"Characterisation of γ-aminobutyric acid B receptor subunits and related proteins in the rat central nervous system"

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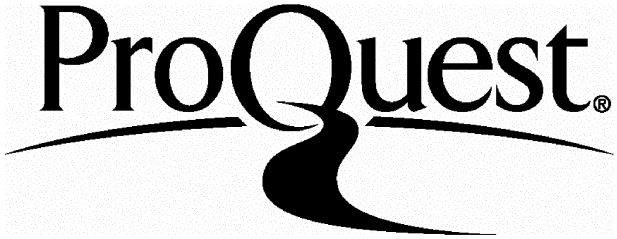

Table of Contents

Acknowledgments

Summary

Chapter 1 – Introduction

Chapter 2 - Materials and Methods

Chapter 3 - Localisation of known $GABA_B$ receptor subunits in the rat CNS

Chapter 4 - Localisation of $GABA_B$ receptor subunits in interneurons and non-neuronal cells in the rat CNS

Chapter 5 - Localisation of a novel $GABA_B$-like protein in the rat CNS

List of Figures

List of Tables

List of Abbreviations

ABC	avidin-biotin complex
Acb	accumbens
AcbC	accumbens Core
AcbSh	accumbens Shell
ATF	Activating transcription factor
B	nucleus basalis (Meynert)
BDNF	brain derived neurotrophic factor
BNST	Bed nucleus of the stria terminalis
BNST	bed nucleus of the stria terminalis
CA1	cornu ammonis 1
CA3	cornu ammonis 3
CACA	cis-4-aminocrotonic acid
CaSR	calcium sensing receptor
CGRP	calcitonin gene related peptide
Cl	claustrum
CNS	central nervous system
CP	complement protein
CPu	caudate putamen
CREB	cAMP response element binding protein
DAB	diaminobenzidine
DMH	dorsal medial hypothalamus
DNA	deoxyribonucleic acid
DR	dorsal raphe
DRG	Dorsal root ganglia
DRG	dorsal root ganglia
EEG	electroencephalogram
EM	electron microscopy
EPSP	excitatory post synaptic potential
ER	endoplasmic reticulum
GABA	γ-aminobutyric acid
GABA-T	GABA α-oxoglutarate transaminase
GAD	Glutamic acid decarboxylase
GAERS	genetic absence epilepsy rat from Strasbourg
GFAP	glial fibrillary acidic protein
gl	granule cell layer of cerebellum
GPCR	G-protein coupled receptor
GST	Glutathione-s-transferase
GST	glutathione-S-transferase
H	habenula
HEK293	human embryonic kidney - 293
Hi	hippocampal formation
InC	inferior colliculus

IP	interpeduncular nucleus
IPSP	inhibitory post synaptic potential
IR	immunoreactivity
KLH	Keyhole lympet haematoxylin
LA	lateral amygdala
LC	locus coeruleus
LD	laterodorsal thalamic nucleus
LHb	lateral habenula
LIV-BP	leusince isoleucine bacterial periplasmic protein
LSD	lateral septal nucleus, dorsal
LSI	lateral septal nucleus, intermediate
LSV	lateral septal nucleus, ventral
MBP	myelin basic protein
MD	mediodorsal thalamic nucleus
MG	medial geniculate nucleus
mGluR	metabotropic glutamate receptor
ml	molecular layer of cerebellum
MLF	medial lateral fasiculus
mRNA	messenger ribonucleic acid
MnR	median raphe
MVe	mesencephalic cells
NGF	nerve growth factor
NSF	N-ethylmaleimide-sensitive factor
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS	phosphate buffered saline
PDZ	PSD-95/discs large/ZO-1
PFA	paraformaldehyde
Pir	piriform cortex
Pn	pontine nuclei
PTX	pertusis toxin
RAIG	retinoic acid inducible receptor
Re	reuniens thalamic nucleus
Rh	rhomboid thalamic nucleus
Rt	reticular thalamic nucleus
SDS	Sodium-dodecyl-sulphate
SG	substantia gelatinosa of spinal cord
SN	substantia nigra
Sp	spinal nucleus
Su	superior colliculus
Th	thalamus
TPMPA	(1,2,5,6-tetrahydropyridin4-yl)-methylphosphonic acid
V	ventral thalamic nucleus
VDB	vertical limb of diagonal band
VH	ventromedial hypothalamus

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Summary

"Characterisation of γ-aminobutyric acid B receptor subunits and related proteins in the rat central nervous system"

Kelly Charles

Since its identification as the major inhibitory neurotransmitter in the central nervous system, γ-aminobutyric acid (GABA) and its receptors have been extensively studied. Thus far two distinct classes of GABA receptors have been identified. Ionotropic $GABA_{A/C}$ receptors are ligand gated ion channels and metabotropic $GABA_B$ receptors are G-protein coupled. Functional $GABA_B$ receptors are composed of two obligate receptor subunits, $GABA_{B1}$ and $GABA_{B2}$. Several splice variants of $GABA_{B1}$ have been indentified ($GABA_{B1a-f}$), though none of the thus far identified splice variants exhibit unique pharmacologies.

Subunit specific and splice variant specific antisera have been generated to probe the biochemistry and protein distribution of these receptor subunits. In this thesis I have utilised such antisera to study the comparative localisation of each receptor subunit throughout the rat central nervous system. These studies revealed that both obligate receptor subunits are expressed in the same regions in the brain, spinal cord and dorsal root ganglia. Co-localisation studies, using confocal microscopy, confirmed that the same neurons within the CA1 subregion of the hippocampus express both $GABA_{B1}$ and $GABA_{B2}$ receptor subunits. At the ultrastructural level I have demonstrated that both receptor subunits are expressed in both pre- and post-synaptic membranes in the CA1 subregion of the hippocampus.

I have also used these antisera to study the expression of these $GABA_B$ receptor subunits in non-neuronal cells. Co-localisation studies revealed that both obligate subunits are expressed in native and cultured astrocytes, as well as in activated microglia in culture. $GABA_B$ receptor subunits were, however, absent from myelin forming cells in the white matter of the rat spinal cord.

A novel putative $GABA_B$ receptor-like protein ($GABA_{BL}$) was recently identified by our group at GlaxoSmithKline. Using immunohistochemistry to map the distribution of $GABA_{BL}$ in the rat central nervous system I have developed antisera to $GABA_{BL}$ and shown that this protein is widely distributed in a manner similar to that of $GABA_{B1/2}$. $GABA_{BL}$ is also expressed in parvalbumin, calbinidin and calretinin positive interneurons within the hippocampus but was absent from astrocytes in this region.

Chapter 1 – Introduction

1.1 GABA

γ-aminobutyric acid (GABA), was first discovered in brain tissue over 50 years ago (Awapara et al., 1950; Undenfriend et al., 1950; Roberts et al., 1950). Enzymatic decarboxylation of glutamic acid by the enzyme glutamic acid decarboxylase (GAD) produces GABA as described by Martin et al., (1993). This enzymatic conversion pathway is shown below:

Figure 1.1 Enzymatic conversion pathway for γ-aminobutyric acid.

GABA is released into the synaptic cleft from vesicles in the pre-synaptic membrane in response to neuronal depolarisation. The released GABA is either

taken up by active transport at the pre- and post-synaptyic sites or into surrounding glia. GABA is converted to succinic semialdehyde by the enzyme, GABA α-oxoglutarate transaminase (GABA-T), in this catabolism glutamate is re-synthesised from α-oxoglutarate. Succinic dehydrogenase completes the cycle by oxidising succinic semialdehyde into succinic acid which then enters the Krebs cycle.

Since its identification, GABA has been classified as the major inhibitory neurotransmitter in the central nervous system (CNS) (Silvotti and Nistri, 1991), and as such is known to play a major role in the maintenance of the excitatory state of the CNS. Due to the neutral ionic properties of GABA it is able to diffuse across the synaptic cleft without interaction with other charged chemicals, to date this is the only neurotransmitter with such properties.

1.2 GABA receptors

1.2.1 Ionotropic $GABA_{A/C}$ receptors

1.2.1.1 $GABA_A$ receptors

The first GABA receptor was identified as a chloride gated-ion channel allosterically modulated by bound GABA to allow for the opening/closing of the channel and subsequent influx of chloride ions, producing hyperpolarisation (Bormann, 1988; Macdonald and Olsen, 1994). This ionophore is part of the ionotropic superfamily of neurotransmitter receptors which are membrane-bound ligand-gated ion channels capable of producing a fast synaptic response upon ligand binding which lasts only milliseconds. The $GABA_A$ receptor is a heteroligomer with a molecular weight range of 220-400kDa according to the subunit composition. Each $GABA_A$ receptor is composed of between 2 and 4 of

five different subunits (α1-6, β1-3, γ1-3, δ, ε and π). Each subunit of this ionophore posesses 4 membrane spaning domains which themselves contain binding sites for GABA.

Several drug classes have been developed with the ability to modulate the activity of the $GABA_A$ channel (for review: Stephenson, 1995). Anxiolytic compounds, such as valium, act at an allosteric binding site within the receptor channel, most widely known as the benzodiazapine binding site. These compounds activate the $GABA_A$ channel causing hyperpolarisation of the target neuron. The $GABA_A$ channel also possesses binding sites for alcohol, barbiturates, neurosteroids and compounds known to be pro-convulsant e.g. pentylenetetrazole. The first antagonist of the $GABA_A$ receptor, bicuculline, a plant derived toxin, was reported in 1970 (Curtis et al.,), although the antagonist-activity of bicuculline was first identified by Tsou in Shanghai but never published (Ong and Kerr, 2000).

However, intracellular microelectrode studies on brain and spinal cord neurons demonstrated a slower and later component of the inhibitory post synaptic potential (IPSP) in response to GABA that could not be explained by the increase in Cl^- conductance alone (Misgeld et al., 1995). It was subsequently discovered that this later component was mediated by a K^+ hyperpolarisation which showed slower kinetics due to the ion channel being indirectly coupled through intracellular G-proteins, unlike the Cl^- ion channel which is an integral part of the GABA receptor.

Localisation studies of the $GABA_A$ receptors have revealed a widespread distribution throughout the central nervous system and periphery (Bowery and Brown, 1974; Bowery et al., 1987). $GABA_A$ receptors are specifically located to

the post-synpatic specialisation where they mediate the fast, rapidly terminating IPSP, and also pre-synaptically where they have an inhibitory effect on neurotransmitter release (Saransaari and Oja, 1994; Curtis et al., 1995).

1.2.1.2 $GABA_C$ receptors

A second class of ionotropic GABA receptors is the $GABA_C$ receptor which was first described in 1984 as a bicuculline insensitive Cl^- ion channel (Drew et al.). Unlike the widespread distribution observed for $GABA_A$ receptors, $GABA_C$ receptors appear to be restricted to the bipolar cells of the retina (Bormann and Feigenspan, 1995). Although one of the subunits cloned thus far has been shown to be expressed in brain, possibly representing central $GABA_C$ receptors (Bormann and Feienspan, 1995). Selective agonists and antagonists have been identified for this receptor. $GABA_C$ receptors are activated by GABA analogues such as cis-4-aminocrotonic acid (CACA) and inhibited by (1,2,5,6-tetrahydropyridin4-yl)-methylphosophonic acid (TPMPA). The $GABA_C$ ion channel is comprised of five subunits, each with four membrane spanning domains, formed of a combination of the three cloned subunits ρ1, ρ2 and ρ3 (Zhang et al., 1995; Ogurusu et al., 1995, 1996). The agonist binding domain for the $GABA_C$ receptors is in the extracellular N-terminal domain. However, unlike $GABA_A$ receptors, $GABA_C$ receptors are insensitive to allosteric modulation by bezodiazapines or barbiturates.

1.2.2 Metabotropic $GABA_B$ receptors

In 1981 Hill and Bowery were the first to identify a GABA receptor which was insensitive to the $GABA_A$ antagonist bicuculline. Application of GABA to a

patch-clamped neuron in the presence of the selective $GABA_A$ receptor antagonist bicuculline only removed the rapid, early component of the typical GABA response, the late, slow hyperpolarisation was still apparent.

Futhermore Hill and Bowery identified that baclofen, an anti-spasticity agent, a lipophilic GABA analogue capable of crossing the blood-brain barrier and able to mimic the actions of GABA (Keberle and Faigle, 1972), was actually a selective agonist for the second component of the GABA IPSP. Hence the $GABA_B$ receptor was first defined. The sensitivity of the $GABA_B$ receptor to baclofen was found to be stereoselective, with the (+) enantiomer 100 times less potent than the (-) enantiomer (Hill and Bowery, 1981, Haas et al., 1985). Further studies confirmed the seperation of the two GABA receptors into subclasses, as the newly identified $GABA_B$ receptor was also found to be insensitive to other $GABA_A$ antagonists such as isoguvacine and was only weakly activated by muscimol (Mott and Lewis, 1994).

Studies investigating the kinetics of the $GABA_B$ response to GABA in the presence of bicuculline prompted Hill and co-workers to identify $GABA_B$ receptors as members of the G-protein coupled super-family (Hill et al, 1984). Activation of $GABA_B$ receptors induced inhibition on post-synaptic membranes via a K^+ mediated hyperpolarisation. This K^+ mediated event could be blocked by pertussis toxin (PTX), a selective guanine-nucleotide inhibitor (Coult and Howells, 1979). The fact that the K^+ response was blocked by PTX suggested that $GABA_B$ receptors are coupled to the G_i/G_o proteins, known to be inhibited specifically by PTX (Hill, 1985). However, for many years after the identification of the $GABA_B$ receptor and its classification as a putative G-protein coupled receptor, the exact molecular structure of the protein remained ellusive depsite

numerous cloning attempts. This was partly due to a complete lack of identified high affinity receptor antagonists which could then be used to isolate the receptor.

1.3 The $GABA_B$ receptors

1.3.1 $GABA_B$ receptor pharmacology

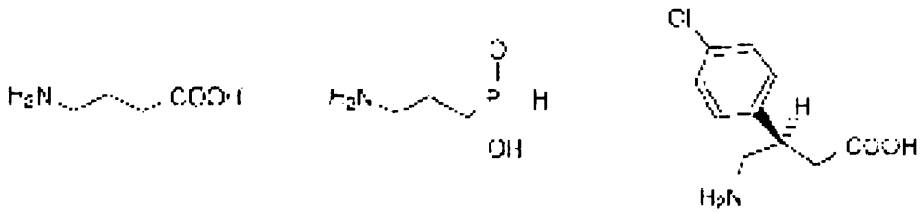
1.3.1.1 $GABA_B$ agonists

The identification of the $GABA_B$ receptor subtype was greatly aided by the use of the GABA mimetic, baclofen, which was developed as a therapeutic agent for muscle relaxation in spasticity. Baclofen, β-[4-chlorophenyl], was therefore the first $GABA_B$ agonist to be identified which allowed the separation of this subgroup from the $GABA_A$ receptor ion channels (Bowery et al., 1980). Following the identification of the $GABA_B$ receptor subgroup much effort was placed on identifying further agonists with greater potency than that afforded by baclofen. This lead to the identification of several phosphinic derivatives of GABA, 3APPA (3-aminopropyl phosphinic acid) and its methyl homologue, AMPPA (SKF97541), which are both up to 100 times more potent at the $GABA_B$ receptor than baclofen (Ong et al., 1990; Seabrook et al., 1990). Since that time the structure-activity relationship of GABA, baclofen and derivatives thereof has not revealed any new advances in compounds with greater affinity (Kerr and Ong, 1995). Indeed it seems that very little structural alteration is possible if one wishes to maintain $GABA_B$ agonist activity. This is particularly noticeable around the backbone region of these compounds where shortening the backbone produces inactive compounds and lengthening it produces compounds with antagonist properties (see Kerr and Ong, 1995). A group at Novartis have afforded considerable efforts to identify further $GABA_B$ agonists and have produced

several other phosphonic derivatives of GABA each with varying potencies, but generally none with greater activity at $GABA_B$ receptors than baclofen (Froestl et al., 1995a,b) (See Figure 1.2).

1.3.1.2 $GABA_B$ antagonists

Identification of the highly selective and potent $GABA_B$ receptor antagonist CGP64213 was critical in the breakthrough which identified the molecular structure of the $GABA_B$ receptor subunit (Kaupmann et al., 1997). However, since the first description of the $GABA_B$ receptors in the late 1980's many groups have sought to produce highly selective $GABA_B$ receptor antagonists. The first antagonist to be indentified with total selectivity over $GABA_A$ was phaclofen, which was produced as a phosphonic derivative of baclofen (Kerr et al., 1987). Subsequently a second selective antagonist, 2-hydroxy saclofen was identified by the same group (Kerr et al., 1988). 2-hydroxy saclofen possesses approximately 10 fold greater potency at $GABA_B$ receptors than phaclofen (Froestl et al., 1992). Hindering the continued development and assessment of phaclofen and 2-hydroxy saclofen was their inability to act centrally when administered systemically. The identification of CGP35348 (*P*-(3-aminopropyl)-*P*-diethoxymethyl-phosphinic acid), which is centrally active, allowed for the first time the assessment *in-vivo* of $GABA_B$ receptor antagonists. However, this compound has low affinity for the $GABA_B$ receptor and therefore few behavioural observations were notable (Bittiger et al., 1990). Despite the identification of many further brain-penetrating selective antagonists none had binding affinities considerably higher than that already observed for saclofen (See Figure 1.3).



GABA 3-APPA Baclofen

Figure 1.2 Structure of three common $GABA_B$ receptor agonists.

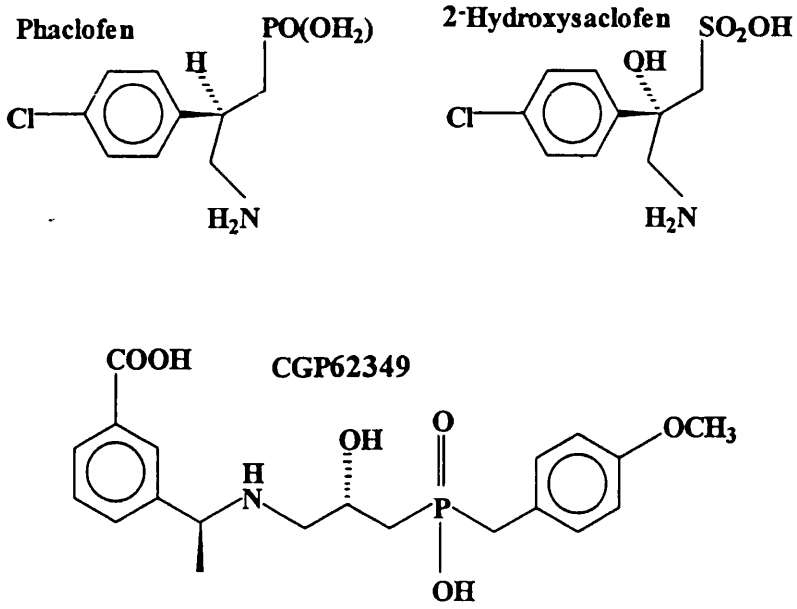


Figure 1.3 Structure of three $GABA_B$ receptor antagonists.

The breakthrough in $GABA_B$ antagonist identification came in 1995 when a new class of compounds was described which had substituted into their existing structures a dichlorobenzene moitey. This resulted in antagonists with nanomolar and subnanomolar affinities (Froestl et al., 1995). A series of compounds, which included CGP64213, revolutionised the field of $GABA_B$ receptor research and allowed the identification of the $GABA_{B1}$ receptor subunit by photoaffinity labelling.

1.3.2 $GABA_{B1}$

The breakthrough in identifying the molecular structure of the $GABA_B$ receptor came in 1997. Kaupmann, and colleagues at Novartis, used the highly selective antagonists CGP64213 and CGP71872 and a photo-affinity labelling technique to identify a protein they called $GABA_{B1}$ obtained from cortical, cerebellar and spinal cord membranes (Kaupmann et al., 1997). $GABA_{B1}$ was found to exist as two alternatively spliced isoforms of 130kDa, $GABA_{B1a}$, and 100kDa, $GABA_{B1b}$, the latter identified by low-stringency hybridisation. Analysis of the cloned $GABA_{B1}$ proteins revealed that they were members of the seven-transmembrane superfamily, and most closely related to the family C GPCRs. Other members of family C include the metabotropic glutamate receptors, Ca^{2+} sensing receptor and pheromone receptors. Family C is set apart from other GPCRs by their large extra-cellular ligand-binding domain.

Further analysis of the $GABA_{B1}$ protein sequences revealed several striking characteristics of this newly identified receptor. The intracellular C-terminal tail of the $GABA_{B1}$ protein contains a coiled-coil domain, bundles of α-

helicees which are wound into a superhelix (Lupas, 1996), which have been identified as dimerization elements. Also present in the C-terminal of the $GABA_{B1}$ proteins is a PDZ domain. PDZ domains (PSD-95/discs large/ZO-1) consist of six β-strands and two α-helices which are capable of binding to the last four to six C-terminal residues in the target interaction protein (Bezprozvanny and Maximov, 2001). As such the PDZ domain in the C-terminal of $GABA_{B1}$ proteins mediates interactions with other proteins and transcription factors such as ATFx and CREB2 (White et al., 2000). The $GABA_{B1a}$ splice variant also contains two sushi-domains in its N-terminal sequence, which are absent in the $GABA_{B1b}$ splice variant (Couve et al., 2000). Sushi-domains, also known as complement protein modules (CPs), are involved in protein/protein recognition events and often are directly implicated in ligand binding (Hawrot et al., 1998). The tandem sushi-domains in the N-terminal of the $GABA_{B1a}$ protein have been reported to interact with an extracellular matrix laminin binding protein, Fibulin (Ginham et al., 2002), however, the $GABA_{B1b}$ splice variant does not possess these sushi-domains.

$GABA_{B1}$ receptor proteins, like those of other family C GPCRs, have strong sequence-homology to the bacterial periplasmic leucine isoleucine-binding protein (LIV-BP). The crystalised structure of the metabotropic glutamate receptor mGluR1 has recently been identified and confirms the proposed "venus fly-trap" model. In the case of mGluR1, glultamate binds in a cleft in the extracellular domain made up of two lobes. Ligand binding causes a conformational shift in the lobes causing them to come closer together into a closed "activated" state. This conformational change results in changes in the nearby transmembrane domains, and as such G-protein signalling can occur

(Kunishima et al., 2000; Tsuchiya et al., 2002). With regard to $GABA_{B1}$, Pin et al have confirmed the "Venus fly-trap" model for ligand binding using *in silico* modelling techniques (Malitschek et al., 1999) (See Figure 1.4).

The cloning of the $GABA_B$ receptor unleashed a multitude of studies investigating the pharmacology of this receptor in recombinant systems. However, it soon became apparent that the expression of either $GABA_{B1a}$ and/or $GABA_{B1b}$ in recombinant systems resulted in a receptor pharmacolgy which, although demonstrating similar agonist rank-order affinities, showed affinities which were 100-150 fold lower than those identified in native tissues (Kaupmann et al., 1997). $GABA_B$ receptor anatagonists, however, displayed normal pharmacologies at the $GABA_{B1a}$ or $GABA_{B1b}$ receptor (Kaupmann et al., 1997). In addition to these discrepancies it was also noted that $GABA_{B1}$ expressed alone in mamalian cells failed to reach the plasma membrane and was instead retained within the endoplasmic reticulum (Couve et al., 1998). This suggested that the $GABA_{B1}$ protein lacked the necessary machinery, chaperones or structure to relocate from the endoplasmic reticulum to the cell surface.

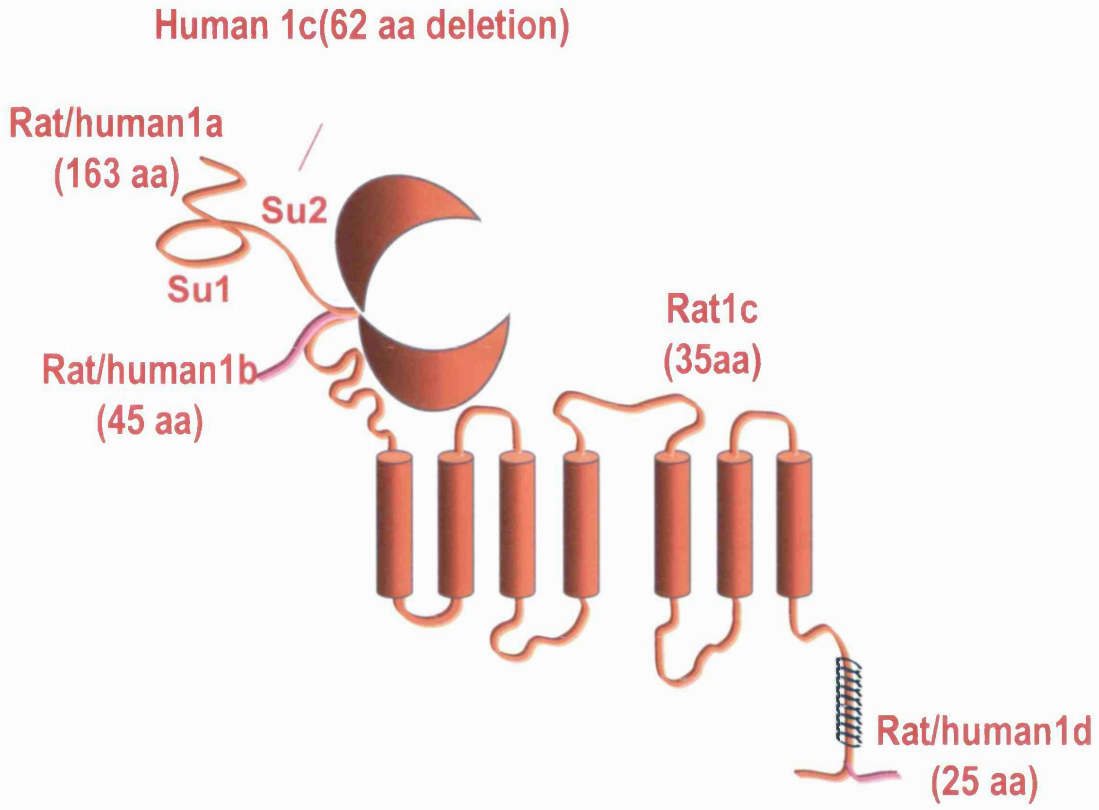


Figure.1.4 Schematic representation of $GABA_{B1}$ splice variants and specific domains. Su = Sushi domains. Green coil- coiled-coil domain.

1.3.3 $GABA_{B2}$

Given the aforementioned discrepancies in $GABA_{B1}$ pharmacology relative to that of native receptors, and the lack of surface expression of $GABA_{B1}$ in mammalian cell transfections a search was initiated by several groups to identify further $GABA_B$ receptors. At the end of 1998, three groups simultaneously reported the cloning of a third $GABA_B$ receptor with sequence homology to $GABA_{B1}$, which was subsequently termed $GABA_{B2}$ (Jones et al., 1998; White et al., 1998; Kaupmann et al., 1998). And followed shortly after by a fourth group the following year (Kuner et al.,1999). All groups identified a novel GPCR with 35% identity to and 54% similarity with the $GABA_{B1}$ receptor. The two $GABA_B$ receptor proteins were expressed in the same tissue regions and co-localised in transfected cells (Jones et al., 1998). Expression of $GABA_{B2}$ in recombinant systems, however, was not sufficient to confer responses to GABA, binding to known high affinity $GABA_B$ antagonists, or coupling to K^+ channels efficiently (Kaupmann et al., 1998). The $GABA_{B2}$ protein was, however, capable of reaching the plasma membrane, and indeed, co-expression of $GABA_{B1}$ and $GABA_{B2}$ in transfected cells resulted in cell surface expression of the $GABA_{B1}$ proteins (White et al., 1998). Functionally, expression of $GABA_{B1}$ and $GABA_{B2}$ in recombinant systems resulted in agonist affinities more closely resembling those observed in native receptors. Two studies have reported the existence of a functional $GABA_{B2}$ receptor in the absence of $GABA_{B1}$, whereby, inhibition of forskolin stimulated cAMP production was observed (Martin et al., 1999; Kuner et al., 1999). However, such findings may possibly be due to the type of expression systems used. For example in superior cervical ganglion cells there is a

low expression of $GABA_{B1}$ which upon microinjection of $GABA_{B2}$ may be sufficient to confir adenylate cyclase inhibition. More simply an overexpression of the receptor proteins in recombinant systems may be sufficient to result in some surface expression and erroneous coupling to effector systems. Indeed studies of $GABA_{B1}$ knock-out animals have shown that there is no $GABA_B$ receptor activity in the presence of $GABA_{B2}$ protein alone, and thus reports of $GABA_{B2}$ homomer function are probably incorrect (Prosser et al., 2000).

Detailed analysis of the protein structure of $GABA_{B2}$ reveals that it too possesses a coiled-coil domain in its intracellular C-terminus and, like $GABA_{B1}$, is homologous to LIV-BPs. Database mining has, however, failed to reveal any further $GABA_{B2}$ splice variants, at least which demonstrate any pharmacological diversity (See Figure 1.5).

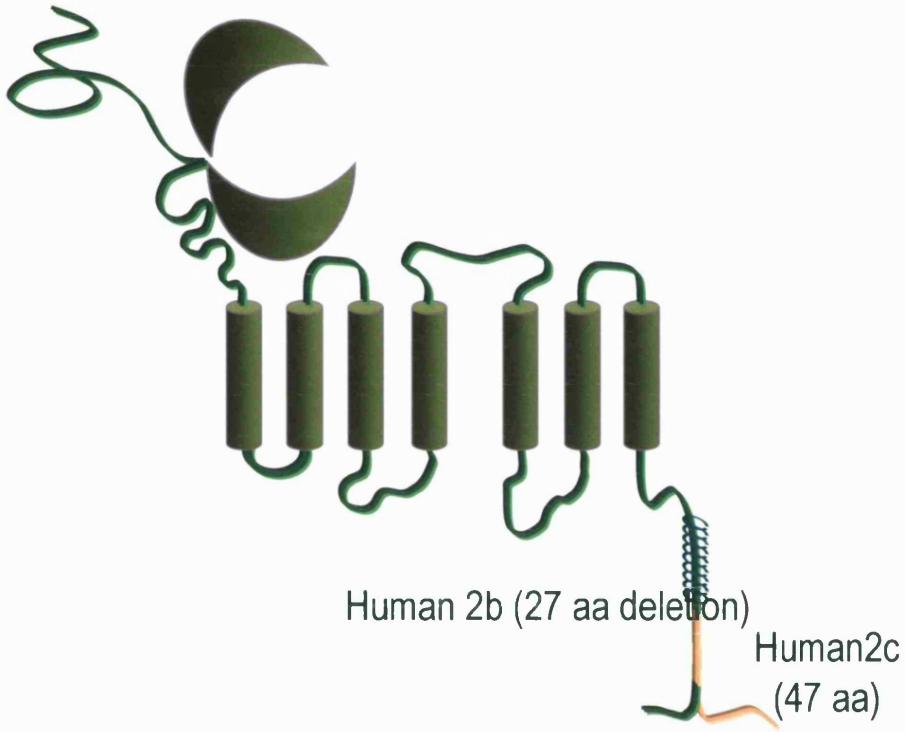


Figure 1.5 Schematic representation of $GABA_{B2}$ protein. Note the absence of the two Sushi domains observed for $GABA_{B1a}$.

1.3.4 Heterodimerisation

Until the identification of the $GABA_{B1}$ and $GABA_{B2}$ receptors, GPCRs had not been reported to form obligate heterodimeric complexes. However, the observations once again of a lack of receptor pharmacology akin to that of native tissues when $GABA_{B2}$ was transfected into mammalian cells prompted the hypothesis that $GABA_{B1}$ and $GABA_{B2}$ may heterodimerise to form a receptor with a pharmacological profile similar to that seen in native cells. Given that the co-expression of $GABA_{B1}$ and $GABA_{B2}$ resulted in cell surface expression it was possible that the two may together form a functional receptor. Indeed co-expression of $GABA_{B1}$ and $GABA_{B2}$ produces a receptor complex capable of binding GABA with affinities similar to that of native receptors. The $GABA_{B1}/GABA_{B2}$ complex activates K^+ channels and inhibits adenyl cyclase activity. Thus the first obligate heterodimeric GPCR was identified.

Since the initial identification of this $GABA_{B1}/GABA_{B2}$ receptor complex, many studies have investigated the associations, agonist binding and signal transduction mechanisms of the heterodimer. The heterodimerisation of the $GABA_{B1}$ and $GABA_{B2}$ receptor subunits is mediated, at least in part, by the coiled-coil domains present in both receptor subunits (White et al., 1998 and see Couve et al., 2000). However, this is not the only point of association between the two proteins, as deletion of either C-terminal does not prevent heterodimer formation. An RXR(R) motif at the bottom of the coiled-coil domain of the $GABA_{B1}$ subunit is primarily responsible for the intracellular retention of the $GABA_{B1}$ subunit within the endoplasmic reticulum (ER). Co-expression with $GABA_{B2}$ which results in dimerisation at, amongst others, the coiled-coil domain,

"masks" this retention motif and therefore allows trafficking of the heterodimer to the cell surface. The role of the $GABA_{B2}$ subunit is therfore to "chaperone" $GABA_{B1}$ to the cell surface where it is then available to bind GABA and signal via G-protein coupling (White et al., 1998). Mutation of the $GABA_{B1}$ subunit to delete the coiled-coil domain and remove the intra-cellular retention sequences results in translocation of the protein to the cell surface. However, this receptor was still non-functional (Calver et al., 2001; Margeta-Mitrovic et al., 2001). It has also been demonstrated that the proposed ligand binding domain of $GABA_{B2}$ is not capable of binding GABA since mutagenesis of the proposed $GABA_{B2}$ binding domain did not alter the ligand binding affinity at the $GABA_{B1}/GABA_{B2}$ receptor complex (Kniazeff et al., 2002).

Examination of the intracellular loops has revealed that in $GABA_{B1}$ there was little homology in the second intracellular loop in terms of overall charged units compared to that of the mGluRs, whereas the $GABA_{B2}$ receptor subunit is much less negatively charged and therefore, like the mGluRs is the more likely of the two receptor subunits to bind G-proteins (Robbins et al., 2001). Mutagenesis studies have revealed that G-protein signalling occurs through the $GABA_{B2}$ receptor subunit, as mutation of the $GABA_{B2}$ subunit to positively charged residues prevented G-protein coupling. Conversely, mutation of the positive residues in $GABA_{B1}$ to negatively charged or neutral amino acids resulted in the ability of $GABA_{B1}$ to bind G-protien (Robbins et al., 2001; Duthey et al., 2001). This work clearly suggests that $GABA_{B2}$ is not merely a chaperone for efficient $GABA_{B1}$ cell surface expression, but is itself critical for mediating G-protein coupling of the $GABA_B$ receptor complex. Further mutagenesis experiments have demonstrated that co-expression of $GABA_{B2}$ with a chimeric protein consisting of

the extracellular $GABA_{B1}$ sequence coupled to the transmembrane sequence of $GABA_{B2}$ is capable of not only binding agonists, but also of signalling G-proteins (Galvez et al., 2001).

The current prediction, therefore, suggests that a side-ways signalling mechanism occurs, whereby GABA binds to the N-terminal "Venus fly-trap" structure on the extracellular $GABA_{B1}$ subunit, inducing a conformational change in the second and third intracellular loops of $GABA_{B2}$ which then potentiates G-protein signalling (Robbins et al., 2001; Duthey et al., 2001 and see Calver et al., 2002) (See Figure 1.6).

Thus, as summarised by Table 1.1, $GABA_{B1}$ and $GABA_{B2}$ alone are non-functional. Heterodimerisation of the two receptor subunits results in fully functional cell surface expressed $GABA_B$ receptors.

$GABA_{B1}$	**$GABA_{B2}$**	**$GABA_{B1}$/$GABA_{B2}$**
Agonist binding affinity 100-150 fold lower than in native systems	No agonist binding affinity	Agonist binding affinity similar to that of native systems
Normal antagonist binding affinity	No antagonist binding affinity	Normal antagonist binding affinity
No coupling to effector systems e.g. K^+, Ca^{2+}	Little, or no, coupling to effector systems	Normal coupling effector systems
No cell surface expression	Expressed at cell surface	Both proteins expressed at cell surface

Table 1.1 Summary of functional roles of $GABA_B$ receptor subunits.

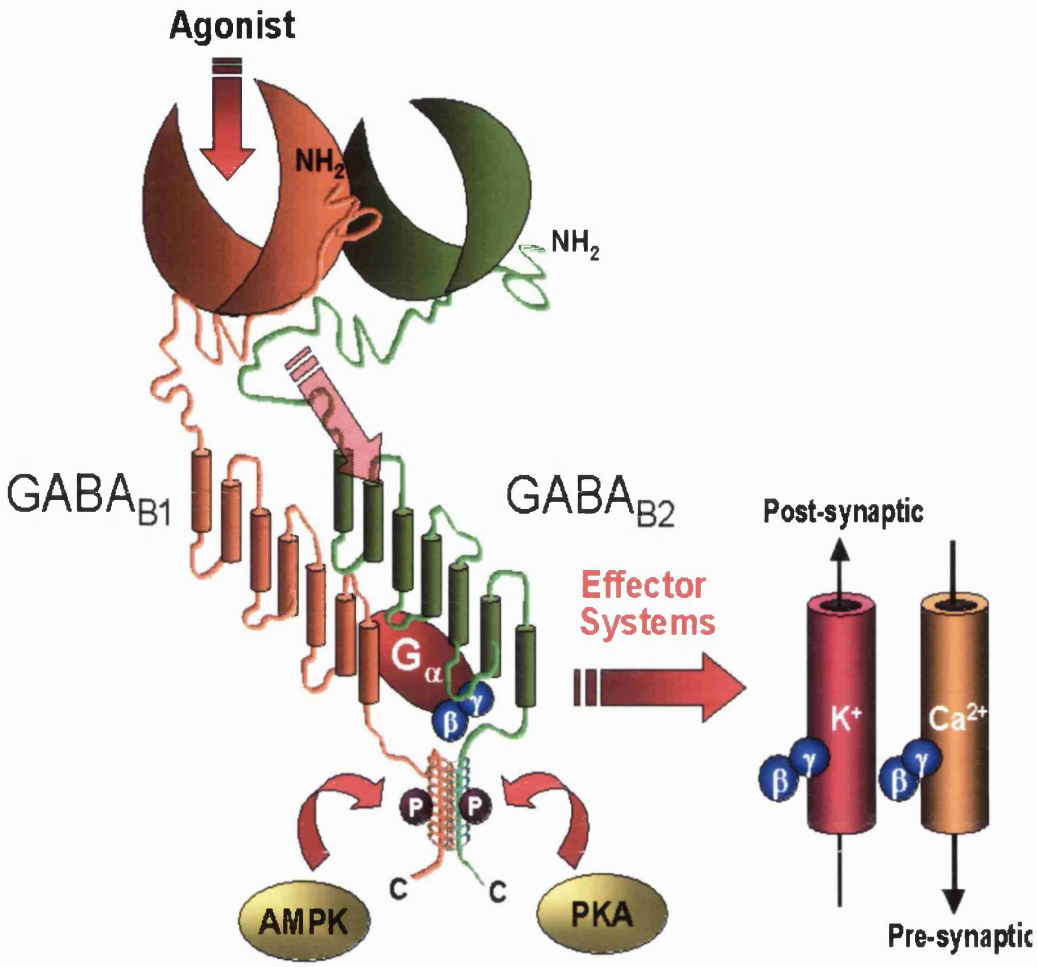


Figure 1.6 Schematic representation of the postulated side-ways signalling model for the $GABA_B$ receptor complex. (Taken from Calver et al., 2003).

1.3.5 Putative additional $GABA_B$ receptor subunits

Prior to its cloning in 1997 the $GABA_B$ receptor had been postulated to comprise a family of sub-types based on the heterogeneity of its pharmacological responses (Mott and Lewis, 1994; Banjeree and Snead, 1995; Teoh et al., 1996; Bonanno et al., 1997; Ong et al., 1998; Phelan, 1999 and see, Bonanno and Raiteri, 1993). The first hint of receptor heterogeneity arose from observations that L-baclofen could interact with two types of $GABA_B$ receptor, one which was phaclofen sensitive and the other phaclofen insensitive. Such observations were made using both electrophysiological recordings and biochemical analyses, whereby phaclofen failed to antagonise baclofen inhibited forskolin-stimulated cAMP formation in neocortical slices, but readily antagonised this effect in other preparations. Regional variation in $GABA_B$ receptor pharmacology has also been reported (Raiteri et al., 1989; Bonnanno and Raiteri, 1992, 1993). Studies of $GABA_B$ autoreceptors in the spinal cord revealed that GABA and 3-APPA (a $GABA_B$ agonist) inhibited GABA release, whereas L-baclofen was a very weak agonist in this structure, yet in the cerebral cortex L-baclofen inhibited GABA release with equal potency to GABA. Together these observations led Bonanno and Raiteri to postulate a heterogenous family of $GABA_B$ receptors based on their pharmacological responses (see Fig. 1.7).

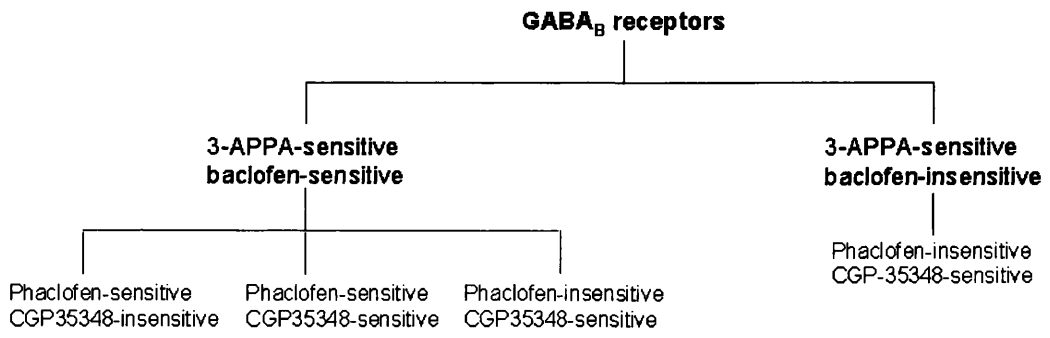


Figure 1.7 Demonstrating the pharmacological diversity of the $GABA_B$ receptor (Taken from Bonanno and Raiteri, TINS, 1993.)

Therefore on this basis of pharmacological diversity alone, $GABA_B$ receptors could be sub-classified into four different $GABA_B$ receptor subtypes. In light of this proposed pharmacological diversity, it is therefore surprising that the recent cloning of the $GABA_{B1}$ and $GABA_{B2}$ receptor subunits, despite the identification of numerous splice variants (Isomoto et al.,1998; Calver et al., 2000), has effectively identified only the $GABA_{B1}/GABA_{B2}$ receptor complex. This $GABA_{B1}/GABA_{B2}$ receptor complex despite thorough analysis and several reports to the contrary (Ng et al., 2001; Bertrand et al., 2001), does not demonstrate any differences in its pharmacological profiles (Lanneau et al., 2001; Jensen et al., 2002), regardless of the splice variant composition of the two heteromeric partners. Much effort has, therefore, been afforded by several groups to try to identify further novel $GABA_B$ receptor subunits. Numerous $GABA_{B1}$ splice variants have been described (Isomoto et al., 1998; Calver et al., 2000; Schwarz et al., 2000; Martin et al., 2001). Of the $GABA_{B1}$ splice variants none have so far displayed any pharmacological differences, although little work has been carried out to confimr this. The most interesting of the $GABA_{B1}$ splice variants is the $GABA_{B1e}$ isoform identified in rat and human (Schwarz et al.,

2000). This isoform encodes only the extracellular ligand binding domain of $GABA_{B1}$ and has been identified as the primary isoform in peripheral tissues. However, $GABA_{B1e}$ does not bind antagonist, activate K^+ channels, or inhibit forskolin induced cAMP production, whether expressed alone or as a heterodimer with $GABA_{B2}$ (Schwarz et al., 2000). Analysis of the $GABA_{B2}$ splice variants revealed that infact these were not real and were probably identified as a result of mRNA degredation (Calver et al., 2000; Martin et al., 2001). Given that the splice variants of the characterised $GABA_{B1}$ receptor subunit did not demonstrate any pharmacological or localisation differences from each other, alternative approaches have been used to identify further $GABA_B$ receptors. Much of this has centered around the use of the yeast-2-hybrid screen where portions of the $GABA_{B1}$ or $GABA_{B2}$ receptors have been used to "fish" for other proteins from cDNA libraries which are capable of interacting at a molecular level with the known $GABA_B$ receptors.

Recently a German based group reported the cloning and functional expression of a novel $GABA_B$ receptor subunit from drosophila (Mezler et al., 2001). This third $GABA_B$ receptor subunit appears to be insect specific, as the group were unable to identify any mammalian counterparts. Baclofen was not capable of producing a functional response at the drosophila $GABA_{B3}$ receptor when expressed alone or in any combination with the other $GABA_B$ receptor subunits. Our group at GlaxoSmithKline subsequently identified a novel human $GABA_B$ receptor-like protein from database mining experiments (Calver et al., 2003). However, as with the drospohila $GABA_{B3}$ subunit, no function has yet been ascribed to this protein when expressed in isolation or in combination with other $GABA_B$ receptor subunits. Thus, further efforts are required to ellucidate the

native ligands or chaperone proteins required for the function of these novel $GABA_B$ receptor-like proteins. Until such ligands or other protein partners are identified, they remain orphan GPCRs.

1.4 Tissue distribution of $GABA_B$ receptors

1.4.1 $GABA_B$ receptors in peripheral tissues

An issue of great debate in the $GABA_B$ receptor field, since the initial cloning of the first receptor subunit five years ago, has been the distribution of these receptors in the CNS and in the periphery. Understandably the distribution of individual $GABA_B$ receptor subunits, at both the mRNA and protein level,was important for identifying regions in which one of the obligate subunits was present in the absence of the other, yet with the known existence of functional receptors. Such a finding would therefore direct the search for novel $GABA_B$ receptor subunits. Analysis at the mRNA level by semi-quantitative Taqman analysis has revealed that in peripheral tissues the $GABA_{B1a}$ and $GABA_{B1b}$ receptor subunits were expressed at relatively abundant levels in spleen, uterus, lung, intestine and prostate (Calver et al., 2000). Functional $GABA_B$ receptors have been described in the lower oesophagal sphincter (Lehmann et al., 1999; Smid and Blackshaw, 2000; Lidmus et al., 2000). However, $GABA_{B2}$ receptor subunit expression was striking in its absence in all peripheral tissues examined (Castelli et al., 1999; Calver et al., 2000). Since tissues, such as the uterus and intestine, have been shown to possess functional $GABA_B$ receptors, the absence of $GABA_{B2}$ is unexpected with respect to the known obligation of $GABA_{B1}$/$GABA_{B2}$ heterodimerisation for functional $GABA_B$ receptors.

1.4.2 $GABA_B$ receptors in the CNS

$GABA_B$ receptors are widely expressed in the CNS, as shown by early autoradiographic studies which demonstrated a widespread distribution of bound radioligands in numerous species (Gehlert et al., 1985; Bowery et al., 1987; Price et al., 1987; Chu et al., 1990; Shaw et al., 1991; Veenman et al., 1994). Following the cloning of the $GABA_{B1}$ and $GABA_{B2}$ receptor subunits many groups have assessed the distribution across numerous tissues and species of the $GABA_{B1}$ receptor mRNA (Bischoff et al., 1999; Calver et al., 2000; Liang et al., 2000) and protein (Margeta-Mitrovic et al., 1999; Sloviter et al., 1999). The $GABA_{B1}$ splice variants, $GABA_{B1a}$ and $GABA_{B1b}$ have also been examined (Fritschy et al., 1999; Poorkhalkali et al., 2000; Princivalle et al., 2000; Towers et al., 2000). The distribution of the $GABA_{B2}$ receptor subunit by mRNA (Durkin et al., 1999; Clark et al., 2000) and protein (Calver et al., 2000; Towers et al., 2000) has also been investigated, though not in direct comparison to $GABA_{B1}$. All localisation techniques have demonstrated a widespread distribution of both receptor subunits throughout the CNS. Indeed, the expression of the receptors is in general agreement with reports of functional $GABA_B$ receptors identified in hippocampus, cortex, cerebellum and spinal cord. In general the distribution of $GABA_{B1}$ and $GABA_{B2}$ receptor subunits in the CNS overlaps, consistent with the obligate heterodimeric nature of the functional receptor. However, there have been some reports of disparities between expression of $GABA_{B1}$ and $GABA_{B2}$ subunits. For example, mRNA analysis revealed the expression of $GABA_{B1}$ but not $GABA_{B2}$ in the caudate putamen (Jones et al., 1998; Kaupmann et al., 1998; Durkin et al., 1999; Clark et al., 2000). Such reports have not been borne out by the more recent

studies of other groups (Billinton et al., 1999; Calver et al., 2000) and thus the lack of co-localisation of the two receptor subunits in this region appears less likely.

Much emphasis has been placed on identifying the synaptic localisation of the $GABA_B$ receptor subunits, in particular the pre- versus post-synaptic localisation of the $GABA_{B1}$ receptor subunit splice variants $GABA_{B1a}$ and $GABA_{B1b}$. Initial mRNA *in-situ* hybridisation studies assessing the relative distribution of $GABA_{B1}$ receptor subunit splice variants suggested that $GABA_{B1a}$ was expressed pre-synaptically whereas $GABA_{B1b}$ was expressed post-synaptically (Bischoff et al., 1999; Liang et al., 2000). However, others have reported contradicting data suggesting that $GABA_{B1a}$ is also expressed post-synaptically and $GABA_{B1b}$ pre-synaptically at both the mRNA and protein levels (Benke et al., 1999; Poorkhalkali et al., 2000). To add further to the controversy it has become apparent that some differences in pre- and post-synaptic localisation may be region specific. In the dorsal root ganglia, Towers et al., (2000) reported that more than 90% of the primary afferent neurons were positive for $GABA_{B1a}$ mRNA, with low expression of this subunit in the dorsal horn of the spinal cord, suggesting a pre-synaptic expression for $GABA_{B1a}$. In agreement with this several groups report pre-synaptic $GABA_{B1a}$ mRNA in the granule cell region of the cerebellum (Kaupmann et al., 1998b; Billinton et al., 1999; Bischoff et al., 1999). The granule cells of the cerebellum project to the molecular layer where they activate the cell bodies of Purkinje cells, thus suggesting a pre-synaptic localisation of $GABA_{B1a}$. However, autoradiographic binding studies of $GABA_B$ receptors suggest the converse, with a high density of labelling in the molecular cell layer, indicating a post-synaptic preponderance in this region for $GABA_B$

receptors. Though, in the thalamo-cortical system, $GABA_{B1a}$ has been described post-synaptically in the cell bodies (Princivalle et al., 2000). More recently still the ultrastructural localisation of the $GABA_B$ subunits has been assessed using subunit specific antisera at the level of the electron microscope. These studies have been performed in the cerebellum (Ige et al., 2000), visual cortex (Gonchar et al., 2001) and basal ganglia (Charara et al., 2000). At this level all groups report the existence of both $GABA_{B1a}$ and $GABA_{B1b}$ in both pre- and post-synpatic membranes. All in all it therefore appears unlikely that differences in $GABA_{B1}$ splice variant expression, pre- or post-synaptically, can account for the pharmacological heterogeneity described previously.

1.5 $GABA_B$ receptors and disease

The $GABA_B$ system is implicated in a wide variety of diseases of the CNS and also of the periphery. Agonists and antagonists of $GABA_B$ receptors have been shown to be efficacious in terms of producing anti-nociception, epileptogenesis, central muscle relaxation and cognitive impairment. In the periphery the $GABA_B$ system is implicated in control of bronchial relaxation, gastric motility, hypotension and cough. Hormonal release has also been linked to $GABA_B$ control, particularly in terms of corticotrophin releasing hormone, melanocyte stimulating hormone and prolactin releasing hormone (see Table 1.2).

Effect	Agonist / Antagonist	Site of action	Possible mechanism(s) of action	Effect of Agonist/ Antagonist on response
Anti-spastic	Agonist	Spinal cord excitatory pre-synaptic terminals	Suppression of release of excitatory neurotransmitter on to neurons. Possible post-synaptic effect on reflex neurons	Antagonist blocks effect
Analgesic	Agonist	Spinal cord C and Aδ sensory primary afferent terminals	Suppression of release of neurotransmitter from small diameter primary afferents	Antagonist blocks Agonist effect and enhances nociception produced by inflammation
Reversal of drug craving	Agonist	Mesolimbic region, probably ventral tegmental area		Antagonist blocks Agonist effect
Anti-asthma	Agonist	Bronchi	Inhibition of neural release of ACh and SP onto airways of smooth muscle, decrease in hypersensitivity reaction	
Anti-absence epilepsy	Antagonist	Thalamo-cortical neurons	Suppression of GABA-mediated Ca^{2+} spikes	Agonist exacerbates seizure activity
Cognitive enhancement	Antagonist	Cerebral cortex/ hippocampus		Agonist produces cognitive impairment in rats
Anti-depressant	Antagonist		Modulation of catecholamine release	Agonist oppopsite action
Neuroprotection	Antagonist		Enhancement of neurotrophin release	
Anxiolytic	Antagonist		Blockade of autoreceptors on GABA terminals	

Table 1.2 Summary of disease in which $GABA_B$ agonists and antagonists have been used. (Adapted from Bowery, "$GABA_B$ Receptors" In: Understanding G protein-coupled receptors and their role in the CNS. Edited by Pangalos and Davies, 2002).

1.5.1 $GABA_B$ and spasticity

The earliest application of a $GABA_B$ receptor agonist was for the treatment of spasticity, in which an increase in release of excitatory neurotransmitters onto the motor neurons of the spinal cord results in the clinical syndrome of spasticity. Baclofen was developed by Ciba-Geigy for the

symptomatic treatment of this disease, and is postulated to act at pre-synaptic heteroreceptors to reduce the release of the excitatory neurotransmitters. However, more recently it has been proposed that baclofen acts post-synaptically to reduce excitation of the target motorneurons (Orsnes et al., 2000). Systemic administration of baclofen does, however, cause untolerable side-effects in some patients including sedation, drowsiness, fatigue, confusion, dizziness, hypertonia and ataxia (Abbruzzese, 2002), and is therefore administered intrathecally. Baclofen is particularly effective at treating the symptomatic spasticity associated with CNS injury, multiple sclerosis, tardive dyskinesia and tetanus (Ochs et al., 1989; Penn and Mangieri, 1993; Albright et al., 1996; Dressler et al., 1997; Auer et al., 1999; Becker et al., 2000; Trampitsch et al., 2000).

It is the action of baclofen at both pre- and post-synaptic receptors in the periphery and in the CNS that is responsible for its large side-effect profile. Compounds designed to specifically target either pre- or post-synaptic receptors should enable a reduction in such side-effects. However, until such time as selective, clean, $GABA_B$ receptor agonists are identified baclofen will remain the drug of choice in treating spasticity.

1.5.2 $GABA_B$ and pain

Baclofen has been associated with antinociception in rodent models and human pain states. For example pain relief is gained in human trigeminal neuralgia with baclofen treatment (Fromm, 1994). Pain syndromes following cerebral ischaemia and spinal cord injury have also been shown to be relieved by baclofen in the clinic, and more recently a report has suggested a role for baclofen in migraine prohylaxis (Hering-Hanit, 1999). What remains unclear is the site of

action of baclofen in treating pain. At the level of the spinal cord $GABA_B$ receptors are located predominantly at the terminals of the small diameter primary afferents known to be sensitised during pain (Price et al., 1987). It is proposed that baclofen acts at these pre-synaptic sites to reduce the release of the sensory, excitatory neurotransmitters Substance P, CGRP and glutamate (Malcangio and Bowery, 1996; Riley et al., 2001). Additionally, at the level of the dorsal root ganglia, baclofen may excert an action at a particular subset of voltage-dependent Ca^{2+} channels, the nifedipine sensitive Ca^{2+} channel (Voisin and Nagy, 2001). However, the action of baclofen in antinociception may also occur in higher brain centres such as the thalamus and cortex (Thomas et al., 1995). Baclofen is believed to inhibit ascending noradrenergic and dopaminergic pathways but to also activate descending noradrenergic pathways possibly via the locus coeruleus (Sawynok, 1984; Fromm, 1994). Baclofen therefore has the ability to act as a double analgesic by reducing ascending excitation and descending excitatory mechanisms which contribute to hyperalgesia. Animal models have shown that baclofen is a potent analgesic in several acute pain models (Przesmycki et al., 1998). However, baclofen appears less consistent in treating inflammatory pain as demonstrated by formalin, carageenan or Freund's complete adjuvant models (Sharma et al., 1993; Dirig and Yaksh, 1995; Shafizadeh et al., 1997). The inability of baclofen to act as an analgesic during ongoing pain may reflect the involvement of $GABA_B$ in episodic and $GABA_A$ in ongoing pain states (Fromm, 1994). Importantly, baclofen's actions as an antinociceptive agent are produced at doses lower than those required to produce muscle relaxation in spasticity. Nevertheless, $GABA_B$ agonists may still reduce motorneuron activity to a sufficient degree to affect the behavioural read-outs utilised in most pre-clinical

models. Thus caution should be used in interpretating such analgesic effects of agonists (Castro-Lopes, 2000). Finally, $GABA_B$ agonists may also work in conjunction with morphine, as they have been shown to enhance some of the analgesic effects of morphine. As such they may be useful in allowing lower levels of administration of morphine which is associated with more severe side-effects (Gordon et al., 1995).

1.5.3 $GABA_B$ and epilepsy

Epilepsy is a common neurological disorder, characterised by spontaneous events, termed siezures, which are generally unprovoked. Partial seizures are a result of spontaneous and synchronised burst firing in specific regions of the brain. They include both absence seizures, whereby patients freeze and gaze into space, and myoclonic, tonic and tonic-clonic seizures types which produce rhythmic contraction of certain muscle groups. Generalised seizures arise from synchronus burst firing which initates in one locus and spreads across the brain resulting in seizures of the myoclonic, tonic and tonic-clonic classifications.

$GABA_B$ receptors are primarily associated with absence epilepsy, where they initiate spike-wave discharges in the thalamus via actiavtion of Ca^{2+} spike generation (Crunelli and Leresche 1991). A genetic rodent model of absence epilepsy, the GAERS rat (Genetic Absence Epilepsy Rat from Strasbourg) has been a useful model in understanding the role of $GABA_B$ in this disease. GAERS rats exhibit spike-wave discharges visible by EEG, which can be dose-dependently reduced by $GABA_B$ receptor antagonists (Marescaux et al., 1992). Further evidence supporting the role of the metabotropic $GABA_B$ receptors in absence seizure is that the G_i/G_o G-protein blocker, pertusis toxin, is capable of

blocking absence seizures in GAERS, and $GABA_B$ receptors have been shown to signal through these G-proteins to allow coupling to Ca^{2+} and K^+ channels. $GABA_B$ receptors have been linked to generalised idiopathic epilepsy in that the gene encoding the $GABA_{B1}$ receptor maps to a chromosomal region which is a major susceptibility locus for common generalised epilepsy (Sander et al., 1999). The major factor preventing the use of $GABA_B$ receptor antagonists in the treatment of absence epilepsy, however, is that $GABA_B$ antagonists at high doses are pro-convulsant in cortical and limbic structures (Vergnes et al., 1997). However, this is not true for all $GABA_B$ receptor antagonists, for example Richards and Bowery (1996) were unable to identify any convulsant activity with the antagonist SCH50911 at doses up to 100-fold higher than those required to block absence seizures. This is yet another example of the heterogeneity associated with $GABA_B$ receptor pharmacology, and which highlights the importance of identifying the molecular determinants of such heterogeneity to allow the full exploitation of the $GABA_B$ system.

More recently several lines of evidence have suggested an excitatory role for GABA in the human sclerotic hippocampus (Cohen et al., 2002). In their studies, the French group, identified depolarising synaptic GABA response in subicular pyramidal cells, and suggest that abberant plastic changes in the subiculum may play a role in the generation of temporal lobe epilepsy. Furtherstill, the generation of $GABA_{B1}$ knock-out animals which display generalised epilepsy has further indicated a role for $GABA_B$ receptors in epilepsy (Prosser et al., 2001; Schuler et al., 2001). Both groups reported that animals lacking the $GABA_{B1}$ receptor subunit gene were viable until the third week of life, and Prosser et al., describe this as resulting in eventual death by approximately

post natal day 19-21. In both instances no $GABA_B$ receptor activity could be identified, implying that the absence of one member of the obligate heterodimer is sufficient to abolish $GABA_B$ receptor function and induce generalised epilepsy.

1.5.4 $GABA_B$ and addiction

The ability of baclofen to reduce drug, alcohol or nicotine craving is an important breakthrough in identifying additional therapeutic benefits of $GABA_B$ agonists (Daoust et al., 1987; File and Andrews, 1993; Colombo et al., 2000). Studies have revealed that rats trained to self-administer a variety of addictive drugs, such as heroin, alcohol or nicotine, and which are then dosed with baclofen, or the selective agonist CGP44532, show a reduction in self-administration of the addictive compounds (Brebner et al., 1999; Xi and Stein, 1999). Baclofen may act by reducing the reinforcing properties of addictive drugs by inhibiting the drug-stimulated release of dopamine into the mesocorticolimbic system. Indeed, when baclofen was injected directly into the ventral tegmental area, the nucleus accumbens or the striatum, significantly reduced drug self-administration was seen with administration into the ventral tegmental area compared with the nucleus accumbens or striatum (Kalivas and Duffy, 1995). Some clinical evidence exists to suggest the effectiveness of baclofen in reducing drug craving. Addolorato et al., (2000) report that baclofen is capable of reducing both alcohol intake and craving in alcoholics, and cocaine addicts show a reduction in drug use when treated with baclofen (Ling et al., 1998). There are reports that the anti-epileptic agent, Gabapentin, is also able to reduce cocaine craving (Myrick et al., 2001). However, despite recent reports by the group of Ng (2001) identifying Gabapentin as an agonist selective for the $GABA_{B1a}/GABA_{B2}$

heterodimer, this has not been confirmed by studies of other groups (Lanneau et al., 2001; Jensen et al., 2002) and the mechanism of action of Gabapentin at $GABA_B$ receptors remains unsubstantiated.

1.5.5 $GABA_B$ and neuroprotection

Whether neuroprotection can be afforded by $GABA_B$ agonists or antagonists is currently debatable. At very high doses baclofen has been shown to be neuroprotective in cerebral ischaemia in the gerbil (Lal et al., 1995) but not in the rat (Rosenbaum et al., 1990). Other studies suggest baclofen is capable of preventing the neurotoxicity of quinolinic acid in the rat hippocampus (Beskid et el., 1999). However, $GABA_B$ receptor activation has also been shown to increase the neurotoxicity of NMDA in murine striatal cultures (Lafoncazal et al., 1999). Other investigators have studied the effectiveness of $GABA_B$ receptor anatgonists in neuroprotection and shown that in the hippocampus, cortex and spinal cord the levels of nerve growth factor (NGF) and brain derived neurotrophic growth factor (BDNF) can be raised upon administration of the selective anatagonists CGP36742, CGP56433A or CGP56999A systemically which may offer neuroprotection (Heese et al., 2000). Thus until the issue of species differences and whether agonists or anatgonists will be suitable is addressed, the potential for $GABA_B$ targeted compounds in neuroprotection remains unclear.

1.5.6 $GABA_B$ and depression

Investigations into a potential role for $GABA_B$ antagonists in treating depression are still unclear. In rodent models the antagonist CGP36742 has been shown to reduce behaviours associated with depression (Nakagawa et al., 1999).

Additionally the antagonist induced increases in nerve growth factors (see 1.5.5 $GABA_B$ and neuroprotection) may also be linked to an anti-depressive action of $GABA_B$ blockade since prescribed anti-depressants have been shown to have similar effects. However, a role for $GABA_B$ agonists in treating depression also exists in that systemic baclofen has been shown to increase the release of serotonin from the raphe nuclei and its associated projection areas, a mechanism similar to that on which the majority of today's anti-depresant therapies are based (Abellan et al., 2000a,b). Further evidence pointing towards the use of $GABA_B$ agonists to treat depression comes from studies in which anti-depressant treatment significantly increased $GABA_B$ binding sites in the rat. This effect was also shown by other anti-depressive treatments such as electro-shock, but was not elicited by anti-psychotics or anxiolytics confirming that this effect is likely specific to depression and not other psychiatric disorders (Lloyd et al., 1985; Szekely et al., 1987; Motohashi, 1992). Indeed, Reserpine, which is associated with the induction of depression lead to significantly reduced $GABA_B$ binding in the frontal cortex (Lloyd et al., 1985). Clinically, baclofen has been shown to reduce panic attack occurance in patients with mood disorders, but is only anti-depressive in some but not all patients (Lloyd et al., 1987; Post et al., 1991).

1.5.7 $GABA_B$ and cognition

Animal models of cognition have revealed that antagonists are capable of improving performance in a number of tasks, thought to be due to an enhancement of cholinergic and glutamatergic neurotransmission (Mondadori et al., 1993, 1994, 1996a,b). Indeed baclofen induced cognitive deficits in rats were reversed by administration of the $GABA_B$ antagonist phaclofen (DeSousa et al.,

1994). Confirming the role of a G-protein coupled system in cognition is the report that baclofen induced amnesia can be prevented by intracerebroventricular administration of the G-protein inhibitor PTX (Galeotti et al., 1998).

1.5.8 $GABA_B$ and schizophrenia

Numerous studies link the involvement of $GABA_B$ with the psychotic syndromes of schizophrenia. At the genetic level, the $GABA_{B1}$ gene is present on a region associated as a major susceptibility locus for schizophrenia. Interestingly, this locus is also associated with generalised epilepsy and there is evidence to suggest that many epileptic sufferers also develop schizophrenic symptoms (Roberts et al., 1990). At the protein level it has been reported that in the hippocampus, entorhinal and inferior temporal cortex there is a reduction in $GABA_B$ immunoreactivity in the principle cells, but protein levels remain unchanged in interneuron populations (Mizukami et al., 2000, 2002). The $GABA_{B1}$ heterozygous mouse demonstrates enhanced pre-pulse inhibition, a marker of anti-psychotic behaviour (Prosser et al., 2001). This data suggests that a $GABA_B$ receptor anatagonist would, therefore, be anti-psychotic. That schizophrenic pateints demonstrate reduced $GABA_B$ protein may either represent a down-regulation of $GABA_B$ in the brain to self-protect against psychosis or may instead represent a result of long-term anti-psychotic treatment which itself has produced a down-regulation in $GABA_B$ as a protective mechanism. However, to date no clinical evidence exists demonstrating an effect of $GABA_B$ agonists or antagonists in treating schizophrenia.

1.5.9 $GABA_B$ and the periphery

As mentioned in Table 1.2 $GABA_B$ is proposed to be involved in a number of peripheral indications including hiccup, bowel function and cough. Baclofen is used in the clinic as a treatment for intractable hiccups (Kumar and Dromerick, 1998; Guelad et al., 1995), a mechanism thought to be mediated at least in part via the raphe magnus (Oshima et al., 1998). Studies of the antitussive nature of baclofen in cats and guinea-pigs (Bolser et al., 1994) have been borne out in man whereby low doses of baclofen reduced cough (Dicpinigaitis and Dobkin, 1997).

1.6 Inhibitory control of the CNS

The function, control and activity of the central nervous system is comprised of a balance between excitation and inhibition. Neurotransmitters and neuropeptides modulate this balance of activity by inducing either excitatory or inhibitory modulation of the target cell. The major excitatory neurotransmitter in the CNS is glutamate which acts by exerting an excitatory post-synaptic potential in the target neuron. A second excitatory neurotransmitter is acetylcholine, whilst other neurotransmitters including noradrenalin, dopamine and serotonin are more frequently described as neuromodulators since their activity can be either excitatory or inhibitory depending on the state of the target cell (Johnston and Amaral, 1998). Inhibitory neurotransmission is principly regulated by the neurotransmitter GABA (γ-aminobutyric acid), though other inhibitory neurotransmitters include glycine and serotonin, acting through the $5HT_3$ receptor, exist.

The widespread distribution of GABAergic neurons throughout the brain is an indication of the wide-ranging inhibition of this neurotransmitter in the CNS.

Much of this thesis focuses on the distribution of the $GABA_B$ receptor subunits in the rat CNS, and thus it is prudent to describe how this might tie in with the neuronal circuitry in which GABA is implicated.

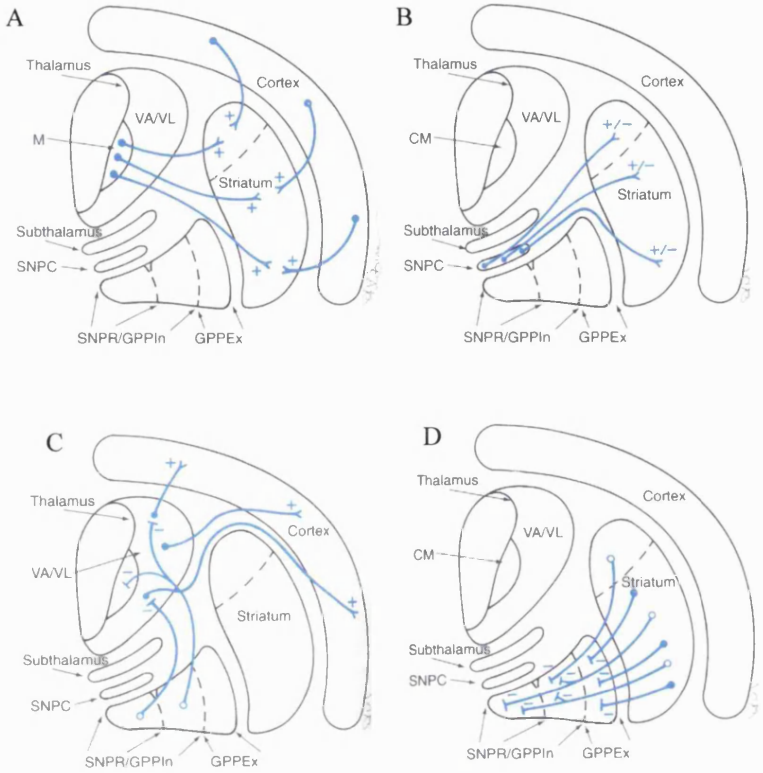
GABA was initially implicated as an inhibitory neurotransmitter which exerted its effects via local circuitry in inhibitory interneurons. GABAergic inhibitory interneurons exist as an heterogenous population of cells throughout the CNS, exhibiting a variety of sizes and morphologies according to their local environment. Interneurons which have been described as possessing GABAergic inhibitory properties include the stellate, basket and Golgi cells of the cerebellum, basket cells of the hippocampus and periglomerular, short axon and granule cells of the olfactory bulb (see Ottersen et al.,1994). As such these GABAergic interneurons are believed to regulate the flow of sensory information (See Figure 1.8).

GABAergic projection neurons are also a prominent mechanism via which GABA exerts modulation over the intrinsic activity of the CNS. GABAergic projection systems include those from the cortex to the globus pallidus, interpeduncular nucleus and substantia nigra pars reticulata. In turn, these nuclei project to the thalamus and superior colliculus. The thalamus then sends a GABAergic projection back to the cortex, thus completing the loop. Other GABAergic and therefore inhibitory loops include that of the habenula and the interpeduncular nucleus, the nucleus accumbens to the substantia nigra and the ventral pallidum to the superior colliculus and mediodorsal thalamic nuclei. Hypothalamic GABAergic projections also extend to the neocortex, and the medial septum innervates the hippocampus via a GABAergic mechanism. At more caudal regions of the CNS, GABAergic projections are found in the

cerebellum whereby the Purkinje cells of the cerebellar cortex project to the deep cerebellar nuclei which in turn send GABAergic fibres to the inferior olive. In turn the mossy fibres which project to the granule cells of the cerebellum are under the control of the pretectal nuclei and zona incerta, again which are GABAergic (see Ottersen et al., 1994). More peripherally, the primary afferent fibres of the dorsal root ganglia are GABAergic and project to the dorsal horn of the spinal cord, specifically to lamina II and IV.

Thus it is fair to say that the GABAergic control over the CNS is wide-spread with inhibitory interneurons controling the local environment and projection pathways spanning large regions of the brain to control distant networks.

Figure 1.8 Schematic diagrams illulstrating a few of the major neuronal pathways in the central nervous system and their excitatory/inhibitory role. A) Cortical and centromedian inputs to the striatum are mainly excitatory. B) The nigrostriatal pathway is both excitatory and inhibitory. C) Showing pathways from the Globus Pallidus and Substantia nigra pars reticulata to the cortex and from the thalamus to the cortex, which contains a mixture of inhibitory and excitatory projections. D) The subthalamic nuclei interconnect with the substantia nigra in a reciprocal excitatory/inhibitory manner. E) The cerebrocerebellar circuitry includes projections from the pons to the cerebellum, and to the cerebral cortex via the red nucleus and the thalamus. Diagrams adapted from Functional Neuroscience by Oswald Stewart.


A
Thalamus
Cortex
VA/VL
M
Striatum
Subthalamus
SNPC
SNPR/GPPIn
GPPEx
B
Thalamus
Cortex
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CM
+/−
+/−
Striatum
+/−
Subthalamus
SNPC
SNPR/GPPIn
GPPEx
C
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Cortex
VA/VL
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Subthalamus
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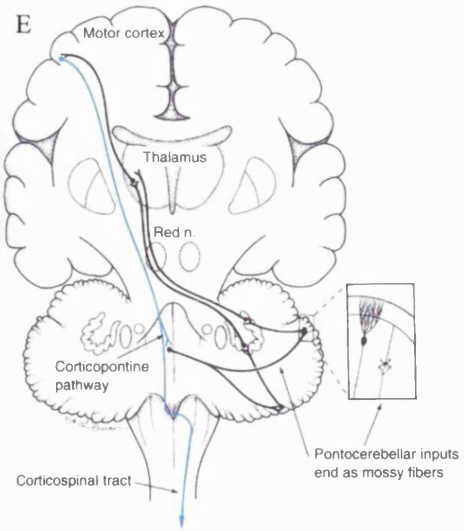

E
Motor cortex
Thalamus
Red n.
Corticopontine
pathway
Corticospinal tract
Pontocerebellar inputs
end as mossy fibers

1.7 Aims of this study

The molecular cloning of the $GABA_B$ receptor subunits in 1997 and 1998 has revealed the cDNA sequence for this ellusive receptor. Studies of the protein distribution of the receptor subunits in the central nervous system are expected to reveal further insights into the role of the $GABA_B$ receptor subunits in physiology. At the commencement of my studies little was known about the comparative localisation of the two main receptor subunits, the types of cells in which they are expressed in the CNS and any other potential additonal proteins which may have a role in $GABA_B$ receptor function. The aims, therefore, of this thesis are:

1) To map the comparative distribution of $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ receptor subunit proteins by immunohistochemistry through the rat central nervous system using subunit specific polyclonal antisera.

2) To identify the non-neuronal cell types in the rat central nervous system which express any or all of the $GABA_B$ receptor subunit proteins by co-localisation using immunohistochemistry.

3) To identify further potential $GABA_B$ receptor subunits and map their distribution in the rat central nervous system using specific polyclonal antisera.

Chapter 2 – Material and Methods

2.1 Cell culture

Cell culture, cell transfection and primary hippocampal culture were kindly carried out by Mrs. D Mitchel and Dr. S. Jourdain.

2.1.1 HEK293, 1321-N1 and COS cells

All work involving the culture of mammalian cells was performed within a class 2 biological saftey cabinet, maintained in an aseptic manner. All liquids aspirated from cell cultures were placed into vessels containing at least 1% virkon. Cells were split from previously grown cultures and placed into 10ml pre-warmed Ca^{2+}/Mg^{2+} free PBS (Gibco) (37°C). Cells were washed in Ca^{2+}/Mg^{2+} free PBS containing 0.25% trypsin (Gibco) at approximately 1-2ml/25cm^2 surface area to detach cells. Trypsin action was stopped by addition of culture medium. Suspended cells were then placed into fresh culture flasks to grow in a CO_2 incubator at 37°C for 4 days.

2.1.1.1 Transient cell transfection

The LipopfectAMINE Plus reagent was used for the transfection of eukaryotic cells as an efficient method requiring lower DNA concentrations. On the day of transfection, cells should be at approximately 50-80% confluence. Prior to transfection the DNA was complexed with the Plus reagent in a serum medium, 3μg of DNA was used for a flask size of 75cm^2. The mixture was incubated for 15min at room temperature. LipofectAMINE reagent was then diluted into serum free media and mixed thoroughly, combined with the pre-complexed DNA and incubated for a further 15min at room temperature. During the second incubation

cells were washed with serum free media and then the DNA-Plus-LipofectAMINE complex was added to the cells and incubated at 37°C for 3h in 5% CO_2. Approximately 5ml of serum containing media was then added to the cell flasks and incubated for 24h at 37°C in 5% CO_2. Cells for Western blot analysis could then be harvested, and those for immunocytochemical studies were re-plated onto poly-L-lysine coated coverslips and allowed to grow overnight.

2.1.2 Primary hippocampal culture

Rat primary hippocampal cell cultures were kindly prepared by Mrs. D. Mitchel as previously described (Skaper et al., 1990). Briefly, the hippocampus was dissected in cold Hanks Balance Salt Solution (HBSS, In Vitrogen) from 18 day gestation Sprague-Dawley rat embryos and transferred to fresh HBSS. Hippocampal tissue was chopped into approximately $2mm^2$ pieces, transferred into 2ml Trypsin-EDTA (In Vitrogen) diluted to 1x in Dulbecco's PBS and incubated for 20mins at 37°C. Tissue pieces were washed twice with 10ml Neurobasal™ (In Vitrogen) containing 10% Foetal Bovine Serum (FBS) and transferred into 2ml growth media (Neurobasal™ containing B27 (In Vitrogen) and 1mM Sodium Pyruvate) before being triturated with a flame fired pasteur pipette to obtain a single cell suspension. The cell suspension was made up to 10ml with growth media and centrifuged at 100g for 5mins at room temperature. The cell pellet was resuspended in 3ml growth media and the cells plated at a density equivalent to 2.5×10^4 cells/cm^2 on Nunc chamber slides which had been previously coated with 50µg/ml Poly-L-Lysine (Sigma) for 1h at room temperature, followed by Neurobasal™ plus 10% FBS for at least 1h at room temperature.

2.2 Tissue preparation

2.2.1 Tissue preparation for Western blot analysis

Fresh frozen tissue was obtained for Western blotting purposes by stunning male Sprague Dawley rats of approximately 250g with a blow to the back of the skull followed by breaking the neck. The skull was then opened, the brain removed and immediately fixed in isopentane at –80°C. Brains were stored at –80°C until homogenisation.

2.2.2 Tissue preparation for immunohistochemistry

Male Sprague-Dawley rats of approximately 250g were terminally anaesthetized with 1ml sodium pentobarbital. The thoracic cavity was then opened and the heart exposed. The descending aorta was clamped to prevent excess fixative reaching the lower body. The left atrium was snipped to allow fluid to exit and a gavage needle was inserted into the right ventricle. Approximately 200ml heparinised saline (0.9% NaCl + 0.1% heparin) was flushed through the animal using a perfusion pump at 12ml/min until the effusate leaving the left atrium ran clear. The source of the perfusion pump was then switched and approximately 400ml 4% paraformaldehyde (PFA) in PBS (pH 7.4) was perfused through the animal. The animals were then decapitated and the brains removed by opening the skull, and gently lifting the brain from the base of the head. The spinal cord and DRG were removed by exposing the dorsal surface of the spine, gently breaking apart the spinal column and slowly lifting the cord away as the dorsal roots were cut free. All tissues were post-fixed in 4% PFA for 24h at room temperature then transfered to 30% sucrose in distilled water (dH_2O)

at 4°C until the tissue sank to the bottom of the vessel, indicating that the water had been displaced by sucrose. Tissues were frozen slowly on card-ice and stored for no longer than four months at –80°C until being removed for sectioning. Brains and spinal cords were cut on a Leica Jung CM3000 cryostat at 35µm at approximately -20°C and were stored free-floating in a cryoprotectant solution (300ml glycerol, 300ml ethylene glycol in 400ml dH_2O) at 4°C. Dorsal root ganglion sections were cut at 14µm, thaw-mounted on to Superfrost Plus Gold slides (BDH), and stored at –80°C. All procedures involving experimental animals were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986 and conformed to GlaxoSmithKline ethical standards.

2.3 Antisera

2.3.1 Primary antisera

All non-commercially available antisera were generated by Research Genetics Inc, Alabama, USA. Polyclonal antisera were raised using synthetic peptides which were synthesised by Research Genetics Inc. and covalently coupled to the carrier keyhole limpet haemocyanin (KLH). Peptides were designed around sequences thought to offer the best antigenicity. Several charged residues (Lysine, Arginine, Aspartic acid and Glutamic acid) within the peptide sequence are desirable, whereas regions predicted to be hydrophobic due to positive charged residues were avoided. The predicted structure of the protein was used to assess membrane spanning regions as these do not offer good antigenicity, preferably then sequences designed to the C- or N-termini were used. If the peptide sequence was designed against the extracellular terminal it was important to avoid regions containing asparagine residues as these often lead to

glycosylation. In addition regions incorporating the signal peptide at the N-terminal were avoided as these sequences are generally lost due to cleavage in the mature protein. Generally it is advisable to design peptide sequences of between 10 and 12 amino acids, however those as short as 8 or as long as 20 a.a. residues have been shown to work. Once a suitable peptide sequence had been designed it was placed into a BLAST search to check for any homology to any other known protein sequences. If the designed sequence did not match any sequences in the public databases it was sent to Research Genetics.

The $GABA_{B2}$ receptor subunit antisera was produced as a GST-fusion protein against a similar sequence to that described by Kaupmann et al., (1998). The entire intracellular C-terminus from the end of the seventh transmembrane spanning domain (a.a's 745-941) was used to generate the protein sequence. This sequence was cloned into a GST containing vector with multiple downstream coding sites. The vector was then transformed into escheridia coli and transduction induced by the application of IPTG. Cells were subsequently lysed and the protein purified through a glutathione column. The resulting GST-protien was used to immunise two rabbits.

The following list is a summary of the peptide sequences used to generate polyclonal antisera against our proteins of interest:

$GABA_{B1a}$ - N-terminal

- **CHPPWEGGIRYRGLTRDQVK** (amino acids 33-51) raised in two rabbits and one sheep.

- **CRGEREVVGPKVRK** raised in two rabbits (this antisera did not work on tissue sections).

$GABA_{B1b}$ – N-terminal

- **HSPHLPRPHPRVPPHPS** (amino acids 30-48) raised in two rabbits and one goat.

$GABA_{B2}$ – N-terminal

- **CTRGAPRPPPSSPP** (worked by ICC but not in Western blot)

- **CEQIRNESLLRP** (very weak signal by ICC, did not work in Western blot or on tissue sections).

- **CSPRHRHVPPS** (did not work in any system).

- a **GST-fusion** protein was generated against the intracellular C-terminus (amino acids 745-941) raised in two rabbits.

$GABA_{BL}$ – N-terminal - sequence

- **CREKLQEVLQE** (amino acids 527-536) raised in two rabbits.

- **PGQELDRRPLHDLC** (did not work on Western blot or tissue sections)

- **CNFKDDLKPTLV** (did not work on Western blot or tissue sections).

Animals were injected with 0.1mg of each peptide-KLH conjugate, or GST-fusion protein, in Freund's complete adjuvant, and boosted four times with the same amount of antigen in incomplete adjuvant. Terminal bleeds were clotted overnight at 4°C and the serum separated from blood cells by centrifugation at 8000g. Antigens were immobilised on activated supports to allow for the affinity purification of antisera. Elution from columns was via a pH gradient and fractions were collected and stored in 0.125 M borate buffer at –80°C.

For each protein target three peptide sequences were designed with two rabbits immunised for each peptide sequence. To characterise each antisera, Western blot analysis was performed on rat brain homogenates and cell lystates from transiently transfected cells. Immunocytochemistry was also performed on transiently transfected and mock-transfected cells to assess the specificity of the antisera for the protein in question. Immunoreactivity was also assessed in rat brain tissue to examine if more than one antisera against a common target were capable of giving a similar pattern of labelling, and indeed if the overall pattern of expression agreed with mRNA or binding studies. In all cases the ability to block the immunoreactive signal was assessed using the immunogenic peptide used to generate the antisera. From this, one antisera from one rabbit was chosen which provided the most robust and specific labelling. Optimal working dilutions were assessed using a dilution gradient for each primary and secondary antisera. In each case the dilutions of both antisera which gave the most robust labelling without excess non-specific background labelling were chosen. The choice of ABC detection system was also assessed as to whether further amplification of the antisera-receptor complex was required. In such cases the ABC elite kit (Vector) or the Tyramide TSA fluorescent kit (NEN) were utilised.

All other primary antisera were obtained from commercial sources or kindly donated:

Antisera	Target	Dilution	Source
Anti-calretinin	Monoclonal against human recombinant calretinin	1:500	Swant, Switzerland
Anti-calbindin	Monoclonal against chicken calbindin	1:500	Swant, Switzerland
Anti-parvalbumin	Monoclonal against carp muscle parvalbumin	1:100	Swant, Switzerland
Anti-glial fibrillary acidic protein (GFAP)	Monoclonal against GFAP purified from pig spinal cord	1:2000	Sigma, UK
Anti-$GABA_{B1}$	Guinea-pig polyclonal against N-terminal of $GABA_{B1}$	1:2500	Chemicon, USA
Anti-$GABA_{B2}$	Guinea-pig polyclonal against C-terminal of $GABA_{B2}$	1:500	Chemicon, USA
Anti-$GABA_{B2}$	Rabbit polyclonal against C-terminal sequence VPPSFRVMVSGL of $GABA_{B2}$	1:400	Dr. P. Emson, Babraham, UK
Anti-myelin basic protein (MBP)	Monoclonal against bovine myelin basic protein	1:200	Chemicon, USA
Anti-ED1	Monoclonal against rat spleen cells	1:100	Chemicon, USA
Anti-CNPase	Monoclonal against Purified human 2', 3'-cyclic nucleotide 3'-phosphodiesterase	1:200	Chemicon, USA

Table 2.1 Primary antisera, sequence, working dilution and supplier.

2.3.2 Secondary antisera

All secondary antisera were sourced commercially as shown below:

Biotinylated	Anti-rabbit Anti-sheep Anti-goat Anti-guinea-pig	1:200	Vector Labs, USA
HRP-conjugated	Anti-rabbit Anti-goat	1:250 1:250	Vector Labs, USA
Alexa-488 Alexa-568 Alexa-633	Anti-mouse Anti-guinea-pig Anti-sheep Anti-rabbit Anti-goat	1:50	Molecular Probes, USA
TSA-FITC	Fluorescein intensification kit	1:50	NEN Life Sciences, USA

Table 2.2 Secondary antisera, working dilutions and suppliers.

2.4 SDS-PAGE and immunoblotting

Crude membranes were prepared from rat brains or cells transfected with the $GABA_B$ receptor subunit cDNA of interest as described previously (Benke et al., 1999; Calver et al., 2000). Membrane preparations were mixed with an equal volume of sample buffer (62.5 mM Tris (pH 6.8), 2% SDS, 25% glycerol, 350 mM DTT) before loading onto SDS-polyacrylamide gels. Discontinuous SDS-PAGE was performed using a 4% polyacrylamide stacking gel and a 7.5% polyacrylamide running gel (Bio-Rad, CA). Proteins were transferred onto PVDF membranes using a semi-dry transfer system (Bio-Rad). After blocking with 5% (w/v) dry nonfat milk in phosphate buffered saline (PBS) containing 0.05% Tween-20, membranes were incubated overnight with affinity purified antibodies at optimal defined working dilutions in blocking solution. Immunoreactive bands were detected with species specific antibody conjugated to horseradish peroxidase

(Chemicon, Temecula, CA, USA) followed by chemiluminescence detection (ECLplus, Amersham, UK).

2.5 Immunocytochemistry

Culture medium was aspirated from wells containing coverslips on which the cells of interest were grown. Cells were washed 2 x 5min in PBS and fixed for 20min in 4% PFA at room temperature. Cells were again washed and stored in PBS at 4°C until use. Cells which were permeabilised were incubated for 30min in 0.1% Triton X-100 in PBS. Cells which were not permeabilised were incubated in PBS during this time. Non-specific antisera labelling was reduced by incubation in a blocking solution containing 10% normal goat serum and 1% BSA in PBS for 1h. Cells were then incubated in primary antisera at optimal working dilutions for 4h at room temp, washed and exposed to Alexa488 conjugated secondary antisera at 1:50 for 1h in the dark. Coverslips were then placed on microscope slides using CitiFluor (Canterbury, UK) as the mounting medium and stored in the dark at 4°C.

2.6 Immunohistochemistry

2.6.1 Peroxidase Immunohistochemistry - single labelling

Immunoreactivity was revealed using the ABC detection system. Sections were washed thoroughly in PBS, permeabilised in 0.1% Triton X-100 and endogenous peroxidase activity removed by 1% H_2O_2 in PBS for free-floating brain and spinal cord, and 1% H_2O_2 in 50% methanol/PBS for slide mounted DRG. Non-specific antibody binding was reduced by pre-incubation in a PBS blocking solution containing 1% bovine serum albumin and 10% normal goat

serum or 10% normal donkey serum for antisera raised in sheep. Sections were incubated with primary antisera at optimally defined working dilutions in blocking solution for 48h at 4°C. After washing in PBS, sections were incubated in biotinylated secondary antisera followed by a peroxidase-conjugated avidin-biotin complex (Vector Labs, at 1:200) and 3,3'-diaminobenzidine tetrachloride (DAB,Vector Labs, at manufacturers recommended dilution) as the colour substrate. Free-floating tissue was slide mounted from dH_2O onto SuperFrost Plus slides (BDH, Lutterworth, UK) and allowed to dry. Slide mounted DRG tissue was rinsed in dH_2O and dried. Sections were dehydrated in 100% ethanol, immersed in Histolene (CellPath, Leeds, UK) for 10min and coverslipped with DPX (BDH).

2.6.2 Fluorescence Immunohistochemistry - Double labelling

For double labelling studies, tissue sections were permeabilised with 0.1% Triton X-100 in PBS followed by a blocking step as described for peroxidase immunohistochemistry. Tissues were then incubated overnight at room temperature with affinity purified anti-$GABA_{B1a}$, $GABA_{B1b}$, $GABA_{B2}$ or $GABA_{BL}$ antisera in blocking solution at optimally defined working dilutions. Tissues were washed thoroughly in PBS. In the dark, direct fluorescent detection was performed using Alexa488 (Molecular Probes, USA) conjugated to the appropriate anti-species antisera in PBS. Sections were then incubated in the second primary antisera of an alternate species at the recommended or optimally defined working dilutions, again over night at room temperature. Following thorough PBS washes the second antisera was detected using Alexa633 (Molecular Probes) conjugated to the appropriate anti-species secondary antisera

in PBS. After washing in PBS, sections were mounted onto SuperFrost Plus slides, coverslipped with Citifluor (Citifluor, Canterbury, UK) and stored in the dark at 4°C prior to confocal microscopy.

In certain instances the direct secondary detection system did not provide optimal fluorescent detection of the receptor in question. In such cases the Tyramide amplification system (NEN) was employed as a means of amplifying the specific labelling but not the background fluorescence. Tissue sections were incubated in TNT (0.1M Tris; 0.15M NaCl; 0.05% Tween-20) and the endogenous peroxidase activity was blocked using 2% H_2O_2 in TNT. Non-specific antisera labelling was blocked with 10% normal goat serum, or normal donkey serum, plus 1% BSA and tissues were then incubated overnight in the primary antisera which required amplification at optimally defined dilutions. Following thorough washing in TNT, sections were incubated for 5min in TNB (0.1M Tris; 0.15M NaCl), then incubated for 1h with an HRP-conjugated secondary at 1:250 in TNT. Sections were then placed in Fl-Tyramide FITC at 1:50 in amplification diluent for 10min in the dark. After washing in TNT, tissues were incubated with the second primary antisera which underwent standard detection as described above.

2.6.3 Antisera control experiments

Control experiments for Western blotting included the pre-absorption of the antisera with its corresponding immunogenic peptide at 1-20μg/ml. The peptide was incubated with each antisera at relative working dilutions for 4h prior to use in Western blotting. In addition untransfected cell lysates were also used as controls in Western blotting.

For immunocytochemical experiments mock-transfected cells, ie those not receiving DNA, were used as a negative control to confirm that no immunoreactivity was observed in cells lacking the protein of interest.

Immunohistochemical control experiments included the substitution of the primary antisera with blocking buffer alone to assess non-specific labelling caused by either the biotinylated secondary antisera or the DAB reaction. Pre-absorption controls were also carried out on all tissues, whereby the primary antisera was incubated with an excess of the immunogenic peptide (10-20μg/ml) at 4°C for 72h prior to the onset of the experiment. The GST-$GABA_{B2}$ fusion protein was used to pre-absorb the $GABA_{B2}$ GST-fusion antisera for 30min prior to incubation with tissue sections. The specificity of the $GABA_{B2}$ GST-fusion antisera was further verified by pre-incubation with 4% GST for 30min prior to incubation with tissue sections to determine the level of immunoreactivity due to GST alone. Specific immunoreactivity would be expected to be observed in regions previously demonstrated to contain the mRNA or protein of interest, thus all labelling was compared to that known from Taqman and autoradiography studies and also any other published findings on the distribution of the protein of interest.

2.7 Image Capture

Single-labelled peroxidase sections were analysed using a Leica DMR microscope equipped with a Leica DC200 digital camera. Images were captured as .tif files and prepared using PaintShop Pro Version 7.0 (JASC, Eden Prairie, MN) for grey scale conversion, contrast and brightness adjustments and cropping.

Fluorescent images were captured using a Leica DMRB Confocal microscope equipped with Leica Confocal softwear. Exposure and threshold levels were defined and images were prepared as maximal projections of, on average, 10 optical sections through the tissue. Confocal controls included the exclusion of each laser during imaging to assess the degree of "bleed-through" from one fluorophore to another. In instances where "bleed-through" occurred the amount of light captured under each laser was altered to reduce cross-over of activation/emission. Confocal images were converted to real colour, saved as .tif files and imported to PaintShop Pro Version 7.0 for re-orientation and cropping.

2.8 Electron Microscopy

2.8.1 Tissue preparation for electron microscopy

Adult male Sprague-Dawley rats of approximately 250g were perfuse-fixed as previously described for immunohistochemistry (section 2.1.2). 4% PFA containing 0.05% glutaraldehyde was used as the fixative. After removal the brains were removed and placed in the same fixation solution for 4h after which time the tissue was sectioned on a vibratome (Leica, UK) at 50μm and stored in PBS.

2.8.2 Immunohistochemistry for electron microscopy

Brain sections were incubated in 10% sucrose for 10 minutes, followed by 20% for 30 minutes and subjected to a freeze-thaw process by immersing in liquid nitrogen three times with thawing between each immersion. Sections were washed in PBS and exposed to primary antisera (same dilutions as described above) overnight at 4°C. After washing in PBS, sections were incubated for a

further 7h in the presence of a biotinylated secondary antisera (1:500, Jackson ImmunoResearch) and then washed and incubated in ExtrAvidin Peroxidase (Sigma, UK) at 1:1600 overnight at 4°C. The colour reaction was carried out using diaminobenzidine at a final concentration of 50mg/100ml in TRIS buffer (pH 7.5), H_2O_2 was then added to a final concentration of 0.1%. Flattened sections were then placed in 1% osmium tetroxide for 45 min and washed with water. Sections were subsequently dehydrated in an ascending series of alcohol washes (50-100%) followed by two washes in propylene oxide for 15 and 5min. Sections were placed in Durcupan resin (Fluka) for 6h and mounted onto standard microscope slides with coverslips prior to incubation in an oven at 60^0C for 48h.

2.8.3 Ultra-sectioning

After identifying the region of interest tissue was cut out and placed onto an ultra-microtome block Ultra-thin tissue sections (70nm) were cut and mounted onto Formvar coated 1mm slotted grids. Sections were then stained with uranyl acetate (1%, 3mins) and lead citrate (3 mins), prior to a dH_2O rinse.

2.8.4 The electron microscope and image capture

Ultra sections were imaged on a Phillips CM10 transmission electron microscope (Leeds University). The resulting negatives were scanned using an AGFA Duoscan or HP ScanJet scanners and digitized before manipulations were carried out in Corel Draw 8/9 or PaintShop Pro V7.0 for contrast and brightness corrections and cropping.

Chapter 3 - Localisation of known $GABA_B$ receptor subunits in the rat CNS

3.1 Introduction

Early distribution studies of the $GABA_B$ receptor were restricted to the use of tritiated GABA or baclofen to assess regions of receptor binding. In 1990 two studies defined the distribution of $GABA_B$ receptors using [^{3}H] GABA in the presence of isoguvacine to block $GABA_A$ receptor binding (Bowery et al., 1990; Chu et al., 1990). Both groups reported a widespread distribution of $GABA_B$ with binding observed in the molecular layer of the cerebellum, the interpeduncular nucleus, frontal cortex, olfactory nucleus and thalamic nuclei, as well as the globus pallidus, temporal cortex, superior colliculus, and substantia gelatinosa of the spinal cord.

The cloning of the $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits has brought about a number of studies in which the expression of $GABA_B$ receptor subunits has been investigated by different methods and with somewhat contradictory findings. Expression of pan $GABA_{B1}$ has been investigated at the mRNA (Bischoff et al., 1999; Liang et al., 2000; Berthele et al., 2001) and protein level (Margeta-Mitrovic et al., 1999; Sloviter et al., 1999; Ige et al., 2000; Ng and Yung, 2001; Gonchar et al., 2001). In addition, four studies have reported data on the distribution of the $GABA_{B1a}$ and $GABA_{B1b}$ splice variants (Fritschy et al., 1999; Poorkhalkali et al., 2000; Princivalle et al., 2000; Towers et al., 2000). Fritschy et al., (1999), however, used a subtractive analysis to determine the distribution of the $GABA_{B1a}$ subunit, due to the lack of a selective $GABA_{B1a}$ antibody at that time. Three other studies have focused on discrete brain regions

such as the cerebellum (Poorkhalkali et al., 2000), piriform cortex (Princivalle et al., 2000) or dorsal horn of the spinal cord (Towers et al., 2000). The localisation of the $GABA_{B2}$ subunit has been characterised by *in situ* hybridisation studies (Durkin et al., 1999; Calver et al., 2000; Clark et al., 2000; Berthele et al., 2001) and reports on $GABA_{B2}$ protein distribution by immunohistochemistry in rat brain and spinal cord (Calver et al., 2000; Towers et al., 2000; Ige et al., 2000; Ng and Yung, 2001). As yet, however, there have been no full comparative reports of the distribution of $GABA_B$ receptor subunits at the protein level in the rat brain. Given that the rat is the rodent of choice for pharmacological studies, knowledge of the precise anatomical localisation of the $GABA_B$ receptor subunits in the rat brain, and reports of any potential discrepancies arising, may significantly aid the understanding of the physiology and pharmacology underlying the $GABA_B$ receptor system.

3.2 Aims of this study

Given the lack of true comparative studies examining the $GABA_B$ receptor subunit distribution in the rat CNS, antisera were raised against epitopes unique to $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ subunits (see Chapter 2.3.1). Following confirmation of the specificity of these antisera a comparative and comprehensive distribution of the protein expression of the $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits in rat brain, spinal cord and dorsal root ganglia was performed using immunohistochemical techniques to specifically label cells and regions expressing any one, two or all of the major $GABA_B$ receptor subunits.

3.3 Results

3.3.1 Characterisation of $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ antisera

Antisera raised in rabbits, sheep and goat were all subjected to stringent characterisation prior to the onset of full distribution studies. Antisera specificity was assessed by immunoblotting of crude rat brain membrane homogenates as well as lysates prepared from transfected cells expressing recombinant $GABA_{B1a}$, $GABA_{B1b}$ or $GABA_{B2}$ subunits (Fig. 3.1). The affinity purified antisera recognised immunoreactive bands of the expected molecular weights for $GABA_{B1a}$ (approximately 130 kDa), $GABA_{B1b}$ (approximately 100 kDa) and $GABA_{B2}$ (approximately 110 kDa) in membranes prepared from either cell extracts expressing the appropriate recombinant receptor subunit or from rat brain homogenates (Fig. 3.1A-C). In rat membrane preparations, the $GABA_{B2}$ antiserum also detected a weak band of lower molecular weight at approximately 80 kDa probably reflecting a degradation product of this receptor subunit. Pre-absorption of each primary antibody with their respective immunogenic peptides completely abolished the recognition of $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ immunoreactive bands in both rat brain homogenates (Fig. 3.1) and transiently transfected cell lysates.

Immunocytochemistry was also performed on transiently transfected HEK293 cells to confirm the specificity of each subunit specific antisera. HEK293 cells expressing $GABA_{B1a}$ showed strong immunofluorescence when anti-$GABA_{B1a}$ antisera were characterised, however in the absence of primary antisera no immunofluorescence was observed (Fig 3.2 A,B). Cells transiently expressing $GABA_{B1b}$ also demonstrated strong immunofluorescence using anti-$GABA_{B1b}$ antisera but not in the absence of primary antisera (Fig. 3.2 C,D).

Likewise $GABA_{B2}$ expressing cells only demonstrated immunofluorescence with $GABA_{B2}$ specific antisera (Fig. 3.2 E,F).

The specificity of each antiserum was also tested immunohistochemically by pre-absorption with 10μg/ml of $GABA_{B1a}$ and $GABA_{B1b}$ immunogenic peptides and 20μg/ml of the C-terminal $GABA_{B2}$ GST-fusion protein before use. All immunoreactive labelling was completely blocked in pre-absorption experiments (Fig. 3.3 middle insets) and, in addition, no staining was observed when primary antisera were omitted from the incubation (Fig. 3.3 top inset). No decrease in IR was observed following pre-incubation of $GABA_{B2}$ antisera with GST, demonstrating that our antiserum was not recognising the GST part of the $GABA_{B2}$ fusion protein (Fig. 3.3 bottom inset).

Figure 3.1 Immunoblot showing identification of $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ subunits. Membranes prepared from untransfected HEK-293T cells (lane 1), HEK-293T cells expressing $GABA_B$ subunits (lane 2) or whole rat brain (lane 3) were analysed by SDS-PAGE (7.5%). Immunoblotting was performed using affinity purified $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ antibodies (1-5mg/ml). $GABA_{B1a}$ migrates at 130 kDa (A), $GABA_{B1b}$ at 100 kDa (B) and $GABA_{B2}$ at 110 kDa (C). Specificity of antibodies was determined by pre-absorption with immunogenic peptides or GST-fusion protein (lane 4 in each panel).

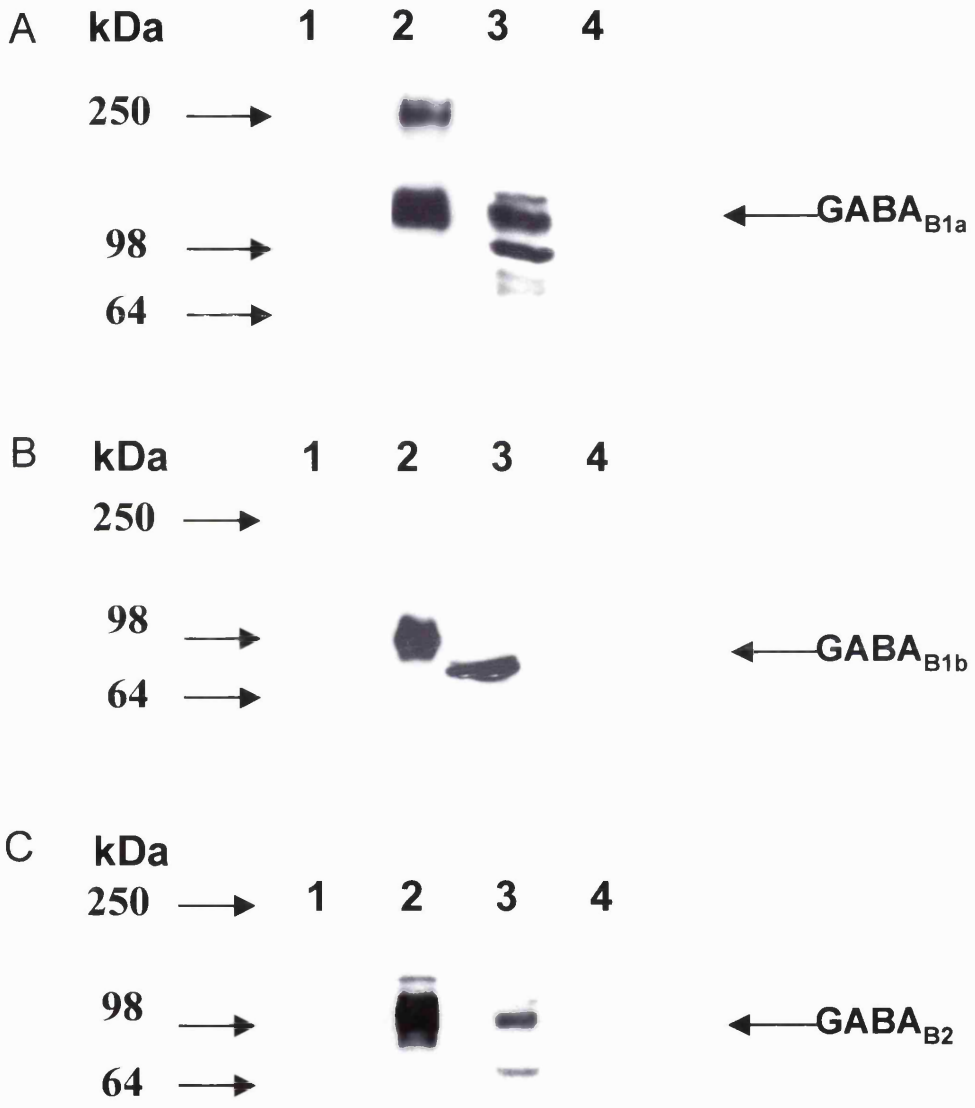

A
kDa
1
2
3
4
250
98
64
GABA$_{B1a}$
B
kDa
1
2
3
4
250
98
64
GABA$_{B1b}$
C
kDa
250
1
2
3
4
98
64
GABA$_{B2}$

Figure 3.2 Fluorescent photomicrographs demonstrating specificity of $GABA_B$ receptor subunit antisera on transiently transfected HEK293 cells. A) HEK293 cells expressing $GABA_{B1a}$ showed strong immunoreactivity with anti-$GABA_{B1a}$ antisera. B) In the absence of primary antisera no $GABA_{B1a}$ IR was observed. C) HEK293 cells expressing $GABA_{B1b}$ demonstrated strong IR with anti-$GABA_{B1b}$ antisera. D) No IR was observed in the absence of $GABA_{B1b}$ specific antisera. E) HEK293 cells expressing $GABA_{B2}$ showed strong IR with subunit specific antisera. F) No IR was seen in the absence of $GABA_{B2}$ antisera. Scale bar = 50μm.

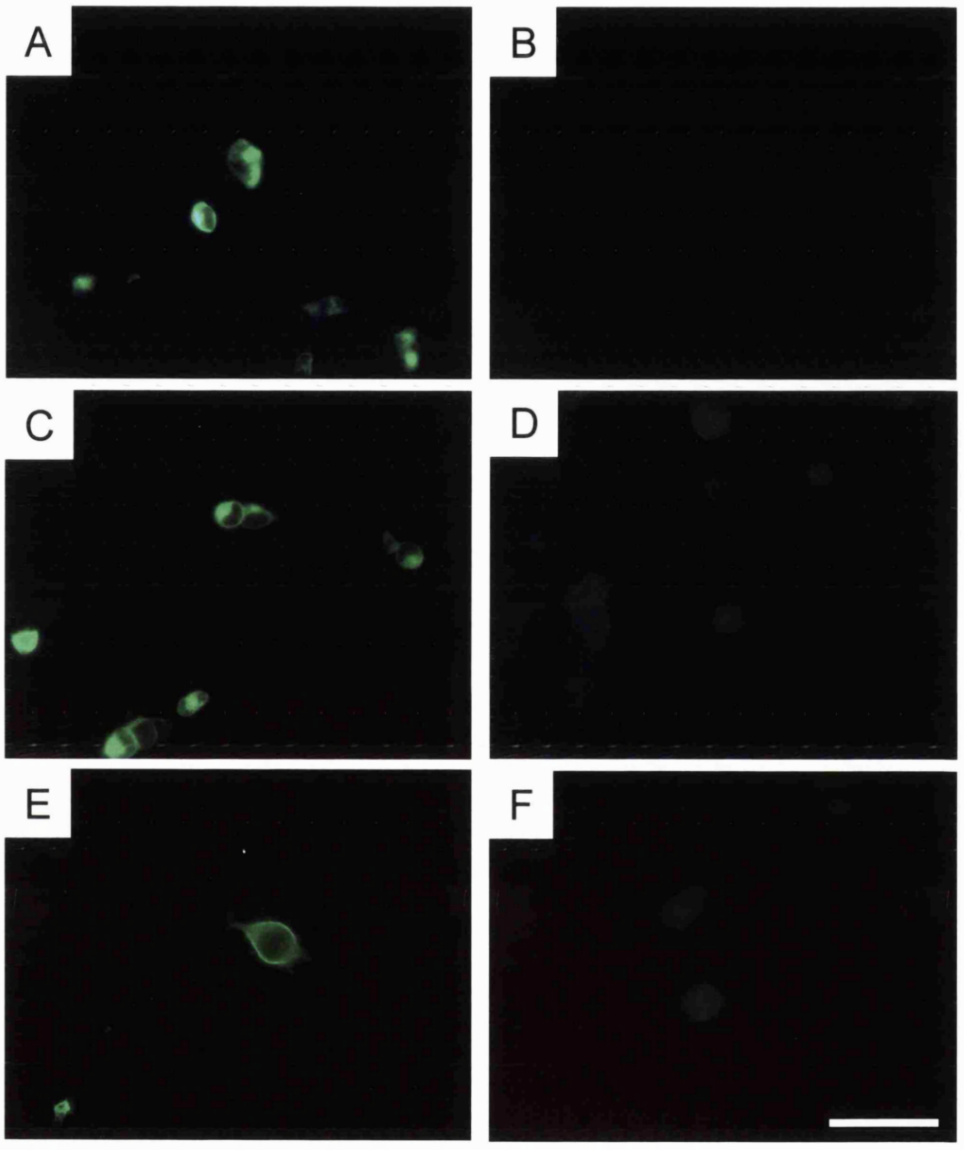
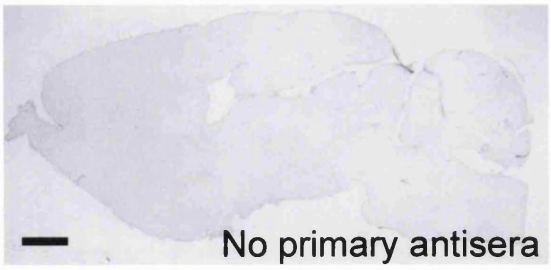
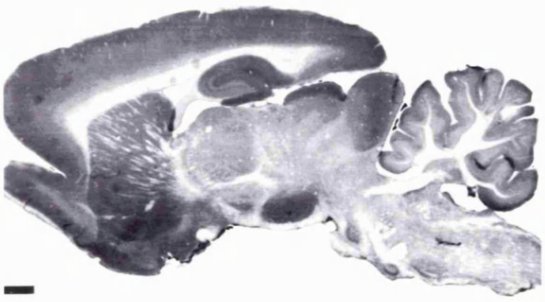

A
B
C
D
E
F

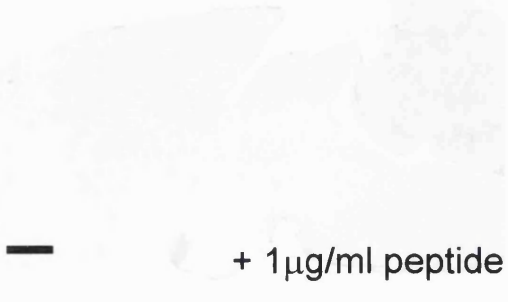
Figure 3.3 Photomicrographs showing $GABA_B$ IR in parasagittal sections of the rat brain. $GABA_{B1a}$ (A) and $GABA_{B1b}$ (B) and $GABA_{B2}$ (C). No IR was observed when primary antisera were omitted from the incubation (Top inset). Selectivity of antisera was determined by pre-absorption the with cognate immunogenic peptide, GST-fusion protein and GST only and completely abolished all IR (insets), save for GST only whereby staining was identical to that seen with antisera alone (bottom inset). Scale bars = 1 mm.



No primary antisera

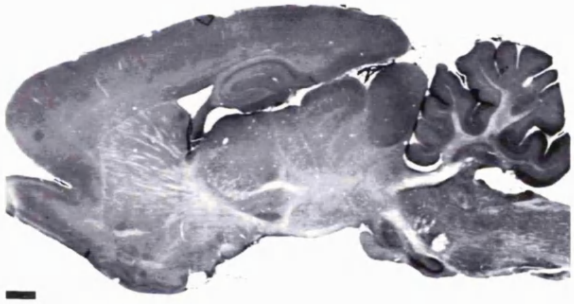
A

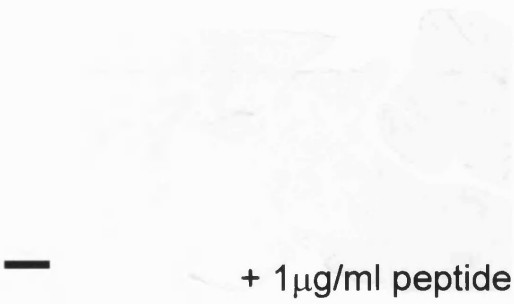




+ 1μg/ml peptide

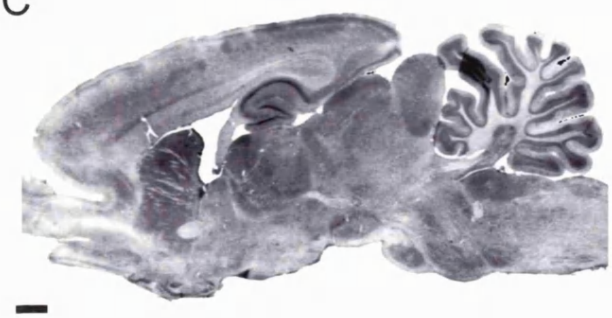
B

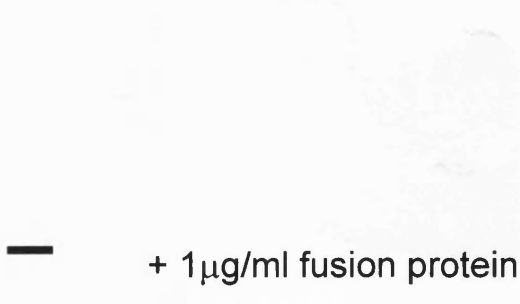




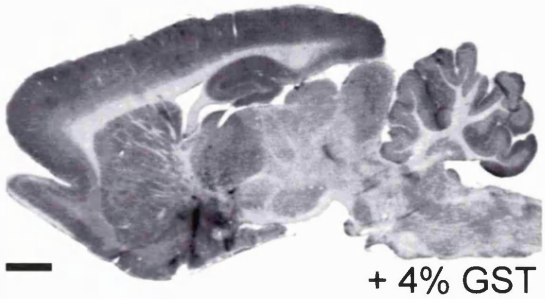
+ 1μg/ml peptide

C





+ 1μg/ml fusion protein



+ 4% GST

3.3.2 General distribution of $GABA_B$ receptor subunits in rat CNS

Para-sagittal sections observed at low magnification and labelled with the $GABA_{B1a}$ and $GABA_{B1b}$ receptor subunit antisera revealed a widespread distribution of $GABA_B$ receptor subunit-like immunoreactivity (IR) throughout the rat brain. $GABA_{B1a}$ (Fig. 3.4A) and $GABA_{B1b}$ IR (Fig. 3.4B) was observed in all neocortical layers with the most dense labelling found in the prefrontal and somatosensory cortices. Dense IR was observed in the caudate-putamen and thalamus, all fields of the hippocampus including the dentate gyrus, midbrain structures including the superior and inferior colliculi and substantia nigra, the pontine nuclei and vestibular nuclei. As expected, dense labelling was also observed throughout the cerebellar cortex. Little $GABA_{B1}$ subunit expression was observed in white matter, the deep mesencephalic nuclei and the hypothalamus. Dense $GABA_{B2}$ receptor IR was seen throughout the neocortex with an expression profile similar to that of the $GABA_{B1}$ subunits (Fig. 3.4C). Very dense labelling was also observed in all hippocampal subfields and the thalamus, as well as in several midbrain structures, including the substantia nigra, and in the pontine and vestibular nuclei. $GABA_{B2}$ IR was also dense in the cerebellar cortex.

A more detailed description of the expression patterns observed for each $GABA_B$ receptor subunit is given in the following sections and is summarised in Tables 3.1-3, based upon analysis of the relative intensities of cell body and neuropil immunoreactivity (IR) for each antibody. IR has been scored as none (-), very low (+), low (++), moderate (+++), dense (++++) or very dense (+++++).

Table 3.1 Semi-quantitative analysis of $GABA_B$ receptor-like IR in the rat cerebral cortex.

Cortical Area	$GABA_B$ Subunit	Cortical Layer I	II	III	IV	V	VI
Orbital	$GABA_{B1a}$	-	++++	++	++	++	+++
	$GABA_{B1b}$	-	++++	+++	+++	+++	+++
	$GABA_{B2}$	-	++++	+	+++	+++	+++
Motor	$GABA_{B1a}$	+	++++	+	++	++	+++
	$GABA_{B1b}$	-	++++	+++	+++	+++	+++
	$GABA_{B2}$	+	++++	++	++	++++	++++
Parietal	$GABA_{B1a}$	-	+++	+++	+++	+++	++
	$GABA_{B1b}$	-	+++	+++	+++	+++	++++
	$GABA_{B2}$	+	++++	+++	++	+++	+++
Visual	$GABA_{B1a}$	-	+++	++	++++	++++	+++
	$GABA_{B1b}$	-	++++	+++	+++	+++	++++
	$GABA_{B2}$	-	++++	+++	++	+++	+++
Piriform	$GABA_{B1a}$	-	+++++	++++	+++	+++	+++
	$GABA_{B1b}$	-	++++	+++	++	++	++
	$GABA_{B2}$	+	+++++	+++	+++	+++	+++
Insular	$GABA_{B1a}$	-	++	+++	+++	+++	+++
	$GABA_{B1b}$	-	++++	+++	+++	++++	+++
	$GABA_{B2}$	+	++++	++++	++	+++	++
Somatosensory	$GABA_{B1a}$	-	+++	+++	++	++++	+++
	$GABA_{B1b}$	-	+++	++	+++	++++	++++
	$GABA_{B2}$	+	+++	+++	++	++++	+++
Auditory	$GABA_{B1a}$	-	+++	+++	+++	++++	+++
	$GABA_{B1b}$	-	+++	+++	+++	+++	+++
	$GABA_{B2}$	-	++++	+++	+++	+++	+++

Table 3.2 Semi-quantitative analysis of $GABA_B$ receptor-like IR in major structures of the rat brain.

Brain Regions	Structure	$GABA_{B1a}$	$GABA_{B1b}$	$GABA_{B2}$
Basal Ganglia	Caudate-Putamen	++++	++++	++++
	Accumbens Shell	+++++	+++++	+++
	Accumbens Core	+++	+++	+++
	Globus Pallidus	++	++	++
Septum		+++	+++	+
BNST		++++	+++	+++
Diagonal band		++	+++	++
	Bas. Meynert n.	+++	+++	++++
Amygdala	Basolateral	++++	++	++++
	Lateral	++	++	+
	Central	++++	++	+++
	Intercalated mass	++++	++	+
Hippocampus	CA1	+++	++++	++++
	CA3	++++	+++	++++
	Dentate gyrus	+++++	+++++	+++++
	interneurons	+++	++++	++
Habenula		+++++	++++	++++
Interpeduncular		++++	++++	++++
Thalamus	Medial	++++	++++	++++
	Lateral	++	++++	++
	Ventral	++++	++++	++++
	Reticular thalamus	++++	++++	++++
Hypothalamus	Dorsal	+++	+++	+++
	Ventromedial	++	+++	++++
	Lateral	+	++++	+++
	Median Eminence	++++	++++	+++++
	Tuber cinereum	++	++	++++
Mid brain	Superior colliculus	++++	+++	++
	Inferior colliculus	+++	+++	++
	Ventral Tegmental a	+++	+++	+++
	Substantia nigra *pc*	++++	++++	+++
	Substantia nigra *pr*	++	+++	+
Pons	Pontine nuclei	++++	++++	+++
Rhombenceph.	Spinal trigeminal n.	++	++	+++
	Locus coeruleus	++++	+++	++
	Vestibular nuclei	++++	+++	+++
	Median raphe nuclei	+	+	+
	Olivary nuclei	+++	+++	++++
Cerebellum	Molecular layer	++	++++	++
	Purkinje cells	++++	+++	++++
	Granule layer	++++	+++++	+++++

Figure 3.4 Photomicrographs showing $GABA_B$ IR in parasagittal sections of the rat brain. $GABA_{B1a}$ (A) and $GABA_{B1b}$ (B) and $GABA_{B2}$ (C). For abbreviations see list. Scale bars = 1 mm.

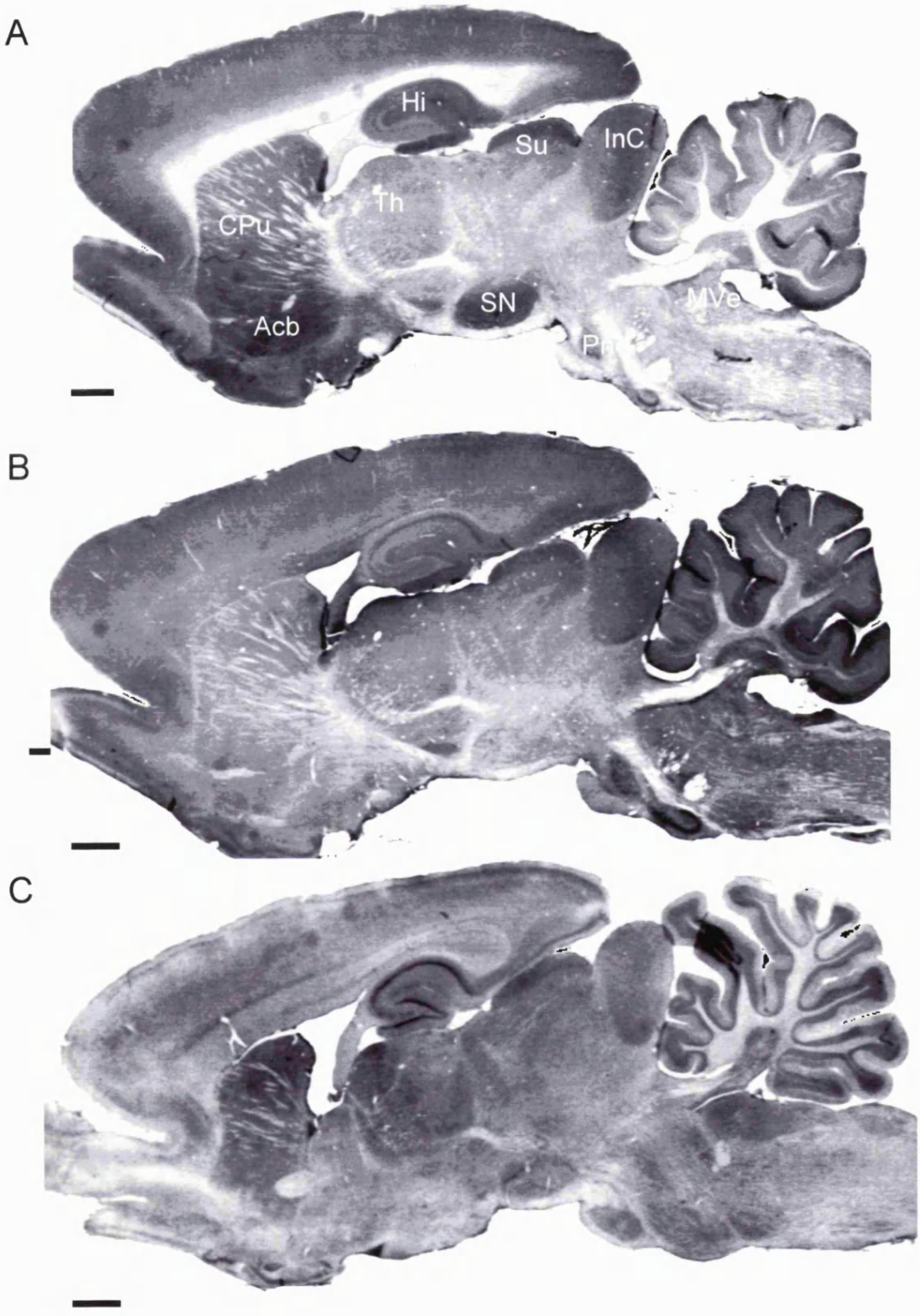

A
Hi
Su
InC
Th
CPu
SN
MVe
Acb
B
C

Table 3.3 Semi-quantitative distribution of $GABA_{B1}$ and $GABA_{B2}$ positive immunostaining in rat lumbar spinal cord.

Lamina	$GABA_{B1a}$	$GABA_{B1b}$	$GABA_{B2}$
I	+++++	+++++	+++++
II	++++	+++++	+++++
III	+++	+++	+++
IV	+++	+++	+++
V	++	+++	++
VI	++	+++	++
VII	+++	++++	+++
VIII	++++	+++	++++
IX	++++	++++	++++
X	++	+	+++

3.3.3 Cerebral cortex

$GABA_B$ receptor subunit IR was detected in neuronal somata and proximal and distal dendrites throughout the neocortex and varied from low to high intensity depending on the region studied, as well as on the particular cortical layer. In general, anatomical distributions of $GABA_{B1}$ and $GABA_{B2}$ subunit expression were coincident (Figs. 3.4 and 3.5 and Table 3.1). In layer I little or no IR was observed, with few $GABA_{B1}$ or $GABA_{B2}$ positive neurones identified (Fig. 3.5). $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ IR was similarly dense in layer II of most cortical areas studied (Fig. 3.4), although the intensity of neuronal IR in the insular cortex was lower for $GABA_{B1a}$ than $GABA_{B1b}$. Labelling in dendrites and cell bodies of neurones in layers III and IV was low-to-moderate for $GABA_{B1a}$ and $GABA_{B1b}$ in most cortical regions studied (Fig. 3.4A and B). A notable difference in $GABA_{B1a}$ from $GABA_{B1b}$ IR, however, was noted in the primary motor cortex which exhibited lower levels of $GABA_{B1a}$ IR. $GABA_{B2}$ IR in somata and dendrites of layers III and IV appeared somewhat lower compared with

$GABA_{B1b}$ IR with the exception of layer IV of the orbital, piriform and auditory cortices, where staining was similar. In layer V, $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ labelling of neurones was not restricted to large pyramidal cell bodies but was also observed in smaller, non-pyramidal interneurones (Fig. 3.5D-F). Subtle differences in $GABA_{B1a}$ and $GABA_{B1b}$ subunit IR were seen between layer V neurones of the orbital and motor cortices. In these regions most $GABA_{B1a}$ IR was observed on small non-pyramidal cells, whereas $GABA_{B1b}$ IR was predominantly expressed on the cell bodies and apical dendrites of large pyramidal neurones. At higher magnification, labelling of apical dendrites could be clearly observed in the majority of layer V pyramidal neurones (Fig. 3.5D-F). Fairly uniform levels of IR in layer VI neurones were observed in all cortical areas studied for both $GABA_{B1}$ and $GABA_{B2}$ subunits, although a more intense cell body IR was seen in layer VIb adjacent to the corpus callosum.

3.3.4 Basal ganglia and nucleus accumbens

Labelling of all $GABA_B$ subunits was observed throughout the basal ganglia (Fig. 3.6). Cellular IR was dense in the caudate-putamen, ventral pallidum and nucleus accumbens with $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ antisera (Fig. 3.6A-C). Immunoreactivity in the nucleus accumbens was observed in the cell bodies, with labelling denser in the shell than in the core of the nucleus with antisera against both $GABA_{B1a}$ and $GABA_{B1b}$ (Fig. 3.6A and B). $GABA_{B2}$ labelling in this region was similar to that seen with either of the $GABA_{B1}$ antisera (Fig. 3.6C). $GABA_{B1a}$ and $GABA_{B1b}$ expression in the caudate-putamen showed dense labelling of the cell bodies of both small-medium spiny neurons as well as larger interneurons throughout this region, although no dendritic labelling was

visible (Fig. 3.6D,E). Similarly, $GABA_{B2}$ IR was observed in the somata of small neurones of the caudate-putamen, though larger interneurons did not appear to be immunoreactive for $GABA_{B2}$ (Fig. 3.6F). Neuropil IR, which could be arising from cortical projection neurons, could be observed with both $GABA_{B1}$ and $GABA_{B2}$ antisera in the caudate-putamen. In the globus pallidus, $GABA_{B1a}$ IR was restricted to cells possessing astrocyte-like morphologies; in contrast $GABA_{B1b}$ and $GABA_{B2}$ IR were seen in cell bodies with neuronal morphology but little labelling was seen in the neuropil. $GABA_B$ subunit IR in the claustrum was moderate with all antisera.

3.3.5 Septal nuclei

$GABA_{B1}$ IR in the septal nuclei was strong in the neuropil and in cell somata (Fig. 3.6A,B). $GABA_{B1a}$ IR was seen extensively in the lateral septal areas as dense neuropil labelling with many positive cell bodies also clearly distinguishable (Fig. 3.6A). The medial septum was mainly devoid of labelled cell bodies, though some neuropil IR was apparent. Moderate cell body IR was observed in the vertical limb of the nucleus of the diagonal band, though in this region neuropil labelling was absent. All subnuclei of the bed nucleus of the stria terminalis (BNST) were densely labelled for $GABA_{B1a}$ (Fig. 3.6A). IR for $GABA_{B1b}$ in the septal nuclei mirrored that seen with $GABA_{B1a}$, although the vertical limb of the diagonal band exhibited stronger $GABA_{B1b}$ neuropil labelling (Fig. 3.6B). $GABA_{B1b}$ IR in the BNST was also not as intense as that seen for $GABA_{B1a}$. Very little cell body labelling was seen in the septum with antisera directed to $GABA_{B2}$ (Fig. 3.6C). No dendritic IR was observed in the vertical limb of the diagonal band, though many labelled somata were seen in this region.

$GABA_{B2}$ IR was apparent throughout the BNST and was particularly dense in the lateral regions of this structure. $GABA_B$ IR was moderate-to-dense in the basal nucleus of Meynert with all antisera (Fig 3.8A-C).

3.3.6 Amygdala

Various subnuclei of the amygdala expressed the $GABA_B$ receptor subunits to different extents. Dense $GABA_{B1a}$ IR was seen in the basolateral nucleus and central amygdaloid nuclei and, to a lesser extent, in the lateral amygdaloid nuclei. Moderate neuropil labelling was seen in the basolateral and central amygdaloid nuclei. Dense $GABA_{B1a}$ IR was also observed in the intercalated cell masses. $GABA_{B1b}$ IR was more uniform throughout the amygdala, with weak dendritic and cell body labelling clearly observable. $GABA_{B2}$ IR was also clearly defined in the basolateral and central amygdaloid nuclei (Table 3.2).

Figure 3.5 Photomicrographs showing $GABA_B$ receptor subunit IR in the somatosensory cortex, $GABA_{B1a}$ (A); $GABA_{B1b}$ (B) and $GABA_{B2}$ (C). Layer V pyramidal neurons (arrows) are immunoreactive for $GABA_{B1a}$ (D); $GABA_{B1b}$ (E) and $GABA_{B2}$ (F) with stained apical dendrites clearly visible (arrowheads). Scale bars A,B,C = 200 μm; D,E,F = 50 μm.

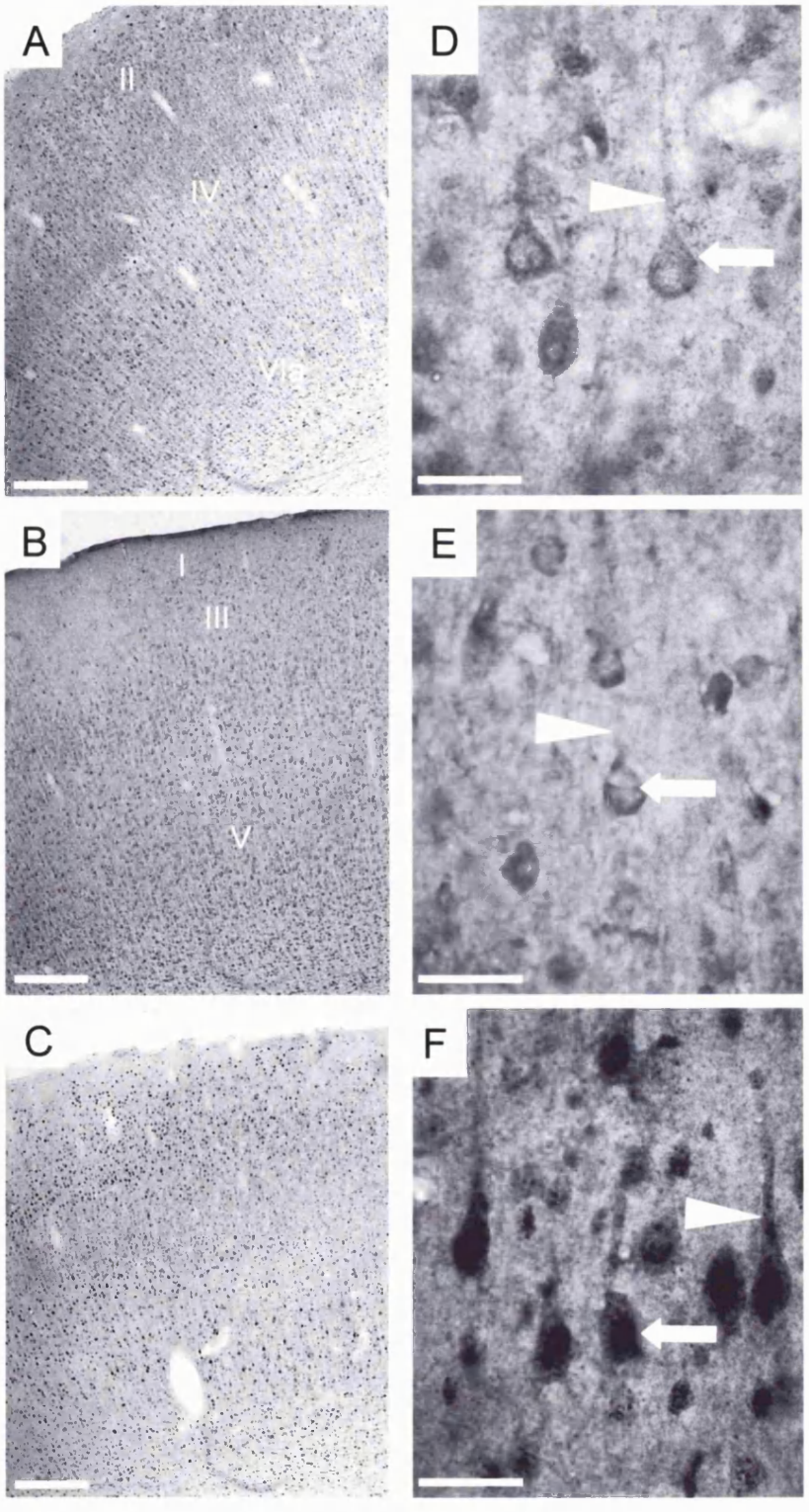
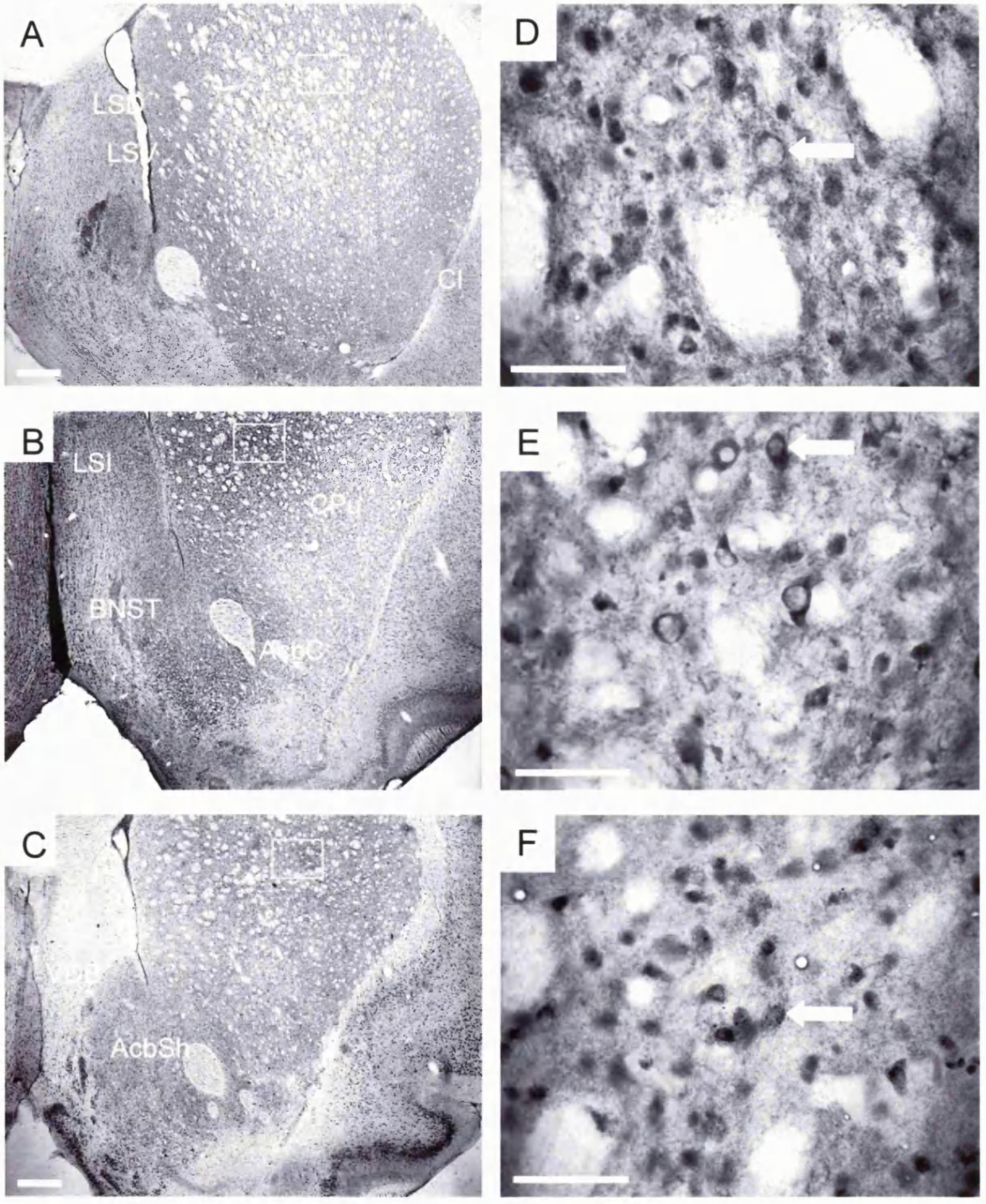

A
II
IV
VI
B
I
III
V
C
D
E
F

Figure 3.6 Photomicrographs showing $GABA_B$ receptor subunit IR in the basal ganglia and septum. $GABA_{B1a}$ (A); $GABA_{B1b}$ (B) and $GABA_{B2}$ (C) IR is present in the caudate-putamen and nucleus accumbens, though $GABA_{B2}$ IR appears absent in the septum. Small spiny neurons (arrows) are immunoreactive for $GABA_{B1a}$ (D); $GABA_{B1b}$ (E) and $GABA_{B2}$ (F) in the caudate-putamen. For abbreviations see list. Scale bars A,B,C = 500 μm; D,E,F = 50 μm.


A
LSD
LSV
CI
D
B
LSI
CPU
BNST
AcbC
E
C
AcbSh
F

3.3.7 Hippocampus and habenula

$GABA_{B1a}$ and $GABA_{B1b}$ IR were observed throughout the hippocampus and both were particularly dense in the somata of pyramidal cells of all subfields and the granule cells of the dentate gyrus (Fig. 3.7). $GABA_{B1a}$ IR was high in CA1-3 subfields. The density of IR in the stratum radiatum and pyramidal cell layers of the CA3 subfield was more intense than that seen in CA1 (Fig. 3.7A). In CA3, $GABA_{B1a}$ IR in the stratum radiatum was mainly dendritic (Fig. 3.7G). $GABA_{B1b}$ IR was strongest in CA1 and weakest in CA3 (Fig. 3.7B). $GABA_{B1b}$ labelling of CA3 showed clear dendritic labelling in the stratum radiatum (Fig. 3.7H). This was similar to CA1 where at high magnification dendritic labelling in the stratum radiatum was clearly seen (Fig. 3.7E). Hippocampi labelled with the $GABA_{B2}$ specific antiserum exhibited a similar expression pattern compared to that seen with $GABA_{B1}$ antiserum, with cell somata labelling being evenly expressed in CA1-3 subfields (Fig. 3.7C), although very little dendritic labelling was observed (Fig. 3.7F, I).

In the dentate gyrus $GABA_{B1a}$ and $GABA_{B1b}$ IR could be clearly seen in granule cell bodies. $GABA_{B1a}$ granule cell labelling was more intense in the superior layer of the dentate gyrus. In contrast, $GABA_{B1b}$ IR was of equal density in both layers of the dentate gyrus and in addition the overall level of cell body IR was higher compared to that of $GABA_{B1a}$ (Fig. 3.7J and 3.7K). $GABA_{B2}$ IR appeared similar to that of $GABA_{B1}$ IR with slightly more intense labelling in the superior layer observed. (Fig. 3.7L). Labelling in the hilus was restricted to cell bodies of interneurons and possibly glial cells for $GABA_{B1a}$ and $GABA_{B1b}$ (Fig. 3.7J and K). Interneurons in the hilus were also immunoreactive for $GABA_{B2}$,

though there were fewer positive interneurons here than those labelled for $GABA_{B1}$ subunits (Fig 3.7L).

Dense-to-very dense $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ IR was observed in the medial and lateral habenula as dense neuropil and cell body labelling (Fig 3.7A-C). In addition, the interpeduncular nucleus, which receives projection neurons from the habenula, was conspicuously labelled with a high level of $GABA_{B1a}$ and $GABA_{B1b}$ neuropil and cell body IR. $GABA_{B2}$ IR was found in many small cell bodies and adjacent neuropil in the interpeduncular nucleus, although neuropil staining was less than that seen with $GABA_{B1}$ antisera.

3.3.8 Thalamus

$GABA_{B1a}$ IR was particularly dense in the cell bodies and neuropil throughout the thalamus (Fig. 3.8A). IR was detected in the majority of nuclei, being most dense in the ventral thalamic nuclei (Fig. 3.8D), reticular thalamus and the mediodorsal nucleus (Fig. 3.8A). Somewhat less dense $GABA_{B1a}$ IR was seen in the lateral dorsal nuclei. $GABA_{B1b}$ IR (Fig. 3.8B,E) was more homogeneous throughout the thalamus with neuropilar and cell body IR observed at similar density in all nuclei. Thalamic $GABA_{B2}$ IR was similar to that seen for $GABA_{B1}$ IR subunits with labelling observed in both neuropil and cell bodies (Fig. 3.8C). The highest levels of expression were detected in the ventral (Fig. 3.8F) and medial thalamic nuclei, with weaker intensities observed in the lateral thalamic nuclei and reticular thalamus.

Figure 3.7 Photomicrographs showing $GABA_B$ receptor subunit IR in the hippocampus and habenula. $GABA_{B1a}$ IR (A) can be seen in CA1 (D), CA3 (G) and dentate gyrus (J). $GABA_{B1b}$ IR (B) can be seen in CA1 (E), CA3 (H) and dentate gyrus (K). $GABA_{B2}$ IR (C) can be seen in CA1 (F), CA3 (I) and dentate gyrus (L). Cell somata staining is shown by arrows and dendritic staining indicated with arrowheads. For abbreviations see list. Scale bars A, B, C = 500 μm and D-I = 50 μm and J-L = 100 μm.

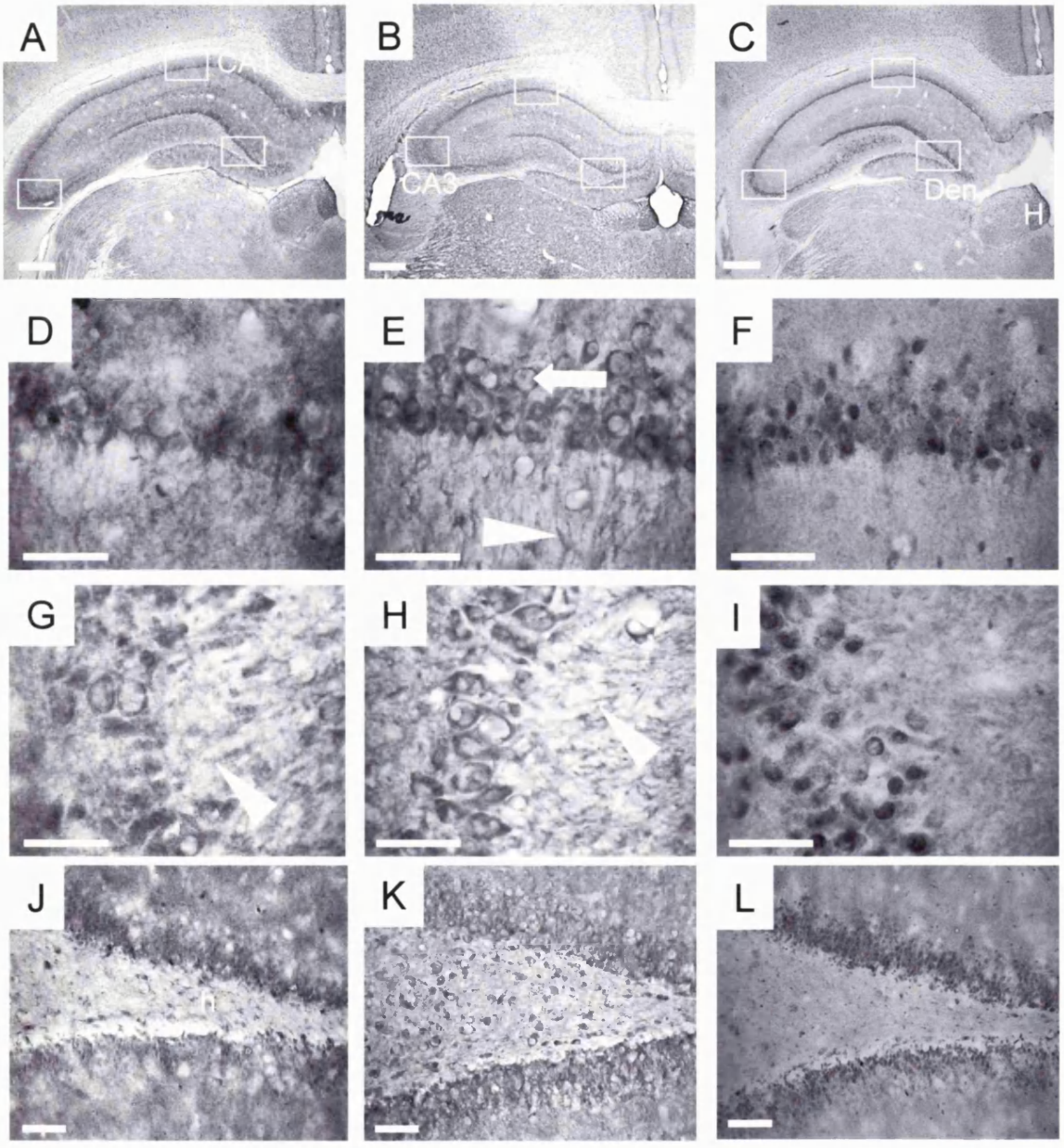

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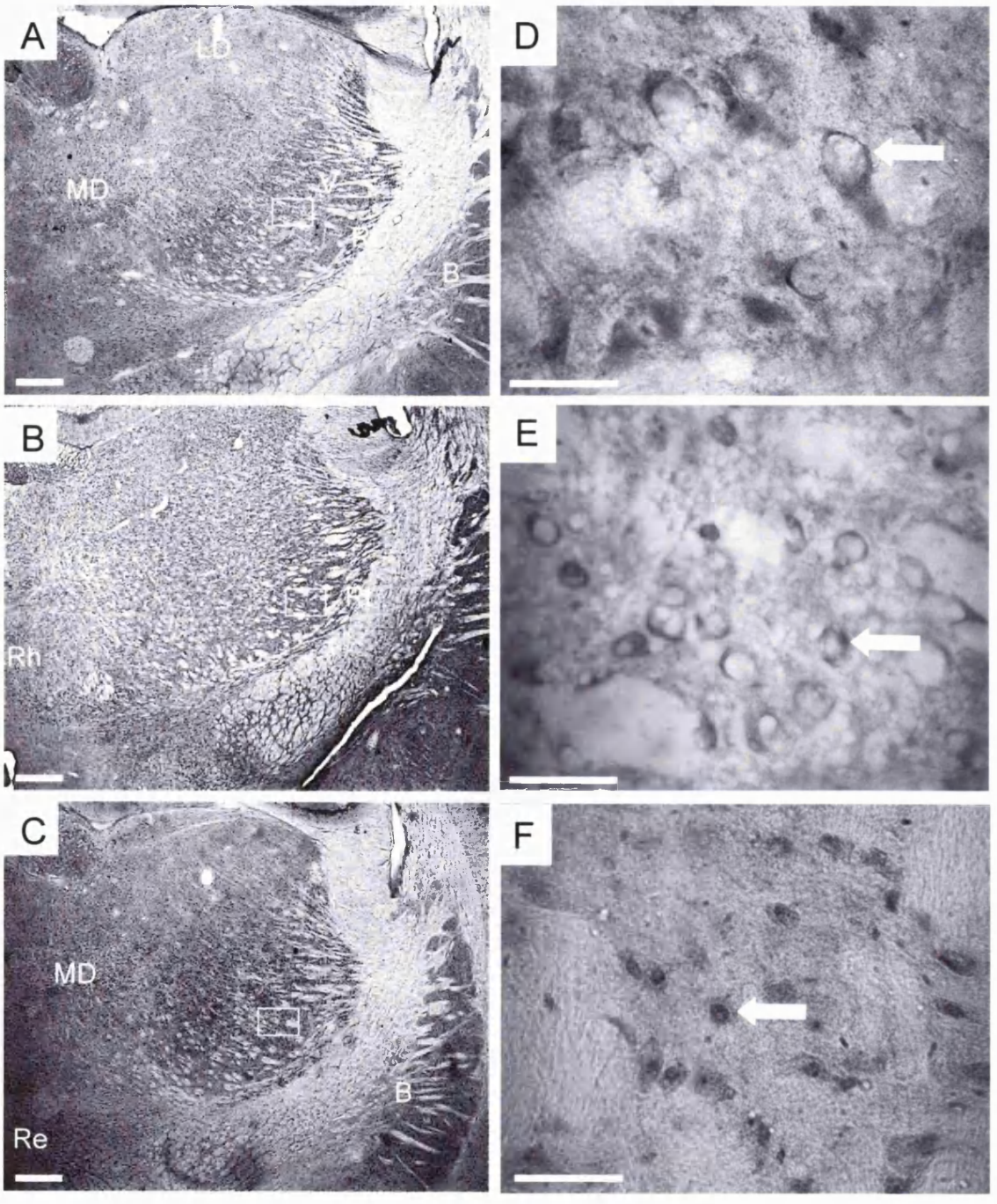
Figure 3.8 Photomicrographs showing $GABA_B$ receptor subunit IR in the thalamus. $GABA_{B1a}$ IR (A), $GABA_{B1b}$ (B) and $GABA_{B2}$ (C) IR can be seen in many thalamic nuclei including the medial dorsal thalamus, the rhomboid nucleus and the reuniens nucleus. $GABA_{B1a}$ (D), $GABA_{B1b}$ IR (E) and $GABA_{B2}$ (F) label neuronal somata (arrows) in the ventrobasal nucleus. For abbreviations see list. Scale bars A,B,C = 500 mm and D,E,F = 50 μm.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3.3.9 Hypothalamus

$GABA_{B1a}$ IR was observed in many nuclei of the hypothalamus and consisted mainly of cell body labelling. $GABA_{B1a}$ IR was moderate-to-dense around the midline structures of the median eminence and dorsal hypothalamic area, and was also observed in the medial tuberal nucleus. Other hypothalamic areas, including the tuber cinereum, had weaker $GABA_{B1a}$ IR restricted to the cell body. $GABA_{B1b}$ IR in cell somata was slightly more intense than $GABA_{B1a}$ IR and restricted mainly to the median eminence, dorsal hypothalamic area and the lateral hypothalamus, though some labelling was also apparent in areas such as the tuber cinereum. $GABA_{B2}$ labelling was denser throughout the hypothalamus, with particularly strong expression in the ventromedial hypothalamus, medial tuberal nucleus, tuber cinereum and the median eminence. In the median eminence, labelled cell bodies could be observed within a densely immunoreactive neuropil. $GABA_{B2}$ labelling was also observed in the lateral hypothalamus, although this labelling was less dense than in other areas (Table 3.2).

3.3.10 Midbrain

$GABA_{B1a}$ IR in the superior colliculus was most dense in the somata and dendrites of the superficial grey (Fig. 3.9A) and optic nerve area. $GABA_{B1a}$ IR was low to moderate in deeper layers including the intermediate layers of the superior colliculus and the inferior collicular nucleus. Dendritic $GABA_{B1b}$ labelling was found in moderate levels in this region, although far fewer positively labelled cells were observed compared to $GABA_{B1a}$ (Fig. 3.9B). $GABA_{B2}$ IR was low-to-moderate in the superior colliculus. There was little

dendritic IR in this area, and relatively few $GABA_{B2}$ IR somata were detected (Fig. 3.9C). In the deep mesencephalic nuclei a few $GABA_{B2}$ positive cells were visible.

In the inferior colliculus $GABA_{B1a}$ IR was moderate in the neuropil and cell bodies of the dorsal cortex and central nucleus. $GABA_{B1b}$ antiserum, however, labelled mainly cell bodies of the dorsal cortex, with cells of the external cortex and central nucleus being more sparsely labelled. $GABA_{B1b}$ neuropil staining was fairly dense in all regions. In contrast, neuropil labelling of the inferior colliculus with the $GABA_{B2}$ antiserum was almost absent. However, many $GABA_{B2}$ positive cell bodies were observed in the dorsal cortex, and somewhat fewer in the external and central nuclei.

In the substantia nigra $GABA_{B1a}$ and $GABA_{B1b}$ IR was apparent in both the pars reticulata and the pars compacta. Neuropil labelling was seen predominantly in the pars reticulata (Fig. 3.9D,E), whereas in the pars compacta densely labelled cell bodies were observed. The white matter of the cerebral peduncle was generally devoid of $GABA_{B1a}$ and $GABA_{B1b}$ IR although some cell body IR, presumably of glial origin, could be seen. Labelling of the substantia nigra with antisera against $GABA_{B2}$ revealed some dendritic IR in the pars reticulata (Fig. 3.9F) and pars compacta. $GABA_{B2}$ IR did not label the white matter of the cerebral peduncle.

$GABA_{B1}$ IR was also observed in the ventral tegmental area. Labelling was restricted mainly to neuronal cell bodies with little neuropil IR observed. $GABA_{B2}$ IR in this region was identical to that seen with $GABA_{B1}$ antisera.

Intense $GABA_{B1a}$ IR was seen in the large cells of the mesencephalic trigeminal nuclei, with dense labelling also evident in the adjacent neuropil. In

contrast, $GABA_{B1b}$ IR and $GABA_{B2}$ IR labelled the large mesencephalic trigeminal cells bodies but not neuropil (Table 3.2).

3.3.11 Pons, rhombencephalon and cerebellum

In monoaminergic areas of the pons, dense staining was observed with $GABA_{B1a}$ and $GABA_{B1b}$ antisera. Very little neuropil IR was seen for $GABA_{B2}$ in the pons, though many positive cell bodies were seen.

Labelling for $GABA_{B1a}$ and $GABA_{B1b}$ receptor subunits was seen in cell somata of the medial ventral periolivary and superior paraolivary nuclei with very little IR in the neuropil. $GABA_{B2}$ antisera produced very dense IR in cells of the medial ventral periolivary and superior paraolivary nuclei, though neuropil labelling was almost absent (Table 3.2).

$GABA_{B1a}$ and $GABA_{B1b}$ IR were observed in the locus coeruleus. $GABA_{B1a}$ and $GABA_{B1b}$ IR labelled cellular processes coursing through this region, and $GABA_{B1b}$ IR was present in small cell bodies in this region. $GABA_{B2}$ IR was not apparent in the neuropil and far fewer positive cell bodies were observed compared with $GABA_{B1}$ labelling (Table 3.2).

All vestibular nuclei exhibited positive neuropil IR for $GABA_{B1a}$, and to a lesser extent for $GABA_{B1b}$. $GABA_{B2}$ showed positive somatic IR and neuropil labelling was much lower than for $GABA_{B1}$ (Table 3.2).

The median raphe nuclei exhibited very little $GABA_{B1}$ IR. $GABA_{B2}$ IR was slightly more intense in cell bodies in this region with a comparable degree of neuropil labelling (Table 3.2).

Labelling of $GABA_{B1a}$ revealed a clear pattern of IR throughout the cerebellar cortex. Dense cell body and neuropil IR was observed in the granule

cell layer. Cell body labelling consistent with that of small granule cell somata and larger displaced golgi cells was observed just below the Purkinje cell layer or deeper within the granule cell layer, although without dendritic labelling it is difficult to confirm their characteristic structure. In contrast, the molecular cell layer was mostly devoid of neuropil and cell body labelling (Fig. 3.10A). $GABA_{B1a}$ IR clearly labelled Purkinje cell bodies and their dendrites (Fig. 3.10A). $GABA_{B1b}$ IR labelled the neuropil of both molecular and granule cell layers (Fig. 3.10B). Neuropil and cell body IR with this antiserum was slightly more intense in the granule cell layer. Purkinje cell labelling with the $GABA_{B1b}$ antiserum was also clearly apparent although immunoreactive dendritic projections were not as clearly evident (Fig. 3.10B). $GABA_{B2}$ IR was particularly striking although there was little labelling in the molecular cell layer (Fig. 3.10C). $GABA_{B2}$ IR was extremely dense in the neuropil and cell bodies of the granule cell layer. In addition, labelling of Purkinje cells and their dendritic branches was evident and appeared similar to that seen for $GABA_{B1a}$ IR (Fig. 3.10C).

In the deep cerebellar nuclei $GABA_{B1a}$ IR and $GABA_{B1b}$ IR was moderately dense both in the neuropil and in cell bodies. The $GABA_{B1a}$ antisera also appeared to label some glial cells in the white matter. $GABA_{B2}$ IR revealed a dense labelling of many cell bodies but was weaker in the neuropil.

Figure 3.9 Photomicrographs showing $GABA_B$ receptor subunit IR in the superficial grey of the superior colliculus and in the substantia nigra pars reticulata. $GABA_{B1a}$ (A); $GABA_{B1b}$ (B) and $GABA_{B2}$ (C) are seen in neuronal somata of the superficial grey of the superior colliculus (arrows). In the substantia nigra pars reticulata $GABA_{B1a}$ and $GABA_{B1b}$ (D,E) are seen in neuronal somata (arrows) and in dendrites (arrow heads). $GABA_{B2}$ shows greater IR in neuronal somata (arrow) compared to $GABA_{B1}$ subunits and is also seen in dendrites (arrow head). Scale bars = 100 μm.

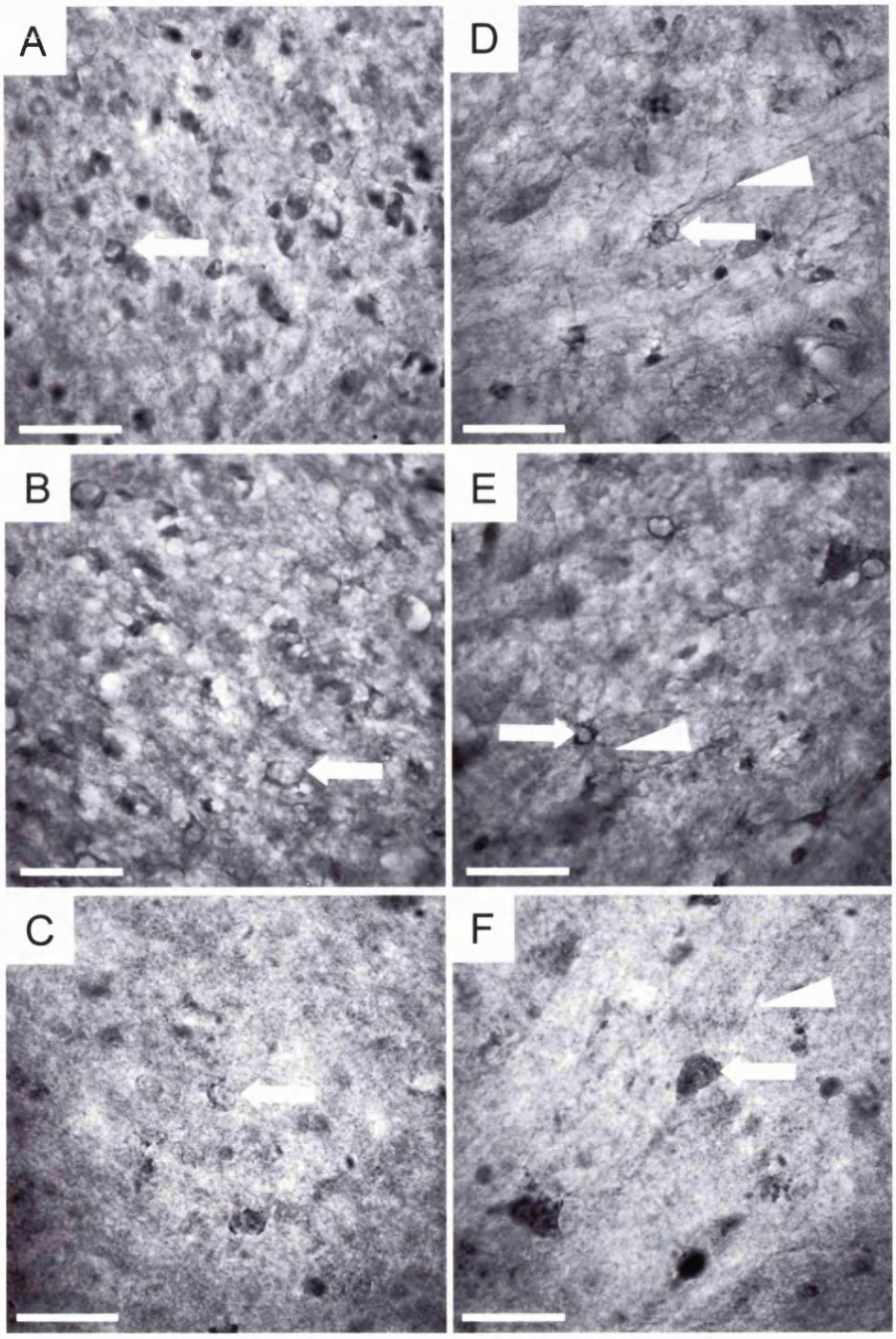
A
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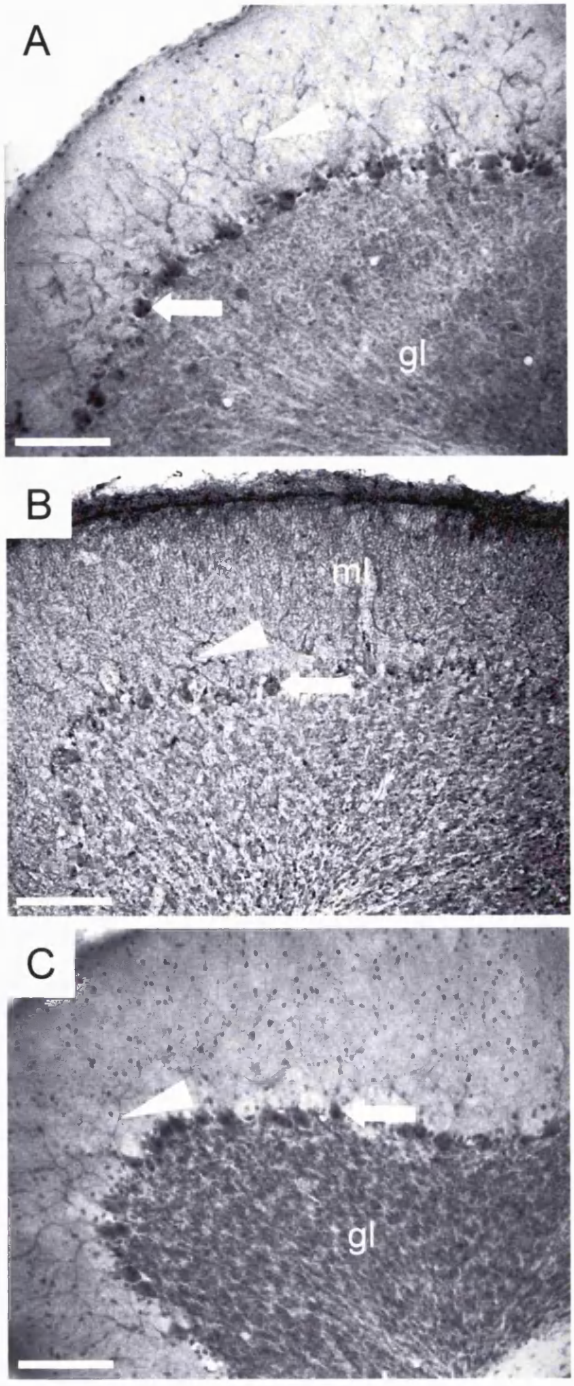
Figure 3.10 Photomicrographs showing $GABA_B$ receptor subunit IR in the cerebellar cortex. $GABA_{B1a}$ IR (A) is seen predominantly in the granule cell layer (gl) and in Purkinje cells (arrows) and Purkinje cell dendrites (arrow heads). $GABA_{B1b}$ IR (B) is present in both the granule cell layer and the molecular layer (ml). Purkinje cells (arrows) and dendrites (arrow heads) are also immunoreactive for $GABA_{B1b}$. The granule cell layer is strikingly immunoreactive for $GABA_{B2}$ (C). $GABA_{B2}$ IR is seen in the somata and dendrites of Purkinje cells (arrows and arrow heads respectively). For abbreviations see list. Scale bars = 100 μm.


A
gl
B
ml
C
gl

3.3.12 Spinal cord

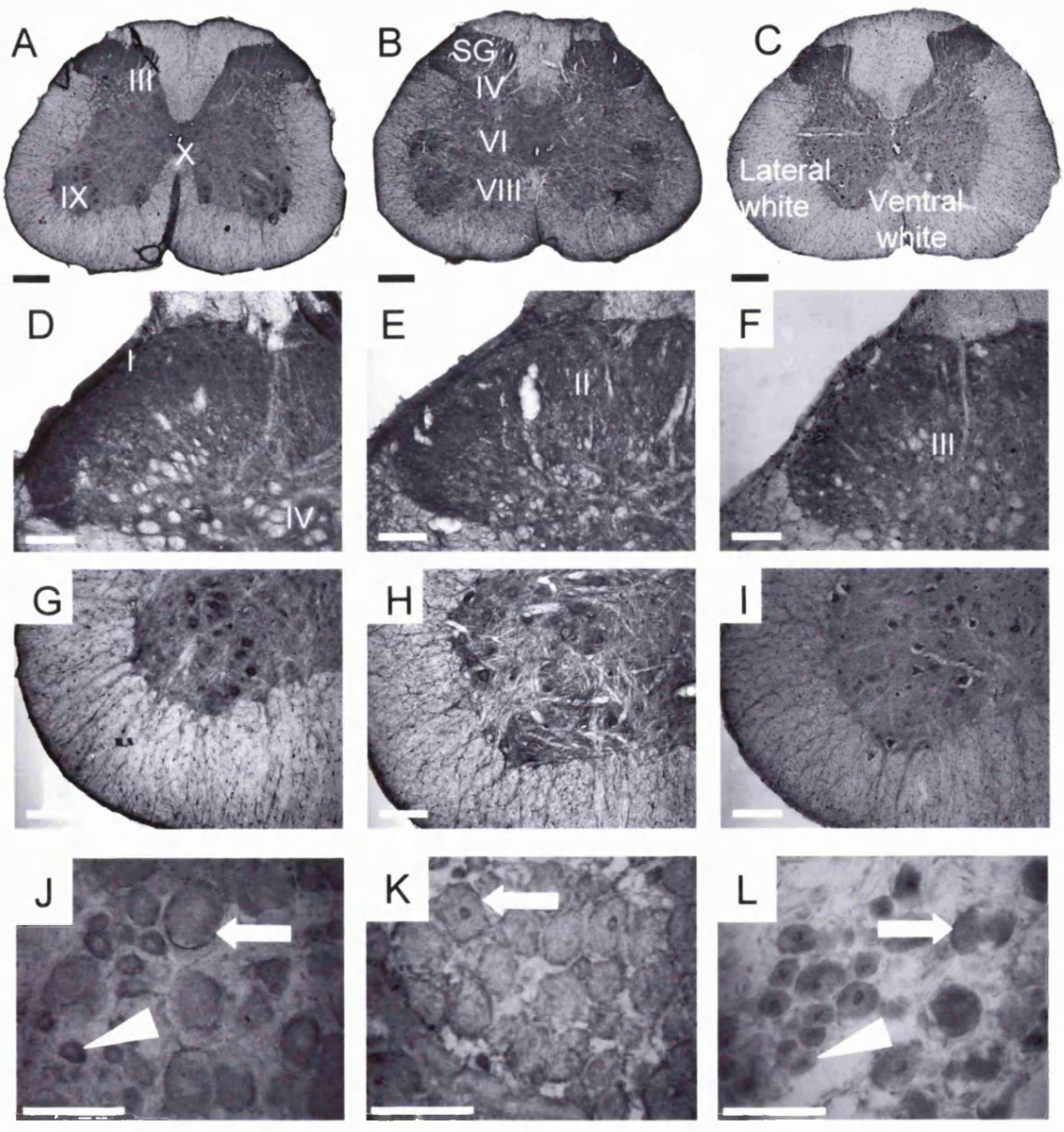
The expression pattern of $GABA_B$ receptor subunits in lumbar spinal cord clearly reflected the laminar organisation of this structure (Fig. 3.11A-C, Table 3.3). $GABA_{B1a}$ IR and $GABA_{B1b}$ IR were very intense in both cell bodies and neuropil of layers I and II (Fig. 3.11D,E). Neuropil labelling in layers III – VI was less intense, though many positive cell bodies were seen. In layers VII – IX $GABA_{B1a}$ and $GABA_{B1b}$ IR were dense in the somata of large motor neurons with dendritic labelling more apparent for the $GABA_{B1b}$ subunit (Fig. 3.11G,H). Some neuropil staining for $GABA_{B1a}$ and $GABA_{B1b}$ was seen in lamina X. $GABA_{B2}$ IR in the spinal cord was highest in the substantia gelatinosa (layers I and II) with dense neuropil and cell body IR being evident (Fig. 3.11F). As with $GABA_{B1}$ IR, $GABA_{B2}$ labelling of cell bodies was apparent in all layers of the spinal cord and there was moderate neuropil labelling. Large motor neuron somata in laminae VIII and IX were also densely stained (Fig. 3.11I). In lamina X, many $GABA_{B2}$ immunopositive cell bodies were observed with a weaker labelling of the surrounding neuropil.

Low-to-moderate white matter IR was seen with both $GABA_{B1a}$ and $GABA_{B1b}$ antisera and was most intense in the ventral and lateral columns. $GABA_{B2}$ IR was lower in the white matter of the spinal cord. In addition, I noted what appeared to be immunopositive motor neuron axons in the white matter of the ventral horn with all $GABA_B$ subunit antisera (Fig. 3.11G-I). This was most evident with the $GABA_{B1a}$ and $GABA_{B1b}$ antisera.

3.3.13 Dorsal root ganglia

Localisation of $GABA_B$ IR in the rat lumbar DRG appeared entirely restricted to perikaryal cytoplasm (Fig. 3.11J-L). Dorsal root ganglion cells are generally divided into two main types; large diameter somata giving rise to large myelinated A-fibres and small diameter neurons associated with unmyelinated C-fibres. $GABA_{B1a}$ IR was found in the cell bodies of both cell groups, with more intense IR observed in some of the small diameter cell bodies (Fig. 3.11J). In contrast, $GABA_{B1b}$ IR appeared restricted to larger diameter cell bodies (Fig. 3.11K). $GABA_{B2}$ IR showed a similar localisation pattern to that seen with $GABA_{B1a}$ IR with staining of both large and small diameter cell bodies (Fig. 3.11L).

Figure 3.11 Photomicrographs showing $GABA_B$ receptor subunit IR in lumbar spinal cord and DRG. $GABA_{B1a}$ IR is shown in spinal cord (A), the dorsal horn (D), and the ventral horn (G). Similar images are shown for $GABA_{B1b}$ IR (B, E and H) and $GABA_{B2}$ IR (C, F and I). $GABA_{B1a}$ (J), $GABA_{B1b}$ (K) and $GABA_{B2}$ (L) IR are also shown in large diameter (arrows) and small diameter (arrowheads) DRG neurons. For abbreviations see list. Scale bars A, B, C = 500 μm; D-L = 100 μm.


A
III
X
IX
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SG
IV
VI
VIII
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Lateral white
Ventral white
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III
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H
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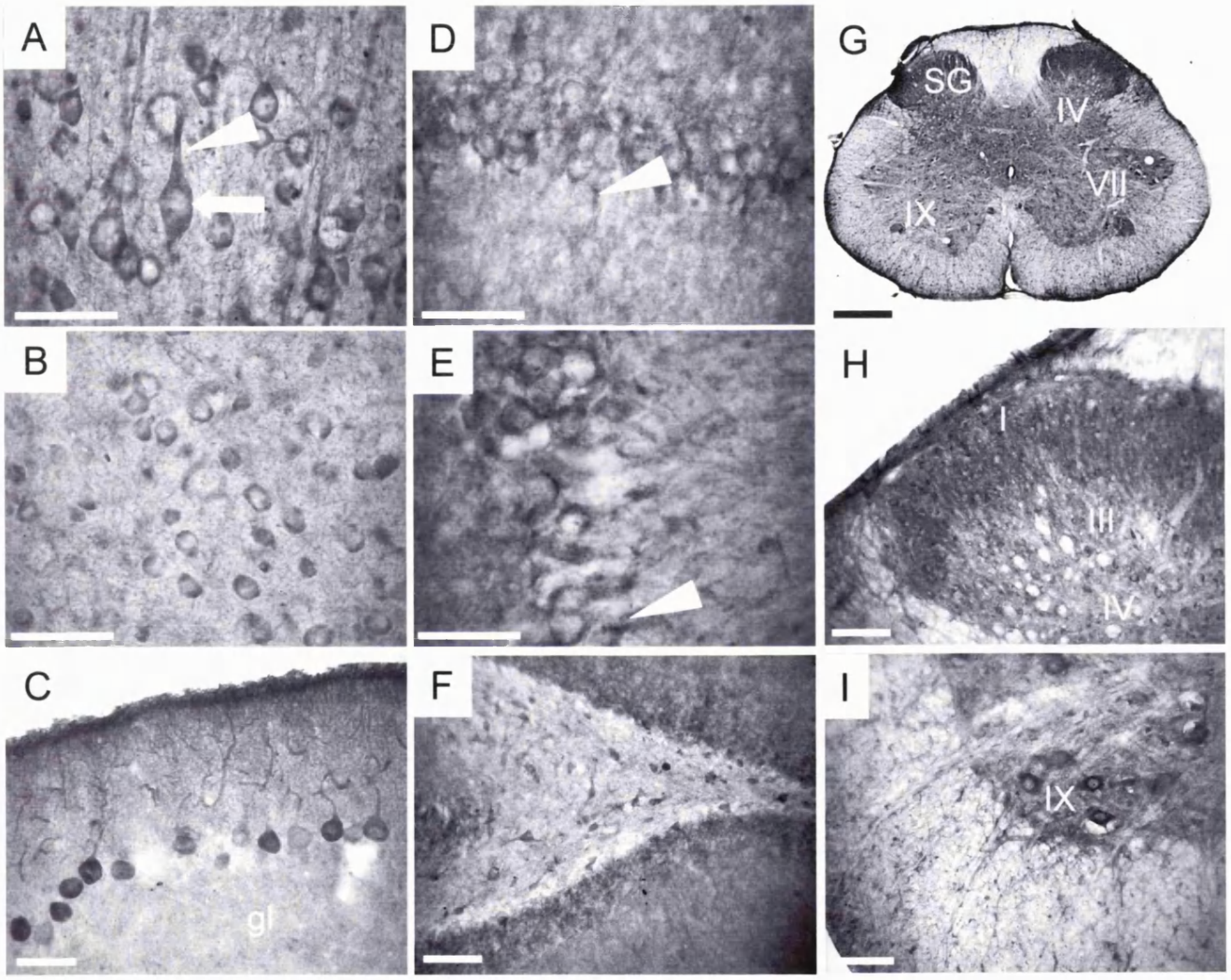
J
K
L

3.3.14 Analysis of a commercially available $GABA_{B1}$ pan antisera

The commercially available $GABA_{B1}$ antisera from Chemicon (Temecula, CA, USA) was also assessed for comparison to our antisera as a specificity control. In general the $GABA_{B1}$ antisera produced immunoreactivity in a similar pattern as that observed with each of our $GABA_{B1}$ subunit specific antisera. In layer V of the motor cortex, $GABA_{B1}$ IR was observed in the membrane and cytoplasm of large pyramidal cells, and also in the proximal area of the apical dendrites (Fig. 3.12A). In the caudate putamen, $GABA_{B1}$ IR was localised to the cell bodies of medium spiny neurons in agreement with that seen with $GABA_{B1a}$ and $GABA_{B1b}$ antisera (Fig. 3.12B). $GABA_{B1}$ IR in the hippocampus mimicked that seen with antisera against the individual splice variants. In the CA1 and CA3 regions, $GABA_{B1}$ IR was observed on the pyramidal cells and also as neuropilic labelling in the proximal apical dendrites (Fig.3.12D,E). $GABA_{B1}$ IR in the dentate gyrus was present on both the upper and lower blades as well as on interneurons within the hilus (Fig. 3.12F). In the spinal cord, labelling of $GABA_{B1}$ positive cells was observed in most layers at different intensities (Fig. 3.12G). In the cerebellum, strong $GABA_{B1}$ IR was seen in the purkinje cell bodies and their dendrites, projecting into the molecular cell layer (Fig. 3.12C). $GABA_{B1}$ IR was observed in the substantia gelatinosa in small cell bodies and in the surrounding neuropil (Fig. 3.12H), in the large motor neurons of lamina IX and in the white matter of the lumbar spinal cord (Fig. 3.12I).

Figure 3.12 Photomicrographs showing examples of $GABA_{B1}$ (Chemicon) IR throughout the rat brain and spinal cord. $GABA_{B1}$ IR in layer V pyramidal cells of the motor cortex (A) in cell bodies (arrows) and apical dendrites (arrowheads), medium spiny neurons of the caudate putamen (B), and cerebellum (C). $GABA_{B1}$ IR in the hippocampus in the CA1 (D) and CA3 (E) subfields, and the dentate gyrus (F). $GABA_{B1}$ IR in the spinal cord (G), substantia gelatinosa (H) and ventral horn (I). Scale bars A,B,C,D,E = 50μm, F,H,I = 100μm and G = 1cm.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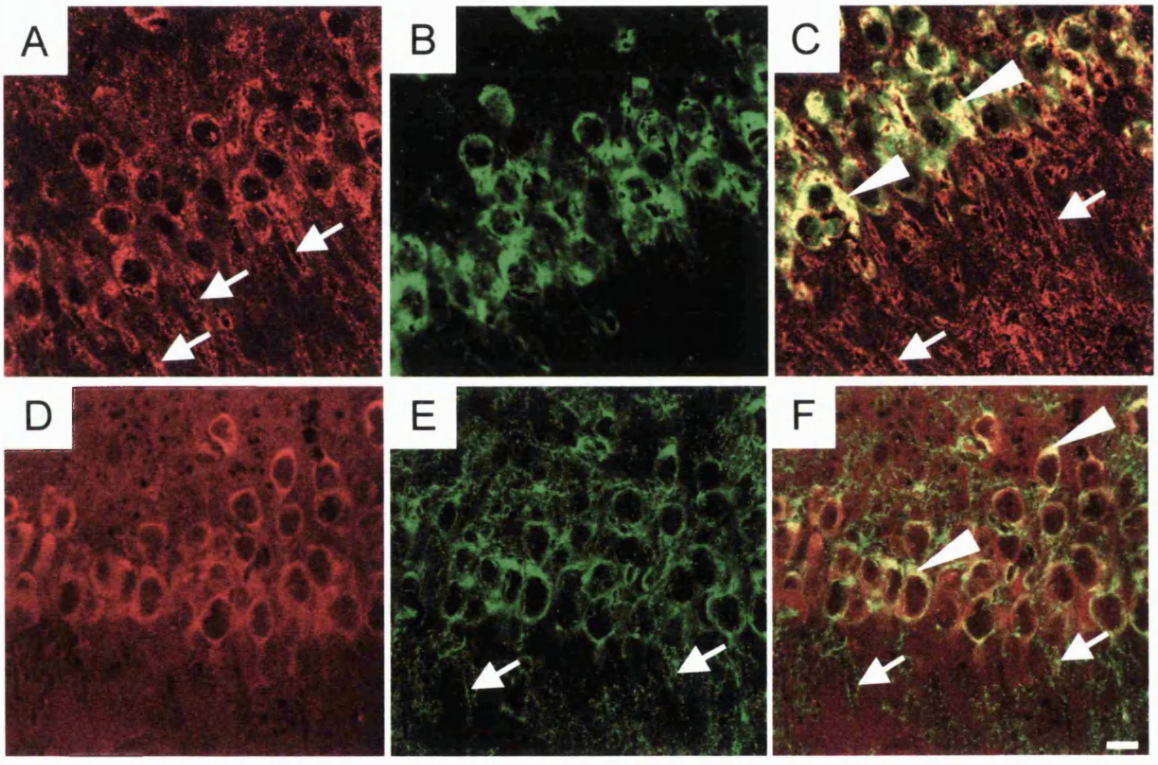
3.3.15 Co-localisation of $GABA_B$ receptor subunits in the hippocampus

$GABA_{B1}$ receptor subunits were co-localised on the same populations of CA1 pyramidal neurons in the rat hippocampus as expected from single labeling data. $GABA_{B1a}$ and $GABA_{B1b}$ receptor subunits were co-expressed in the large pyramidal cell neurons of the CA1 region as shown by confocal fluorescence microscopy (Fig. 3.13A-C). $GABA_{B1a}$ expression was observed in the cell bodies of the CA1 pyramidal cells and also in the proximal dendrites of these cells projecting into the stratum radiatum, in agreement with peroxidase data. $GABA_{B1b}$ was also expresseed on the cell bodies of the CA1 pyramidal neurons, however $GABA_{B1b}$ did not appear to be expressed in the proximal dendrites of these cells. The confocal overlay of the two fluorescent channels demonstrates the co-expression of the two $GABA_{B1}$ receptor subunits, as seen by the "yellow" signal. The merged image clearly shows the lack of $GABA_{B1b}$ labelling in the proximal dendrites as these are labelled red for the $GABA_{B1a}$ channel. $GABA_{B2}$ expression was also observed by immunofluorescence in the CA1 pyramidal neurons (Fig. 3.13D-F), in the same cells that express $GABA_{B1a}$. As observed previously $GABA_{B1a}$ was localised to the cell body and also to the proximal dendrites of the CA1 pyramidal cells. $GABA_{B2}$, however, was localised only in the cell somata and not the dendrites. In general the labelling of all $GABA_B$ subunits in the CA1 of the hippocampus by immunofluorescence was in agreement with the labelling observed using peroxidase detection.

Figure 3.13 Confocal photomicrographs showing $GABA_B$ receptor subunit co-localisations in the CA1 stratum pyramidale of the rat hippocampus. $GABA_{B1a}$ (A) was expressed in the principle pyramidal neuron cell bodies of the CA1 subregion, and was also visible on the proximal dendrites of the pyramidal cells (arrows), $GABA_{B1b}$ was also present on the same pyramidal cells (B) as shown by the merged channels (C) (arrowheads). Note that $GABA_{B1b}$ does not appear to be expressed on the proximal dendrites of these cells. $GABA_{B2}$ (D) was expressed in the cell bodies of the CA1 pyramidal cells, as was $GABA_{B1a}$ (E), the two receptor subunits were co-expressed on the same cell bodies (arrowheads) (F), though $GABA_{B1a}$ was present on proximal dendrites (arrows) apparently in the absence of $GABA_{B2}$. Scale bar = 20μm.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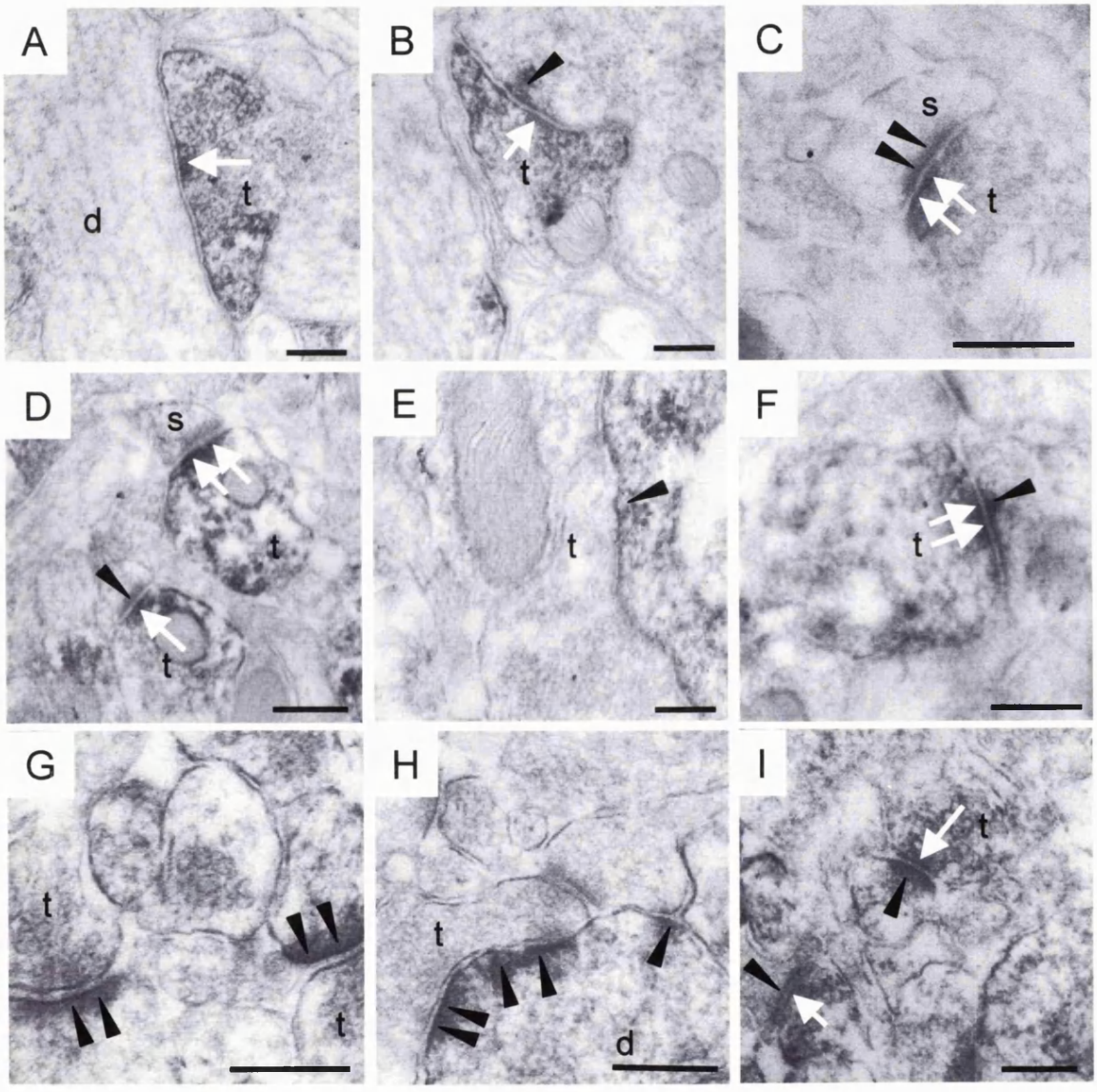
3.3.16 Synaptic localisation in the hippocampal CA1 subregion.

Peroxidase detection immunohistochemistry was carried out on rat hippocampal sections and analysed by electron microscopy. The border between the pyramidal cell layer and the stratum radiatum was examined for the existence of reaction product in pre- and post-synpatic regions and for the type of synapse on which the receptor subunits were expressed. Expression of $GABA_{B1a}$ was noted on pre- and post-synaptic membranes in the CA1 subregion of the hippocampus (Fig. 3.14A-C). Pre-synaptic axo-dendritic labelling as well as pre-synaptic labelling onto a dendritic spine heads were observed. $GABA_{B1a}$ was also seen to be present post-synaptically in symmetric inhibitory synapses, and was also observed on the pre-synaptic membrane. The expression of $GABA_{B1b}$ in the CA1 subregion at the ultra-structural level showed little difference from that observed with $GABA_{B1a}$ (Fig. 3.14D-F). $GABA_{B1b}$ was expressed in the pre- and post-synaptic membranes of excitatory and inhibitory synapses. Pre-synaptic localisation was also observed on dendritic spine heads (Fig. 3.14D). The ultrastructural localisation of the $GABA_{B2}$ receptor subunit was similar to that of $GABA_{B1}$ receptor subunits as would be expected given the pre-requisite of heterodimerisation for this receptor to be functional. $GABA_{B2}$ was therefore localised on pre- and post-synaptic membranes of inhibitory and excitatory synapses. Post-synaptic dendritic expression of $GABA_{B2}$ as well as pre-synpatic expression onto a spinehead was observed (Fig. 3.14G-I).

Figure 3.14 Electron microscope photomicrographs demonstrating the pre- and post-synaptic localisation of $GABA_B$ receptor subunits in the CA1 subregion of the rat hipppocampus. $GABA_{B1a}$ was expressed pre-synaptically on axo-dendritic terminals (A), $GABA_{B1a}$ IR was detected in pre-synaptic terminals (white arrow) forming a symmetric type junction with somata in the cell body layer. IR was also detected adjacent to the post-synaptic membrane (B). $GABA_{B1a}$ IR wasl also detected in pre-synaptic terminals forming asymmetric type junctions with spine heads, and are therefore likely to be excitatory (C). $GABA_{B1b}$ expression was seen in the excitatory terminals apposed to a spine head, forming excitatory, asymmetric, synapses (D). Post-synaptic expression of $GABA_{B1b}$ was also observed in an inhibitory synapse onto a pyramidal cell somata (E). In an inhibitory synapse in the CA1, $GABA_{B1b}$ expression was noted in the pre-synaptic terminal and also in the post-synaptic specialisation (F). $GABA_{B2}$ expression was also present in the CA1 subregion. $GABA_{B2}$ expression was noted on two post-synaptic specialisations in close proximity to each other (G), both synapses appear to be excitatory. Post-synaptic $GABA_{B2}$ expression was also observed on an axo-dendritic synapse (H). $GABA_{B2}$ was also expressed pre-synaptically in inhibitory and excitatory terminals in the same field of view (I). S = spine, d = dendrite, t = terminal, black arrowhead = post-synaptic receptor expression, white arrow = pre-synaptic receptor expression. Scale bars = 0.25μm.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3.4 Discussion

3.4.1 Experimental considerations

In this study I report the immunohistochemical distribution of the two principle N-terminal splice variants of the $GABA_{B1}$ subunit, $GABA_{B1a}$ and $GABA_{B1b}$, and the $GABA_{B2}$ subunit in rat brain, spinal cord and DRG using antisera selective for each subunit. Although the epitopes selected for our $GABA_{B1a}$ and $GABA_{B1b}$ antisera were specific for these splice variants, it should be noted that other $GABA_{B1}$ splice variants have also been reported that cannot be differentiated with these tools (Isomoto et al., 1998; Calver et al., 2000 and see figure 1.4). Therefore, although I discuss the expression of $GABA_{B1a}$ and $GABA_{B1b}$ subunits these antisera may also recognise other additional $GABA_{B1}$ splice variants, although it is important to note that so far none of the other splice variants of the $GABA_{B1}$ subunit has altered or distinct pharmacology when expressed in recombinant systems (Wood et al., 2000). In addition, my studies using a commercially available $GABA_{B1}$ antisera (Chemicon) revealed a similar pattern of staining when compared our own, in-house generated, $GABA_{B1a}$ and $GABA_{B1b}$ antisera, giving further confidence in the specificity of these antisera. The $GABA_{B2}$ antisera used in this study is believed to be specific to all known $GABA_{B2}$ splice variants, as for $GABA_{B1}$ there is no distinct pharmacology reported for any of these splice variants. However, further splice variants may yet be discovered and hence this antisera may not represent all $GABA_{B2}$ receptor subunits. Differences I have observed between results of this study and others studies examining $GABA_{B1}$ IR may be predominantly due to differences in the epitopes selected. Although all of the studies discussed have demonstrated the relative selectivity of their antisera it is feasible that treatment of tissue sections

with various chemicals and fixatives can alter the conformation and binding conditions of the antibody target proteins. Importantly it is also possible that other splice variants of $GABA_{B1}$ or $GABA_{B2}$ subunits remain to be identified which may or may not be recognised by each of these particular antisera. It is also worth considering the different sensitivities of labelling kits used. In general I have used the avidin-biotin complex (ABC) kit from Vector to bind the secondary antisera and provide a substrate for the diaminobenzidine (DAB) colour substrate. However, such kits, both the ABC and the DAB, are dispensed through dropers and may therefore not be exact in the volume dispensed in each drop. Indeed the DAB kit is particularly prone to variability. However, I have used the safe kit system instead of alternative methods due to the potential carcinogenic properties of the DAB substance. For fluorescence work extra amplification was required for the $GABA_{B1b}$ and the $GABA_{B2}$ antisera, although not for the $GABA_{B1a}$ antisera. Thus comparing relative intensities of labelling between these polyclonal antisera is open to interpretation.

Much effort has been placed on ensuring that the distribution of labelling with these antisera is in agreement with known receptor distribution. Classically the distribution of the $GABA_B$ receptor has been described using autoradiographical binding studies. However, as described in chapter 1.3.4, the current hypothesis for $GABA_B$ binding and signalling is that ligands bind $GABA_{B1}$ and signal through $GABA_{B2}$. Therefore, the binding described by autoradiography is only representative of $GABA_{B1}$ receptors. That said, however, $GABA_{B1}$ receptors do not reach the cell surface unless in the presence of $GABA_{B2}$, or other as yet unidentified chaperone proteins. Thus it is even prudent

to place some caution on the interpretation of autoradiographical data in comparison to the protein distribution of the individual receptor subunits.

Interpretation of the data presented here must therefore be performed cautiously with a mind to considerations of technique, tissue preparation and the unknown status of further unidentified splice variants. Nevertheless, as much effort as possible has been afforded to confirm the specificity and selectivity of these antisera for the protien target to which they were designed. Thus, at present, full confidence is placed in these antisera and the results obtained here.

3.4.2 $GABA_B$ receptor subunit distribution in the rat CNS

$GABA_{B1}$ and $GABA_{B2}$ subunit expression was found throughout many regions of the rat brain and spinal cord in general agreement with previous studies looking at $GABA_{B1}$ (Bischoff et al., 1999; Liang et al., 2000) and $GABA_{B2}$ mRNA expression (Durkin et al., 1999; Clark et al., 2000), $GABA_{B1}$ protein expression (Fritschy et al., 1999; Margeta-Mitrovic et al., 1999; Poorkhalkali et al., 2000; Towers et al., 2001) or $GABA_{B2}$ protein expression (Ige et al., 2000; Billinton et al., 2000; Princivalle et al., 2001; Ng and Yung, 2001a/b; Davis et al., 2002). Furthermore, these studies match well with $GABA_B$ receptor radioligand binding studies suggesting that $GABA_{B1a}$ or $GABA_{B1b}$ expressed with $GABA_{B2}$ can account for most if not all $GABA_B$ binding sites in brain and spinal cord (Bowery et al., 1984; Bowery et al., 1987; Bischoff et al., 1999; Chu et al., 2000). High levels of $GABA_{B1a}$ and $GABA_{B1b}$ subunit expression were found in many areas including the neocortex, hippocampus, most thalamic nuclei, habenula, interpeduncular nucleus, shell of the nucleus accumbens and cerebellum. $GABA_{B2}$ IR was also widespread with highest expression detected in the

neocortex, hippocampus, habenula, and median eminence of the hypothalamus and cerebellum. This generally correlates well with areas of highest mRNA expression, although interestingly we also detected moderate $GABA_{B2}$ IR in areas where mRNA expression has been reported to be very low such as the caudate-putamen, the nucleus accumbens and the hypothalamus (Durkin et al., 1999; Clark et al., 2000). Such differences may be due to either the relative sensitivity of the immunoperoxidase technique or may be related to the rate of mRNA turnover. However, in agreement with our observations other groups have also reported the expression of $GABA_{B2}$ in the caudate-putamen (Ng et al., 2001; Billinton et al., 2000). Indeed co-localisation studies in this chapter reveal that $GABA_{B1}$ and $GABA_{B2}$ receptor subunits always exist co-incidently. In the hippocampus $GABA_{B1a}$ and $GABA_{B2}$ were co-localised to the same cells in the CA1 stratum pyramidale. Interestingly, however, $GABA_{B1a}$ expression was observed by immunfluorescence on the proximal dendrites of the CA1 pyramidal cells in the absence of $GABA_{B2}$. This could possibly reflect a true absence of the second part of the obligate heterodimer from these dendrites. However, functional $GABA_B$ receptors have been recorded from these dendrites (Newberry and Nicoll, 1985). Therefore, the more likely scenario is that the density of $GABA_{B2}$ subunits in the proximal dendrites is below the detection limit of the subunit antisera used.

The subcellular pattern of $GABA_B$ receptor subunit expression observed in this study was generally consistent with various functional studies performed on these receptors. For example excitatory neurons of the dorsal thalamus known to be modulated by GABA (Liu et al., 1995) were immunopositive for both $GABA_{B1}$ and $GABA_{B2}$ subunits. Inhibitory interneurons of the reticular thalamus, known to be GABAergic (Arcelli et al., 1996) were found here to be $GABA_B$ positive along

with the adjacent neuropil, suggesting that these receptors may have an autoreceptor function. In the hypothalamus, another area exhibiting both $GABA_{B1}$ and $GABA_{B2}$ IR in our study, both pre- and postsynaptic $GABA_B$ receptors have been demonstrated to regulate the activation of transient calcium currents (Obrietan and Van den Pol 1999). Baclofen has been shown to inhibit voltage-gated calcium currents in Purkinje cells of the cerebellum (Mintz and Bean 1993) and locus coeruleus neurones (Olpe et al., 1988). Both these areas exhibit $GABA_{B1}$ and $GABA_{B2}$ IR in the present study. In the basal ganglia the output pathways, as well as the intrinsic synaptic circuits, are predominantly GABAergic (Parent and Hazarati 1995b). There is abundant pharmacological and electrophysiological evidence demonstrating the importance of $GABA_B$ receptors in regulating release of GABA as well as other neurotransmitters in the striatum (Sing 1990; Arias-Montano et al., 1992), globus pallidus (Sing 1990) and substantia nigra (Sing 1990; Chan et al., 1998). These findings demonstrate expression of both $GABA_{B1}$ and $GABA_{B2}$ subunits in the basal ganglia consistent with the presence of both pre- and post-synaptic hetero- and autoreceptor function. Interestingly, the substantia nigra pars compacta was one of the areas with most intense cell body labelling for $GABA_{B1}$ and $GABA_{B2}$ suggesting a robust postsynaptic expression of $GABA_B$ receptors in this region. This is supported by mRNA studies (Bischoff et al., 1999; Liang et al., 2000) and a recent immunohistochemical report in primates (Charara et al., 2000) but does not agree with a previous autoradiographic study that described predominant binding in the substantia nigra pars reticulata (Bowery et al., 1987).

$GABA_B$ binding studies in the neocortex suggested that the highest levels of receptor expression are generally found in layers I-IV (Bowery et al., 1987;

Bischoff et al., 1999), although mRNA expression of $GABA_{B1a}$ and $GABA_{B1b}$ transcripts were highly expressed in all cortical layers (Bischoff et al., 1999; Liang et al., 2000). In agreement with the mRNA observations I found $GABA_{B1}$ and $GABA_{B2}$ variants expressed in cell bodies and dendrites throughout the neocortex with highest levels of expression generally seen in layer II and layer V.

Involvement of $GABA_B$ receptors in cognitive function has previously been demonstrated with pre- and post-synaptic receptor activation being shown to regulate hippocampal neuronal activity and the induction of long term potentiation (Davies et al., 1991). In accord with these observations I found $GABA_{B1}$ and $GABA_{B2}$ receptor subunit expression widespread in all hippocampal subfields studied. $GABA_{B1a}$ and $GABA_{B1b}$ subunits were highly expressed in the somata of pyramidal cells of CA1 in agreement with previous studies that used C-terminal pan $GABA_{B1}$ antisera, to recognise both $GABA_{B1a}$ and $GABA_{B1b}$ (Margeta-Mitrovic et al., 1999; Sloviter et al., 1999) and with mRNA expression data in this region (Bischoff et al., 1999; Liang et al., 2000). However, these observations are in contrast to autoradiographic studies that used selective $GABA_B$ ligands to show a lack of specific binding to the pyramidal cell layer (Bowery et al., 1984; Bischoff et al., 1999; Sloviter et al., 1999). These results could previously have been explained by a lack of $GABA_{B2}$ subunit expression in CA1 pyramidal cells. However, this no longer seems true as $GABA_{B2}$ was also densely expressed in pyramidal cells of CA1, in agreement with mRNA expression data for this subunit (Durkin et al., 1999; Calver et al., 2000; Clark et al., 2000). In addition, I observed a clear expression of $GABA_{B1a}$ and $GABA_{B1b}$ subunits in the strata radiatum and oriens of CA1, that is in contrast to the

findings of Margeta-Mitrovic et al., (1999). Nevertheless, these findings are in accord with the functional observations of Newberry and Nicoll (1985) who observed an increase in currents induced in the dendritic fields of CA1 following application of $GABA_B$ agonists at the CA1 pyramidal cell somata. In CA3 I observed higher levels of $GABA_{B1a}$ and $GABA_{B2}$ expression compared with $GABA_{B1b}$ in both the somata of pyramidal cells and adjacent neuropil. This expression of the two $GABA_{B1}$ splice variants is in agreement with the findings of Poorkhalkali et al., (2000) and with $GABA_{B1}$ subunit mRNA expression studies (Bischoff et al., 1999; Liang et al., 2000). In contrast, groups using pan-$GABA_{B1}$ antisera detected abundant neuropil staining in CA3 but a complete lack of pyramidal cell body staining (Fritschy et al., 1999; Margeta-Mitrovic et al., 1999; Sloviter et al., 1999), although in my hands the pan $GABA_{B1}$ antisera from Chemicon, raised against the carboxy domain of the $GABA_{B1}$ receptor, labelled pyramidal cell bodies in the CA1-3 subregions (Fig. 3.2.8). Although data from these latter studies agree with autoradiographic ligand binding studies, functional studies have demonstrated the clear presence of $GABA_B$-mediated currents from CA3 pyramidal cells stimulated by both pre- and post-synaptic receptors (Okuhura and Beck 1994; Sodickson and Bean 1996; Scanziani 2000). In addition, a recent study of $GABA_{B1}$ and $GABA_{B2}$ receptor subunit expression in human brain agrees well with our findings of higher $GABA_B$ subunit expression in CA3 than CA1 (Billinton et al., 2000). All three $GABA_B$ subunits were expressed in granule cells and interneurones of the dentate gyrus in agreement with mRNA data but in contrast to ligand binding studies (Bischoff et al., 1999; Sloviter et al., 1999). Here the data would therefore suggest that ligand binding

studies in the rat hippocampus may be detecting only a subset of $GABA_B$ receptors and that some unknown factors may be important in determining ligand binding to $GABA_B$ receptors in this region.

The cerebellum is an area of the brain demonstrated to be particularly rich in $GABA_B$ receptors. In the granule cell layer $GABA_{B1a}$ and $GABA_{B2}$ receptor subunits appear to be more highly expressed than $GABA_{B1b}$. This is in agreement with $GABA_{B1a}$ and $GABA_{B1b}$ mRNA (Liang et al., 2000), and protein (Poorkhalkali et al., 2000) studies but in contrast to reports using pan-$GABA_{B1}$ antisera (Billinton et al., 1999; Margeta-Mitrovic et al., 1999; Sloviter et al., 1999). It is interesting to note that whilst ligand binding studies detected high levels of binding in the molecular layer and almost a complete absence of binding in the granule cell layer, there appears to be abundant expression of $GABA_{B1}$ and $GABA_{B2}$ mRNA and protein in the latter region (Bowery et al., 1987; Bischoff et al., 1999; Sloviter et al., 1999; Clark et al., 2000). The molecular layer contains Purkinje cell dendrites receiving excitatory afferents from parallel and climbing fibres and inhibitory afferents from stellate cells (Ito 1984). $GABA_{B1a}$ and $GABA_{B2}$ subunit IR in this region was highest in the small cell bodies of radial glia and/or stellate cells with $GABA_{B1b}$ IR being more noticeable in the neuropil.

Much effort has been made in recent studies to differentiate between the pre- and postsynaptic distribution of $GABA_{B1a}$ and $GABA_{B1b}$ splice variants using different techniques. Results appear quite contradictory however, with some studies suggesting $GABA_{B1a}$ as predominantly postsynaptic (Benke et al., 1999; Poorkhalkali et al., 2000) or presynaptic (Billinton et al., 1999; Bischoff et al., 1999; Fritschy et al., 1999; Liang et al., 2000). These data at the light microscope

level are consistent with both suggestions with $GABA_{B1a}$ and/or $GABA_{B1b}$ subunits forming both pre- and postsynaptic receptors. $GABA_{B1}$ and $GABA_{B2}$ immunostaining of Purkinje cell bodies and dendrites extending into the molecular layer was demonstrated with all three antisera, as would be predicted from mRNA (Bischoff et al., 1999; Liang et al., 2000) and functional studies (Turgeon and Albin 1993; Vigot and Batini 1997; Wojcik and Neff 1990). I was, however, unable to detect more abundant levels of the $GABA_{B1a}$ subunit relative to $GABA_{B1b}$ as had been previously reported (Bischoff et al., 1999; Fritschy et al., 1999; Liang et al., 2000; Poorkhalkali et al., 2000).

Confocal microscopy using double labelling techniques for $GABA_{B1a}$ and $GABA_{B1b}$ in the hippocampus also demonstrated a near total overlap of receptor subunits in the pyramidal cells. However, $GABA_{B1a}$ immunofluorescence was observed in the proximal dendrites of these cells in the absence of $GABA_{B1b}$, though as discussed previoulsy for $GABA_{B2}$, this may be due to the inability of the antisera to detect the protein rather than an absence of the protein in the dendrites, and given that analysis at the electron microscope revealed the expression of $GABA_{B1b}$ in dendritic spines in this region it is also possible the fluorescent detection system is not sensitive enough for detecting the protein in the dendrites.

In collaboration with Dr.J.Deuchars at Leeds University, I have performed electron microscopy in the hippocampus in an attempt to address the issue of the pre- versus post-synaptic localisation of each of the $GABA_B$ receptor subunits and if, indeed, there is any difference between the expression of $GABA_{B1a}$ and $GABA_{B1b}$ subunits. This study shows that both $GABA_{B1a}$ and $GABA_{B1b}$ are expressed in both pre- and post-synaptic membranes of inhibitory and excitatory

synapses in the hippocampus. $GABA_{B2}$ also showed a similar pattern of localisation and thus we were unable even at the ultrastructural level to observe differences in the subunit localisations that may have helped explain the diverse pharmacological nature of the $GABA_B$ receptor. Others have reported the ultra-structural localisation of the $GABA_{B1}$ and $GABA_{B2}$ receptor subunits in regions including the cerebellum (Ige et al., 2000), basal ganglia (Charara et al., 2000) and visual cortex (Gonchar et al., 2001). None of these reports have investigated the subcellular localisation of the two $GABA_{B1}$ splice variants, instead they have examined the pre- and post-synaptic localisation of $GABA_{B1}$ using antisera selective for both splice variants. Electrophysiological measurements would suggest that most $GABA_B$ receptors are situated extra-synaptically on the pre- and post-synaptic membranes (Isaacson et al., 1993), and this hypothesis is in agreement with the localisation of the $GABA_B$ receptor subunits in the cerebellum according to Ige et al., (2000). Two other reports, however, suggest that $GABA_B$ receptors are located directly at the synapse (Charara et al., 2000; Gonchar et al., 2001), although Gonchar et al., also report observing extra-synaptic receptor localisation. Importantly all the studies reported, to date, have not only examined different regions of the CNS, but also different species. The studies of Charara et al., were carried out in the basal ganglia of monkeys, whereas those of Gonchar et al., were carried out in the rat visual cortex. Another critical factor in the comparison of these studies is the utilisation of different polyclonal antisera. All the studies reported have utilised C-terminal antisera to examine the ultrastructure of $GABA_B$ receptor expression, however, each antisera has been raised to different receptor epitopes. These differences may allow or prevent antisera binding to the receptor subunit depending on the conformation of the protein in its

specific synaptic localisation. The nature of the synapse within which the $GABA_B$ receptors were expressed according to Charara et al., and Gonchar et al., also agrees with our data, whereby pre- and post-synaptic localisation was observed in both excitatory and inhibitory synapses.

$GABA_B$ receptor agonists have a well-documented antinociceptive activity in animal models of pain (Malcangio and Bowery, 1996; Couve et al., 2000). In the spinal cord our $GABA_B$ antisera labelled cell bodies and processes of the superficial layers of the dorsal horn with a slightly more pronounced expression of the $GABA_{B1b}$ subunit (compared with the other subunits) in this area. These findings are consistent with ligand binding studies (Price et al., 1987), $GABA_{B1}$ immunohistochemical studies (Poorkhalkali et al., 2000; Towers et al., 2000), and $GABA_{B2}$ mRNA studies (Durkin et al., 1999; Calver et al., 2000). Large motor neurons and axons originating within the ventral horn were also immunopositive with each of the $GABA_{B1}$ and $GABA_{B2}$ antisera in contrast to a previous report in which only $GABA_{B1a}$ IR was observed in these cells (Poorkhalkali et al., 2000). My data are in agreement with the observations of Margeta-Mitrovic et al. (1999), however, and suggest that regulation of these neurons is not only mediated by a presynaptic inhibition of glutamatergic input (Misgeld et al., 1995) but also by postsynaptic $GABA_B$ receptor mechanisms. In DRG $GABA_{B1a}$ and $GABA_{B2}$ IR was observed in large and small diameter cells whereas $GABA_{B1b}$ was more commonly associated only with large diameter cells. These observations suggest that $GABA_{B1a}$ and $GABA_{B1b}$ subunits can form functional pre- and post-synaptic receptors in the spinal cord in the presence of $GABA_{B2}$. These may be either pre-synaptic heteroreceptors from excitatory Aδ and C (small diameter) or Aα and Aβ

(large diameter) primary afferents or postsynaptic autoreceptors from GABAergic neurons in the spinal cord (Bernardi et al., 1995).

It was also of interest to note that in some instances I observed an unusual nuclear staining in some cells with both $GABA_{B1}$ and $GABA_{B2}$ antisera. This was not observed in all brain regions but instead appeared restricted to certain areas and cell types such as cell bodies of the DRG and some cortical pyramidal cells. Importantly a second but distinct anti-$GABA_{B2}$ antiserum that we raised against a C-terminal peptide not only yielded a similar pattern of protein localisation throughout the rat brain and spinal cord but also a comparable nuclear staining. Similar observations have been reported by others using a number of different $GABA_B$ selective antisera at the light and electron microscope level (Poorkhalkali et al., 2000; Gonchar et al., 2001; Bowery N. G., personal communication). Although it is possible that this nuclear staining may be artifactual in nature, it may not be so surprising if one considers that $GABA_B$ receptors have been implicated in the regulation of gene transcription and have been demonstrated to associate via their C-terminal domains with nuclear transcription factors such as the activating transcription factor ATF-4 (Nehring et al., 2000). Indeed, analysis at the electron microscope level revealed the presence of immunoreactivity for all $GABA_B$ receptor subunits in the nucleus of neurons in the CA1 subregion of the hippocampus. Such a finding, combined with the ATF-4 interaction, suggests that this labelling is in fact not artifactual but represents the presence of $GABA_B$ receptor subunits in the nucleus, though their function in this cellular compartment remains to be confirmed.

In conclusion this study demonstrates the anatomically overlapping expression of two $GABA_{B1}$ splice variants with $GABA_{B2}$ throughout the CNS.

This suggests that both $GABA_{B1a}$ and $GABA_{B1b}$ subunits can form pre- and postsynaptic receptors in association with a $GABA_{B2}$ receptor subunit.

Chapter 4 - Characterisation of $GABA_B$ receptor subunits on glial cell subpopulations in the rat CNS

4.1 Introduction

The expression of $GABA_B$ receptor subunits in non-neuronal cells has remained largely unexplored. $GABA_B$ immunoreactive (IR) cells in the white matter tracts of rat brain and spinal cord with $GABA_B$ antisera have been reported (Margeta-Mitrovic et al., 1999; Charles et al, 2001). Binding of GABA to cultured astrocytes in the presence of unlabelled bicuculline has been demonstrated (Hosli and Hosli, 1990). And at the functional level, several reports have proposed roles for $GABA_B$ receptors in non-neuronal cells of the CNS, such as astrocytes. In particular Albrecht et al., (1986) demonstrated a reduction in $[Ca^{2+}]$ efflux in response to GABA or baclofen in rat cortical astrocyte cultures in the presence of the $GABA_A$ receptor antagonist bicuculline. Furthermore, baclofen but not the $GABA_A$ receptor agonist isoguvacine induced increases in intracellular Ca^{2+} (Nilsson et al., 1993) and inhibited the release of endogenous benzodiazepines from astrocytes (Patte et al., 1999). To date, however, the subunit composition of these functional $GABA_B$ receptors remains unexplored. Are the functional receptors in glia composed of the same subunits as those on neurons or are these cells a possible source of additional, as yet unidentified, $GABA_B$ receptor subunits?

4.2 Aims of this study

Using $GABA_B$ receptor subunit specific antisera I aim to assess, for the first time, the $GABA_B$ receptor subunit expression profile on astrocytes, microglia

and oligodendrocytes at both the light and electron microscope level in the rat CNS.

4.3 Results

4.3.1 Expression of $GABA_B$ receptor subunits in astrocytes

$GABA_B$ receptor subunit expression on astrocytes in the hippocampus, was assessed by co-localisation with the astrocytic marker glial fibrillary acidic protein (GFAP). Co-localisation was determined by a clear overlap of fluorescent GFAP and $GABA_B$ specific immunoreactive (IR) signals. GFAP positive astrocytes were clearly labeled throughout the rat brain in regions including the hippocampus, cortex, cerebellum and spinal cord. $GABA_{B1a}$ expression was observed in these areas on neurons as well as on cells resembling astrocytes. Co-localisation of $GABA_{B1a}$ IR was observed on all GFAP positive astrocytes, as illustrated for the CA1 subregion of the rat hippocampus (Fig. 4.1A-C). $GABA_{B1b}$ and $GABA_{B2}$ receptor subunit expression was very similar to that of $GABA_{B1a}$ expression with postivie IR being observed on GFAP positive astrocytes throughout the hippocampus (Fig. 4.1D-I). The hippocampal pattern of co-localisation was preserved throughout the brain such that $GABA_{B1}$ and $GABA_{B2}$ subunits were observed on GFAP positive cells in the cerebral cortex, cerebellum and spinal cord.

Given these observations I next used electron microscopy to further evaluate the ultrastructural localisation of $GABA_B$ receptor subunits on glial cells in the CA1 subregion of the hippocampus. $GABA_B$ subunit immunoreactivity was detected as a dark, electron dense diaminobenzidine reaction product. As expected from previous studies, immunoreactivity for all subunits could be detected in the

postsynaptic membranes at synapses identified as inhibitory by their symmetric morphology and location on dendritic shafts or somata. (e.g. Fig. 4.2D), but not postsynaptically at asymmetric, presumably excitatory, synaptic junctions (e.g Fig. 4.2A). With respect to glia, immunoreactivity for all $GABA_B$ subunits was present on glial cell bodies and processes throughout the CA1 stratum pyramidale and stratum radiatum (Fig. 4.2). At synaptic junctions $GABA_B$ subunit immunoreactivity was detected in astrocyte processes surrounding or adjacent to the synapses (Fig. 4.2). These synapses were either of the symmetric type onto dendritic shafts (Fig. 4.2B, D, H) or asymmetric type onto dendritic spine heads (Fig. 4.2A, C, E, F, G, I).

To investigate protein expression of astrocytes in an "activated" state I examined $GABA_B$ receptor subunit expression in primary astrocyte cultures derived from E18 rat pups, since the steps used to prepare these dissociated primary cultures result in astrocyte activation. Confocal microscopy demonstrated that $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits were all expressed on every GFAP positive astrocytes in this activated system (Fig. 4.3). Specifically the $GABA_{B1a}$ (Fig. 4.3A-C) and $GABA_{B1b}$ (Fig. 4.3D-F) receptor subunits were expressed on the cell body, processes and microprojections of the GFAP positive astrocytes whereas the $GABA_{B2}$ receptor subunit was observed throughout the GFAP positive astrocyte (Fig. 4.3G-I).

Figure 4.1 Confocal photomicrographs showing $GABA_B$ receptor subunit expression on glial fibrilary acidic protein (GFAP) positive astrocytes in the CA1 subregion of the rat hippocampus. $GABA_{B1a}$ (A) was expressed on GFAP +ve astrocytes (arrowheads) (B) in the CA1 stratum pyramidale (C). $GABA_{B1b}$ (D) was also expressed on GFAP +ve astrocytes (E,F). $GABA_{B2}$ (G) was observed in on pyramidal cells of the CA1, GFAP positive astrocytes were observed in the same region (H) and $GABA_{B2}$ could be seen to be expressed on astrocytes (I). Scale bar = 20μm.

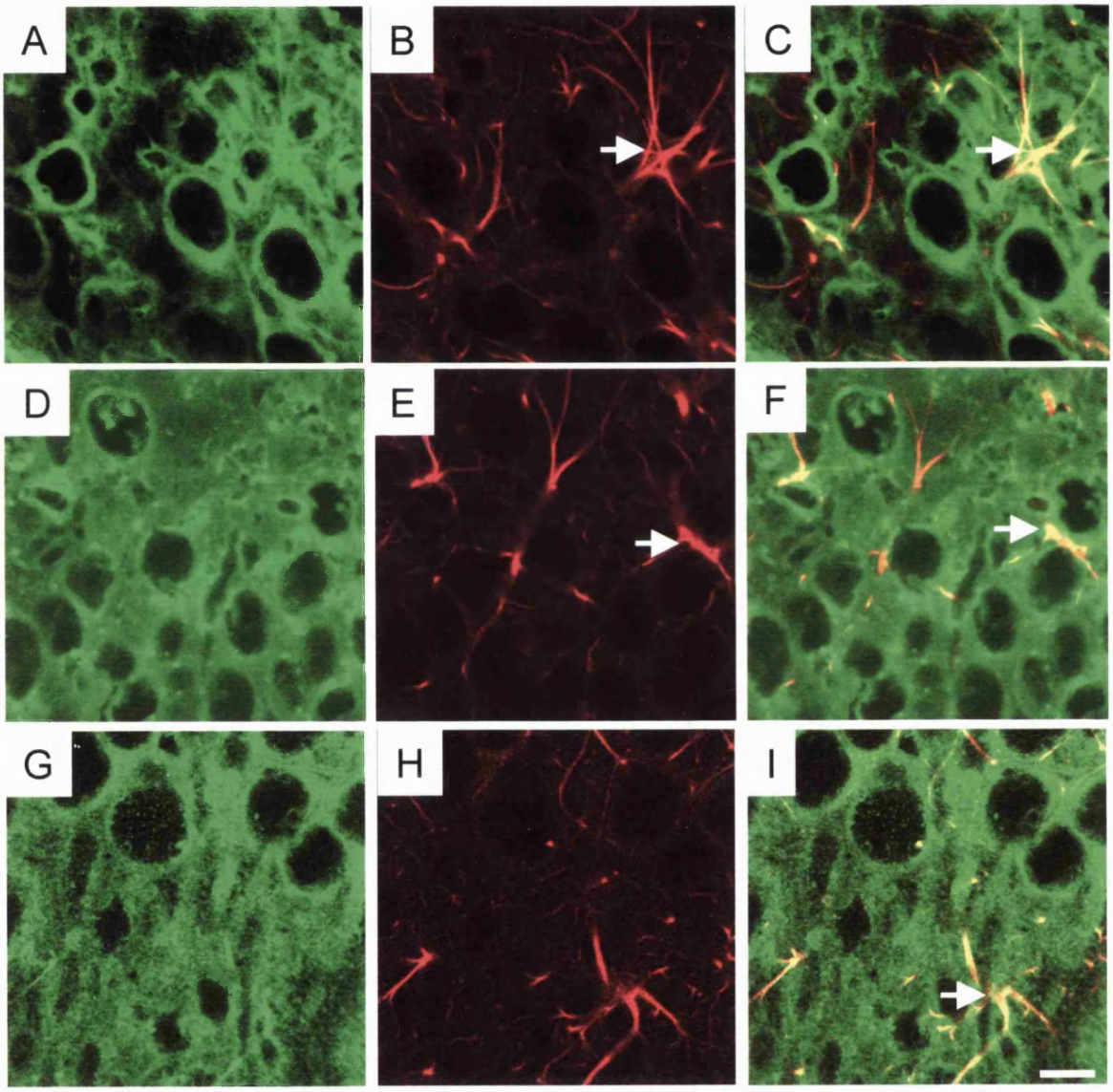
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Figure 4.2 Electron microscope photomicrographs demonstrating expression of $GABA_B$ receptor subunits on glial cells in the CA1 subregion of the rat hippocampus. $GABA_{B1a}$ receptor subunit antisera (A-C) produced an electron dense reaction product in glial processes (g) and cell bodies. $GABA_{B1a}$ receptor subunit IR was observed in the post synaptic specialisation (arrowheads) of symmetric synapses adjacent to excitatory terminals (t) forming synapses with dendritic spines (A,C) or shafts (B). $GABA_{B1b}$ receptor subunit labelling produced a similar pattern of electron dense reaction product (D-F) in glial processes adjacent to symmetric (D) and asymmetric (E,F) synaptic specialisations. Anti-$GABA_{B2}$ receptor subunit antisera (G-I) also labelled cell bodies of glia and processes adjacent to symmetric (H) and asymmetric type terminals. Scale bars = 0.25μm.

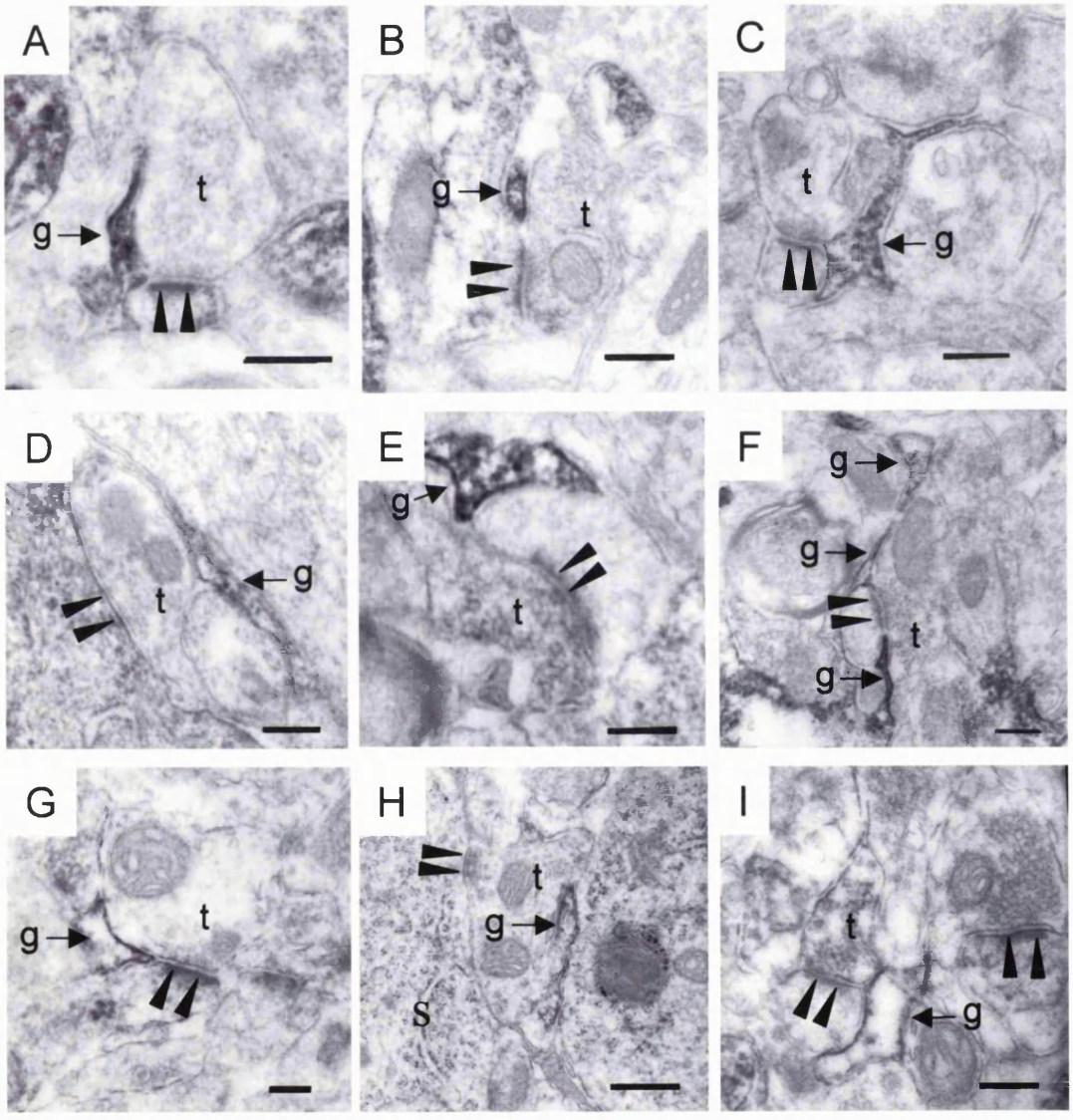
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Figure 4.3 Confocal photomicrographs showing co-localisation of $GABA_B$ receptor subunits with GFAP positive astrocytes in cultured rat hippocampal cells. $GABA_{B1a}$ (A) co-localised with GFAP (B) in cultured astrocytes as shown by the overlay in (C). $GABA_{B1b}$ (D) was also present in GFAP positive astrocytes in culture (E,F) as was $GABA_{B2}$ (G) on astrocytes (H, I), however the $GABA_{B2}$ antisera appeared to label preferentially the cell body of the astrocytes rather than their processes. Scale bar = 20μm.

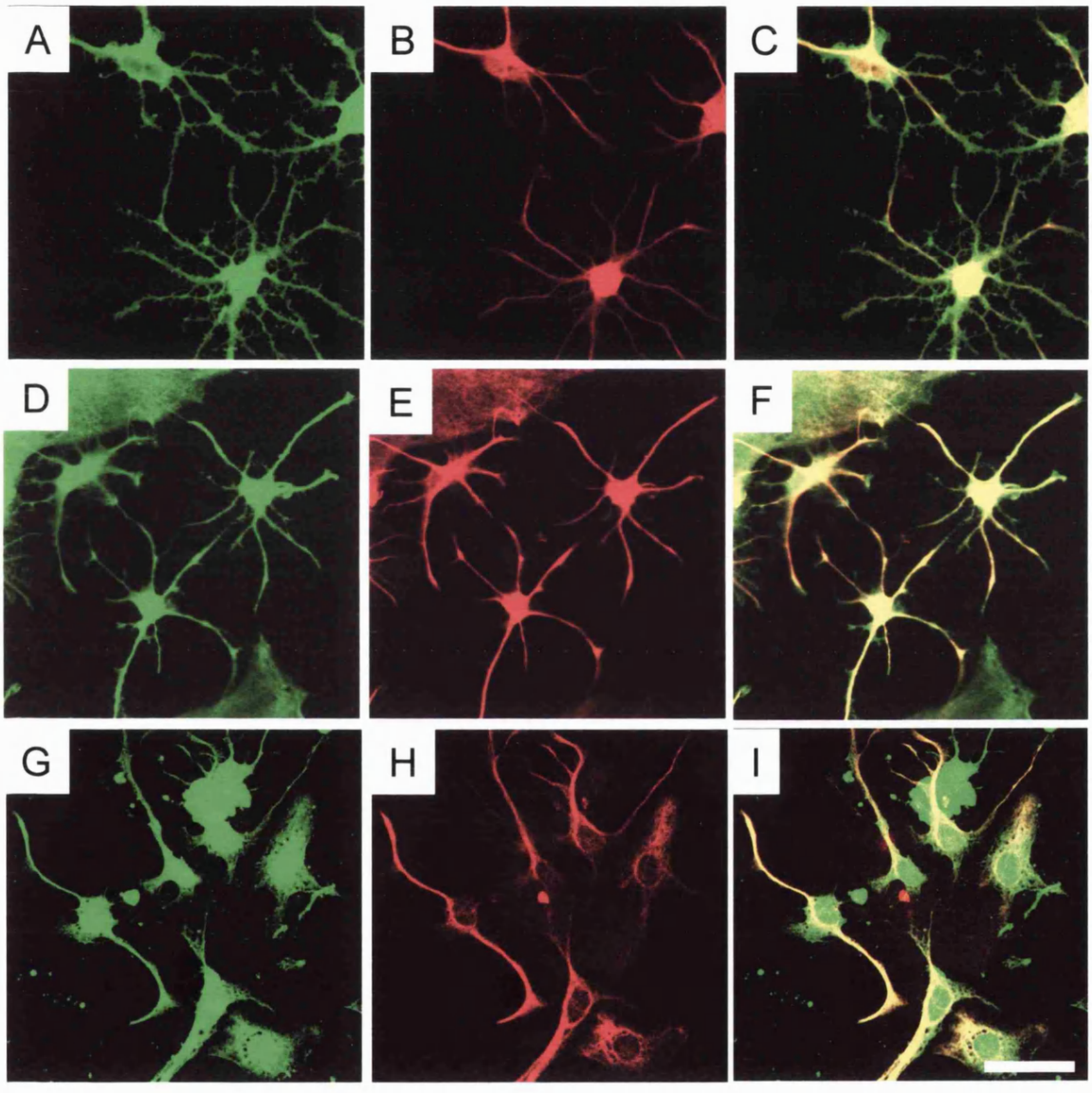
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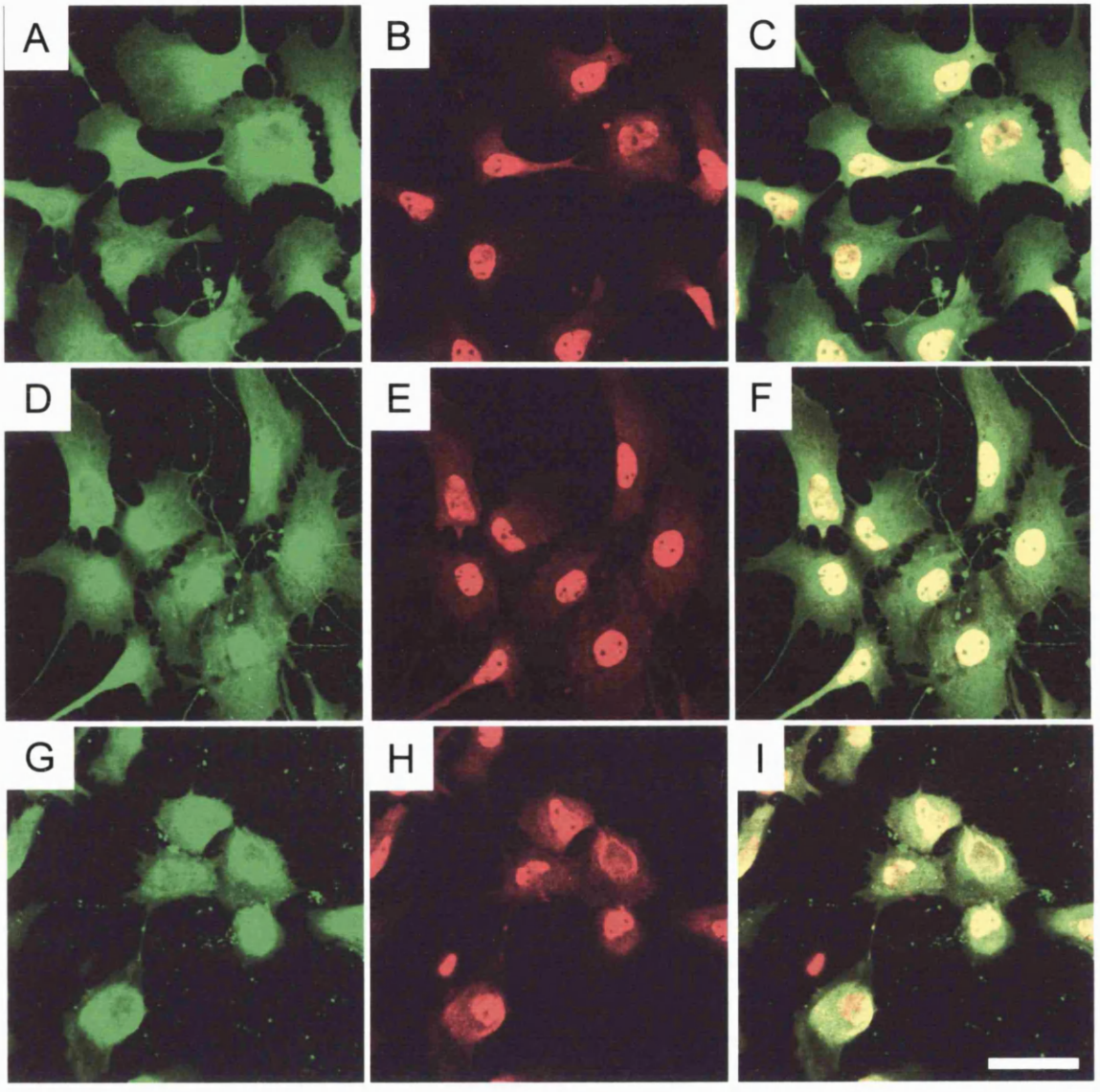
Figure 4.4 Confocal photomicroraphs demonstrating co-localisation of $GABA_B$ receptor subunits in ED1-positive macrophages in rat primary hippocampal cultures. $GABA_{B1a}$ (A) was expressed strongly on ED1 (B) positive macrophages, as shown by the merged image (C). $GABA_{B1b}$ (D) was also present on macrophages (E,F). $GABA_{B2}$ (G) was expressed on all ED1 positive macrophages (H,I) from rat hippocampal cultures. All three $GABA_B$ receptor subunits were expressed at approximately equal intensity and in all macrophages within this preparation. Scale bar = 20μm.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B
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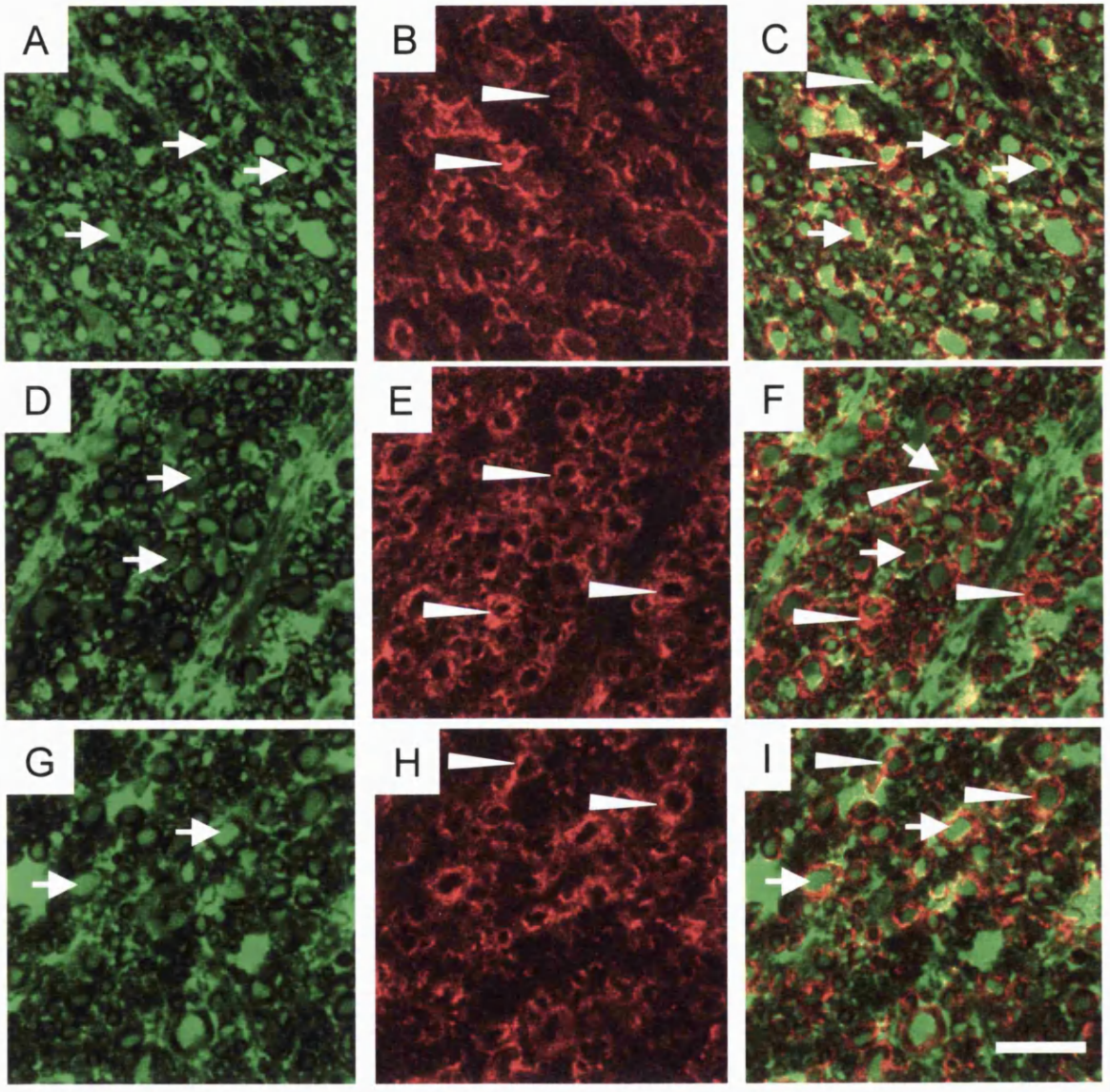
Figure 4.5 Confocal photomicrographs demonstrating $GABA_B$ receptor subunit co-localistaion on myelin basic protein (MBP) positive profiles in white matter of the rat ventral lumbar spinal cord. $GABA_{B1a}$ (A) was present on fibre bundles (arrows) contained within MBP positive (B) sheathing in the white matter (arrowheads), though the two proteins did not co-localise as shown by the separation of the red and green channels (C), instead MBP could be seen to surround the $GABA_B$ positive fibres. $GABA_{B1b}$ (D) and $GABA_{B2}$ (G) were also separate from the MBP sheathing (E,H) as no co-localisation was observed (F,I). Scale bar = 10μm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4.3.2 Expression of $GABA_B$ receptor subunits on microglia

It is well documented that microglial expression in brain is sparse under normal conditions and only readily detectable following injury since MHC antigens are significantly down-regulated in the quiescent state and hence are unavailable to antisera binding (Strit et al., 1989; Perry et al., 1993). I chose therefore to study $GABA_B$ receptor subunit expression on "activated" microglia dissociated cultures prepared from E18 rat hippocampus using ED1 as a marker of "activated" microglia. In agreement with previous reports, ED1 preferentially labeled the nucleus and, to a lesser extent, the cytoplasm of all microglial cells. $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits were strongly expressed on all activated microglia with IR being observed throughout the cell (Fig. 4.4).

4.3.3 Expression of $GABA_B$ receptor subunits on oligodendrocytes

Finally I investigated the expression of $GABA_B$ receptor subunits in myelin producing oligodendrocytes in the spinal cord. These cells were identified using an antibody specific for the myelin marker MBP (Myelin basic protein). Immunofluorescent labeling of white matter spinal cord tracts revealed a complete lack of co-localisation of any of the $GABA_B$ receptor subunits with MBP (Fig. 4.5). Thus, $GABA_{B1a}$ receptor subunit expression (Fig. 4.5A) was detected in the neuronal fibre tracts of the ventral horn white matter, whereas MBP IR was observed in the myelin rings surrounding the fibre tracts (Fig. 4.5B). As such, no significant merging of the two fluorescent signals was observed (Fig. 4.5C). $GABA_{B1b}$ (Fig. 4.5D) and $GABA_{B2}$ (Fig. 4.5G) receptor subunits were also expressed in the fibre tracts and were surrounded by a sheath of MBP-positive

myelin encasing each tract but on no occasion was co-localisation of the two proteins observed (Fig. 4.5 F and I).

4.4 Discussion

4.4.1 Experimental considerations

The $GABA_B$ receptor subunit antisera used in this study have been characterised previously for specificity (see Chapter 3). However, here I have used commercially available antisera targeted against non-neuronal cell markers such as glial fibrillary acidic protein, ED1 and myelin basic protein. The specificity of these monoclonal antisera has been reported previously (Sigma Product number G3893; Miampamba and Sharkey, 1999 and Cai et al., 2001). Due to the nature of these monoclonal antisera it is unlikely that any non-specific immunoreactivity will occur, however, as with all immunohistochemistry the pattern of labelling obtained should be cautiously interpreted. Given that these monoclonal antisera all labelled only the cells which they are predicted to bind it is assumed that they are specific to their target.

4.4.2 Glial cell $GABA_B$ receptors

Here I have demonstrated for the first time that both $GABA_{B1}$ and $GABA_{B2}$ receptor subunits are expressed on astrocytes and microglia but not on oligodendrocytes. Over the past 15 years our understanding of glia in the CNS has dramatically changed. Glia are no longer thought to play a minor functional role in the CNS, and are now considered critical for supporting neuronal function in the adult and developing brain, providing structural frameworks, contributing to the immune system of the CNS, responding to stimuli such as

neurotransmitters and hormones, interacting directly with synapses and co-ordinating activity within sets of neurons (Barres, 1991; Verkhratsky and Kettenmann, 1996; Ridet et al., 1997; Pfreiger and Barres, 1996 and 1997; Araque et al., 1999; Fields and Stevens-Graham, 2002). In turn, neuronal activity is involved in regulating a wide range of glial functions such as proliferation, differentiation and myelination (Field and Stevens-Graham, 2002). To this end numerous neurotransmitter receptors including glutamatergic, GABAergic, adrenergic, purinergic, serotonergic, muscarinic and peptidergic receptors have been shown to be functionally expressed on glial cells (Verkhratsky and Kettenmann, 1996; Porter and McCarthy, 1996 and 1997). In astrocytes, intracellular Ca^{2+} rises have been observed in response to a variety of agonists and neurotransmitters including carbachol, histamine, noradrenaline, vasopressin, substance P, serotonin and glutamate. However, the percentage of astrocytes displaying these responses were variable (Albrecht et al., 1986; Hosli and Hosli, 1990; Inagaki et al., 1991).

In this study, I have used immunohistochemistry to demonstrate the expression of the three principle $GABA_B$ receptor subunits, $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ in astrocytes. Expression was observed in both native brain sections as well as in "activated" astrocyte primary cultures. All three receptor subunits were co-expressed in these cells, consistent with reports documenting functional $GABA_B$ receptors on astrocytes. In this respect, the presence of $GABA_B$ receptors on astrocytes was first described by Albrecht and colleagues (1986), who reported that GABA and baclofen were capable of inducing Ca^{2+} transients in astrocytes cultured from rat cortex. The presence of $GABA_B$ receptors in astrocyte cultures has since been demonstrated in other CNS regions, including cerebellum, brain

stem and spinal cord by some (Hosli and Hosli, 1990) but not others (Butt and Jennings, 1994). In contrast, it has also been suggested that the inhibitory responses observed in glioma cells, in contact with neurons, are mediated through $GABA_A$ receptors, although no assessment of $GABA_B$ receptor mediated responses were made in this study and it is likely that glioma cells represent a quite specialised and atypical cell population in this study (Synowitz et al. 2001).

Nevertheless, this data does suggest that astrocytes express the components required to make a functional heteromeric $GABA_B$ receptor. Given this observation, our findings raise the question as to what role these receptors may play in astrocyte function? Importantly all $GABA_B$ receptor subunits studied were expressed on astrocytes both in brain sections and in *in vitro* primary cultures. Astrocytes present in normal brain would be perceived to be in a "resting" state compared to those in culture which more closely represent astrocytes in an "activated" state (for review see Porter and McCarthy, 1997). These findings suggest that $GABA_B$ receptor expression is independent of astrocyte activation state and therefore that $GABA_B$ receptors more likely play a role in the tonic modulation of astrocyte function that may include cross-talk to neurons and other glia via gap-junctions (Araque et al., 1999), alteration of astrocytic morphology via a Ca^{2+} mediated mechanism (Verkhratsky and Kettenmann, 1996), and uptake or release of neurotransmitters.

In respect to glial involvement in neurotransmission the EM data presented in this study show $GABA_B$ receptor containing astrocytes in close apposition to synapses displaying characteristic location and morphology of both excitatory and inhibitory connections. The role of $GABA_B$ receptors on glia at inhibitory junctions has been shown directly by experimentation where $GABA_B$

receptors mediate rises in calcium in glial cells following GABA release from inhibitory neuron terminals (Kang et al., 1998). Interestingly, the rise in intracellular calcium in glial cells results in glutamate release which acts on excitatory amino acid receptors to facilitate release from inhibitory terminals (Kang et al., 1998) and reduces the probability of failure of inhibitory post-synaptic currents (IPSCs) generated in pyramidal neurons during periods of repetitive interneuronal firing. Therefore, the $GABA_B$ receptors on glial cells may act to 'sense' extracellular GABA and effectively potentiate GABA mediated inhibition; this role is presumably relatively independent of the "resting" or "activated" state of the astrocyte given the high level of expression of $GABA_B$ receptors in both states.

Turning to excitatory synapses it has been reported by Araque and colleagues in 1998 that increases in astrocytic $[Ca^{2+}]_i$ induce the release of glutamate and initiate spontaneous inward currents in neighbouring hippocampal neurones in culture (see Hassinger et al., 1995). Theoretically, activation of $GABA_B$ receptors may be one stimulus that initiates such an intracellular Ca^{2+} rise, generating these postsynaptic currents. However, this leaves open the question as to where the GABA that activates astrocytic $GABA_B$ receptors comes from? The obvious answer to this question is from spillover from distantly located GABAergic synapses (Isaccson et al 1993), a scenario that occurs during periods of repetitive interneuronal activity generating sufficient GABA release to overwhelm GABA uptake mechanisms. What this means physiologically, and whether glial cells specifically localize $GABA_B$ receptors on processes that are in close proximity to specific synaptic structures, as has recently been observed with $GABA_A$ receptors in the cerebellum (Riquelme et al., 2002), needs further

evaluation and may help us to further understand the relationship between astrocytes and neurons within the CNS.

Irrespective of the outcome of such investigations, it is likely that other glial cell types also exhibit complex interactions with neurons that relie on activation of G-protein coupled receptors. In this respect, microglia express a number of metabotropic receptors including bradykinin B2, purinergic P2Y and endothelin receptors, which when activated result in intracellular Ca^{2+} influx (Verkhratsky and Kettenmann, 1996). Here we report that $GABA_B$ receptors are also expressed on primary microglial cultures exhibiting the lysosomal monocyte / macrophage marker, ED1 (Kullberg et al., 2001). This marker is expressed on "activated" microglia and is not detected in microglia in a quiescent "resting" state. All three $GABA_B$ receptor subunits were co-expressed in primary mircoglial cells *in vitro* as confirmed by immunoblotting and immunohistochemistry in a recent abstract by Kuhn et al (2002). Although each of the subunits appeared to be expressed throughout the microglial cell, high levels of expression were observed in intracellular compartments. Functional analysis has revealed that these $GABA_B$ receptors are capable of generating (a) outwardly rectifying K^+ currents and (b) transient increases in intracellular Ca^{2+} (Kuhn et al., 2002); both effects presumably mediated by receptors located within the plasma membrane of the microglia. This leaves open the question: what is the functional role, if any, of the receptors expressed intracellularly? Interestingly, the C-terminal tail of $GABA_{B1}$ does interact with nuclear transcription factors such as ATF-4 and CREB-2 (White et al., 2000; Vernon et al., 2001). Thus, intracellular $GABA_B$ receptors may regulate the activity of transcription factors following microglial activation in a similar way to that described for the JAK-STAT pathway in which

ligand-receptor interaction induces a signalling cascade via JAK and STAT proteins which results in transcriptional events (Cattaneo et al., 1999). This transcriptional activation within microglia may for example induce a number of morphological or phagocytic changes that may play a role in the well documented effects of activated microglia in brain defence, immune-protection and regeneration (Kreutzberg, 1996, Nakajima and Kohsaka, 2001).

In summary, I have demonstrated for the first time the expression of the three principle $GABA_B$ receptors subunits in non-neuronal cells in the rat CNS. $GABA_B$ receptor subunits are expressed at the protein level in astrocytes and activated microglia but not oligodendrocytes, and reflect the existence of functional $GABA_B$ receptors in these two glial cell populations. It is curious that no $GABA_B$ receptors were identified on the myelin forming Schwann cells of the peripheral nervous system, since these cells are believed to perform all the function of astrocytes and oligodendrocytes in these regions (Fields and Stevens-Graham, 2002). Further studies will now be required to fully elucidate the functional role(s) that $GABA_B$ receptors play in these cells.

Chapter 5 - Localisation of a novel $GABA_B$-like protein in the rat CNS

5.1 Introduction

Historically, $GABA_B$ receptors are heterogeneous in nature based on their pharmacology (Ong and Kerr, 1990; Bonnano and Raiteri, 1993; Kerr and Ong, 1995). Thus far no other subunits have been identified and of the reported splice variants for $GABA_{B1}$ none appear to confer any pharmacological $GABA_B$ receptor diversity (Calver et al., 2000; Isomoto et al., 1998; Martin et al., 2001; Schwarz et al., 2000). $GABA_{B1}$ has also been shown to be expressed in the absence of $GABA_{B2}$ in a number of peripheral tissues (Calver et al., 2000), despite the presence of functional peripheral receptors (Ong and Kerr, 1990), further strengthening the argument for the existence of additional $GABA_B$ receptor subunits. As a result, significant efforts have been afforded to the discovery of additional mammalian $GABA_B$ subunits. A recent report has identified a putative third $GABA_B$ receptor subunit in drosophila, termed $GABA_{B3}$ (Mezler et al., 2001), though as yet no functional activity has been demonstrated. A novel GPCR with homology to both $GABA_{B1}$ and $GABA_{B2}$ that has been termed $GABA_{BL}$, has been identified by database mining, cloned and expressed as the human, mouse and rat orthologues of $GABA_{BL}$ (Accession numbers: AF488739, AF488740, AF488741, respectively). $GABA_{BL}$ is a 814 a.a. protein in its mature state. $GABA_{BL}$ is 27.4% and 31% identical to $GABA_{B1}$ and $GABA_{B2}$ respectively, and the $hGABA_{BL}$ is 32% identical to that of the drosophila $GABA_{B3}$ protein (Calver et al., 2003). Calver et al., constructed a phylogenetic tree of the family C GPCRs,

which demonstrates that $GABA_{BL}$ is indeed a member of this family, and is most like the $GABA_B$ receptor subfamily (See Figure 5.1).

Although this receptor bears significant sequence homology to $GABA_B$ subunits, functional studies have thus far failed to demonstrate any modulation of receptor binding or signalling when $GABA_{BL}$ is expressed transiently in cells with the two known $GABA_B$ receptor subunits (Calver et al., 2003; Kaupmann et al., FENS 2002). The CNS distribution of this putative $GABA_B$-like receptor may provide further clues as to its potential role in the CNS.

5.2 Aims of this study

Specific rabbit polyclonal antisera were raised against the C-terminus of this novel orphan family C GPCR, and their specificity confirmed against the protein sequence, with the aim of mapping the distribution of $GABA_{BL}$ in the rat CNS for the first time.

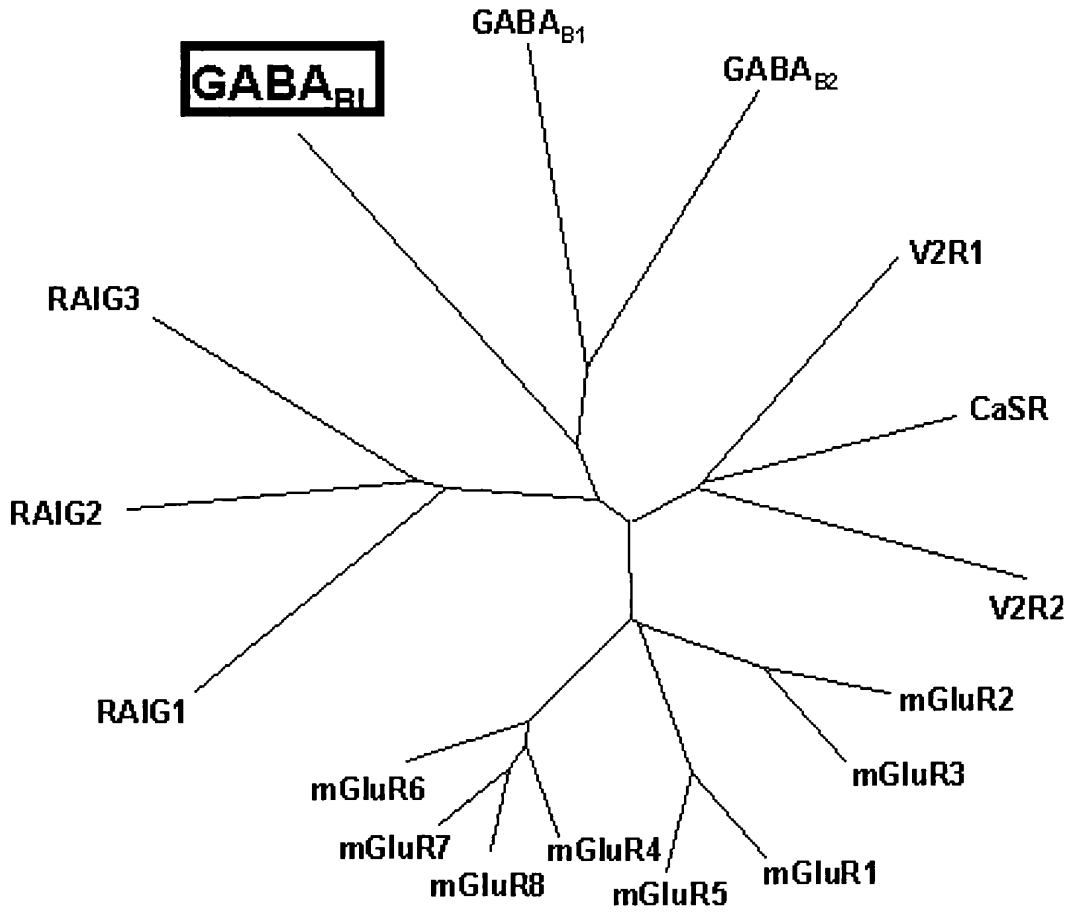


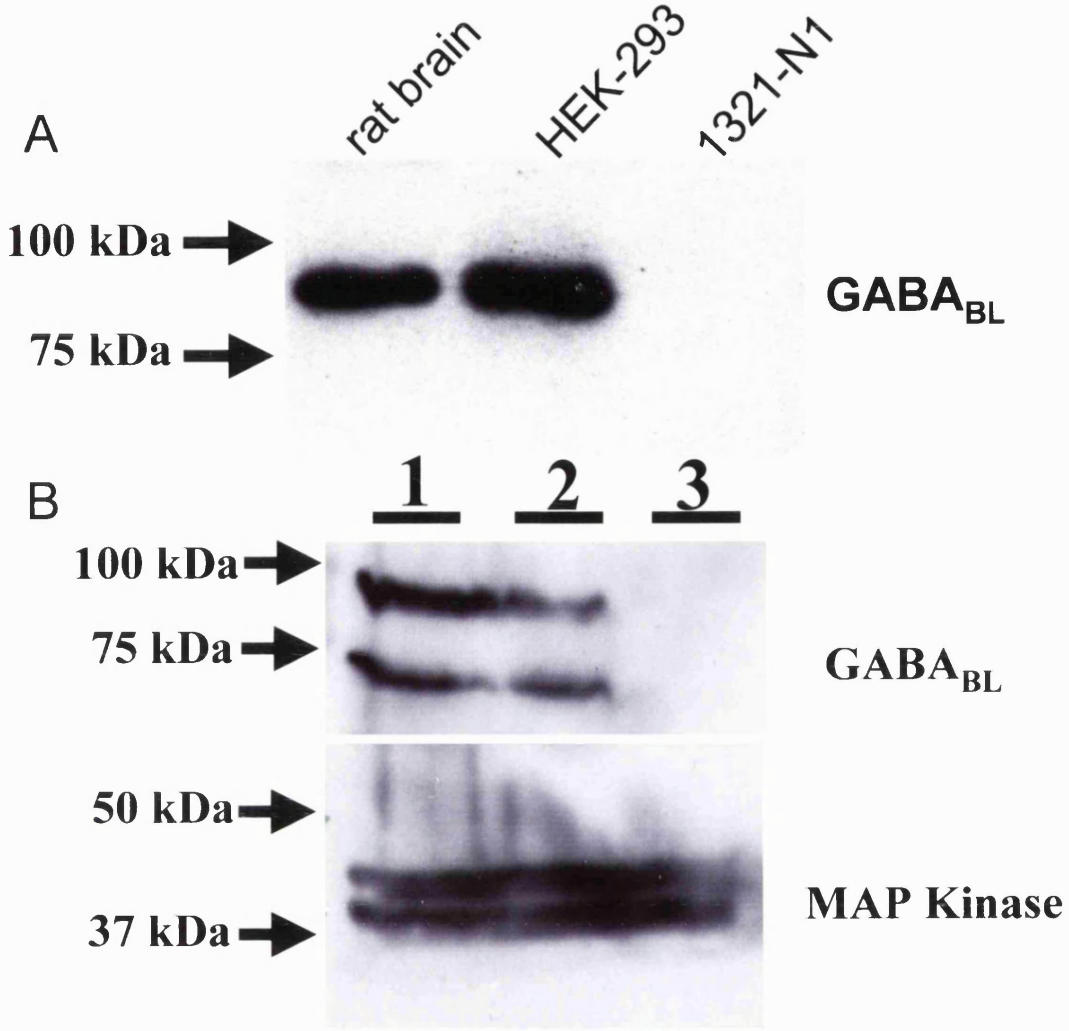
Figure 5. 1 Phylogram showing $GABA_{BL}$ with other family C GPCRs, prepared using CLUSTALW with standard parameters. RAIG1,2,3 are a family of retinoic acid inducible receptors. CaSR is the calcium sensing receptor and V2R1 and V2R2 are putative pheromone receptors. (Adapted from Calver et al., 2003).

5.3 Results

5.3.1 Characterisation of the $GABA_{BL}$ antisera

Three peptides were designed against the $GABA_{BL}$ protein sequence and each peptide used to immunise two rabbits. The six resulting antisera were analysed by Western blot, immunocytochemistry and immunohistochemistry. The antisera which showed the best selectivity in terms of correct molecular weight by Wetsern blot, and ability to pre-absorb the signal was further characterised for its distribution in the rat CNS. By Western blot analysis anti-$GABA_{BL}$ antisera yielded a single immunoreactive band at 90kDa, the expected molecular weight of this putative receptor protein (Fig. 5.2A). The immunoreactive band of $GABA_{BL}$ at 90kDa was present in rat brain homogenates and also in untransfected HEK-293 cells which express this protein constitutively. 1321/N1 cells were identified by Taqman mRNA analysis as not expressing $GABA_{BL}$, and this was confirmed by Western blot analysis, whereby an absence of any immunoreactive bands with the anti-$GABA_{BL}$ antisera was noted. The specificity of the anti-$GABA_{BL}$ antisera was also confirmed by using rat brain homogenates from wildtype, heterozygous and null mutant animals (Fig. 5.2B). No $GABA_{BL}$ labelling was observed in the null mutant membranes, although loading of protein was equal for all three, as confirmed by immunoblotting with an anti-MAP kinase antisera. Specificity of the $GABA_{BL}$ antisera was also confirmed by peptide pre-absorption in tissue sections. All $GABA_{BL}$ immunoreactivity was abolished by pre-incubation of the antisera with the cognate peptide for 72h prior to incubation with tissue (Fig.5.2C).

Figure 5.2 A) Immunoblot probed with antisera to $GABA_{BL}$ in whole rat brain membranes; HEK-293 cell membranes and 1321-N1 cell membranes. B) Specificity of the $GABA_{BL}$ antisera was confirmed by immunoblotting on rat brain membranes from wildtype, heterozygous and null mutant animals (lanes 1,2,3 respectively). No $GABA_{BL}$ labelling was observed in null mutant animals. Equal loading of protein was confirmed for all genotypes by immunoblotting for MAP kinase which showed equal labelling intensity for all three genotypes. C) Parasaggital sections of rat brain demonstrating $GABA_{BL}$ protein expression in many regions including the piriform cortex, hippocampus, pons and granule cell layer of the cerebellum, and a consecutive section demonstrating the abolition of all immunoreactivity by preabsorption with the immunogenic peptide (10mg/ml).


A
rat brain
HEK-293
1321-N1
100 kDa
75 kDa
GABA$_{BL}$
B
1
2
3
100 kDa
75 kDa
GABA$_{BL}$
50 kDa
37 kDa
MAP Kinase
C

5.3.2 General distribution of $GABA_{BL}$ in the rat CNS

$GABA_{BL}$ immunoreactivity was widely distributed throughout the rat brain and spinal cord, as observed in parasagittal and coronal sections (Fig. 5.3). $GABA_{BL}$ protein expression was seen throughout the neocortex, layers II-VI, the caudate putamen, thalamus and hypothalamus. $GABA_{BL}$ IR was particularly intense in the pyramidal cell layers of the hippocampus (CA1-3) and also in the granule cells of the dentate gyrus. The superficial gray region of the superior colliculus also demonstrated $GABA_{BL}$ IR, as did the reticular and pontine nuclei, the locus coeruleus and the facial nucleus. Immunoreactivity was conspicuous in the granule cell layer of the cerebellum, and in the deep cerebellar nuclei.

More detailed results are given below. Semi-quantitative results of $GABA_{BL}$ IR are summarised in Tables 5.1-3 and are based upon relative labelling intensities. Immunoreactivity has been scored as none(-), very low (+), low (++), moderate (+++), dense (++++) and very dense (+++++).

Table 5. 1 Semi-quantitative analysis of $GABA_{BL}$ protein expression in the rat cortex.

Cortical Area	Cortical Layer					
	I	II	III	IV	V	VI
Orbital	+	++++	+++	++++	++++	++++
Motor	+	+++	+++	++++	++++	+++
Parietal	+	++++	+++	++++	+++	++
Visual	+	+++++	+++	++++	+++	++
Piriform	+	+++++	+++	++	+++	+++
Insular	+	+++	+++	++++	++++	+++
Somatosensory	+	++++	+++	+++++	+++	++
Auditory	+	+++	+++	++++	+++	+++

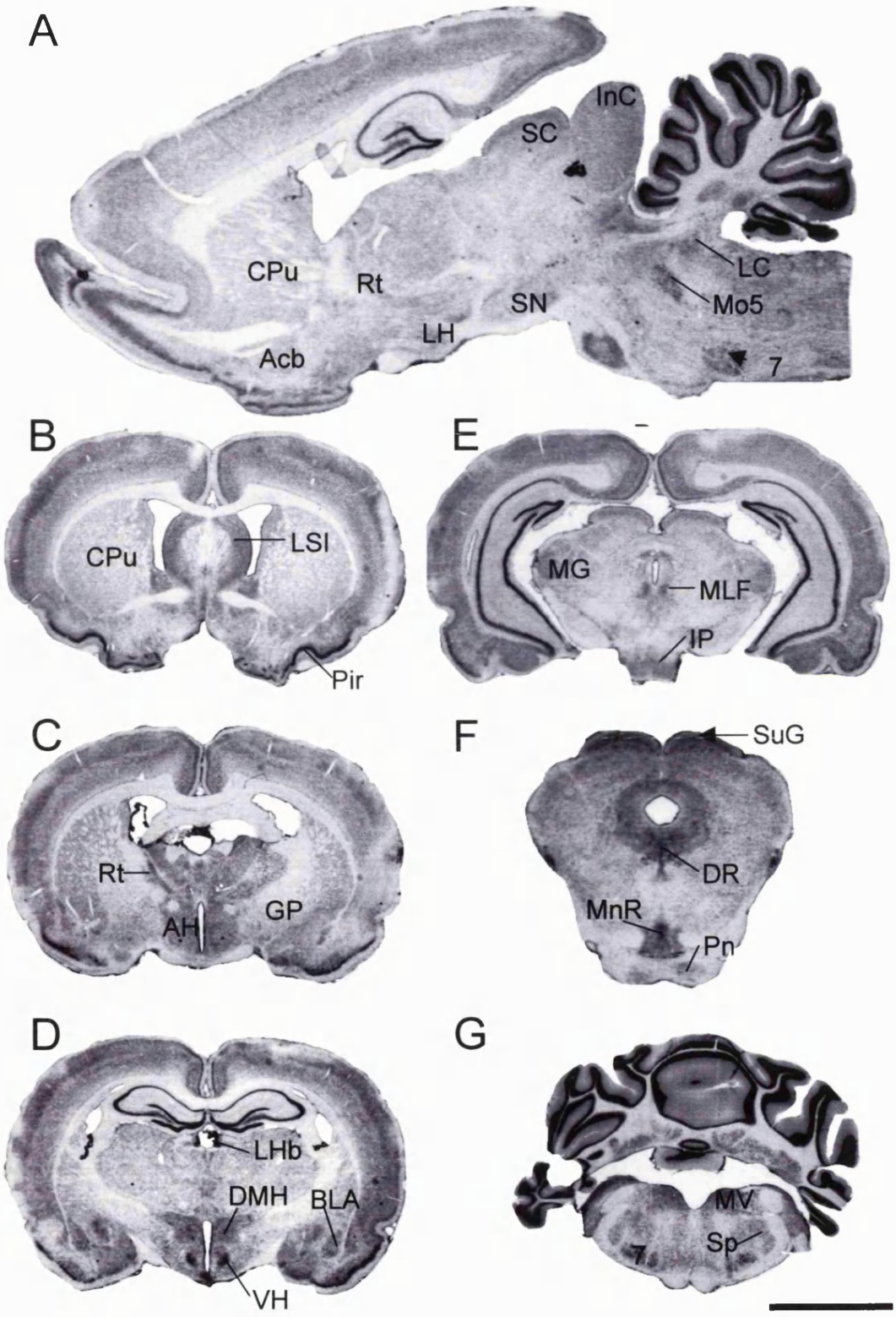
Table 5.2 Semi-quantitative analysis of $GABA_{BL}$ protein expression in the rat brain.

Brain Regions	Structure	$GABA_{BL}$
Basal Ganglia	Caudate-Putamen	+++
	Accumbens Shell	++++
	Accumbens Core	+++
	Globus Pallidus	++
Septum/BNST		++++/++
Diagonal band		+
	Bas. Meynert n.	+
Amygdala	Basolateral	++++
	Lateral	+++
	Central	+++
	Intercalated mass	++++
Hippocampus	CA1	++++
	CA3	++++
	Dentate gyrus	+++++
Habenula	Medial/lateral	+++++/+++
Interpeduncular		+++++
Thalamus	Medial	++++
	Lateral	++++
	Ventral	++++
	Reticular thalamus	+++
Hypothalamus	Dorsal	++++
	Ventromedial	++++
	Lateral	+++
	Arcuate	++++
	Tuber cinereum	+++
Mid brain	Superior colliculus	++++
	Inferior colliculus	++++
	Interpeduncular n.	++++
	Ventral Tegmental a	+++
	Substantia nigra *pc*	+++
	Substantia nigra *pr*	++++
Pons	Pontine nuclei	+++++
Rhombenceph.	Spinal trigeminal n.	++++
	Locus coeruleus	+++
	Vestibular nuclei	++++
	Dorsal raphe nuclei	+++++
	Median raphe nuclei	+++
	Olivary nuclei	+++++
Cerebellum	Molecular layer	++
	Purkinje cells	++++
	Granule layer	++++

Table 5.3 **Semi-quantitative distribution of $GABA_{BL}$ protein expression in control rat lumbar spinal cord.**

Lamina	$GABA_{B1a}$
I	++++
II	++++
III	+++
IV	+++
V	++
VI	++
VII	+++
VIII	+++
IX	++++
X	++++

Figure 5.3 Photomicrographs showing $GABA_{BL}$ protein expression in parasagittal and coronal sections throughout the rat brain. $GABA_{BL}$ was highly expressed in the hippocampus (A,D,E), piriform cortex (B,C,D), hypothalamus (C,D), interpeduncular nucleus (E), superficial grey of the superior colliculus (A, F), raphe nuclei (F) and was very conspicuous in the cerebellum (A,G). For abbreviations see list. Scale bar = 5mm.


A
InC
SC
CPu
Rt
SN
LC
Mo5
LH
Acb
7
B
CPu
LSI
Pir
E
MG
MLF
IP
C
Rt
AH
GP
F
SuG
DR
MnR
Pn
D
LHb
DMH
BLA
VH
G
MV
Sp
7

5.3.3 Cerebral cortex

$GABA_{BL}$ IR was detected throughout the neocortex in neuronal somata, though the intensity of IR varied according to the cortical layer and region studied (Table 5.1). Throughout all cortical areas studied layer I showed only minimal IR for $GABA_{BL}$ protein in small scattered cell body profiles consistent with the sparse occurance of neurons in this layer. Layer II of all cortical areas, in contrast, showed moderate to dense IR for $GABA_{BL}$ receptor protein, with the visual and piriform corticies demonstrating very dense $GABA_{BL}$ IR. The large pyramidal neurons of layer IV were also dense in their $GABA_{BL}$ protein expression, except in the somatosensory cortex where IR was very dense (Fig. 5.4A) and the piriform cortex where $GABA_{BL}$ IR was low. Notably there was a total absence of dendritic labelling in the large pyramidal neurons, instead IR was located in the membrane and cytoplasm of the soma, a trend which was observed throughout all cortical layers and regions. Protein expression in layers V and VI was variable according to the region studied. Parietal, visual, piriform and somatosensory cortices demonstrated low $GABA_{BL}$ protein expression, in contrast to dense IR in orbital cortex.

5.3.4 Basal ganglia, nucleus accumbens and septal nuclei

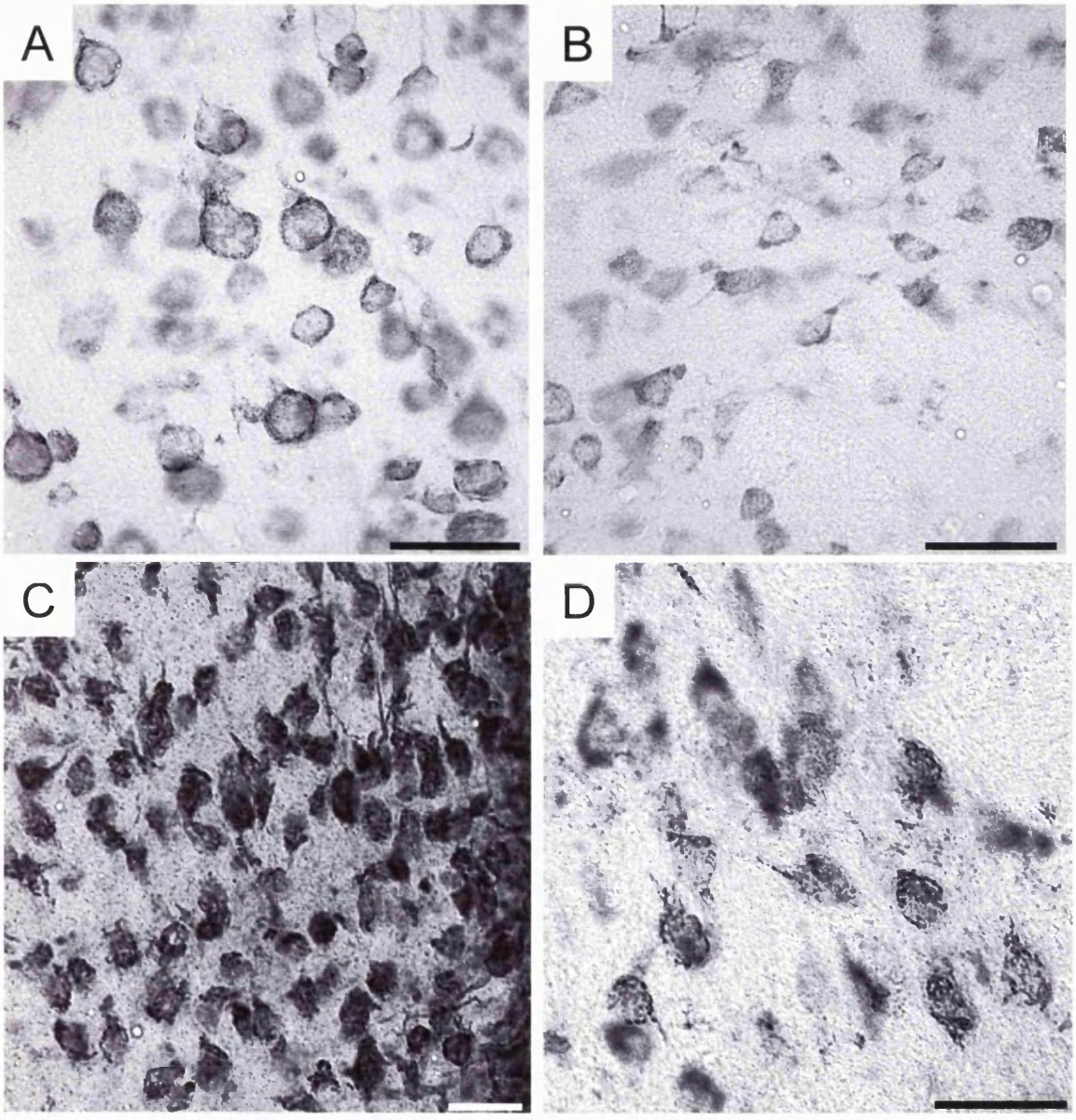
$GABA_{BL}$ IR was noted throughout the regions of the basal ganglia and nucleus accumbens of the rat (Fig. 5.4A,B). In the caudate putamen IR was restricted to the cell somata of neurons with a cell diameter consistant with that of medium spiny interneurons (20μm) (Fig. 5.4B). Labelling in these cells was mainly restricted to the cell membrane, with a weak cytoplasmic expression also noted. $GABA_{BL}$ IR was also observed in the nucleus accumbens, though the shell

demonstrated dense IR in contrast to the core where protein expression was moderate. In both regions of the accumbens, $GABA_{BL}$ IR was restricted to the membrane of cell bodies. Fewer somata were labelled in the globus pallidus than in other regions of the basal ganglia. $GABA_{BL}$ IR in the globus pallidus was once again restricted to cell membranes, though the actual intensity of this staining was greater than seen in the caudate putamen. Septal $GABA_{BL}$ IR was dense in the lateral septum (Fig. 5.3B) and was expressed mainly in cell bodies but also to a lesser degree in the neuropil. $GABA_{BL}$ IR in the bed nucleus of the stria terminalis (BNST) was moderate.

5.3.5 Amygdala

$GABA_{BL}$ immunoreactivity in the amygdala varried according to subnuclei (see Table 5.2 and Fig. 5.3D). In the basolateral amygdala $GABA_{BL}$ IR was dense, and was located primarily to the cell membrane of cell bodies. The intercalated masses also showed dense $GABA_{BL}$ IR, however the lateral and central nuclei demonstrated only moderate $GABA_{BL}$ IR, again in a cell body restricted manner.

Figure 5.4 Photomicrographs showing $GABA_{BL}$ immunoreactivity in layer V of the somatosensory cortex (A), medium spiny neurons of the caudate putamen (B), arcuate nucleus of the hypothalamus (C), and the reticular thalamic nucleus (D). Scale bars = 50μm.


A
B
C
D

5.3.6 Hippocampus and habenula

In the hippocampus $GABA_{BL}$ IR was striking in its dense to very dense labelling of the pyramidal and granule cells respectively (Fig. 5.3D,E and Fig. 5.5). The pyramidal cells of the CA1 sub region were densely IR for $GABA_{BL}$ with labelling restricted to the cell body layer. In the stratum oriens and radiatum of the CA1 region $GABA_{BL}$ IR was restricted to only very few interneurons, with no dendritic or neuropilic labelling observed (Fig. 5.5A). A similar pattern of IR was also noted in the CA3 pyramidal cells with dense $GABA_{BL}$ IR restricted to the cell body layer (Fig. 5.5B). $GABA_{BL}$ IR was very dense in the dentate gyrus with IR in the cell bodies of granule cells of the gyrus, and also in occasional interneurons in the hilus (Fig. 5.5C). The habenula was very strongly immunoreactive for $GABA_{BL}$ in the medial nucleus (Fig. 5.3D), with only moderate IR in the lateral region. The interpeduncular nucleus of the mid brain, to which the habenula projects, was also dense for $GABA_{BL}$ protein expression (Fig. 5.3E).

$GABA_{BL}$ protein was expressed on parvalbumin positive interneurons in the CA1 stratum pyramidale of the hippocampus, predominantly in cell bodies (Fig. 5.6A-C). $GABA_{BL}$ was also expressed on calbindin positive interneurons in the hippocampus (Fig. 5.6D-F) but was not expressed on the calbindin positive mossy fibres in this region. Calretinin positive interneurons also contain $GABA_{BL}$ protein as demonstrated in the CA1 region (Fig. 5.7A-C). However, GFAP positive astrocytes in the hippocampus do not contain $GABA_{BL}$ protein (Fig. 5.7D-F).

Figure 5.5 Photomicrographs showing $GABA_{BL}$ protein expression in the rat hippocampus. $GABA_{BL}$ IR was dense in the CA1 (A), CA3 (B) and in the dentate granule layer and hilus (h) (C). Scale bars = 50μm.

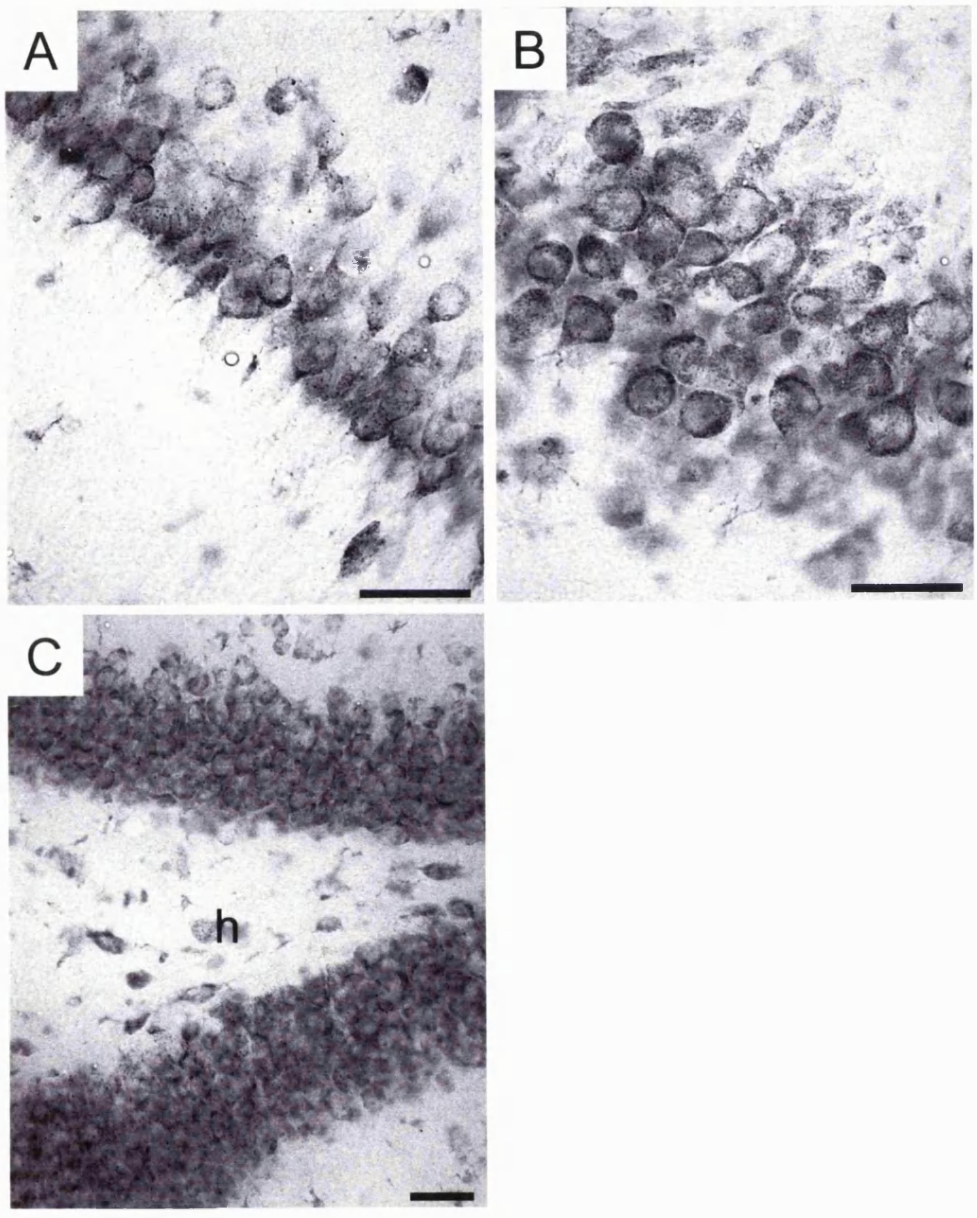

A
B
C
h

Figure 5.6 Confocal images showing $GABA_{BL}$ protein co-localisation in the rat hippocampus. $GABA_{BL}$ was expressed in the CA1 stratum pyramidale (A) as were occasional Parvalbumin positive interneurons (B), the merged image (C) shows $GABA_{BL}$ protein expressed on a parvalbumin positive interneuron, arrows. CA3 stratum pyramidale cells expressed $GABA_{BL}$ protein (D), the stratum radiatum of the CA3 expressed Calbindin positive projections (E), the merged image (F) shows co-localisation of $GABA_{BL}$ and Calbindin in scattered interneurons, arrows, but no co-localisation in the mossy fibers which synapse onto the pyramidal neurons of the CA3. Scale bars = 20μm.

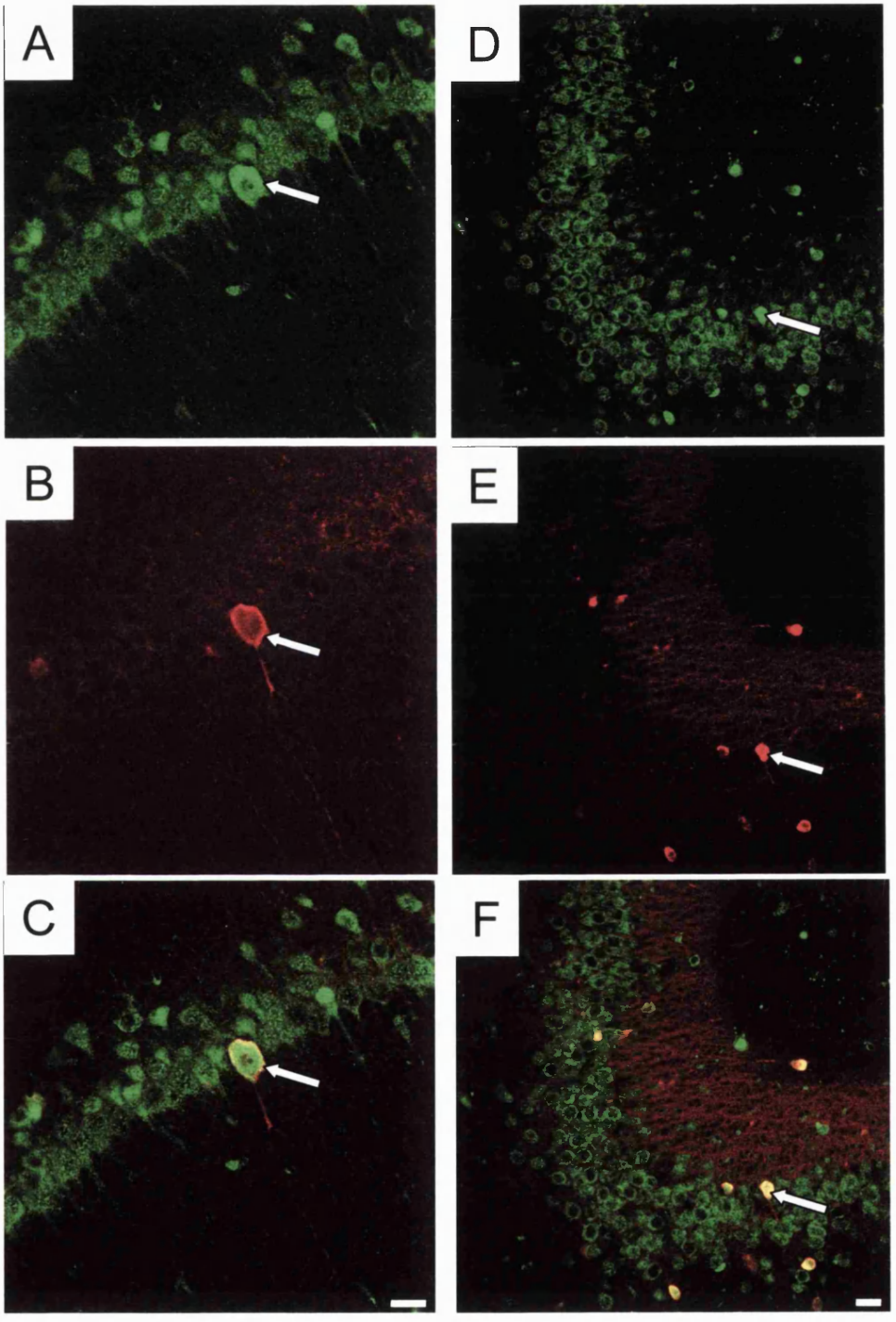

A
D
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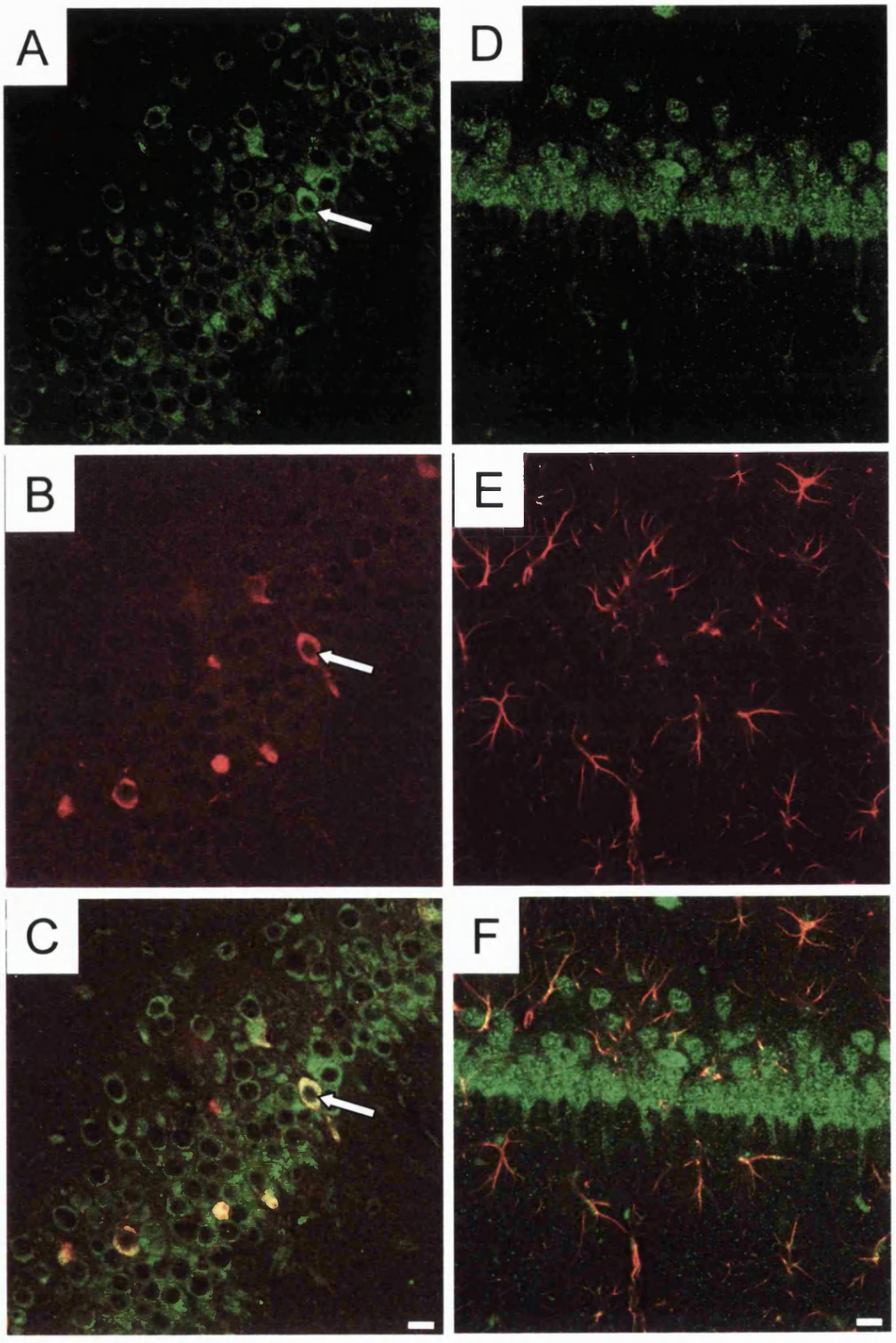
Figure 5.7 Confocal images showing the co-localisation of $GABA_{BL}$ with the interneuron marker calretinin and the astrocytic marker GFAP. $GABA_{BL}$ (A) was expressed in the CA1 in the pyramidal cells and also in the calretinin positive interneurons (B), as shown by the yellow cells in (C), see arrows. $GABA_{BL}$ (D) protein was not expressed in GFAP positive astrocytes in the CA1 (E), or any other region of the hippocampus, as shown by the clear separation of colours (F). Scale bars = 20μm.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5.3.7 Thalamus

Most regions of the thalamus demonstrated moderate to dense $GABA_{BL}$ IR (Fig. 5.4D). Medial thalamic nuclei were densely immunoreactive for $GABA_{BL}$ as were the lateral and ventral nuclei, with labelling mainly observed in the cell body membrane. The reticular thalamus demonstrated moderate $GABA_{BL}$ IR (Fig. 5.4D), here $GABA_{BL}$ protein was expressed in cell bodies and little or no dendritic or neuropilic IR was observed.

5.3.8 Hypothalmus

Expression levels of $GABA_{BL}$ in the rat hypothalamus were moderate to dense according to the nucleus (see Table 5.2 and Fig. 5.4C,D). The dorsal and ventromedial hypothalamic nuclei both demonstrated dense $GABA_{BL}$ IR as did the median eminence. Immunoreactivity in the arcuate nucleus was also dense (Fig. 5.4C) with $GABA_{BL}$ protein observed in the cell membrane and cytoplasm. Moderate $GABA_{BL}$ expression was noted in the lateral nucleus and in the tuber cinereum. In general $GABA_{BL}$ IR was present in cell bodies but not in dendrites or neuropil throughout the hypothalamus.

5.3.9 Midbrain structures

$GABA_{BL}$ IR varied in the superior colliculus in a layer specific manner. In the superficial grey layer $GABA_{BL}$ IR was very dense, whereas IR was moderate in all other areas, though all exhibited only cell body labelling (Fig. 5.3E,F). In the inferior colliculus $GABA_{BL}$ IR was homogenous throughout all regions in the neuronal somata (Fig. 5.3A,F). $GABA_{BL}$ IR in the substantia nigra was dense in the pars reticulata, whereas it was only moderate in the pars compacta. In both

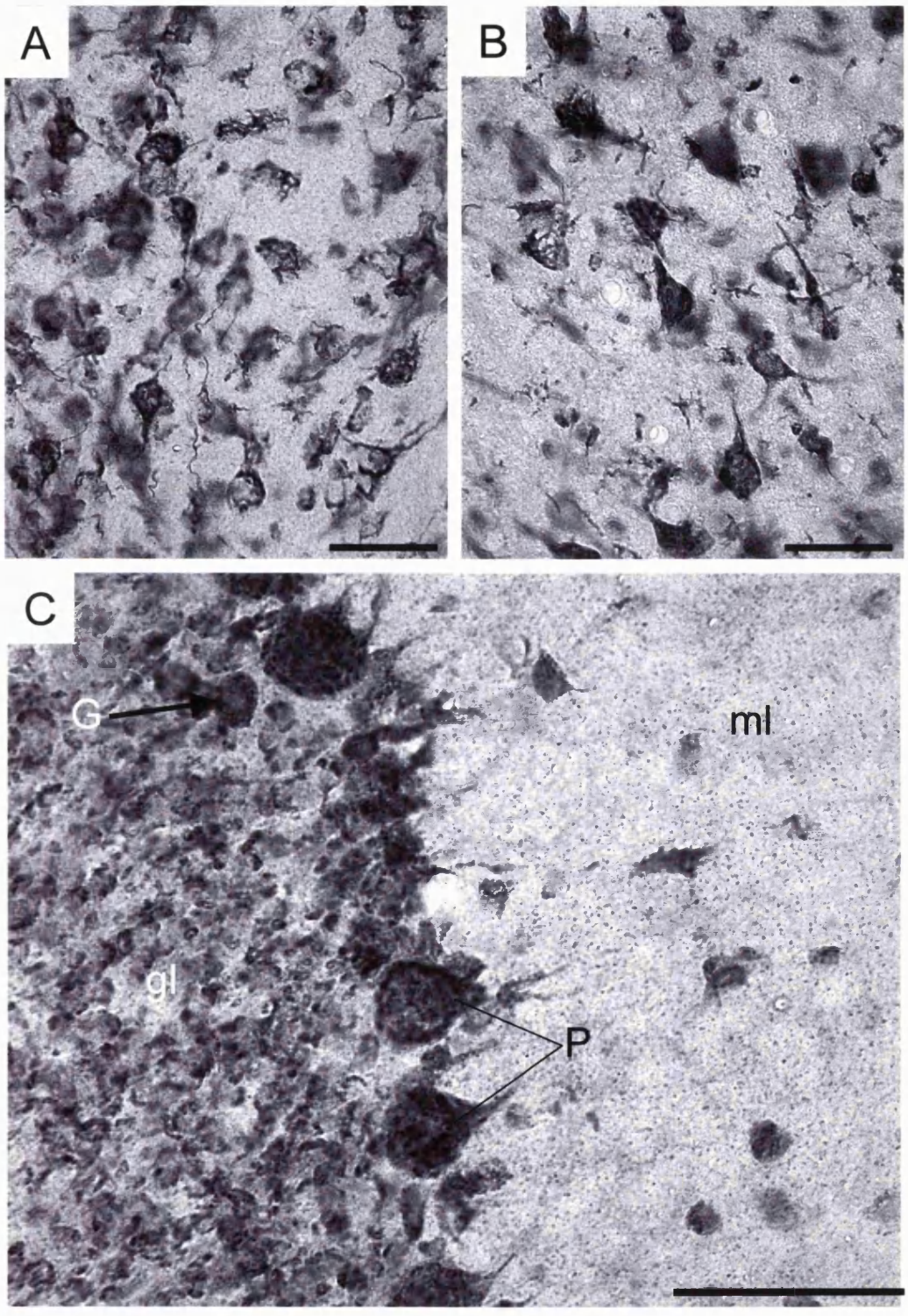
nuclei of the substantia nigra $GABA_{BL}$ labelling was restricted to cell bodies with no neuropilic IR observed. $GABA_{BL}$ labelling of the ventral tegmental area was also noted, in this region of the midbrain IR was moderate in cell body profiles only.

5.3.10 Pons, rhombencephalon and cerebellum

In the monoaminergic neurons of the pons $GABA_{BL}$ IR was very dense (Fig. 5.4A,F) being restricted once again to cell bodies. In the spinal trigeminal and vestibular nuclei dense $GABA_{BL}$ IR was noted, though the locus coeruleus exhibited only moderate IR. Raphe nuclei exhibited varrying intensities of $GABA_{BL}$ IR, with the dorsal raphe demonstrating very dense protein expression (Fig. 5.4F and Fig. 5.8A), moderate $GABA_{BL}$ IR was also present in the median raphe.

The cerebellum demonstrated low to dense $GABA_{BL}$ IR depending on the layer. The molecular layer demonstrated only low $GABA_{BL}$ IR which was restricted to small cell bodies, presumably those of stellate cells (Fig. 5.8C). The Purkinje cells revealed dense $GABA_{BL}$ protein expression with IR restricted to the cell body and none noted in the dendrites projecting into the molecular cell layer. Dense $GABA_{BL}$ IR was observed in the granule cell layer of the cerebellum (Fig. 5.8C). The deep cerebellar nuclei also exhibited dense $GABA_{BL}$ IR again in cell bodies but not dendrites or neuropil.

Figure 5.8 Photomicrographs showing $GABA_{BL}$ IR in the hind brain. $GABA_{BL}$ IR was dense in the dorsal raphe (A), the nucleus of the solitary tract (B). In the cerebellum the granule cell layer (gl) and Purkinje cells were densely immunoreactive for $GABA_{BL}$ (C), a few scattered cells, presumably stellate cell bodies, in the molecular cell layer (ml) also showed $GABA_{BL}$ IR. Scale bars = 50μm.


A
B
C
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ml
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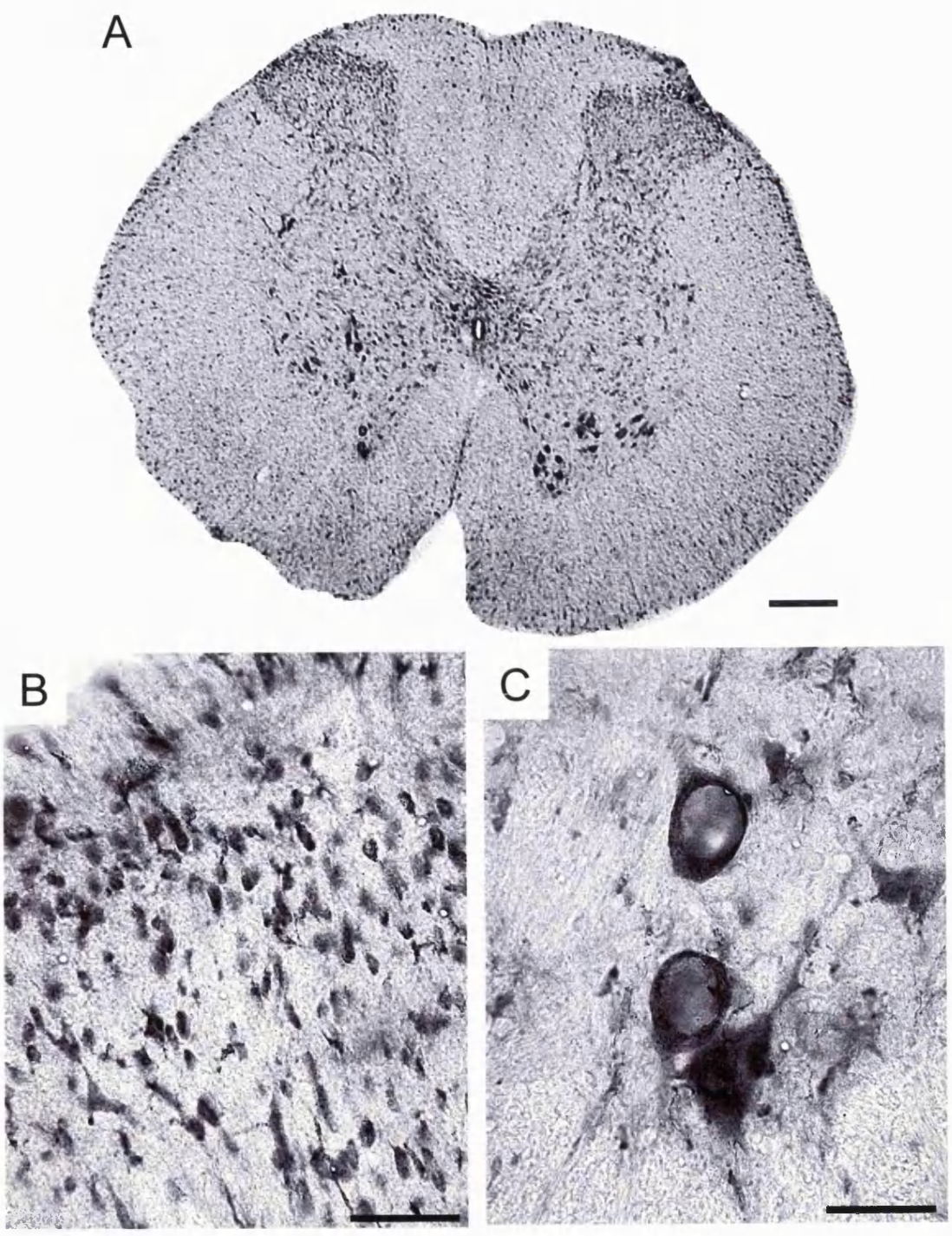
P

5.3.11 Spinal cord

$GABA_{BL}$ protein expression in the spinal cord was wide spread (Table 5.3 and Fig. 5.9A). Superficial laminae of the dorsal horn exhibited dense $GABA_{BL}$ IR in small cell bodies with no neuropilic expression noted (Fig 5.9B). In the deeper laminae $GABA_{BL}$ IR was less intense and in laminae V and VI was only low. The deep laminae of the ventral horn expressed moderate to dense $GABA_{BL}$ IR, with the large motor neurons of lamina IX demonstrating dense membrane bound IR (Fig. 5.9C). Lamina X around the central canal also exhibited dense protein expression. $GABA_{BL}$ IR was also observed in the white matter of the spinal cord.

Figure 5.9 Photomicrographs showing $GABA_{BL}$ protein expression in the rat spinal cord (A). $GABA_{BL}$ IR was dense in the dorsal horn (B) and also in the ventral horn motor neurons (C). Some white matter IR was also evident (A). Scale bars A = 5mm, B,C = 50μm.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5.4 Discussion

5.4.1 Experimental considerations

As discussed in Chapter 3, the use of polyclonal antisera for determining the localisation of novel proteins should be undertaken cautiously with the inclusion of as many controls as available to establish the specificity of the antisera for the protein in question. The cell-type Taqman studies initially performed demonstrated that the $GABA_{BL}$ mRNA was endogenously expressed in a number of cell lines including HEK-293 cells, although it was absent in 1321-N1 cells and COS cells (Calver et al., 2003). Western blot analysis was therefore performed on cell lysates from 1321-N1 cells and HEK-293 cells as a positive control, as well as whole rat brain homogenates. As shown in Fig. 5.2 $GABA_{BL}$ protein was not detected in the 1321-N1 cell lystates but was clearly detectable in HEK-293 and whole brain lysates, and ran at the expected size of 90kD. There was also a total absence of $GABA_{BL}$ labelling by immunoblot on $GABA_{BL}$ null mutant rat brain homogenate preparations, $GABA_{BL}$ labeling was strong in the wild type and heterozygotes, and the immunoblot with MAP kinase antisera confirmed that similar amounts of protein had been loaded. The specificity of $GABA_{BL}$ was also confirmed on tissue sections by means of preabsorption with the immunogenic peptide, in which instance all $GABA_{BL}$ immunoreactive labelling was abolished (Fig. 5.2). That the protein can be detected by Western analysis at the correct molecular weight and can be successfully competed out using the cognate peptide both by Western blot and immunohistochemistry, therefore suggests that the antisera is specific to the $GABA_{BL}$ protein in question. The distribution of $GABA_{BL}$ in the rat brain was in agreement with that observed by Taqman analysis (Calver et al., 2003) and was also in agreement with *in-situ*

hybridisation data as presented at the $GABA_B$ symposium, FENS 2002, by K. Kaupman from the Novartis group.

5.4.2 Distribution of $GABA_{BL}$ in the rat CNS

The required heterodimerisation of two distinct $GABA_B$ receptor subunits for the formation of functional receptors coupled with the observed discrepancies between tissue expression in the two known subunits has led to an intense search for other novel subunits. A novel orphan seven transmembrane receptor with homology to and structural characteristics of the $GABA_B$ receptor subunits and the recently reported Drosophila "$GABA_{B3}$" subunit (Mezzler et al., 2001), has been identified through database mining. The protein expression of $GABA_{BL}$ IR in the rat brain and spinal cord was reminiscent of that previously observed with antisera specific to $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits (see Chapter 3) and was in agreement with $GABA_{BL}$ mRNA analysis (Calver et al., 2003; Kaupmann et al., 2002). Highest levels of $GABA_{BL}$ immunoreactivity were seen in areas which also demonstrated high levels of $GABA_B$ receptor subunits, namely the cortex, thalamus, hypothalamus, hippocampus, the granule cell layer of the cerebellum and throughout the spinal cord, which correlates well with previous Taqman data for the $GABA_{BL}$ orphan receptor (Calver et al., 2003). Areas known to be modulated by GABA also demonstrated $GABA_{BL}$ protein expression. For example the inhibitory interneurons of the reticular thalamus which are known to be GABAergic and invloved in the thalamo-cortical loop (Arcelli et al., 1996), expressed moderate levels of $GABA_{BL}$ in cell soma, although no IR was observed in the neuropil. Transient calcium current activation has been shown to be regulated by pre- and post-synaptic $GABA_B$ receptors in the

hypothalamus (Obrietan and Van den Pol, 1999), and dense $GABA_{BL}$ IR was observed in such regions. In addition, an area of intense $GABA_{BL}$ immunoreactivity in this study was the cerebellum, the Purkinje cells of which show inhibited voltage-gated calcium currents upon application of baclofen, a $GABA_B$ agonist (Mintz and Bean, 1993).

Throughout the rat brain and spinal cord $GABA_{BL}$ protein expression was observed on neuronal cell body profiles and not in the surrounding neuropil, a pattern of localisation indicative of post-synaptic receptor expression. However, $GABA_{BL}$ was expressed in the medial habenula which projects via the fasiculus retroflexus to the interpeduncular nucleus, which also demonstrated dense $GABA_{BL}$ IR. Expression in these structures may indicate both pre- and post-synaptic expression. However, the exact pre- and post-synaptic localisation of this putative receptor remains to be determined by ultrastructural analysis at the electron microscope level.

Clues as to the potential inhibitory/excitatory nature of this orphan receptor can be gleaned from its cerebellar distribution. The Purkinje cells which receive excitatory input from ascending mossy fibres demonstrate strong $GABA_{BL}$ protein expression, as do the granule cells which act as excitatory interneurons. Golgi cells situated close to the Purkinje cells in the granule cell layer act as inhibitory interneurons. $GABA_{BL}$ IR was observed on these interneurons suggesting a potential inhibitory role of $GABA_{BL}$ in this system. Further evidence pointing towards an inhibitory role of this orphan receptor is its expression on inhibitory interneurons of the hippocampus. $GABA_{BL}$ protein was expressed on parvalbumin-positive inhibitory interneurons in all regions of the hippocampus, which represent approximately half of the GABAergic neurons in

the principle cell layers of the hippocampus (Freund and Buzsaki, 1996). Parvalbumin-positive interneurons represent basket or chandelier cells, however, parvalbumin does not account for all of these inhibitory interneurons. Importantly parvalbumin neurons are known to form gap junctions between other parvalbumin-positive neurons or neurons of unknown origin (Freund and Buzsaki, 1996). The presence of $GABA_{BL}$ on these gap junction forming interneurons may be of significance to the treatment of neurological diseases characterised by a disruption to normal excitatory/inhibitory balances such as the epilepsies.

Calbindin, an intracellular Ca^{2+}-binding protein which represents a marker of a different subset of interneurons in the hippocampus, is found throughout the dentate gyrus, including the mossy fibres of these dentate granule cells, and does not represent basket or chandelier interneurons. Calbindin positive neurons are believed to innervate pyramidal cell dendrites, consistant with the mossy fibre labelling we have observed in this study. Calbindin positive interneurons are also found throughout the CA1-3 subregions and represent GABAergic interneurons with distant projections (Freund and Buzsaki, 1996). In the CA3 subregion calbindin positive interneurons were observed in the stratum oriens, pyramidale and radiatum. These interneurons were found to contain $GABA_{BL}$ protein as seen by immunofluorescence. The calretinin positive interneurons visualised in the CA1 region of the rat hippocampus by immunofluorescence probably represent the spine-free sub-population, which are described as "interneurons specialised to innervate other interneurons" (Freund and Buzsaki, 1996). $GABA_{BL}$ protein expression was observed on these interneurons in the CA1 and also with calretinin positive interneurons in the CA3 which probably represent the spiny sub-

population of calretinin positive interneurons thought to innervate principal cell dendrites but with mossy fibre innervation.

$GABA_B$ receptor subunits are expressed on GFAP positive astrocytes in the hippocampus (Hosli and Hosli, 1990; Nilsson et al., 1993; Patte et al., 1999) (and see Chapter 4). In contrast, $GABA_{BL}$ protein was not observed on such cells and therefore we assume $GABA_{BL}$ has no influence on normal astrocytic function.

In conclusion, the distribution of a novel $GABA_B$-like receptor by immunohistochemistry in the rat CNS has been demonstrated. $GABA_{BL}$ is widely distributed with highest intestity of immunoreactivity seen in the hippocampus, amygdala, habenula, hypothalamus, raphe nuclei, cerebellum and dorsal horn of the spinal cord. Functional assessment of this receptor has, however, failed to produce a pharmacological response to $GABA_B$ ligands even when co-transfected with $GABA_{B1}$ or $GABA_{B2}$ (Calver et al., 2002). The possibility remains that this orphan receptor heterodimerises with an as yet undiscovered protein to form a functional receptor, alternatively the ligand for this novel $GABA_B$-like receptor is indeed not GABA but one yet to be identified. The distribution of $GABA_{BL}$ in the rodent may provide valuable clues as to its potential role in physiology and may direct studies for the identification of potential protein partners and may yet yield the pharmacological diversity that has been previously described.

Chapter 6 - General Discussion

The preceeding chapters have incorporated discussions as to the significance of the data obtained for the localisation of the $GABA_B$ receptor subunits, the non-neuronal localisation of these subunits, and the first characterisation of a novel $GABA_B$-like receptor. The aim of this discussion is to bridge these chapters, drawing synergies between the CNS localisation of the $GABA_B$ and $GABA_B$-like receptors and their potential relevence in physiology and disease.

Since the initial cloning of the first $GABA_{B1}$ and $GABA_{B2}$ receptor subunits, much work has gone on to understand the interactions between these two proteins that result in the formation of a functional receptor. The unique and obligate heterodimerisation of the $GABA_{B1}$ and $GABA_{B2}$ receptor subunits is now well documented. Cell surface expression is dependent on heterodimerisation, whereby the protein-protein interaction of $GABA_{B1}$ and $GABA_{B2}$ masks an endoplasmic reticulum retention motif (di-leucine and RSRR) at the C-terminal tail and allows trafficking of the heterodimeric complex to the cell surface (Margeta-Mitrovic et al., 2000; Calver et al., 2001; Grunewald et al., 2002; Pagano et al., 2001). Heterodimer formation is not only critical for cell surface expression of $GABA_{B1}$, but also for G-protein signalling. GABA binds to the $GABA_{B1}$ receptor subunit in the "venus fly-trap" ligand binding domain resulting in a conformational change in the heterodimer and then allows the $GABA_{B2}$ receptor subunit to couple to the inhibitory G-protein ($G_{i/o}$) and subsequent downstream signalling cascades (Duthey et al., 2001; Robbins et al., 2001). It is therefore not surprising that, as described in Chapter 3, all three principle $GABA_B$ receptor subunits were found to co-localise in the same regions and cell

populations. Confocal co-localisation analysis revealed that both $GABA_{B1a}$ and $GABA_{B1b}$ receptor subunits co-localise to the same cell, as do $GABA_{B1}$ and $GABA_{B2}$ receptor subunits. Furthermore, in Chapter 4 I have demonstrated that $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ are also all expressed in astrocytes and microglia in the rat CNS. It was hoped that localisation analysis using subunit specific antisera would reveal subtle differences in distribution which could then be used as a basis to develop heterodimer specific compounds with selective pharmacological profiles. Indeed, early reports of differences in the pre- versus post-synaptic localisation of the $GABA_{B1a}$ and $GABA_{B1b}$ receptor subunits suggested that agonists and antagonists which could be developed specifically for each heterodimeric complex could be used to target specific physiological functions without serious side-effects (Benke et al., 1999; Billinton et al., 1999; Bischoff et al., 1999; Fritschy et al., 1999, Liang et al., 2000; Poorkhalkali et al., 2000). However, these reports speculating the pre- versus post-synaptic localisation proved contradictory and also region dependent. In this study, I have demonstrated at the ultrastructural level that $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ are expressed at both the pre-synaptic terminus and in the post-synaptic membrane in the CA1 subregion of the hippocampus. I have not been able to demonstrate a difference between any of the receptor subunits and their synaptic localisation (see Chapter 3). This data has been coroborated in other regions including the cerebellum (Ige et al., 2000), visual cortex (Gonchar et al., 2001) and basal ganglia (Charara et al., 2000), with all studies demonstrating a pre- and post-synaptic expression of each receptor subunit.

At the pharmacological level much excitement was caused by several publications describing the anti-convulsant compound gabapentin as being a

selective agonist of the $GABA_{B1a}/GABA_{B2}$ heterodimer (Ng et al., 2001; Bertrand et al., 2001). However, despite the efforts of several laboratories these results have not been coroborated and it is now concluded that gabapentin is not a $GABA_B$ receptor agonist, let alone a $GABA_{B1a}/GABA_{B2}$ heterodimer specific agonist (Lanneau et al., 2001; Jensen et al., 2002). To date no differences in the synaptic localisation of the two main heterodimers $GABA_{B1a}/GABA_{B2}$ or $GABA_{B1b}/GABA_{B2}$, and no heterodimer subtype specific agonist or antagonist have been identified. This therefore raises the question as to whether the two $GABA_{B1}$ receptor subunits have any distinct functional significance or whether they are merely consequences of random genetic splicing. The glial localisation of the $GABA_B$ receptors would also agree with this. In this study I have seen no evidence of a difference in non-neuronal cell expression for either $GABA_{B1a}$, $GABA_{B1b}$ or $GABA_{B2}$. Cells expressing one receptor subunit would appear to express all receptor subunits, thus the splicing of the $GABA_{B1}$ receptor subunit confers no diversity in terms of neuronal and non-neuronal cell expression.

The absence of differences in localisation and pharmacological selectivity between heterodimer subtypes does not however explain the rich pharmacological heterogeneity observed in native systems (Bonnano and Raiteri, 1993). Such heterogeneity is particularly pronounced for the autoreceptors of the cerebral cortex and spinal cord. Cortical autoreceptors are sensitive to phaclofen but insensitive to CGP35348, however the converse is true of the spinal cod autoreceptors (Bonnano and Raiteri, 1993). Therefore it is natural to assume that as yet not all $GABA_B$ receptor subunits or chaperone proteins have been indentified. Since the initial description of the $GABA_B$ receptor as an obligate heterodimer much effort has been afforded to the search for novel protein partners

which may offer differing pharmacology from that described for $GABA_{B1}$/$GABA_{B2}$. However, despite intense efforts by many groups using diverse fields of research such as yeast-2-hybrid screening and proteomic database mining no novel receptor subunits have as yet been identified. Studies in drosophila have, however, revealed a third putative $GABA_B$ receptor subunit, D-$GABA_{B3}$ (Mezler et al., 2001). *In-situ* hybridisation studies, however, demonstrated that D-$GABA_{B3}$ is expressed in a unique pattern in drosophila compared to D-$GABA_{B1}$ and D-$GABA_{B2}$. Further still, D-$GABA_{B3}$ was not functional in any system in combination with either of the other D-$GABA_B$ receptor subunits or when expressed alone. In Chpater 5, I have decsribed for the first time the CNS distribution of a putative novel $GABA_B$-like receptor protein, $GABA_{BL}$. Since its initial identification by database mining, the human, rat and mouse orthologues of $GABA_{BL}$ have been cloned and expressed in a multitude of functional systems, alone or in combination with either of the other $GABA_B$ receptor subunits, and indeed as chimeras of $GABA_{BL}$ and $GABA_{B1/2}$ (Calver et al., 2003). Disappointingly these studies have so far failed to demonstrate any functional activity for $GABA_{BL}$ despite screening against a multitude of GABA-like compounds. It is possible that the ligand for $GABA_{BL}$ is not GABA-like. However, it is also possible that further receptor subunits or chaperone proteins exists that to date remain ellusive, but which may confer $GABA_B$ like activity to this $GABA_{BL}$ protein. Until that time this protein will remain classified as an orphan $GABA_B$-like GPCR. Despite this, I have mapped the distribution of $GABA_{BL}$ in the rat CNS using $GABA_{BL}$-specific polyclonal antisera. As described in Chapter 5, $GABA_{BL}$ is widely expressed throughout the rat CNS in a pattern very similar to that reported in Chapter 3 for the three principle $GABA_B$

receptor subunits. This distinguishes the novel $GABA_B$ receptor cloned in drosophila from the human, rat and mouse receptor cloned here and described as $GABA_{BL}$, since in drosophila there was a disparity between the distribution of D-$GABA_{B1/2}$ and D-$GABA_{B3}$ (Mezler et al., 2001). Thus, so far the identification of novel functional $GABA_B$-like receptor proteins has failed to account for any of the pharmacological heterogeneity of *in vivo* $GABA_B$ receptors.

It is also possible that further $GABA_B$ receptor subunits do not exist. Three separate groups have bred $GABA_B$ null mutant mice, all of which displayed a total absence of pre- or post-synaptic responses to GABA or GABA agonists (Prosser et al., 2001; Schuler et al., 2001; Lehmann et al., 2002). In all cases the mice developed severe epilepsy within four weeks of age. However the severity and age of onset of epilepsy did vary between studies, and probably reflects differences in the background mouse strains used. That no $GABA_B$ response could be identified on these animals, even in the presence of the $GABA_{B2}$ receptor subunit, casts doubt on the potential existence of additional subunits, as these would have been expected to account for at least part of the $GABA_B$ receptor response to GABA. Indeed, $GABA_{B2}$ receptor protein levels were significantly downregulated in these animals, suggesting that heterodimer formation is essential for the stability of this subunit in a native system (Prosser et al., 2001; Schuler et al., 2001). However, it is worth remembering that functional $GABA_B$ receptors have been identified in certain peripheral tissues in the absence of the $GABA_{B2}$ receptor subunit. This suggests that in such regions, other proteins are present which confer functionality on the $GABA_{B1}$ receptor. In light of this, the finding that no $GABA_B$ response could be detected in the ileum or urinary bladder in $GABA_{B1}$ null mutant mice suggests that any potential partner or chaperone

protein in the periphery is not capable of forming functional $GABA_B$ receptors in its own right (Sanger et al., 2002).

It may then be prudent to assess intracellular effector systems which may instead account for the diversity in pharmacological responses *in vivo*. For example protein kinases, such as protein kinase A and protein kinase C have both been shown to modulate $GABA_B$ receptor function (Taniyama et al., 1992; Yoshimura et al., 1995). The cloning of the $GABA_B$ receptor subunits has allowed investigations into the exact residues within the receptor subunits which act as consensus sites for kinases. Recently it has been shown that serine 892 in the C-terminal tail of the $GABA_{B2}$ receptor subunit is a substrate for protein kinase A, which results in an enhancement of receptor stability at the cell membrane upon agonist activation (Couve et al. 2002). Such an observation sets $GABA_B$ receptor phosphorylation apart from that known for other GPCRs, as it is generally accepted that agonist-induced phosphorylation of GPCRs results in negative modulation by way of receptor internalisation, and eventual downregulation of receptor density (Tsao and Von Zastrow, 2000). As to whether other intracellular kinases interact with either of the $GABA_B$ receptor subunits remains to be seen. If indeed kinases are key to the activation/inactivation of $GABA_B$ receptors they may represent a novel mechanism of modulating $GABA_B$ receptor function without the side-effects expected to be associated with direct $GABA_B$ receptor agonists or antagonists.

In addition to the kinase family, other proteins or intracellular enzymes may be involved in producing the pharmacological heterogeneity of the $GABA_B$ receptor. Yeast-2-hybrid studies have focused predominantly on interactions between the $GABA_B$ receptor subunit C-termini and other proteins. These studies

have revealed interactions with specific members of the 14-3-3 family of intracellular proteins (Couve et al., 2001). Such an interaction is suggested to prevent the normal heterodimerisation of $GABA_{B1}$ and $GABA_{B2}$ by binding with the C-terminal of the $GABA_{B1}$ receptor subunit. The family of 14-3-3 proteins have previously been shown to modulate G-protein activity (Benzing et al., 2000), and to modulate synaptic transmission via potassium channels in drosophila (Zhou et al., 1999).

The C-terminus of $GABA_{B1}$ has also been shown by several groups to interact with the transcription factor CREB2, also known as ATF4 (Nehring et al., 2000; White et al., 2000; Vernon et al., 2001). However, controversy surrounds these reports since two groups report directly apposing effects of the $GABA_B$-CREB2 interaction. One group report that CREB2 re-locates to the nucleus from the cytoplasm upon baclofen stimulation (White et al., 2000). In contrast, Vernon et al., (2001) showed CREB2 migrating from the nucleus into the cytoplasm upon baclofen stimulation. Such differences are hard to explain, except in the context of different neuronal populations being used to study this phenomenon. The nuclear expression of this transcription factor and its interaction with the $GABA_{B1}$ C-terminal does, at least partly, help to explain the phenomenon of intracellular/nuclear staining seen in this thesis and by other groups (Poorkhalkali et al., 2000; Princivalle et al., personal communication). If $GABA_B$ receptor heterodimers are interacting with a nuclear transcription factor via the C-terminal portion of $GABA_{B1}$ it is not suprising that intracellular and perinuclear labelling was observed. This would be made even more easy to visualise by the necessary permeabilisation of the tissue sections to allow antisera penetrance. Thus antisera would label not only membrane expressed $GABA_B$ receptors but also those

internalised or those relocated to within the nucleus. Nuclear labelling with antisera is frequently associated with non-specificity and as such is often dismissed as a false-positive. In the case of $GABA_B$, however, and its now known interaction with nuclear proteins, this labelling is probably a fair reflection of the distribution of $GABA_B$ receptors throughout the neuron.

$GABA_{B2}$ is also reported to interact with a number of intracellular proteins. A protein containing multiple PDZ domains, MUPP-1, which has also been shown to interact with the C-terminal of the $5HT_{2c}$ GPCR, reportedly interacts with the C-terminus of the $GABA_{B2}$ protein (Ige et al., 2001). The C-terminal tail of the $GABA_{B2}$ protein also interacts with N-ethylmaleimide-sensitive factor (NSF) (White et al., 2002). NSF is believed to play a crucial role in intracellular trafficking, and interestingly interacts with the ionotropic GABA receptor, $GABA_A$ (Kittler et al., 2001). This NSF/$GABA_A$ interaction is mediated through the microtubule-associated protein, GABARAP, and as such may provide a mechanism for the specific membrane targeting of the $GABA_B$ receptor. A further structural protein, β-filamin, has also been reported to interact with $GABA_{B2}$, and probably accounts for another way of linking the $GABA_B$ receptor to the cytoskeleton (White et al., 2002).

Other family C GPCRs are reported to interact with intracellular proteins. The mGluR subfamily interact with the PDZ-domain containing Homer family (Tu et al., 1998; Dev et al., 2001; Hirbec et al., 2002). Interestingly the $GABA_{B2}$ receptor subunit C-terminus contains a sequence very similar to that of the Homer-binding consensus, PPXXFR. This would suggest that $GABA_{B2}$ is capable of binding Homer proteins, which may also explain the interaction of $GABA_B$ and mGluR in the cerebellum (Hirono et al., 2001). However, unpublished results

suggest that $GABA_{B2}$ does not in fact bind Homer proteins (Pangalos et al., personal communication), and thus this site, PPSFR, is either redundant or plays a different, as yet unidentified, role.

With regard to extracellular interactions of the $GABA_B$ receptors much less is known. Thus far the majority of Yeast-2-hybrid screening performed has been done on the intracellular C-terminal due mainly to this being the coiled-coil containing region shown to modify intracellular retention. Only one piece of evidence points towards extracellular interactions. The sushi-domains of $GABA_{B1a}$ reportedly interact with an extracellular matrix protein, Fibulin (Ginham et al., 2002). Such an interaction may be important in anchoring the receptor to the correct synaptic or extra-synaptic region and as such play a significant role in pharmacological diversity.

It is now becoming apparent that identifying specific agonists or antagonists with blood brain barrier penetrance and high receptor affinity is not only challenging, but also these compounds may be fraught with side-effects. One area to potentially exploit $GABA_B$ pharmacology with minimal side-effects is the development of allosteric modulators specific for the $GABA_B$ receptor. These compounds do not have any activity in their own right, instead they serve to enhance agonist affinity only when and where the neurotransmitter is released. The first positive allosteric modulators of the $GABA_B$ receptor were reported in 2001 (Urwyler et al.). The compound CGP7930 [2,6-Di-*tert*-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol] and its aldehyde analog CGP13501 potentiated GABA-induced effects in functional assays such as GTPγS binding assays and cellular activation assays such as modultaion of inwardly activating K^+ channels and Ca^{2+} signaling. Such effects were present not only in recombinant systems but

also rat brain membrane preparations, and importantly neither compound had any activity when applied in the absence of GABA (Urwyler et al., 2001). No difference was observed in the potency of the modulatory effect with either the $GABA_{B1a}/GABA_{B2}$ or the $GABA_{B1b}/GABA_{B2}$ heterodimer complexes suggesting that these compounds do not exert their effects in the extracellular domain of $GABA_{B1}$ or the sushi binding domains of $GABA_{B1a}$. More recently the same group have reported the identification of a further $GABA_B$ specific allosteric modulator GS39783 (N,N'-Dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine) which has equal potency to CGP7930 but greater efficacy for the $GABA_B$ receptor (Urwyler et al., 2002). Importantly CGP7930 and GS39783 were efficacious in an *in vivo* model studying striatal dopamine release, but only when administered in conjunction with L-baclofen and not in isolation. Although the exact site of action of these compounds is as yet unclear it is feasible to assume that these modulators may act either in the ligand binding domain of $GABA_{B1}$, in that of $GABA_{B2}$ which has been shown to not bind GABA (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Couve et al., 2000; Kniazeff et al., 2002), or indeed in the transmembrane domains of the receptor complex. The identification of these and no doubt other allosteric modultors may pave the way for novel, safe pharmacological interventions of the $GABA_B$ system for the treatment of amongst others epilepsy, pain and depression.

Since commencing this thesis much has been learnt about the molecular pharmacology of this unique GPCR. Many studies have investigated the distribution of the receptor subunits. Others have discovered novel proteins which interact with one or other of the receptor subunits, with potential intra- and extracellular consequences. Others have ascribed further roles to kinases, which

themselves distinguish $GABA_B$ receptors from other GPCRs. Allosteric modulators have been identified which act as agonists only in the presence of GABA and therefore may in time serve as a major therapeutic advantage to current therapies. Further still novel $GABA_B$-like proteins have been identified, and though no functions have yet been ascribed for these putative novel receptors, further studies may yet yield $GABA_{B3}$.

Chapter 7 – Bibliography

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