstrated that clozapine inhibited [^{35}S]TBPS binding at $\alpha1\beta2\gamma2_L$ receptors and was most effective at reversing the inhibitory effect of GABA on [^{35}S]TBPS binding at the $\alpha1\beta1\gamma2$ receptor complex, followed by the $\alpha1\beta2\gamma2$ and $\alpha1\beta3\gamma2$ complexes. Thus, clozapine's action is selective for subsets of the $GABA_A$ receptor complex.

Changes in GABA receptors in basal ganglia structures following typical neuroleptic treatment are thought to contribute to the extrapyramidal side effects of the drugs, since parallel changes have not been detected with administration of clozapine, which does not demonstrate such side effects (Delfs *et al.*, 1995; See & Lynch, 1995; See *et al.*, 1990). In total, neuroleptics have a predominantly inhibitory effect on GABAergic transmission, they inhibit binding to GABA receptors *in vitro*, and prolonged neuroleptic treatment in some cases downregulates GABA receptor expression. Thus, neuroleptics may influence GABA activity to effectively compensate for alterations in GABA transmission in schizophrenia.

4.7.5 Interactions Between GABAergic and Other Neurotransmitter Systems in Schizophrenia

4.7.5.1 GABAergic/dopaminergic interactions

Roberts (1972) proposed that reduced GABAergic function with a consequent imbalance between GABAergic and dopaminergic transmission could play a primary pathogenetic role in schizophrenia. Evidence exists for a role of GABA in controlling the release of dopamine. GABA receptor antagonism increases dopamine release in the prefrontal cortex (Santiago *et al.*, 1993), while the $GABA_B$ receptor agonist, baclofen, reduces dopamine release in the nucleus accumbens (Fuxe *et al.*, 1975). Thus, GABA appears to mediate the inhibition of dopamine activity (Wassef *et al.*, 1999). The putative reduction in GABAergic inhibitory control of dopaminergic neurons in

schizophrenia may lead to overactivity of these neurons, which is compensated for by the introduction of dopamine D_2 receptor antagonists (Carlson, 1991; van Kammen, 1977).

Alternatively the primary pathology may lie in the dopaminergic modulation of GABA. Increased dopamine neurotransmission mediated by repeated amphetamine administration decreases GAD_{67} mRNA expression and GABA release in the nucleus accumbens, (Lindefors *et al.*, 1992; Lindefors, 1993), globus pallidus (Floran *et al.*, 1997), and striatum (Girault *et al.*, 1986). This may also be the case in other brain areas, providing a clue to the pathophysiological process in schizophrenia. Hypothesised dopaminergic overactivity may lead to decreased GAD mRNA and reduced GABA release, with a resultant hypothesised GABAergic hypoactivity.

Dopamine D_2 receptor stimulation was found to decrease GABA release *in vivo* (Floran *et al.*, 1997; Girault *et al.*, 1987; Reid *et al.*, 1990). GABA levels were elevated in the rat striatum after injection of GABA into the substantia nigra, an effect that was reversed by the D_2 receptor agonist, pergolide (Reid *et al.*, 1990). D_2 receptor agonists decreased [^{3}H]GABA release in the striatum and globus pallidus, and this action was blocked by the D_2 receptor antagonist, sulpiride (Floran *et al.*, 1997; Girault *et al.*, 1986). Therefore it follows that D_2 receptor blockade, such as that which occurs with neuroleptic drugs, increases GABA release, which may form part of the therapeutic effect of neuroleptics (Lindefors, 1993). In fact, chronic haloperidol administration increased GABA-immunoreactivity in the rat prefrontal cortex, implying that haloperidol increases the concentration of GABA, probably by inhibiting the inhibitory effect of dopamine on GABA release (Vincent *et al.*, 1994). Additionally, D_2 receptor antagonists, clozapine and haloperidol, increased GABA release in the rat striatum, and haloperidol elevated GABA release in the globus pallidus (Drew *et al.*, 1990). Moreover, continuous exposure to dopamine D_2 receptor antagonists EEQD and fluphenazine elevated GAD mRNA in mouse striatum, nucleus accumbens, frontal and parietal cortices, indicative of an increase in GABA synthesis in these regions (Qin *et al.*, 1993).

Dopaminergic axons arising from the mesencephalon terminate on GABAergic interneurons in the rat medial prefrontal cortex and limbic system (Benes *et al.*, 1993;

Vincent *et al.*, 1993), specifically on the parvalbumin-containing basket and chandelier cells (Sesack *et al.*, 1995). These afferents exert an inhibitory effect, thus exacerbating a deficiency in GABAergic neurotransmission (Benes *et al.*, 1992b; Benes, 2002). Moreover, dopamine D_2 and D_4 receptors and mRNAs occur on GABAergic interneurons in rat prefrontal cortex (Vincent *et al.*, 1993; Vysokanov *et al.*, 1998) and on chandelier cells in primate brain (Mrzljak *et al.*, 1996). These findings are significant in light of the well-recognised role of dopamine D_2 and D_4 receptors in the mechanism of neuroleptic drug action. Dopamine receptor antagonism may permit normal synthesis and release of GABA, alleviating symptoms of schizophrenia derived from deficient GABAergic neurotransmission (Benes *et al.*, 1992b; Benes, 2002).

4.7.5.2 GABAergic/serotoninergic interactions

Serotoninergic neurons arising in the brainstem synapse on GABAergic interneurons in the cortex (Abi-Saab *et al.*, 1999). 5-HT_2 receptors are located on GABAergic interneurons in rat brain (Morilak *et al.*, 1993), where they are important in modulating GABA release (Dean, 2001). Moreover, 5-HT_2 receptors have been shown to regulate extracellular GABA levels in the prefrontal cortex, such that administration of a 5-HT_2 receptor agonist increases extracellular GABA content (Abi-Saab *et al.*, 1999). Hence, like dopamine transmission, serotonin modulation of cortical GABAergic transmission may fail in schizophrenia (Benes, 2002). Abnormal convergence of subcortical dopaminergic and serotoninergic inputs onto cortical GABAergic interneurons may also affect GABAergic influence on glutamatergic pyramidal cell activity (Benes, 2002).

4.7.5.3 GABAergic/glutamatergic interactions

An imbalance between inhibitory GABAergic and excitatory glutamatergic transmission appears to be involved in schizophrenia (Bonnano & Raiteri, 1992; Meldrum, 1982). GABA counterbalances glutamate-mediated neuronal excitation and it is thought that abnormalities in the interaction between these two systems might disrupt several brain functions, including cognitive function (Costa, 1992). An equilibrium between glutamatergic and GABAergic synaptic strength may play a role in the regulation of neocortical cognitive function (Costa, 1992). Thus, GABA may be inefficiently modulating glutamatergic activity. Inhibitory GABAergic interneurons synapse upon glutamatergic pyramidal cells and GABA receptors are localised on pyramidal cells

(Benes & Berretta, 2001; Deakin *et al.*, 1997). In a rat hippocampal model, it was demonstrated that pyramidal cells activate GABAergic inhibitory neurons via NMDA receptors, and the GABAergic cells in turn inhibit the pyramidal cells, forming a recurrent inhibitory circuit (McCarley *et al.*, 1999). McCarley and associates (1999) suggested that some symptoms of schizophrenia are consistent with a failure of this recurrent inhibitory circuit in the neocortex. Thus deficits in interneurons that usually maintain inhibitory control of pyramidal cells may lead to a dysfunction of the glutamatergic pathway, with a resultant loss of pyramidal cell stability and excessive excitatory output (Benes & Berretta, 2001; Reynolds *et al.*, 2002).

Descending glutamatergic efferents stimulate GABAergic outflow from the nucleus accumbens, striatum and hippocampus (Thornberg & Saklad, 1996), three structures implicated in the pathophysiology of schizophrenia. The postulated impaired glutamatergic transmission may reduce GABA outflow from these regions, resulting in neuronal disinhibition, and translating to symptoms of schizophrenia (Thornberg & Saklad, 1996). Olney and Farber (1995) hypothesised that NMDA receptors located on GABAergic interneurons may be hypoactive in certain circuits, resulting in disinhibition. At the same time, glutamatergic feedback onto dopaminergic neurons in the mesencephalon may also be impaired. As mentioned in section 4.7.5.1, dopamine afferents from the midbrain synapse upon D_2 receptors located on GABAergic neurons in the nucleus accumbens, striatum, hippocampus and cortex to inhibit GABA release (Benes *et al.*, 1993; Sesack *et al.*, 1995; Thornberg & Saklad, 1996; Vincent *et al.*, 1993). Therefore, an additive decrease in GABA release may occur through aberrant glutamatergic modulation of these neurotransmitters, resulting in further neuronal disinhibition which may permit unmodulated stimulatory activity to flood corticolimbic regions, and development of schizophrenic symptoms (Olney & Farber, 1995; Thornberg & Saklad, 1996).

4.8 CONCLUSIONS

While not all findings are entirely consistent, compelling evidence exists for a disturbance in GABAergic neurotransmission in the brain in schizophrenia, whereby presynaptic GABAergic function appears to be downregulated. Moreover, the findings are in key brain regions implicated in the symptoms of schizophrenia, namely the

prefrontal and temporal cortices and medial temporal lobe structures. Inconsistencies in published findings may represent, in part, paucity in the accurate diagnosis of schizophrenia, especially in earlier studies. The current practice of diagnosis on the basis of stringent, standardised criteria has ameliorated this problem, allowing researchers to comment with confidence on findings from research samples that represent the schizophrenic population.

Decreased GAD activity, changes in receptor densities, altered neuronal numbers, and decreased markers for GABAergic transmission all support Roberts (1972) original hypothesis of a deficit in inhibition mediated by GABA in the pathogenesis of schizophrenia. Schizophrenia is essentially "a disease of neuronal connectivity" (Andreasen, 2000). The evidence points to aberrant cortical GABA-mediated transmission and cortico-cortical connections, especially in the supragranular cortex. Supragranular cortical layers project predominantly to other cortical regions and are thus important for associative, integrative processing (Lee & Tobin, 1995; Zilles, 1991), disruptions of which may lead to the disruptions of higher cognition observed in schizophrenia (Akbarian *et al.*, 1995a; Lee & Tobin, 1995). The implications of a disturbance in GABAergic transmission are considerable.

Since GABA is ubiquitous throughout the CNS and plays an important role in counterbalancing glutamate-mediated neuronal excitation, abnormalities in the interaction between these two systems might disrupt several brain functions, including cognitive function (Costa, 1992). Deficient synaptic activity, whether from a reduction in cell size, number or degree of arborisation, resulting in decreased connectivity between interneurons and pyramidal cells, would have gross consequences on the excitatory activity of pyramidal projection neurons, playing a potential role in the pathophysiology of schizophrenia. Reduced synaptic contact between excitatory pyramidal and inhibitory local circuit neurons would result in a loss of inhibitory tone, with consequent disinhibition of excitatory pyramidal cells, leading to increased excitatory output to other areas of the brain (Benes *et al.*, 1996b). An up-regulation of post-synaptic GABA receptors may compensate for reduced inhibition to some extent (Benes *et al.*, 1996b).

Alternately, or concurrently, the interaction between dopamine and GABA may be compromised by a loss of inhibitory tone in schizophrenia. It is thought that decreased GABAergic function may lead to increased dopaminergic activity. Dopamine receptors are localised to GABAergic interneurons in rat and primate brain (Mrzljak *et al.*, 1996; Vincent *et al.*, 1993). Dopaminergic afferents also terminate on cortical GABA interneurons to exert an inhibitory effect, thus exacerbating a deficiency in GABAergic neurotransmission (Benes *et al.*, 1992b). It has been suggested that neuroleptic-mediated dopamine receptor antagonism may permit normal release of GABA, alleviating symptoms of schizophrenia effected by deficient GABAergic neurotransmission (Benes *et al.*, 1992b). GABAergic transmission is vital in modulating cortical activity, thus altered activity would disrupt normal neural processing, resulting in an imbalance in other systems, and symptoms of schizophrenia.

4.9 AIMS

Post-mortem studies aim to define the neural substrates of schizophrenia, corresponding perhaps to subtle changes in mRNA and protein expression, the effects of which may be profoundly disruptive to neural function. Through this research the aim is, therefore, to better understand and further characterise the potential role of GABA in the symptoms of schizophrenia, by assessing changes in the distribution and density of ionotropic GABA receptors in the cortex of individuals with schizophrenia compared to matched controls. The expression of ionotropic GABA receptors in *Xenopus* oocytes further permits the assessment of neuroleptic drug activity at these receptors, in an attempt to define whether the drugs may be contributing any therapeutic effect via direct modulation of the receptors.

IONOTROPIC GABA RECEPTORS IN SCHIZOPHRENIA
CHAPTER 5: *IN SITU* HYBRIDISATION HISTOCHEMISTRY

5.1 INTRODUCTION AND AIMS

5.1.1 General Introduction

A review of the literature grossly implicates the GABAergic system in schizophrenia, both at the pre- and post-synaptic levels. Aberrations in GABAergic transmission may thus contribute to the pathophysiology of the disease. For this reason, further investigation into changes in synaptic elements of the GABAergic system is required. The GABA receptors present important targets for research in schizophrenia, owing to their key role in mediating inhibitory synaptic transmission, and their pharmacological relevance.

5.1.2 Brain Regions Investigated

The primary areas of dysfunction in schizophrenia appear to be the temporal and frontal lobes (Deakin *et al.*, 1989; Ohnuma *et al.*, 1999; Reynolds, 1991; Syvälahti, 1994). Furthermore, many of the gross and chemical neuropathological changes documented in schizophrenia are confined to the left hemisphere (Bogerts *et al.*, 1990b; Reynolds, 1991; Shenton *et al.*, 1992; Syvälahti, 1994; Weinberger *et al.*, 1992). Since schizophrenia involves the disruption of higher order cognitive functions, the association cortex is most likely affected. Thus, for the current study, two regions of association cortex were chosen for investigation; the middle frontal cortex and the superior temporal gyrus. Figure 5.1 is adapted from the Atlas of the Human Brain (Mai *et al.*, 1997) and demonstrates the location of the superior temporal gyrus (STG) and middle frontal cortex (MFG) in a coronal section of the human brain. Both the middle frontal cortex, comprising part of the dorsolateral prefrontal cortex, and the superior temporal gyrus are heteromodal association cortex, functioning to coordinate cross-modal sensory and motor activities, and are specifically affected in schizophrenia (Schlaepfer *et al.*, 1994).

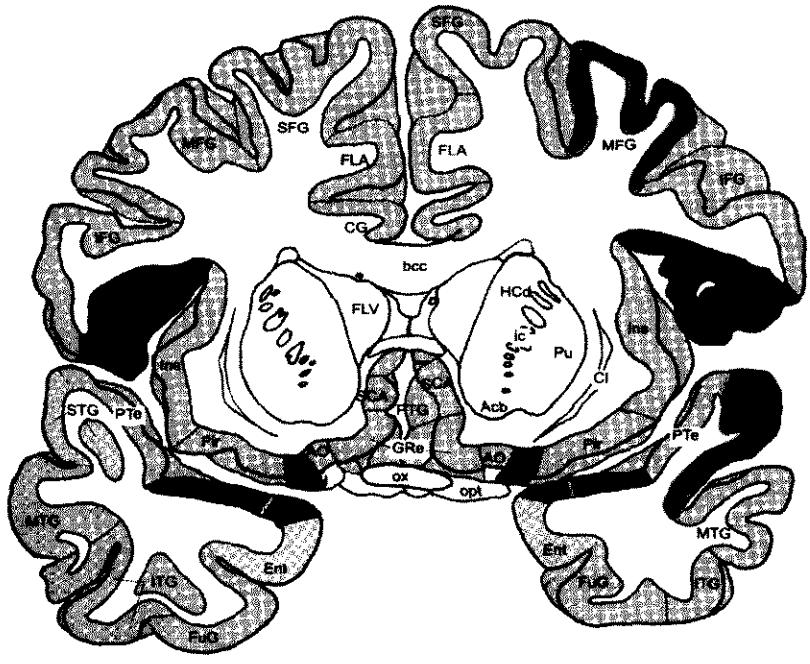


Figure 5.1: Coronal section of human brain displaying regions investigated in the present study. Middle frontal cortex is highlighted in red. Superior temporal gyrus is highlighted in blue. Image adapted from The Atlas of the Human Brain, Figure 7p (Mai *et al.*, 1997).

5.1.2.1 Middle frontal cortex

The middle frontal cortex lies on the lateral aspect of the frontal cortex between the superior and inferior frontal gyri (Crespo-Facorro *et al.*, 1999). The posterior region of the middle frontal gyrus contains Brodmann's area 8, the frontal eye field (Crespo-Facorro *et al.*, 1999). The anterior portion is part of the prefrontal association cortex, most likely corresponding to Brodmann's area 46, a component of the dorsolateral prefrontal cortex (Akbarian *et al.*, 1995b; Crespo-Facorro *et al.*, 1999; Selemon *et al.*, 1998). This region is essential for social behaviours, motor planning and cognitive processing, including analysis of visual and auditory information, semantic and spatial memory processing and attention (Akbarian *et al.*, 1995b; Kupfermann, 1991; Lahti *et al.*, 2001; Zilles, 1990). The prefrontal cortex is widely implicated in the pathophysiology of schizophrenia, since disruptions to this region result in abnormal social conduct, inadequate decision-making and planning, poor temporal memory, avolition and apathy (Damasio *et al.*, 1990; Deakin, 1994; Kolb & Whishaw, 1996; Weinberger *et al.*, 1994), symptoms similar to those observed in schizophrenia.

Functional imaging studies implicate the middle frontal cortex, as well as the wider dorsolateral prefrontal cortex in the symptoms of schizophrenia. Reduced cerebral blood

flow to the middle frontal cortex was observed in schizophrenic patients exhibiting primarily negative symptoms compared to controls at rest and during an auditory recognition task (Lahti *et al.*, 2001). The reduction in blood flow did not correspond to a decrease in performance on tasks. The Wisconsin Card Sorting Test (WCST) is considered to measure frontal lobe "executive" functioning, which relies on the ability to access appropriate information and recognise the correct experience necessary to modify one's behaviour (Weinberger *et al.*, 1986; Weinberger *et al.*, 1994). Patients with schizophrenia perform poorly on this test, with a pattern characteristic of patients exhibiting frontal lobe damage, suggestive of a reduced ability to make critical judgements required for autonomous functioning (Bunney *et al.*, 1993; Weinberger *et al.*, 1986; Weinberger *et al.*, 1992; Weinberger *et al.*, 1994). Moreover, concurrent measurements of regional cerebral blood flow and oxygen utilisation demonstrate decreased activation of the dorsolateral prefrontal cortex during the WCST (Bunney *et al.*, 1997; Weinberger *et al.*, 1986; Weinberger *et al.*, 1992). The dorsolateral prefrontal cortex, especially Brodmann's area 46, is implicated in WCST from lesion studies, and this region is "hypofrontal" in neuroimaging experiments with schizophrenic subjects (Bunney *et al.*, 1997; Weinberger *et al.*, 1986; Weinberger *et al.*, 1994). Menon and colleagues (2001) further demonstrated reduced activation of the dorsolateral prefrontal cortex in schizophrenic subjects compared to controls during a working memory task, corresponding with poorer task performance.

Structural magnetic resonance imaging (MRI) studies have shown a reduction in grey matter volume of the middle frontal cortex in schizophrenic patients relative to controls (Goldstein *et al.*, 1999; Schlaepfer *et al.*, 1994). Neuroimaging studies, however, often fail to identify subtle differences between schizophrenic and control subjects, which can be detected by post-mortem experiments (Goldstein *et al.*, 1999).

While a number of post-mortem studies have assessed GABA receptors and markers of GABAergic transmission in the dorsolateral prefrontal cortex of schizophrenic brain (see Chapter 4, section 4.7.4), few have adequately defined the anatomical location of tissue investigated. Akbarian *et al.* (1995b) demonstrated non-significant decreases in $GABA_A$ receptor subunit mRNA expression in middle frontal cortex of post-mortem schizophrenic brains compared to controls. Other documented changes in the GABA

system in Brodmann's area 46 were reductions in parvalbumin- and calbindin-immunoreactivity, indicating decreased GABAergic cell number or function in schizophrenic brains (Beasley *et al.*, 2002; Reynolds *et al.*, 2002).

5.1.2.2 Superior temporal gyrus

The superior temporal gyrus (STG) extends from the temporal pole to the posterior end of the Sylvian fissure, and corresponds to Brodmann's areas 41, 42 and 22 (Kim *et al.*, 2000; Kolb & Whishaw, 1996). Areas 41 and 42, or posterior STG, constitute the primary auditory cortex, while Area 22, or anterior STG, is implicated in auditory processing, as a known part of the auditory association cortex (Barr & Kiernen, 1993; Craven, 2002; Deakin, 1994; Kolb & Whishaw, 1996; Zilles, 1990). The superior temporal gyrus has been studied in schizophrenia, as disruptions in neural processing in this region of the brain are thought to contribute to disturbances of auditory sensation and perception, as well as language comprehension, present in the disorder (Barta *et al.*, 1990; Franzén & Ingvar, 1975a; Kolb & Whishaw, 1996; Le Corre *et al.*, 2000; Shenton *et al.*, 1992).

Structural MRI studies revealed changes in the superior temporal gyrus in schizophrenia, notably a reduction in volume of the STG (Bryant *et al.*, 1999; Matsumoto *et al.*, 2001; Shenton *et al.*, 1992; Sigmundsson *et al.*, 2001; Woodruff *et al.*, 1997). Reduced volume of the left posterior STG was correlated to severity of thought disorder, such that as volume decreased, severity of thought disorder increased (Shenton *et al.*, 1992). A significant decrease in volume of the posterior, but not anterior, STG was also observed in first episode schizophrenics (Hirayasu *et al.*, 1998). Volume of superior temporal gyrus grey matter was reduced in schizophrenic patients compared to controls (Gur *et al.*, 2000; Marsh *et al.*, 1997; Schlaepfer *et al.*, 1994; Sigmundsson *et al.*, 2001). Marsh *et al.* (1997) found a correlation between psychotic symptoms and reduced volume of left posterior STG grey matter. Barta and colleagues (1990; 1997) encountered smaller anterior STG volumes in both early and late life onset schizophrenics. Decreased STG volumes correlated with auditory hallucinations, so that as volume decreased, severity of auditory hallucinations increased (Barta *et al.*, 1990). This finding was replicated by Levitan and colleagues (1999), who showed that increased severity of hallucinations was associated with smaller left anterior STG

volumes. Matsumoto and associates (2001) also demonstrated a negative correlation between STG volume and hallucinatory behaviour scores. Some researchers failed to demonstrate a change in STG volume measured by MRI in schizophrenic patients (Goldstein *et al.*, 1999; Roy *et al.*, 1998; Vita *et al.*, 1995). Inconsistencies may have arisen through sampling, whether total or grey matter volume measurements were taken, and methods of segmentation (Vita *et al.*, 1995). Post-mortem studies have confirmed a decrease in STG volume in schizophrenic subjects relative to controls (Highley *et al.*, 1999), indicating that this is indeed a characteristic of schizophrenia.

Functional abnormalities in the superior temporal gyrus in schizophrenic subjects have been revealed through functional MRI. Wible and colleagues (2001) showed decreased activation of the STG in schizophrenic subjects during a mismatch negativity task compared to controls, attesting to abnormal auditory processing in schizophrenia. A number of experiments assessing P300 evoked potentials have found reduced amplitude and increased latency of these potentials in schizophrenic subjects (Benes & Berretta, 2001). McCarley and associates (2002) demonstrated a reduction in P300 evoked potential amplitude corresponding to reduced left posterior STG volume in first-episode schizophrenic patients. These changes are related to a reduced capacity to habituate to selective attentional responses, conceivably reflecting diminished GABAergic inhibitory modulation (Benes & Berretta, 2001), and abnormalities in language and auditory processing in schizophrenia (McCarley *et al.*, 2002). Such impairments in the superior temporal gyrus may account for the observed inability of patients with schizophrenia to filter out extraneous information (Benes & Berretta, 2001), resulting in over-inclusivity and cognitive overload.

Post-mortem evidence exists for altered GABAergic transmission in the superior temporal gyrus in schizophrenia. Kiuchi *et al.* (1989) observed increased [^{3}H]flunitrazepam binding to benzodiazepine-sensitive $GABA_A$ receptors in the superior temporal gyrus of schizophrenic subjects compared to controls. This increase in binding was limited to the type 1 benzodiazepine receptors. Type 1 benzodiazepine receptors contain the $\alpha 1$ subunit while type 2 receptors contain either the $\alpha 2$, $\alpha 3$ or the $\alpha 5$ subunit (Pandey *et al.*, 1997).

5.1.2.3 Dorsolateral PFC and STG connectivity

Cognitive deficits observed in schizophrenia are thought to reflect an aberration in prefrontal-temporal cortical connections (Huntsman *et al.*, 1998; Weinberger *et al.*, 1992) and a 'dysregulation' of coordinated activity between the regions (Egan & Weinberger, 1997). Woodruff and colleagues (1997) in fact demonstrated, using structural MRI, a lack of association between dorsolateral PFC and STG volumes in schizophrenic subjects. The volume correlation between these two regions was apparent in control subjects, suggesting that in schizophrenia, a loss of reciprocal connectivity may occur between the dorsolateral PFC and STG (Woodruff *et al.*, 1997). The uncinate fasciculus connects the orbitofrontal cortex with the anterior temporal cortex, as well as the amygdala and dorsomedial thalamus (Deakin, 1994; Raleigh & Steklis, 1981). This circuit is implicated in social cognition, the human capacity to infer intention and influence others (Deakin, 1994). Moreover, the dorsolateral prefrontal cortex in particular receives major projections from the inferior parietal and superior temporal association areas (Weickert & Kleinman, 1998; Woodruff *et al.*, 1997) and pyramidal cells of the dorsolateral prefrontal cortex project to cortical association areas including the superior temporal gyrus (Pierri *et al.*, 2001; Woodruff *et al.*, 1997).

Lesions to the dorsolateral and orbital prefrontal cortices and anterior temporal cortex of primates result in an inability to comprehend the meaning of affiliative behaviours, ignorance to grooming, agnosia, social isolation and reduced social awareness (Deakin, 1994; Franzen & Myers, 1973; Raleigh & Steklis, 1981). Furthermore, lesions to the prefrontal cortex in humans are associated with abnormal social conduct, inadequate decision-making and planning, impoverished speech, avolition, apathy, anhedonia and perseverative behaviours (Andreasen *et al.*, 1986; Damasio *et al.*, 1990; Deakin, 1994; Weinberger *et al.*, 1986; Wolkin *et al.*, 1992). Similarities between these findings and the symptoms of schizophrenia imply that schizophrenia appears to involve a dysfunction in the neural circuitry underlying social cognition (Deakin, 1994). Impaired fronto-temporal connectivity may be required for manifestation of the symptoms of schizophrenia (Deakin *et al.*, 1997). In general, it appears that a disruption in connectivity between heteromodal cortical association areas such as the dorsolateral prefrontal cortex and superior temporal gyrus disturbs complex human behaviours and integration of functions (Sanfilipo *et al.*, 2000).

5.1.3 *In Situ* Hybridisation Histochemistry

The study of gene expression can facilitate understanding of how candidate genes may be involved in a disorder through their pattern of expression and alteration in the disease state (Harrison & Pearson, 1995). In this way, genetic abnormalities may be related to pathological phenotype (Harrison & Pearson, 1995), which is especially advantageous in the study of uniquely human disorders (Barton *et al.*, 1993) such as schizophrenia. *In situ* hybridisation histochemistry on post-mortem brain tissue can be used to determine the distribution and density of mRNAs in normal control subjects compared to pathological cases. The translation and distribution of the encoded polypeptides are presumably affected in parallel (Harrison & Pearson, 1995). The advantage of *in situ* hybridisation is that, not only does it provide an indication of the genetic alterations in pathological states, it furnishes information about subtle changes in receptor subunit gene expression, which cannot be obtained using other techniques such as quantitative receptor autoradiography. While the use of post-mortem tissue limits determination of the functional consequences of molecular changes (Dean, 2001), it is the only means currently available to obtain anatomical resolution of subtle alterations in schizophrenia (Lewis, 1995).

5.1.4 Aims

The specific aim of the present study was to determine whether expression of ionotropic GABA receptor subunits is changed in schizophrenia, by comparing subunit mRNA density in post-mortem schizophrenic brain to that in matched control tissue. Two regions of the heteromodal association cortex were investigated to ascertain whether abnormalities in GABA receptor expression contribute to aberrant neural processing in these regions in schizophrenia. To this end, the putative role of GABA receptors in the symptomatology of schizophrenia can be investigated, in view of the receptors as potential targets for therapeutic intervention.

$GABA_A$ and $GABA_C$ receptors are thought to be involved in cortical development (Behar *et al.*, 2000; Martina *et al.*, 1995; Strata & Cherubini, 1994). In light of the neurodevelopmental hypothesis of schizophrenia, which maintains that schizophrenia

arises from abnormal cortical development, the ionotropic GABA receptors are ideal candidates for study in schizophrenia.

Potential changes in $GABA_C$ receptor subunit mRNAs in schizophrenia were probed in the current study. The $GABA_C$ receptors are novel, exhibiting many similarities to the $GABA_A$ receptors, and little investigation has been carried out on the subunits in human brain, let alone in pathological states.

Potential changes in the $GABA_A$ receptor $\alpha 1$ and $\beta 3$ subunits were also investigated. The $\alpha 1$ subunit is most widely distributed in the CNS (Laurie *et al.*, 1992; McKernan & Whiting, 1996; Persohn *et al.*, 1992; Wisden *et al.*, 1992) and studies have reported changes in this subunit mRNA density and protein distribution in various pathological states, including schizophrenia (Akbarian *et al.*, 1995b; Hawkins & Dodd, 2000; Impagnatiello *et al.*, 1998; Ohnuma *et al.*, 1999; Pesold *et al.*, 1998). The $\beta 3$ subunit is less widely distributed or investigated, and warrants examination in schizophrenia, considering its role in normal neurological functioning based on subunit knockout studies (DeLorey *et al.*, 1998; Homanics *et al.*, 1997; Rudolph *et al.*, 2001; Ugarte *et al.*, 2000). The diversity in function and expression patterns of different $GABA_A$ receptors makes the subunits suitable for investigation in a genetically complex disease such as schizophrenia (Byerley *et al.*, 1995).

5.2 Materials and Methods

Materials and methods follow those of Chapter 2, *in situ* hybridisation histochemistry. Matched pair tissue measuring all subunits was processed simultaneously.

5.2.1 Subjects

Subjects with a history of schizophrenia were matched with controls with no history of neuropsychiatric disorder for gender, age, agonal state and post-mortem interval. Pairwise matching of schizophrenic and control subjects minimises the influence of such confounding variables on the data obtained (Lewis, 1995). Six matched pairs were used, with tissue from both brain regions coming from the same subject. All tissue was taken from the left hemisphere and the mean pH of the tissue was 6.18, indicating adequate

quality of mRNA (Harrison & Pearson, 1995; Kingsbury *et al.*, 1995). Post-mortem toxicology testing did not detect the presence of neuroleptics in three of the patients with schizophrenia. One of the six controls was a suicide control, matching criteria for control type 1, while the remaining controls matched criteria for control type 2 (outlined in Appendix 1). Tissue from the same subjects had been utilised in a previous study assessing NMDA receptors (Le Corre *et al.*, 2000). Table 5.1 provides a summary of the subjects' gender, mean age and mean post-mortem interval. Table iv, Appendix 6, provides extensive subject information, including cause of death, agonal state, diagnosis, tissue pH, drug use and medication history.

Table 5.1: Subject information. Data are expressed as mean±S.D.

Psychiatric diagnosis	Gender	Mean age	PMI
Schizophrenia	5 male, 1 female	46.7 ± 16.3 years	24.6 ± 13.7 hours
Control	5 male, 1 female	48.3 ± 13.8 years	20.3 ± 9.2 hours

5.2.2 Statistics

The Kolmogorov-Smirnov (KS) test of Normality was performed using GraphPad Prism 3.0 (GraphPad Inc., San Diego, CA, USA) to ensure data fit a Guassian distribution for subsequent statistical analyses.

Pearson product moment correlation analysis was performed using GraphPad Prism 3.0 between densities for each probe (in nCi/g tissue equivalent) and subject variables, including post-mortem interval, tissue pH, subject age at death and agonal state (designated numerically as 1=good, 2=average and 3=fair) in each brain region. Information on post-mortem interval, tissue pH, subject age and agonal state is provided in table iv, Appendix 6.

To determine whether significant changes in mRNA levels occurred between schizophrenic and matched control subjects, sense or competition labelling densities were first subtracted from antisense values to take background into account. Since the ratio of grey to white matter can vary between sections, where grey matter could be delineated, this was quantified separately, in addition to whole section measurements. Subsequent mRNA densities (nCi/g tissue equivalent) between control and case subjects for each probe were compared using two way repeated measures ANOVAs with

SuperANOVA version 1.11 (Abacus Concepts, Inc, Berkeley, CA, USA). Diagnosis (control or schizophrenia) was the between-subject factor and region was the within-subject factor. The dependent variable was density of mRNA (nCi/g). If either of the main effects (diagnosis or region) or the interaction between diagnosis and region were statistically significant ($p<0.05$), means comparison contrasts were performed and the significance of the contrasts analysed using the Scheffe F test with SuperANOVA version 1.11. Data are expressed as mean and standard error of the mean (mean±s.e.mean).

5.3 RESULTS

5.3.1 Correlation Analysis

Results from Pearson product moment correlations are presented in tables v and vi, Appendix 7. No statistically significant correlation was found between subject variables and probe densities in either the middle frontal cortex or superior temporal gyrus. This indicates that post-mortem interval, tissue pH, subject age at death and agonal state did not influence mRNA densities in the current study.

5.3.2 Repeated Measures ANOVA Results

$GABA_A$ and $GABA_C$ receptor subunit mRNAs could be detected above background in the middle frontal cortex and superior temporal gyrus of control subjects, described in Chapter 2, section 2.4.6. ANOVA summary tables, interaction plots and means comparison contrast results from the repeated measures ANOVAs performed are presented in Appendix 8. Repeated measures ANOVAs for all subunits did not render any statistically significant difference in mRNA densities for the main effect of diagnosis, thus there was no statistically significant difference between mRNA densities in control and schizophrenic tissue when holding brain region constant. Nonetheless, the main effect of diagnosis for the β3 subunit when grey matter was assessed was only marginally non-significant ($F=4.631$, $df=1$, $p=0.057$), as seen in table xv, Appendix 8.

No statistically significant difference in mRNA densities was observed for the main effect of region with $GABA_C$ receptor ρ1 and ρ2 subunits, nor for the interaction

between diagnosis and region (see tables vii and viii, Appendix 8). Moreover, the interaction between diagnosis and region was not statistically significant for the $GABA_A$ receptor $\alpha 1$ and $\beta 3$ subunit mRNAs when measured in whole section or grey matter, as seen in tables ix, xi, xiii, and xv, Appendix 8. A lack of interaction (crossover) between bars in the interaction plots graphically demonstrates this finding (see figures iii, iv, v and vi, Appendix 8).

A statistically significant difference in mRNA densities was observed with the $\alpha 1$ subunit for the main effect of region in both whole section and grey matter measurements ($F=15.248$ $df=1$, $p<0.01$ and $F=14.729$, $df=1$, $p<0.01$ respectively), as shown in tables ix and xi, Appendix 8. A statistically significant difference in mRNA densities was also observed with the $\beta 3$ subunit for the main effect of region in both whole section and grey matter measurements ($F=5.958$, $df=1$, $p=0.03$ and $F=22.541$, $df=1$, $p<0.01$ respectively), as seen in tables xiii and xv, Appendix 8. This suggests that there was a statistically significant difference in mRNA densities for the $\alpha 1$ and $\beta 3$ subunits between the middle frontal cortex and superior temporal gyrus. Since the main effect of diagnosis and the interaction between diagnosis and region were not statistically significant, however, this difference was not due to differences between control and schizophrenic mRNA densities in the regions investigated.

5.3.3 Middle Frontal Cortex

Despite the lack of statistically significant differences in mRNA densities between control and schizophrenic brain, obvious trends in the data deserve description. Figure 5.2 portrays mRNA density for each GABA receptor subunit measured in control and schizophrenic brain. As grey matter was not delineated in sections hybridised with $GABA_C$ receptor $\rho 1$ and $\rho 2$ subunit probes, whole section measurements only were taken. For tabular results giving specific density measurements, refer to table xvii, Appendix 9.

In summary, $GABA_C$ receptor $\rho 1$ subunit mRNA density was increased 75% on average in the middle frontal cortex of schizophrenic brain compared to matched controls (schizophrenics, 0.57 ± 0.33 nCi/g; controls, 0.14 ± 0.09 nCi/g). There did not appear to

be any difference (8% change) in ρ2 subunit mRNA density between the two groups (schizophrenics, 1.15±0.50 nCi/g; controls, 1.25±0.64 nCi/g). The $GABA_A$ receptor α1 subunit transcript exhibited greater density in the middle frontal cortex of schizophrenic tissue compared to controls, when measured as whole sections (32%; schizophrenics, 6.02±1.10 nCi/g; controls, 4.06±1.07 nCi/g). However, when grey matter alone was measured, this increase was only small (16%; schizophrenics, 7.91±1.55 nCi/g; controls, 6.67±1.61 nCi/g). On the other hand, β3 subunit mRNA was decreased on average in schizophrenic tissue compared to controls, when measured both as whole sections (33%; schizophrenics, 2.26±0.50 nCi/g; controls, 3.41±0.75 nCi/g), and grey matter only (31%; schizophrenics, 3.34±0.54 nCi/g; controls, 4.88±0.80 nCi/g). The data in figure 5.2 is represented in a different way in figure 5.3, showing the spread of mRNA densities for each subunit tested in middle frontal cortex of control versus schizophrenic subjects.

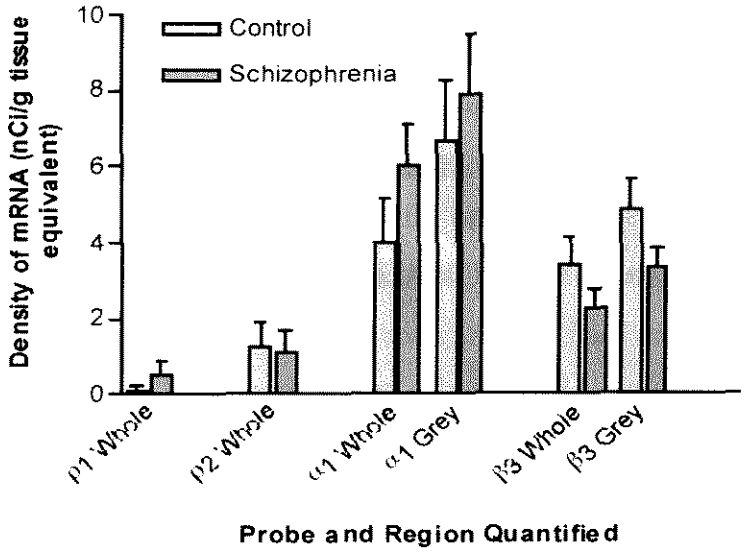
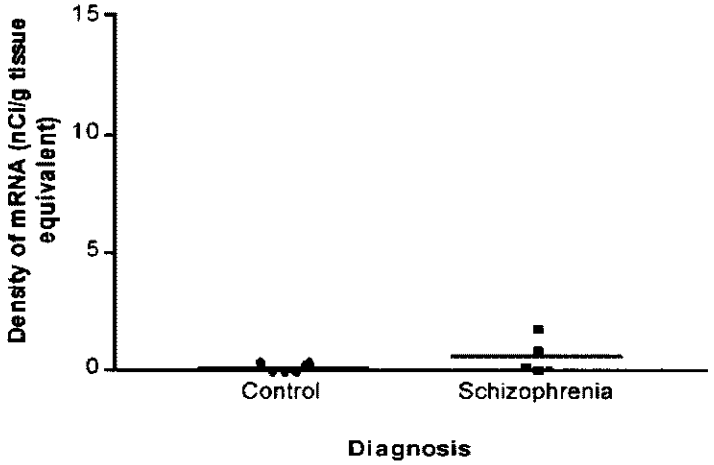


Figure 5.2: Bar graph representing density of mRNA for each probe (indicated) in the middle frontal cortex of control and schizophrenic brain. Data are expressed as mean±s.e.mean. Data were quantified using whole section optical density, and grey matter optical density for α1 and β3 subunits, indicated by Whole and Grey on the x axis.

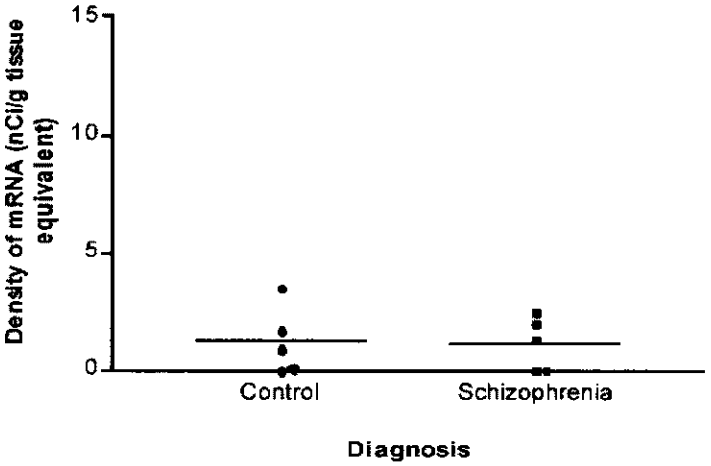
The trends described above, with the exception of the ρ1 subunit mRNA, are evident in autoradiograph images of middle frontal cortical sections from control and schizophrenic subjects that have been hybridised with the $GABA_A$ and $GABA_C$ receptor subunit probes, seen in figures 5.4 and 5.5 respectively. Figure 5.4 furnishes images of middle frontal cortical sections from matched pairs, demonstrating specific signal achieved by hybridisation with $GABA_A$ receptor antisense probes. Both autoradiographic macroscopic images and micrographs are provided. The difference in grey to white matter ratio between subjects is evident from the macroscopic images, emphasising the importance of quantifying grey matter density alone.

A comparison of figures 5.4A and 5.4B shows an increase in $GABA_A$ receptor $\alpha 1$ subunit mRNA density in the middle frontal cortex of the subject with schizophrenia compared to the matched control. This is supported by the microscopic images (5.4C and 5.4D) which clearly indicate elevated silver grain density in the grey matter of schizophrenic tissue compared to matched control tissue. Selective clustering of silver grains around cell soma reinforces the specific nature of the mRNA labelling. Figures 5.4E and 5.4F show the converse to be true for the $GABA_A$ receptor $\beta 3$ subunit mRNA, which is observed to be reduced in the subject with schizophrenia compared to the matched control. Again, microscopic examination showed a corresponding decrease in silver grain density around cell soma in schizophrenic tissue compared to control tissue, depicted in figures 5.4G and 5.4H.

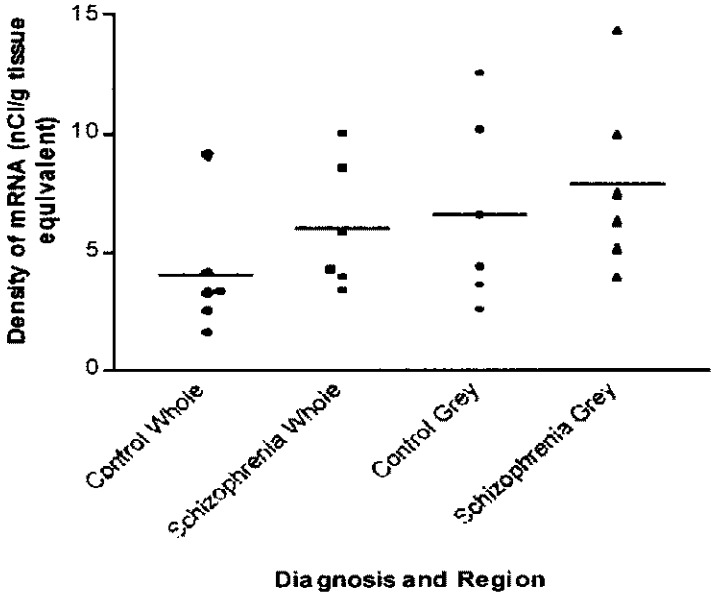
The lack of delineated grey matter in sections hybridised with the $GABA_C$ receptor subunit probes is apparent in macroscopic autoradiograph images provided in figure 5.5. As a consequence, qualitative and quantitative comparison of signal intensity between control and schizophrenic subjects is difficult. mRNA density was quantified from whole sections, therefore the increase in signal observed for the $\rho 1$ subunit probe and no change in signal for the $\rho 2$ subunit probe may be an artefact of measurement, rather than reflecting a true change, or lack thereof, in mRNA density in schizophrenic subjects compared to controls. The levels of $\rho 1$ subunit mRNA appear to be so low that one cannot be certain whether the reported increase reflects any major difference between control and case tissue. Microscopic examination of the sections showed a low level of silver grains in both schizophrenic and control tissue, seen, for example, in figures 5.5C and 5.5D. Since this density was not quantified, the microscopic images do not support or refute the quantitative finding of an increase in mRNA density for the $\rho 1$ subunit, or no change in density for the $\rho 2$ subunit in schizophrenic compared to matched control tissue.



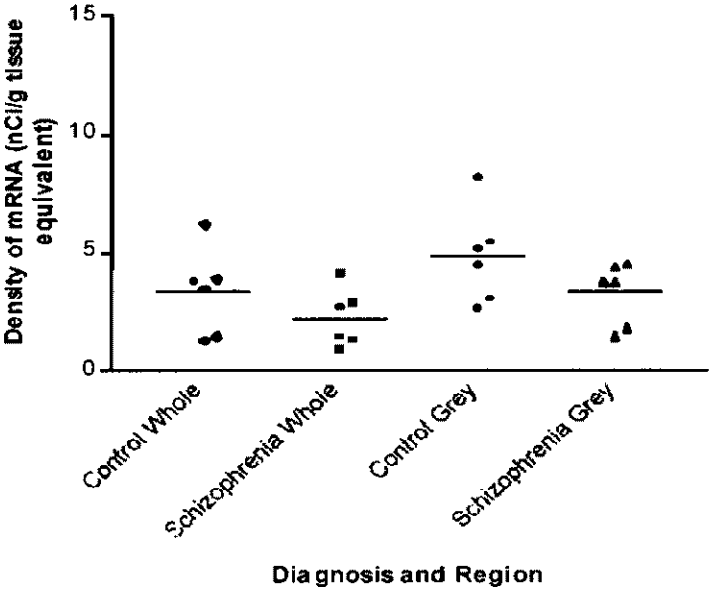
A. $GABA_C$ receptor ρ1 subunit mRNA



B. $GABA_C$ receptor ρ2 subunit mRNA



C. $GABA_A$ receptor α1 subunit mRNA



D. $GABA_A$ receptor β3 subunit mRNA

Figure 5.3: Column graphs representing the spread of mean mRNA densities in the middle frontal cortex of subjects with schizophrenia and matched controls. **A**. $GABA_C$ receptor ρ1 subunit mRNA densities (n=5). **B**. $GABA_C$ receptor ρ2 subunit mRNA densities (n=5). **C**. $GABA_A$ receptor α1 subunit mRNA densities (n=6). **D**. $GABA_A$ receptor β3 subunit mRNA densities (n=6). Data are expressed such that each point represents the mean mRNA density for a single subject. Mean density of mRNA for the entire subject group (eg: control or schizophrenia) is indicated by the horizontal bar. $GABA_A$ receptor α1 and β3 subunit mRNA densities were measured both from the whole section and from grey matter only, indicated by Whole or Grey on the x axes.

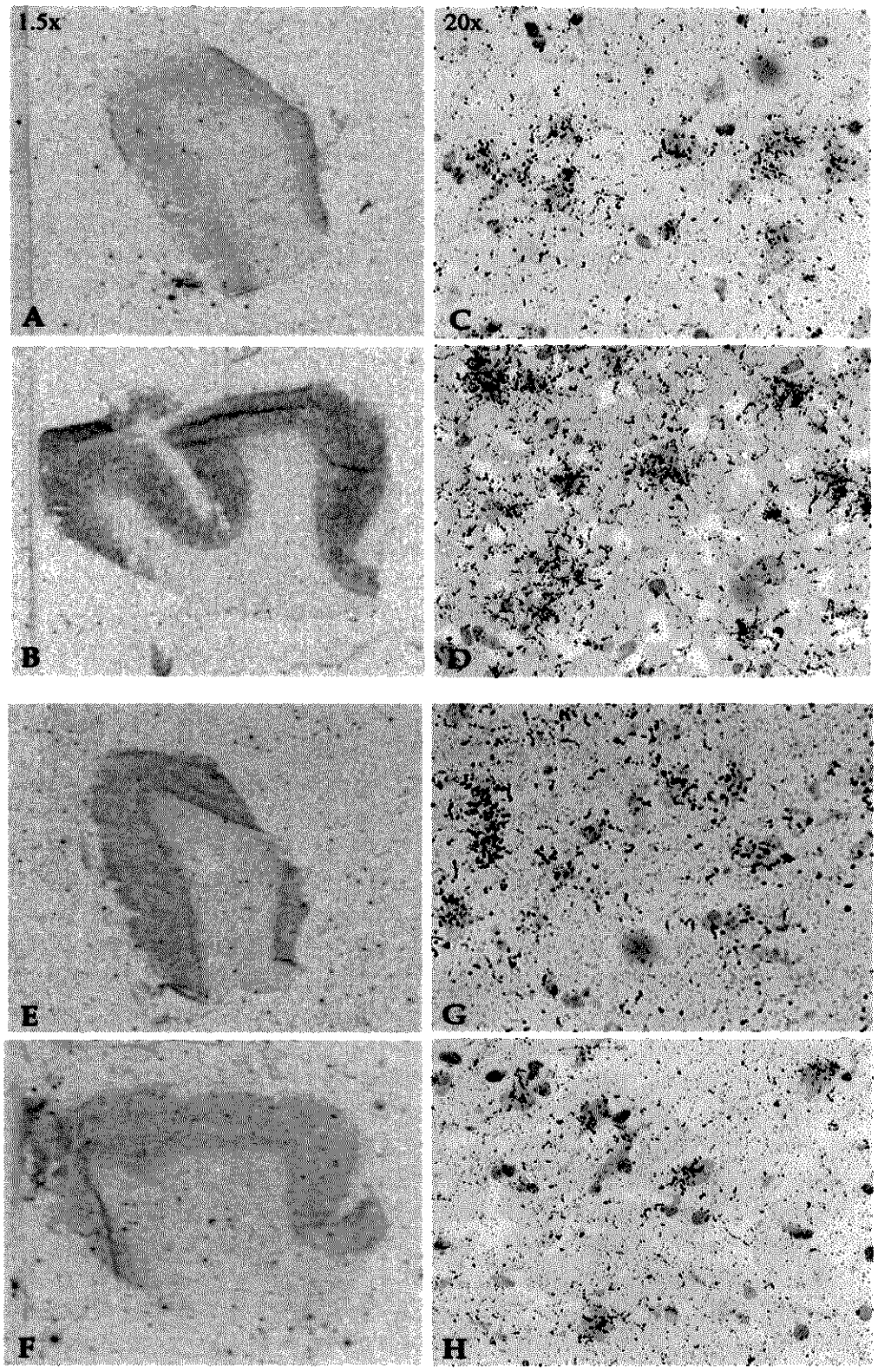


Figure 5.4: Autoradiogram and micrograph images of middle frontal cortical sections showing densities of $GABA_A$ receptor mRNAs in control and schizophrenic brain. **A.** & **C.** α1 subunit density and labelling in control tissue. **B.** & **D.** α1 subunit density and labelling in schizophrenic brain. **E.** & **G.** β3 subunit density and labelling in control tissue. **F.** & **H.** β3 subunit density and labelling in schizophrenic brain. Images shown for each subunit are from matched pairs. Images 1.5x macroscopic and 20x microscopic magnification

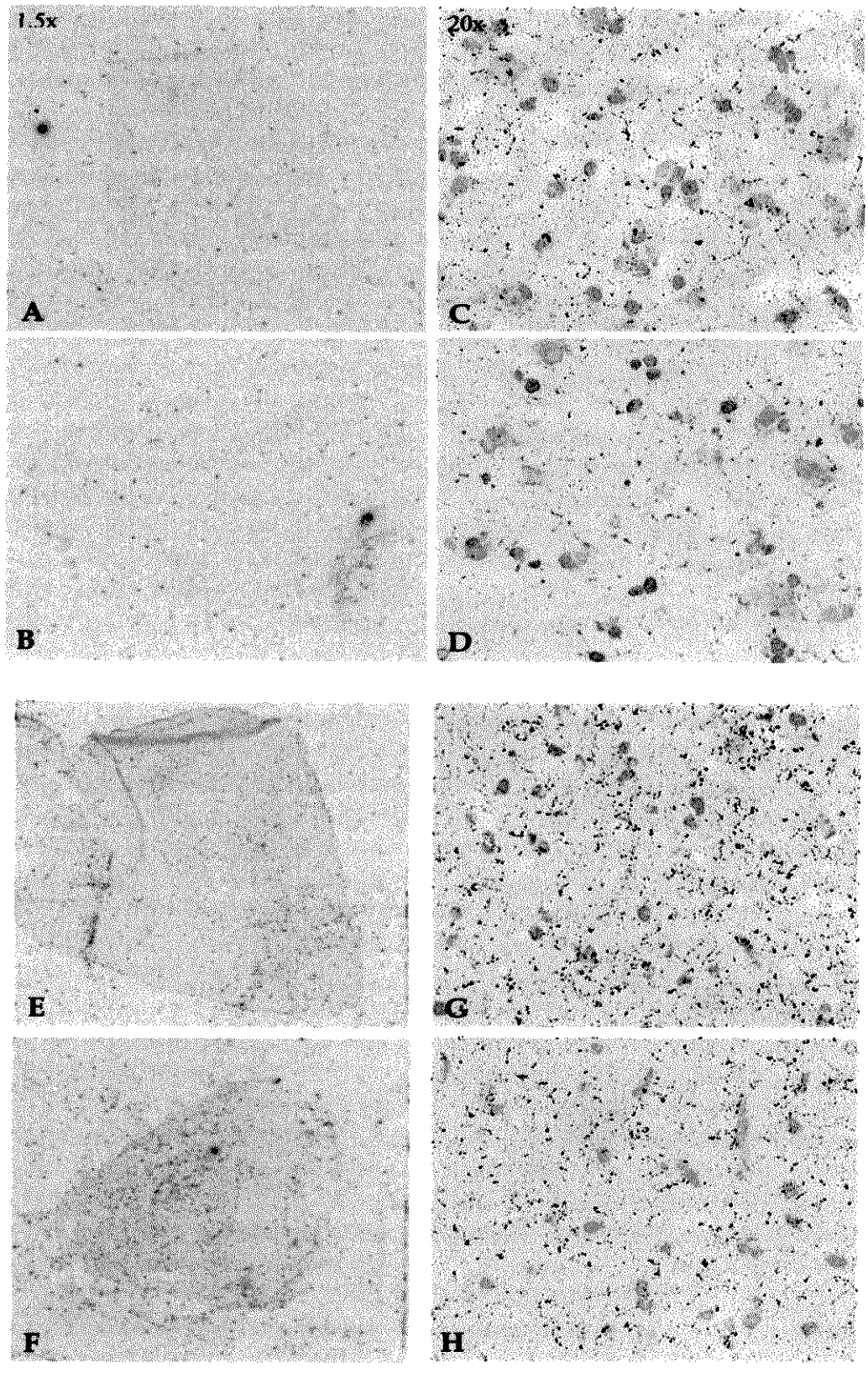


Figure 5.5: Autoradiogram and micrograph images of middle frontal cortical sections showing densities of $GABA_C$ receptor mRNAs in control and schizophrenic brain. **A.** & **C.** ρ1 subunit density and labelling in control tissue. **B.** & **D.** ρ1 subunit density and labelling in schizophrenic brain. **E.** & **G.** ρ2 subunit density and labelling in control tissue. **F.** & **H.** ρ2 subunit density and labelling in schizophrenic brain. Images shown for each subunit are from matched pairs. Images 1.5x macroscopic and 20x microscopic magnification

5.3.4 Superior Temporal Gyrus

Again, despite the failure to obtain statistically significant differences in mRNA densities between control and schizophrenic brain, obvious trends in the data deserve description. Figure 5.6 depicts mRNA density for each GABA receptor subunit measured in the superior temporal gyrus of control and schizophrenic brain. As grey matter was not delineated in sections hybridised with $GABA_C$ receptor ρ1 and ρ2 subunit probes, whole section measurements only were taken. For tabular results giving specific density measurements, refer to table xviii, Appendix 9.

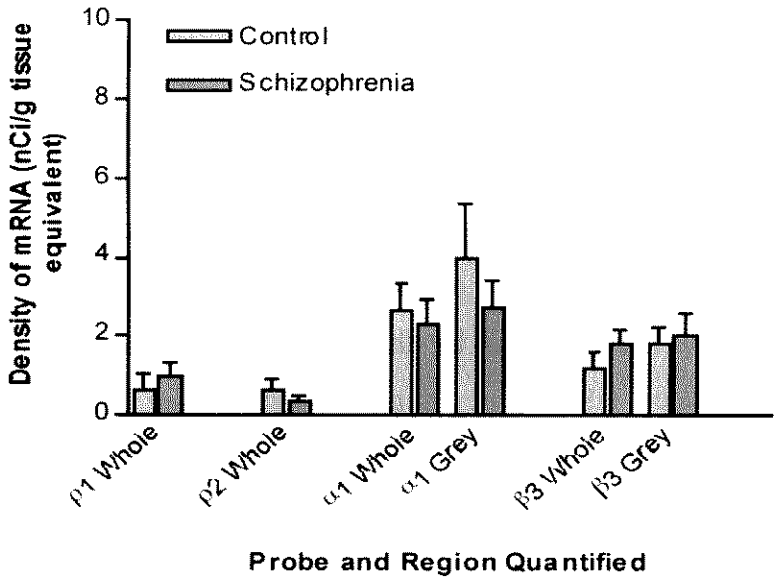


Figure 5.6: Bar graph representing density of mRNA for each probe (indicated) in the superior temporal gyrus of control and schizophrenic brain. Data are expressed as mean±s.e.mean. Data were quantified using whole section optical density, and grey matter optical density for α1 and β3 subunits, indicated by Whole and Grey on the x axis.

In summary, $GABA_C$ receptor ρ1 subunit mRNA density appeared to be increased (30%) on average in the STG of schizophrenic brain compared to matched controls (schizophrenics, 0.98±0.36 nCi/g; controls, 0.68±0.40 nCi/g). ρ2 Subunit mRNA density appeared to be reduced by 42% on average in the patient population compared to matched controls (schizophrenics, 0.39±0.13 nCi/g; controls, 0.68±0.23 nCi/g). The $GABA_A$ receptor α1 subunit transcript exhibited a trivial decrease in density in the STG of schizophrenic subjects compared to controls when measured as whole sections (14%; schizophrenics, 2.30±0.60 nCi/g; controls, 2.68±0.65 nCi/g). However, when taking only grey matter into account, this decrease was moderate (31%; schizophrenics, 2.75±0.67 nCi/g; controls, 4.00±1.35 nCi/g). The β3 subunit transcript was apparently increased on average in the schizophrenic cases compared to controls, when measured as whole sections (32%; schizophrenics, 1.82±0.38 nCi/g; controls, 1.23±0.37 nCi/g),

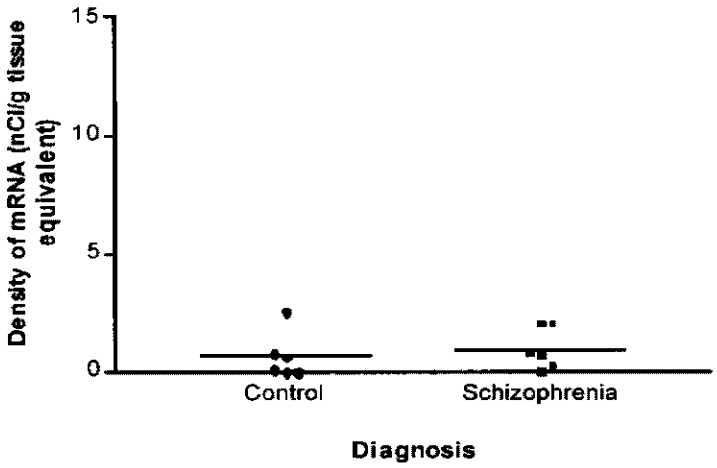
although there appeared to be little difference when measured in grey matter alone (13%; schizophrenics, 2.07±0.50 nCi/g; controls, 1.81±0.44 nCi/g). The data in figure 5.6 are represented in a different way in figure 5.7, indicating the spread of mRNA densities for each subunit tested in superior temporal gyrus of control versus schizophrenic subjects.

The trends described above are evident in autoradiograph images of superior temporal gyrus sections from control and schizophrenic subjects that have been hybridised with the $GABA_A$ and $GABA_C$ receptor subunit probes, seen in figures 5.8 and 5.9 respectively. Figures 5.8 and 5.9 provide images of superior temporal gyrus sections from matched pairs, demonstrating specific signal achieved by hybridisation with antisense probes. Both autoradiographic macroscopic images and micrographs are provided.

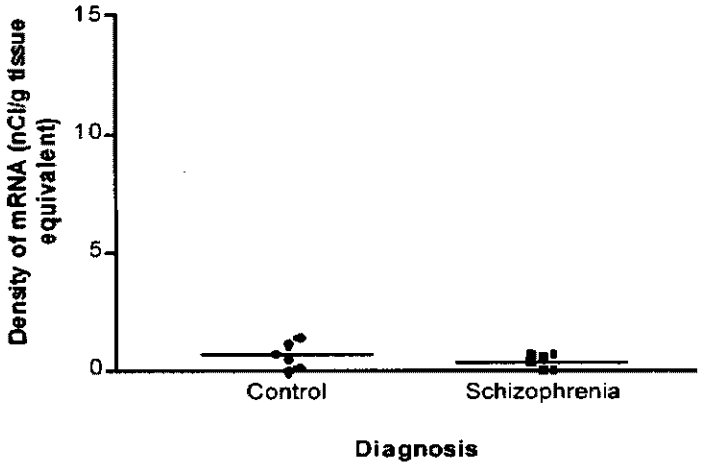
A comparison of figures 5.8A and 5.8B shows a decrease in $GABA_A$ receptor $\alpha 1$ subunit mRNA density in the superior temporal gyrus of the subject with schizophrenia compared to the matched control. This is supported by the microscopic images (5.8C and 5.8D) which clearly indicate reduced silver grain density in the grey matter of schizophrenic tissue compared to matched control tissue. Figures 5.8E and 5.8F show the opposite to be true for the $GABA_A$ receptor $\beta 3$ subunit mRNA, which is observed to be increased in the subject with schizophrenia compared to the matched control. Again, microscopic examination showed a corresponding elevation in silver grain density around cell soma in schizophrenic tissue compared to control tissue, depicted in figures 5.8G and 5.8H.

As for the middle frontal cortex sections, the lack of delineated grey matter in sections hybridised with the $GABA_C$ receptor subunit probes is apparent in macroscopic autoradiograph images provided in figure 5.9, making qualitative and quantitative comparison of signal intensity between control and schizophrenic subjects difficult. The levels of $\rho 1$ and $\rho 2$ subunit mRNA appear to be so low that one cannot be certain whether the reported changes in expression reflect any major difference between control and case tissue. Microscopic examination of the sections showed a low level of silver grains in both schizophrenic and control tissue, seen, for example, in figures 5.9G and 5.9H. Since this density was not quantified, the microscopic images do not support or

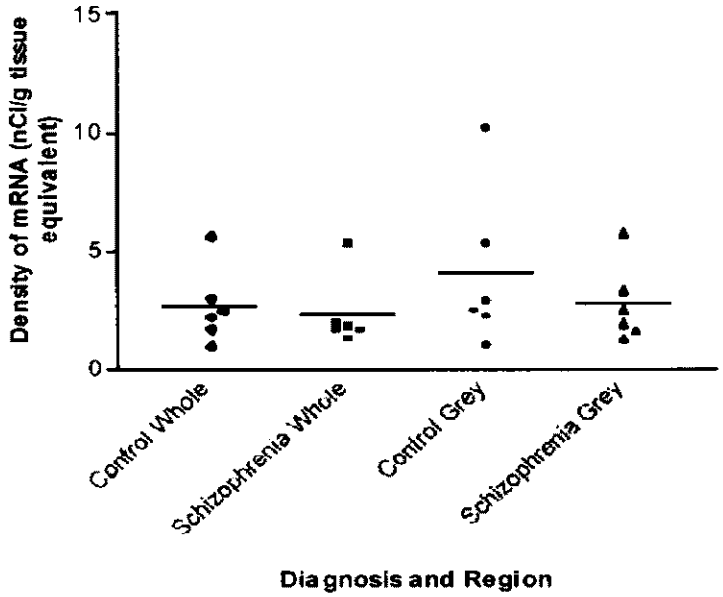
refute the quantitative finding of an increase in mRNA density for the ρ1 subunit, or decrease in density for the ρ2 subunit in schizophrenic compared to matched control tissue.



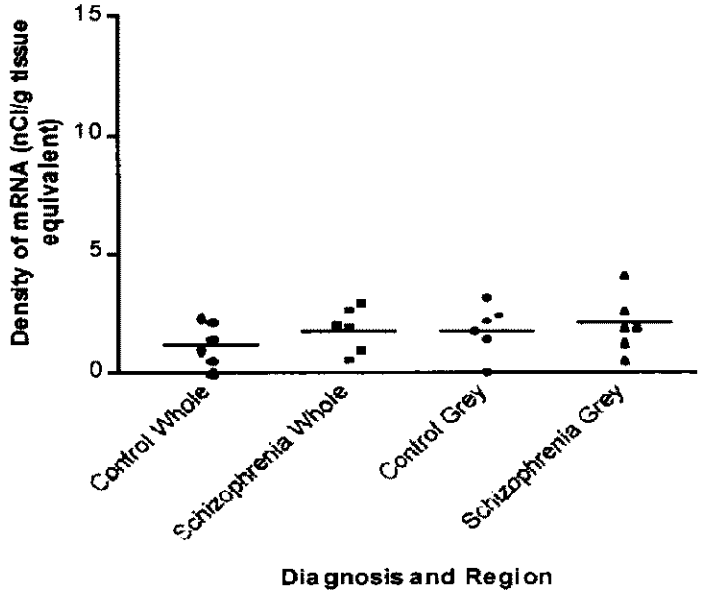
A. $GABA_C$ receptor ρ1 subunit mRNA



B. $GABA_C$ receptor ρ2 subunit mRNA



C. $GABA_A$ receptor α1 subunit mRNA



D. $GABA_A$ receptor β3 subunit mRNA

Figure 5.7: Column graphs representing the spread of mean mRNA densities in the superior temporal gyrus of subjects with schizophrenia and matched controls. **A**. $GABA_C$ receptor ρ1 subunit mRNA densities (n=6). **B**. $GABA_C$ receptor ρ2 subunit mRNA densities (n=6). **C**. $GABA_A$ receptor α1 subunit mRNA densities (n=6). **D**. $GABA_A$ receptor β3 subunit mRNA densities (n=6). Data are expressed such that each point represents the mean mRNA density for a single subject. Mean density of mRNA for the entire subject group (eg: control or schizophrenia) is indicated by the horizontal bar. $GABA_A$ receptor α1 and β3 subunit mRNA densities were measured both from the whole section and from grey matter only, indicated by Whole or Grey on the x axes.

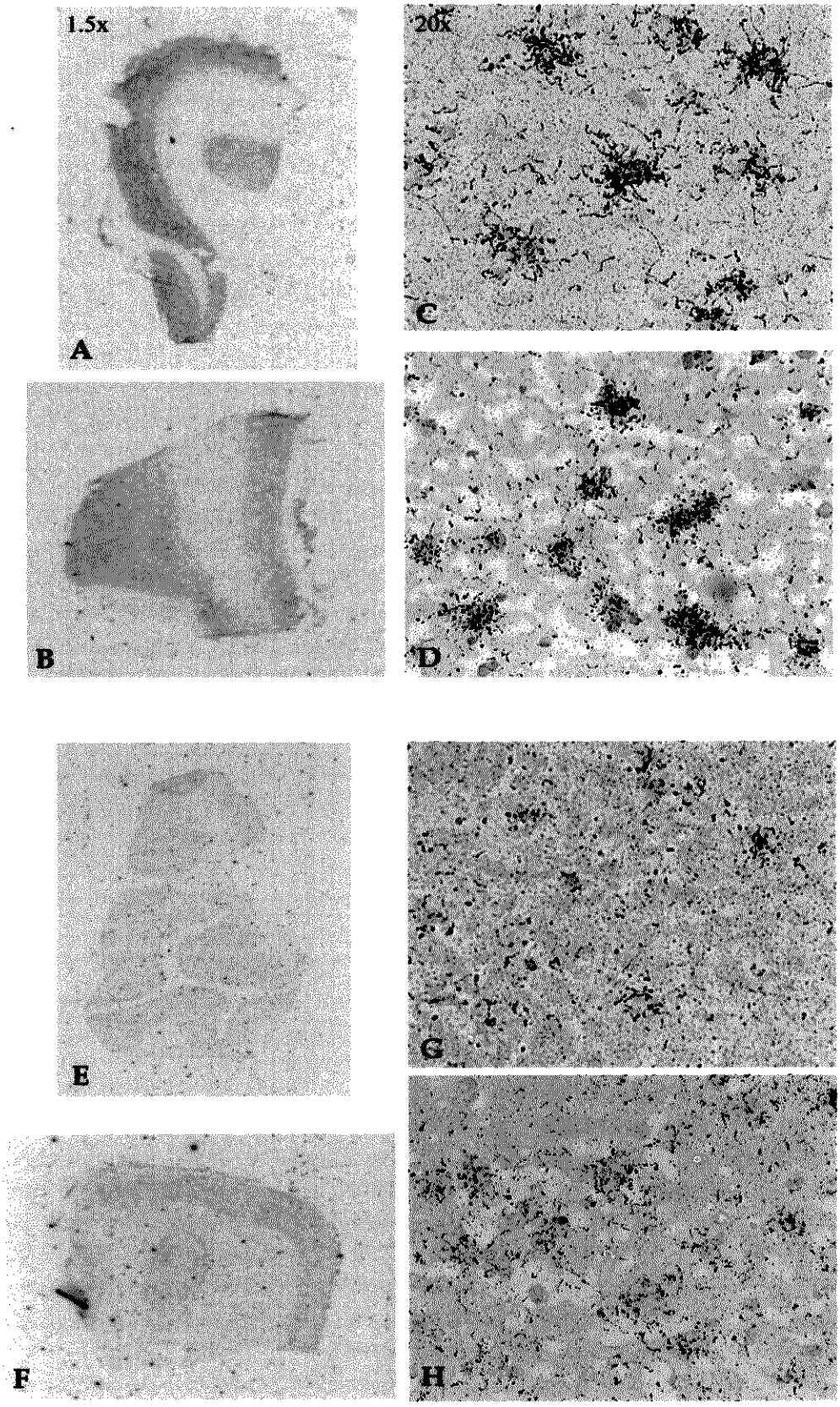


Figure 5.8: Autoradiogram and micrograph images of superior temporal gyrus sections showing densities of $GABA_A$ receptor mRNAs in control and schizophrenic brain. **A.** & **C.** α1 subunit density and labelling in control tissue. **B.** & **D.** α1 subunit density and labelling in schizophrenic brain. **E.** & **G.** β3 subunit density and labelling in control tissue. **F.** & **H.** β3 subunit density and labelling in schizophrenic brain. Images shown for each subunit are from matched pairs. Images 1.5x macroscopic and 20x microscopic magnification

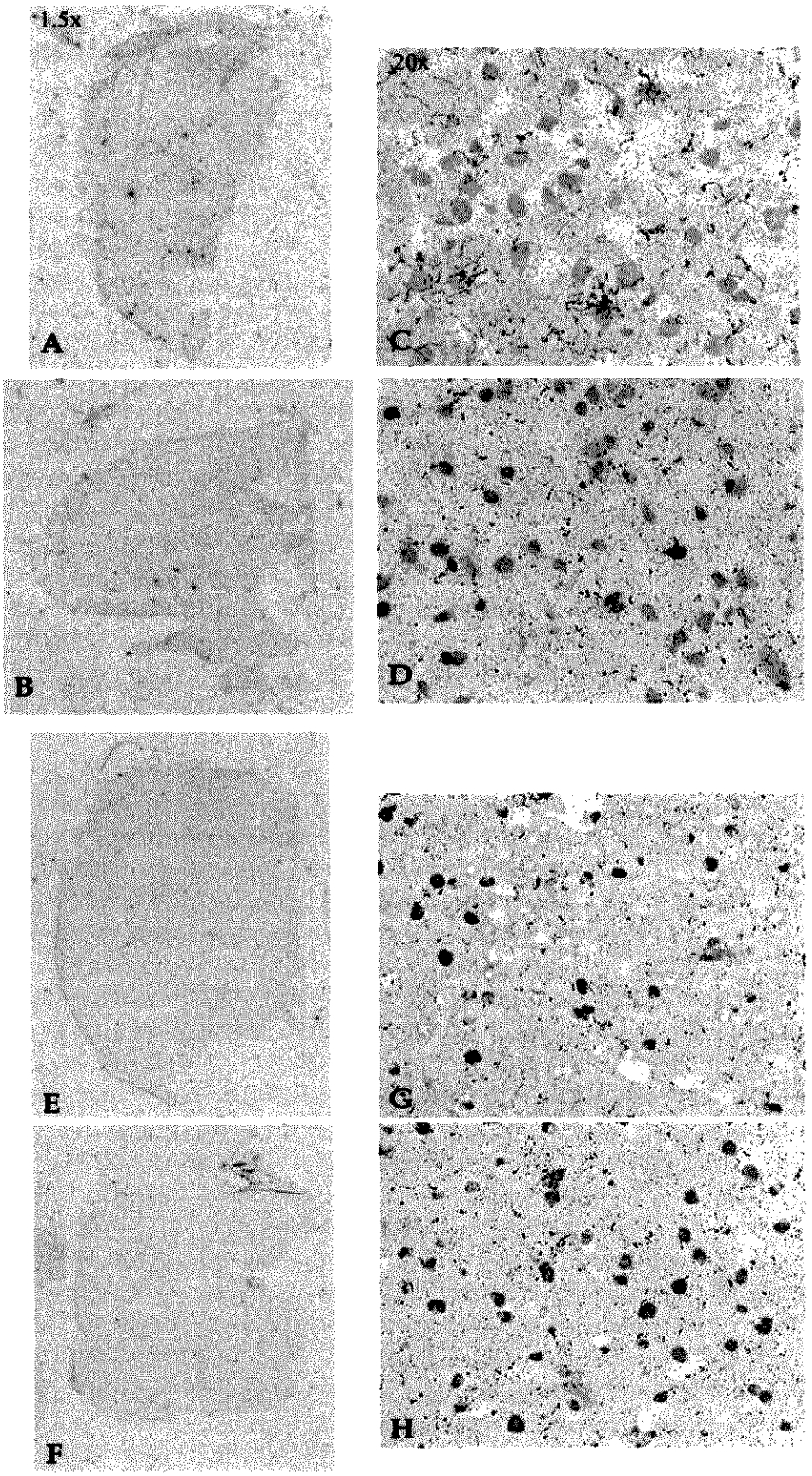


Figure 5.9: Autoradiogram and micrograph images of superior temporal gyrus sections showing densities of $GABA_C$ receptor mRNAs in control and schizophrenic brain. **A.** & **C.** ρ1 subunit density and labelling in control tissue. **B.** & **D.** ρ1 subunit density and labelling in schizophrenic brain. **E.** & **G.** ρ2 subunit density and labelling in control tissue. **F.** & **H.** ρ2 subunit density and labelling in schizophrenic brain. Images shown for each subunit are from matched pairs. Images 1.5x macroscopic and 20x microscopic magnification

5.4 DISCUSSION

Results from the present *in situ* hybridisation study demonstrate changes in $GABA_A$ and $GABA_C$ receptor subunit mRNA expression in post-mortem brain of individuals who suffered schizophrenia. The observed changes were not statistically significant, however the apparent trends deserve consideration in light of previous research and possible implications in the symptomatology of schizophrenia.

A trend for $GABA_A$ receptor $\alpha 1$ subunit mRNA existed, with an apparent average increase in mRNA density in the middle frontal cortex and mean reduction in mRNA density in the superior temporal gyrus in schizophrenia compared to control tissue.

The observed increase in $GABA_A$ receptor $\alpha 1$ subunit mRNA in the schizophrenic middle frontal cortex is in keeping with the literature. Ohnuma *et al.* (1999) found a selective and discrete increase in $\alpha 1$ subunit mRNA density measured by *in situ* hybridisation in the dorsolateral prefrontal cortex (Brodmann's area 9) in schizophrenic brain compared to controls. Impagnatiello *et al.* (1998) also showed an increase in $\alpha 1$ subunit mRNA expression in prefrontal cortex of post-mortem schizophrenic brain. Further, Pesold and colleagues (1998) demonstrated increased immunolabelling of α subunit protein in the prefrontal cortex of schizophrenic subjects. Using RT-PCR, they further revealed that $\alpha 1$ subunit mRNA was increased in this region in schizophrenia. The findings of a non-significant decrease in $\alpha 1$ subunit mRNA in the middle frontal cortex by Akbarian and colleagues (1995b), however, do not support these results.

The observed trend in $\alpha 1$ subunit mRNA expression is also compatible with prior ligand binding studies which showed increased [^{3}H]muscimol binding in the prefrontal cortex of schizophrenic brain compared to controls (Benes *et al.*, 1996b; Hanada *et al.*, 1987). Fénelon and Herbison (1996) demonstrated that the expression of $\alpha 1$ subunit mRNA in rat brain is sensitive to endogenous GABA levels, which suggests that the changes in $\alpha 1$ subunit mRNA in schizophrenia might result from changes in GABA concentration in the synapse (Ohnuma *et al.*, 1999). In their experiments, Fénelon and Herbison (1996) used γ-vinyl GABA to inhibit GABA catabolism, increasing GABA concentrations in the brain. This lead to reduced expression of the $\alpha 1$ subunit transcript in the globus

pallidus. In the case of schizophrenia, the opposite may occur, where decreased GABA concentration in the synapse results in increased α1 subunit transcription. The increase in [^{3}H]muscimol binding and increased α1 subunit mRNA expression in the PFC is thought to be a compensatory mechanism for functional reduction in GABA (Akbarian *et al.*, 1995b; Ohnuma *et al.*, 1999). Such findings are consistent with decreased GABAergic transmission in the PFC in schizophrenia. GABA is known to regulate GABA subunit transcription (Fénelon and Herbison, 1996). If GABA synthesis or release is compromised, subunit transcription may be altered to compensate.

The α1 subunit has not previously been studied in the superior temporal gyrus, therefore the trend for a decrease in subunit expression reported here may be the first of its kind. Using the same tissue as that employed in the present study, Le Corre and colleagues (2000) demonstrated an increase in NMDA receptor mRNA density in the superior temporal gyrus, specifically in the NR1a splice variant. This is evidence of an imbalance between GABAergic and glutamatergic transmission in the schizophrenic brain, contributed by reciprocal changes in receptor subunit expression.

$GABA_A$ receptor subunit transcript expression is known to be regulated by NMDA receptor activation (Memo *et al.*, 1991). Memo and coworkers (1991) found that in rat cerebellar granule cell cultures, antagonism of NMDA receptors decreased expression of $GABA_A$ receptor α1, β1, β2 and γ2 subunit mRNAs, while NMDA receptor stimulation increased α1, α4, β1, β2 and γ2 subunit expression. NMDA receptor activation was not found to affect β3 subunit mRNA expression. In addition, an animal model of schizophrenia using PCP to induce a psychotic-like state in rats, demonstrated a down-regulation of $GABA_A$ receptor α1 subunit mRNA after acute PCP administration (Abe *et al.*, 2000). The mRNAs were measured in 17 brain regions, and significant decreases in expression were found in the cingulate and temporal cortices and the hippocampus for the α1 subunit. A corresponding decrease in [^{35}S]TBPS binding was observed in the cerebral cortices and cerebellum, suggesting reduced $GABA_A$ receptor density. NMDA receptor antagonism provides a model of NMDA receptor hypofunction. Thus, NMDA receptor hypofunction appears to result in reduced GABA receptor expression in selected brain regions. The literature described above is compatible with the results of Le Corre *et al.* (2000) in the STG. One might expect an

upregulation of NMDA receptor subunits in this region to compensate for receptor hypofunction, with a consequent decrease in GABA receptor subunit expression.

In the present study, there was a non-significant trend towards a reduction in $GABA_A$ receptor β3 subunit mRNA expression in the middle frontal cortex of schizophrenic subjects relative to controls. This contrasts to the non-significant trend towards an increase in α1 subunit mRNA expression in the same region. The apparent reduction in β3 subunit transcripts in schizophrenic middle frontal cortex does not concur with prior studies, which show increased immunolabelling of β2/3 subunit peptide labelling (Pesold *et al.*, 1998) and increased [^{3}H]muscimol binding (Benes *et al.*, 1992b; Benes *et al.*, 1996b; Dean *et al.*, 1998; Dean *et al.*, 1999; Hanada *et al.*, 1987) in the prefrontal cortex of schizophrenic subjects. However, in an animal model of schizophrenia using phencyclidine (PCP) to induce a psychotic-like state in rats, down-regulation of $GABA_A$ receptor β2 and β3 subunit mRNA expression was observed after chronic PCP administration (Abe *et al.*, 2000). Decreases in expression of β3 subunit mRNA were encountered in the caudate-putamen, cerebellum, cingulate, parietal and temporal cortices, although not the frontal cortex. The apparent reduction in β3 subunit mRNA expression in the middle frontal cortex in the current investigation is also consistent with putative NMDA receptor hypoactivity in schizophrenia.

In contrast to the reduction seen in the middle frontal cortex, the $GABA_A$ receptor β3 subunit mRNA was non-significantly increased on average in the superior temporal cortex of schizophrenic subjects compared to controls. As with the α1 subunit, to our knowledge this is the first report of such an observation, albeit a trend in data. Increased β3 subunit mRNA expression in the STG is not consistent with findings of increased NR1a subunit expression in the same tissue, assuming NMDA receptor subunits are increased to compensate for receptor hypoactivity in the schizophrenic superior temporal gyrus. However, GABA receptor subunit mRNA expression may be differentially modulated by NMDA receptor activity. For example, NMDA receptor activation was not found to affect β3 subunit mRNA expression in rat cerebellar granule cells (Memo *et al.*, 1991). Moreover, NMDA receptor subunit and splice variant mRNA expression also appears to vary in the schizophrenic STG. Grimwood and associates (1999) showed that the NR2B-containing NMDA receptors are selectively

up-regulated in the superior temporal cortex (Brodmann's area 22) of schizophrenic brain, while the NR2A-containing receptors are not. Since NMDA receptors are comprised of at least one NR1 subunit combined with at least one NR2 subunit (Grimwood *et al.*, 1999), the NR1a splice variant may combine with NR2B receptors, thus be upregulated in parallel. Humphries and associates (1996) demonstrated reduced NR1 subunit mRNA expression in tissue homogenates from superior temporal gyrus of schizophrenic brain. The differences in these findings may reflect the samples chosen, since NR1 subunit mRNA loss was found exclusively in subjects with cognitive impairment in the study of Humphries *et al.* (1996). Moreover, Le Corre and colleagues (2000) studied splice variants of the NR1 subunit, which may be differentially expressed in schizophrenia, a factor which the study of NR1 subunits in total does not address.

It is evident that the complex neural modifications in schizophrenia do not mirror a simple rat model of reduced GABA receptor mRNA expression following NMDA receptor antagonism. Partial deletion of the gene encoding the NMDA NR1 isoform lead to behavioural abnormalities in mice that represented symptoms of schizophrenia, including antisocial behaviour (Mohn *et al.*, 1999). Such a model provides potent evidence for reduced NMDA receptor activity in the pathophysiology of schizophrenia. This animal model of NMDA receptor knockdown would be of great use in the investigation of the putative NMDA/GABA receptor imbalance in schizophrenia, for example, by studying the NMDA NR1 splice variants and $GABA_A$ receptor subunit mRNAs.

The parallel differences in the changes observed between $\alpha 1$ and $\beta 3$ subunit mRNA expression in the middle frontal cortex and superior temporal gyrus in the current study are surprising, and may be explained by the fact that the subunits generally form distinct receptors. As mentioned previously, $\alpha 1$ subunits precipitate and colocalise most commonly with $\beta 2$ and $\gamma 2$ subunits in the mammalian brain (McKernan & Whiting, 1996; Persohn *et al.*, 1992; Sieghart, 2000; Wisden *et al.*, 1992). These subunits form a high affinity benzodiazepine-sensitive receptor complex (Pritchett *et al.*, 1989). $\beta 3$ subunits, on the other hand, appear to precipitate and colocalise predominantly with $\alpha 2$ and $\gamma 2$ subunits (McKernan & Whiting, 1996; Persohn *et al.*, 1992; Sieghart, 2000;

Wisden *et al.*, 1992). Certainly, the α1 and β3 subunit transcripts have been shown to colocalise (Fénelon & Herbison, 1996; Persohn *et al.*, 1992; Wisden *et al.*, 1992), however differential localisation of the subunits means they occur in different receptor compositions in different regions of the brain.

Fénelon & Herbison (1996) demonstrated regional and subunit-specific modulation by endogenous GABA of $GABA_A$ receptor subunit mRNA expression in rat brain. For example, while α1 subunit transcripts were reduced in the globus pallidus following elevated GABA levels, the γ2 and β2 subunit mRNAs were increased. Perhaps a decrease in one subunit is compensated for by an increase in others in the schizophrenic brain, with an alteration in the functional characteristics of the $GABA_A$ receptors, hence aberrant information processing. These speculations highlight the need to investigate the specific cellular localisation of the subunit mRNAs and polypeptides to see if they colocalise or are expressed in different parts of the neurons or different neural subpopulations. For instance, a specific loss of cells expressing $GABA_A$ receptors containing the β3 subunit may be occurring in the middle frontal cortex which accounts for the decrease in β3 subunit mRNA, while cells expressing α1 subunit-containing receptors are spared.

While single subunit changes have been observed, we do not know whether the expression of receptors containing these subunits is correspondingly altered. The *in vivo* functional significance of potential single subunit changes within the $GABA_A$ receptor complex is not known (Fénelon & Herbison, 1996). Subunit composition alters sensitivity to GABA and allosteric modulators (refer to chapter 1). Therefore changes in subunit expression in schizophrenia might be expected to alter GABAergic transmission. Changes in subunit density may reflect important changes in receptor composition or number. Furthermore, alterations in GABA receptor subunit transcript expression may reflect an imbalance between GABAergic and glutamatergic neurotransmission and subsequent integrative cortical functions, which may thus present as symptoms of schizophrenia. Abnormal GABAergic/glutamatergic neurotransmission in the middle frontal cortex may contribute to abnormalities in the many functions subserved by the dorsolateral prefrontal cortex, including inappropriate and abnormal social behaviours, poor memory, avolition and apathy witnessed in

schizophrenia. Altered GABAergic/glutamatergic neurotransmission in the superior temporal gyrus in schizophrenia may lead to abnormalities in language and auditory processing which have been linked to this region of the brain.

The pattern in schizophrenic tissue of increased α1 mRNA expression in the middle frontal cortex and concurrent decreased density in the superior temporal gyrus, and the converse with β3 subunit mRNA, may mirror changes in connectivity between the prefrontal cortex and superior temporal gyrus in schizophrenia. As mentioned in the introduction, the prefrontal cortex and superior temporal gyrus are intricately and reciprocally connected. Altered connectivity between the prefrontal and superior temporal cortex in schizophrenic brain may be due to structural changes in white matter, or neurochemical changes in the GABAergic system alone, or, as mentioned in preceding paragraphs, the intricate interplay between GABAergic and glutamatergic innervation. The differential effects this may have on the $GABA_A$ receptor subunits, however, is uncertain. Increasing the sample size to ascertain whether the observed pattern prevails with statistical significance is required, however, before further speculation can be made.

Given that the $GABA_C$ receptors are relatively novel and their role in the CNS has not yet been defined, little speculation can be made about their potential role in the pathophysiology of schizophrenia, based on the current findings. $GABA_C$ receptor ρ1 subunit mRNA was found to be somewhat increased in the middle frontal cortex, with an apparent small increase in the superior temporal gyrus of schizophrenic brains compared to control tissue. The ρ2 subunit, on the other hand, displayed a trend for reduced expression in the superior temporal gyrus in schizophrenic subjects relative to controls. However, as seen in chapter 2, delineated grey matter was rarely seen in sections hybridised with the ρ subunits in macroscopic autoradiograph images. As a consequence, qualitative comparison of signal intensity between control and schizophrenic subjects is difficult. mRNA density was quantified from whole sections, therefore the alterations in signal intensities may well be an artefact of measurement, rather than reflecting a true change, or lack thereof, in mRNA density in schizophrenic subjects. Microscopic examination of middle frontal and superior temporal cortex sections, as seen in the micrographs in figures 5.5 and 5.9, further demonstrates a low level of silver grains and a lack of specific labelling of neurons in both schizophrenic and

control tissue hybridised with the $GABA_C$ receptor probes. Since density of silver grains was not quantified, the microscopic images do not support or refute the findings of an increase in mRNA density for the ρ1 subunit in the middle frontal cortex, and decrease in ρ1 and increase in ρ2 subunit mRNA expression in the superior temporal gyrus of schizophrenic compared to matched control brains.

While altered gene expression suggests functional properties of the receptors may be altered, we cannot determine from the current study whether the observed changes in mRNA density were due to altered expression of mRNA or to changes in cell numbers. This is also important in light of post-mortem and structural imaging studies showing reduced volume of the dorsolateral prefrontal and superior temporal cortices in schizophrenic subjects. Quantification of cell numbers as well as identification of cell types expressing the genes must therefore be performed to clarify this crucial point.

While it is tempting to comment on the trends observed in the current study, these comments remain speculative in the absence of statistical significance. Lack of statistical significance may be due to a number of factors, the most obvious of which are the restricted number of sections obtained for each subject and small number of subjects. The effect size in this investigation was obviously too small to be determined as significant with the sample size used.

The large variability in mRNA expression and integrity between human subjects is also an important factor, as it may mask minor changes in mRNA in disease states (Harrison & Pearson, 1995). Minor quantitative differences between one situation and another are difficult to reveal in human tissue, unless large sample sizes are considered and cases are selected according to parameters of overall RNA content or integrity (Barton *et al.*, 1993). Furthermore, while case-control studies should take into account confounding variables, prevalent post-mortem cases were used in the current investigation, therefore drug use and lifestyle factors which increase inter-subject variability were not extensively controlled for.

The lack of statistical significance may be explained by the fact that the reported average findings in the present study were not apparent in all matched pairs. Since

schizophrenia represents a group of disorders, which may encompass several causative biochemical mechanisms, such heterogeneity in results may be expected. Small increases and decreases in GABA receptor subunit mRNAs therefore may not represent a global pathology, but may only be apparent in a subgroup of patients displaying particular symptomatology, or corresponding to a specific type of schizophrenia. Increasing the sample size is one measure to investigate whether this is the case, and factoring in the prevalent symptomatology for each patient such that schizophrenia types are accounted for.

In the present examination, whole section and entire grey matter measurements were used to assess changes in mRNA expression in schizophrenia. Such an assessment does not account for changes in specific laminae. Thus, quantitation at the laminar level, that is, for each cortical layer, may yield statistically significant differences in mRNA density in schizophrenic subjects compared to controls that may have been diluted in the assessment of total grey matter.

Yield and integrity of RNA may be compromised in some neuropathological states (Higgins & Mah, 1989), therefore it may be difficult to draw conclusions on the basis of a single experimental technique of *in situ* hybridisation, without confirmation using other techniques. However, it is known that mRNA degradation occurs "essentially at random" in normal and pathological brain tissue (Higgins & Mah, 1989). Thus, potential changes in mRNA density are anticipated to arise from neuropathology involved in the disease process and symptomatology, and not through mRNA degradation. Ultimately, immunohistochemical studies on the same regions from the same subjects need to be undertaken to assess whether changes in mRNA expression are paralleled by changes in polypeptide expression. Alterations in mRNA expression are expected to result in alterations in polypeptide expression. This is not always the case, however. For example, in a study employing both RT-PCR to measure mRNA expression and immunohistochemistry to measure polypeptide expression of $GABA_A$ receptor subunits in alcoholic brain, Dodd & Lewohl (1998) found little correlation between mRNA and polypeptide expression. Such a discrepancy may be accounted for by post-transcriptional or post-translational factors (Dodd & Lewohl, 1998). Polypeptide expression was not measured in the present study, therefore one cannot

state whether the observed changes in mRNA expression were mirrored by changes in protein expression. It is thus essential to confirm *in situ* expression studies using another technique such as RT-PCR, Northern blotting or microarrays, and to apply immunohistochemistry to the same regions from the same subjects to assess changes in protein expression.

When studying changes in human disease states, therein lies the difficulty in acquiring tissue from early in the disease process, which precludes the option of studying gene expression corresponding only to primary disease-related changes (Higgins & Mah, 1989). A major difficulty is acquiring tissue from subjects who have not been exposed to drug intervention. Therefore, as with all post-mortem studies utilising tissue from schizophrenic patients, we cannot disregard the influence of neuroleptics. As reviewed in chapter 4, neuroleptics generally have an inhibitory effect on GABAergic transmission, inhibiting binding to GABA receptors, and prolonged neuroleptic treatment in some cases downregulates GABA receptor expression. Thus, neuroleptics may influence GABA activity and alter GABA receptor expression. Experiments with animals to test the effects of chronic neuroleptic administration on GABA receptor subunit mRNA expression using *in situ* hybridisation are therefore necessary to ascertain whether altered subunit mRNA density is due to neuroleptic treatment or the primary disease process.

5.4.1 Conclusions

The main findings of this study were a trend towards increased $GABA_A$ receptor $\alpha 1$ subunit mRNA expression in the middle frontal cortex and decreased expression in the superior temporal gyrus, while the $\beta 3$ subunit displayed a trend towards decreased density in the middle frontal cortex and increased expression in the superior temporal gyrus of schizophrenic brain compared to matched control tissue. It is difficult to explain the disparate findings with the two $GABA_A$ receptor subunits investigated in the present study, and to reconcile these findings with the literature. The findings do, however, point to regional variations in subunit expression in schizophrenic subjects compared to controls, and underscore the complexity of neurochemical changes in schizophrenia.

Only trends were observed in the present study. This study therefore requires replication to increase sample size and obtain statistical significance. Another cohort of subjects with psychosis, such as bipolar disorder, also need to be investigated to ascertain whether the observed changes in GABA receptors are specific to schizophrenia.

The regulatory effects of NMDA receptor activity on GABA receptor transcript expression (Memo *et al.*, 1991) suggest that a reciprocal change in NMDA receptor and GABA receptor expression occurs in schizophrenia. Changes in NMDA receptors, postulated in Olney and Farber's (1995) hypothesis of NMDA receptor hypoactivity in schizophrenia, may therefore have enduring consequences on GABA receptor expression. It would be of great interest to investigate further the effects of NMDA receptor hypoactivity on GABA receptor mRNA expression using a mouse model of NR1 subunit knockdown.

The current data are preliminary, and further investigation is required before substantial conclusions can be drawn. Nonetheless, the observed trends in $GABA_A$ receptor mRNA expression patterns discovered in the current study point to a role for the $GABA_A$ receptors in the symptoms of schizophrenia, particularly those stemming from dysfunctions in the prefrontal cortex and superior temporal gyrus, and the connections between these two brain regions.

IONOTROPIC GABA RECEPTORS IN SCHIZOPHRENIA
CHAPTER 6: EFFECTS OF NEUROLEPTIC DRUGS ON IONOTROPIC GABA RECEPTORS EXPRESSED IN *XENOPUS* OOCYTES

6.1 INTRODUCTION AND AIMS

6.1.1 Introduction to the Method

Xenopus laevis oocytes are large, single cells in meiotic prophase, therefore actively translating RNA and synthesising proteins (Colman, 1984; Gurdon & Wickens, 1983). This makes them ideal for electrophysiological experiments as their large size permits easy injection of RNA and expression of exogenous receptors.

Injection of exogenous receptor mRNA transcribed from cloned cDNA into the cytoplasm of Stage V-VI *Xenopus* oocytes leads to expression of functional proteins in the oocyte membrane (Goldin, 1993; Kushner *et al.*, 1989; Sherman-Gold, 1993; Sumikawa *et al.*, 1989). Miledi and colleagues (1982) first expressed functional ion channels in *Xenopus* oocytes after injecting RNA isolated from rat brain. Many researchers have subsequently demonstrated expression of various types of ion channels and receptors in *Xenopus* oocytes following microinjection of poly(A)$^+$ RNA or cRNA transcribed from cloned cDNA which had been isolated from appropriate tissue (Polenzani *et al.*, 1991).

Xenopus oocytes are ideal expression systems as only mature and properly processed transcripts tend to be translated and expressed (Gurdon & Wickens, 1983) and translation occurs very efficiently (Colman, 1984). Furthermore, *Xenopus* oocytes carry out post-translational modifications of polypeptides and can direct proteins to the membrane for insertion in the correct orientation (Barnard *et al.*, 1984; Barnard *et al.*, 1987; Colman, 1984; Houamed *et al.*, 1984; Gurdon & Wickens, 1983; Kushner *et al.*, 1989). Hence, proteins synthesised from non-amphibian transcripts are suitable for assessment in this expression system (Gurdon & Wickens, 1983), acquiring activity similar to that seen in native preparations (Colman, 1984). This is advantageous for examination of ligand-gated ion channel properties in isolation from other *in vivo*

responses (Barnard *et al.*, 1987; Houamed *et al.*, 1984; Goldin, 1993; Sumikawa *et al.*, 1989).

The functional properties of ligand-gated ion channels expressed in *Xenopus* oocytes can be assessed by two-electrode voltage clamp electrophysiology techniques (Goldin, 1993; Sumikawa *et al.*, 1989). In two-electrode voltage clamp experiments, two electrodes are inserted into the cell and membrane voltage is controlled, or clamped, with one electrode while the transmembrane current required to maintain the voltage is measured with the other electrode (Fraser *et al.*, 1993; Sherman-Gold, 1993; Sumikawa *et al.*, 1989).

Oocyte preparation requires the removal of follicle cells surrounding the oocyte with collagenase (Colman, 1984; Fraser *et al.*, 1993; Goldin, 1993; Kushner *et al.*, 1989; Sumikawa *et al.*, 1989). Since follicle cells are electrically coupled to the oocyte and possess receptors and ion channels which confer responses in the oocyte (Fraser *et al.*, 1993; Kushner *et al.*, 1989; Miledi & Woodward, 1989; Sumikawa *et al.*, 1989), removal of these cells excludes some endogenous responses (Goldin, 1993; Miledi & Woodward, 1988). This is beneficial for the study of ion channels where the presence of other channels and receptors may confound responses under study. It is necessary to exclude the effects of novel pharmacological agents on endogenous channels and receptors intrinsic to the oocyte by testing the agents on oocytes that have not been injected, or those injected with water (Kushner *et al.*, 1989).

For recording from ligand-gated ion channels, membrane voltage is generally clamped at -60mV (Fraser *et al.*, 1983; Kushner *et al.*, 1989). Since the reversal potential for chloride ions is approximately -20mV in *Xenopus* oocytes (Miledi & Woodward, 1988), at a membrane potential of -60mV, the amplitude of the inward whole-cell current produced by outward flow of Cl^- through the $GABA_A$ receptor channels is used to measure activation of the receptor. This inward current is used as a direct measure of drug effects at receptors.

Xenopus oocytes thus provide a robust and reliable vector for expression of functional proteins in isolation which can be manipulated pharmacologically using two-electrode

voltage clamp electrophysiogy techniques. Expression of receptors in oocytes is useful for determination of the pharmacological status of novel compounds. Furthermore, altering subunit combinations for injection may facilitate evaluation of drug effects on receptors comprised of differing subunit combinations (Kushner *et al.*, 1989).

6.1.2 Ionotropic GABA Receptors Expressed in *Xenopus* oocytes

Xenopus oocytes are particularly suitable for the study of functional properties of ligand gated ion channels (Fraser *et al.*, 1993; Goldin, 1993). Injection of total or poly(A) mRNA isolated from rat or chick brain into *Xenopus* oocytes produced receptors that were sensitive to GABA and conducted chloride ions (Barnard *et al.*, 1984; Houamed *et al.*, 1984; Smart *et al.*, 1983). Ionotropic GABA receptors assembled correctly in the oocyte membrane and displayed pharmacological properties similar to those observed with native receptors (Barnard *et al.*, 1984; Barnard *et al.*, 1987; Houamed *et al.*, 1984; Smart *et al.*, 1983).

Cloning of ionotropic GABA receptor subunit cDNAs from rat, bovine, human and chick retina and CNS, and their subsequent expression in *Xenopus* oocytes, has allowed researchers to determine subunit combinations for the formation of functional receptor subtypes, and to characterise the pharmacological and physiological properties of the different subtypes. Importantly, this technology permits the investigation of pharmacological sensitivities of ionotropic GABA receptors. In the current research, the effects of various compounds on $GABA_A$ receptors expressed in *Xenopus* oocytes were evaluated.

6.1.3 Neuroleptics

Neuroleptics are used in the treatment of schizophrenia and were so called for their ability to induce catalepsy in rats (Meltzer, 1995). Neuroleptics are classified as either typical or atypical. Typical neuroleptic drugs fall into a number of chemical classes, including the phenothiazines (such as chlorpromazine and fluphenazine) and butyrophenones (including haloperidol and droperidol) (Rang *et al.*, 1999). Figure 6.1 depicts the chemical structures of chlorpromazine (A) and haloperidol (C). Typical

neuroleptics are rather non-selective, demonstrating activity at a range of receptor types including histamine, acetylcholine and adrenergic receptors (Rang *et al.*, 1999; Stahl & Wets, 1988). The therapeutic efficacy of neuroleptics, however, is believed to be mediated by their selective antagonism of dopamine D_2 receptors (Creese *et al.*, 1976). Nonetheless, such broad activity of the typical neuroleptics leads to a multiplicity of side effects, notably movement disorders, referred to as extrapyramidal side effects (Stahl & Wets, 1988). While the use of typical neuroleptics has been eclipsed by the advent of atypical neuroleptics, they are still prescribed in contemporary psychiatry.

The advent of an entirely new drug, clozapine, which belongs to the dibenzodiazepine class of compounds, prompted the division of neuroleptics into typical and atypical. This distinction was made on the basis of the "atypical" action of clozapine, the chemical structure of which is depicted in figure 6.1. Other atypical neuroleptics include the benzamide and benzixazole classes of compounds (Rang *et al.*, 1999; Stahl & Wets, 1988). By definition, atypical neuroleptics do not produce the extrapyramidal side effects commonly encountered with the use of typical neuroleptics and appear to be more selective for dopamine receptors in the mesolimbic pathway (Meltzer, 1995; Meltzer, 1999; Stahl & Wets, 1988). Another defining feature of the atypical neuroleptics is their high affinity for some classes of 5-HT receptors (Meltzer, 1999). Atypical neuroleptics are more effective in patients who fail to respond to typical neuroleptics and in reducing the negative symptoms of schizophrenia (Dean, 2001; Holmes, 1997; Meltzer, 1999; Sawa & Snyder, 2002).

Previous research on the effects of neuroleptics on GABAergic transmission, and in particular, $GABA_A$ receptors, has been detailed in section 4.7.4.14, chapter 4). In summary, chlorpromazine, the prototypical phenothiazine neuroleptic, has been widely investigated using a number of assays, with varied effects on GABAergic transmission. Haloperidol has also been extensively investigated in various assays of GABAergic transmission, owing to its structural similarity to GABA. Again, the effects of haloperidol have been inconsistent. Of the atypical neuroleptic compounds, clozapine and olanzapine are the most widely investigated. Clozapine generally inhibits the effects of GABA, displaces binding to $GABA_A$ receptors, and decreases expression of $GABA_A$ receptors after chronic administration. Together, the data suggest that

neuroleptics may influence GABA activity by inhibiting $GABA_A$ receptors directly or indirectly. Such activity implies that neuroleptics may effectively compensate for alterations in GABA transmission in schizophrenia.

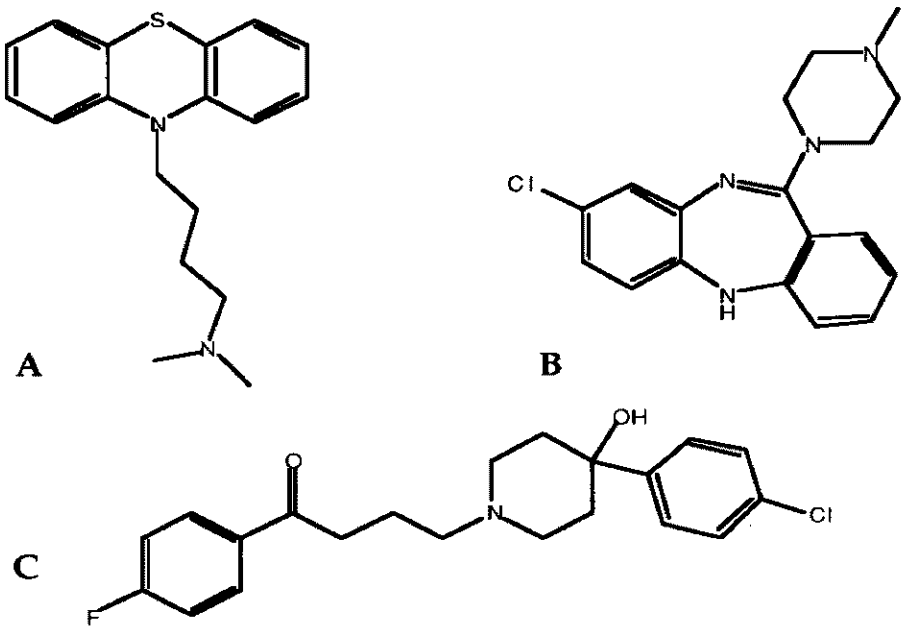


Figure 6.1: Chemical structures of (A) chlorpromazine, (B) clozapine and (C) haloperidol.

6.1.5 Aims

It is apparent that previous studies examining the effects of neuroleptics on $GABA_A$ receptors have focussed predominantly on *in vivo* and *ex vivo* techniques. Few studies to date have used mammalian cell lines and *Xenopus* oocytes to assess neuroleptic action *in vitro*. Expression of neural receptor subtypes in *Xenopus* oocytes and measurement of drug activity at the receptors using electrophysiological techniques presents a reliable method of evaluating drug action on neural receptors. *Xenopus* oocytes permit analysis of drug effects directly on receptors, in isolation from other transmitter systems. The aims of this study were, therefore, to determine whether neuroleptics produce direct effects at the ionotropic $GABA_A$ receptors using recombinant cRNA in the *Xenopus* oocyte cell expression system.

6.2 MATERIALS

6.2.1 *Xenopus* Oocytes

Xenopus laevis frogs were acquired from The African *Xenopus* Facility, Republic of South Africa, and housed in the Department of Veterinary Science at the University of Sydney. Oocytes were obtained from the *Xenopus laevis* frogs in the manner described in section 6.3.3.

6.2.2 GABA Receptor Subunit cDNA

Human α1, β2 and $\gamma 2_{Long}$ cDNA encapsulated in the pcDM8 vector (Stratogene, Lajolla, CA, USA) were a gift from Dr Paul Whiting (Department of Biochemistry and Molecular Biology, Neuroscience Research Centre, Merck, Sharp and Dohme Research Laboratories, Harlow, Essex, UK).

6.2.3 Drugs

γ-Amino-n-butyric acid (γ-aminobutyric acid, or GABA), 8-Chloro-11-[4-methyl-1-piperazinyl]-5H-dibenzo[b,e] [1,4]-diazepine (clozapine) and α-(Hydroxymethyl)benzeneacetic acid 8-methyl-8-azabicyclo(3.2.1)oct-3-yl ester tropine tropate (atropine sulfate) were obtained from Sigma-Aldrich (St Louis, MO, USA). 4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone (haloperidol hydrochloride) and ([R-(R*S*)]-5-(6,8-dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro -6,6-dimethyl-1,3-dioxolo[4,5-g]isoquindinium chloride ((-)-bicuculline methochloride) were purchased from Tocris Cookson (Avonmouth, Bristol, UK). 2-Chloro-10-[3-dimethylaminopropyl]phenothiazine (chlorpromazine HCl) was supplied by ICN Biochemicals (Costa Mesa, CA, USA).

6.2.4 Reagents and Chemicals

Restriction enzyme NOT1 was purchased from Promega Corporation (Madison, WI, USA). Collagenase A was obtained from Boehringer Mannheim (Mannheim, Germany).

Diethyl pyrocarbonate (DEPC), dimethyl sulfoxide (DMSO), absolute ethanol (molecular biology grade), phenol:chloroform:isoamyl alcohol (25:24:1), HEPES, LB broth tablets, gentamycin, theophylline, agarose, pyruvic acid (sodium salt) and 3-aminobenzoic acid ethyl ester were purchased from Sigma-Aldrich (St Louis, MO, USA).

Potassium chloride was purchased from Fisons Scientific Equipment (Loughborough, England). Magnesium chloride and calcium chloride solution were supplied by BDH Laboratory Supplies (Poole, England). Chloroform (analytical reagent grade) and sodium acetate were obtained from APS Finechem (Seven Hills, NSW, Australia).

6.3 METHODS

6.3.1 Linearisation of cDNA

E. Coli containing $\alpha 1$, $\beta 2$ and $\gamma 2_L$ cDNA encapsulated in the relevant plasmid vector were cultured in autoclaved LB Broth and released from the plasmid using the Wizard®Plus Minipreps kit (Promega Corporation, Madison, WI, USA). cDNA was linearised with restriction endonuclease NOT1 for 2 hours at 37°C. The linearised cDNA was then purified through phenol/chloroform extraction, followed by a chloroform wash, and precipitated with ethanol and 10% sodium acetate v/v (pH 5.2). Following this, the precipitated cDNA was washed with 70% ethanol and resuspended in DEPC-treated water.

6.3.2 Synthesis of GABA Receptor mRNA from Cloned cDNA

mRNA was transcribed from the linearised cDNA using T7 RNA Polymerase and capped with 5'7-methylguanosine using the mMESSAGE mMACHINE kit (Ambion Inc., Austin, TX, USA). Resultant mRNA was purified through phenol/chloroform extraction, followed by chloroform wash, then precipitated with ethanol and 33% ammonium acetate v/v (pH 5.2). mRNA was then washed with 70% ethanol and resuspended in RNase-free water. mRNA concentration was estimated by running samples of mRNA in 0.9% agarose gel containing ethidium bromide for 30 minutes to 1

hour. Samples were then screened on a UV spectrometer for the level of illumination produced by ethidium bromide as confirmation of a reasonable mRNA yield.

6.3.3 Isolation of Oocytes from *Xenopus leavis*

The following procedures were performed by Dr Hue Tran, Kong Li and Dr Erica Campbell of the Department of Pharmacology, University of Sydney. Female *Xenopus laevis* were anaesthetised with 0.17% 3-aminobenzoic acid ethyl ester and a lobe of the ovaries removed. Lobes were thoroughly rinsed with Ca^{2+}-free OR-2 buffer (82.5mM NaCl, 2mM KCl, 1mM $MgCl_2.6H_2O$, 5mM HEPES, pH 7.5) and treated with Collagenase A (2mg/mL in OR-2) for 2 hours to separate oocytes from connective tissue and follicular cells. Released oocytes were then rinsed in ND96 storage solution (96mM NaCl, 2mM KCl, 1mM $MgCl_2.6H_2O$, 1.8mM $CaCl_2$, 5mM HEPES, pH 7.5, supplemented with 2.5mM pyruvate, 0.5mM theophylline and 50μM/mL gentamycin). Stage V to VI oocytes were collected and stored at 16°C in ND96 storage solution with constant mixing on an orbital shaker.

6.3.4 Expression of GABA Receptor mRNA in *Xenopus* Oocytes

Micropipettes for injecting mRNA were fabricated on a flaming/brown micropipette puller (Sutter Instruments Inc., USA) and the tips were broken to the correct diameter of 15-20μm using a micromanipulator (W.R. Prior & Co., LTD, Hertfordshire, England). mRNA was diluted with DEPC treated water and drawn up into the micropipette under negative pressure. A total of 50ng/50nL of $\alpha 1$, $\beta 2$ and $\gamma 2_L$ mRNA in the ratio 1:1:1 was injected into the cytoplasm of defolliculated Stage V *Xenopus* oocytes using a 'Nanoject' (Drummond Scientific Co., Broomali, PA, USA). After injection, oocytes were stored at 16°C in ND96 storage solution on an orbital shaker. Oocytes were washed with ND96 storage solution up to 5 days subsequent to injection and dead or matured cells were removed.

6.3.5 Recording from Expressed GABA Receptors in *Xenopus* Oocytes

Two to six days after injection with mRNA, receptor activity was measured by two-electrode voltage clamp recording using a Geneclamp 500 amplifier (Axon Instruments

Inc., Foster City, CA, USA) and a MacLab 2e recorder (ADInstruments, Castle Hill, NSW, Australia). Oocytes were placed in a cell bath and voltage clamped using two micropipettes containing 3M KCl. Micropipettes were made using a micropipette puller (Narishige Scientific Instrument Lab, Tokyo, Japan). The membrane potential was clamped at -60mV. The cell bath was continually superfused with ND96 recording solution (96mM NaCl, 2mM KCl, 1mM $MgCl_2.6H_2O$, 1.8mM $CaCl_2$, 5mM HEPES, pH 7.5) through gravity feed from a reservoir. Solution was drawn through tubing from the bath by a vacuum pump connected to a waste flask. Concentrations of compounds being tested on the functional receptors formed from mRNA in the *Xenopus* oocytes were superfused into the cell bath containing the clamped oocyte. Oocytes were first screened for functional receptor formation by the addition of 100μM GABA to the cell bath. Amplitude of current (in nA) produced by activation of the chloride channels of the receptors was recorded using the MacLab 2e recorder onto files of Chart version 3.6.3 software (ADInstruments, Castle Hill, NSW, Australia) on a Macintosh computer for off-line analysis.

Drugs were initially weighed out and dissolved in distilled water or DMSO to make stock concentrations of 100mM. From stock solutions the drugs were diluted using ND96 recording solution to the required concentration for superfusion into the cell bath. GABA was further diluted to a 1mM stock solution and diluted with ND96 recording solution to the appropriate concentration for application to the cell bath. Concentrations of drugs for dose-response recording were made up freshly each day of experimentation. For all solutions at high concentrations, the pH was determined using universal litmus paper. All solutions had a pH of 7.5 to 7.55 (the pH of ND96 recording solution).

Agonists were applied in increasing concentrations to the cell bath until the maximal current was attained, to generate a current dose-response relationship. Antagonists were applied in the presence of agonist to the cell bath until the maximal current was reached to generate a current dose-response relationship with increasing concentrations of antagonist. Drugs were washed out of the cell bath by superfusion of ND96 recording solution until the baseline (holding current) was recovered and remained stable. For each drug dose-response, a minimum of 3 cells was recorded from to ensure an accurate and

replicable dose-response relationship was produced. Where DMSO was used to make up solutions, it was tested alone and in the presence of drug. The final concentration of DMSO applied was 0.8% v/v.

6.3.6 Analysis of Electrophysiological Data

Current dose-response relationships for agonists and antagonists were measured by recording the peak amplitude of current obtained for each concentration of drug. Any recorded effect of the DMSO component alone on cell current was subtracted from current elicited by drugs in the presence of DMSO. Dose-response data were standardised by calculating the ratio, I/I_{max}, where I is the peak amplitude of current at a given concentration of drug, and I_{max} is the maximal current generated by GABA at individual oocytes. Data are expressed as the mean current (nA) or ratio of the maximal GABA response (I/I_{max}) ± standard error of the mean (s.e.mean).

6.3.6.1 EC_{50} values

EC_{50} values were calculated from dose-response data by fitting ratios of maximal GABA current as a function of agonist concentration by least squares method to the sigmoid dose-response equation in GraphPad Prism 3.0 (GraphPad Inc., San Diego, CA, USA) on a Macintosh computer. Data was fit to the sigmoid dose-response and sigmoid dose-response with variable slope equations, and the best fit was determined by GraphPad Prism 3.0. The sigmoid dose-response curve equation is:

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogEC_{50}-X))})$$

The sigmoid dose-response equation with variable slope takes into account a Hill coefficient that differs from unity, and is given:

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogEC_{50}-X)*HillSlope)})$$

In these equations, Y is the peak current at a given concentration of agonist, Bottom is the baseline response, Top is the maximal current generated by the concentration of agonist, EC_{50} is the effective dose which activates 50% of the maximal current for individual cells, X is the logarithm of agonist concentration, and HillSlope refers to the Hill coefficient generated.

6.3.6.2 IC_{50} values

IC_{50} values were calculated using the one-site competition equation in GraphPad Prism 3.0. The one site-competition curve equation is:

$$Y=Bottom + (Top-Bottom)/(1+10^{(X-LogEC_{50})})$$

where Y is the peak current at a given concentration of agonist and antagonist, Bottom is the minimal concentration, Top is the maximal current generated by the concentration of agonist, EC_{50} is the effective dose which antagonises 50% of the maximal current for individual cells, and X is the logarithm of antagonist concentration. The sigmoid dose-response and sigmoid dose-response with variable slope equations were also calculated to determine the best fit, as with the EC_{50} data.

The EC_{50} and IC_{50} values were expressed as mean ± s.e.mean or mean with reported 95% confidence interval.

6.3.6.3 Statistical analysis

Repeated measures one-way ANOVA was implemented to ascertain the effects of increasing doses of drug at $GABA_A$ receptors expressed in *Xenopus* oocytes. The dependent variable was current expressed as a proportion of the maximal response (I/I_{max}), and the independent variable was dose of drug (μM). If the ANOVA was significant, post-hoc Tukey-Kramer contrasts were computed to assess which doses significantly altered the response to GABA or produced significant intrinsic effects. Two-way ANOVA was performed to compare the effects of drugs to control responses. For example, to test whether drug effects at oocytes injected with $\alpha1\beta2\gamma2_L$ cRNA were significantly different to those effects observed on control oocytes not injected with cRNA, or whether bicuculline significantly reduced the response to drugs. In two-way ANOVAs, the dependent variable was current (nA) and the independent variables were concentration and oocytes (injected or control) or drug (alone or in the presence of bicuculline). If the overall ANOVA was significant, post-hoc Bonferroni contrasts were carried out to determine which variables produced the significant effects. A p value of <0.05 was deemed statistically significant. All statistical analyses were performed using GraphPad Prism 3.0.

6.4 RESULTS

6.4.1 Effects of Drugs on Uninjected Oocytes

Administration of GABA at concentrations up to 100μM and clozapine at concentrations up to 600μM to the cell bath containing control oocytes that had not been injected with $GABA_A$ receptor cRNA failed to elicit any change in holding current (baseline) of these cells (data not shown). Uninjected oocytes did, however, display sensitivity to chlorpromazine (n=9) and haloperidol (n=7), to a very small extent. When compared to the effects on cells injected with $GABA_A$ receptor cRNA, chlorpromazine and haloperidol produced a similar response, although at lower currents. ANOVA summary tables and Bonferroni post-hoc contrast results are given in Appendix 10. Figures 6.4 and 6.6 present the dose response curves of chlorpromazine and haloperidol respectively at uninjected oocytes compared with their effects at cells injected with $GABA_A$ receptor cRNA. Data are presented as mean ± s.e.mean current in nA, since a maximal response could not be obtained with the concentrations used.

Tables xix and xx in Appendix 10 show that there is a significant main effect of oocytes ($F_{0.05,1}$ = 14.16, $p<0.01$) and concentration ($F_{0.05,4}$ = 9.168, $p<0.01$) of chlorpromazine, but no significant interaction between the two effects ($F_{0.05,4}$ = 1.275, $p=0.285$). Thus, chlorpromazine produced a dose-dependent increase in response at both oocytes expressing $GABA_A$ receptors and uninjected oocytes, and the effect of chlorpromazine at $GABA_A$ receptors was significantly different to uninjected oocytes. The lack of a significant interaction implies that the effect of drugs on oocytes (injected versus control) is not the same at all concentrations tested.

Tables xxi and xxii in Appendix 10 show that there was a significant main effect of haloperidol concentration ($F_{0.05,4}$ = 3.328, $p=0.018$), while the main effect of oocytes ($F_{0.05,1}$ = 2.137, $p=0.151$) and the interaction between concentration and oocytes $F_{0.05,4}$ = 0.03, $p=0.998$) were not significant. Thus, haloperidol produced a significant dose-dependent effect at oocytes, but there was no difference between the effect at control oocytes and those injected with $GABA_A$ receptor cRNA.

6.4.2 Effect of GABA on Oocytes Injected with $\alpha1\beta2\gamma2_L$ cRNA

Application of increasing concentrations of GABA to the cell bath containing oocytes expressing $GABA_A$ receptor cRNA produced a dose-dependent change in the amplitude of whole cell current (I/I_{max}), as illustrated in figure 6.2. A one-way repeated measures ANOVA demonstrated that the dose-dependent effect of GABA was statistically significant ($F_{0.05, 4} = 261.6$, $p<0.01$), as shown in tables xxiii and xxiv, Appendix 10.

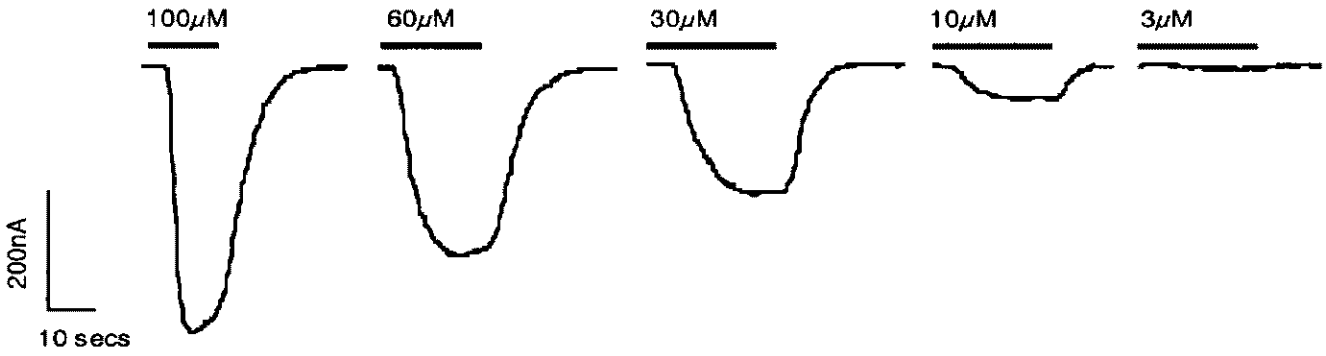


Figure 6.2: Dose-response relationship between current (in nA) and differing concentrations of GABA (as indicated) at $\alpha1\beta2\gamma2_L$ receptors in *Xenopus* oocytes.

Figure 6.3 depicts the log dose-response curve generated by GABA at $\alpha1\beta2\gamma2_L$ receptors in *Xenopus* oocytes (n=13), expressed as mean proportion of the maximal GABA response (I/I_{max}) ± s.e.mean. From the curve, the EC_{50} of GABA was 20.75 ± 1.11μM (95% CI 18.67-23.05), with a Hill coefficient of 1.6 ± 0.2.

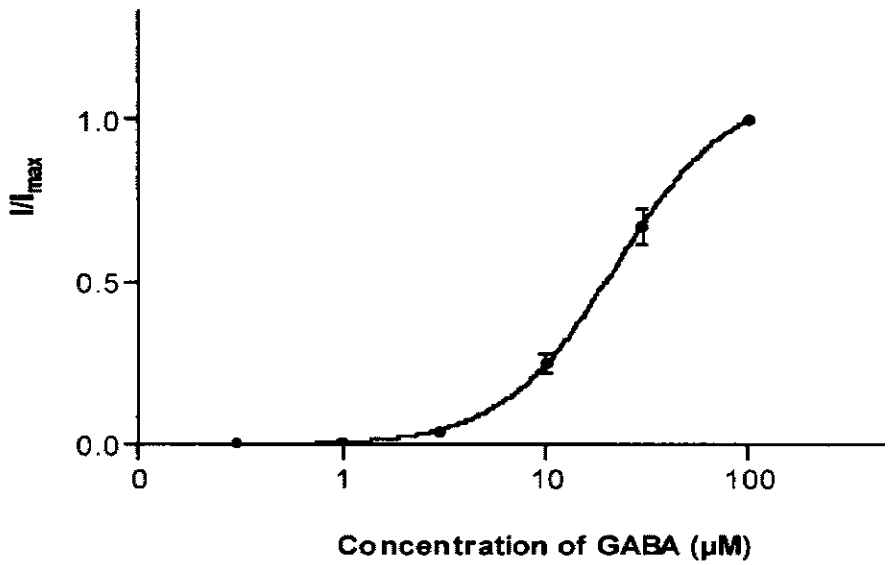


Figure 6.3: Dose-response curve of GABA at $\alpha1\beta2\gamma2_L$ receptors in *Xenopus* oocytes (n=13). Data are expressed as mean proportion of the maximal response of 100μM GABA (I/I_{max}) ± s.e.mean (indicated by error bars).

6.4.3 Effect of Chlorpromazine on Oocytes Injected with $\alpha1\beta2\gamma2_L$ cRNA

6.4.3.1 Chlorpromazine alone

Addition of increasing concentrations of chlorpromazine to the cell bath containing oocytes expressing $GABA_A$ receptor cRNA (n=13) produced a dose-dependent change in the amplitude of whole cell current (nA), as illustrated in figure 6.4. This dose-dependent increase in response was determined to be statistically significant ($F_{0.05,4}$ = 19.55, $p<0.01$) when analysed using one-way repeated measures ANOVA, the results of which are presented in tables xxv and xxvi, Appendix 10. A maximal response could not be ascertained owing to the high concentrations of chlorpromazine required, thus a valid EC_{50} could not be determined. At concentrations above 600μM chlorpromazine appeared to fall out of solution. No desensitisation was observed with administration of chlorpromazine, although compared to GABA, chlorpromazine had a longer onset and much longer offset, requiring long washout times. Subsequent application of GABA after a short washout period, even where current had returned to baseline, resulted in a reduced response to GABA in some oocytes. At concentrations over 600μM, application of chlorpromazine in some instances yielded a rapid decrease in current that failed to recover even upon extended washout periods.

Since chlorpromazine produced an effect at uninjected oocytes, 2μM atropine was added to the cell bath in the presence of chlorpromazine to assess whether the effects of chlorpromazine were due to action at endogenous muscarinic cholinergic receptors. Application of 2μM atropine in the presence of chlorpromazine, however, failed to reduce the response to chlorpromazine at control oocytes (data not shown). 2μM atropine is known to abolish muscarinic receptor activity.

6.4.3.2 Chlorpromazine in the presence of GABA

Co-application of chlorpromazine with 10μM GABA to *Xenopus* oocytes expressing $\alpha1\beta2\gamma2_L$ receptors (n=6) did not produce any alteration to the GABA-induced response (data not shown). 10μM GABA was chosen as this sub-EC_{50} concentration would permit observation of even small effects of chlorpromazine.

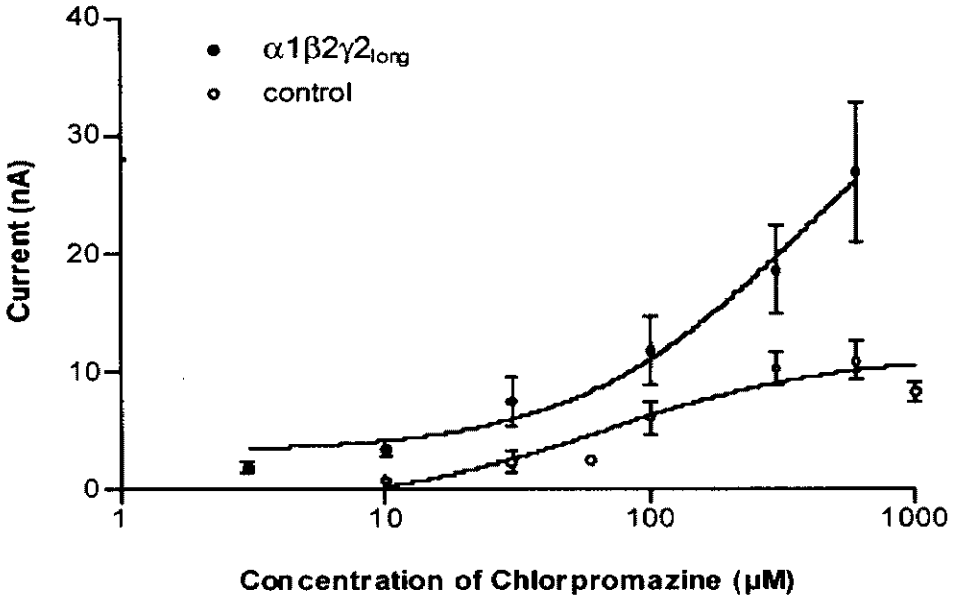


Figure 6.4: Dose-response curve of chlorpromazine at *Xenopus* oocytes injected with $\alpha1\beta2\gamma2_L$ cRNA (n=13) or at control oocytes not injected with cRNA (n=9). Data are expressed as mean current (nA) ± s.e.mean (indicated by error bars).

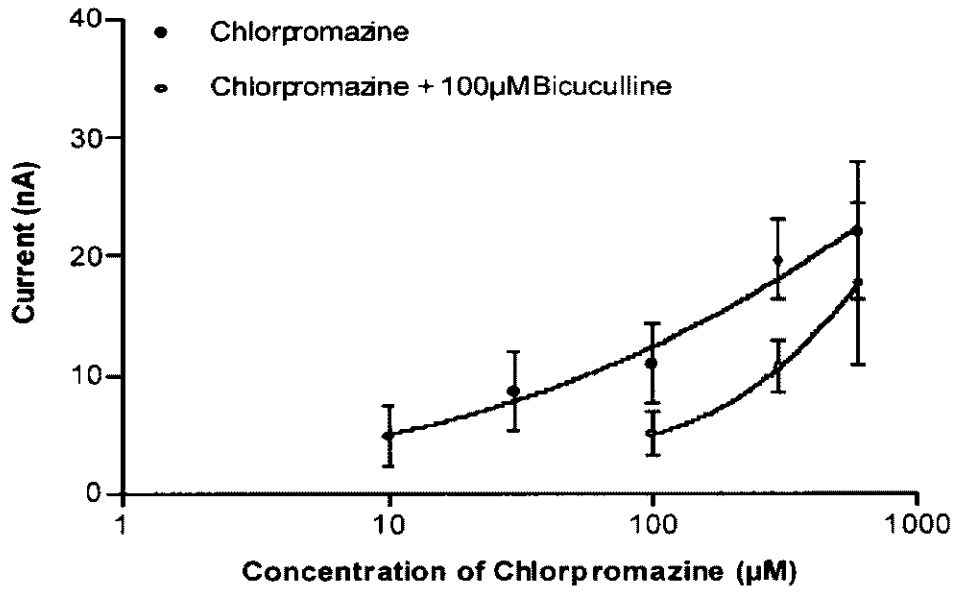


Figure 6.5: Dose-response curve of chlorpromazine alone and in the presence of 100μM bicuculline (n=4) at $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes. Data are expressed as mean current (nA) ± s.e.mean (indicated by error bars).

6.4.3.3 Chlorpromazine in the presence of bicuculline

Co-application of 100μM bicuculline with 100, 300 and 600μM chlorpromazine to the cell bath reduced the response to chlorpromazine (n=4). Figure 6.5 depicts this graphically. A two-way ANOVA showed that the main effects of concentration ($F_{0.05,2}$ = 3.001, p=0.08), drug (presence or absence of bicuculline) ($F_{0.05,1}$ = 2.513, p=0.135) and interaction between these variables ($F_{0.05,2}$ = 0.113, p=0.894) failed to produce statistical significance. Thus, the bicuculline-mediated reduction of chlorpromazine response at $\alpha1\beta2\gamma2_L$ receptors was not statistically significant. The ANOVA summary is given in table xxvii, Appendix 10.

6.4.4 Effect of Haloperidol on Oocytes Injected with $\alpha1\beta2\gamma2_L$ cRNA

6.4.4.1 Haloperidol alone

Addition of increasing concentrations of haloperidol to oocytes expressing $GABA_A$ receptor cRNA (n=9) produced a dose-dependent change in the amplitude of whole cell current (nA), as illustrated in figure 6.6. This dose-dependent increase in response was determined to be statistically significant ($F_{0.05,4} = 4.535$, $p<0.01$) when analysed using one-way repeated measures ANOVA, the results of which are presented in tables xxviii and xxix, Appendix 10. A maximal response could not be ascertained owing to the high concentrations of haloperidol required, thus a valid EC_{50} could not be determined. No desensitisation was observed with administration of haloperidol.

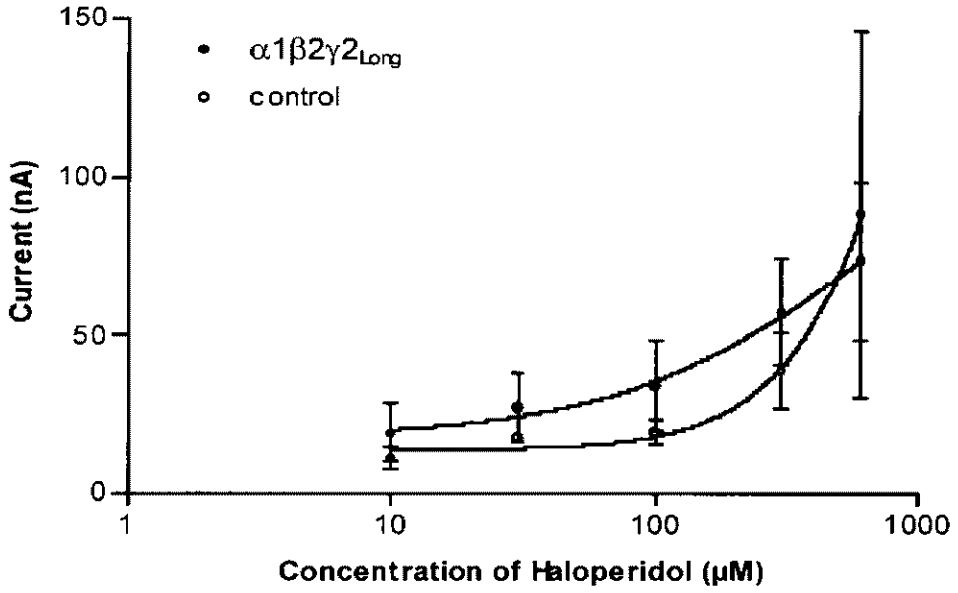


Figure 6.6: Dose-response curve of haloperidol at *Xenopus* oocytes injected with $\alpha1\beta2\gamma2_L$ cRNA (n=9) or at control oocytes not injected with cRNA (n=7). Data are expressed as mean current (nA) ± s.e.mean (indicated by error bars).

6.4.4.2 Haloperidol in the presence of GABA

Co-application of haloperidol with 10μM GABA to *Xenopus* oocytes expressing $\alpha1\beta2\gamma2_L$ receptors (n=6) did not produce any alteration to the GABA-induced response (data not shown).

6.4.4.3 Haloperidol in the presence of bicuculline

Co-application of 100μM bicuculline with varying concentrations of haloperidol to the cell bath reduced the response to haloperidol (n=3) to a small extent. Figure 6.7 depicts this graphically. A two-way ANOVA showed that the main effects of concentration ($F_{0.05,4} = 2.524$, $p=0.073$), drug (presence or absence of bicuculline) ($F_{0.05,1} = 0.161$, $p=0.693$) and interaction between these variables ($F_{0.05,4} = 0.028$, $p=0.998$) failed to

produce statistical significance. Thus, the bicuculline-mediated reduction of haloperidol response at $\alpha1\beta2\gamma2_L$ receptors was not statistically significant. The ANOVA summary is given in table xxx, Appendix 10.

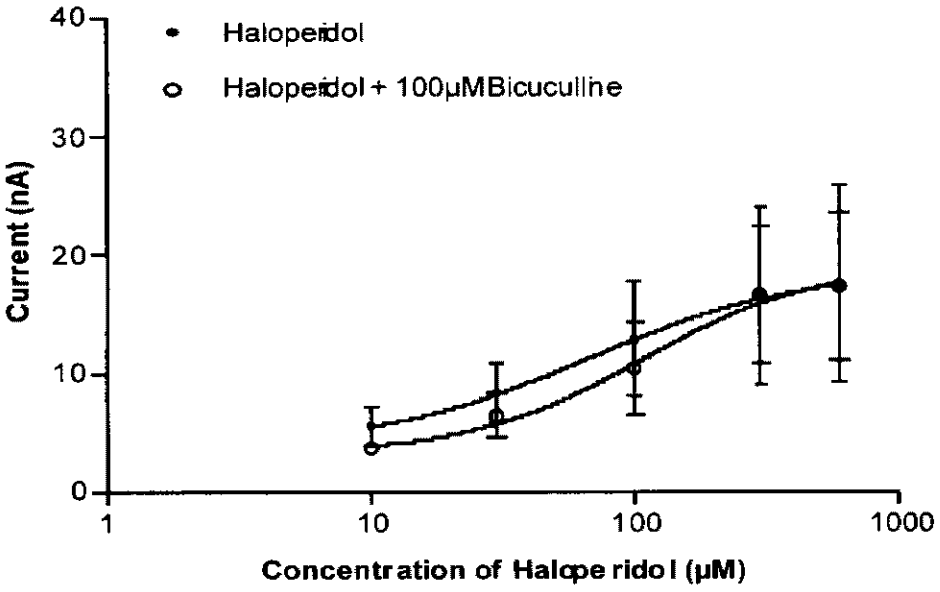


Figure 6.7: Dose-response curve of haloperidol alone and in the presence of 100μM bicuculline (n=3) at $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes. Data are expressed as mean current (nA) ± s.e.mean (indicated by error bars).

6.4.5 Effect of Clozapine on Oocytes Injected with $\alpha1\beta2\gamma2_L$ cRNA

6.4.5.1 Clozapine alone

Administration of clozapine to oocytes injected with $GABA_A$ receptor cRNA (n=6) failed to elicit any change in holding current (baseline) of these cells (data not shown).

6.4.5.2 Clozapine in the presence of GABA

Co-application of clozapine with 10μM GABA to *Xenopus* oocytes expressing $\alpha1\beta2\gamma2_L$ receptors (n=10) altered the GABA-induced response. A one-way repeated measures ANOVA indicated that clozapine inhibited the response to GABA in a dose-dependent manner that was statistically significant ($F_{0.05,4}$ = 46.66, $p<0.01$). The ANOVA summary is provided in table xxxi, Appendix 10. Figure 6.8 depicts the dose-dependent inhibition by clozapine of the current elicited by 10μM GABA at $\alpha1\beta2\gamma2_L$ receptors.

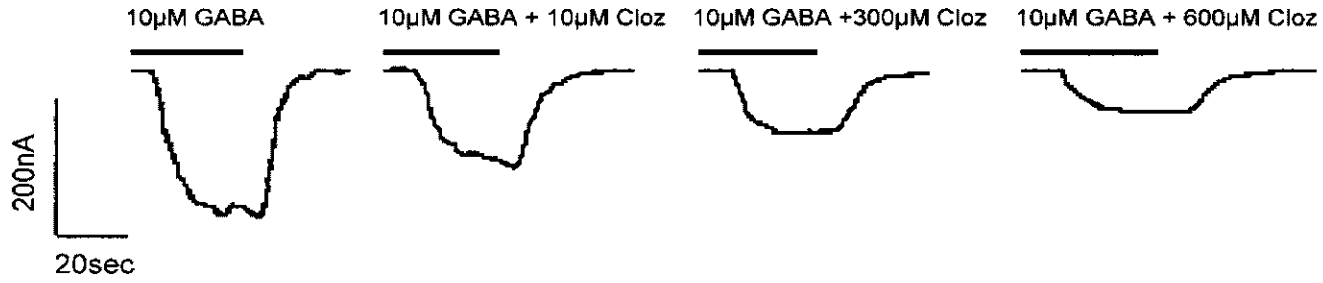


Figure 6.8: Dose-response relationship between current (in nA) and 10μM GABA in the presence of differing concentrations of clozapine (as indicated) at $\alpha1\beta2\gamma2_L$ receptors in *Xenopus* oocytes.

A log dose-response of clozapine representing inhibition by clozapine of the maximal response elicited by 10μM GABA at $\alpha1\beta2\gamma2_L$ receptors in *Xenopus* oocytes (n=10) gave an IC_{50} of 314.77 ± 2.27μM (95% CI 138.45-715.65). The curve generated by clozapine in the presence of 10μM GABA is presented in figure 6.9. Comparison of fits showed that the dose-response curve with standard slope fit the data best, denoting that the slope (Hill coefficient) is unity. This is indicative of a single binding site.

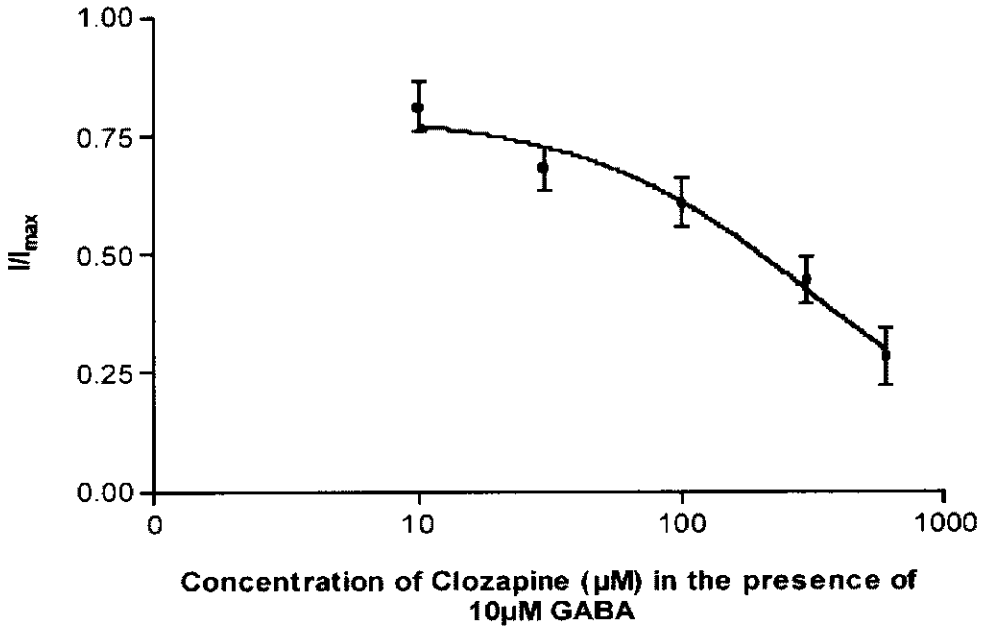


Figure 6.9: Dose-response curve of clozapine at varying concentrations in the presence of 10μM GABA at $\alpha1\beta2\gamma2_L$ receptors in *Xenopus* oocytes (n=10). Data are expressed as mean proportion of the maximal response of 10μM GABA (I/I_{max}) ± s.e.mean (indicated by error bars).

6.5 DISCUSSION

The aims of these experiments were to determine whether certain typical and atypical neuroleptic drugs produce effects at the ionotropic GABA receptors. For this purpose, $GABA_A$ receptors comprised of the $\alpha1\beta2\gamma2_L$ subunit combination were expressed in *Xenopus* oocytes. The $\alpha1\beta2\gamma2$ receptor combination is the most abundant in the mammalian CNS (Laurie *et al.*, 1992; McKernan & Whiting, 1996; Persohn *et al.*, 1992;

Sieghart, 2000; Wisden *et al.*, 1992), making this receptor subtype an ideal candidate for assessment of drug effects.

6.5.1 Actions of GABA

The action of GABA on *Xenopus* oocytes injected with $\alpha1\beta2\gamma2_L$ receptors proved that the receptors were indeed expressed in the oocyte membrane and could be recorded from successfully. An EC_{50} of 20.75 ± 1.11μM (95% CI 18.67-23.05), with a Hill coefficient of 1.56 ± 0.22 concurs with previous studies using the same subunit combination in the same expression system (Amin & Weiss, 1994 and unpublished observations).

6.5.2 Actions of Chlorpromazine

Chlorpromazine produced significant dose-dependent effects at control oocytes that were not injected with cRNA, suggesting action at endogenous receptors contained within the oocyte membrane. *Xenopus* oocytes express a number of endogenous receptors, including muscarinic acetylcholine, β-adrenergic, dopamine and serotonin receptors, as well as a number of ion channels and pumps (Fraser *et al.*, 1993). The response of muscarinic acetylcholine receptors can be abolished with low concentrations of atropine (Barnard *et al.*, 1984). In the present study, addition of 2μM atropine failed to antagonise the response to chlorpromazine, indicating that it was not acting at endogenous muscarinic acetylcholine receptors. Naturally, chlorpromazine may have had some affinity for the endogenous dopamine and serotonin receptors, however one would not expect to see agonist activity at these receptor types, considering it is a dopamine receptor antagonist. Quamme (1997) in fact demonstrated an effect of chlorpromazine on native *Xenopus* oocytes using two-electrode voltage clamp techniques. Application of chlorpromazine was found to dose-dependently and reversibly stimulate endogenous conductances in the presence of external chloride. It was concluded that chlorpromazine activated endogenous phenothiazine receptors which induced Ca^{2+}-dependent Cl^- channel activity (Quamme, 1997). Results with chlorpromazine in the current study were similar to those of Quamme (1997), and may potentially be explained by a comparable mechanism.

It was further shown in the present examination that chlorpromazine elicited activity at $GABA_A$ receptors expressed in *Xenopus* oocytes, displaying a statistically significant dose-dependent increase in current. Judging by the shape of the dose-response curve generated by chlorpromazine, it appeared to act as a very weak partial agonist. This response was also statistically significantly greater than the response of control oocytes to chlorpromazine, as shown in figure 6.4, indicating a $GABA_A$ receptor-specific action. Chlorpromazine did not attenuate nor antagonise the response of $GABA_A$ receptors to GABA, thus it did not act as an allosteric modulator of GABA activity. Owing to the large number of known binding sites on the $\alpha1\beta2\gamma2_L$ $GABA_A$ receptor, it was not feasible to test all potential sites to which chlorpromazine may have bound. However, 100μM bicuculline was added to the recording solution to assess whether chlorpromazine was binding to the GABA recognition site. High concentrations of chlorpromazine were blocked by 100μM bicuculline, although not significantly, possibly owing to the small sample size. Nonetheless, 100μM bicuculline is known to abolish the effects of GABA at $GABA_A$ receptors (Bormann & Feigenspan, 1995), yet it did not abolish the effect of chlorpromazine, therefore chlorpromazine must be eliciting its effects via an alternate mechanism.

Findings in the current study of agonist-like activity of chlorpromazine are not consistent with previous examinations. The literature has generally proved chlorpromazine to be a non-competitive inhibitor of GABA activity at GABA receptors at concentrations similar to those used here. Chlorpromazine has been shown to inhibit GABA currents by reducing GABA binding and increasing the unbinding rate for GABA (Mozrzymas *et al.*, 1999). Thus, it may affect the free energy required for a conformational change in the receptor by acting anywhere within the ion channel (Flood & Coates, 2002; Zorumski & Yang, 1988). While the current findings are unusual, prior studies were not conducted on *Xenopus* oocytes. Membrane properties of the *Xenopus* oocytes may be different to those of mammalian cells, so that chlorpromazine produces contrary effects. The research of Quamme (1997) also points to a non-GABAergic effect of chlorpromazine on *Xenopus* oocytes, as chlorpromazine stimulated endogenous Ca^{2+}-dependent Cl^- channel activity in oocytes. Chlorpromazine is 'a cationic amphipathic compound that is known to increase the open probability of mechanosensitive ion channels (Martinac *et al.*, 1990). Its high lipid solubility may

facilitate insertion into the cell membrane, with a consequent alteration in membrane shape and ion permeability (Casado & Ascher, 1998; Martinac *et al.*, 1990). Thus, $GABA_A$ receptor channels formed in the *Xenopus* oocyte membrane may be sensitive to stretch stimuli such as that incurred by chlorpromazine, where the alteration in membrane shape results in altered ion permeability.

The effect of chlorpromazine on the $\alpha1\beta2\gamma2_L$ subclass of $GABA_A$ receptors may be similar to the action of other $GABA_A$ receptor modulators, such as steroids and barbiturates. For example, barbiturates at high concentrations are known to mimic the action of GABA, opening the channel in the absence of GABA (Macdonald & Olsen, 1994). It may be worth pursuing the effects of chlorpromazine at these binding sites, however given that such high concentrations were necessary to elicit such weak activity, there appears to be no therapeutic relevance to these results. At the concentrations used, chlorpromazine is not thought to contribute to side effects through GABA modulation either. In conclusion, we may assert that at equivalent therapeutic doses, chlorpromazine has no effect on the $\alpha1\beta2\gamma2_L$ subtype of the $GABA_A$ receptors.

6.5.3 Actions of Haloperidol

Owing to the structural similarity between haloperidol and GABA, it has been suggested that some of the antipsychotic effect of haloperidol could be attributed to activity at GABA receptors (Janssen, 1967). It became clear from the results that haloperidol does not exhibit direct activity at the $\alpha1\beta2\gamma2_L$ subclass of $GABA_A$ receptors. Haloperidol produced significant dose-dependent, albeit weak, agonist effects at control oocytes that were not injected with cRNA, suggesting action at endogenous receptors contained within the oocyte membrane. Furthermore, the dose-dependent agonist activity of haloperidol at *Xenopus* oocytes expressing $\alpha1\beta2\gamma2_L$ receptors was not significantly different to the control response, nor could it be blocked by 100μM bicuculline. These results do not entirely preclude the possibility that the observed weak agonist effect of haloperidol were mediated by another of the many allosteric modulatory binding sites on the $\alpha1\beta2\gamma2_L$ receptors, although activity at these sites usually requires the presence of GABA. Thus, one may assume that the observed response to haloperidol was mediated by some endogenous receptor or ion channel expressed by *Xenopus* oocytes.

A limitation with the use of the *Xenopus* oocytes is that they express electrophysiological responses which may interfere with responses under study from exogenous RNA (Goldin, 1993; Kushner *et al.*, 1989). Haloperidol may have a very weak effect at the $GABA_A$ receptors which was masked by its activity at endogenously expressed receptors. In any case, the effect of haloperidol, like chlorpromazine, was too weak to be of clinical relevance.

The present results are in keeping with the literature, where haloperidol was found to have no effect alone nor on GABA-induced responses (Yokota *et al.*, 2002; Zorumski & Yang, 1988). Moreover, haloperidol at low concentrations inhibited GABA-induced depolarisation of cat primary afferent neurons, while at concentrations above 10μM, GABA-induced depolarisation was enhanced (Higashi *et al.*, 1981). The authors concluded that this biphasic effect was not direct, rather, haloperidol modulated GABA receptor activity by altering neuronal membrane properties. Droperidol, another butyrophenone compound used for neuroleptanaesthesia, was shown to inhibit GABA at α1β1γ2 receptors expressed in *Xenopus* oocytes with an IC_{50} of 12.6 nM (Flood & Coates, 2002). This inhibitory effect reversed at 5μM, and at high concentrations (100μM), droperidol activated the receptors in the absence of GABA (Flood & Coates, 2002), much like the effect observed in the present study. However, unlike in the current study, uninjected oocytes were not activated by droperidol. The activation properties of droperidol were not thought to be agonistic, since low GABA concentrations did not produce larger currents in the presence of droperidol as would be expected (Flood & Coates, 2002). Thus, butyrophenones appear to indirectly alter GABA activity, perhaps through modification of the cell membrane (Higashi *et al.*, 1981).

Although the effects of haloperidol on α1β2γ2L receptors expressed in *Xenopus* oocytes in the present study were not significantly different to those on uninjected oocytes, the currents elicited in injected oocytes were still greater than those elicited in uninjected oocytes. Therefore, haloperidol may have had an effect on the $GABA_A$ receptors in a manner similar to that observed in prior studies. Since the lowest concentration of haloperidol used was 10μM, potential inhibitory effects were not

investigated. Thus, lower concentrations of haloperidol should be tested for inhibitory action. It is tempting to think that chlorpromazine may be having a similar biphasic response on the $GABA_A$ receptors expressed in *Xenopus* oocytes. Investigation of lower concentrations of chlorpromazine is therefore also required.

6.5.4 Actions of Clozapine

Clozapine inhibited the response to 10μM GABA in a statistically significant dose-dependent manner, confirming its activity as an antagonist at $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes. The resultant IC_{50} for clozapine was 314.77 ± 2.27μM (95% CI 138.45-715.65), indicating that it is a weak antagonist. The curve of best fit followed the standard dose-response curve with a Hill slope of 1. This indicates that the binding of clozapine to $\alpha1\beta2\gamma2_L$ receptors follows the law of mass action for binding to a single site. Thus, it is likely that clozapine binds to the same site as GABA at $\alpha1\beta2\gamma2_L$ receptors.

The results with clozapine are congruous with those in the literature. Displacement by clozapine of [^{3}H]muscimol and [^{3}H]SR 95531 binding to $GABA_A$ receptors in rat cortical and cerebellar membrane preparations (Korpi et al., 1995) confirms its activity at the GABA site on $GABA_A$ receptors. Inhibition of GABA-induced currents in rat neural preparations and picrotoxin-sensitive GABA-induced ^{36}Cl flux into brain vesicles further substantiates clozapine's action as an antagonist (Korpi *et al.*, 1995; Michel & Trudeau, 2000; Yokota *et al.*, 2002). Finally, clozapine was found to inhibit GABA-evoked Cl^- currents at recombinant $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes at concentrations between 10 and 300μM (Asproni *et al.*, 2002).

Regional differences in the extent of clozapine's effects suggested subtype selectivity of the drug's action. Using HEK293 cells expressing different combinations of $GABA_A$ receptor subunits, Korpi *et al.* (1995) demonstrated that clozapine inhibited [^{35}S]TBPS binding at $\alpha1\beta2\gamma2_L$ receptors and was most effective at reversing the inhibitory effect of GABA on [^{35}S]TBPS binding at the $\alpha1\beta1\gamma2$ receptor complex, followed by the $\alpha1\beta2\gamma2$ and $\alpha1\beta3\gamma2$ complexes. Since the affinity of clozapine for the $GABA_A$ receptor is contingent upon the subunit composition of the receptor, it would be interesting to

assess the compound on different $GABA_A$ receptor subunit combinations expressed in *Xenopus* oocytes. Specifically, it is necessary to examine the effects of clozapine at the $\alpha1\beta1\gamma2_L$ subunit combination expressed in *Xenopus* oocytes, to determine whether clozapine is more potent at these receptors as Korpi and colleagues (1995) reported using HEK293 cells.

Clinically relevant concentrations of clozapine in the brain are between 0.8 and 72μM (Michel & Trudeau, 2000; Squires and Saederup, 1997). In order to assess whether clozapine binds to the $GABA_A$ receptors at therapeutically significant concentrations, the apparent binding constant must be determined. Nonetheless, even if the concentration of clozapine required to antagonise $GABA_A$ receptors in the present experiment was higher than therapeutic doses, the observed activity of clozapine may be important in overdose and drug interactions. A serious consideration with the use of clozapine is its ability to lower the seizure threshold, which is thought to occur through antagonism of GABA (Michel & Trudeau, 2000; Yokota *et al.*, 2002). The activity of clozapine observed in the current thesis may therefore be pertinent to both the side effects and the clinical application of the drug. The direct action of clozapine at GABA receptors may ameliorate symptoms contributed by an aberration in GABA transmission.

6.5.5 Conclusions

Chlorpromazine was found to activate the $\alpha1\beta2\gamma2_L$ $GABA_A$ receptors in a dose-dependent manner. While the present findings with chlorpromazine did not support previous results showing chlorpromazine to be a non-competitive inhibitor of $GABA_A$ receptor activity, they do suggest an alternate mechanism of chlorpromazine action. The compound may be acting on the Cl^- channel (Flood & Coates, 2002; Quamme, 1997; Zorumski & Yang, 1988) or incurring a stretch stimulus to alter membrane shape, thus ion permeability (Casado & Ascher, 1998; Martinac *et al.*, 1990).

Haloperidol was not shown to influence responses of $\alpha1\beta2\gamma2_L$ $GABA_A$ receptors expressed in *Xenopus* oocytes, contrary to previous studies which proved it to be a non-competitive inhibitor of GABA (Korpi *et al.*, 1995; Sasaki *et al.*, 1997; Yokota *et al.*,

2002). However, some experiments failed to demonstrate an effect of haloperidol on GABA receptors (Yokota *et al.*, 2002; Zorumski & Yang, 1988), thus its structural similarity to GABA does not necessitate activity at the GABA receptors.

Consistent with prior studies, clozapine was also shown to antagonise $\alpha1\beta2\gamma2_L$ $GABA_A$ receptors expressed in *Xenopus* oocytes. Such activity may be important for both the side effects and the therapeutic efficacy of clozapine in patients with schizophrenia.

In conclusion, this study has shown that different chemical classes of typical and atypical neuroleptics have varying actions at $GABA_A$ receptors. While the effects, or lack thereof, of chlorpromazine and haloperidol were too weak to be of therapeutic significance, the direct effect of clozapine may contribute to the improved efficacy of this drug in the treatment of schizophrenia.

CHAPTER 7

CONCLUSION

$GABA_C$ receptors are the least studied of the three major classes of GABA receptors. Their presence and molecular composition in the mammalian CNS has not been fully substantiated. The current study aimed, in part, to map $GABA_C$ receptors in rat CNS using *in situ* hybridisation and immunohistochemistry. Use of rat brain permitted optimisation of procedures, while providing information on the location and relative density of the $GABA_C$ receptors. At the same time, two $GABA_A$ receptor subunits were explored as positive controls using these techniques, since their presence in the CNS is prolific and well documented.

The $GABA_C$ receptor $\rho 1$ and $\rho 2$ subunit mRNAs were detected in rat retina and cerebellum, and the $\rho 2$ subunit transcript was encountered in some regions of the rat forebrain and midbrain using *in situ* hybridisation histochemistry. These findings are consistent with previous reports of $\rho 1$ and $\rho 2$ subunits in rat retina and CNS, and justify the use of the probes designed for the present thesis in detecting the target mRNA. $\rho 3$ Subunit mRNA, on the other hand, could not be detected in retina or CNS. The $GABA_C$ receptor protein was observed in the retina and weakly in the cerebellum, however it could not be detected in rat forebrain using immunohistochemistry.

$GABA_A$ receptor $\alpha 1$ and $\beta 3$ subunit probes provided a consistent signal in rat brain, and were therefore useful positive controls. These subunit transcripts were widely distributed throughout rat brain, in agreement with the literature. $GABA_A$ receptor $\beta 2/\beta 3$ subunit protein was also present in all regions of the rat brain investigated, supporting findings from *in situ* hybridisation studies.

Successful mapping of ionotropic GABA receptors in rat brain permitted optimisation of techniques and transferral to the human brain. Accordingly, the next step was to investigate the $GABA_A$ and $GABA_C$ receptors in human brain, prior to their assessment in post-mortem schizophrenic cases. *In situ* hybridisation techniques were employed for this purpose owing to their success with rat brain and sensitivity to low levels of transcript. Although only small sample sizes were used for each region of the

brain investigated, giving way to large variability, the $GABA_A$ receptor subunit mRNAs were detected in regions of the human brain previously not reported, including the superior temporal gyrus, temporal pole, amygdala and primary visual cortex. This study therefore extends the literature on the location of these transcripts in human brain. In general, the $GABA_C$ receptor subunit mRNAs could not be detected above background levels in the areas examined, with the exception of the cerebellum. The capacity to identify these transcripts at the regional and cellular levels can furnish valuable information on the ionotropic GABA receptors in human brain.

Mapping techniques are necessary to evaluate the relative density and distribution of receptors and their subunits, but they do not provide conclusive evidence of the presence of functional receptors. Mapping experiments alone therefore make it difficult to assess the function of the receptors, although they do provide some idea of the physiological roles of receptors on the basis of their regional distribution. Ultimate confirmation of the presence of $GABA_C$ receptors in mammalian brain must be ascertained from functional studies such as electrophysiological recording from cells expressing $GABA_C$ receptors in the CNS.

Electrophysiological studies have shown $GABA_C$ receptors in various regions of the mammalian CNS (Arakawa & Okada, 1988; Boller & Schmidt, 2001; Johnston *et al.*, 1975; Martina *et al.*, 1995; Pasternack *et al.*, 1999; Strata & Cherubini, 1994). These presumably $GABA_C$ receptors generally displayed properties different to those of $GABA_C$ receptors in the retina, suggesting the non-retinal $GABA_C$ receptors are distinct. The findings from the present investigation indicate that the non-retinal $GABA_C$ receptors are comprised of ρ2 subunits, since ρ2 subunits predominated in the CNS. Although rat ρ2 subunits cloned from retina do not form functional homo-oligomeric receptors *in vitro* (Zhang *et al.*, 1995), this may not be the case in rat brain *in vivo*. Rather, in the absence of ρ1 or ρ3 subunits, ρ2 subunits may form functional homooligomeric receptors in the CNS, or pseudo-heterooligomeric receptors with a novel ρ subunit or splice variants. Some researchers have suggested, the ρ subunits combine with $GABA_A$ receptor subunits. This seems unlikely in light of evidence that ρ subunits do not co-immunoprecipitate or co-assemble *in vitro* with $GABA_A$ receptor subunits (Hackam *et al.*, 1998; Kusama *et al.*, 1993a; Shimada *et al.*, 1992). Thus,

functional *in vivo* assays with selective pharmacological agents and electrophysiological techniques such as those employed by Yeh and coworkers (1996) in rat retina, coupled with mapping techniques, are necessary to correlate the presence of $GABA_C$ receptor subunits with functional $GABA_C$ receptors in the rat brain. Such experiments can provide information on the molecular composition of $GABA_C$ receptors in the CNS.

Since difficulties were encountered in demonstrating immunoreactivity to the $GABA_C$ receptor in rat forebrain using immunohistochemistry, quantitative receptor autoradiography using selective $GABA_C$ receptor agents such as the recently available [^{3}H]P4MPA should be undertaken. Such studies may shed further light on the distribution and density of the $GABA_C$ receptor binding sites in rat and human brain, supporting *in situ* hybridisation studies through the detection of functional proteins.

The current investigation went on to further characterise the potential role of GABA in the symptoms of schizophrenia. Having successfully employed *in situ* hybridisation techniques in human brain, changes in the distribution and density of ionotropic GABA receptors were assessed in regions of heteromodal association cortex of individuals with schizophrenia compared to matched controls. In individuals with schizophrenia, a trend was observed for increased expression of $GABA_A$ receptor $\alpha 1$ subunit mRNA in the middle frontal cortex and $\beta 3$ subunit mRNA in the superior temporal gyrus. A converse decrease in expression of $\alpha 1$ subunit mRNA in the superior temporal gyrus, and $\beta 3$ subunit mRNA in the middle frontal cortex was encountered. These findings point to regional variations in subunit expression in schizophrenic subjects compared to controls and underscore the complexity of neurochemical changes in schizophrenia. Altered patterns of gene expression may have functional consequences if subunit or receptor expression is subsequently abnormal.

Since changes were observed in the expression of $GABA_A$ receptor mRNAs in the schizophrenic cortex, the investigation moved on to assess neuroleptic drug activity at human $GABA_A$ receptors expressed in *Xenopus* oocytes. Neuroleptic drugs are known to alter the density of GABA receptor binding sites in rat studies, and it has been proposed that they may contribute therapeutic effects via direct modulation of these receptors. Chlorpromazine was found to weakly activate human recombinant $\alpha 1\beta 2\gamma 2_L$

$GABA_A$ receptors in a dose-dependent manner, an effect which was not significantly blocked by co-administration of bicuculline, the selective $GABA_A$ receptor antagonist. Haloperidol failed to influence responses of $\alpha1\beta2\gamma2_L$ $GABA_A$ receptors expressed in *Xenopus* oocytes. A very notable finding was antagonism of the $GABA_A$ receptors by clozapine. This action may ameliorate symptoms of schizophrenia contributed by an aberration in GABA transmission, or contribute to the side effects of clozapine treatment in schizophrenic patients.

While recording from functional recombinant $GABA_A$ receptors provided information on neuroleptic activity at these receptors, animal studies are still necessary to investigate whether clozapine treatment alters the expression pattern of $GABA_A$ receptor subunits in association cortex. This would allow assessment of whether clozapine contributes to the altered expression pattern of GABA receptor mRNA observed in schizophrenic cortex.

Further work is required before definitive conclusions can be drawn from the data. Quantification of immunohistochemical procedures is required. Qualitative analysis is adequate for peptides that are expressed at high levels. However, where proteins exist at low densities, quantification is necessary to ensure immunolabelling is present above background levels. The current research also emphasises the need to quantify the density of transcripts at the cellular level from *in situ* hybridisation experiments using image analysis. Since the cellular level of analysis is far more sensitive to the detection of transcripts expressed at low levels, quantification of the $GABA_C$ receptor subunit transcripts would be useful in determining their presence. Moreover, it would be advantageous to identify cell types to which subunit transcripts were localised to ascertain pre- and/or post-synaptic distribution. Finally, increasing the sample size in studies utilising human brain will reduce measures of variability, and hopefully yield statistical significance to the trends observed in the data.

Overall, results from the current study replicate and extend the literature on the distribution and density of ionotropic GABA receptors in the rat and human brain. Furthermore, key regions of the human brain involved in the genesis of schizophrenic symptoms demonstrated regional variations in the expression of $GABA_A$ receptor

subunit mRNAs. These regions of the brain have not previously been examined for changes in ionotropic GABA receptor subunit expression, with the single exception of Akbarian and colleagues (1995b), who probed schizophrenic middle frontal cortex. The inhibitory effect of clozapine on human recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes further emphasises a role for the GABAergic system in aspects of schizophrenia. Together, these results point not only to a role for ionotropic GABA receptors in the normal functioning of mammalian brain, but also in the pathology and treatment of schizophrenia.

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APPENDIX 1

Inclusion/Exclusion Criteria for Cases, from the New South Wales/NISAD Tissue Resource Centre Guidelines for Researchers

Inclusion/exclusion criteria for schizophrenia cases:

- diagnosis of schizophrenia on P79a or narrative by police is suggestive of mental illness
- age: 18 years and over
- no significant history of drug or alcohol abuse, epilepsy, developmental disorder or head injury at time of death
- no recent history of medical illness that may affect results or agonal state, no neurological disorders
- no recent cerebral stroke (within 5 years)
- postmortem interval <48 hours
- proven infectious cases excluded
- tissue on gross examination has no obvious abnormalities

Inclusion/exclusion criteria for control type 1 – death by suicide

- no known history of major psychiatric illness
- mode of death – suicide
- age: 18 years and over
- no significant history of drug or alcohol abuse, epilepsy, developmental disorder or head injury at time of death
- no recent history of medical illness that may affect results or agonal state, no neurological disorders
- no recent cerebral stroke (within 5 years)
- postmortem interval <48 hours
- proven infectious cases excluded
- tissue on gross examination has no obvious abnormalities

Inclusion/exclusion criteria for control type 2 – sudden death

- psychiatrically well
- mode of death – sudden
- age: 18 years and over
- no significant history of drug or alcohol abuse, epilepsy, developmental disorder or head injury at time of death
- no recent history of medical illness that may affect results or agonal state, no neurological disorders
- no recent cerebral stroke (within 5 years)
- postmortem interval <48 hours
- proven infectious cases excluded

tissue on gross examination has no obvious abnormalities

APPENDIX 2

NISAD Tissue Resource Centre

Neuroscience Institute of Schizophrenia and Allied Disorders

The Tissue Resource Centre
Department of Pathology, The University of Sydney, Blackburn Building D06, Sydney 2006.
Tel: (02) 9351 2410, Fax: (02) 9351 3429, Pager: (02) 9982 6174, Email: marms@pathology.usyd.edu.au

REQUEST FOR TISSUE

Date .....

Name ..... Title .....

Department ..... Institution .....

Address .....

.....

Telephone ( ) ..... Fax ( ) .....

E-mail Address .....

Have you ever requested tissue from the NISAD Tissue Resource Centre? Yes / No

Research Project

Title .....

Funding Source ..... Grant Number ..... Ethics Approval No: .....

(Please Note: Material cannot be forwarded without ethics approval)

Goal of the research - please give a short explanation of project on a separate page.

Tissue Request

Type: (fixed, fresh frozen, paraffin blocks, slides) .....

Name of Disorder (Schizophrenia/Depression/Control etc) .....

Quantity ..... Unaffected (Control) ..... Disorder: .....

Pathological area: .....

Age Limits ..... Sex: ..... Ethnic Origin: .....

Post mortem delay (range): .....

Other requirements .....

.....

Transportation details .....

.....

The following person may be contacted for further information ..... Tel: .....

If significant consumable costs are incurred in the access of tissue, this will have to be offset by funds from: .....

Signed: .....

Authorised by Prof. Clive Harper ..... **Date:** .....

PLEASE NOTE: Your request cannot be processed until the disclaimer on the following page is signed.

DISCLAIMER

"I hereby acknowledge that I have read and understood the Guidelines for Researchers Booklet.

The Neuroscience Institute of Schizophrenia and Allied Disorders (NISAD) and the NHMRC Network for Brain Research into Mental Health (the NHMRC Network) have taken all reasonable precautions to screen the material for infectious diseases. However, NISAD and the NHMRC Network accept no responsibility for the transmission of any disease as a result of the use of any materials supplied by NISAD and the NHMRC Network.

NISAD and the NHMRC Network must be acknowledged in all publications and presentations arising from the use of material and authorship may be sought in some cases.

Any material accessed from NISAD and the NHMRC Network remains the property of NISAD and the NHMRC Network. I acknowledge that the material will only be used for the project identified on the Request for Tissue Form. I also understand that the material must be returned to NISAD and the NHMRC Network upon the completion of the project or if requested at any time by NISAD or the NHMRC Network".

Name: .....

Signed: ..... **Date:** .....

Signed: ..... **Date:** .....

Prof. Clive Harper

APPENDIX 3

In Situ Hybridisation Solutions

All solutions are made with autoclaved DEPC-treated milliQ water in autoclaved DEPC-treated glassware or black polyacetal staining boxes using autoclaved pipette tips. Solutions follow those implemented by Heath *et al.* (1996) and LeCorre *et al.* (2000).

3.1 0.1M DEPC-treated water

1mL diethylpyrocarbonate was added to 1L milliQ water and the solution shaken vigorously

Solution was left for 24 hours at room temperature then autoclaved

3.2 2% Silane solution

1L of 2% silane solution was made by the addition of 20mL silane to 980mL acetone and mixed thoroughly

3.3 0.1M Triethanolamine HCl (TEA)

For a 1L solution, 19.5g TEA and 9g NaCl were mixed with 1L water

For a 250mL solution, 4.625g TEA and 2.25g NaCl were mixed with 250mL water

pH was adjusted to 8 with 10M NaOH

3.4 PBS solution made with PBS tablets (ICN Biochemicals)

10 PBS tablets were dissolved in 1L of DEPC-treated water according to instructions provided by the manufacturer

3.5 4% Paraformaldehyde solution

1L of 4% paraformaldehyde was made by the addition of 40g paraformaldehyde to 1L PBS solution

The solution was heated and stirred in a fumehood, ensuring the temperature did not rise above 60°C (as this causes the paraformaldehyde to re-polymerize)

The solution was filtered through Whatman filter paper #1 and cooled rapidly on ice before use

3.6 Ethanol solutions

250mL of 70% solution were made by mixing 175mL of 100% ethanol with 75mL of water

250mL of 80% solution were made by mixing 200mL of 100% ethanol with 50mL of water

500mL of 95% solution were made by mixing 450mL of 100% ethanol with 50mL of water

3.7 20x Standard Citrate Saline (SSC)

175.3g NaCl and 88.2g Sodium Citrate were mixed with 800mL autoclaved DEPC-treated water

pH was adjusted to 7.0 with 10N NaOH, and autoclaved DEPC-treated water was added to make 1L

The solution was stored at 4°C

3.8 50x Denhardt's Solutions

5g ficoll, 5g polyvinylpyrrolidone and 5g bovine serum albumin (BSA) were mixed with 500mL autoclaved DEPC-treated water

The solution was filtered through a 0.2µm disposable Nalgene filter, then aliquoted in 1mL aliquots into autoclaved microfuge tubes and stored at –20°C

3.9 100% Deionized Formamide

50mL formamide were stirred with5g BioRad AG501-X8, 20-50 mesh ion exchange resin for 30 minutes at room temperature

The solution was filtered twice into beakers through Whatman filter paper (#1), the aliquoted in 25mL into sterile conical tubes and stored at -20°C

3.10 Denatured Herring Sperm DNA

100mg herring sperm DNA were mixed with 10mL autoclaved DEPC-treated water and stirred on a magnetic stirrer for 2-4 hours until dissolved

The herring sperm DNA solution was sheared by sonication for 30-60 seconds, boiled for 10 minutes, then cooled rapidly on ice

Solution was aliquoted in 1mL aliquots into autoclaved microfuge tubes and stored at -20°C

3.11 0.5M Ethylenediaminetetraacetic acid (EDTA)

93.05g EDTA were stirred vigorously with400mL autoclaved DEPC-treated water until dissolved

NaOH solid pellets were added to the solution to assist dissolving until pH 8.0 was reached

Further autoclaved DEPC-treated water was added to make a final volume of 500mL, and the solution was stored at -20°C

3.12 0.2M Phosphate Buffer

0.2M phosphate buffer is comprised of 1 part Solution A and 4 parts Solution B

To make solution A, 27.6g NaH_2PO_4 (sodium phosphate monobasic, anhydrous) were dissolved in 1L autoclaved DEPC-treated water

To make Solution B, 28.4g Na_2HPO_4 (sodium phosphate dibasic, anhydrous) were dissolved in 1L autoclaved DEPC-treated water

3.13 0.02M Phosphate Buffer

50mL of 0.02M phosphate buffer were made by mixing 5mL stock 0.2M phosphate buffer with 45mL autoclaved DEPC-treated water

3.14 1M Dithiothreitol (DTT)

5g DTT were mixed with 32.4mL autoclaved DEPC-treated water

The solution was aliquoted in 1mL lots into autoclaved 1.6mL microfuge tubes and stored at -20°C.

3.15 Reagent A

200µL of 0.5M EDTA, 0.186g of TEA and 1.576g of Tris HCl were added to 100mL autoclaved DEPC-treated water

pH was adjusted to 7.7 using 10N NaOH, and the solution then store in sterile 50mL conical tubes at 4°C

3.16 50% Formamide

500mL of 4x SSC were made up using 100mL of 20x SSC in 400mL of autoclaved DEPC-treated water

500mL of formamide were mixed with 500mL 4x SSC mix then stored at 4°C.

In Situ Hybridization Buffer

10mL of 20x SSC, 1mL of 50x Denhardt's solution and 25mL of deionized formamide were added to a 100mL beaker, with the stirrer and heat on (about 50°C)

5g dextran sulfate were weighed in a sterile container and added slowly to the solution in the beaker

The beaker was covered and the solution allowed to stir for 30-45 minutes

Herring sperm DNA was heated for 5 minutes in a boiling water bath then cooled rapidly on ice before addition of 1mL (10mg/mL) to the solution in the beaker

6mg of heparin were weighed into a sterile container and added to the solution in the beaker

500µL (10mg/mL) Poly(A) were added to the solution in the beaker

Once all dextran sulfate was dissolved, the volume of the solution was made up to 50mL with 20mM phosphate buffer, mixed thoroughly, then poured into a sterile 50mL conical tube and stored at -20°C

APPENDIX 4

Table i: Results of dependent samples Student's *t*-tests performed between sense and antisense probes for rat retina experiments (Chapter 2). * denotes statistical significance.

Probe pair	Observed t value	Degrees of freedom	p value
ρ1 sense and antisense	4.203	3	0.02*
ρ2 sense and antisense	3.838	3	0.03*
α1 sense and antisense	2.704	3	0.07
β3 sense and antisense	2.448	3	0.09

Table ii: Results of dependent samples Student's *t*-tests performed between sense and antisense probes for rat brain mapping experiments (Chapter 2). * denotes statistical significance.

Probe pair	Region	Observed t value	Degrees of freedom	p value
ρ1 sense and antisense	Anterior forebrain bregma 3.70 to 0.48mm	3.330	3	0.04*
	Medial forebrain bregma -2.3 to -6.8mm	4.496	4	0.01*
	Posterior forebrain/midbrain bregma -9.8 to -13.8mm	2.386	4	0.07
	cervical spinal cord	1.220	1	0.44
ρ2 sense and antisense	Anterior forebrain bregma 3.70 to 0.48mm	3.095	2	0.09
	Medial forebrain bregma -2.3 to -6.8mm	2.095	5	0.09
	Posterior forebrain/midbrain bregma -9.8 to -13.8mm	2.427	5	0.06
	cervical spinal cord	1.535	2	0.26
ρ3 sense and antisense	Anterior forebrain bregma 3.70 to 0.48mm	0.889	4	0.42
	Medial forebrain bregma -2.3 to -6.8mm	0.053	3	0.96
	Posterior forebrain/midbrain bregma -9.8 to -13.8mm	0.963	2	0.44
α1 sense and antisense	Anterior forebrain bregma 3.70 to 0.48mm	4.958	4	0.01*
	Medial forebrain bregma -2.3 to -6.8mm	4.123	4	0.015*
	Posterior forebrain/midbrain bregma -9.8 to -13.8mm	2.127	3	0.12
	cervical spinal cord	1.077	2	0.37
β3 sense and antisense	Anterior forebrain bregma 3.70 to 0.48mm	9.381	4	<0.01*
	Medial forebrain bregma -2.3 to -6.8mm	7.015	5	<0.01*
	Posterior forebrain/midbrain bregma -9.8 to -13.8mm	3.282	2	0.08
	cervical spinal cord	3.708	2	0.065

Table iii: Results of dependent samples student t-tests performed between sense, antisense and antisense plus excess unlabelled probe signals for human brain mapping experiments (Chapter 2). * denotes statistical significance.

Probe pair	Region	Observed t value	Degrees of freedom	p value
ρ1 sense and antisense	Middle frontal cortex	0.693	4	0.526
	Hippocampus	0.842	1	0.554
	Amygdala (CeA)	19.79	1	0.032*
	Polar temporal cortex	0.937	1	0.521
	Superior temporal gyrus	0.201	4	0.850
	Primary visual cortex	0.673	5	0.531
	Cerebellum	0.953	1	0.515
ρ1 antisense and antisense competition	Middle frontal cortex	1.735	4	0.158
	Amygdala (CeA)	2.642	1	0.232
	Temporal cortex	1.330	1	0.411
	Superior temporal gyrus	1.454	5	0.206
	Cerebellum	0.665	1	0.626
ρ2 sense and antisense	Middle frontal cortex	1.824	4	0.142
	Hippocampus	0.674	2	0.570
	Amygdala (CeA)	4.823	1	0.130
	Polar temporal cortex	0.769	1	0.583
	Superior temporal gyrus	1.606	4	0.184
	Primary visual cortex	1.568	4	0.192
	Cerebellum	1.821	1	0.320
ρ2 antisense and antisense competition	Middle frontal cortex	0.788	4	0.475
	Amygdala (CeA)	2.217	1	0.270
	Temporal cortex	7.694	1	0.082
	Superior temporal gyrus	2.934	5	0.032*
	Cerebellum	3.666	1	0.170
α1 sense and antisense	Middle frontal cortex	3.855	5	0.012*
	Hippocampus	326.9	1	0.002*
	Amygdala (CeA)	0.069	1	0.956
	Polar temporal cortex	1.381	1	0.399
	Superior temporal gyrus	1.853	5	0.123
	Primary visual cortex	2.678	4	0.055
	Cerebellum	3.336	1	0.185
α1 antisense and antisense competition	Middle frontal cortex	5.091	4	0.007*
	Amygdala (CeA)	1.004	1	0.499
	Temporal cortex	3.976	1	0.157
	Superior temporal gyrus	3.552	5	0.016*
	Cerebellum	5.474	1	0.115
β3 sense and antisense	Middle frontal cortex	2.533	5	0.052
	Hippocampus	1.691	2	0.233
	Amygdala (CeA)	1.089	1	0.476
	Polar temporal cortex	1.979	1	0.298
	Superior temporal gyrus	1.309	5	0.248
	Primary visual cortex	2.351	2	0.143
	Cerebellum	1.259	1	0.427
β3 antisense and antisense competition	Middle frontal cortex	3.331	4	0.029*
	Amygdala (CeA)	1.434	1	0.388
	Temporal cortex	2.228	1	0.269
	Superior temporal gyrus	2.009	5	0.101
	Cerebellum	7.791	1	0.081

APPENDIX 5

Immunohistochemistry Solutions

5.1 0.9% Saline solution

9g NaCl were dissolved in 1L deionised water

5.2 PBS solution made with PBS tablets (ICN Biochemicals)

10 PBS tablets were dissolved in 1L of DEPC-treated water according to instructions provided by the manufacturer

5.3 4% Paraformaldehyde solution

500mL of 4% paraformaldehyde was made by the addition of 20g paraformaldehyde to 500mL PBS solution

The solution was heated and stirred in a fumehood, ensuring the temperature did not rise above 60°C (as this causes the paraformaldehyde to re-polymerize)

The solution was filtered through Whatman filter paper #1 and cooled rapidly on ice before use

5.4 15% Sucrose solution

15mg sucrose were dissolved in 100mL deionised water (final concentration 438.2mM), then stored at 4°C

5.5 30% Sucrose solution

30mg sucrose were dissolved in 100mL deionised water (final concentration 876.4mM), then stored at 4°C

5.6 0.1M Sodium citrate buffer

29.41gm of sodium citrate were dissolve in 1L of milliQ water

Solution was adjusted to pH 6 with the addition of 1M HCl and stored at 4°C

5.7 0.2M Phosphate buffer, pH 7.4

22.99g NaH_2PO_4 (sodium phosphate monobasic, anhydrous) and 4.55g Na_2HPO_4 (sodium phosphate dibasic, anhydrous) were dissolved in 1L of deionised water then stored at 4°C

5.8 0.01M Phosphate buffered saline (PBS), pH 7.4

8.76g NaCl, 0.20g KCl and 50mL 0.2M phosphate buffer (pH 7.4) were dissolved in 1L deionised water and stored at 4°C

5.9 0.1M Tris buffered saline (TBS)

4.5g NaCl and 121.1g TRIZMA base were dissolved in 800mL deionised water

The pH was adjusted to 7.4 with the addition of 1M HCl and the volume adjusted to 1L with deionised water. TBS was stored at 4°C

5.10 Blocking solution

The final volume of solution for immediate use contained the following in 0.1M TBS or 0.01M PBS:

10% normal serum (v/v)
1% BSA (w/v)
1% Triton X (v/v)

5.11 Primary antibody base solution

The final volume of solution for immediate use contained the following in 0.1M TBS or 0.01M PBS:

3% normal serum (v/v)
1% Triton X (v/v)

5.11 Secondary antibody base solution

The final volume of solution for immediate use contained the following in 0.1M TBS or 0.01M PBS:

1% normal serum (v/v)
1% Triton X (v/v)

5.12 PBS/glycerol mountant

25mL of 0.01M PBS were mixed thoroughly with 25 mL glycerol and stored at room temperature

5.13 3% Hydrogen peroxide solution

100µL of 30% "neat" H2O2 were diluted in 900µL of milliQ water and stored at 4°C

5.14 0.02% Diaminobenzidine (DAB) solution

10mg DAB in tablet form were dissolved in 50mL deionised water then filtered through Whatman's filter paper #4 into a container on ice and cooled for 15 minutes before use

APPENDIX 6

Table iv: Subject information. Subjects are listed in pairs such that each control is matched to each patient in the order presented. Information provided by the New South Wales Tissue Resource Centre courtesy of the Tissue Resource Centre Coordinator.

Case number	Gender	Age	Diagnosis	Agonal state	Date of death	Post-mortem delay	Cause of death	Brain pH	Drug use	Medication history
12886	Male	26	Suicide control	1- Good	17/11/97	36 hours	Carbon monoxide poisoning	6.65	Nil known	Nil known
12887	Male	27	Chronic paranoid schizophrenia (9 year history)	1- Good	18-19/11/97	21-33 hours	Suicide by hanging	6.25	Cigarettes (heavy), THC (heavy before 1991), ampheta-mines and heroin (not since 1991)	Haloperidol, thioridazine, cogentin
12858	Male	55	Control	3- Fair	9/1/96	20 hours	Cardiac arrest		Nil known	Salbutamol, steroids
12884	Male	51	Chronic residual schizophrenia (first admission 1973)	2- Average	25/8/97	21 hours	Ischaemic heart disease	6.02	Moderate alcohol use	Trifluperazine, cogentin, fluphenazine, thioridazine
12862	Female	52	Control	3- Fair	12/2/96	9.5 hours	Ischaemic heart disease	5.75	Nil known	Nil known
12878	Female	51	Chronic residual schizophrenia (first diagnosed at age 35)	3- Fair	13/2/97	12 hours	Lithium toxicity and multiple organ failure	5.42	Nil known	Fluphenazine, benztropine, anatensol, lithium, risperidone, haloperidol, zoloft
12889	Male	37	Control	1- Good	19/1/98	21 hours	Ischaemic heart disease		Nil known	Nil known
13113	Male	27	Chronic undifferentiated schizophrenia (first treated 1994)	1- Good	11/8/98	36 hours	Myocarditis	6.22	THC (regular until 1994), LSD and psylocibin (not since 1994), moderate alcohol use	Clozapine, olanzapine, risperidone, haloperidol, cogentin

12870	Male	60	Control	1- Good	22/7/96	13 hours	Acute myocardial infarction		Nil known	Antihyperten-sives
12845	Male	67	Chronic undifferentiated schizophrenia (first admission 1957)	2- Average	9/4/98	5 hours	Ischaemic heart disease	6.36	1.5L beer per day	Thioridazine, thiamine, fluphenazine
13082	Male	60	Suicide control	1- Good	19/7/98	22 hours	Suicide by hanging	6.75	Moderate alcohol use	Nil
12918	Male	57	Chronic paranoid schizophrenia (26 year history)	3- Fair	26-27/4/98	33-38 hours	Consequences of myocardial scarring		10-20mg alcohol per day, cigarettes	Fluphenazine, concordin, trifluperazine, artane, olanzapine, zoloft, thioridazine, prothiaden, conversil, notvilix, plendil, dothienpin, keflex, amoxil

APPENDIX 7

Table v: Results from Pearson product moment correlations between middle frontal cortex data expressed as final density (nCi/g tissue equivalent) and subject variables: post-mortem interval (PMI), tissue pH, subject age at death and agonal state (numerically designated 1=good, 2=average, 3=fair).

Subunit	Control/Schiz	Variable	Correlation (r)	R^2	p value
ρ1	Control	PMI	-0.378	0.143	0.53
		pH	n too small		
		Age	0.799	0.639	0.10
		Agonal state	-0.353	0.125	0.56
	Schizophrenia	PMI	0.031	0.001	0.96
		pH	0.337	0.114	0.66
		Age	0.664	0.441	0.22
		Agonal state	0.474	0.225	0.42
ρ2	Control	PMI	-0.378	0.143	0.53
		pH	n too small		
		Age	0.799	0.639	0.10
		Agonal state	-0.353	0.125	0.56
	Schizophrenia	PMI	0.031	0.001	0.96
		pH	0.337	0.114	0.66
		Age	0.664	0.441	0.22
		Agonal state	0.474	0.225	0.42
α1 whole section and grey matter	Control	PMI	-0.464	0.215	0.35
		pH	n too small		
		Age	0.657	0.431	0.16
		Agonal state	-0.414	0.171	0.41
	Schizophrenia	PMI	0.004	0.000	0.99
		pH	0.177	0.031	0.77
		Age	0.570	0.325	0.24
		Agonal state	0.478	0.228	0.34
β3 whole section and grey matter	Control	PMI	-0.464	0.215	0.35
		pH	n too small		
		Age	0.657	0.431	0.16
		Agonal state	-0.414	0.171	0.41
	Schizophrenia	PMI	0.004	0.000	0.99
		pH	0.177	0.031	0.77
		Age	0.570	0.325	0.24
		Agonal state	0.478	0.228	0.34

Table vi: Results from Pearson product moment correlations between superior temporal gyrus data expressed as final density (nCi/g tissue equivalent) and subject variables: post-mortem interval (PMI), tissue pH, subject age at death and agonal state (numerically designated 1=good, 2=average, 3=fair).

Subunit	Control/Schiz	Variable	Correlation (r)	R^2	p value
ρ1	Control	PMI	-0.464	0.215	0.35
		pH	n too small		
		Age	0.657	0.431	0.16
		Agonal state	-0.414	0.171	0.41
	Schizophrenia	PMI	0.004	0.000	0.99
		pH	0.177	0.031	0.77
		Age	0.570	0.325	0.24
		Agonal state	0.478	0.228	0.34
ρ2	Control	PMI	-0.464	0.215	0.35
		pH	n too small		
		Age	0.657	0.431	0.16
		Agonal state	-0.414	0.171	0.41
	Schizophrenia	PMI	0.004	0.000	0.99
		pH	0.177	0.031	0.77
		Age	0.570	0.325	0.24
		Agonal state	0.478	0.228	0.34
α1 whole section and grey matter	Control	PMI	-0.464	0.215	0.35
		pH	n too small		
		Age	0.657	0.431	0.16
		Agonal state	-0.414	0.171	0.41
	Schizophrenia	PMI	0.004	0.000	0.99
		pH	0.177	0.031	0.77
		Age	0.570	0.325	0.24
		Agonal state	0.478	0.228	0.34
β3 whole section and grey matter	Control	PMI	-0.464	0.215	0.35
		pH	n too small		
		Age	0.657	0.431	0.16
		Agonal state	-0.414	0.171	0.41
	Schizophrenia	PMI	0.004	0.000	0.99
		pH	0.177	0.031	0.77
		Age	0.570	0.325	0.24
		Agonal state	0.478	0.228	0.34

APPENDIX 8

Table vii: ANOVA summary table for $GABA_C$ receptor ρ1 subunit mRNA in control and schizophrenic brain. Group refers to the main effect of diagnosis. Region refers to the main effect of region (compacted variables for repeated measures).

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Group	1	0.651	0.651	1.322	0.2834
Subject(Group)	8	3.939	0.492		
Region	1	1.959	1.959	2.703	0.1388
Region * Group	1	0.021	0.021	0.029	0.8681
Region * Subject(Group)	8	5.798	0.725		

Dependent: Rho1

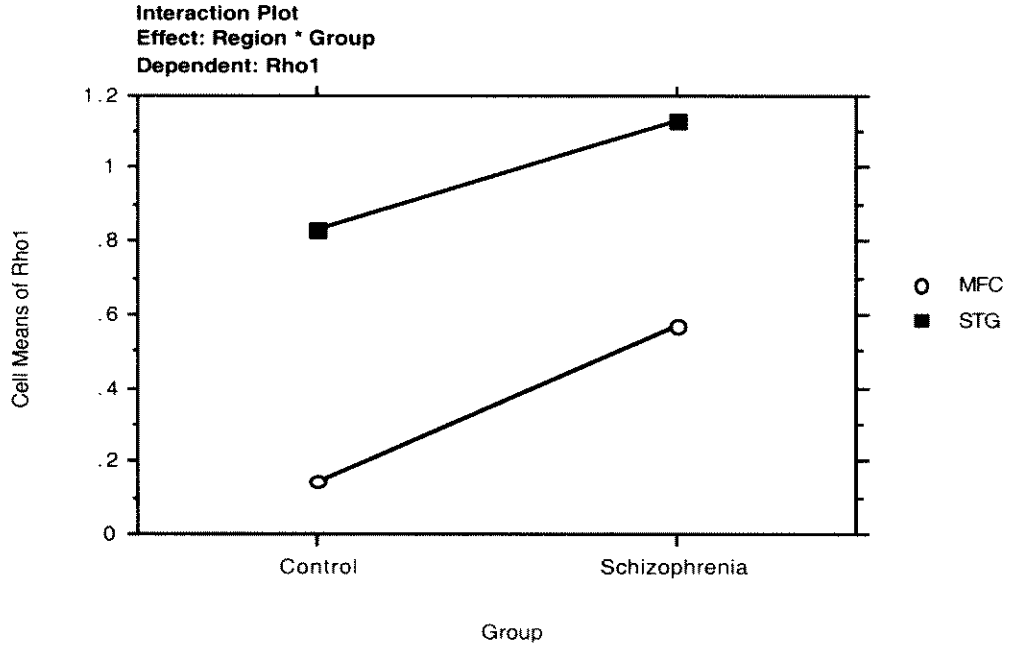


Figure i: Interaction plot for $GABA_C$ receptor ρ1 subunit mRNA in control and schizophrenic brain. Group refers to the main effect of diagnosis. Region refers to the main effect of region. MFC is the middle frontal cortex. STG is the superior temporal gyrus.

Table viii: ANOVA summary table for $GABA_C$ receptor ρ2 subunit mRNA in control and schizophrenic brain. Group refers to the main effect of diagnosis. Region refers to the main effect of region (compacted variables for repeated measures).

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Group	1	0.431	0.431	0.303	0.5969
Subject(Group)	8	11.364	1.421		
Region	1	1.935	1.935	4.297	0.0719
Region * Group	1	0.176	0.176	0.392	0.5488
Region * Subject(Group)	8	3.603	0.45		

Dependent: Rho2

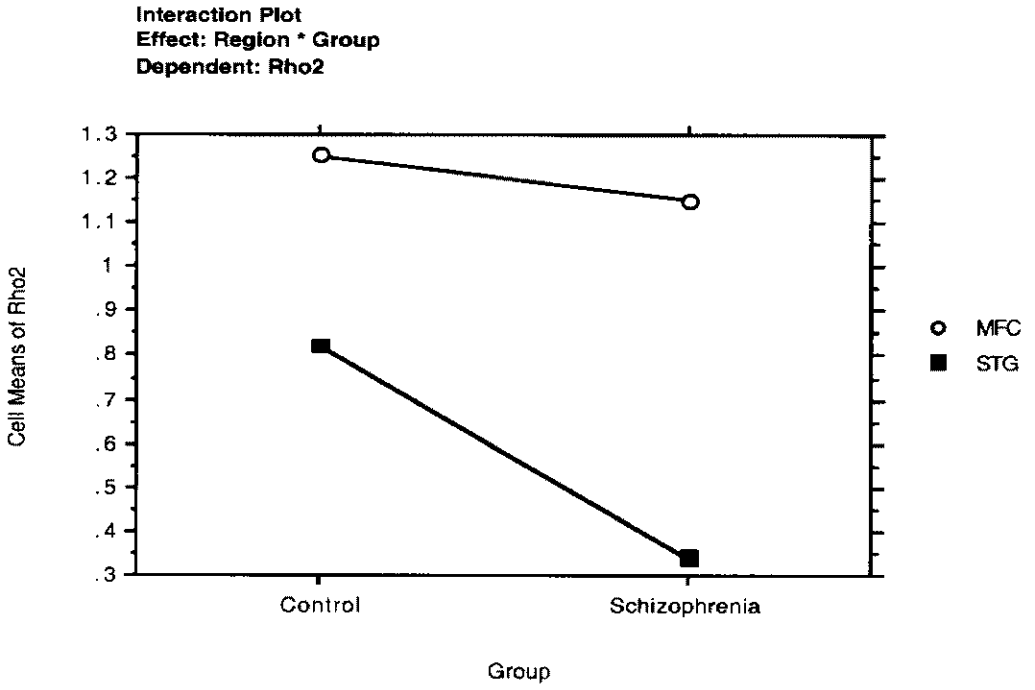


Figure ii: Interaction plot for $GABA_C$ receptor ρ2 subunit mRNA in control and schizophrenic brain. Group refers to the main effect of diagnosis. Region refers to the main effect of region. MFC is the middle frontal cortex. STG is the superior temporal gyrus.

Table ix: ANOVA summary table for $GABA_A$ receptor α1 subunit mRNA in control and schizophrenic brain from whole section measurements. Group refers to the main effect of diagnosis. Region refers to the main effect of region (compacted variables for repeated measures).

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Group	1	3.76	3.76	0.542	0.4785
Subject(Group)	10	69.369	6.937		
Region	1	38.877	38.877	15.248	0.0029
Region * Group	1	8.234	8.234	3.23	0.1025
Region * Subject(Group)	10	25.497	2.55		

Dependent: Alpha1 Whole

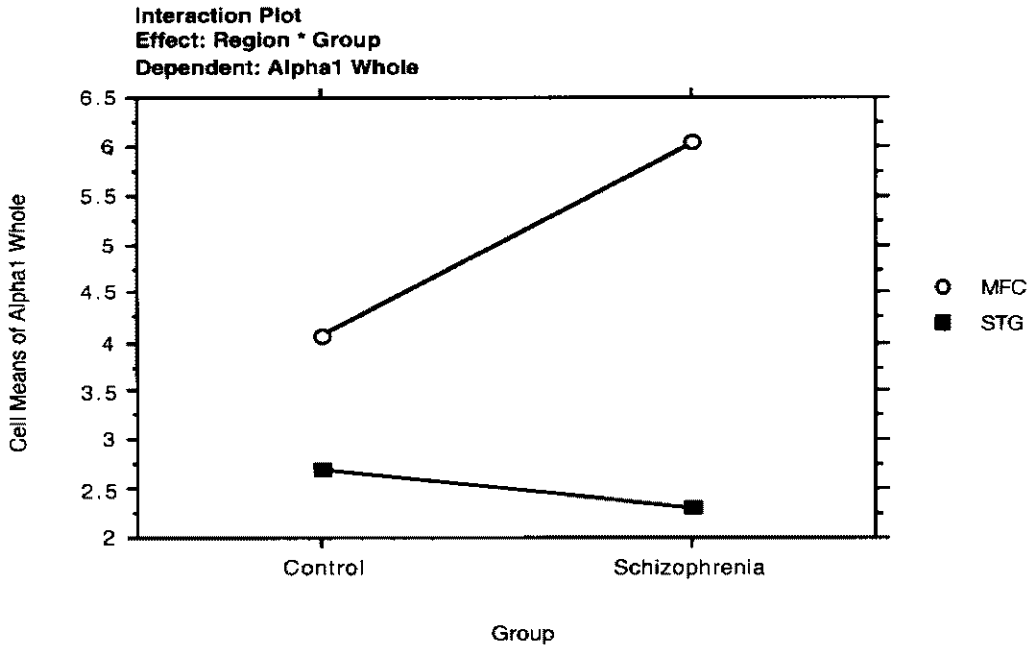


Figure iii: Interaction plot for $GABA_A$ receptor $\alpha 1$ subunit mRNA in control and schizophrenic brain from whole section measurements. Group refers to the main effect of diagnosis. Region refers to the main effect of region. MFC is the middle frontal cortex. STG is the superior temporal gyrus.

Table x: Means comparison contrast for main effect of region (MFC vs STG) for $GABA_A$ receptor $\alpha 1$ subunit mRNA from whole section measurements in schizophrenic and control brain. The post-hoc Scheffe F test for statistical significance could not be performed as the main effect of region was a compacted variable (repeated measures).

Comparison 1
Effect: Region
Dependent: Alpha1 Whole

	Cell Weight
MFC	1.000
STG	-1.000

df	1
Sum of Squares	38.877
Mean Square	38.877
F-Value	15.248
P-Value	.0029
G-G	.0029
H-F	.0029

Table xi: ANOVA summary table for $GABA_A$ receptor $\alpha 1$ subunit mRNA in control and schizophrenic brain from grey matter measurements. Group refers to the main effect of diagnosis. Region refers to the main effect of region (compacted variables for repeated measures).

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Group	1	1.82E-04	1.82E-04	1.16E-05	0.9973
Subject(Group)	10	156.178	15.618		
Region	1	91.76	91.76	14.729	0.0033
Region * Group	1	9.308	9.308	1.494	0.2496
Region * Subject(Group)	10	62.297	6.23		
Dependent: Alpha1 Grey					

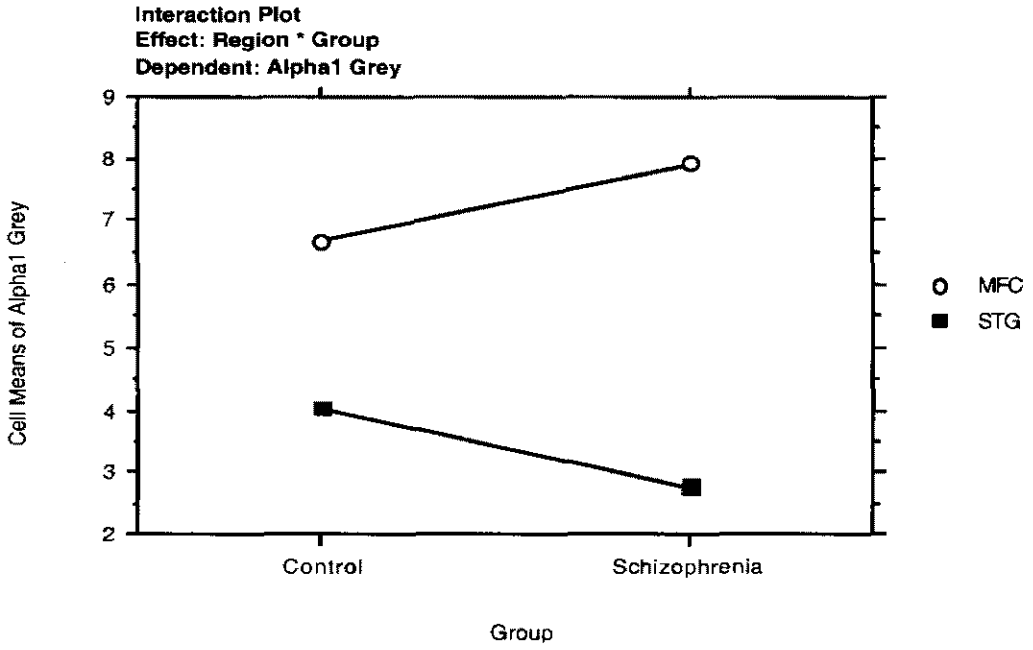


Figure iv: Interaction plot for $GABA_A$ receptor $\alpha 1$ subunit mRNA in control and schizophrenic brain from grey matter measurements. Group refers to the main effect of diagnosis. Region refers to the main effect of region. MFC is the middle frontal cortex. STG is the superior temporal gyrus.

Table xii: Means comparison contrast for main effect of region (MFC vs STG) for $GABA_A$ receptor $\alpha 1$ subunit mRNA from grey matter measurements in schizophrenic and control brain. The post-hoc Scheffe F test for statistical significance could not be performed as the main effect of region was a compacted variable (repeated measures).

Comparison 1
Effect: Region
Dependent: Alpha1 Grey

	Cell Weight
MFC	1.000
STG	-1.000

df	1
Sum of Squares	91.760
Mean Square	91.760
F-Value	14.729
P-Value	.0033
G-G	.0033
H-F	.0033

Table xiii: ANOVA summary table for $GABA_A$ receptor $\beta 3$ subunit mRNA in control and schizophrenic brain from whole section measurements. Group refers to the main effect of diagnosis. Region refers to the main effect of region (compacted variables for repeated measures).

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Group	1	0.473	0.473	0.306	0.5922
Subject(Group)	10	15.434	1.543		
Region	1	10.315	10.315	5.958	0.0348
Region * Group	1	4.521	4.521	2.611	0.1372
Region * Subject(Group)	10	17.314	1.731		

Dependent: Beta3 Whole

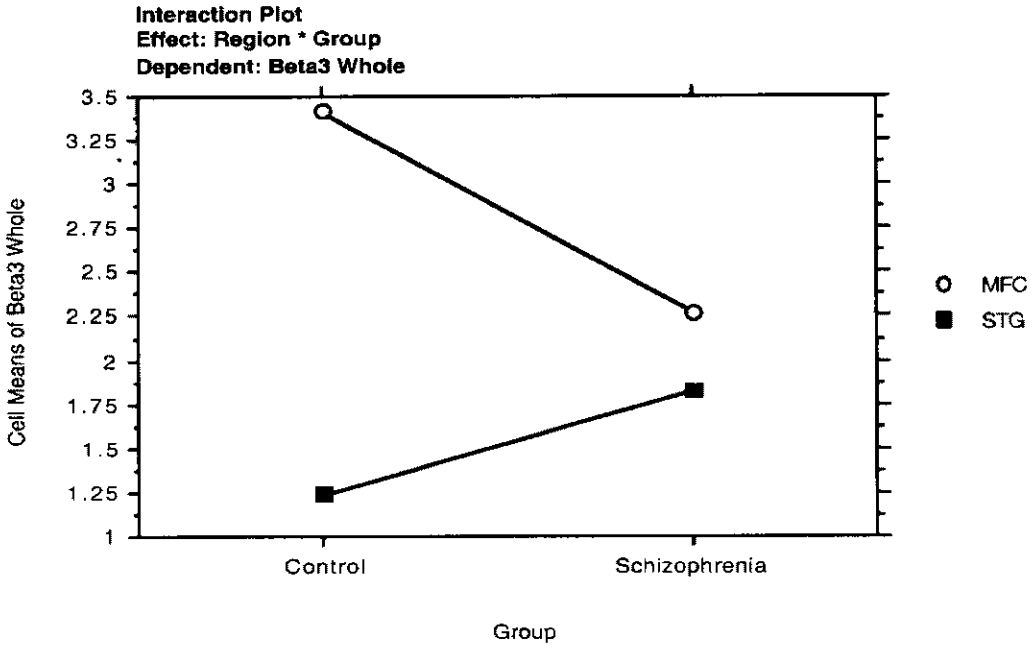


Figure v: Interaction plot for $GABA_A$ receptor β3 subunit mRNA in control and schizophrenic brain from whole section measurements. Group refers to the main effect of diagnosis. Region refers to the main effect of region. MFC is the middle frontal cortex. STG is the superior temporal gyrus.

Table xiv: Means comparison contrast for main effect of region (MFC vs STG) for $GABA_A$ receptor β3 subunit mRNA from whole section measurements in schizophrenic and control brain. The post-hoc Scheffe F test for statistical significance could not be performed as the main effect of region was a compacted variable (repeated measures).

Comparison 1
Effect: Region
Dependent: Beta3 Whole

	Cell Weight
MFC	1.000
STG	-1.000

df	1
Sum of Squares	10.315
Mean Square	10.315
F-Value	5.958
P-Value	.0348
G-G	.0348
H-F	.0348

Table xv: ANOVA summary table for $GABA_A$ receptor β3 subunit mRNA in control and schizophrenic brain from grey matter measurements. Group refers to the main effect of diagnosis. Region refers to the main effect of region (compacted variables for repeated measures).

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Group	1	31.218	31.218	4.631	0.0569
Subject(Group)	10	67.411	6.741		
Region	1	87.318	87.318	22.541	0.0008
Region * Group	1	3.39	3.39	0.875	0.3716
Region * Subject(Group)	10	38.738	3.874		

Dependent: Beta3 Grey

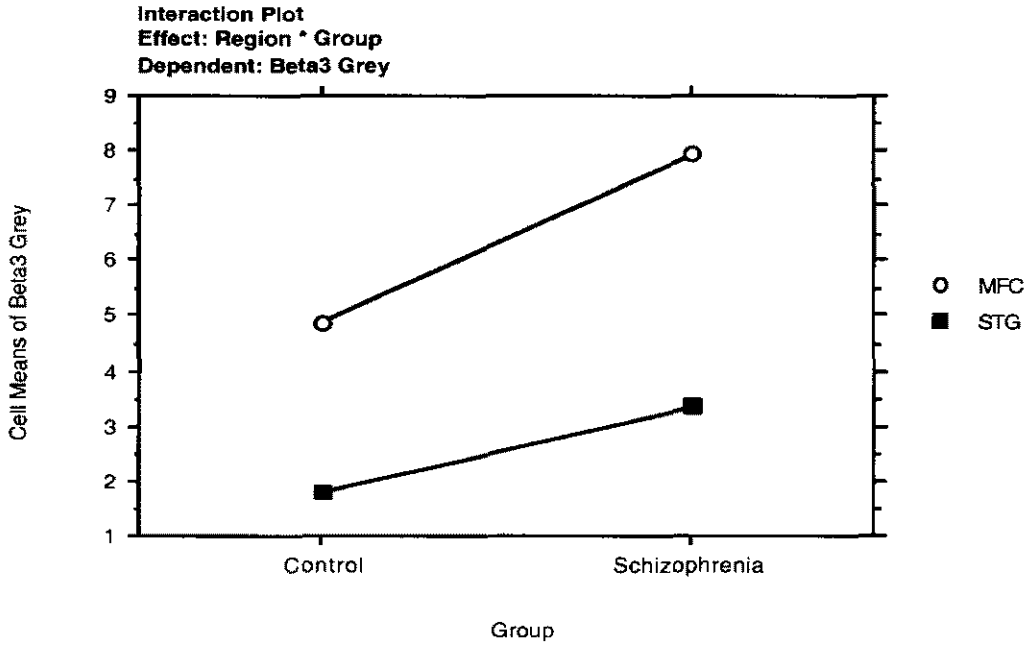


Figure vi: Interaction plot for $GABA_A$ receptor β3 subunit mRNA in control and schizophrenic brain from grey matter measurements. Group refers to the main effect of diagnosis. Region refers to the main effect of region. MFC is the middle frontal cortex. STG is the superior temporal gyrus.

Table xvi: Means comparison contrast for main effect of region (MFC vs STG) for $GABA_A$ receptor β3 subunit mRNA from grey matter measurements in schizophrenic and control brain. The post-hoc Scheffe F test for statistical significance could not be performed as the main effect of region was a compacted variable (repeated measures).

Comparison 1
Effect: Region
Dependent: Beta3 Grey

	Cell Weight
MFC	1.000
STG	-1.000

df	1
Sum of Squares	87.318
Mean Square	87.318
F-Value	22.541
P-Value	.0008
G-G	.0008
H-F	.0008

APPENDIX 9

Table xvii: Density of mRNA for $GABA_C$ receptor ρ1 and ρ2 subunits and $GABA_A$ receptor α1 and β3 subunits in middle frontal cortex of schizophrenic compared to matched control brains (n=5 for $GABA_C$ receptor subunits, n=6 for $GABA_C$ receptor subunits). Data are expressed as mean±s.e.mean nCi/g tissue equivalent.

Subunit	Control	Schizophrenia	% Change
ρ1	0.142±0.087	0.568±0.332	75% increase
ρ2	1.255±0.645	1.150±0.506	8% decrease
α1 Whole	4.058±1.074	6.021±1.105	32% increase
α1 Grey	6.671±1.610	7.912±1.551	16% increase
β3 Whole	3.414±0.747	2.266±0.497	33% decrease
β3 Grey	4.878±0.805	3.344±0.541	31% decrease

Table xviii: Density of mRNA for $GABA_C$ receptor ρ1 and ρ2 subunits and $GABA_A$ receptor α1 and β3 subunits in superior temporal gyrus of schizophrenic compared to matched control brains (n=6). Data are expressed as mean±s.e.mean nCi/g tissue equivalent.

Subunit	Control	Schizophrenia	% Change
ρ1	0.685±0.399	0.981±0.357	30% increase
ρ2	0.684±0.233	0.395±0.136	42% decrease
α1 Whole	2.684±0.652	2.304±0.602	14% decrease
α1 Grey	4.006±1.356	2.755±0.667	31% decrease
β3 Whole	1.235±0.372	1.823±0.384	32% increase
β3 Grey	1.815±0.437	2.076±0.501	13% increase

APPENDIX 10

Table xix: ANOVA summary table for chlorpromazine at oocytes injected with $\alpha 1\beta 2\gamma 2_L$ cRNA and control oocytes not injected with cRNA. A two-way ANOVA was performed with current (nA) as the dependent variable and oocytes and concentration as the independent variables. * indicates statistical significance.

Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	4	503.3	125.8	1.275	0.2854
Oocytes	1	1427	1427	14.46	P<0.01*
Concentration	4	3620	905	9.168	P<0.01*
Residual	95	9378	98.71		

Table xx: Bonferroni post-hoc contrasts for chlorpromazine at oocytes injected with $\alpha 1\beta 2\gamma 2_L$ cRNA compared to control oocytes not injected with cRNA. * indicates statistical significance.

Concentration	GABAA	Control	Difference
10	3.5	0.8333	2.667
30	7.5	2.063	5.438
100	11.85	6.167	5.679
300	18.62	10.28	8.338
600	26.9	10.94	15.96

Concentration	Difference	*t*	P value
10	2.667	0.5438	P>0.05
30	5.438	1.218	P>0.05
100	5.679	1.318	P>0.05
300	8.338	1.935	P>0.05
600	15.96	3.642	P<0.01*

Table xxi: ANOVA table for haloperidol at oocytes injected with $\alpha 1\beta 2\gamma 2_L$ cRNA and control oocytes not injected with cRNA. A two-way ANOVA was performed with current (nA) as the dependent variable and oocytes and concentration as the independent variables. * indicates statistical significance.

Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	4	145.3	36.33	0.03001	0.9982
Oocytes	1	2587	2587	2.137	0.1506
Concentration	4	16120	4029	3.328	0.0178*
Residual	46	55690	1211		

Table xxii: Bonferroni post-hoc contrasts for haloperidol at oocytes injected with $\alpha 1\beta 2\gamma 2_L$ cRNA compared to control oocytes not injected with cRNA.

Concentration	GABAA	Control	Difference	95% CI of diff.
10μM	16.14	2.4	13.74	-68.49 to 41.00
30μM	23.86	9.667	14.19	-78.71 to 50.33
100μM	30.93	16.21	14.71	-64.69 to 35.26
300μM	51.66	30.67	20.99	-84.29 to 42.31
600μM	66.58	57.17	9.417	-75.53 to 56.69

Concentration	Difference	*t*	P value	Summary
10μM	13.74	0.6745	P>0.05	ns
30μM	14.19	0.591	P>0.05	ns
100μM	14.71	0.7911	P>0.05	ns
300μM	20.99	0.891	P>0.05	ns
600μM	9.417	0.3827	P>0.05	ns

Table xxiii: ANOVA summary table for GABA at $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes (n=13). A one-way repeated measures ANOVA was performed with response (I/I_{max}) as the dependent variable and concentration of GABA (μM) as the independent variable.

ANOVA Table	SS	df	MS	F
Treatment (between columns)	9.747	4	2.437	261.6
Individual (between rows)	0.3081	12	0.02568	
Residual (random)	0.447	48	0.009314	
Total	10.5	64		

Table xxiv: Tukey's multiple comparisons post-hoc tests for differing concentrations of GABA at $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes. * indicates statistical significance.

Tukey's Multiple Comparison	Mean Diff.	q	P value	95% CI of diff
1μM vs 3μM	-0.02571	0.9605	P > 0.05	-0.1332 to 0.08174
1μM vs 10μM	-0.2698	10.08	P < 0.001*	-0.3773 to -0.1624
1μM vs 30μM	-0.6987	26.1	P < 0.001*	-0.8061 to -0.5912
1μM vs 100μM	-0.9862	36.85	P < 0.001*	-1.094 to -0.8788
3μM vs 10μM	-0.2441	9.12	P < 0.001*	-0.3516 to -0.1367
3μM vs 30μM	-0.673	25.14	P < 0.001*	-0.7804 to -0.5655
3μM vs 100μM	-0.9605	35.89	P < 0.001*	-1.068 to -0.8531
10μM vs 30μM	-0.4288	16.02	P < 0.001*	-0.5363 to -0.3214
10μM vs 100μM	-0.7164	26.76	P < 0.001*	-0.8238 to -0.6089
30μM vs 100μM	-0.2875	10.74	P < 0.001*	-0.3950 to -0.1801

Table xxv: ANOVA summary table for chlorpromazine at $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes (n=13). A one-way repeated measures ANOVA was performed with current (nA) as the dependent variable and concentration of chlorpromazine as the independent repeated variable.

ANOVA Table	SS	df	MS	F
Treatment (between columns)	4476	4	1119	19.55
Individual (between rows)	6129	12	510.8	
Residual (random)	2747	48	57.23	
Total	13350	64		

Table xxvi: Tukey's multiple comparisons post-hoc tests for differing concentrations of chlorpromazine at $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes. * indicates statistical significance.

Tukey's Multiple Comparison	Mean Diff.	q	P value	95% CI of diff
10μM vs 30μM	-4	1.906	P > 0.05	-12.42 to 4.422
10μM vs 100μM	-8.346	3.978	P > 0.05	-16.77 to 0.07634
10μM vs 300μM	-15.12	7.204	P < 0.001*	-23.54 to -6.693
10μM vs 600μM	-23.4	11.15	P < 0.001*	-31.82 to -14.98
30μM vs 100μM	-4.346	2.071	P > 0.05	-12.77 to 4.076
30μM vs 300μM	-11.12	5.298	P < 0.01*	-19.54 to -2.693
30μM vs 600μM	-19.4	9.246	P < 0.001*	-27.82 to -10.98
100μM vs 300μM	-6.769	3.226	P > 0.05	-15.19 to 1.653
100μM vs 600μM	-15.05	7.175	P < 0.001*	-23.48 to -6.631
300μM vs 600μM	-8.285	3.948	P > 0.05	-16.71 to 0.1379

Table xxvii: ANOVA summary table for chlorpromazine versus chlorpromazine in the presence of 100μM bicuculline at oocytes injected with $\alpha1\beta2\gamma2_L$ cRNA (n=4). A two-way ANOVA was performed with current (nA) as the dependent variable and chlorpromazine versus chlorpromazine + bicuculline and concentration as the independent variables.

Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	2	18.47	9.234	0.1133	0.8937
Drug	1	204.8	204.8	2.513	0.1352
Concentration	2	489.1	244.5	3.001	0.0823
Residual	14	1141	81.48		

Table xxviii: ANOVA summary table for haloperidol at $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes (n=9). A one-way repeated measures ANOVA was performed with current (nA) as the dependent variable and concentration of haloperidol as the independent repeated variable.

ANOVA Table	SS	df	MS	F
Treatment (between columns)	10460	4	2616	4.535
Individual (between rows)	23150	6	3858	
Residual (random)	13840	24	576.8	
Total	47450	34		

Table xxix: Tukey's multiple comparisons post-hoc tests for differing concentrations of haloperidol at $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes. * indicates statistical significance.

Tukey's Multiple Comparison	Mean Diff.	q	P value	95% CI of diff
10μM vs 30μM	-7.714	0.8498	P > 0.05	-45.53 to 30.10
10μM vs 100μM	-14.79	1.629	P > 0.05	-52.60 to 23.03
10μM vs 300μM	-20.02	2.206	P > 0.05	-57.84 to 17.79
10μM vs 600μM	-50.44	5.557	P < 0.01	-88.26 to -12.62
30μM vs 100μM	-7.071	0.779	P > 0.05	-44.89 to 30.75
30μM vs 300μM	-12.31	1.356	P > 0.05	-50.13 to 25.51
30μM vs 600μM	-42.73	4.707	P < 0.05	-80.54 to -4.908
100μM vs 300μM	-5.239	0.5771	P > 0.05	-43.06 to 32.58
100μM vs 600μM	-35.65	3.928	P > 0.05	-73.47 to 2.163
300μM vs 600μM	-30.42	3.351	P > 0.05	-68.23 to 7.402

Table xxx: ANOVA summary table for haloperidol versus haloperidol in the presence of 100μM bicuculline at oocytes injected with $\alpha1\beta2\gamma2_L$ cRNA (n=3). A two-way ANOVA was performed with current (nA) as the dependent variable and haloperidol versus haloperidol + bicuculline and concentration as the independent variables.

Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	4	8.467	2.117	0.02826	0.9983
Drug	1	12.03	12.03	0.1606	0.6928
Concentration	4	756.3	189.1	2.524	0.0731
Residual	20	1498	74.91		

Table xxxi: ANOVA summary table for clozapine in the presence of 10μM GABA at $\alpha1\beta2\gamma2_L$ receptors expressed in *Xenopus* oocytes (n=10). A one-way repeated measures ANOVA was performed with response (I/I_{max}) as the dependent variable and concentration of clozapine (μM) as the independent variable.

ANOVA Table	SS	df	MS	F
Treatment (between columns)	1.639	4	0.4097	46.66
Individual (between rows)	0.6074	8	0.07592	
Residual (random)	0.2809	32	0.008779	
Total	2.527	44		

Table xxxii: Tukey's multiple comparisons post-hoc tests for differing concentrations of clozapine in the presence of 10μM GABA. * indicates statistical significance.

Tukey's Multiple Comparison	Mean Diff.	q	P value	95% CI of diff
10μM vs 30μM	0.1272	4.074	P > 0.05	-0.0004784 to 0.255
10μM vs 100μM	0.2238	7.167	P < 0.001*	0.09611 to 0.3516
10μM vs 300μM	0.3801	12.17	P < 0.001*	0.2524 to 0.5078
10μM vs 600μM	0.545	17.45	P < 0.001*	0.4173 to 0.6727
30μM vs 100μM	0.09659	3.092	P > 0.05	-0.03114 to 0.2243
30μM vs 300μM	0.2528	8.095	P < 0.001*	0.1251 to 0.3806
30μM vs 600μM	0.4178	13.38	P < 0.001*	0.2901 to 0.5455
100μM vs 300μM	0.1562	5.003	P < 0.05*	0.02853 to 0.2840
100μM vs 600μM	0.3212	10.28	P < 0.001*	0.1935 to 0.4489
300μM vs 600μM	0.1649	5.281	P < 0.01*	0.03723 to 0.2927

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