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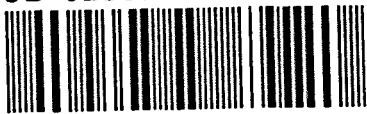
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**MOLECULAR AND ONTOGENIC ANALYSIS OF  
THE MAMMALIAN GABA<sub>A</sub> RECEPTOR**

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The Royal Free Hospital  
London

A dissertation submitted for the degree of Doctor of  
Philosophy of the Open University

1998

AUTHOR'S No: Pa251085

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## **Preface**

The work presented in this thesis was performed entirely by the author, except as acknowledged, at the MRC Molecular Neurobiology Unit, Hills Road, Cambridge between October 1989 and November 1992. This thesis has not been previously submitted for a degree or diploma at this or any other institution.

Margaret L. Sutherland  
Cambridge  
March 1998

## **Dedication**

**I would like to dedicate this work to the memory of my father**

**Russell H. Sutherland**

**1907-1988**

## **Acknowledgments**

I would like to thank Professor Eric Barnard and Professor Wayne Davies for their encouragement and endurance during the preparation of this thesis. I would like to thank Drs. Sara Lummis and Thora Glencorse for their friendship and encouragement throughout my doctoral training and beyond.

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Table 6.2

Summary of  $K_d$  and  $B_{max}$  values for receptors containing wildtype subunits only or with wildtype subunits and GABA<sub>A</sub>  $\alpha$  or  $\beta$  subunits with point mutations in the putative M1 transmembrane domain.

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## Abbreviations

Ac	accumbens nucleus
AM	anteromedial thalamic nucleus
AV	anteroventral thalamic nucleus
BIC	nucleus brachium inferior colliculus
bp	base pair
BSt	bed nucleus stria terminalis
CA1-3	fields CA1-3 of Ammon's horn
cb	cerebellar primordium
Cb	cerebellum
cDNA	complementary DNA
CeL	central amygdaloid nucleus, lateral
CeM	central amygdaloid nucleus, medial
CG	central gray
CGD	central gray, dorsal
CIC	central nucleus inferior colliculus
Cl	claustrum
co	cortical plate
CPu	caudate putamen
Ctx	cortex
d	diencephalon
DG	dentate gyrus
DpWh	deep white layer of superior colliculus
drg	dorsal root ganglia
D3V	dorsal third ventricle
Ent	entorhinal cortex
Fi	fimbria hippocampi
GABA	$\gamma$ -aminobutyric acid
Gcl	granule cell layer
GP	globus pallidus
he	heart
hi	hippocampus (embryonic)
Hi	hippocampus
Ic	inferior colliculus
InCo	intercollicular nucleus
InG	intermediate gray layer superior colliculus
IMD	intermediodorsal thalamic nucleus
kb	kilobase



kDa	kilodalton
l	liver
La	lateral amygdaloid nucleus
LD	laterodorsal thalamic nucleus
LHb	lateral habenular nucleus
LP	lateral posterior thalamic nucleus
LS	lateral septal nucleus
LSD	lateral septal nucleus, dorsal
m	medulla oblongata
mb	mid brain
Mcl	molecular cell layer
MD	mediodorsal thalamic nucleus
MHb	medial habenular nucleus
MG	medial geniculate nucleus
ol	olfactory lobe
OP	optic nerve layer superior colliculus
OPT	olivary pretectal nucleus
OT	nucleus optic tract
PaS	parasubiculum
PBS	phosphate buffered saline
PCom	nucleus posterior commissure
PCR	polymerase chain reaction
PF	parafascicular thalamic nucleus
PrS	presubiculum
PT	paratenial thalamic nucleus
PV	paraventricular thalamic nucleus
PVA	paraventricular thalamic nucleus, anterior
Re	reuniens thalamic nucleus
Rh	rhomboid thalamic nucleus
RLi	rostral linear nucleus raphe
Rt	reticular thalamic nucleus
Sb	subiculum
sc	spinal cord (embryonic)
Sc	superior colliculus
SuG	subgeniculate nucleus
t	thymus
TBS	Tris buffered saline
TS	triangular septal nucleus
tt	tenia tecta

Tu	olfactory tubercle
VL	ventrolateral thalamic nucleus
VPL	ventral posterolateral thalamic nucleus
VPM	ventral posteromedial thalamic nucleus

## Summary

$\gamma$ -aminobutyric acid is the major inhibitory neurotransmitter in the adult mammalian central nervous system (CNS) and may also play a neurotrophic role during CNS development. Diversification of GABA<sub>A</sub> receptor mediated responses are in part a result of variation in subunit composition in the receptor complex. This variation arises both from the number of different subtypes of GABA<sub>A</sub> receptor subunits ( $\alpha$ 1-6,  $\beta$ 1-4,  $\gamma$ 1-3,  $\delta$ 1,  $\rho$ 1-3,  $\epsilon$ ,  $\rho$ ), as well as from post-transcriptional processes such as RNA splicing. In this thesis, I have investigated the developmental onset of GABA<sub>A</sub> receptor gene expression and the distribution and temporal expression of GABA<sub>A</sub> receptor subunit mRNAs and  $\gamma$ 2 splice variants within the developing and adult murine CNS.

Preliminary studies using S1 nuclease protection analysis demonstrated that  $\alpha$ 1,  $\beta$ 3 and  $\gamma$ 2 were the predominant subtypes of GABA<sub>A</sub> receptor subunits expressed at embryonic day 14 and in the adult murine CNS. *In situ* hybridisation analysis demonstrated overlapping but distinct spatial and temporal patterns of GABA<sub>A</sub> subunit mRNA expression during postnatal development and in the adult murine CNS. Analysis of  $\gamma$ 2 mRNA splice variants demonstrated that the  $\gamma$ 2S transcript is the predominant  $\gamma$ 2 mRNA expressed during latter stages of embryogenesis, while the  $\gamma$ 2L transcript is the predominant  $\gamma$ 2 isoform present in the adult CNS.

Since there is a 29 to 47 percent amino acid identity among the various GABA<sub>A</sub> receptor subunits, I have also demonstrated through site-directed mutagenesis studies, that changes in a conserved amino acid in the cysteine loop of the bovine  $\alpha$ 1 GABA<sub>A</sub> receptor subunit resulted in a loss of agonist and antagonist binding (D149N), while a change in a conserved amino acid in the M1 transmembrane domain of the bovine  $\alpha$ 1 GABA<sub>A</sub> receptor subunit resulted in loss of agonist binding and reduction in the  $B_{\max}$  and  $K_d$  for antagonist binding (P243A). These results are in contrast to the effect of identical mutations in the bovine  $\beta$ 1 subunit and suggest that if the pentameric GABA<sub>A</sub> receptor assembly is composed of ( $\alpha$ 1)<sub>2</sub>( $\beta$ 1)<sub>1</sub>( $\gamma$ 2)<sub>2</sub>, then changes in highly conserved amino acids in the  $\alpha$ 1 receptor subunit would have a greater distortion on the structure of the receptor complex.

# **Chapter 1**

## **General Introduction**

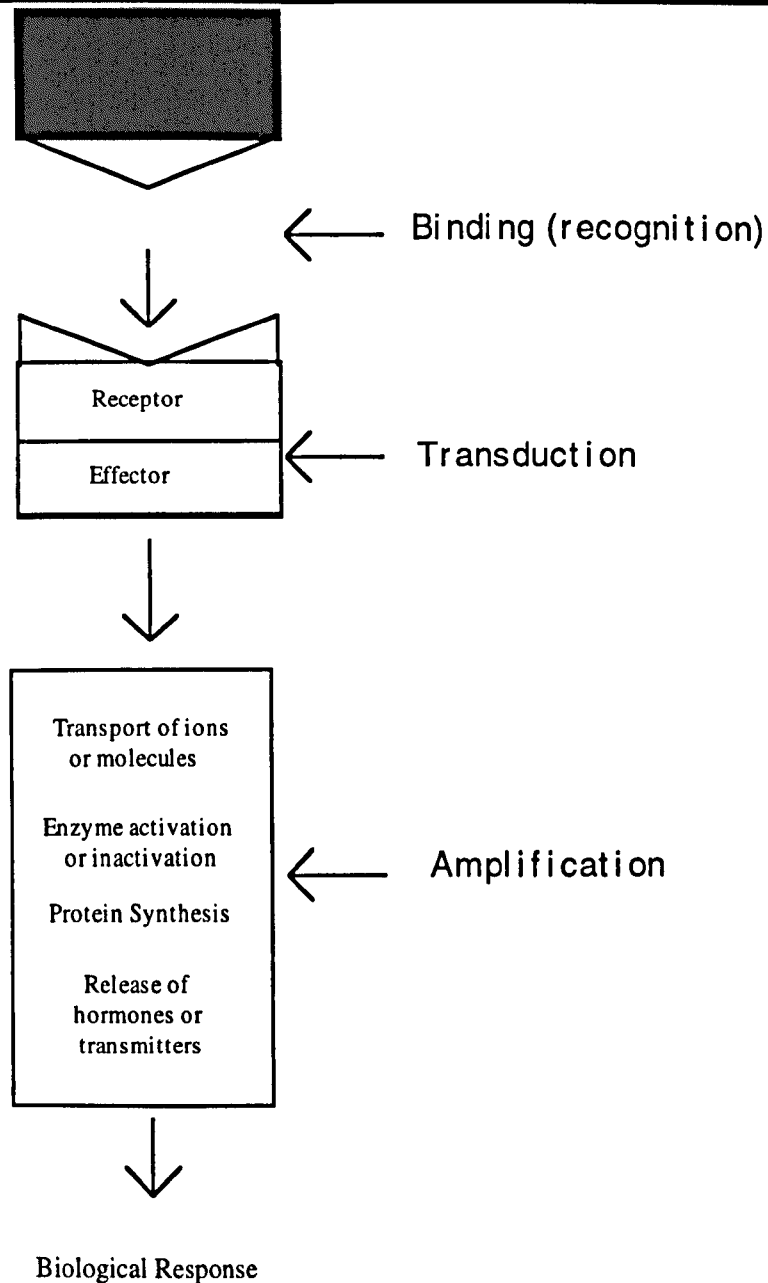
*Immense numbers of individual units, the neurons, completely independent, simply in contact with each other, make up the nervous system.*

*Santiago Ramon y Cajal, Histology of the Nervous System*

## 1.1 COMMUNICATION

The ability of the central nervous system to respond to environmental stimuli is dependent on an elaborate communication network, within and among the individual neurons. Although sharing many characteristics with cells found in other tissues and organs, neurons have several specialized features which effect the flow of information between cells. These include the axon, specialized for intercellular information transfer, the dendrite, a site where signals are often received from other neurons, and the synapse, the point of information transfer between neurons (Levitan and Kaczmarek, 1991). Electrical signals or action potentials arriving at the pre-synaptic terminals are converted into chemical signals through the release of neurotransmitters into the synaptic cleft. The neurotransmitters interact with molecules on both pre- and post-synaptic elements of the synapse to elicit a series of electrical and/or biochemical signals, which act through effector systems to generate a biological response (Figure 1.1). The accurate communication of information requires a high degree of specificity, both at the pre- and postsynaptic sites. This precision is accomplished by a variety of specialized proteins termed receptors.

When a cell is receiving synaptic input, the processing of information is achieved by neurotransmitters, interacting with receptors in the plasma membrane of the neuron, and by depolarization or hyperpolarization of the membrane, or via activation of G-protein coupled receptor mechanisms. Many of the receptors are associated with pores or channels which span the lipid bilayer and allow selective flow of ions at a controlled rate across the membrane. These are known as ligand-gated ion channels. Table 1.1 illustrates the ion conductance characteristics of various ligand-gated and voltage-gated ion channels and the number of transmembrane spanning regions associated with the channel topology.



**Figure 1.1 Schematic Model of Ligand-Receptor Interaction**

Extracellular signals, such as hormones, neurotransmitters and drugs, are molecules which exhibit selective binding interactions with receptors in the plasma membrane. The binding of the molecule, in this example, is coupled with an element which initiates a biological response, such as an ion channel. The activation of the ion channel results in the flow of selective ions across the membrane, resulting in either depolarization or hyperpolarization of the membrane. The biological response is seen in the excitation or inhibition of synaptic activity.

Receptor or Channel	Ion Selectivity	TM Domains	References
Potassium	K <sup>+</sup>	6	Miller, 1991
Calcium	Ca <sup>2+</sup>	24	Catterall, 1991
Sodium	Na <sup>+</sup>	24	Catterall, 1991
GABA <sub>A</sub>	Cl <sup>-</sup> , HCO <sub>3</sub> <sup>-</sup>	4	Schofield, <i>et al.</i> , 1987
Glycine	Cl <sup>-</sup> , HCO <sub>3</sub> <sup>-</sup>	4	Langosch, Becker, Betz, 1990
ACh muscle nicotinic	Na <sup>+</sup> , K <sup>+</sup>	4	Galzi, <i>et al.</i> , 1991b
ACh neuronal nicotinic	Na <sup>+</sup> , K <sup>+</sup> , Ca <sup>2+</sup>	4	Galzi, <i>et al.</i> , 1991b
Glutamate non-NMDA	Na <sup>+</sup> , K <sup>+</sup> , Ca <sup>2+</sup>	4	Hollmann, <i>et al.</i> , 1989
Glutamate NMDA	Na <sup>+</sup> , K <sup>+</sup> , Ca <sup>2+</sup>	4	Wisden and Seeburg, 1993
5-HT <sub>3</sub>	Na <sup>+</sup> , K <sup>+</sup>	4	Maricq, <i>et al.</i> , 1991

**Table 1.1 Ion Selectivity of Transmitter-Gated and Voltage-Gated Ion Channels**

*TM domains indicates the number of putative transmembrane spanning domains associated with the receptors listed. The receptors listed are associated with ion channels which are modulated through a gating mechanism, by an endogenous, extracellular molecule or by changes in the membrane resting potential. (Modified from Barnard, 1992)*

## 1.2 ION CHANNELS

The diversity and plasticity of the central nervous system derives not only from the different types of receptors and channels found, but also in the developmental regulation, alternative and differential expression of the mRNAs, and in the post-translational modification of the encoded polypeptides. For every class of ion channel receptor so far identified, these factors play a role in the diversity and hence heterogeneity observed among the various functional receptor subtypes. This heterogeneity is discussed below for the two classes of receptors, with specific emphasis on the GABA<sub>A</sub> ion channel receptor, which is the subject of study in this thesis.

The gating mechanisms of ion channels and receptors define two broad classifications: 1) P-domain ion channels, which are voltage-gated and include potassium, calcium and sodium voltage-sensitive receptors and 2) ligand-gated ion channel receptors which include neuronal and muscle nicotinic acetylcholine,  $\gamma$ -aminobutyric acid (GABA), glycine, serotonin (5-HT<sub>3</sub>), and glutamate (N-methyl-D-aspartate (NMDA) and AMPA( $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid)/kainate).

### 1.2.1 P-Domain Channels

The voltage-gated ion channels are responsible for the generation of action potentials in electrically excitable cells ( $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ), participate in the regulatory processes in non-excitable cells ( $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ) and control the resting potential and excitability of the membrane ( $\text{K}^+$ ) (Reviewed by Catterall, 1991). The ion conductance activity of voltage-gated ion channels is both rapid and highly selective. The gating activity of voltage-gated channels is described by two rate processes: 1) activation which determines the rate of channel opening and; 2) inactivation which determines the rate of channel closure. Voltage-gated ion channels are not directly regulated by known physiological ligands, but are targets for numerous neurotoxins, therapeutically useful drugs, and can be modulated via second messenger systems.

### 1.2.2 Calcium Channels

Calcium channels (T-, L-, N- P- Q- and R-type) can be distinguished according to their voltage dependence, single-channel properties, ionic selectivity and pharmacological profiles (Snutch *et al.*, 1991; Zhang *et al.*, 1993; Randall & Tsien 1995). The subunit composition of the L-type  $\text{Ca}^{2+}$  channel from skeletal muscle consists of four different subunits,  $\alpha 1$ ,  $\alpha 2$ - $\delta$ ,  $\beta$  and  $\gamma$  (Campbell, Leung and Imagawa, 1988; Catterall, Seager and Takahashi, 1988). Molecular cloning has determined the complementary deoxyribonucleic acid (cDNA) sequences of a subfamily of  $\alpha 1$  subunit related proteins and  $\alpha 2$ ,  $\beta$ , and  $\gamma$  (Snutch *et al.*, 1990, 1991; Ellis *et al.*, 1988; Ruth *et al.*, 1989; Jay *et al.*, 1990). Expression of functional DHP-sensitive L-type  $\text{Ca}^{2+}$  channels is seen in *Xenopus* oocytes, injected with complementary ribonucleic acid (cRNA) from the cloned  $\alpha 1$  calcium channel receptor subunit (Mikami *et al.*, 1989; Biel *et al.*, 1990). The heterogeneity within the subfamily of  $\alpha 1$  related proteins (6 genes), and the functional expression in *Xenopus* oocytes, suggest that the  $\alpha 1$ -subunit is the central functional component of the receptor complex. Mutations introduced into single residues of the  $\alpha 1$  subunit lead to dramatic alterations in the affinity of calcium channels for calcium ions (Yang *et al.*, 1993), further suggesting that this subunit forms the channel pore. The mRNA of  $\alpha 1$ -subunits encode a protein of 212-273 kD, with four homologous domains, each with six putative  $\alpha$ -helical transmembrane segments. Rat brain  $\text{Ca}^{2+}$  channel  $\alpha 1$  subunits, rbC-1 and rbC-11 differ by



a three amino acid insert in the putative cytoplasmic loop between domains II and III and by a twenty-eight amino acid substitution corresponding to the third transmembrane segment (S3) of the fourth domain. These distinct forms of the  $\alpha 1$  subunit are generated by alternative splicing and are differentially expressed in the rat CNS (Snutch *et al.*, 1991). Further diversity is introduced by posttranslational proteolytic cleavage (De Jongh *et al.*, 1991). The  $\alpha 1$ -subunits are present in a complex with the non-membrane spanning  $\beta$ -subunit of 55 kD and a glycosylated transmembrane  $\gamma$ -subunit of 30 kD. The  $\alpha 1$ -,  $\beta$ -,  $\gamma$ -complex interacts with a disulfide-linked glycoprotein complex of  $\alpha 2$ - and  $\delta$ -subunits. The  $\alpha 2$ - and  $\delta$ -subunits derive from a single gene, whose protein product is proteolytically cleaved to yield the disulphide-linked  $\alpha 2$ - and  $\delta$ -polypeptides (De Jongh, Warner, and Catterall, 1990). While the  $\alpha 1$  subunits are capable of generating functional calcium currents when expressed in isolation, coinjection of  $\alpha 1$  with  $\beta$ -,  $\gamma$ - and  $\alpha 2/\delta$  leads to a much greater level of channel expression. The  $\beta$  subunit can alter the kinetics and voltage dependence of  $\alpha 1$  subunits (McClesky 1994).

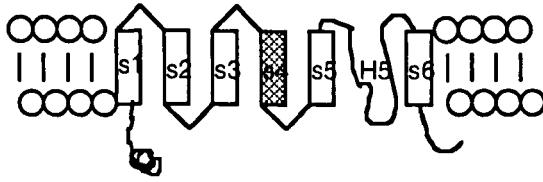
### 1.2.3 Voltage-Gated Sodium Channels

Voltage-gated sodium channels are large membrane glycoprotein complexes composed of a 230-270 kD  $\alpha$ -subunit (Agnew *et al.*, 1980). There are a number of distinct  $\alpha$  subunits (Suzuki *et al.*, 1988; Gautron *et al.*, 1992), and additional diversity is added by splice variants (Mandel 1992). Brain and muscle voltage-gated sodium channels also contain one to two smaller subunits (Hartshorne and Catterall, 1981; Barchi, 1983). The  $\beta 1$ - (36kD) and  $\beta 2$ - (33kD) subunits are found in the brain and the  $\beta 3$ - (38kD) subunit is found in muscle. The stoichiometry of channels expressed in the brain appears to be  $\alpha\beta 1\beta 2$ , with the  $\beta 2$ -subunit covalently bound to the  $\alpha$ -subunit through a disulphide bridge (Hartshorne *et al.*, 1982). Complementary DNA sequences have been identified for the  $\text{Na}^+$  channel  $\alpha$ -subunit from eel electroplax, rat brain, skeletal muscle and heart (Noda *et al.*, 1984, 1986a; Kayano *et al.*, 1988; Trimmer *et al.*, 1989; Auld *et al.*, 1988; Kallen, *et al.*, 1990; Rogart *et al.*, 1989). From the cDNA sequences the predicted topology of the  $\alpha$ -subunit resembles the topology described for the  $\text{Ca}^{2+}$  channel  $\alpha$ -subunit, which is composed of four homologous domains with six proposed transmembrane segments per domain. Expression of the sodium channel  $\alpha$ -subunit in *Xenopus* oocytes results in functional channels, but inactivation rates and gating properties are different from those seen *in vivo* (Trimmer *et al.*, 1989; Auld *et al.*, 1988; Goldin *et al.*, 1986; Noda *et al.*, 1986b). Transient expression of  $\alpha$ -subunits in mammalian cells gives rise to sodium channels with normal physiological properties and normal toxin sensitivity (Catterall, Seagar, Takahashi, 1988). These channels can also be phosphorylated by protein kinase A and C (Li *et al.*, 1992; Numann *et al.*, 1991) leading to functional modulation.

### 1.2.4 Voltage-Gated Potassium Channels

Four complementary deoxyribonucleic acid (cDNA) sequences of outwardly-rectifying potassium channel subunits have been identified in *Drosophila* and encode channels with unique activation and inactivation kinetics (*Shal*, *Shab*, *Shaker* and *Shaw*) (Butler, *et al.*, 1989; Wei, *et al.*, 1990; Papazian, *et al.*, 1987; Pongs, *et al.*, 1988; Kamb, Tseng-Crank and Tanouye, 1988). Mammalian homologues of these *Drosophila* genes have been identified, as well as subunit variants (Pak, *et al.*, 1991a, 1991b; Frech, *et al.*, 1989; Yokoyama, *et al.*, 1989; Roberds and Tamkun, 1991). Diversification and related plasticity within a subfamily of potassium channels is best described in the *Shaker* potassium channel gene family in vertebrates and invertebrates. To date there have been 12 different mammalian *Shaker* subfamily genes identified, suggesting diversification through gene duplication (Rudy *et al.*, 1991; Jan and Jan, 1990). In contrast, only one *Shaker Drosophila* gene has been cloned. However, the *Drosophila Shaker* mRNA can undergo multiple alternative-splice events, thus generating a number of gene products (Salkoff, *et al.*, 1992). Like sodium and calcium channels these  $\alpha$  subunits can express functional channels *in vitro*. Recently an auxiliary potassium channel subunit has been cloned, the  $\beta$  subunit (Scott *et al.*, 1994). Three  $\beta$  subunits have been isolated to date (Scott *et al.*, 1994; Morales *et al.*, 1995).

The topology of the potassium channel is outlined in Figure 1.2. The receptor subunit has 6 predicted transmembrane domains, which span the lipid bilayer. The potassium channel is more elementary than the sodium or calcium channels, and may be representative of an ancestral voltage-gated channel from which these three channels derive (Hille, 1992).  $\alpha$  subunits can form ion conducting pores but require the combination of four  $\alpha$  subunits in a tetrameric oligomer. The  $\beta$  subunit is a cytoplasmic protein that copurifies with pore-forming subunits. Some *Shaker*-type potassium channels have a unique inactivation mechanism (ball and chain), which has been localized to the first twenty amino acids at the 5'-terminus, through deletion mutagenesis analysis and peptide replacement experiments (Hoshi, Zagotta and Aldrich, 1990). An alternative method of inactivation involves the  $\beta$  subunit (Rettig *et al.*, 1994). The presence of  $\beta$  subunits can convert non-inactivating  $\alpha$  subunits into inactivating subunits. The hyperkinetic locus in *Drosophila* codes for a potassium channel  $\beta$  subunit (Chouinard *et al.*, 1995).



**Figure 1.2 Membrane topology of a subunit of the Voltage-gated Potassium Channel**

The potassium channel receptor subunit has six putative transmembrane spanning domains represented by cylinders (S1-6). The receptor has sites for glycosylation and neurotoxin binding on its extracellular surface and can be phosphorylated by protein kinases on its intracellular face. The S4 transmembrane domain contains positively charged amino acids and acts as the voltage sensor to detect changes in transmembrane electrical potential, which when detected result in a conformational change of the channel to the active, ion conducting state. The H5 region lines the channel or pore when subunits are in a tetraoligomeric structure.

### 1.3 SYNAPTIC EXCITATION AND INHIBITION IN THE CENTRAL NERVOUS SYSTEM

#### 1.3.1 Synaptic Excitation

Rapid excitatory synaptic transmission within the central nervous system is primarily mediated by acetylcholine and glutamate-activated receptors. Excitation results in the depolarization of the membrane and may lead to neuronal firing.

#### 1.3.2 Acetylcholine Receptor

The nicotinic acetylcholine receptor can be divided into two classes based on localization and subunit composition. These are: 1) the muscle acetylcholine receptors (AChRs) found at the neuromuscular junction and 2) the neuronal AChRs which are localized in the peripheral and central nervous systems. The mammalian muscle AChRs are transmembrane glycoproteins of 290 kD, that are composed of four different subunits ( $\alpha$ ,  $\beta$ ,  $\gamma/\epsilon$ ,  $\delta$ ) stoichiometrically arranged as either  $\alpha 2\beta\gamma\delta$ , in the case of the mammalian embryonic skeletal muscle or  $\alpha 2\beta\epsilon\delta$  as in the adult skeletal muscle (Noda *et al.*, 1983; Tanabe *et al.*, 1984; Takai *et al.*, 1984; Kubo *et al.*, 1985; Shibahara *et al.*, 1985; LaPolla, Mayne and Davidson, 1984; Boulter *et al.*, 1985). It is also possible that naturally occurring receptors which characterize distinct channel classes in embryonic muscle may be formed from only three different subunits, where the  $\gamma$  subunit is absent from the complex (Charnet, Labarca, and Lester, 1992, Liu and Brehm, 1993). Alternative-splicing of the human muscle AChR  $\alpha$  subunit mRNA generates two isoforms which differ by an additional exon of 25 base pairs, within the proposed extracellular domain (Beeson *et al.*, 1990). In the adult muscle, the AChR is present predominantly at the neuromuscular

junction, whereas in fetal muscle, as well as in denervated adult muscle, the AChR is also found in the non-junctional membrane (Mishina *et al.*, 1986). The junctional and non-junctional types of AChR channels differ in conductance and gating properties and these differences are attributable to the differences between the primary sequences of the  $\gamma$  and  $\epsilon$  subunits (Mishina *et al.*, 1986).

The small area of a mature muscle fibre that is contacted by motor nerve terminals is highly specialized. The hallmark of the synaptic region is the cluster of AChRs organized along the tops of the junctional folds at a packing density of about 10,000 receptors per square micrometer of membrane (Fertuck and Salpeter, 1976). Suppression of extrajunctional AChR subunit mRNA expression during development of the neuromuscular junction is regulated by muscle activity induced by the innervating axon (Klarsfeld and Changeux, 1985; Goldman, Brenner, and Heinemann, 1988). The gene encoding the  $\epsilon$  subunit of adult-type receptors, however, is unique in that it is induced in endplate-associated nuclei as a result of muscle innervation (Brenner, Witzemann and Sakmann, 1990), but is not regulated by muscle activity. In this situation it has been proposed that the basal lamina serves as a type of memory for the muscle, conferring nerve-dependent properties on a noninnervated fiber (Goldman, Carlson and Staple, 1991). The  $\epsilon$  subunit also plays a key role in the assembly of the AChR complex. Chimaeric  $\beta$  receptor subunits in which the N-terminal and C-terminal extracellular domains are derived from the  $\epsilon$  subunit have demonstrated that a heterodimer with an  $\alpha$  subunit, which is part of the pathway for subunit assembly, could only form when an extracellular domain of the  $\epsilon$  subunit was present (Yu and Hall, 1991).

The muscle AChR from *Torpedo* is the only hetero-oligomeric ion channel receptor where diffraction electron microscopy has successfully resolved a 0.9 nm structure (Unwin, 1993, Unwin, 1995). This structure is consistent with a quasi-symmetrical arrangement of the five subunits around the central channel. Three rods, presumed to be  $\alpha$ -helices, are evident in the extracellular domains of each subunit. The three helices in each  $\alpha$ -subunit were hypothesized to form an ACh-binding site. A single, kinked rod is seen in the membrane-spanning domain of each subunit and is hypothesized to be a kinked  $\alpha$ -helix formed by the M2 transmembrane domain.

Neuronal nAChRs are either homologous (as demonstrated in the expression of the  $\alpha 7$  subunit in *Xenopus* oocytes) or heterologous pentamers, with a predicted conformation of  $\alpha 2\beta 3$  (Cooper, Couturier, and Ballivet, 1991). To date eight  $\alpha$  and three  $\beta$  neuronal subunit cDNAs have been cloned and sequenced, and through *in situ* hybridization shown to have complex overlapping patterns of expression (reviewed by Deneris *et al.*, 1991; Sargent 1993; McGehee and Role, 1995). The  $\alpha 4\beta 2$  combination is the most common in the mammalian central nervous system (Sargent 1993). Although, the neuronal AChR  $\alpha$  and  $\beta$  subunit polypeptides show 40 to 50 percent sequence identity to the muscle AChR

subunit polypeptides (Boulter *et al.*, 1990), the two types of receptor display different pharmacological and functional properties (Reviewed by Deneris *et al.*, 1991 and Léna and Changeux, 1993), the most significant being the differences in  $\alpha$ -bungarotoxin binding. Recently isolated neuronal subunits  $\alpha 7$ ,  $\alpha 8$  and  $\alpha 9$  do however show  $\alpha$ -bungarotoxin sensitivity. Mutagenesis studies have identified key residues important in the binding of  $\alpha$ -bungarotoxin as prolines 194 and 197 and aromatic residues at 187-189 (Kachalsky *et al.*, 1995).

### 1.3.3 Glutamate Receptor

Glutamate mediates fast excitatory neurotransmission by activating cation-selective channels with distinct gating kinetics, ionic permeabilities and conductances, and pharmacological properties (Wisden and Seeburg, 1993). The vertebrate glutamate receptors have been classified into three general groups: 1) AMPA/kainate receptors, which are transmitter-gated, ionotropic ion channels; 2) the NMDA receptor, another ionotropic receptor type, which has multiple and specific allosteric modulatory sites and 3) the metabotropic glutamate receptor, a G-protein coupled receptor, which can be activated by glutamate and quisqualate to stimulate inositol phospholipid metabolism (Monaghan, Bridge and Cotman, 1989) (reviewed by Barnard, 1992). Cloning of the corresponding cDNA for the metabotropic glutamate receptor (Masu *et al.*, 1991) has demonstrated that it is a single polypeptide with seven hydrophobic transmembrane domains and with no structural relationship to the ionotropic glutamate receptors.

A different class of glutamate-binding proteins was isolated from the CNS of the frog *Rana pipiens* (Hampson and Wenthold, 1988) and from chicken cerebellum (Gregor *et al.*, 1989). The cDNAs (Wada *et al.*, 1989; Gregor *et al.*, 1989) encoding these proteins were isolated and expressed in transfected cells. The expressed protein could bind kainate, but was not functional in terms of ion conductance, and hence was referred to as a kainate binding protein. The kainate binding proteins share a 20 to 40 % identity with members of the ligand-gated ion channel superfamily. These receptors were originally proposed to maintain the four transmembrane topology of the family, but do not conserve the cysteine loop, M1 proline and M2 threonine/serine-rich domains characteristic of this family (family characteristics are discussed in the GABA section 1.4.5) (Barnard, 1992; Gasic and Heinemann, 1991). New evidence suggests that AMPA/kainate receptors may have a three transmembrane topology.

AMPA receptors, which consist of GluR -A to -D (GluR-1 to -4) subunits constitute a high affinity AMPA/low affinity kainate receptor type (Wisden and Seeburg, 1993; Gasic and Hollmann, 1992). In patch clamp experiments most native neurons exhibit AMPA receptor-mediated currents with linear or outwardly rectifying current-voltage relationships (Jonas and Sakmann, 1992). In a transient expression system, the native characteristics of

the AMPA-receptor mediated currents is only achieved if GluR-B is co-expressed with the GluR-A, -C or -D subunits, suggesting that the GluR-B subunit dominates the properties of ion flow and that most native AMPA receptors are hetero-oligomeric assemblies of these subunits (Jonas and Sakmann, 1992; Hollmann, Hartley and Heinemann, 1991; Verdoorn, *et al.*, 1991). The GluR-B transcript is thought to undergo an RNA editing event which changes the conserved glutamine found in the other receptors to an arginine at the QRN site and represents the first demonstration that RNA editing occurs in the brain (Sommer *et al.*, 1991). GluR-5 and GluR-6 are subject to RNA editing in the M2 transmembrane domain and in the case of GluR-6 also at two sites in the M1 transmembrane domain (Sommer and Seeburg, 1992; Sommer *et al.*, 1991, Kohler *et al.*, 1993). In this case, however, conversion of an arginine(R) to glutamine(Q) in M2 leads to low calcium permeability. Mutations in homologous regions of M1 for AMPA receptors does not lead to changes in calcium permeability. RNA-editing at other sites in the AMPA receptor leads to changes in kinetic properties (Lomeli *et al.*, 1994).

GluR-A,-B,-C and -D subunit also exist in one of two molecular forms, that differ in the sequence of a segment preceding the most C-terminal transmembrane region. These segments arise through alternative use of two exonic sequences present in the primary transcripts of members of this gene family and are designated Flip and Flop (Sommer *et al.*, 1990). Another subfamily of excitatory amino acid receptors, the high-affinity kainate receptors can consist of KA-1 or -2 and/or GluR-5, -6 or -7 subunits. In a transient expression system, the homomeric GluR-5 (Q) channel, functionally resembles the native kainate receptor found on dorsal root ganglia (DRG) cells, (Sommer *et al.*, 1992; Huettner, 1990) suggesting that these subunits may occur naturally as homomeric proteins (Wisden and Seeburg, 1993).

Expression cloning and homology screening have identified five subunits of the NMDA receptor (NMDA-R1 and NMDA-R2A, -R2B, -R2C and -R2D) (Yamazaki, *et al.*, 1992; Monyer *et al.*, 1992; Meguro *et al.*, 1992; Kutsuwada *et al.*, 1992; Ikeda *et al.*, 1992). NR1 mRNA is found in virtually every neuron, and hence it has been suggested that this subunit should be viewed as a common constituent of all NMDA receptor channels (Wisden and Seeburg, 1993). The NR1 subunit also has at least seven distinct splice forms which are derived from alternative-splicing events in the large amino-terminal extracellular domain, and in the amino-terminal hydrophilic moiety (Anantharam *et al.*, 1992; Durand *et al.*, 1992; Hollmann *et al.*, 1993; Nakanishi, Axel, and Schneider, 1992; Sugihara *et al.*, 1992). Subunit switching, as in the case of the muscle nicotinic acetylcholine receptor may also play a role in the progressive changes in the properties of NMDA receptor channels in the developing nervous system (Carmignoto and Vicini, 1992; Hestrin, 1992; Laurie & Seeburg 1994).

## 1.4 SYNAPTIC INHIBITION

Synaptic inhibition in the central nervous system of vertebrates is primarily mediated by ligand- and voltage-gated K<sup>+</sup> channels, and two amino acid neurotransmitter gated chloride channels, the GABA and glycine receptors (Krnjevic, 1974). Glycine is the major inhibitory neurotransmitter in spinal cord and brain stem, whereas GABA predominates in other parts of the brain. Binding of these two amino acids to specific ligand-gated receptors increases the chloride conductance of the post synaptic membrane, and thus produces hyperpolarization and hence inhibition of neuronal firing (Coombs, Eccles and Fatt, 1955). GABA can also activate a G-protein coupled GABA<sub>B</sub> receptor that is linked to a potassium channel (Bowery, 1989).

### 1.4.1 Glycine Receptors

The convulsive alkaloid strychnine selectively antagonizes glycine, but not GABA-mediated inhibitory responses (Aprison, Lipkowitz and Simon, 1987). Glycine-displaceable <sup>3</sup>H-strychnine binding has been widely used to investigate and localize the glycine receptor in different regions of the CNS (Young and Snyder, 1973; Zarbin, Wamsley and Kuhar, 1981; Probst, Cortes and Palacios, 1986). From <sup>3</sup>H-strychnine binding data, glycine receptor deficiencies have been implicated in the pathogenesis of spasticity (Hall *et al.*, 1979; White and Heller, 1982; Becker *et al.*, 1992) and the loss of motor control. Recently mutations in the glycine receptor have been implicated in spasticity (Kingsmore *et al.* 1994; Ryan *et al.*, 1994).

The glycine receptor has been purified from the spinal cord of various mammalian species by affinity chromatography on aminostychnine-agarose, and shown to be a large glycoprotein containing polypeptides of relative molecular masses (Mr) 48,000 (48 kD), 58,000 (58 kD), and 93,000 (93 kD). The cDNA sequences of four  $\alpha$  subunits (48 kD) and one  $\beta$  (58 kD) glycine receptor subunit have been determined (Grenningloh *et al.*, 1987, 1990; Langosch, Becker and Betz, 1990), and share from 47 percent to 79 percent identity with each other. The 93 kD polypeptide is a peripheral membrane protein, which is not glycosylated and which by immuno-electron microscopy is seen to be cytoplasmic and separate from the receptor/channel structure. Photo-affinity labeling demonstrated that the  $\alpha$  subunit contained the strychnine binding site (Graham, Pfeiffer and Betz, 1983), and from antibody immunoprecipitation of the native receptor complex, a pentameric structure of  $\alpha_3\beta_2$  has been proposed (Langosch, Becker and Betz, 1990).

Homo-oligomer glycine-activated chloride channels are produced when either  $\beta$  or  $\alpha$  subunits alone are expressed in *Xenopus* oocytes (Grenningloh *et al.*, 1990). An expressed  $\alpha\beta$  combination shows no difference in function from  $\alpha$  alone (Grenningloh *et al.*, 1990), although the  $\beta$  subunit transcript is more widely distributed in the adult nervous system, and during development than the mRNA for any of the  $\alpha$  isoforms, suggesting that a

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constant  $\beta$  is combined alone (strychnine-insensitive, glycine-gated) or with one or more  $\alpha$  subunits (strychnine-sensitive, glycine-gated) to form a receptor complex. A strychnine-insensitive glycine binding site also exists on the NMDA receptor and must be occupied by glycine to enable activation of the complex by NMDA.

The expression of genes encoding the  $\alpha$  subunits is both regionally and developmentally regulated.  $\alpha 1$  glycine receptor subunit mRNA is present in the adult CNS, whereas the  $\alpha 3$  glycine receptor subunit transcript is present predominantly, in the CNS, during postnatal development (Becker, Hoch and Betz, 1988; Kuhse, Schmieden, and Betz, 1990a).  $\alpha 2$  glycine receptor subunit mRNAs are the principal glycine receptor subunit transcripts present during embryonic CNS development (Kuhse, Schmieden, and Betz, 1990b). Further glycine receptor subtype diversity, in the CNS, occurs through alternative splicing of glycine receptor  $\alpha 1$ - and  $\alpha 2$ -subunit transcripts (Kuhse *et al.*, 1991; Malosio *et al.*, 1991). Two  $\alpha 1$ - subunit transcripts are generated by the use of alternative 3' acceptor splice sites within the proposed intracellular loop (Malosio *et al.*, 1991). The longer transcript, designated  $\alpha 1^{ins}$ , has 8 additional amino acids that encode a potential phosphorylation site. Preliminary experiments have shown that both subunit variants are expressed in similar regions of the nervous system, but whether they are contained within the same receptor complex is not known (Malosio *et al.*, 1991). Alternative splicing of the glycine receptor  $\alpha 2$  subunit ( $\alpha 2$  and  $\alpha 2^*$ ) occurs by alternative exon usage (Kuhse *et al.*, 1991). The two alternative exons both encode 22 amino acids, of which 20 are identical, within the putative extracellular loop region. The function of these two polypeptides is unclear although it has been suggested that these variants may play a role in synaptogenesis (Kuhse *et al.*, 1991) and in the immunity of neonatal rats against strychnine poisoning. *In vitro* mutagenesis has demonstrated that a single amino acid change from a glycine to glutamate residue results in the low affinity binding of strychnine to the  $\alpha 2^*$  variant (Kuhse, Schmieden, and Betz, 1990b).

## 1.4.2 GABA Receptors

The amino acid GABA acid is the major inhibitory neurotransmitter in the vertebrate central nervous system, and is present at 30 percent of all synapses (Bloom and Iversen, 1971). The brain content of GABA is also 200- to 1000-fold greater than that of other neurotransmitters such as dopamine, noradrenaline, ACh, and serotonin. As such, the GABA neuronal network is probably the largest in the central nervous system (Squires, 1988). Receptors that bind GABA can be divided into three categories, GABA<sub>A</sub>, GABA<sub>B</sub> and GABA<sub>C</sub>, based on pharmacological distinctions (Table 1.2). The GABA<sub>A</sub> receptor complex forms an ion channel permeable to both chloride and to a lesser extent bicarbonate anions (Bormann, Hamill, and Sakmann 1987; Kaila and Voipio, 1987). The GABA<sub>A</sub> receptor complex has binding sites for at least five different classes of



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pharmacological agent, including bicuculline (BIC), a competitive antagonist of GABA (reviewed by Olsen and Venter, 1986; Stephenson, 1988). The GABA<sub>A</sub> receptor is insensitive to baclofen (BAC), an analogue of GABA, active at the GABA<sub>B</sub> receptor (Enna, 1988).

A second receptor type, termed the GABA<sub>B</sub> receptor, although widespread, is generally less abundant than the GABA<sub>A</sub> type in mammalian brain (Bowery, Hudson and Price, 1987) and appears to be coupled to Ca<sup>2+</sup> and K<sup>+</sup> channel conductances (reviewed by Bowery, 1989). GABA<sub>B</sub> receptors are insensitive to bicuculline, selectively activated by baclofen (Hill and Bowery, 1981) and are thought to be members of the G-protein coupled, seven transmembrane domain, receptor superfamily. Whereas GABA<sub>A</sub> receptors are thought to be predominantly postsynaptic, GABA<sub>B</sub> receptors are found both pre- and post-synaptically (reviewed by Sivilotti and Nistri, 1991) and appear to be involved with presynaptic events such as neurotransmitter release (Curits *et al.*, 1970; Enna and Snyder, 1977). In the lethargic mouse model of absence epilepsy, an increased number of GABA<sub>B</sub> binding sites have been observed suggesting that GABA<sub>B</sub> receptors may play a role in the expression of absence seizures in this animal model (Lin, Cao, and Hosford, 1993). Recently, Kaupmann *et al.*, (1997) have reported the expression cloning of a GABA<sub>B</sub> receptor cDNA that shares sequence similarity with metabotropic glutamate receptor cDNAs.

An additional class of GABA receptor sites, which is both bicuculline- and baclofen-insensitive, has been suggested from binding studies (Drew, Johnston and Weatherby, 1984; Drew and Johnston, 1992). Electrophysiological experiments have shown that this third class, denoted GABA<sub>C</sub>, is present in rodent retina, frog optic tectum and homologous regions of the guinea pig brain. Like the GABA<sub>A</sub> receptor, the GABA responses mediated by this receptor appear to be dependent on extracellular Cl<sup>-</sup> (Sivilotti and Nistri, 1991).

#### 1.4.2.1 GABA<sub>A</sub> Receptors

The GABA<sub>A</sub> receptor has binding sites for several pharmacological agents (for reviews see Olsen and Venter, 1986; Schumacher and McEwen, 1989; Sivilotti and Nistri, 1991), and their actions and clinical use reflect the importance of inhibitory pathways for normal function. In addition to the recognition site for GABA, the natural ligand, the receptor has a number of modulatory binding sites for the anxiolytic benzodiazepines (Enna, 1988), the convulsant channel blockers picrotoxin (Olsen, 1982) and t-butyl bicyclophosphorothionate (TBPS) (Squires *et al.*, 1983), the anti-convulsant barbiturates, such as pentobarbital (Barnard, 1988) and steroids (Callachan *et al.*, 1987). Each of these sites behaves in an allosteric manner and hence as a consequence, binding at one site will affect the binding affinity at another. However, not all GABA<sub>A</sub> receptors appear to exhibit

all the modulatory sites (reviewed in Schumacher and McEwen, 1989; and Sivilotti and Nistri, 1991).

Receptor	GABA <sub>A</sub> - competitive site	GABA <sub>A</sub> - benzodiazepine modulatory site	GABA <sub>B</sub>	GABA <sub>C</sub>
Selective Agonist	isoguvacine muscimol GABA	flunitrazepam zolpidem CGS9896	L-baclofen 3-aminopropyl -phosphinic acid GABA	muscimol GABA cis-4- aminoacro- tonic acid
Inverse Agonist	N/A	DMCM	N/A	N/A
Selective Antagonist	bicuculline SR95531	flumazenil (Ro 15- 1788)	saclofen CGP35348 2-hydroxy -saclofen	
Radio-ligand	[ <sup>3</sup> H]muscimol [ <sup>3</sup> H]SR95531	[ <sup>3</sup> H]flunitrazepam [ <sup>3</sup> H]zolpidem [ <sup>3</sup> H]flumazenil	[3H]L-baclofen [3H] 3-amino- propyl-phosphinic acid	
Channel Blocker	TBPS Picrotoxin	TBPS Picrotoxin		
Subunit Involvement	α, β	α, γ	G protein-coupled	ρ1,2
Effector Pathway	integral membrane Cl <sup>-</sup> channel	facilitates GABA <sub>A</sub> -gated Cl <sup>-</sup> channel opening	cAMP +/- K <sup>+</sup> channel + Ca <sup>2+</sup> channel -	

**Table 1.2 GABA Receptor Pharmacology**

*GABA receptors can be divided into 3 groups based on pharmacological characterization.*

### 1.4.2 GABA<sub>A</sub> Pharmacology

Agonist binding to the GABA<sub>A</sub> receptor increases the neuronal membrane conductance to chloride ions, generally resulting in membrane hyperpolarization and reduced neuronal excitability, a phenomenon termed synaptic inhibition. Ligand binding studies of GABA and GABA receptor agonists have described at least two distinct binding sites differing in

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affinity for the agonist ligand (reviewed in Sivilotti and Nistri, 1991). The high affinity binding site has an equilibrium constant (kD) between 3 to 30 nM while the kD of the low affinity site ranges from 100 to 300 nM. GABA binding sites from different brain regions, different mammalian species, and animals of different ages all show this heterogeneity in affinity. It was believed that these sites represent different affinities of the ligands to different conformational states of the receptor (de Blas, Vitorica, and Friedrich, 1988; Olsen *et al.*, 1984). Since it is known that micromolar concentrations of GABA are required to produce physiological responses (Barker and Mathers, 1981; Segal and Barker, 1984), this would correspond to activation of the channel by the binding of GABA to the low affinity site. Moreover, the concentration of GABA in the brain is such that all the high affinity sites are occupied in the resting state. The high affinity state may correspond to a desensitized or inactive form of the receptor (Agey and Dunn, 1989), as has been observed for the nicotinic AChR (Changeux, Devillers-Thiery and Chemouilli, 1984).

Benzodiazepine agonists bind at a location removed from the GABA binding site and potentiate the GABA induced neuronal inhibition by increasing the frequency of channel opening (MacDonald and Barker, 1977). Benzodiazepine agonist binding to the benzodiazepine sites modulates the interaction of GABA agonists at the GABA site by increasing the affinity of the GABA binding site for agonists (Tallman *et al.*, 1978). The binding of the benzodiazepine antagonist Ro 15-1788 (flumazenil) is not affected by GABA (Möhler and Richards, 1981). Inverse agonists (imidazopyridines, triazolopyridazines and  $\beta$ -carbolines) have revealed a heterogeneity of benzodiazepine binding. In general, BZI sites bind  $\beta$ -carbolines and CL218872, and predominate in the cerebellum, while BZII sites show little affinity for these drugs (reviewed by Doble and Martin, 1992), and are found primarily in the cortex, hippocampus and spinal cord (Braestrup and Nielsen, 1981). Recently it has been postulated that this pharmacological classification of benzodiazepine binding is an oversimplification and that at least three subtypes are required to explain the GABA-benzodiazepine interactions *in vitro*. The benzodiazepine Ro 5-4868 binds to a distinct class of receptor site in the kidney, and other tissues, including the brain. Interestingly, a peripheral benzodiazepine receptor (PKBS) has been cloned (Sprenkel, Werner, and Seeburg 1989), although the physiological importance of these sites is unknown. There also exists a population of GABA<sub>A</sub> receptors that lack benzodiazepine binding sites (Unnerstall *et al.*, 1981) and yet are immunologically similar to benzodiazepine-coupled GABA<sub>A</sub> receptors (de Blas, Vitorica, and Friedrich, 1988).

Recently a binding site for the benzodiazepine GYKI 52466 has been found on an AMPA-type glutamate receptor (Zorumski *et al.*, 1993). This benzodiazepine has muscle relaxant and anticonvulsant properties not attributable to a GABA<sub>A</sub> receptor-mediated mechanism. Zorumski and coworkers demonstrated that GYKI 52466 acts

noncompetitively to block ion currents and associated excitotoxicity, including ischemic neuronal degeneration, mediated through AMPA receptors. This inhibition is antagonized by drugs that inhibit AMPA receptor desensitization and further demonstrates that not all benzodiazepine receptors are GABA<sub>A</sub> receptors.

Barbiturates modify the binding of GABA agonists to the GABA<sub>A</sub> receptor causing increased mean channel open time (Study and Barker, 1981) and increasing the maximal number of binding sites ( $B_{max}$ ) for GABA (Schumacher and McEwen, 1989). Barbiturates have further been shown to increase the affinity of benzodiazepine agonists and reduce the binding of inverse agonists at the benzodiazepine site (Wong *et al.*, 1984). Inverse agonists show a lower affinity in the presence of GABA (Braestrup and Nielsen, 1981), antagonise the sedative effects of the benzodiazepines and act as convulsants.

Picrotoxin and TBPS are competitive inhibitors of one another (Lawrence and Casida, 1983) and the binding of these channel blockers can inhibit and be inhibited by barbiturate binding (Squires *et al.*, 1983). TBPS binding is additionally inhibited by GABA, in the presence of chloride ions (Abel, Blume, and Garrett, 1989), and steroids (Harrison *et al.*, 1987).

Steroid hormones such as oestrogens, progesterones, corticosteroids, androgens (reviewed by Schumacher and McEwen, 1989) and neurosteroids (Puia *et al.*, 1990) modulate GABA<sub>A</sub> receptor neurotransmission by increasing the affinity of GABA agonists for the GABA site (Harrison *et al.*, 1987). Steroids prolong the duration of channel open time in a manner similar to barbiturates. Neuroactive steroids are natural or synthetic steroids that rapidly alter the excitability of neurons and can have positive or negative modulatory effects on GABA<sub>A</sub>-activated chloride conductance. Neurosteroids are classified as agonists, such as allopregnanolone and alfaxalone, or antagonists such as sulphate esters of pregnenolone (Paul and Purdy, 1992).

### 1.4.3 Biochemistry of the GABA<sub>A</sub> Receptor

The GABA<sub>A</sub> receptor protein has been solubilized from bovine cerebral cortex in the zwitterionic derivative of cholic acid, CHAPS (Stephenson, Watkins, and Olsen 1982) and purified on a benzodiazepine affinity column (Schofield *et al.*, 1987; reviewed by Stephenson, 1988). The purified complex had both high and low affinity sites for GABA and binding sites for benzodiazepines. When the GABA receptor protein was analyzed by SDS-PAGE (polyacrylamide gel electrophoresis), two polypeptide bands of 53kDa ( $\alpha$ ) and 57kDa ( $\beta$ ) were observed (Sigel *et al.*, 1983; Sigel and Barnard, 1984). The mobility shifts observed after endoglycosidase F treatment and SDS-PAGE analysis (Mamalaki, Stephenson, and Barnard, 1987) indicated that both the  $\alpha$  and  $\beta$  subunits were glycosylated. In membrane preparations, [<sup>3</sup>H] flunitrazepam specifically photoaffinity labels polypeptides of 50kDa (Möhler, Battersby and Richards, 1980; Sieghart and

Karobath, 1980). In the purified receptor preparations, it is the  $\alpha$ -subunit that is specifically photoaffinity-labelled with flunitrazepam and is thus considered to carry the benzodiazepine binding site (Stephenson *et al.*, 1986). However, in experiments where higher concentrations of flunitrazepam were used, some incorporation of radioactivity was also found in the  $\beta$  subunit of purified bovine and porcine cerebral cortical receptors (Sigel *et al.*, 1983; Kirkness and Turner, 1986). [ $^3\text{H}$ ]muscimol photoaffinity labels a polypeptide from rat cerebellar membrane preparations with an apparent molecular size similar to that photolabelled by flunitrazepam (Cavalla and Neff, 1985). Photoaffinity labelling studies with [ $^3\text{H}$ ] muscimol on purified GABA<sub>A</sub> receptor preparations, however, have shown that it is the  $\beta$ -subunit that is specifically labeled with [ $^3\text{H}$ ] muscimol (Casalotti, Stephenson, and Barnard, 1986; Deng, Ransom, and Olsen 1986). As reviewed by Stephenson in 1988, this ambiguity may have arisen due to the slight (4kDa) difference between the  $\alpha$  and  $\beta$  polypeptides, and the sometimes poor resolution of these bands by SDS-PAGE analysis. It was in part this poor resolution by SDS-PAGE of large polypeptides with small differences in molecular size, which resulted in the initial observation that the GABA<sub>A</sub> receptor was composed of only two types of subunits. Later studies which incorporated higher resolution SDS-PAGE revealed that these two bands could be resolved further (Sieghart, 1989; Fuchs and Sieghart, 1989), displayed differing affinities for various ligands (Fuchs, Möhler, and Sieghart, 1988), and could be detected in different brain regions or developmental stages.

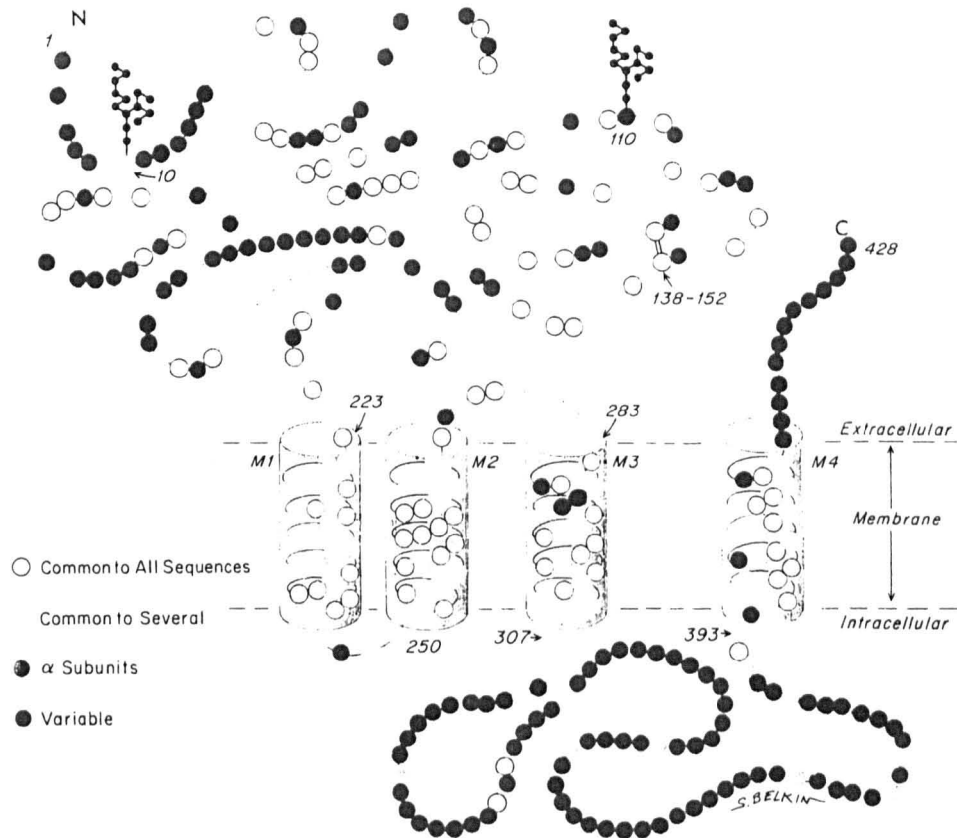
Full and partial purification of the GABA<sub>A</sub> receptor enabled the production of monoclonal and polyclonal antibodies (Schoch *et al.*, 1985; Haring *et al.*, 1985; Stephenson *et al.*, 1986; Mamalaki, Stephenson, and Barnard, 1987). Monoclonal antibodies raised against a partially purified GABA<sub>A</sub> receptor from bovine cerebral cortex (Schoch *et al.*, 1985) distinguished four epitopes of the receptor which were defined by a combination of immunoblotting, species specificity and competition in a solid phase antibody binding assay (Haring *et al.*, 1985). In particular, western blot analysis indicated that monoclonal antibody bd-17 was  $\beta$  subunit-specific and monoclonal antibody bd-24 was  $\alpha$  subunit-specific. Both bd-17 and bd-24 co-immunoprecipitated [ $^3\text{H}$ ]muscimol and [ $^3\text{H}$ ]flunitrazepam binding sites from partially purified receptor preparations, thus demonstrating that the  $\alpha$  and  $\beta$  subunit were integral to the same oligomeric structure (Schoch *et al.*, 1985). Antibody cross-reactivity between human and bovine brain preparations was demonstrated and generated identical patterns of distribution throughout the central nervous system (Richards, Möhler and Haefly, 1986; Richards *et al.*, 1987; Schoch *et al.*, 1985). These observations suggested that the GABA<sub>A</sub> receptor population was homogeneous, however on comparison of the bd17 and [ $^3\text{H}$ ]muscimol binding and [ $^3\text{H}$ ]Ro 15-1788 labeling in the rat brain, areas of the thalamus and cerebellum showed an inverse correlation between antibody and benzodiazepine labelling (Schoch *et al.*, 1985;

Richards, Möhler and Haefly, 1986; Richards et al., 1987). Antibody bd-28, which can recognize  $\alpha$ ,  $\beta$  and  $\gamma$  polypeptides, labeled three polypeptides of 51, 53 and 59 kDa on western blots of [ $^3\text{H}$ ]flunitrazepam photolabeled purified receptors, suggesting that bd-28 recognized at least three different  $\alpha$  polypeptides, which shared a common epitope (Fuchs, Möhler and Sieghart, 1988). Further studies with bd-17 also found that the antibody could recognize three distinct polypeptides of 51, 53 and 55 kDa, two of which were previously labeled with bd-28. Taken together, these results indicated a limited biochemical/molecular heterogeneity consistent with the previously described pharmacological heterogeneity.

#### 1.4.4 Isolation of GABA<sub>A</sub> Receptor cDNAs

The GABA receptor was purified using a benzodiazepine affinity column, and cleaved with cyanogen bromide. The HPLC-isolated peptide fragments were then gas-phase microsequenced, and the resulting amino acid sequences were used to design synthetic oligonucleotide probes. Bovine brain and calf cerebral cortex cDNA libraries were screened with these probes, resulting in the isolation of cDNAs encoding an  $\alpha$  and a  $\beta$  subunit (Schofield *et al.*, 1987). The deduced amino acid sequences shared 35% sequence identity and their predicted hydropathy profiles exhibited similarities with the proposed topologies of the nicotinic acetylcholine and glycine receptors, suggesting a common evolutionary origin. This shared sequence and organizational identity indicated that the GABA<sub>A</sub> receptor was part of a ligand-gated ion channel superfamily (Barnard, Darlison, and Seeburg, 1987; Grenningloh *et al.*, 1987; Schofield *et al.*, 1987).

The sequence identity shared between the  $\alpha$  and  $\beta$  subunits of the GABA<sub>A</sub> receptor, and the concurrent application of molecular biology to the study of the vertebrate brain GABA<sub>A</sub> receptor, has resulted in a rapid increase in the identification of a number of different subunits and subunit isoforms. Degenerate oligonucleotides and GABA<sub>A</sub> receptor subunit cDNAs have been used as probes to identify new and species-related sequences. As a result of these experiments, several distinct subunit classes ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ), and subunit isoforms ( $\alpha 1-6$ ,  $\beta 1-4$ ,  $\gamma 1-4$ ,  $\delta 1$ ,  $\rho 1-3$ ,  $\epsilon 1$  and  $\tau 1$ ) of the vertebrate GABA<sub>A</sub> receptor have been identified (Bateson *et al.*, 1990, 1991a; Bateson, Lasham and Darlison, 1991; Garrett *et al.*, 1988; Glencorse, Bateson and Darlison, 1990, 1991, 1992; Kato, 1990; Keir, Deitech and Sikela, 1991; Kirkness *et al.*, 1991; Kofuji *et al.*, 1991; Levitan *et al.*, 1988a; Lüddens *et al.*, 1990; Montpied *et al.*, 1989; Pritchett, Ludden and Seeburg, 1989; Pritchett and Seeburg, 1990; Schofield *et al.*, 1987, 1989; Sommer *et al.*, 1990a; Whiting, McKernan, Iversen 1990; Wilson-Shaw *et al.*, 1991; Ymer *et al.*, 1989a, b, 1990; Cutting *et al.*, 1991; Wang, T.L., Guggino, W.B. and Cutting, G.R., 1994; Ogurusu, T. and Shingai, R., 1996; Davies *et al.*, 1997; Hedblom and Kirkness, 1997).



**Figure 1.3 Schematic Model of the Proposed Secondary Structure of the Rat  $\alpha 1$  GABA<sub>A</sub> Receptor Subunit**

The amino acids are numbered according to Krestchatisky et al., 1989. The colour code indicates the identity shared among the  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 4$ ,  $\beta 1$ ,  $\beta 3$ ,  $\gamma 2S$ , and  $\delta$  rat GABA<sub>A</sub> receptor subunits. White represents an identity common to all the subunits mentioned above, gray represents an amino acid in a position common to several subunits, black indicates amino acid identity shared by only the alpha subunits, and red indicates the variable positions for amino acid identity among the subunit sequences. The four putative transmembrane domains (M1 to M4) are represented as  $\alpha$ -helices (for current views refer to Chapter 6). The NH<sub>2</sub>- and COOH- termini are shown as "N" and "C" respectively. N-linked glycosylation sites in the NH<sub>2</sub>-terminus are indicated by small branched black circles. (Reproduced from Olsen and Tobin, 1990)

The subunit rho with isoforms  $\rho 1$ ,  $\rho 2$  and  $\rho 3$ , is highly expressed in the human retina and shares 30 to 38 percent identity with other GABA<sub>A</sub> receptor subunits (Cutting *et al.*, 1991, 1992). Pharmacological characterization of the  $\rho$  class of receptors suggests a novel type of bicuculine-, baclofen-, and benzodiazepine-insensitive GABA receptor that might represent the GABA<sub>C</sub> (non-A, non-B) class (Cutting *et al.*, 1991; Shimada, Cutting and Uhl, 1992). Invertebrate forms of the GABA<sub>A</sub> receptor have been identified in *Lymnaea*, *Drosophila* and *C. elegans* (Harvey *et al.*, 1991; McIntire, Jorgensen and Horvitz, 1993).

#### 1.4.5 A Ligand-Gated Ion Channel Superfamily

As mentioned previously, the GABA<sub>A</sub> receptor subunits share topology and sequence similarities with the nicotinic AChR, 5-HT<sub>3</sub>, and glycine receptors, and a more limited identity with subunits of the AMPA/kainate glutamate receptor. Within the lipid bilayer the GABA<sub>A</sub> receptor subunit polypeptides, like the acetylcholine receptor subunit polypeptides, are believed to form a heteropentameric structure. Figure 1.3 outlines the predicted topology of the  $\alpha 1$  subunit polypeptide of the rat GABA<sub>A</sub> receptor. In common with other members of the superfamily, the GABA<sub>A</sub> receptor polypeptides contain a signal sequence, suggesting that the amino-terminus is likely to be extracellular. The mature subunits contain four hydrophobic sequences that are proposed to be membrane-spanning domains, termed M1-M4. The intracellular region between M3 and M4 is the area of greatest diversity among the GABA<sub>A</sub> receptor subunits. Consensus sequence sites for protein phosphorylation are located within the cytoplasmic domains of the  $\gamma 2$ , an  $\alpha$  and several  $\beta$  GABA<sub>A</sub> receptor subunits (Moss *et al.*, 1992; Whiting, McKernan, Iversen 1990; Sweetnam *et al.*, 1988; Browning *et al.*, 1990). The extended hydrophilic extracellular region features several potential sites for N-linked glycosylation, and a proposed loop structure formed by disulphide bonding between two cysteine residues. NMDA, AMPA and kainate receptors differ significantly from other members of the ligand-gated ion channel superfamily. The differences include, overall length, lack of dicysteine loop structure, absence of conserved M1 proline, and fewer threonine/serine residues in the M2 region.

#### 1.4.6 Further GABA<sub>A</sub> Receptor Subunit Diversification

Alternative-splicing of primary gene transcripts has been demonstrated for the human, bovine, chick, rat and mouse  $\gamma 2$  and the chick  $\beta 4$  GABA<sub>A</sub> receptor subunit mRNAs (Whiting, McKernan, Iversen, 1990; Kofuji *et al.*, 1991; Glencorse, Bateson and Darlison, 1992; Bateson, Lasham and Darlison, 1991). The GABA<sub>A</sub> receptor  $\gamma 2$ -subunit gene transcripts are alternatively spliced to generate two mRNAs that differ by the presence or absence of 24 base pairs. The subunits that are encoded by these mRNAs are termed  $\gamma 2L$  and  $\gamma 2S$ . Analysis of the gene structure of the  $\gamma 2$  subunit revealed that the 24 base pair



sequence is encoded in a separate exon contained within the cytoplasmic loop domain. The alternatively-expressed exon carries 8 amino acids (Leu-Leu-Arg-Met-Phe-Ser-Phe-Lys) which encode a consensus site for protein kinase C phosphorylation. At present it is unclear whether this exonic sequence acts as a modulatory site for the action of ethanol on the GABA<sub>A</sub> receptor complex (Wafford *et al.*, 1991; Sigel, Baur and Malherbe, 1993). The chick  $\beta$ 4-subunit variants are generated by the differential choice of 5'-donor splice sites in the  $\beta$ 4-subunit gene and differ by the presence ( $\beta$ 4') or absence ( $\beta$ 4) of four amino acids in the proposed intracellular loop (Bateson, Lasham and Darlison, 1991).

### 1.4.7 Subunit Homology

All GABA<sub>A</sub> receptor polypeptide sequences identified to date share sequence identity to each other and to other members of the superfamily (Barnard, Darlison, and Seeburg, 1987). The  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  subunit polypeptides exhibit about 30 to 45% sequence identity at the amino acid level (Figure 1.4), which is increased to 50% with the inclusion of conservative amino acid residue substitutions. The percentage identity between each of two of the four subunits is shown in Table 1.3.

	$\beta$ 1	$\gamma$ 2	$\delta$
$\alpha$ 1	33%	47%	29%
$\beta$ 1	-	34%	37%
$\gamma$ 2	-	-	35%

**Table 1.3. Percentage of amino acid sequence identity between the rat GABA<sub>A</sub> receptor  $\alpha$ 1-,  $\beta$ 1-,  $\gamma$ 2-, and  $\delta$ -subunit polypeptides.**

The percentage identities were determined using the alignment shown in Figure 1.4. The rat  $\alpha$ 1-subunit polypeptide is from Lolait *et al.*, (1989), the rat  $\beta$ 1-subunit polypeptide sequence is from Ymer *et al.*, (1989b) and the rat  $\gamma$ 2- and  $\delta$ - subunit polypeptide sequences are from Shivers *et al.*, (1989).

The percentage identity comparison reveals that  $\alpha$ - and  $\gamma$ -subunit sequences are more closely related to each other than either is to  $\beta$ - or  $\delta$ -subunit sequences. This may reflect the evolutionary origins of the genes encoding each of the subunits. It has been suggested that the members of the superfamily of ligand-gated ion channels originated from a common ancestor (Schofield *et al.*, 1987). Arbas *et al.*, (1991) proposed the ancestor of the superfamily to be a ligand-gated membrane ion channel which evolved preferences for acetylcholine, GABA and glycine. In support of this idea, other members of the superfamily, for example the glycine receptor  $\alpha$ 1 subunit (Grenningloh *et al.*, 1987), and the nicotinic AChR  $\alpha$ 1 subunit (Noda *et al.*, 1983), exhibit approximately 34% and 20%

**Figure 1.4**

***Figure 1.4 Amino acid sequence comparisons of rat GABA<sub>A</sub> receptor  $\alpha$ 1,  $\beta$ 1,  $\gamma$ 2 and  $\delta$  subunits.***

Alignment of the deduced amino acid sequences of the  $\alpha$ 1 (A1) (Lolait et al., 1989),  $\beta$ 1 (B1) (Ymer et al., 1989),  $\gamma$ 2 (G2) and  $\delta$  (D1) (Shivers et al., 1989) subunits was performed using the computer program MULTALIGN (Barton and Sternberg, 1987). Gaps are introduced into the sequences to maximise the alignment and negative numbers refer to signal peptide residues. Identical residues, at the same position in all four sequences, are highlighted. The proposed transmembrane domains (M1-M4) are indicated by solid lines above the sequences. The proposed disulphide bonded loop region is shown by the dashed line.

A1	-27	MKKSPGLSDYLWAWTLFLSTLTGRSYG
B1	-25	MWTVQNRESLGLLSFPVMIAMVCCA
G2	-38	MSSPNTWSTGSTVYSPVFSQKMTLWILLLLSLYPGFTS
D1	-16	MDVLGWLLPLLLLST
A1	1	QPSQDELKDNTTVFTR-----ILDRLDGIDNRLTPGLSERV
B1	1	HSANEPSNMSYVKET-----VDRLKGYDIRLRPDFGPP
G2	1	QKSDDDYEDYASNKTWVLTTPKVPEGDVTVILNNILEGYDNKLRPDICVKP
D1	1	QPHHGARAMNDIGDYVG---SNLEISWLPNLDGLMEGYARNFRPGIGPP
A1	38	TEVKTDIFVTSFGPVSDHDEETIDVFFRQSWKDEKFKFGPMTVTRNN
B1	36	VDVGMRIDVASIDMVSEVNDYDLTMYFQQSKKIKRLSYSGIPLNLTADN
G2	51	TLIHTDMYVNSIGPVNAINKEYLIDIFFAQTQYDRKFNSTIKVERLNS
D1	48	VNVALALEVASIDHISEANMEYEMTVFLHRAWRSPLSYNHTNETLGLDS
A1	88	LMASKITDQFFFRSFKADAHWITTPRMLRIWNRVLTLLTIDAE
B1	86	RVADQLVDPYFLDNKSFVHGVTVKRMIHLHPDGTVLYGLKIPITAA
G2	101	NMVGKIWIPDFFFRSFKADAHWITTPRMLRIWNRVLTLLTIDAE
D1	98	RFVDKLWLEDFIVNAKVLVHDVTVRNKLERLQPDQVILYSIITSTVA
A1	138	QPMHLEDFMDAHAQPKFGSTATRAEVVYETREPARSVVVAEDGSR
B1	136	QMDLRRYFLDEQNGTDEIESGTTDDIEFYNGGEGA--VTGVNKIEI
G2	151	QQLQLHNFEMDEHSPLEFSSYGPREEIVYQWKRSSVE--VGDTRSWRI
D1	148	QMDLAKYEMDEQEQMIDLESVGYSSDIVYYWSENQEQ--IHGLDRLCT
A1	188	NYDLLGQTVDSGIVQSSST--EYVVMTHHAKKIGYFVITLTCIMT
B1	184	POFSIVDYKMKVSKKVEFTT--GAYPRLSLSERIKENIGYFILOTMMSTLI
G2	199	YQFSFVGLRNTTEVVKTTT--QDYVMSVYEDLSRMRGYFTICTYIFCTLI
D1	196	AQFTTITSYRFTELMNFKSAGQFPRLSLHEQLRNRGVYIIQSYMSVLL
A1	237	VILQSEFLNRESVPAITVFEVQVQVNSISANSKQVAYATM
B1	233	TILSWSEWINYDASAEVVALEITVTLVSTHLETTKIPYVKID
G2	248	VVLSWVSEWINKDAVPAITSLCITVSTMTLSTIARKSLPKVSYVTAM
D1	246	VAMSWVSTWISQAAPVAVSLCITVSTMTLMSARSSLRASAIAKLD
A1	287	WFIAVYAPVTSALIEFATVNYF--TKRGYAWDGKSVVPEKPKVVDPLI
B1	283	IYLMGFVVEVLALEAFVNYIFFGKGPQKKGAGKQDQSANENNLEMN
G2	298	LFVSVGFIEVESALVEYGTLHYFVSN-----RKPSKDKDKKK
D1	296	VYFWICVVEVLALEAFVYAFAHFNAD-----YRKKRRAKVKVT
A1	335	KKNNTYAPTATSYPNLRGDPGLATIAKSATIEP-----
B1	333	KVQVDAHGNILLSTLEIRNETSGSEVLTGVGDPKTTMYSYDSASIQYRKP
G2	335	KNPAPTIDIRPRSATIOQMNATHLQERDEEYGYECLDHKDCASFF-----
D1	333	K-PRAEMDVRNAIVLFLSLSAAGVSQELAISRRQGRVPGNLMGYSYRSV---
A1	370	-----KEVKPETKPEPKKT-----FNSVSKIIRLSIAF
B1	383	MSSREGYGRALDRHGAHSGRIRRRASQLKVLIPDLTDVNSILKWSLMEF
G2	380	---CCFEDCRTGAWRHGRIHIRIAK-----MDSYARIF
D1	380	---EVEAKKEGGVPPGGPGGIRSRLKPI-----DADTIDIYAAVE
A1	400	PLLGIFNLVVAATLNREPQLKAPTHQ
B1	433	PIITSLFNVVWLYIVH
G2	412	PTATCLFNLVVWSLYL
D1	418	PAATAAVNIIWAAYTM

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identity, respectively, to the GABA<sub>A</sub> receptor subunits. Arbas *et al.*, (1991) believe that the first to diverge, and therefore the most ancient member of this family, was the acetylcholine-gated ion channel. This was followed by GABA<sub>A</sub>/glycine receptor divergence.

The amino acid identity of different subtype polypeptides is generally greater than 70% (reviewed by Betz, 1990). Subtype-specific sequences are mainly within the proposed intracellular domain of the mature polypeptide. Subtype-specific functions or structure are associated with the highly conserved amino acids among polypeptides of a particular subtype, located on the cytoplasmic sides of transmembrane domains, M3 and M4 (Kato, 1990; Ymer *et al.*, 1990; Wilson-Shaw *et al.*, 1991). Although the extracellular domains of GABA<sub>A</sub> receptor isoforms of a specific subtype exhibit a high degree of amino acid identity, within this region, it is the nonhomologous sites which contribute to the differences in allosteric modulation exhibited by the subunit variants.

#### 1.4.8 GABA<sub>A</sub> Receptor Subunit Gene Chromosomal Localizations

The chromosomal localizations of several human GABA<sub>A</sub> receptor subunit genes have been determined and are listed in Table 1.4 with the mouse syntology. The  $\alpha 3$  GABA<sub>A</sub> receptor subunit gene is on the mouse X chromosome and has been mapped near the spontaneous X-linked mouse mutant, trembly (*Ty*) (Taylor *et al.*, 1978). The trembly males develop tremors at two weeks of age and later experience seizures. In humans, one form of inheritable manic depression (MAFD2) is X-linked and has been mapped close to color-blindness and G6PD genetic markers (Baron *et al.*, 1987; Del Zompo *et al.*, 1984; Mendlewicz *et al.*, 1987), in the region of the human  $\alpha 3$  gene. Other neurological disorders which map within the Xq28 region include adrenoleukodystrophy, Emery-Dreifuss muscular dystrophy, spastic paraplegia, myotubular (centronuclear) myopathy and hydrocephalus (Rowland, 1992). GABA<sub>A</sub> receptor subunit genes,  $\beta 1$  and  $\alpha 2$ , with localizations that encompass p12-p13 bands on human chromosome 4, map onto mouse chromosome 5, in a region that contains a neurological mutant mouse locus, tilted (*tlt*) (Buckle *et al.*, 1989). The susceptibility locus for schizophrenia is located on the 5q arm (Sherrington *et al.*, 1988), as are the  $\gamma 2$  and  $\alpha 1$  GABA<sub>A</sub> receptor subunit genes (Buckle *et al.*, 1989). The  $\beta 3$ ,  $\alpha 5$ ,  $\alpha 4$  and  $\gamma 3$  GABA<sub>A</sub> receptor subunit genes map to human chromosome 15 (Wagstaff *et al.*, 1991; LaLande and Sikela, unpublished results; Danciger, Farber and Kozak, 1993; Brilliant *et al.*, 1994). Recently a spontaneous mouse mutant locus on chromosome 7 was shown to be a deletion of the  $\gamma 3$ ,  $\alpha 5$ , and  $\beta 3$  GABA<sub>A</sub> receptor subunit genes (Brilliant *et al.*, 1994). The locus is syntenic with human chromosome 15q11-q13. Prader-Willi syndrome and Angelmann syndrome genes localized in the area of 15q11, on chromosome 15 (Wagstaff *et al.*, 1991). Another murine neonatally lethal mutation of the *p* locus, includes only the GABA<sub>A</sub> receptor  $\beta 3$  subunit

Subunit	Chromosome	Species	Location	Reference	Mouse Equivalent
$\alpha 1$	5	Human	5q31.1-q33.2	Buckle, <i>et al.</i> , 1989	11
$\alpha 2$	4	Human	4p12-p13	Buckle, <i>et al.</i> , 1989	5
$\alpha 3$	X	Human	Xq28	Buckle, <i>et al.</i> , 1989	N.D.
$\alpha 4$	15	Human	15q11-13	Danciger, Farber, Kozak, 1993	7
$\alpha 5$	15	Human	near $\beta 3$	Marc LaLande, James M. Sikela, Un-published	7
$\beta 1$	4	Human	4p12-p13	Buckle, <i>et al.</i> , 1989	5
$\beta 3$	15	Human	15q11-13	Wagstaff, <i>et al.</i> , 1991	7
$\gamma 1$	4	Human	4p14-q21.1	Wilcox, <i>et al.</i> , 1992	[3,5,19]
$\gamma 2$	5	Human	5q31.1-q33.2	Wilcox, <i>et al.</i> , 1992	11
$\delta 1$	1	Human	1p	Sommer, <i>et al.</i> , 1990a	[1,3,4,5]

**Table 1.4 Localization of Human GABA<sub>A</sub> Receptor Subunit Genes**

Locations of GABA<sub>A</sub> receptor subunit genes on human chromosomes are listed. The mouse chromosomal localizations are based on known syntologies with human chromosomes.

gene (Culiat *et al.*, 1993). Complementation and RNA expression data suggest that the deficiency in the  $\beta 3$  GABA<sub>A</sub> receptor subunit gene contributes to the clefting defects observed in mice homozygous for this mutation and is consistent with earlier observations that GABA or its agonist diazepam interfer with normal palate development in mice (Wee and Zimmerman, 1983).

The co-localization of  $\alpha 1$ ,  $\gamma 2$  GABA<sub>A</sub> receptor subunit genes to chromosome 5,  $\alpha 2$ ,  $\beta 1$  and  $\gamma 1$  GABA<sub>A</sub> receptor subunit genes to chromosome 4 and  $\beta 3$ ,  $\alpha 5$ ,  $\alpha 4$  and  $\gamma 3$  to chromosome 15 suggests coordinated expression, possibly through shared regulatory regions for these co-localised subunits.

### 1.4.9 Functional Properties Conferred by Specific Subunits

GABA<sub>A</sub> receptors may be constructed *in vitro* by the transient expression of combinations of  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  and  $\pi$  subunits in *Xenopus* oocyte or mammalian cell expression systems. Both the pharmacological and electrophysiological properties of these subunit combinations, and the specific contribution of different subunits to these parameters, have been studied, and the results from several labs are discussed below.

#### 1.4.9.1 $\alpha$ Subunit

The greatest diversity among GABA<sub>A</sub> receptor subunit isoforms is observed for the  $\alpha$  subunit. It has been suggested that these subunit isoforms determine different functional subtypes of the GABA<sub>A</sub> receptor in the brain (Seeburg *et al.*, 1990). The GABA<sub>A</sub> receptor subtypes are defined by their subunit composition and by their affinities for various allosteric ligands (reviewed by Lüddens and Wisden, 1991; Doble and Martin, 1992).

Initial studies using *Xenopus* oocytes demonstrated that expression of bovine  $\alpha 1$ , 2 or 3 GABA<sub>A</sub> receptor subunits with the bovine  $\beta 1$  subunit produced receptor subtypes which could be distinguished by their apparent sensitivity to GABA (Levitan *et al.*, 1988a, b). Transient expression experiments using a human kidney 293 cell line demonstrated that  $\alpha x \beta 1 \gamma 2$  recombinant receptors, where x is 1, 2 or 3 displayed unique affinities for the triazolopyridazine CI218-872, and the  $\beta$ -carboline, BCCE (Pritchett, Lüddens, and Seeburg, 1989). The high affinity of the  $\alpha 1 \beta 1 \gamma 2$  receptor complexes for CI218-872 and BCCE is characteristic of a benzodiazepine (BZI) type-pharmacology, whereas the low affinity of the  $\alpha 2 \beta 1 \gamma 2$  and  $\alpha 3 \beta 1 \gamma 2$  receptor complexes for these ligands resembled a BZII type-pharmacology. Receptors containing the  $\alpha 3$  subunit exhibited greater GABA potentiation of benzodiazepine binding than did the receptors containing the  $\alpha 2$  subunit, thereby demonstrating the existence of subtypes within the BZII class of GABA<sub>A</sub> receptors (Pritchett, Lüddens and Seeburg, 1989).

The presence of the  $\alpha 5$  GABA<sub>A</sub> receptor subunit in a coexpression experiment with  $\beta 1 \gamma 2$  subunits conferred a BZII like-pharmacology different to that seen for the other type

II variants. These receptors displayed a higher affinity for CI218-872 than receptors containing either the  $\alpha 2$  or  $\alpha 3$  subunits, but a lower affinity for CI218-872, when compared with  $\alpha 1$  containing receptor complexes (Pritchett and Seeburg, 1990). Further heterogeneity within the BZII class of receptors occurs in the pharmacological profile of the imidazopyridin, zolpidem. GABA<sub>A</sub> receptors containing the  $\alpha 5$  isoform have a much lower affinity to zolpidem than do receptor complexes containing either the  $\alpha 2$  or  $\alpha 3$  subunits (Pritchett and Seeburg, 1990).

Recombinant receptors containing the  $\alpha 4$  or  $\alpha 6$  subunits exhibit a disparate pharmacology from GABA<sub>A</sub> type I and type II receptors. These novel receptors bind the benzodiazepine 'alcohol antagonist' Ro15-4513 with high affinity, but fail to bind benzodiazepine agonists (Luddens *et al.*, 1990; Wisden *et al.*, 1991b). In addition, the Ro15-4513 binding is poorly, if at all, displaced by diazepam indicating that these receptors may be similar to the previously described GABA<sub>A</sub> receptors that lack benzodiazepine binding sites (Unnerstall *et al.*, 1981). However, approximately one-third of the  $\alpha 6$  subunit containing receptor complexes were shown to be diazepam sensitive and may therefore represent a pharmacologically distinct class of GABA<sub>A</sub> receptors (Luddens *et al.*, 1990).

#### 1.4.9.2 $\beta$ Subunits

Ligand binding studies have implicated the  $\beta$  subunit as the GABA agonist binding site (Casalotti *et al.*, 1986). However, in both *Xenopus* oocyte and mammalian expression systems, some bovine and rat  $\alpha$ ,  $\gamma$  and  $\delta$  homomeric channels have been shown to be gated by GABA, implying that the GABA agonist binding site is not unique to  $\beta$  subunits (Blair *et al.*, 1988; Ymer *et al.*, 1989a; Shivers *et al.*, 1989). Mutational analysis suggests that certain substitutions in the  $\alpha$  subunit can have a much more dramatic effect on GABA affinity than alteration in homologous residues in the  $\beta$  subunit (Sigel *et al.*, 1992). Interestingly these mutations also decreased the affinity of the  $\alpha$  subunit for competitive antagonists, such as bicuculline, while homologous substitutions in  $\beta$  or  $\gamma$  subunits did not. Initial studies in *Xenopus* oocyte system with coexpression of  $\alpha 1\beta_y$ , where  $y$  is 1, 2 or 3, bimeric receptor combinations suggested that the beta subunits were functionally interchangeable and that substitution of either the  $\beta 2$  or  $\beta 3$  subunit for the  $\beta 1$  subunit did not change the qualitative properties of the expressed GABA<sub>A</sub> receptors (Lolait *et al.*, 1989; Ymer *et al.*, 1989b). However, later studies using suggest that the replacement of  $\beta 2$  or the  $\beta 1$  subunit in a given recombinant receptor results in a decreased affinity for GABA and in an increased sensitivity to diazepam (Sigel *et al.*, 1990). In addition, it has been suggested that natural GABA<sub>A</sub> receptors containing either  $\beta 2$  or  $\beta 3$  subunits differ in their affinity to GABA analogues, and pentobarbital (Bureau and Olsen, 1990).



### 1.4.9.3 $\gamma$ Subunits

Recombinant receptors expressed in heterologous systems using  $\alpha$  and  $\beta$  subunit combinations were found to display many of the properties of native GABA<sub>A</sub> receptors, but to lack consistent benzodiazepine potentiation (Schofield *et al.*, 1987, Levitan *et al.*, 1988b; Pritchett *et al.*, 1989; Malherbe *et al.*, 1990a).

Coexpression of the  $\gamma 2$  subunit with  $\alpha 1$  and  $\beta 1$  subunits, in an *Xenopus* oocyte system produced GABA<sub>A</sub> receptors displaying high affinity binding for the central benzodiazepine receptor ligands (Pritchett *et al.*, 1989). Thus, although the  $\alpha$  subunits determine the different affinities of the benzodiazepine ligands in GABA<sub>A</sub> receptor subtypes, the  $\gamma$  subunit is proposed to contribute to the formation of the benzodiazepine allosteric site, or to induce a conformational change on the receptor complex, making the binding site more accessible to benzodiazepine ligands (Pritchett *et al.*, 1989; Stephenson, Duggan, Pollard, 1990). Targeted disruption of the  $\gamma 2$  gene in mice leads to animals with a 94% reduction in benzodiazepine binding sites, and GABA receptors that are insensitive to benzodiazepines (Gunther *et al.*, 1995). Additionally, the  $\gamma$  subunits have been shown to reduce sensitivity to a non-competitive block of channel conductance by zinc ions (Draguhn *et al.*, 1990; Herb *et al.*, 1992; Smart *et al.*, 1991).

The  $\gamma$  subunit variants have been shown to differ in the extent to which they modify the functional properties of GABA<sub>A</sub> receptors. Specifically, when recombinant receptors are expressed in *Xenopus* oocytes, the  $\gamma 1$  subunit confers a markedly reduced affinity for the benzodiazepine antagonist Ro15-1788, and the inverse agonist DMCM, compared to those containing  $\gamma 2$  (Ymer *et al.*, 1990). This loss in affinity may be linked to the increase in GABA evoked current produced in  $\alpha x \beta 1 \gamma 1$  complexes. Thus the positive modulation conferred by these compounds define the unusual pharmacological profile of  $\alpha \beta \gamma 1$  receptors (Ymer *et al.*, 1990; Puia *et al.*, 1991).

In contrast, recombinant receptors containing the  $\gamma 3$  subunit are sensitive to DMCM, as are  $\gamma 2$  containing receptors, but differ in their responses to diazepam and zolpidem (Knoflach *et al.*, 1991; Herb *et al.*, 1992). Therefore, replacement of the  $\gamma 2$  subunit with either the  $\gamma 1$  or  $\gamma 3$  subunits, in recombinant receptors, results in disparate pharmacological profiles (Ymer *et al.*, 1990; Herb *et al.*, 1992).

### 1.4.9.4 $\delta$ subunit

Homo-oligomeric recombinant receptors with the  $\delta$  subunit have been shown to form GABA-gated bicuculline and picrotoxin sensitive channels. In one study, replacement of the  $\gamma 2$  subunit in recombinant  $\alpha 1 \beta 1 \gamma 2$  receptors with the  $\delta$  subunit, resulted in the abolition of benzodiazepine sensitivity (Shivers *et al.*, 1989). From these results it has been suggested that the  $\delta$  subunit may contribute to the formation of GABA<sub>A</sub> receptors that lack benzodiazepine binding sites (Shivers *et al.*, 1989; Wisden *et al.*, 1992). HEK-293 cells

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co-transfected with  $\alpha$ ,  $\beta$  and  $\delta$  GABA<sub>A</sub> receptor subunit cDNAs exhibit GABA evoked chloride currents that are not potentiated by anaesthetic steroids (Zhu *et al.*, 1996).

#### 1.4.9.5 $\rho$ subunits

A novel class of receptors for GABA, termed GABA<sub>C</sub> have been identified in the retinae of a variety of species including white perch, catfish, hybrid bass, salamander, rat and cow (Polenzani, Woodward and Miledi, 1991; Feigenspan, Wassle, Bormann, 1993; Qian and Dowling, 1993; Lukasiewicz, Maple and Werblin, 1994; Takahashi, Miyoshi and Kaneko, 1994; Qian and Dowling, 1995). Retina GABA<sub>C</sub> receptors are present on rat and tiger salamander bipolar cells (Feigenspan, Wassle, Bormann, 1993; Feigenspan and Bormann, 1994) and on horizontal cells of the white perch (Qian and Dowling, 1993, 1994). Recent cloning experiments have identified 3 novel GABA receptor subunits that when expressed in *Xenopus* oocytes form functional homo- and hetero-oligomeric Cl<sup>-</sup> channels, with unique pharmacological properties that correlate with the bicuculine- and baclofen-insensitive GABA<sub>C</sub> receptors characterized in retinae. The  $\rho$  subunits share a 30 to 38 percent identity with GABA<sub>A</sub> receptor subunits (Cutting *et al.*, 1991; 1992).

Contrary to the initial view that  $\rho$  represented a retinal-specific GABA receptor isoform, Boue-Grabot *et al.*, (1998) have demonstrated by *in situ* hybridization analysis the co-localise of  $\rho$ 1-3 mRNAs in the superficial gray layer of the superior coliculus and in cerebellar Purkinje cells. Although  $\rho$  subunits can form homomeric GABA-activated chloride channels that are bicuculline-barbiturate- and benzodiazepine-insensitive in *Xenopus* oocytes, the co-localisation of these subunits suggest that  $\rho$  subunits may form either homomeric or heteromeric GABA<sub>C</sub> receptors *in vivo*. Homo-oligomeric  $\rho$ 1 and  $\rho$ 2 receptors are less sensitive to muscimol and picrotoxin and desensitize slower than GABA<sub>A</sub> receptors. Like GABA<sub>A</sub> receptor complexes composed of  $\alpha$  and  $\beta$  subunits only, GABA-activated chloride currents generated by  $\rho$ 1 and  $\rho$ 2 homo- and hetero-oligomer expression in *Xenopus* oocytes are reversibly inhibited by ZnCl<sub>2</sub> (Wang, Guggino and Cutting, 1994).

The  $\rho$ 1 subunit mRNA is alternatively-spliced in the amino terminal extracellular domain resulting in either a non-functional receptor (150 amino acid deletion) or a functional GABA<sub>C</sub>-like receptor (17 amino acid deletion) when expressed in *Xenopus* oocytes (Martínez-Torres *et al.*, 1998). Co-immunoprecipitation studies with truncated versions of the  $\rho$ 1 subunit have demonstrated that the N-terminal regions of  $\rho$  subunits contain signals for both homo- and hetero-oligomeric assembly (Hackam *et al.*, 1997). The generation of chimeric receptors consisting of glycine receptor  $\alpha$ 1 subunit and GABA<sub>C</sub>  $\rho$ 1 subunit sequences has lead to the identification of two amino acid residues in transmembranes 2 and 3 that are critical for allosteric modulation of both GABA<sub>A</sub> and glycine receptors by alcohols and two volatile anaesthetics (Mihic *et al.*, 1997).

#### 1.4.9.6 $\epsilon$ Subunit

A novel GABA<sub>A</sub> receptor gene ( $\epsilon$ ) has recently been identified on human chromosome Xq28 (Wilke *et al.*, 1997). The  $\epsilon$  subunit is clustered with the  $\alpha 3$  and  $\beta 4$  GABA<sub>A</sub> receptor subunit genes in an approximately 0.8 megabase interval on Xq28 and in candidate regions for early onset parkinsonism (Waisman syndrome) (Laxova *et al.*, 1985; Gregg *et al.*, 1991) and X-linked mental retardation (MRX3) (Gedeon *et al.*, 1991; Nordstrom *et al.*, 1992). The  $\epsilon$  subunit shares the highest amino acid identity (49 percent) with the  $\gamma 4$  GABA<sub>A</sub> receptor subunit and therefore represents a new class of GABA<sub>A</sub> receptor subunit. The genomic organization of the  $\epsilon$  subunit gene differs from other GABA<sub>A</sub> receptor genes due to the loss of the third intron/exon boundary that is otherwise highly conserved among GABA<sub>A</sub> receptor subunit genes (Wilke *et al.*, 1997). The position of the last intron of the  $\epsilon$  subunit gene is conserved with the deduced position of an intron in  $\gamma 2$  subunit genes.

Northern blot analysis indicates that the  $\epsilon$  GABA<sub>A</sub> receptor subunit transcript is abundant in heart and placenta, but is not detectable in whole brain RNA (Wilke *et al.*, 1997). The  $\epsilon$  subunit transcript is alternatively spliced at several positions that yield truncated protein sequences in most tissues. This form of alternative splicing may function to down-regulate expression of the  $\epsilon$  subunit in these tissues or to create truncated protein variants with unknown function. In another study, Davies *et al.*, (1997) used Northern blot analysis of dissected brain regions to demonstrate an enrichment of the  $\epsilon$  subunit transcript in amygdala, thalamus and subthalamic nucleus. The subthalamic nucleus receives major GABA-mediated inputs, with particularly dense projections from the globus pallidus.

HEK-293 cells transfected with the  $\epsilon$  subunit only did not express binding sites for [<sup>3</sup>H] muscimol or [<sup>35</sup>S] TBPS and failed to exhibit chloride currents in response to GABA or glycine. HEK-293 cells transfected with a combination of  $\alpha$ ,  $\beta$  and  $\epsilon$  subunits exhibited GABA-activated chloride currents, that were not potentiated by benzodiazepines, pregnanolone or intravenous anaesthetic agents such as pentobarbital and propofol (Davies *et al.*, 1997). In contrast, the activating effects of pregnanolone, propofol and pentobarbital were unaffected by the presence of the  $\epsilon$  subunit and supports the view that the two responses (potential and activation) are mediated by different anaesthetic binding sites on the GABA<sub>A</sub> receptor complex (Davies *et al.*, 1997).

#### 1.4.9.7 $\pi$ Subunit

Recently a sixth class of GABA<sub>A</sub> receptor subunit has been cloned,  $\pi$ , that shares a 30 to 40 percent amino acid identity with known GABA<sub>A</sub> receptor family members and is most closely related to the  $\beta$  subunits. Both rat and human  $\pi$  subunits have been characterised and share a 93 percent amino acid sequence identity (Hedblom and Kirkness, 1997). The  $\pi$  subunit polypeptide has a predicted four transmembrane spanning topology,

with five sites for potential N-linked glycosylation in the extracellular loop that also includes the putative cysteine loop which is a characteristic of all other GABA<sub>A</sub> receptor subunits.  $\pi$  transcripts are localized in the hippocampus and temporal cortex as well as in peripheral tissues including the uterus, prostate, ovaries, placenta, gall bladder, lung, and small intestine (Heblom and Kirkness, 1997). The transcript for the  $\pi$  GABA<sub>A</sub> receptor subunit was particularly enriched in uterine tissue.

Expression of the  $\pi$  subunit alone in HEK-293 cells resulted in no discernible binding sites for [<sup>35</sup>S]TBPS, [<sup>3</sup>H] muscimol, and [<sup>3</sup>H] Ro15-1788 demonstrating an inability of the  $\pi$  subunit to form homo-oligomers. Expression of the GABA<sub>A</sub> receptor  $\beta$ 3 subunit, which can assemble homomeric chloride channels that bind [<sup>35</sup>S] TBPS with high affinity, with the  $\pi$  subunit had no significant effects on the  $K_d$  and  $B_{max}$  values for [<sup>35</sup>S] TBPS binding to transfected cell membranes. Displacement of [<sup>35</sup>S] TBPS binding by pentobarbital yielded similar  $K_i$  values for the two membrane preparations, however displacement of [<sup>35</sup>S] TBPS binding by pregnanolone demonstrated a significant reduction in sensitivity to pregnanolone in the receptor population containing the  $\pi$  subunit (Hedblom and Kirkness, 1997). Since the actions of pregnanolone at GABA<sub>A</sub> receptors have been proposed to regulate uterine motility by inhibiting contractions (Majewska and Vaupel, 1991; Putnam *et al.*, 1991), the identification of a novel class of GABA<sub>A</sub> receptors in the uterus may provide new insights into the role of GABA<sub>A</sub> receptors in this process.

#### 1.4.9.7 Null Mutations in GABA<sub>A</sub> Receptor Subunits

Genetic modification of GABA<sub>A</sub> receptor subunit genes by the introduction of null mutations into the germline have provided insight into the functional roles of these genes *in vivo*. To date null mutations have been generated in genes for the  $\alpha$ 6,  $\gamma$ 2,  $\beta$ 3 and  $\delta$  GABA<sub>A</sub> receptor subunits and a GABA synthesising enzyme GAD 67 (Jones *et al.*, 1997; Homanics *et al.*, 1997a; Günther *et al.*, 1995; Homanics *et al.*, 1997b; Homanics *et al.*, 1998; Condie *et al.*, 1997). Knockout of the  $\alpha$ 6 gene results in animals that are grossly normal in motor skills and cerebellar cortical cytoarchitecture. Analysis of cerebellar granule cells from these mice demonstrates a severe deficit in high affinity [<sup>3</sup>H] muscimol and [<sup>3</sup>H] SR 95531 binding to GABA sites and inverse agonist binding of [<sup>3</sup>H]Ro15-4513 (Mäkelä *et al.*, 1997). In a similar animal model of an  $\alpha$ 6 GABA<sub>A</sub> receptor null mutation, Jones *et al.*, (1997) demonstrated that loss of  $\alpha$ 6 GABA<sub>A</sub> receptor gene expression had a profound influence on the expression of the  $\delta$  GABA<sub>A</sub> receptor subunit polypeptide. In the mutant animals, wild-type levels of  $\delta$  subunit mRNA were present, but there was an almost complete loss of  $\delta$  subunit immunoreactivity, suggesting that there is an association of the  $\alpha$ 6 and  $\delta$  subunits which may aid in trafficking of this receptor complex to the cell surface, as well as provide protection against proteolytic degradation of the  $\delta$  subunit monomer.

When a null mutation was introduced into the  $\delta$  subunit gene, there was a reduction in high affinity binding of [ $^3$ H] muscimol in the forebrain and cerebellum, confirming the high affinity of GABA sites in the  $\delta$  subunit-containing receptors. In contrast to the  $\alpha 6$  null mutation, there was an increase in [ $^3$ H] Ro15-4513 binding in the forebrain and cerebellum suggesting that either  $\delta$  subunits normally prevent the assembly of  $\gamma 2$  subunits with the  $\alpha 6$  subunit or that in the presence of the  $\delta$  subunit, the  $\gamma 2$  subunit is unable to form normal benzodiazepine sites (Homanics *et al.*, 1998). Most of the increase in [ $^3$ H] Ro15-4513 binding was diazepam insensitive indicating the presence of  $\alpha 4$  subunits (Homanics *et al.*, 1998).

A null mutation in the  $\gamma 2$  GABA<sub>A</sub> receptor subunit gene resulted in a 94 percent loss of benzodiazepine binding sites in neonatal brain, although there was only a slight reduction in the number of GABA sites (Günther *et al.*, 1995). The single channel main conductance level and Hill coefficient were reduced to values consistent with recombinant GABA<sub>A</sub> receptors composed of  $\alpha$  and  $\beta$  subunits. The GABA response was potentiated by pentobarbital but not by flunitrazepam in neonatal DGR neurons. Loss of the  $\gamma 2$  GABA<sub>A</sub> receptor subunit demonstrated no obvious embryonic phenotype, however during postnatal development reduced GABA<sub>A</sub> receptor function was associated with retarded growth, sensorimotor dysfunction and a drastically reduced life span (Günther *et al.*, 1995).

The  $\beta 3$  subunit is an essential component of the GABA<sub>A</sub> receptor in many brain regions and especially during development. Generation of a null mutation in the  $\beta 3$  subunit gene by homologous recombination results in an approximately fifty percent reduction in [ $^3$ H] muscimol binding in the cortex and thalamus (Homanics *et al.*, 1997). [ $^3$ H] Ro15-4513 binding was decreased in the hippocampus, thalamus, basal nuclei, and frontoparietal cortex and demonstrates that another subunit such as  $\beta 2$  is unable to substitute for the  $\beta 3$  subunit. Electrophysiological analysis of DRG neurons from the homozygous null  $\beta 3$  mutant mouse demonstrated a dramatic eighty percent reduction in the maximal amplitude of GABA-activated chloride currents and suggests that the majority of the GABA<sub>A</sub> receptors on the cell somas of isolated DRG sensory neurons in the neonatal mouse contain the  $\beta 3$  subunit and is consistent with *in situ* hybridization studies in the neonatal rat (Homanics *et al.*, 1997). Most  $\beta 3$ -deficient mice die as neonates and some neonatal mortality is accompanied by cleft palate. The  $\beta 3$  null mutant mice that survive are runted until weaning but achieve normal body size by adulthood, although with a reduced life span. The adult  $\beta 3$  mutant mice are hyperactive and hyperresponsive to human contact and other sensory stimuli. The mice have difficulty swimming, walking on grids and fall off platforms and rotarods, although they do not have a jerky gait. The  $\beta 3$ -deficient mice display frequent myoclonus and occasional epileptic seizures. Hyperactivity, lack of coordination, and seizures are consistent with reduced presynaptic

inhibition in spinal cord and impaired inhibition in higher cortical centers (Homanics *et al.*, 1997).

Mice with a homozygous null mutation in the *Gad67* gene, which encodes an enzyme, responsible for GABA synthesis, glutamic acid decarboxylase, have defects in the formation of the palate, a phenotype which is also evident in  $\beta 3$ -deficient mice (Condie *et al.*, 1997).

#### 1.4.11 Subunit Composition of GABA<sub>A</sub> Receptors *In vivo*

Unlike the muscle acetylcholine receptor, the quaternary structure of the GABA<sub>A</sub> receptor has not been elucidated. It is, however, possible to construct putative subunit combinations, through *in situ* histochemical localisation of mRNA and immunocytochemical data.

The significant sequence identity among the GABA<sub>A</sub> receptor subunits and to an even greater extent among subunit variants has encumbered the preparation of subunit-specific antibodies. It is only recently that subunit-specific antibodies have been available for immunocytochemical studies (Benke *et al.*, 1991a, 1991b; Zimprich *et al.*, 1991), which so far have only implicated the existence of one possible GABA<sub>A</sub> receptor complex,  $\alpha 1\beta 2\gamma 2$  (Benke *et al.*, 1991b). Immunoprecipitation studies have shown that the  $\alpha 1$  subunit together with either  $\beta 2$  or  $\beta 3$  subunits are constituents of 80% of the native GABA<sub>A</sub> receptors in bovine and rat brain, and that these  $\alpha 1$ ,  $\beta 2$  or  $\beta 3$  combinations co-exist with the  $\gamma 2$  subunit in 20% to 30% of native receptors (Benke *et al.*, 1991b). The results of immunoprecipitation studies have also described several other possible GABA<sub>A</sub> receptor subtype combinations: [1]  $\alpha 1\beta 1\gamma 2$ , [2]  $\alpha 2\beta 1\gamma 2$ , [3]  $\alpha 3\beta 1\gamma 2$ , and [4]  $\alpha 5\beta 1\gamma 2$  (McKernan *et al.*, 1991). Although the majority of receptors immunoprecipitated from membranes contain only one  $\alpha$  subunit isoform, a minority have been shown to contain more than one  $\alpha$  subunit per receptor complex (Duggan, Pollard, Stephenson, 1991; Lüddens, Killisch and Seeburg, 1991; McKernan *et al.*, 1991b).

mRNA localization studies represent an indirect approach for addressing the question of GABA<sub>A</sub> receptor subunit composition. Where the mRNA expression patterns of two or more genes coincide, the receptor complex is presumed to co-assemble from the subunits present. Whether the mRNA abundance and distribution correlates with protein levels and localization has yet to be determined.

*In situ* hybridization studies have demonstrated the differential distribution of the 13 isolated rat GABA<sub>A</sub> receptor transcripts in embryonic, postnatal and adult brain (Wisden *et al.*, 1992; Laurie *et al.*, 1992; Mohler *et al.*, 1991; Killisch *et al.*, 1991). mRNA localization studies of rat GABA<sub>A</sub> receptor transcripts have also implicated the existence of several possible GABA<sub>A</sub> receptor subtype subunit combinations in specific brain regions: [1]  $\alpha 1\beta 2\gamma 2$  (Laurie, Wisden, Seeburg, 1992; Lüddens and Wisden, 1991;

Gambaran *et al.*, 1991; Pritchett *et al.*, 1989; Shivers *et al.*, 1989; Wisden *et al.*, 1992), [2]  $\alpha 1\beta 1\gamma 2$  (Malherbe *et al.*, 1990b), [3]  $\alpha 1\beta 2\gamma 3$  (Herb *et al.*, 1992), [4]  $\alpha 2\beta 3\gamma x$  (Persohn, Malherbe and Richards, 1991; Wisden *et al.*, 1992), [5]  $\alpha 5\beta 1\gamma x$  (Wisden *et al.*, 1992), [6]  $\alpha 1\alpha 4\beta 2\delta$  (Wisden *et al.*, 1992), [7]  $\alpha 1\alpha 4\beta 2$  (Wisden *et al.*, 1992), [8]  $\alpha 6\beta x\gamma 2$  and [9]  $\alpha 1\alpha 6\beta x\delta$  (Laurie, Wisden and Seeburg, 1992a).

A more direct approach has been recently developed that uses a combination of transcriptional amplification and PCR amplification to measure RNA levels in single neurons (Sheng *et al.*, 1994). While this method still does not provide evidence of functional assembly it does greatly increase the sensitivity of RNA detection.

The immunoprecipitation, *in situ* histochemical and immunocytochemical studies described above have all identified the  $\alpha 1\beta 2\gamma 2$  combination, as the major receptor subtype in the adult brain. Co-expression experiments with  $\alpha 1$ ,  $\beta 2$  and  $\gamma 2$  subunits have resulted in GABA<sub>A</sub> receptors which display high-affinity type 1 benzodiazepine binding (Pritchett *et al.*, 1989) and have channel properties similar, but not identical, to a subset of native GABA<sub>A</sub> receptors (Verdoorn *et al.*, 1990). Two of the other proposed subtypes,  $\alpha 1\beta 1\gamma 2$  and  $\alpha 5\beta 1\gamma x$ , are common to two of the three approaches used to investigate the *in vivo* GABA<sub>A</sub> receptor subunit composition. The  $\alpha 1\beta 1\gamma 2$  subtype exhibits a pharmacologically and functionally similar profile to the  $\alpha 1\beta 2\gamma 2$  subtype (Malherbe *et al.*, 1990b; Pritchett *et al.*, 1989), while the  $\alpha 5\beta 1\gamma x$  subtype produces GABA<sub>A</sub> receptors which display sensitivity to benzodiazepine receptor ligands but differ in their functional properties (Herb *et al.*, 1992; Knoflach *et al.*, 1991; Pritchett and Seeburg, 1990).

## 1.5 Conclusions

This chapter has attempted to outline a small part of the elaborate communication network required by the mammalian central nervous system to enable responses to environmental stimuli. Pre- and post-synaptic receptors are responsible for the accurate transmission of information between cells. The diversity of responses required for such communication among cells is accomplished by heterogeneity within receptor classes. Receptor heterogeneity can be derived from several sources: [1] the number and types of subunits available to form the receptor complex, [2] different combinations of subunits and hence the emergence of subtypes of receptors, [3] alternative-splicing of subunit mRNAs, [4] RNA editing of subunit mRNA and [5] allelic variation, as has been demonstrated for the D4 subunit of the human dopamine receptor (Van Tol *et al.*, 1992). The regulation of heterogeneity is controlled predominantly through the developmental and co-ordinated expression of receptor subunits and subunit variants (Schofield, Shivers, Seeburg, 1990).

## 1.6 Research Project

GABA<sub>A</sub> receptor mediated responses are a classical example of the diversity that can be generated through the number of subunit genes and the possible combinatorial arrangements of these subunits within the receptor complex. As GABA is the major inhibitory neurotransmitter in the adult mammalian CNS (Bloom and Iversen, 1971) and may also play a neurotrophic role during development, the elucidation of temporal patterns of subunit mRNA expression is central towards an understanding of the development of system heterogeneity and hence neuronal plasticity.

The aims of this project are: [1] to investigate the temporal patterns of GABA receptor subunit mRNA expression in the murine central nervous system and [2] to study the structure-function relationship of subunit amino acid conservation in ligand binding studies. The mouse was chosen because it is currently the only mammalian system in which both *in vitro* and *in vivo* genetic manipulations can be performed. Although the scope of this project concerns *in vitro* studies only, the information derived from these experiments should provide a useful reference for future *in vivo* studies where the generation of null mutants or the over-expression of specific subunit genes may be considered.

GABA<sub>A</sub> receptor subunit genes exhibit a 30 to 40 percent amino acid sequence identity between subunits and a 70 to 90 percent sequence identity among subunit variants. When GABA<sub>A</sub> receptor subunit sequences are compared in a broader context with subunit sequences from other ligand-gated ion channels belonging to the ligand-gated ion channel superfamily, sequence identity decreases. There are however, specific amino acids in the putative cytoplasmic loop and transmembrane domains M1 and M2, which are conserved throughout the ligand-gated ion channel superfamily. We have chosen several of these highly conserved amino acids as positions for site-directed mutagenesis, and subsequent ligand binding analysis. We hypothesize that these sites may play key roles in subunit interactions involved in; pentameric subunit receptor assembly and in the formation of cooperative ligand binding domains. The results of these experiments may provide important insights into the structure-function relationship of the ligand-gated ion channel superfamily.



# **Chapter 2**

## **Materials and Methods**

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## CHAPTER 2: MATERIALS AND METHODS

### 2.1 MATERIALS

#### 2.1.1 Bacterial Strains

The strains of bacteria used in this work are listed below. They are discussed in greater depth and referenced in Sambrook *et al.*, (1989).

##### TG-1:

*supE, hsd*  $\Delta 5$ , *thi*  $\Delta(lac-proAB)F[traD36proAB^+lacI^qlacZ\Delta M15]$  An *EcoK*-derivative of JM101 that neither modifies or restricts transfected DNA and supports growth of vectors carrying amber mutations. Used for the propagation of M13 recombinants.

##### CJ236:

*dut1, ung1, thi-1, relA1/pCJ105(cam^F')* A *dut<sup>-</sup> ung<sup>-</sup>* strain used to prepare uracil-containing DNA for site-directed mutagenesis experiments (Kunkel, Roberts and Zakour, 1987). pCJ105 carries F' and *cam<sup>r</sup>*; growth of CJ236 in the presence of chloroamphenicol selects for the retention of F'.

##### MC1061/p3:

*ara139, \Delta(ara, leu)7696, galU, glaK, \Delta lacX74, hsr<sup>-</sup>, hsr<sup>m+</sup>, strA, deoR<sup>+</sup>, {p3:amber amp<sup>r</sup>, amber tet<sup>r</sup>, Km<sup>r</sup>}* A P3 containing strain designed to support replication of plasmid DNA's which encode the tyrosine tRNA suppressor (synthetic *supF* gene).

All strains were grown in 2YT (1.6% bacto-tryptone, 1.0% bacto-yeast extract, 0.09 M NaCl, pH7.0), at 37°C .

#### 2.1.1.2 Mammalian Cell Line

Human embryonic kidney 293 cells (ATCC CRL 1573) were a generous gift of Karen Haddington. The 293 cells were grown in Dulbecco's MEM/Nutrient Mix, F12(1:1) supplemented with 1% L-Glutamine, 1X Streptomycin/Penicillin and 10% fetal calf serum, at 37°C in 8% CO<sub>2</sub>.

Media for growth of bacteria and tissue culture were prepared by staff of the media kitchen in the MRC Laboratory of Molecular Biology.

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## 2.1.2 Enzymes, Chemicals, Radioisotopes and Vectors

### 2.1.2.1 Enzymes

All enzymes were purchased from New England Biolabs with the following exceptions. T4 DNA ligase, RAV2 reverse transcriptase, S1 nuclease and terminal transferase (used in the direct sequencing protocol, Lasham *et al.*, 1992) were purchased from Boehringer Mannheim. T4 gene32 protein was purchased from United States Biochemical Corp. Terminal transferase (used to label probes for Northern blots and *in situ* hybridization), T7 DNA polymerase and the Pharmacia oligo labelling kit were purchased from Pharmacia LKB. SP6 RNA polymerase, T7 RNA polymerase, T3 RNA polymerase, RNasin, Reagent kit for *in vitro* transcription and Taq DNA polymerase were purchased from Promega. Taq DNA polymerase was also purchased from Perkin Elmer Cetus. DNaseI, RNaseA and lysozyme were purchased from Sigma. All enzymes were used according to manufacturers specifications.

### 2.1.2.2 Chemicals

All chemicals were BDH analytical grade with the following exceptions. Acrylamide and bisacrylamide were BDH electrophoresis grade and sodium dodecyl sulphate (SDS) and diethylpyrocarbonate (DEPC) were BDH biochemical grade. The Prep-A-Gene, DNA purification matrix kit was purchased from Biorad. Ethidium bromide (EtBr), putrescine, polyethylene glycol (PEG) 4000, polyvinylpyrrolidone (PVP), sorbitol, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside (X-gal), heparin, poly-L lysine, poly A and trypsin, tissue culture grade were purchased from Sigma. Water-saturated phenol was purchased from Rathburn Chemicals Ltd. Deoxynucleotide triphosphates (dNTPs), dideoxynucleotide triphosphates (ddNTPs), adenosine ribonucleotide triphosphate (rATP), random hexamers (pd(N)<sub>6</sub>) and Sephadex G-25 were purchased from Pharmacia LKB. Formamide and guanidine thiocyanate were purchased from Fluka. Dulbecco's MEM/Nutrient Mix, F12(1:1) with L-glutamine, 10X streptomycin/penicillin and fetal calf serum were purchased from GIBCO BRL. The dGTP reagent kit for sequencing with Sequenase was purchased from United States Biochemical Corporation. Ultra pure grade reagents purchased from BRL were agarose, low melting point (LMP) agarose, cesium chloride, and urea. Dithiothreitol (DTT), N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), spermidine and spermine were purchased from Calbiochem. Yeast tRNA and glycogen were purchased from Boehringer Mannheim. K5 emulsion was purchased from Ilford Scientific Products. D19 developer was purchased from Kodak. Chloroform, methanol and N-butanol were purchased from R.P. Normapur. 95% and 100% absolute ethanol (EtOH) was purchased from Hayman Ltd.

### 2.1.2.3 Radioisotopes

$\alpha$ - $^{32}\text{P}$  dCTP (3000 Ci/mmol),  $\gamma$ - $^{32}\text{P}$  ATP (>5000 Ci/mmol) and  $\alpha$ - $^{35}\text{S}$  dATP (800 Ci/mmol) were purchased from Amersham.  $\alpha$ - $^{35}\text{S}$  dATP (1200 Ci/mmol) and  $\alpha$ - $^{32}\text{P}$  dATP (6000 Ci/mmol) were purchased from New England Nuclear. [ $^3\text{H}$ ] muscimol (23 Ci/mmol), [ $^3\text{H}$ ] flunitrazepam (83 Ci/mmol), [ $^3\text{H}$ ] Ro15-1788 (78 Ci/mmol), isoquavine, GABA, CL218 828, clonazepam, and flunitrazepam were generous gifts from I.L. Martin.

### 2.1.2.4 Vectors

**M13mp18:** M13 is a filamentous, F-pilus-specific, *E. coli* bacteriophage. A polylinker for the subcloning of DNA fragments with blunt, 3' or 5' overhangs resulting from restriction endonuclease cleavage has been introduced 5' of the  $\beta$ -galactosidase sequence. Insertion of DNA into the polylinker results in inactivation of the  $\beta$ -galactosidase  $\alpha$ -fragment coding sequence which renders the resulting transformed colony or plaque unable to utilize the X-gal chlorogenic substrate and hence remains white.

**Bluescript KS+:** pBluescript KS+ is a 2,961 base pair phagemid derived from PUC19. The vector possesses an f1 phage origin, a *colE1* origin and T3 and T7 promoters flanking a novel polylinker containing 21 unique restriction sites. The f1 phage origin allows single strand rescue, via helper phage infection, which produces a single-strand DNA which can be used for site-directed mutagenesis or DNA sequencing. The vector was purchased from Stratagene.

**pSP65:** pSP65 is a 3,005 base pair plasmid containing an SP6 RNA polymerase promoter, 5' of a polylinker containing 11 unique restriction sites. Other features of this vector are described by Melton *et al.*, (1984). The vector was purchased from Promega.

**pCDM8:** pCDM8 is a 4,500 base pair eukaryotic expression for cDNA cloning analysis in *E. coli* and eukaryotic systems. pCDM8 has a human CMV promoter and enhancer for transient expression in mammalian cells, SV40 and polyoma viral origins of replication for expression in mammalian cell lines which have been infected with SV40 or Polyoma viruses or in cell lines which carry the large T antigen, a *ColE1*-like origin of replication for expression in *E. coli*, the *supF* gene for the rescue of amber mutations which acts as a selectable marker for transformed *E. coli* cells, an M13 origin for single strand rescue via helper phage infection and T7 and SP6 RNA promoters. pCDM8 was a generous gift of Bill Wisden.

**pRc/CMV:** pRc/CMV is a 5,400 base pair eukaryotic expression vector designed for high level stable expression in eukaryotic cells. pRc/CMV has a human cytomegalovirus promoter and enhancer for the expression of cloned cDNAs in mammalian cell lines, a neomycin gene for the selection of stable transfectants, a ColE1 origin of replication for expression in *E. coli*, an M13 origin for single strand rescue via helper phage infection and T7 and SP6 RNA promoters. pRc/CMV was purchased from British Biotechnology, a United Kingdom distributor for Invitrogen.

### 2.1.2.5 Oligonucleotide probes and PCR primers

All oligonucleotides (Tables 2.1, 2.2, 2.3 and 2.4) were synthesised on a Pharmacia:LKB Gene Assembler Plus.

Probe Name/Subunit	Sequence	Amino Acid Change	References
<b>Cys loop</b> MGD1079/alpha1	5' <u>agggcaggcatgggcat</u> <u>fc</u> atagggaaatcctc'3	Asp149-Asn149	Schofield <i>et al.</i> , (1987)
MGD1085/beta1	5' <u>cgaagat</u> atcctctg <u>aat</u> gaacaaaactgcacc'3	Asp146-Asn146	"
MGD1082/beta1	5' <u>cgaagat</u> atcctctg <u>gag</u> gaacaaaactgcacc'3	Asp146-Glu146	"
<b>TM1</b> MGD1154/alpha1	5'aactgtcattatgca <u>agcc</u> aggtatgtttgaat'3	Pro233-Ala233	Schofield <i>et al.</i> , (1987)
MGD1149/beta1	5'ttgcaaactacatg <u>gctt</u> ccacactgattaca'3	Pro228-Ala228	"

**Table 2.1** *Oligonucleotides designed for mutagenesis of bovine GABA<sub>A</sub> receptor subunit cDNA sequences.*

*The oligonucleotides were used for in vitro mutagenesis as described by Kunkel, Roberts and Zakour, (1987). The nucleotides involved in the amino acid change are underlined and highlighted in bold print.*

Probe Name/Subunit	Sequence	Amino Acids	Reference
MNU1479 alpha1	5'ggggtcaccctggctaagttagggtatagctggttgctgtagg'3(A)	342-356	Khrestchatisky <i>et al.</i> , (1989)
MNU1502 alpha2	5'ggcaacggctacagcatagggcgttctgtatcatgacggagcc'3(A)	340-344	Khrestchatisky <i>et al.</i> , (1991)
MNU1503 alpha3	5'aggtggttcccactatgtgaaggtggtgctgttttcttggtg'3(A)	361-375	Malherbe <i>et al.</i> , (1990a)
MNU1577 alpha4	5'gagtggtttcagaagactcctgggcagcggtggtcttgctggaat'3(A)	380-395	Wisden <i>et al.</i> , 1991b
MNU1480 alpha5	5'attcccagtcctgctggaagctgctcttgggatgttgagg'3(A)	355-369	Khrestchatisky <i>et al.</i> , (1989)
MNU1505 alpha6	5'agctgattcttcttcagatgtacttgga gtcagagtgcacaac'3(A)	342-356	Malherbe <i>et al.</i> , (1990a)
MNU1506 beta1	5'gcctgtccagccctgctccgaagccctcacggctgctcagtggt'3(A)	382-396	Luddens <i>et al.</i> , (1990)
MNU1509 beta2	5'tcgttccagggcgttgccgcaaaaactatgcctaggcaaccagc'3(A)	382-396	Ymer <i>et al.</i> , (1989b)
MNU1512 beta3	5'tctgtctcccatgtaccgccatgcccttcctgggcatgctctg'3(A)	380-394	Ymer <i>et al.</i> , (1989b)
MNU1515 gamma1	5'catgggaatcagagtagatccagcatggagacctggggataccgaagt'3(A)	340-354	Ymer <i>et al.</i> , (1989b)
MNU1516 gamma2T	5'gttcatttgatcggtgctgatctgggacggatatcaatggtagg'3(A)	339-353	Ymer <i>et al.</i> , (1990)
MNU15 gamma3	5'ttagagggtgcttgaaggcttattcgatcaggaaatccatctgtt'3(A)	343-358	Shivers <i>et al.</i> , (1989) Herb <i>et al.</i> , (1992)

**Table 2.2** *Oligonucleotides designed to rat GABA<sub>A</sub> subunit cDNA sequences.*

*The oligonucleotides were used for S1 protection experiments and as subunit specific-probes for Southern blot analysis. The gamma2T oligonucleotide can recognize both the long and short forms of the gamma2 subunit. The oligonucleotides are in an antisense configuration (A).*

Probe Name/Subunit	Sequence	Amino Acid	Reference
MLS4000 alpha1	5'gggtcaccctggctaagttaggggtat agctggttgctgtagga'3(A)	369-383	Keir, <i>et al.</i> , (1991)
MNU1616/ alpha2	5'aggatctttgaaagattcggggcatagt tggaacagctaccgc'3(A)	340-355	Chapter 3 and Burt, unpublished results
MNU1617/ alpha3	5'gagaactcagtatcctggccagattgat aggataggtggtacc'3(A)	373-385	Chapter 3 and Burt, unpublished results
MLS4001/ alpha6	5'ggcctcagaagatggaacgatgggcaa agtcagagagctgattct'3(A)	383-398	Kato, (1990)
MNU1508/ beta1	5'tggcactctggtcttggctcgcctcctt ttctgagggcct'3(A)	N/A	Chapter 3
MNU1507/ beta3	5'acgatcattctggccttggtctcttcc gcaagcttcttctgcc'3(A)	N/A	Chapter 3
MNU1526/ gamma1	5'ccctccaacactggtagccataatcatct tccccttgggcaa3'(A)	N/A	Chapter 3
MGD740/ gamma2T	5'tagccatattctcatcctctctgaagg gggtggcattgttcatt'3(A)	351-367	Kofuji <i>et al.</i> , (1991)
MGD1273/ gamma2L	5'atggtagggcctgaaggaaaacatcc gaagaagagggttttc'3(A)	334-341	"
MGD1274/ gamma2S	5'ggacgaatatcaatggtaggggcaggg ttttctcttttct'3(A)	330-345	"
MGD1352/ gamma3	5'ggaattgttaacgtgatcacaggtg gtgggggcctcatgc'3(A)	363-377	Knoflach <i>et al.</i> , (1991)
MLS4002/ delta1	5'accatgaggtcccaggaccggcct tgacgacgggagatagc'3(A)	352-366	Sommer <i>et al.</i> , (1990a)

**Table 2.3** Oligonucleotides designed to mouse GABA<sub>A</sub> subunit cDNA sequences.

The oligonucleotides were used for *in situ* hybridization experiments. The gamma2T oligonucleotide can recognize both the long and short forms of the gamma2 subunit. The oligonucleotides are in an antisense configuration (A).

Degenerate Oligos Name/Subunits	Sequence	GABA cDNA coding region	Reference
MNU1455 alpha1,2,3,4,5,6	5'tagagtggatccatgac(ac)ac(tca)(t c)t (gac)ag(tc)atcagtc'3	TM2	Chapter 3
MNU1491 alpha1,2,3,5	5'ctagatgagctctt(tc)a(ac)ata(ct)gt (gt)gccca(ag)ta(ag)ac'3	TM4	Chapter 3
MNU1489 alpha4,6	5'ctagatgagctcga(at)a(ag)gtaaac (at)a(tc)cca(ag)ta(gc)ac'3	TM4	Chapter 3
MLS4004 beta1,2,3	5'tagagtggatccgt(gca)ct(gc)ac(c ga)atgac(act)ac(ac)atca'3	TM2	Chapter 3
MLS4003 beta1,2,3	5'ctagatgagctc(ga)t(tg)(atc)acata gta(ca)a(ga)cca(ag)ta(ga)ac'3	TM4	Chapter 3
MNU1494 gamma1,2,3	5'tagagtggatccatcac(tc)ac(gt)gt( ctg)(ct)t(ga)ac(tc)atgac'3	TM2	Chapter 3
MNU1495 gamma1,2,3	5'ctaactgagctc(ca)(ga)accca(ag)t a(ga)ac(ac)ag(ga)ttgaaca'3	TM4	Chapter 3

**Table 2.4** *Base-degenerate oligonucleotide sequences based on known rat GABA<sub>A</sub> cDNA sequences.*

*The oligonucleotides are degenerate at the positions indicated by brackets and were used in PCR amplification experiments to identify corresponding mouse sequences. The design and rationale of these degenerate nucleotide sequences is outlined in Chapter 3.*

## 2.2 METHODS

### 2.2.1 DNA Preparation

#### 2.2.1.1 Plasmid DNA Mini Preparation

This procedure is briefly outlined below and can be found in greater detail in Sambrook *et al.*, (1989). 2 millilitres (ml) of 2 YT with the appropriate antibiotic were inoculated with a single colony and grown overnight at 300 revolutions per minute (rpm), 37°C, in a New Brunswick incubator. Cells were harvested by centrifugation for 5 minutes in a Beckman microfuge. The pellet was resuspended in 100 microlitres (µl) of lysis buffer (50 mM glucose, 10 mM EDTA) and incubated at room temperature for 5 minutes. 200 µl of 0.2 M NaOH, 0.1% SDS was added and the reaction was gently mixed by inversion of the tube several times. 150 µl of 5M potassium acetate, pH 4.5 was quickly added and the solutions were briefly mixed by inverting the tubes and vortexing. The reaction was then centrifuged in a Beckman microfuge for 10 minutes. 400 µl of supernatant was removed and extracted once with an equal volume of phenol/chloroform (50:50). The DNA was pelleted by the



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addition of 1 ml of 95 % EtOH, placed in dry ice for 10 minutes followed by centrifugation for 10 minutes in a Beckman microfuge. The DNA pellets were washed with 70 % EtOH, dried under vacuum and resuspended in 25  $\mu$ l of dH<sub>2</sub>O.

This procedure gave plasmid DNA of a quality which could be restriction digested, sequenced.

### 2.2.1.2 Large Scale Plasmid DNA Preparation

This procedure is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). A single colony was inoculated into 500 ml of 2YT with the appropriate antibiotic (100  $\mu$ g/ml ampicillin for Bluescript KS+, pSP65 and pHEI with CAT and 100  $\mu$ g/ml ampicillin, 12.5  $\mu$ g/ml tetracyclin, and 50  $\mu$ g/ml kanamycin for pCDM8) and shaken at 300 rpm in a New Brunswick incubator at 37<sup>o</sup> C, overnight. The cells were harvested by centrifugation at 4<sup>o</sup> C in a GSA rotor at 6K rpm for 10 minutes. The cell pellets were resuspended in 10 ml of lysis solution (10% sucrose, 5 mM EDTA, 50 mM Tris, pH 7.5 and 2 mg/ml of lysozyme) and placed on ice for 5 minutes. 20 ml of 0.2M NaOH, 0.2% SDS was added and the suspension was vortexed briefly before 15 ml of 3M NaOAc, pH 4.5 was added. The suspension was again vortexed briefly and then centrifuged at 8K rpm, 4<sup>o</sup> C for 10 minutes in a GSA rotor. The supernatant was collected and 0.6 volumes of isopropanol were added. This suspension was centrifuged at 8K rpm, 4<sup>o</sup> C for 10 minutes in a GSA rotor. The pellet was resuspended in 9 ml of TE (10 mM Tris, pH 7.5 and 1 mM EDTA) and 1 gram of cesium chloride per gram of solution. 200  $\mu$ l of 10 mg/ml ethidium bromide (EtBr) was added and the solution was transferred to Beckman Quick Seal tubes, prior to centrifugation in a NVT65 rotor at 60K rpm, 18<sup>o</sup> C for 8 hours. Plasmid bands were extracted with an 18 gauge needle. Et Br was removed with 3 to 4 butanol extractions. The plasmid was collected by suspension in 8 ml of dH<sub>2</sub>O, 0.8 ml of 3M NaOAc, pH 5.2 and 16 ml of 95% EtOH, followed by a 30 minute incubation on ice, then a 30 minute incubation in dry ice and finally centrifugation for 30 minutes in a SS34 rotor at 8K rpm, 4<sup>o</sup>C. The DNA pellet was washed twice with 70% EtOH and resuspended in dH<sub>2</sub>O. The OD<sub>260</sub> was determined and the purity of the DNA was checked by electrophoresis on an 0.7% agarose gel.

The quality of this DNA was suitable for restriction endonuclease digestion, sequencing, *in vitro* transcription and mammalian cell transfection.

### 2.2.1.3 Single-Strand DNA Preparation

#### 2.2.1.3.1 Single-stranded M13 DNA Preparation

Single-strand DNA from M13 phage was prepared as described by Vieira and Messing (1987).

#### 2.2.1.3.2 Single-stranded Bluescript KS<sup>+</sup> DNA Preparation

A single colony was inoculated into 2 mls of 2YT containing 100 µg/ml of ampicillin and approximately  $1 \times 10^9$  pfu/ml of M13K07 helper phage (prepared as described by Vieira and Messing, (1987)) and shaken in a New Brunswick incubator at 37° C for 1 hour. Kanamycin was then added to a final concentration of 5 µg/ml and the culture was grown overnight with aeration at 37° C. The cells were harvested by centrifugation in a Beckman microfuge for 10 minutes. The supernatant was transferred to a fresh tube with 10% PEG6000, 2.5 M NaCl, and 15 mM EDTA, pH 8.0 and incubated on ice for 2 hours. The phage were pelleted by centrifugation in a Beckman microfuge for 15 minutes. The supernatant was carefully removed and the inner walls of the microfuge tube were wiped prior to resuspending the pellet in 100 µl of single strand extraction buffer (5 mM NaCl, 10 mM TrisHCl, pH 8.0, 1 mM EDTA, 0.2% Sarkosyl and 50 µg/ml of Proteinase K). The reaction was incubated at 55° C for 30 minutes followed by phenol/chloroform (50:50) inactivation. The aqueous phase was removed and EtOH precipitated. The DNA pellet was washed with 70% EtOH, vacuum dried and resuspended in 30 µl of TE.

## 2.2.2 RNA Preparation

### 2.2.2.1 Small Scale RNA Preparation

For RNA preparation from less than 0.5 grams of tissue or from  $10^6$  cells the following method was used and is a modification of the method described by Chomczynski and Sacchi (1987). 100 µl of solution D (4 M guanidine thiocyanate, 12.5 mM EDTA, 50 mM TrisHCl, 2% sodium N-lauryl sarcosinate and 140 mM β-mercaptoethanol) was added to an eppendorf tube with cells or tissue and vortexed. In the case of large embryos, the tissue and solution were syringed to obtain a homogeneous suspension. 10 µl of 2M Na acetate, pH 4.0 was added and the suspension was phenol extracted by adding 100 µl of H<sub>2</sub>O-saturated phenol (no pH adjustment), 100 µl of chloroform/IAA (isoamyl alcohol) 49:1, vigorously mixed, placed on ice for 15 minutes and spun in a microfuge at 4° C for 15 minutes. The aqueous phase was placed in a new tube and if dealing with less than  $10^5$  cells or 1 mg of tissue, 1 µl of RNase-free glycogen at 20 µg/ml was added prior to EtOH precipitation. The RNA pellet was washed in 70% EtOH, dried and resuspended in 10 to 100 µl of DEPC-treated H<sub>2</sub>O.

### 2.2.2.2 Tissue Isolation and Large Scale RNA Preparation

This protocol is a modification of methods described by Glisin *et al.*, (1974) and Ullrich *et al.*, (1977) and is outlined in greater detail in Sambrook *et al.*, (1989). Whole brains were removed from freshly killed animals, immediately placed in liquid nitrogen and then stored at  $-70^{\circ}\text{C}$ . 1 to 5 grams of frozen tissue was ground under liquid nitrogen using a mortar and pestle. The ground tissue was suspended in 12 ml of guanidine thiocyanate solution (4M guanidine thiocyanate, 12.5 mM EDTA, pH 8.0, 50 mM TrisHCl, 2% sodium N-lauryl sarcosinate and 140 mM 2-mercaptoethanol), briefly homogenised with a Brinkman polytron homogeniser and then centrifuged at 5K rpm, for 10 min in a Sorvall SS34 rotor, at  $4^{\circ}\text{C}$ . The supernatant was carefully layered over a 5.7M cesium chloride, 0.1M EDTA, pH8.0 cushion and spun for 18 hours, at 32K rpm, in a Beckman SW40 rotor at  $4^{\circ}\text{C}$ . The RNA pellet was washed twice with 95% EtOH and suspended in 1 ml of HES buffer (10 mM Hepes, 5 mM EDTA, pH 8.0 and 0.1% SDS). The RNA was EtOH precipitated, phenol extracted, reprecipitated and finally suspended in DEPC (diethylpyrocarbonate)-H<sub>2</sub>O, at a concentration of 1 mg/ml. Total RNA was used for Northern blot analysis and S1 nuclease protection. For 1st strand synthesis, poly A+ mRNA was prepared by the method of Sambrook *et al.*, (1989) with the following modifications. Total RNA was heated at  $65^{\circ}\text{C}$  in elution buffer (10 mM Hepes, 1 mM EDTA and 0.1% SDS) for 5 minutes, at which time 5 M NaCl was added to a final concentration of 0.4 M NaCl. The RNA solution was repeatedly applied to an oligo dT cellulose column (up to 6 times). The column was washed with 25 ml of binding buffer (10 mM Hepes, 1 mM EDTA, pH 8.0, 0.1% SDS and 0.4 M NaCl) and the RNA was eluted from the column with eight 0.5 ml volumes of elution buffer, which had been preheated to  $65^{\circ}\text{C}$ . The 260 nm absorbance of the 0.5 ml aliquots was measured and the appropriate aliquots were pooled and EtOH precipitated.

### 2.2.2.3 *In Vitro* Transcription of cRNA

10  $\mu\text{g}$  of plasmid DNA containing various bovine GABA<sub>A</sub> subunit cDNAs were digested with the appropriate restriction endonuclease, following the manufacturer's instructions, for at least 6 hours. Complete linearisation of the plasmid was checked by electrophoresing an aliquot on an 0.7% agarose (BRL/ultrapure) 1XTAE (0.04 M Tris-acetate and 0.001 M EDTA, pH 8.0) gel. The linearised plasmid was phenol/chloroform (50:50) extracted, EtOH precipitated, washed in 70% EtOH, vacuum dried and resuspended in dH<sub>2</sub>O at a final concentration of 1  $\mu\text{g}/\mu\text{l}$ . 1 to 2  $\mu\text{g}$  of DNA were added to a 60  $\mu\text{l}$  reaction containing 1X transcription buffer, 10 mM DTT, 2.0 units of RNasin, 1 mM of each rNTP (ribonucleotide triphosphate), 50  $\mu\text{M}$  of pGppG, and 60 units of T7, T3 or SP6 RNA polymerase. The reaction was incubated at  $37^{\circ}\text{C}$  for 90 minutes to 2 hours. 1.5 units of RNasin and 3 units

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of RNase-free DNase was added and the reaction was allowed to incubate at 37° C for a further 5 minutes. At this time the reaction was stopped by phenol/chloroform (50:50) extraction, followed by EtOH precipitation. The RNA pellet was washed with 70% EtOH, air dried and resuspended in 20 µl of DEPC-treated H<sub>2</sub>O. 5 µl of this RNA was electrophoresed on a 1.5% agarose, 2.2 M formaldehyde, 1X MOPS (3-(N-morpholino)propanesulfonic acid gel, as described by Sambrook *et al.*, (1989), to check the quantity and integrity of the sample. If a poly A tail was to be added to the RNA, then the remaining 15 µl of the RNA sample was added to a 85 µl reaction containing 100 µM rATP, 2.5 units of RNasin, 5 mM DTT, 1X Poly A buffer (50 mM Tris-HCl, pH 7.9, 10 mM MgCl<sub>2</sub>, 2.5 mM MnCl<sub>2</sub>, 250 mM NaCl) and 5 units of *E. coli* poly A polymerase (BRL) and incubated at 37° C for 2 hours. The reaction was terminated by phenol/chloroform (50:50) extraction. The aqueous phase was precipitated with 0.1 volumes of 3M K acetate, pH 5.2 and 3 volumes of EtOH. After centrifugation in a microfuge for 15 minutes at 4° C, the RNA pellet was washed with 70% EtOH, air dried and resuspended in 15 µl of DEPC-treated H<sub>2</sub>O. This procedure routinely produced between 10 to 15 µg of cRNA, which could be used for *Xenopus* oocyte injection or for *in vitro* translation.

## 2.3 DNA MANIPULATION

### 2.3.1 PCR Applications

#### 2.3.1.1 First-Strand cDNA Synthesis

Five micrograms of poly A+ mRNA, which had been heat denatured at 65° C for 10 minutes and then snap cooled, was added to a 25 µl reaction containing 1X transcription buffer (50 mM Tris-HCl, pH 8.3, 140 mM KCl, 10 mM MgCl<sub>2</sub>), 1 mM of each dNTP, 4 mM DTT (dithiothreitol) 13 units of RNasin, 1 nM of random hexanucleotides (Pharmacia) and 40 units of RAV-2 (avian myeloblastosis virus) reverse transcriptase. The reaction was incubated at 42° C for 2 hours followed by heat inactivation at 70° C for 10 minutes. The 1st strand cDNA was separated from the remaining hexanucleotides by adding 65 µl of DEPC-treated H<sub>2</sub>O, 0.1 volumes of 3 M Na acetate, pH 5.2 and 0.6 volumes of isopropanol to the reaction, followed by incubation on ice for 15 minutes and finally centrifugation in a microfuge for 20 minutes. This precipitation was repeated. The 1st strand cDNA pellet was washed in 70% EtOH, vacuum dried and resuspended in 25 µl of DEPC-treated H<sub>2</sub>O.

#### 2.3.1.2 PCR (Polymerase Chain Reaction) Amplification

PCR reactions were routinely conducted in a 50 µl volume containing 1X PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 10 to 20 pmoles each of a forward and reverse primer,

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2 mM MgCl<sub>2</sub>, 200 μM dNTPs, 1 to 5 μl of template and 0.3 units of Taq polymerase (Promega for degenerate PCR and Perkin Elmer Cetus for 3' and 5' termini modification).

Templates used in the PCR reaction were:

1. First strand cDNA
2. Plasmid DNA containing cloned GABA<sub>A</sub> receptor subunit cDNAs

### 2.3.1.3 Southern Blotting

Four μl of 6X agarose gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll 400) was added to 10 μl of PCR product prior to loading onto 1.0% agarose gels containing 0.5 μg/ml of EtBr. Following electrophoresis the gels were photographed, denatured in 1.5 M NaCl, 0.5 M NaCl for 30 minutes, rinsed briefly in dH<sub>2</sub>O and neutralized for two 15 minute intervals in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5. The DNA was transferred onto Hybond-N filters (Amersham International) in 10X SSC (1X SSC: 150 mM NaCl, 15 mM Na citrate) overnight according to the method of Southern (1976). After transfer the well positions were marked and the filter was washed briefly in 2X SSPE, air dried and UV cross-linked using a Statagene UV Stratalinker<sup>TM</sup>2400 (120 Joules/254 nm).

### 2.3.1.4 Filter Hybridisation

50 to 100 pmoles of oligonucleotide was labeled in a 50 μl reaction containing 1X kinase buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 100 μM spermidine-HCl, 100 μM EDTA), 30 μCi of [ $\gamma$ -<sup>32</sup>P]ATP and 10 units of T4 polynucleotide kinase (New England Biolabs). The reaction was incubated at 37° C for 2 to 3 hours and heat inactivated at 70° C for 10 minutes. The unreacted isotope was removed by spinning the sample through a Sephadex G25 column as outlined in Sambrook *et al.*, (1989) and the specific activity of the sample was determined by Cerenkov counting. The labeled probe was added to the hybridization solution (5X Denhardt's, 6X SSC, ) at a concentration of 2 x 10<sup>6</sup> dpm/ml and the hybridization was continued at 42° C for 24 hours. After hybridisation was completed, the filter was washed 2 times in 500 ml of 2 x SSC, 0.1% SDS, at room temperature, followed by two washes for 30 minutes each in 1 X SSC, 0.1% SDS, at 55° C. The filters were exposed to preflashed Fuji-RX X-ray film, with an intensifying screen, at -70° C for 2 to 24 hours.

## 2.3.2 Cloning

### 2.3.2.1 Preparation of Vectors

**Bluescript KS+ constructs:** 3 μg of vector was digested with either 10 units of *Eco*R1 or 10 units of both *Bam*H1 and *Xba*1 for 6 hours at 37° C, following the manufacturers specifications for the enzymes. The enzymes were heat inactivated by incubation at 70° C

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for 10 minutes followed by calf alkaline phosphatase (CIP) (Boehringer Mannheim) treatment as outlined by Sambrook *et al.*, (1989). The CIP was inactivated by phenol/chloroform (50:50) extraction and the vector DNA was EtOH precipitated, washed with 70% EtOH, vacuum dried and resuspended in 20  $\mu$ l of H<sub>2</sub>O. The bovine alpha ( $\alpha$ ) 1 and beta ( $\beta$ ) 1 cDNAs were isolated from the pSP65 constructs described by Schofield *et al.*, (1987), by restriction digestion of the alpha 1 construct with *EcoRI* and restriction digestion of the beta 1 construct with *BamHI* and *XbaI*. The fragments were isolated from a 0.8% TAE agarose gel and further purified using a Biorad Prep-a-gene kit, as outlined by the manufacturer. 100 ng of vector and approximately 50 to 100 ng of cDNA insert were added to a ligation reaction containing 1 X ligase buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, and 10 mM DTT), 1 mM rATP and 1 unit of T4 DNA ligase (Boehringer Mannheim). The reaction was incubated at 14<sup>o</sup> C for 24 hours. One quarter to one half of the reaction was transformed in to competent E. Coli TG1 cells and after incubation at 37<sup>o</sup> C for 30 to 60 minutes, with gently shaking, the transformed cells were plated onto 2YT agar plates containing 100  $\mu$ g/ml of ampicillin and the plates were incubated overnight at 37<sup>o</sup> C. Mini prep plasmid DNA was prepared as outlined in section 2.2.1.1 and the products were analysed by electrophoresis on a 0.8% agarose gel containing 0.5  $\mu$ g/ml of ethidium bromide and visualized under UV illumination.

**pCDM8 and pRcCMV constructs:** The vector DNA was prepared as described above, with the following exceptions. 3  $\mu$ g of vector DNA was digested with either 10 units of *HindIII* or 10 units of *XbaI*, at 37<sup>o</sup> C for 8 hours. The 5' and 3' termini of mutant and wild-type bovine alpha 1 and beta 1 cDNAs cloned into Bluescript KS+ were modified by PCR amplification with forward and reverse primers containing a *HindIII* restriction endonuclease site. 10 ng of template and 10 pmoles of each primer were included in a PCR reaction as outlined in section 2.3.1.2. The reaction was phenol/chloroform (50:50) extracted and isopropanol precipitated. An aliquot of the products was checked on a 0.8% 1X TAE agarose gel, prior to purification of the remaining products with a Prep-a gene kit (Pharmacia), as specified by the manufacturer. The products were then incubated in a 50  $\mu$ l reaction with 40 units of *HindIII*, overnight at 37<sup>o</sup> C. The enzyme was heat inactivated by incubation at 70<sup>o</sup> C for 10 minutes. One fifth of the digested product was added to a ligation reaction as outlined above. In the case of pCDM8 the ligated products were transformed into competent MC1061 cells and the transformed cells were plated onto 2YT agar plates containing 100  $\mu$ g/ml of ampicillin and 12.5  $\mu$ g/ml of tetracycline.

**M13mp18 constructs:** Vector DNA was prepared as described above with the following exception. M13mp19 DNA was digested with 10 units each of *BamHI* and *SacI* for 6 hours at 37<sup>o</sup> C. 5  $\mu$ l of 1st strand cDNA and 20 pmoles of both forward and reverse degenerate

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primers were included in a PCR reaction as outlined in section 2.3.1.2. 10  $\mu$ l of each PCR reaction was run on a 0.8% LMP agarose gel and the products were excised under UV illumination. The resulting gel slices were incubated at 70 $^{\circ}$  C for 15 minutes and 5  $\mu$ l of the melted slices was added to a 15  $\mu$ l restriction reaction containing 10 units of both *Bam*HI and *Sac*I. This reaction was incubated at 37 $^{\circ}$  C, overnight. The enzymes were heat inactivated and 5  $\mu$ l of this reaction was added to 15  $\mu$ l of a ligation reaction as outlined above. After 24 hours at 14 $^{\circ}$  C one half of the ligation reaction was transformed into competent TG1 cells and the transformed cells were pipetted into 3 ml of prewarmed top agar (50 $^{\circ}$  C) containing 20  $\mu$ l of 20mg/ml IPTG (isopropylthio- $\beta$ -D-galactoside) and 20  $\mu$ l of 20 mg/ml Xgal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside). The 3 ml of top agar was then poured onto 2YT agar plates, which were incubated at 37 $^{\circ}$  C overnight.

### 2.3.2.2 Competent Cells

A single colony of cells was inoculated into 100 ml of 2YT and grown at 37 $^{\circ}$  C in a New Brunswick incubator to an OD600 of 0.6 to 0.8. The cells were harvested by centrifugation, at 4 $^{\circ}$  C in a Centronix clinical centrifuge at 3,000 rpm for 10 minutes. The cell pellet was resuspended in 20 ml of ice cold 50 mM CaCl<sub>2</sub> and left on ice for 2 hours. The cells were again harvested by centrifugation at 4 $^{\circ}$  C in a Centronix clinical centrifuge at 3,000 rpm for 10 minutes and then resuspended in 7 ml of ice cold 50 mM CaCl<sub>2</sub>. After preparation the competent cells could routinely be used for up to 5 days.

### 2.3.3 Site-Directed Mutagenesis

A single colony of *E. coli* CJ236 transformed cells was inoculated into 10 mls of 2YT medium containing 100  $\mu$ g/ml ampicillin and 30  $\mu$ g/ml chloramphenicol and shaken in a New Brunswick incubator at 300 rpm. After three hours, the culture was inoculated with 2 X 10<sup>7</sup> pfu of K07 helper phage and incubated at 37 $^{\circ}$ C for a further 8 hours. Single-stranded DNA was prepared as described in section 2.2.1.3.2. The single-stranded DNA was analyzed on a 0.8% TAE agarose gel and quantitated by measuring the absorbance at 260 nm. Primers for mutagenesis were phosphorylated at the 5' terminus. 100 pmoles of mutagenic oligonucleotide (10 pmoles/ml) was added to 1mM rATP, 5mM DTT, 50 mM Tris-HCl, pH 8, 10 mM MgCl<sub>2</sub> and 10 units of T4 polynucleotide kinase (Biolabs) and incubated for 30 minutes at 37 $^{\circ}$  C. The enzyme was heat inactivated at 70 $^{\circ}$  C for 10 minutes. To anneal template to primer, 1 to 2  $\mu$ g of template and 5 pmoles of primer in 20 mM Tris-HCl, pH 7.4, 2 mM MgCl<sub>2</sub> and 50 mM NaCl (final volume 10  $\mu$ l) were heated in a 0.5 ml eppendorf tube placed a water-filled 15 ml polypropylene culture tube at 70 $^{\circ}$  C. One microlitre of dH<sub>2</sub>O replaced the 5 pmoles of primer as the control to determine the amount of unsubstituted template present in the reaction. After two minutes the culture tube was removed and the reaction was allowed to cool to room temperature. After cooling, the

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eppendorf tubes were removed from the culture tubes, spun briefly in an eppendorf centrifuge and placed on ice. One microlitre of 10X synthesis buffer (5 mM of each dNTP, 10 mM rATP, 100 mM Tris-HCl, pH 7.4, 50 mM MgCl<sub>2</sub> and 20 mM DTT ), 10 units of T4 DNA polymerase (Biolabs), and 5 units of T4 DNA ligase (Boehringer Mannheim) were added to the annealed reaction and left on ice for 5 minutes prior to incubation at room temperature for 5 minutes and finally at 37° C for 90 minutes. The reaction was stopped by the addition of 90 µl of dH<sub>2</sub>O and freezing at -20° C. Ten microliters of the mutagenesis reaction was used to transform competent *E.coli* TG2 cells prepared as described in section 2.3.3.2. The transformed cells were plated on 2YT agar plates with 100 µg/ml ampicillin and grown at 37° C, overnight. Only unsubstituted templates and newly synthesized template can be replicated in TG2 cells.

### 2.3.4 DNA Sequencing

A Sequenase II (USB) DNA sequencing kit was used to sequence PCR products cloned into the Bluescript KS+ vector. Direct sequencing, which is a PCR-based method essentially as outlined by Lasham *et al.*, (1992) was used to verify cDNAs cloned into the pCDM8 and pRcCMV expression vectors.

### 2.4 Transient Expression in 293 Cells

293 cells were plated at a concentration of 4 X 10<sup>6</sup> cells per 10 centimetre plate in 10 mls of Dulbecco's MEM /Nutrient Mis, F12 (1:1), supplemented with 1% L-glutamine, 1X streptomycin/penicillin and 10 % fetal calf serum and incubated overnight at 37° C in a 5 % CO<sub>2</sub> incubator. After overnight incubation, plates of cells which had reached approximately 80 % confluence were used for transfections. 15 µg of each cDNA construct was added to 0.5 ml of 250 mM CaCl<sub>2</sub> and mixed gently prior to the addition of 0.5 ml of 2X BBS buffer, pH 6.95 (50 mM N-NBis(2-hydroxy-ethyl)-2-amino-ethanesulfonic acid, 280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>). After incubation at room temperature for 15 minutes, 1 ml of the calcium phosphate/DNA was added per plate of cells and incubated for 48 hours at 37° C in a 3% CO<sub>2</sub> incubator.

Transfected 293 cell were harvested after 48 hours by washing the cell 2X in ice cold TBS (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.6 mM KCl). The cells were transferred into 10 ml of TBS and collected at 4° C by centrifugation, for 10 minutes at 5K rpm in a clinical centrifuge. The cells were lysed by homogenisation in 25 mM Tris buffer. The membranes were collected by centrifugation in a Sorvall SS34 rotor at 20K rpm for 20 minutes at 4° C. The membrane pellet was washed 3X in ice cold TBS and resuspended to a final concentration of 1 mg/ml in ice cold TBS.



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## 2.5 S1 Nuclease Protection

The S1 nuclease protection assay is essentially as outlined by O'Donovan et al, (1991).

### 2.5.1 Oligonucleotide Preparation

Oligonucleotides used for S1 nuclease protection were gel purified. Approximately, one third of an oligonucleotide synthesis was suspended in formamide loading buffer, without bromophenol blue or xylene cyanol and heated at 85° C for 10 minutes prior to loading onto a prerun 10% polyacrylamide gel. The gel was run until the bromophenol blue dye was approximately two thirds of the distance from the top of the gel. The major product was visualized by UV shadowing and excised from the gel. The oligonucleotide was eluted from the gel slice by soaking the slice in 3M ammonium acetate, pH 5.4, for 3 to 8 hours. The eluted oligo was desalted over a C18 sep-pack, which had been pretreated with 100 percent acetonitrile and washed with 10 mls of H<sub>2</sub>O. The bound oligonucleotide was eluted from the column with a 60:40 mixture of acetonitrile:H<sub>2</sub>O, dried in a Speedvac and resuspended in 50 µl of DEPC-treated H<sub>2</sub>O prior to quantitation at 260 nm.

### 2.5.2 S1 Nuclease Reaction

10 pmoles of purified oligonucleotide were end labelled with polynucleotide kinase and  $\gamma^{32}\text{P}$ -ATP in a reaction outlined in section 2.3.3.1 for 1 hour, at 37° C. 5 to 40 µg of total RNA and an equal amount of tRNA were ethanol precipitated and the pellets after centrifugation were air dried. The pelleted RNA was resuspended in 30 µl of hybridization buffer (probe concentration: 1 pmole/ml in 0.4M NaCl, 40 mM Hepes, pH 6.4 and 1 mM EDTA), heated at 90° C for 3 minutes and incubated at 70° C for 2 hours. Excess probe was removed by the addition of 300 µl of S1 nuclease buffer (S1 nuclease 120 U/ml, 4.5 mM zinc sulphate, 50 mM sodium acetate, pH 5.2, 0.3 M sodium chloride and 10 µg/ml single stranded DNA) and digestion at 37° C for 15 minutes. 300 µl of the reaction was transferred to a fresh tube and inactivated with 48 µl of 4 M ammonium acetate, 0.1 M EDTA. Double-stranded hybrids were precipitated with ethanol, resuspended in 20 µl of formamide running buffer and denatured at 90° C for 2 minutes. The denatured samples were loaded onto a pre-electrophoresed 10% polyacrylamide gel and run until the bromophenol blue band was approximately two thirds of the distance from the top of the gel. The gels were fixed and dried prior to exposure to Kodak X-Omat film from one to twenty four hours, at -70° C, with an intensifying screen.

## 2.6 Localization of mRNA distribution in mouse brain

Messenger RNA (mRNA) hybridization (*in situ* hybridisation) was performed essentially as in Wisden *et al.*, (1992).

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### 2.6.1 Section preparation

Mice were killed by spinal dislocation and their brains were rapidly removed, frozen on dry ice, wrapped in parafilm (Fisons) to prevent freeze-drying, and stored at  $-70^{\circ}\text{C}$  until sections were ready to be prepared. Horizontal serial sections ( $12\mu\text{m}$ ) were cut, using a cryostat, and thaw-mounted onto poly-L-lysine-coated slides. Sections were dried for 30 min at room temperature before being fixed for 5 min in 4% (w/v) paraformaldehyde in 1 x phosphate-buffered saline (PBS; 130 mM sodium chloride, 7 mM disodium hydrogen orthophosphate, 3 mM sodium dihydrogen orthophosphate). After washing twice for 2 min in 1 x PBS, sections were dehydrated for 5 min in 70% (v/v) ethanol and 5 min in 95% (v/v) ethanol, and stored in 95% (v/v) ethanol at  $4^{\circ}\text{C}$  until use.

### 2.6.2 Oligonucleotide probes

Transcript-specific, antisense, 45-base oligonucleotide probes (Table 2.3) were 3' end-labelled using terminal deoxynucleotidyl transferase (Pharmacia) and [ $\alpha$ - $^{35}\text{S}$ ]dATP (1300 Ci/mmol, New England Nuclear) to a specific activity of  $\sim 10^9$  dpm/mg. Labelled probes were purified on a 1-ml Sephadex G-25 spun-column (Sambrook *et al.*, 1989) prior to use, to remove unincorporated radiolabelled nucleotides.

### 2.6.3 mRNA localization

All hybridisations were standardized such that each contained  $2.0 \times 10^3$  dpm/ml hybridization buffer (50% (v/v) formamide, 4 x SSC, 25 mM sodium phosphate (pH 7.0), 1 mM sodium pyrophosphate, 20 mM dithiothreitol, 5 x Denhardt's solution, 10% (w/v) dextran sulphate, 200  $\mu\text{g}/\text{ml}$  acid-alkali hydrolyzed salmon sperm DNA, 100  $\mu\text{g}/\text{ml}$  polyadenylic acid, 120  $\mu\text{g}/\text{ml}$  heparin). Sections were hybridized in 200 $\mu\text{l}$  buffer, under parafilm coverslips, overnight at  $42^{\circ}\text{C}$ . Control sections were hybridized in the presence of a 30-fold molar excess of the appropriate unlabelled oligonucleotide. After hybridisation, coverslips were removed in 1 x SSC at room temperature, and the sections were washed in 0.3x SSC for 60 min at  $55^{\circ}\text{C}$ . They were rinsed at room temperature in 1x SSC, followed by a rinse in 0.1x SSC, dehydrated sequentially in 70% (v/v) and 95% (v/v) ethanol and air-dried. All wash solutions contained 10 mM dithiothreitol. Sections were exposed to Kodak X-Omat film at room temperature for 15 days before being dipped in Ilford K-5 emulsion. After exposure for 50 days at  $4^{\circ}\text{C}$ , sections were processed in Kodak D19 developer for 2 min at  $17^{\circ}\text{C}$ , briefly rinsed in distilled water, fixed in 30% (w/v) sodium thiosulphate for 4 min, and finally washed in distilled water for 10 min. Sections were dried overnight at room temperature before being counterstained with 0.05% (w/v) thionin, differentiated in an ascending ethanol series (70%, 95% and 100% (v/v)), cleared in Histo-clear (National Diagnostics), and mounted in DPX (BDH).

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#### 2.6.4 Photography

Autoradiographic images generated on X-ray film were printed with an enlarger directly onto Kodak Kodabrome II RC photographic paper (grade F5 M), giving reverse images. All sections were photographed onto Kodak Tech-Pan film using a Leitz microscope and 35mm camera with lightfield and darkfield condensers at 200 ASA and 1600 ASA settings respectively. Negatives were processed using Kodak HC-110 developer at 20°C for 4.5 minutes. Both lightfield and darkfield negatives were printed onto Kodak Kodabrome II RC photographic paper (grade F5 M). Scale bars were determined using a precalibrated graticule.

# **Chapter 3**

## **Developmental Expression I**

### 3.1 Introduction

Development of the mouse nervous system begins during embryogenesis and is completed during early postnatal development. During the postnatal period, activity-dependent processes play a critical role in cell migration, differentiation and division, and synapse formation. Activity-dependent development has been extensively investigated in cortical structures where the normal progression of synapse formation can be disrupted either by sensory deprivation or by neuropharmacological manipulations, such as tetrodotoxin implants or chronic treatment with NMDA receptor antagonists. During embryonic development, trophic factors and cell adhesion molecules play an important role in cell differentiation, and the growth and guidance of neuronal processes. Recently, however, studies on embryonic development have also focused on molecules more normally associated with synaptic transmission in the adult central nervous system. Ion channels, particularly potassium channels, have recently been implicated in the processes of neuronal differentiation in cultured neurons (Jones and Ribera, 1994), and in granule cell migration *in vivo* in the autosomal recessive mouse mutant *weaver*. Neurotransmitters and their appropriate receptor complexes may also play an important part in neuronal development. Concentration gradients of neurotransmitter can in some circumstances, play an important role in growth cone guidance. Since GABA is the major inhibitory neurotransmitter of the adult central nervous system it is reasonable to suppose that GABA might also play a role during nervous system development.

Several developmental studies of the nervous system including the superior cervical ganglia and cerebral cortex, have demonstrated that GABA can exhibit trophic activities (Wolff, Joo and Dames, 1978; Wolff, Rickman and Chronwall, 1979). Furthermore, studies on the effects of GABA and GABA receptor agonists and antagonists in neuroblastoma cell cultures and on certain neuronal cell types in the retina, cortex and cerebellum have demonstrated a major role for GABA in synapse formation and cell differentiation (Spoerri and Wolff, 1981; Spoerri, 1988; Hansen *et al.*, 1987). GABA-induced chemotaxis has been demonstrated in rat embryonic spinal cord cultures where neurons migrate toward picomolar concentrations of NGF and femtomolar concentrations of GABA beginning at embryonic day 13 (Behar *et al.*, 1994). The neurons in the microchemotaxis assay exhibited chemotactic responses to NGF, while GABA stimulated chemokinesis (increased random movement). GABA chemokinesis was mimicked by muscimol and inhibited by bicuculline and picrotoxin, suggesting GABA motility signals were mediated by GABA receptors (Behar *et al.*, 1994). Immunohistochemical analysis of GABA immunoreactivity in E13 spinal cord sections demonstrated that GABA localised in fibers close to the target destinations of migrating neurons, suggesting diffusible gradients of GABA for migratory cells (Behar *et al.*, 1994). GABA stimulated chemokinesis involves both GABA<sub>A</sub> and GABA<sub>B</sub> signaling pathways (Behar *et al.*, 1995). In the embryonic and

postnatal CNS, GABA<sub>A</sub> receptors have been demonstrated using biochemical, electrophysiological and immunocytochemical methodologies (Meier *et al.*, 1991; Fiszman *et al.*, 1990; Killisch *et al.*, 1991).

Although several *in situ* hybridization studies have examined the expression of GABA<sub>A</sub> receptor subunit mRNAs during embryogenesis, the starting point for such studies has been E15.5 in the rat, a time point that corresponds to approximately E13.5 in the mouse (Wisden *et al.*, 1992; Poulter *et al.*, 1993). This chapter is a preliminary investigation into the developmental pattern of GABA<sub>A</sub> receptor subunit mRNA expression and describes the early embryonic detection of GABA<sub>A</sub> receptor subunit mRNAs in the mouse, using degenerate RT-PCR and Southern blot analysis. Quantitation of various GABA<sub>A</sub> receptor subunit mRNAs was accomplished using S1 nuclease protection analysis, during late embryonic and throughout postnatal CNS development.

## 3.2 Results

### 3.2.1 Experimental Rationale

To identify the presence of GABA<sub>A</sub> receptor subunit mRNAs during early stages of nervous system development, it was necessary to use reverse transcription (RT)-PCR, due to the very small percentage of nervous system tissue present in the embryo during early embryogenesis. Degenerate oligonucleotides were designed based on rat sequences and were used in the RT-PCR experiments because of the lack of murine sequences for GABA<sub>A</sub> receptor subunits at the time of the experiment. Southern blot hybridization analysis was used to identify specific GABA<sub>A</sub> receptor subunit sequences amplified from the PCR reactions.

To identify specific GABA<sub>A</sub> receptor subunit sequences derived from PCR amplification, forward and reverse PCR primers were designed to amplify a region of nonhomology among all GABA<sub>A</sub> receptor subunit cDNAs. Among GABA<sub>A</sub> receptor subunit cDNA sequences, the putative intracellular loop between transmembrane domains 3 and 4 is the most heterogeneous and therefore an excellent target sequence for the specific identification of GABA<sub>A</sub> receptor subunit mRNAs. In contrast, the sequences encoding transmembrane domains 3 and 4 are highly conserved among GABA<sub>A</sub> receptor subunits and therefore are excellent regions for the design of degenerate oligonucleotide PCR primers. Degenerate oligonucleotides were therefore designed to sequences within transmembrane domain 3 (forward primers) and transmembrane domain 4 (reverse primers), as illustrated in Figure 3.1.

### 3.2.2 Degenerate Oligonucleotide Design

Oligonucleotides for PCR had temperatures of melting (T<sub>m</sub>s) between 58°C and 74°C, as determined by the formula ( $2^{\circ}\text{C} \times \# \text{ of A/T nucleotides} + 4^{\circ}\text{C} \times \# \text{ of G/C nucleotides}$ ). The

difference in thermal stability between A/T and G/C base pairs is based on the higher degree of hydrogen bonding between G/C base pairs. The degree of degeneracy of each oligonucleotide is the product of the number of bases present at each degenerate site. The degree of degeneracy ranged from 64 to 192. As illustrated in Figure 3.1 amino acid alignments were used initially to determine the most homologous regions among rat receptor subunit sequences and then the corresponding DNA sequences were selected based on the above mentioned criteria ( $T_m$  and degree of degeneracy). Only one set of degenerate primers for each of the  $\beta$  (1,2,3) and  $\gamma$ (1,2,3) subunit alignments was required to meet both the  $T_m$  and degeneracy guidelines. In the case of the  $\alpha$  subunit sequences, however, two reverse primers were required to maintain the limitations on the degree of sequence degeneracy for the PCR primers (listed in Table 2.4). As well as the GABA<sub>A</sub> receptor subunit sequences, each PCR primer also contains one restriction endonuclease site followed by six nucleotides. The six nucleotides at the end of each primer are positioned such that the restriction sites are not at the immediate ends of the PCR products. This positioning is based on optimization of cleavage (NewEngland Biolabs, technical notes) from an end with the restriction enzymes listed above. The restriction sites for *EcoRI* (forward primers) and *SacI* (reverse primers) are used for directional subcloning of PCR products into the phage vector, M13mp18 for subsequent sequence analysis.

### 3.2.3 RT-PCR and Southern blot analysis of GABA<sub>A</sub> receptor subunit mRNAs

First strand cDNA was reverse transcribed with RAV-2 reverse transcriptase from either total or polyA<sup>+</sup> mRNA preparations as described in Methods 2.3.1.1. Polymerase chain reactions contained 5  $\mu$ l of the first strand cDNA, 0.3 units of Taq polymerase (Promega), 10 pmoles of each of a degenerate forward and reverse primer, in a volume of 50  $\mu$ l with final concentrations of 2 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 50 mM KCl, and 20 mM Tris-HCl. The reactions were heat denatured for 5 minutes prior to 30 cycles of 1 minute at 90°C, 2 minutes at 50°C and 1 minute at 72°C. After the 30 cycles were completed, one final extension was performed at 72°C for 7 minutes. The PCR products were electrophoresed on a 0.8% LMP agarose gel and subcloned into M13mp18 as described in Methods 2.3.2.1 and 2.3.2.2. Blue/white selection was used to choose plaques for subsequent DNA sequencing experiments. Introduction of foreign DNA into the multiple cloning sites of the M13 vector results in interruption of complementation provided by the  $\alpha$  fragment of the  $\beta$  galactosidase gene carried by the M13 plasmid. Single stranded M13mp18 template DNA was prepared as described by Vieira and Messing (1987) and dideoxynucleotide sequencing was performed using a Sequenase II DNA sequencing kit (USB) and the M13 universal forward sequencing primer.





alignments of the corresponding rat cDNA sequences. The rat  $\gamma 1$  amino acid sequence is above the DNA alignment for codon reference. A consensus sequence is listed below the alignment and includes sites of degeneracy. Asterisks are placed above the sequence used in the PCR primer and the degree of degeneracy (96X) is calculated below the consensus sequence.

Figure 3.2 illustrates the PCR products detected by ethidium bromide staining of the electrophoresed 0.7% agarose gels. As shown in Figure 3.2 the presence and amount of PCR product generated was dependent on the primer set used. For example, in PCR reactions where degenerate oligonucleotides for the detection of GABA<sub>A</sub> receptor  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\alpha 5$  subunit mRNAs were used, ethidium bromide staining detected PCR products at all developmental stages studied. In contrast, in PCR reactions where degenerate oligonucleotides were used for the detection of GABA<sub>A</sub> receptor  $\alpha 4$  and  $\alpha 6$  mRNAs, only PCR products from adult first strand cDNA were visualized by ethidium bromide staining. PCR products are present at varying amounts in all developmental stages tested from PCR reactions incorporating degenerate oligonucleotides for the detection of GABA<sub>A</sub> receptor  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$  subunit mRNAs. There was no detectable ethidium bromide staining of PCR products derived from the amplification of first strand templates derived from E7.5, E8.5, E12 and E14 total mRNAs using degenerate oligonucleotides designed for the detection of GABA<sub>A</sub> receptor  $\gamma 1$ ,  $\gamma 2$ , and  $\gamma 3$  subunit mRNAs (Figure 3.2).

Subunit	Gestation Days				Postnatal Days				
	7.5	8.5	9.5	12	14	1	7	14	Adult
$\alpha 1$	+	+	+	+	+	+	+	+	+
$\alpha 2$	+	+	+	+	-	+	-	+	-
$\alpha 3$	+	+	+	+	+	+	+	+	+
$\alpha 4$	-	-	+	-	-	+	+	+	+
$\alpha 5$	-	-	-	-	-	+	+	+	+
$\alpha 6$	-	-	+	+	+	+	+	+	+
$\beta 1$	-	-	+	+	+	+	+	+	+
$\beta 2$	-	-	-	-	-	+	+	+	+
$\beta 3$	-	-	-	-	+	-	+	+	+
$\gamma 1$	-	-	-	-	-	+	-	+	+
$\gamma 2$	+	+	+	+	+	+	+	+	+
$\gamma 3$	-	-	-	-	-	-	-	+	+

**Table 3.1** Southern blot analysis of RT-PCR detection experiments for GABA<sub>A</sub> receptor subunit mRNA expression during murine CNS

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**development.** Hybridisation signals were obtained from Southern blot analysis with oligonucleotide probes specific for the GABA<sub>A</sub> receptor  $\alpha 1$ -,  $\alpha 2$ -,  $\alpha 3$ -,  $\alpha 4$ -,  $\alpha 5$ -,  $\alpha 6$ -,  $\beta 1$ -,  $\beta 2$ -,  $\beta 3$ -,  $\gamma 1$ -,  $\gamma 2$ -, and  $\gamma 3$ -subunit cDNAs and are scored as positive (+) or negative (-).

Further analysis of the DNA products generated from degenerate-oligonucleotide driven PCR are shown in Figures 3.3 through 3.6 and results are summarized in Table 3.1. Figure 3.2 shows that although there was abundant product from each amplification reaction, as evidenced by ethidium bromide staining, not all subunit cDNAs to which the degenerate oligonucleotides were designed, are detectable at each stage in development. In Figure 3.3, the  $\alpha 1$  GABA<sub>A</sub> receptor subunit mRNA was represented throughout development, while Southern blot analysis failed to detect the  $\alpha 2$  subunit mRNA of the GABA<sub>A</sub> receptor in E14, P7 and adult first strand cDNA templates. The  $\alpha 3$  subunit mRNA of the GABA<sub>A</sub> receptor was detected throughout embryonic and postnatal development, by Southern blot analysis. In Figure 3.4, a P<sup>32</sup>-labeled rat  $\alpha 4$  GABA<sub>A</sub> receptor subunit oligonucleotide probe detected specific murine  $\alpha 4$  subunit mRNA amplified from the E8.5, P0, P7, P14 and adult first strand templates. The  $\alpha 6$  rat GABA<sub>A</sub> receptor subunit oligonucleotide probe detected mRNAs amplified from the E9.5, E12.5, E14.5, P0, P7, P14 and adult first strand templates (Figure 3.4), while the  $\alpha 5$  GABA<sub>A</sub> receptor subunit oligonucleotide probe only detected  $\alpha 5$  subunit mRNA amplified from postnatal and adult first strand templates (Figure 3.4).

In Figure 3.5, Southern blot analysis detected murine  $\beta 1$  GABA<sub>A</sub> receptor subunit mRNA amplified from E12.5, E14.5, P0, P7, P14 and adult first strand cDNA templates. A  $\beta 2$  specific subunit mRNA was amplified from postnatal and adult first strand templates (Figure 3.5), while a  $\beta 3$  specific GABA<sub>A</sub> receptor subunit mRNA was identified from the amplification of E14, P7, P14 and adult first strand cDNA templates (Figure 3.5).

Southern blot analysis with a rat  $\gamma 1$  GABA<sub>A</sub> receptor subunit probe identified murine  $\gamma 1$  subunit specific mRNA from amplification of P0, P14 and adult first strand cDNA templates (Figure 3.6). Murine  $\gamma 2$  GABA<sub>A</sub> receptor subunit mRNA was detected in all first strand cDNA templates amplified (Figure 3.6), while a  $\gamma 3$  GABA<sub>A</sub> receptor subunit mRNA was detected in DNA amplified from P14 and adult cDNA first strand templates, only (Figure 3.6). Southern blot analysis with rat  $\alpha 6$  and  $\beta 2$  GABA<sub>A</sub> receptor subunit oligonucleotide probes detected more than one PCR product in DNA amplified from P14 ( $\alpha 6$ ) and adult ( $\alpha 6$ ,  $\beta 2$ ) first strand cDNA templates (Figures 3.4 and 3.5).

### 3.2.4 Sequence Analysis of Embryonic PCR products

#### Partial Mouse $\alpha 1$ GABA<sub>A</sub> receptor subunit cDNA sequences

L I K K N N T Y A P T A T S Y T P N L  
 A1.1 TCTCATTAAGAAAAACAACACATATGCTCCTACAGCAACCAGCTATAACCCCTAACTTA  
 A1.2 TCATTAAGAAAAACAACACATATGCTCCTACAGCAACCAGCTATAACCCCTAACTTA  
 A1.3 TCATTAAGAAAAACAACACATATGCTCCTACAGCAACCAGCTATAACCCCTAACTTA  
 A1.4 TCTCATTAAGAAAAACAACACATATGCTCCCACAGCAACCAGCTATAACCCCTAACTTA  
 A1.5 TCTCATTAAGAAAAACAACACATATGCTCCTACAGCAACCAGCTATAACCCCTAACTTA

A R G D P G L A T I A K S A T I E P K E  
 A1.1 GCCAGGGGTGACCCCGGCTTGGCAACTATTGCTAAAAGTGCGACCATAGAACCGAAAG  
 A1.2 GCCAGGGGTGACCCCGGCTTGGCAACTATTGCTAAAAGTGCGACCATAGAACCGAAAG  
 A1.3 GCCAGGGGTGACCC GGCTTGGCAACTATTGCTAAAAGTGCGACCATAGAACCGAAAG  
 A1.4 GCCAGGGGTGACCCCGGCTTGGCAACTATTGCTAAAAGTGCGACCATAGAACCGAAAG  
 A1.5 GCCAGGGGTGACCCCGGCTTGGCAACTATTGCTAAAAGTGCGACCATAGAACCGAAAG

Y K P E T K P P E P K K T F N S V S K  
 A1.1 AAGTCAAGCCTGAGACAAAACCACCAGAACCCAAGAAAAC TTTAACAGCGTCAGCAA  
 A1.2 AAGTCAAGCCTGAGACAAAACCACCAGAACCCAAGAAAACCTTTAACAGCGT AGCAA  
 A1.3 AAGTCAAGCCTGAGACAAAACCACCAGAACCC  
 A1.4 AAGTCAAGCCTGAGACAAAACCACCAGAACCCAAGAAAACCTTTAACAGCGTCAGCAA  
 A1.5 AAGTCAAGCCTGAGACAAAACCACCAGAACCCAAGAAAACCTTTAACTGCGTCAGCAA

I D R L S R I A F  
 A1.1 AATCGACCG CTG T AAGAATAGC TT  
 A1.2 AATCGACCGACTG TCAAGAATAGCCTT  
 A1.3  
 A1.4 AATCGACCGACTGCTCAAGAATAGCCTTTCG  
 A1.5 AATCGACCG CTG T

#### Partial Mouse $\alpha 3$ GABA<sub>A</sub> receptor subunit cDNA sequences

M T T L S I S A R N S L P K V A Y A T A  
 A3.1 ATGACAACGCTGAGTATCAGTGCCAGAACTCTTTACCTAAAGTGGCATAACGCGACGG

M D W E M A V C Y A F V F S A L I E F  
 A3.1 CCATGGACTGGTTTCATGGCCGTCTGTTATGCCTTTGTATTTTCTGCACTGATTGAATT

A T V N Y F T K R S W A W E G K K  
 A3.1 TGCTACTGTCAACTACTTCACCAAGCGGAGTTGGGCTTGGGAAGGCAAGAAGG

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**Partial Mouse  $\beta 1$  GABA<sub>A</sub> receptor subunit cDNA sequences**

K I P Y V K A I D I Y L M G C F V F V  
 B1.1 GAAGATCCCTTACGTCAAAGCGATTGACATCTATCTCATGGGCTGTTTTGTGTTTGTG  
 B1.2 GAAAGCGATTGACATCTATCTCATGGGCTGTTTTGTGTTTGTG  
 B1.3  
  
 F L A L L E Y A F V N Y I F F G K G P Q  
 B1.1 TTCCTGGCTCTACTGGAATATGCTTTTGTCAATTACATTTTCTTCGGAAAAGGCCCTC  
 B1.2 TTCCTGGCTCTACTGGAATATGCTTTTGTCAATTACATTTTCTTCGGAAAAGGCCCTC  
 B1.3 TTCTTCGGAAAAGGCCCTC  
  
 K K G A S K Q D Q S A N F K N R L E M  
 B1.1 AGAAAAGGGAGCGAGCAAACAAGACCAGAGTGCCAATGAAAAGAACAGACTGGAGAT  
 B1.2 AGAAAAGGGAGCGAGCAAACAAGACCAGAGTGCCAATGAAAAGAACAGACTGGAGAT  
 B1.3 AGAAAAGGGAGCGAGCAAACAAGACCAGAGTGCCAATGAAAAGAACAGACTGGAGAT  
  
 N K V Q V D  
 B1.1 GA CAAAGTC AGTTGTGCCACGCA TATCTCT  
 B1.2 GAAACACAGTCCAAGTTGACCCACGCAATATCTCTCA  
 B1.3 GAA CAAAGTC AGTTGACGCACGCAATATCTCTCA

**Partial Mouse  $\beta 3$  GABA<sub>A</sub> receptor subunit cDNA sequences**

K I P Y V K A I D M Y L M G C F V F V  
 B3.1 AAAATTCCCTATGTCAAAGCCATCGACATG TACCTGATGGGCTGCTTTGGTCTTTGT  
 B3.2 AAAGCCATCGACATGATACCTGATGGGCTGCTTTG TCTTTTG  
 B3.3 AAAATTCCCTATGTCAAAGCCATCGACATG TACCTGATGGGCTGCTTTG TCTTTGT  
 B3.4 AAAATTCCCTATGTCAAAGCCATCGACATG TACCTGATGGGCTGCTTTG TCTTTGT  
 B3.5 AAAATTCCCTATGTCAAAGCCATCGACATG TACCTGATGGGCTGCTTTG TCTTTGT  
  
 F I A L L E Y A F V N Y I F F G R G P  
 B3.1 ATTCCTGGCACTTCTGGAGTATGCCTTTGTCAACTACATTTTCTTTGGAAGAGGTTC  
 B3.2 TATTCCTGGCACTTCTGGAGTACGCCTTTGTCAACTACATTTTCTTTGGAAGAGGTCC  
 B3.3 ATTCCTGGCACTTCTGGAGTACGCCTTTGTCAACTACATTTTCTTTGGAAGAGGTCC  
 B3.4 ATTCCTGGCACTTCTGGAGTATGCCTTTGTCAACTACATTTTCTTTGGAAGAGGTCC  
 B3.5 ATTCCTGGCACTTCTGGAGTATGCCTTTGTCAACTACATTTTCTTTGGAAGAGGTCC  
  
 Q R Q K K L A E K T A K A K N D R S K  
 B3.1 CCCAAAGGCAGAAGAAGCTTGCGGAGAAGACAGCCAAGGCCAAGAATGATCGTTCTA  
 B3.2 CCAAAGGCAGAAGAAGCTTGCGGAGAAGACAGCCAAGG AAGAATGATCGT  
 B3.3 CAAAGGCAGAAGAAGCTTGCG AGAAGACAGCCAAGGCCAAGAATGATCGT CTA  
 B3.4 CAAAGGCAGAAGAAGCTTGCGGAGAAGACAGCCAAGGCCAAGAATGATCG  
 B3.5 CAAAGGCAGAAGAAGCTTGCGGAGAAGACAGCCAAGGCCAAGAATGATGCTTCTA

S E I N R V D A H G N  
 B3.1 AGAGTCAAATAAACCCGGGTGGATGCTCACGGGAAT  
 B3.2  
 B3.3 AGAG  
 B3.4  
 B3.5 AGAGTCAAATAAAC GGTGGATGCTCACGG AATA

### Partial Mouse $\gamma 1$ GABA<sub>A</sub> receptor subunit cDNA sequences

T L I P M N T I S L P Q G F D D Y G Y  
 G1.1 ACT CTCATTCCCATGAACACCATTTCTTTGCCACAAGGGGAAGATGATTATGGCTAC  
 G1.2 ACTTCTCATTCCCATGAACAGCATTCTTTGCCACAAGGGGAAGATGATTATGGCTAC  
 G1.3 ACT CTCATTCCCATGAACAGCATTCTTTGCCACAAGGGGAAGATGATTATGGCTAC  
 G1.4 ACT CTCATTCCCATGAACAGCATTCTTTGCCGCAAGGGGAAGATGATTATGGCTAC

Q C L E G K D C T S F F C C F D G C R T  
 G1.1 CAGTGTTTGGAGGGCAAAGACTGTACTAGCTTTTTCTGTTGCTTGCATGACTGCAGAA  
 G1.2 CAGTGTTTGGAGGGCAAAGACTGTACTAGCTTTTTCTGTTGCTTCGATGACTGCAGAA  
 G1.3 CAGTGTTTGGAGGGCAAAGACTGTACTAGCTTTTTCTGTTGCTTCGATGACTGCAGAA  
 G1.4 CAGTGTTTGGAGGGCAAAGACTGTACTAGCTTTTTCTGTTGCTTCGATGACTGCAAAA

G S W R E G R I H I R I A K I D S Y S  
 G1.1 C GGGTCCTGGAGGGAAGGGCGGATACACATACGCATTGCAAAAATTGACTCCTACTC  
 G1.2 CTGGGTCCTGGAGGGAAGGGCGGATACACATACGCATTGCAAAAATTGACT CTACTC  
 G1.3 CTGGGTCCTGGAGGG  
 G1.4 CTGGGTCCTGGAGGG

R I F F Q Q L C L F N W  
 G1.1 CAGAATCTTTTTCCAACAGCTTT GC TTGTTCAACTGG  
 G1.2 CAGAATCTTTTTCCAACAGCTTTTGCCTTGTTCACCT

**Figure 3.7 Partial Sequences of Murine GABA<sub>A</sub> receptor Subunit cDNAs.** First strand cDNAs synthesised from total RNA isolated from 9.5 and 14 day embryos were used in PCR amplifications with degenerate oligonucleotide primers. The PCR products were subcloned into the M13mp18 vector and templates were sequenced using a Sequenase II DNA sequencing kit (USB) and the universal M13 forward sequencing primer. Single letter amino acid classification is shown above the first nucleotide of each codon. Underlined sequences were used in the design of probes for *in situ* hybridisation.

To verify that the results from Southern blot analysis, PCR products from the amplification of sequences between TM2 and TM4 were identified for the mouse  $\alpha 1$ ,  $\alpha 3$ ,  $\beta 1$ ,  $\beta 3$  and  $\gamma 1$  GABA<sub>A</sub> receptor subunit cDNAs (Figure 3.7) and previously reported sequences for  $\alpha 2$ ,  $\alpha 6$ ,  $\gamma 2$ ,  $\gamma 3$  and  $\delta 1$  mouse GABA<sub>A</sub> receptor subunit cDNAs. In this sequencing analysis, sequences for  $\alpha 4$ ,  $\alpha 5$  and  $\beta 2$  GABA<sub>A</sub> receptor subunit cDNAs were

not detected. The absence of sequences for the  $\alpha 4$ ,  $\alpha 5$ ,  $\beta 2$ , were confirmed by Southern blot analysis of the PCR products from the amplification of first strand cDNAs synthesised from 9.5 and 14 day embryonic total RNA.

Several M13 clones were obtained for each of the  $\alpha 2$ ,  $\alpha 3$ ,  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$ ,  $\gamma 1$  and  $\delta 1$  partial GABA<sub>A</sub> receptor subunit cDNA sequences and amino acid alignments showed that the amino acid code remained constant. However, at the third position in several codons variability in the nucleotide sequences was observed. This variability in DNA sequence probably reflects allelic variation, rather than PCR artefacts, since the same amino acid and nucleotide positions were always affected.

### 3.2.5 S1 Nuclease Analysis of Developmental Murine GABA<sub>A</sub> Receptor Subunit mRNA Expression

Although RT-PCR with degenerate oligonucleotide primers and subsequent Southern blot analysis can identify the presence of specific GABA<sub>A</sub> receptor subunit mRNAs during development, it is not a quantitative method. S1 nuclease protection was therefore used to quantitate 4  $\alpha$ , 2  $\beta$  and 3  $\gamma$  GABA<sub>A</sub> receptor subunit mRNAs during embryonic and postnatal stages of CNS development. Total RNA was prepared as described in Chapter 2: Materials and Methods (Section 2.2.2.2) from whole embryos or whole brain. Three to four early stage embryos were required for the preparation of quantities of RNA necessary for the S1 experiments. Oligonucleotides listed in Table 2.2 were gel purified and P<sup>32</sup>-labeled as outlined in Method 2.5.1. S1 nuclease reactions were performed as outlined in Chapter 2: Materials and Methods (Section 2.5.2) and run on non-denaturing pre-electrophoresed 10 percent polyacrylamide gels. After electrophoresis, the gels were dried and exposed to pre-flashed Fuji RX X-ray film (Figures 3.8, 3.9 and 3.10). S1 nuclease experiments were repeated three times for each oligo and RNA sample used and quantified by densitometry of autoradiograms of the GABA<sub>A</sub> receptor subunit and  $\beta$ -actin mRNA products. Since  $\beta$ -actin mRNA levels are not developmentally regulated, ratios of the densitometric values of GABA<sub>A</sub> receptor subunit mRNA to  $\beta$ -actin mRNA were used to calculate the changes in GABA<sub>A</sub> receptor subunit mRNAs through development. Use of the GABA<sub>A</sub> receptor subunit mRNA to  $\beta$ -actin mRNA ratio accounts for differences which may exist in the amount of total RNA used in each S1 nuclease reaction. The ratios of GABA<sub>A</sub> receptor subunit mRNA to  $\beta$ -actin subunit mRNA were plotted throughout embryonic and postnatal CNS development and are summarized in Figure 3.11.

Ratios of  $\alpha 1$  GABA<sub>A</sub> receptor subunit mRNA to  $\beta$ -actin subunit mRNA were consistently higher than those for the  $\alpha 2$ ,  $\alpha 3$  and  $\alpha 6$  GABA<sub>A</sub> receptor subunit mRNAs, throughout the stages of CNS development studied and in the adult brain.  $\alpha 1$  GABA<sub>A</sub> receptor subunit to  $\beta$ -actin subunit mRNA ratios were lowest at E14 and peaked around P0 before decreasing to the adult level. The ratio of  $\alpha 2$  GABA<sub>A</sub> receptor subunit mRNA to  $\beta$ -

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actin subunit mRNA was highest at E14 and decreased throughout postnatal development to adult levels. Both  $\alpha 3$  and  $\alpha 6$  GABA<sub>A</sub> receptor mRNA to  $\beta$ -actin subunit mRNA ratios were low in the 14 day embryo and throughout CNS postnatal development.

Ratios of  $\beta 3$  GABA<sub>A</sub> receptor subunit mRNA to  $\beta$ -actin subunit mRNA were consistently higher than the ratios of  $\beta 1$  GABA<sub>A</sub> receptor subunit mRNA to  $\beta$ -actin subunit mRNA, throughout CNS postnatal development and in the adult brain.  $\beta 3$  GABA<sub>A</sub> receptor subunit mRNA to  $\beta$ -actin ratios increased incrementally from E14 to the adult level. After birth, ratios of  $\beta 1$  GABA<sub>A</sub> receptor subunit mRNA to  $\beta$ -actin subunit mRNA peaked at P7 and decreased by P14 to the level of  $\beta 1$  mRNA present in the adult .

Ratios of  $\gamma 2$  GABA<sub>A</sub> receptor subunit mRNA to  $\beta$ -actin subunit mRNA were consistently higher than the ratios of  $\gamma 1$  and  $\gamma 3$  GABA<sub>A</sub> receptor subunit mRNAs to  $\beta$ -actin subunit mRNA, throughout CNS development and in the adult murine brain. After birth, ratios of  $\gamma 1$ ,  $\gamma 2$  and  $\gamma 3$  GABA<sub>A</sub> receptor subunit mRNAs to  $\beta$ -actin subunit mRNA increased incrementally to adult levels.

### 3.3 Discussion

During mouse embryogenesis, rapid morphological changes occur between embryonic days 7.5 and 14.5 (Figure 3.12). Beginning at about 7.5 days of gestation the mouse embryo shows signs of organ differentiation (Figure 3.12A), including the thickening of the ectoderm overlaying the notochord and paraxial mesoderm, which fold to form the neural tube, the rudiments of the mouse CNS. Closure of the neural tube begins at about E8.5 (Figure 3.12B) along with the development of somites and neuromeres, which indicate the beginning segmentation of the muscles, skeleton, brain and spinal cord. Closure of the neural tube continues in an anterior to posterior direction and is completed by about E10 (Rugh, 1991). At 9.5 days of gestation (Figure 3.12C) four major divisions of the brain (prosencephalon, mesencephalon, metencephalon, and myelencephalon) are apparent, with the prosencephalon and tail in close approximation. In fact, the head of the 9.5 day mouse embryo has developed well in advance of the rest of the body. Twenty-one to 25 somites are apparent through the skin surface (Figure 3.12C), with the more anterior somites always the most developed. By 14.5 days of gestation (Figure 3.12D), the skeletal and nervous systems are still in a stage of rapid development. The susceptibility of the nervous system during this stage of embryogenesis is due to rapid cell division, differentiation and migration .

To investigate the presence of GABA<sub>A</sub> receptor subunit mRNAs during this early period of nervous system development, degenerate oligonucleotides were designed to known rat GABA<sub>A</sub> receptor subunit cDNA sequences within the highly conserved regions of putative TM2s and TM4s. First strand cDNA templates were prepared from various whole embryo stages and postnatal and adult whole brain total mRNAs. At E7.5, before closure of the neural tube,  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 6$  and  $\gamma 2$  GABA<sub>A</sub> receptor subunit mRNAs were detected by

PCR with degenerate oligonucleotides and subsequent Southern blot analysis. *In vitro* transient expression studies in HEK293 cells have demonstrated that co-expression of  $\alpha 1$  and  $\gamma 2$  GABA<sub>A</sub> receptor subunit cDNAs can result in functional GABA<sub>A</sub> receptors with unique pharmacological properties. At E8.5, when closure of the neural tube has begun, as well as early rhombomere organization,  $\alpha$ ,  $\beta$  and  $\gamma$  GABA<sub>A</sub> receptor subunit mRNAs were detected by PCR with degenerate oligonucleotides and subsequent Southern blot analysis. Neither  $\gamma 3$  nor  $\gamma 1$  GABA<sub>A</sub> receptor subunit mRNAs were detected during embryogenesis using this method.

The very early detection (E7.5) of GABA<sub>A</sub> receptor subunits supports the hypothesis that during CNS development, GABA<sub>A</sub> receptors may participate in trophic activities. This trophic activity of GABA<sub>A</sub> receptors is believed to correlate with carrier-mediated release of GABA through a reverse uptake mechanism present in the plasma membranes of specialized growth cones (Gordon-Weeks, Lockerbie and Pearce, 1984). Growth cones are highly motile expansions at the ends of growing axons and dendrites and play key roles in synaptogenesis by making the initial contact with the postsynaptic neuron and by themselves becoming the presynaptic terminal. Recognition of an appropriate synaptic partner transforms the growth cone into either a presynaptic terminal, in the case of axonal growth cones (Rees, 1978) or into a postsynaptic element, in the case of dendritic growth cones (Vaughn *et al.*, 1974). Taylor and Gordon-Weeks, (1991) have demonstrated that a major portion of the K<sup>+</sup>-stimulated, Ca<sup>+</sup>-independent release of GABA from growth cones is mediated by a reversal of the GABA transporter, using uptake inhibitors to demonstrate this mechanism. During the transformation from a highly motile growth cone to a sessile synaptic element, the biochemical machinery for neurotransmission must develop.

Electrical activity plays an important role in synaptogenesis and neuronal differentiation (Nelson *et al.*, 1990). The excitation level depends on the temporal activation of both excitatory and inhibitory pathways. However, stimulation of immature inhibitory pathways can evoke excitation rather than inhibition (reviewed by Cherubini, Gaiarsa and Ben Ari, 1991). Cl<sup>-</sup>-mediated depolarizing potentials have been recorded in spinal motoneurons of newborn rats (Fulton *et al.*, 1980; Takahashi, 1984; Jahr and Yoshioka, 1986) and embryonic chicks (Obata, Oide and Tanaka, 1978; Velumian, 1984; O'Donovan, 1989). In motoneurons of E16-E17 embryos, both strychnine and bicuculline were capable of blocking dorsal root ganglia evoked potentials. These GABA and glycine induced, Cl<sup>-</sup>-mediated depolarizing responses gradually decrease during embryonic development and hence may be attributable to temporal changes in the expression of glycine and GABA<sub>A</sub> receptor subunit mRNAs, since suppression of this response results from neither a decrease in neuronal excitability nor the inhibition of glutamate receptors (Wu, Ziskind-Conhaim and Sweet, 1992). The chemotropic response of microdissected rat E13.5 cortical plate cells (95% neurons) and immature ventricular zone cells (60% precursors) was differentially modulated



by micromolar and femtomolar concentrations of GABA, respectively (Behar *et al.*, 1998). Behar *et al.*, (1998) demonstrated that GABA could promote motility via G-protein activation (response blocked by pertussis toxin) and arrest attractant-induced migration via GABA<sub>A</sub> receptor mediated depolarization.

Degenerate oligonucleotide derived PCR results reported in this chapter demonstrate the rapidly changing temporal expression of GABA<sub>A</sub> receptor subunit mRNAs during embryonic development and support the role potential embryonic GABA<sub>A</sub> receptor complexes (E7.5-9.5:  $\alpha 1\gamma 2$ ,  $\alpha 3\gamma 2$ ,  $\alpha 2\gamma 2$ ,  $\alpha 4\gamma 2$ ,  $\alpha 6\gamma 2$ ; E12.5-14.5:  $\alpha 1\beta 1\gamma 2$ ,  $\alpha 3\beta 1\gamma 2$ ,  $\alpha 6\beta 1\gamma 2$ ), may play in elicitation of network excitability. At E15 in the rat, *in situ* hybridization analysis has demonstrated that transcripts for GABA<sub>A</sub> receptor  $\alpha 3$ ,  $\beta 3$ , and  $\gamma 2$  subunits were found exclusively in the mantle zone, while those for  $\alpha 4$ ,  $\beta 1$  and  $\gamma 1$  were predominantly detected in the inner half of the germinal or ventricular zone (Ma and Barker, 1995). In the E15 germinal matrix of the lumbar spinal cord,  $\alpha 4$  subunit mRNA was much more abundant in the receding intermediate plate, which contains mostly postmitotic cells, that in the alar plate comprised of many DNA-synthesising cells and suggests that cells in the ventricular zone may express GABA<sub>A</sub> receptor  $\alpha 4$ ,  $\beta 1$  and  $\gamma 1$  subunit mRNAs at the premigratory stage, just after completing cell division (Ma and Barker, 1995).

The results of S1 nuclease protection experiments indicate that during postnatal CNS development,  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2$  subunits are the most abundant of the GABA<sub>A</sub> receptor subunit mRNAs examined. At postnatal day 1 and postnatal day 7, the abundance of  $\alpha$  GABA<sub>A</sub> receptor subunit mRNA was  $\alpha 1 > \alpha 2 > \alpha 3 > \alpha 6$ , whereas at postnatal day 14 and in the adult brain, the abundance of  $\alpha$  GABA<sub>A</sub> receptor subunit mRNA was  $\alpha 1 > \alpha 2 \geq \alpha 6 > \alpha 3$ . Throughout postnatal development the abundance of  $\beta$  and  $\gamma$  GABA<sub>A</sub> receptor subunit mRNAs was  $\beta 3 > \beta 1$  and  $\gamma 2 > \gamma 1 > \gamma 3$ . The predominance of  $\beta 3$  and  $\gamma 2$  GABA<sub>A</sub> receptor subunit mRNAs are in agreement with Northern blot analysis of mRNA levels by MacLennan *et al.*, 1991. Autoradiographs of Northern blot analysis also indicate that rat and bovine  $\beta 2$  and  $\beta 3$  mRNAs are considerably more abundant than  $\beta 1$  mRNA in the adult brain, and  $\beta 2$  mRNA was considerably more abundant than  $\beta 3$  mRNA in the adult CNS (Ymer *et al.*, 1989b). Bovolín *et al.*, (1992) demonstrated by competitive PCR analysis with mimic DNA as an internal standard, that during postnatal development, the levels of  $\alpha 5$  mRNA are considerably lower than those of  $\alpha 1$  mRNA and decrease from 0.08 fmol/ $\mu$ g total RNA at P0 to less than 0.02 fmol/ $\mu$ g of total RNA in the adult rat cerebellum. In contrast  $\alpha 1$  mRNA in the P0 rat cerebellum starts at about 0.1 fmol/ $\mu$ g of total RNA and increases to 2 fmol/ $\mu$ g of total RNA in the adult. When looking at primary cultures of rat cerebellar granule cells, Bovolín *et al.*, (1992) observed that the GABA<sub>A</sub> receptor subunit  $\alpha 5$  mRNA peaked later in the development (8 days) of the primary cultures.

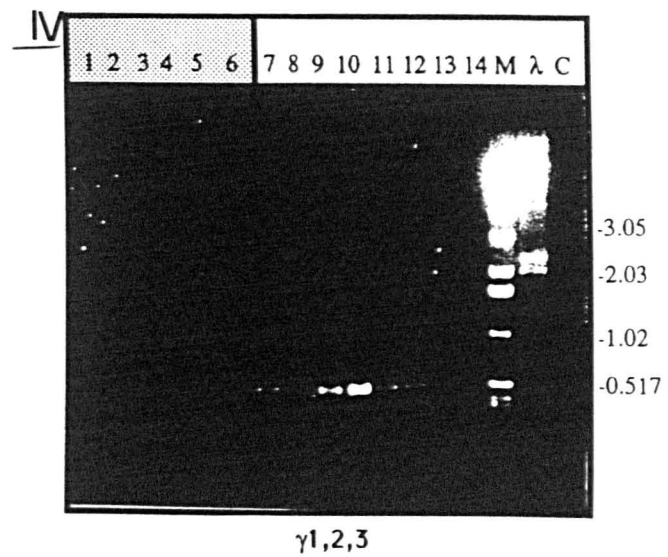
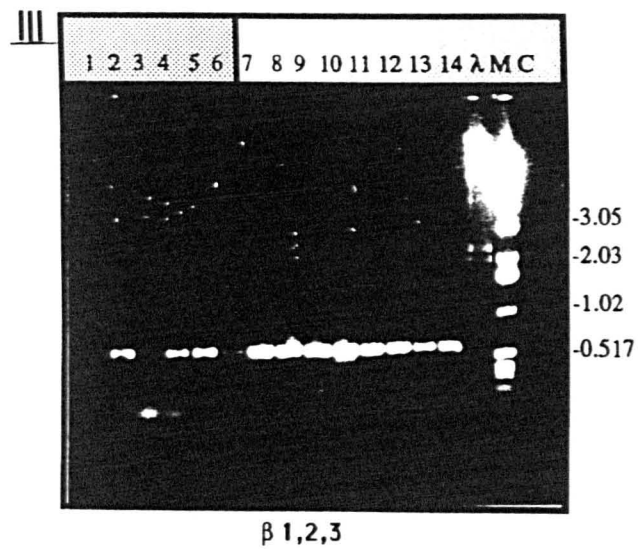
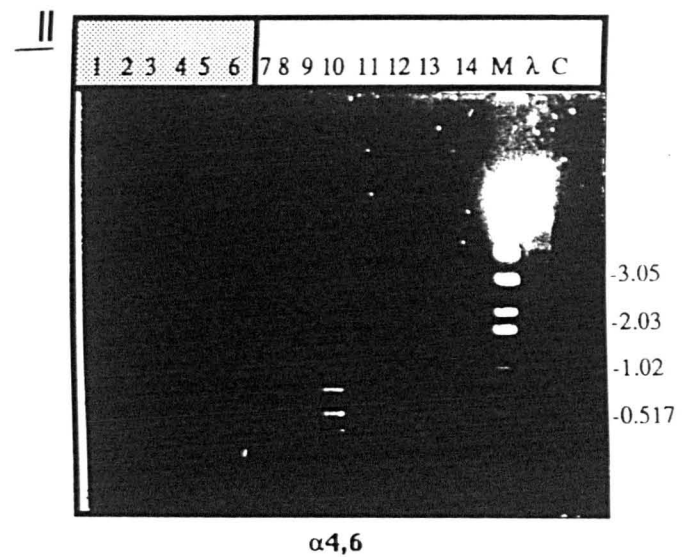
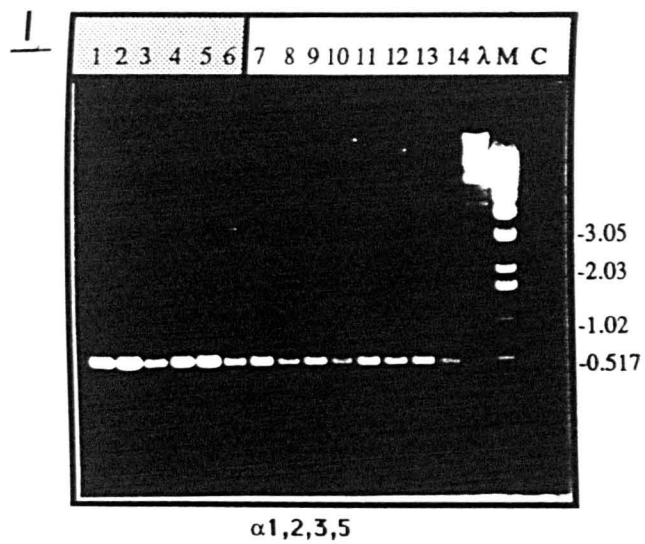
In this chapter, differences exist between RT-PCR and S1 nuclease protection assay in the determination of the presence or absence of  $\alpha 2$ ,  $\gamma 1$  and  $\gamma 2$  GABA<sub>A</sub> subunit mRNAs at

E14.5. RT-PCR analysis failed to detect mRNA for the  $\alpha 2$ ,  $\gamma 1$ , and  $\gamma 2$  GABA<sub>A</sub> receptor subunits, whereas S1 nuclease analysis detected the presence of all three GABA<sub>A</sub> receptor subunit transcripts at E14.5. These discrepancies are probably due to variabilities in the sensitivity of the methodologies employed. The same preparation of E14.5 total mRNA was used in both the RT-PCR amplification and the S1 nuclease protection analysis. The ability of degenerate oligonucleotide PCR primers to detect  $\alpha 2$ ,  $\gamma 1$  and  $\gamma 2$  GABA<sub>A</sub> receptor subunit mRNAs may be dependent upon both the abundance of these specific mRNAs and the number and abundance of competing templates. The RT-PCR methodology also requires conversion of mRNA into first strand cDNA templates which again may be less efficient for less abundant transcripts.

Unlike glycine and muscle acetylcholine receptors that have unique embryonic subtypes, GABA<sub>A</sub> receptor subunits demonstrate differential patterns of embryonic and postnatal mRNA expression. It is evident from the results presented in this chapter, that GABA<sub>A</sub> receptor  $\alpha 1$ ,  $\alpha 3$  and  $\gamma 2$  subunit mRNAs are present during critical periods of CNS embryonic development ( $\alpha 1$  was not detected during early rat embryogenesis). It further appears from the preliminary studies presented in this chapter, that GABA<sub>A</sub> receptor  $\alpha 5$ ,  $\beta 2$ ,  $\gamma 1$  and  $\gamma 3$  subunit mRNAs are primarily expressed during postnatal development and could contribute to the plasticity of the GABA response in the adult brain. Alternative-splicing of the GABA<sub>A</sub> receptor subunit  $\gamma 2$  mRNA as well as variation in the embryonic and postnatal GABA<sub>A</sub> receptor subunit composition may account for the trophic activity of GABA during murine embryonic CNS development.

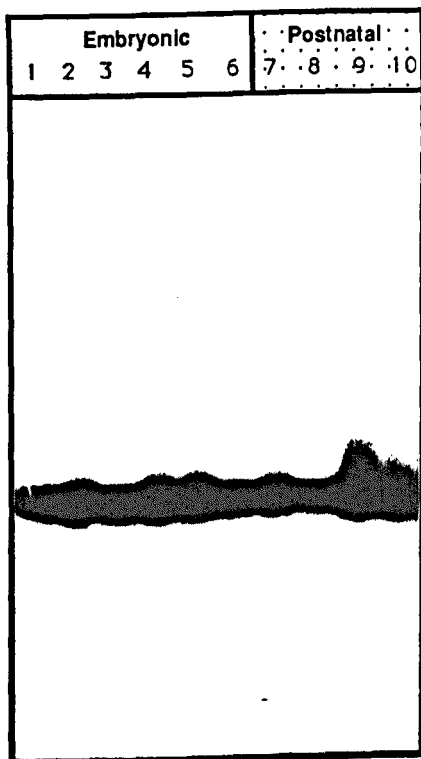
**Figure 3.2**

**Figure 3.2 Gel electrophoresis and ethidium bromide staining of PCR products.** 0.8 percent TAE agarose gel analysis of PCR products derived from the amplification of first strand cDNA reversed transcribed from embryonic, whole brain postnatal, adult kidney and cell line total RNA as well as embryonic 12 day and adult whole brain poly A+ mRNA. Embryonic stages lanes 1-6: 1) E7.5; 2) E8.5; 3) E9.5; 4) E12; 5) E12 poly A+ mRNA; 6) E14. Postnatal developmental stages lanes 7-10: 7) P1; 8) P7; 9) P14; 10) adult; 11) adult kidney; 12) adult liver; 13) adult retina; 14) adult spleen; M) 1kb ladder;  $\lambda$ )  $\lambda$  DNA digested with restriction endonuclease *Hind* III; C) control reaction with H<sub>2</sub>O as template. 3.05, 2.03, 1.02 and 0.517 kb DNA size markers. **I:** Degenerate oligonucleotides for GABA<sub>A</sub> receptor  $\alpha$ 1,2,3,5 subunits were used in the PCR reaction; **II:** degenerate oligonucleotides for GABA<sub>A</sub> receptor  $\alpha$ 4,5 subunits were used in the PCR reaction; **III:** degenerate oligonucleotides for GABA<sub>A</sub> receptor  $\beta$ 1,2,3 subunits were used in the PCR reaction; **IV:** degenerate oligonucleotides for GABA<sub>A</sub> receptor  $\gamma$ 1,2,3 subunits were used in the PCR reaction.

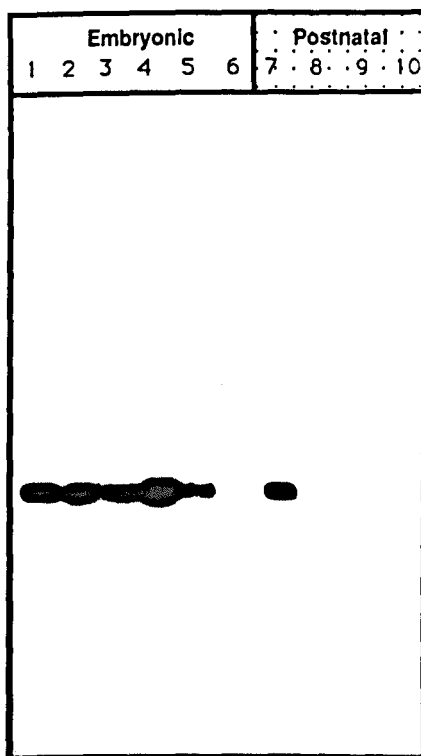


**Figure 3.3**

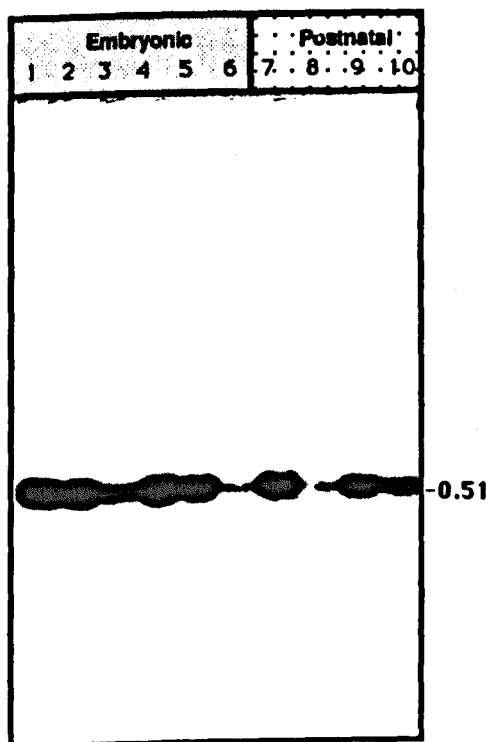
**Figure 3.3 Southern blot analysis of PCR products with GABA<sub>A</sub> receptor subunit specific oligonucleotide probes.** Each lane was loaded with 10 µl of a PCR reaction containing degenerate oligonucleotide primers designed to the conserved regions of transmembranes 2 and 4 for GABA<sub>A</sub> receptor α1, 2, 3 and 5 subunits. First strand cDNA from embryonic and postnatal stages of development were used in the PCR reactions. Prior to hybridization with <sup>32</sup>P-labeled GABA<sub>A</sub> receptor subunit specific oligonucleotide probes, PCR products electrophoresed through a 0.8% agarose gel were immobilized onto Hybond N membranes. PCR products from embryonic stages, lanes 1-6: 1) E7.5; 2) E8.5; 3) E9.5; 4) E12; 5) E12 poly A+ mRNA; 6) E14. PCR products for postnatal developmental stages, lanes 7-10: 7) P0; 8) P7; 9) P14; 10) adult. There was no signal observed in control lanes that represent PCR reactions without a DNA template (results not shown). DNA size markers 0.46 0.51, 0.96 kb.



$\alpha 1$



$\alpha 2$



$\alpha 3$



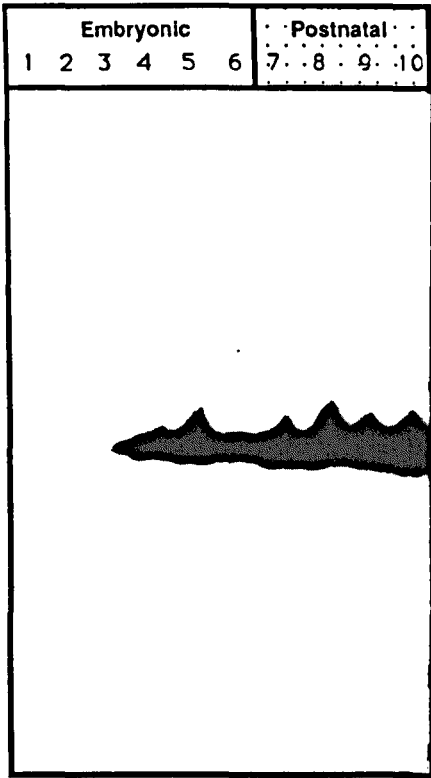
**Figure 3.4**

**Figure 3.4 Southern blot analysis of PCR products with GABA<sub>A</sub> receptor subunit specific oligonucleotide probes.** Each lane of a 0.8% agarose gel was loaded with 10 µl of a PCR reaction containing degenerate oligonucleotide primers designed to the conserved regions of transmembranes 2 and 4 for either GABA<sub>A</sub> receptor α4 and 6 subunits (A,B) or GABA<sub>A</sub> receptor α1,2,3 and 5 subunits (C). First strand cDNA from embryonic and postnatal stages of development were used in the PCR reactions. Prior to hybridization with <sup>32</sup>P-labeled GABA<sub>A</sub> receptor subunit specific oligonucleotide probes, PCR products electrophoresed through a 0.8% agarose gel were immobilized onto Hybond N membranes. PCR products from embryonic stages, lanes 1-6: 1) E7.5; 2) E8.5; 3) E9.5; 4) E12; 5) E12 poly A+ mRNA; 6) E14. PCR products for postnatal developmental stages, lanes 7-10: 7) P0; 8) P7; 9) P14; 10) adult. There was no signal observed in control lanes that represent PCR reactions without a DNA template (results not shown). DNA size markers 0.41, 0.44, 0.7, 0.9 kb.

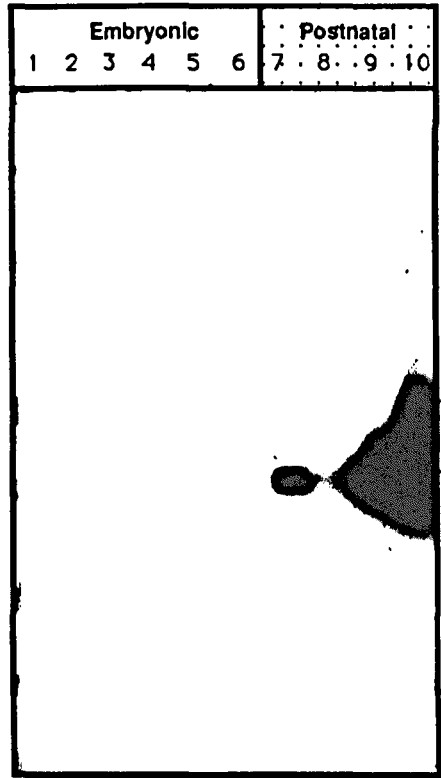


**Figure 3.5**

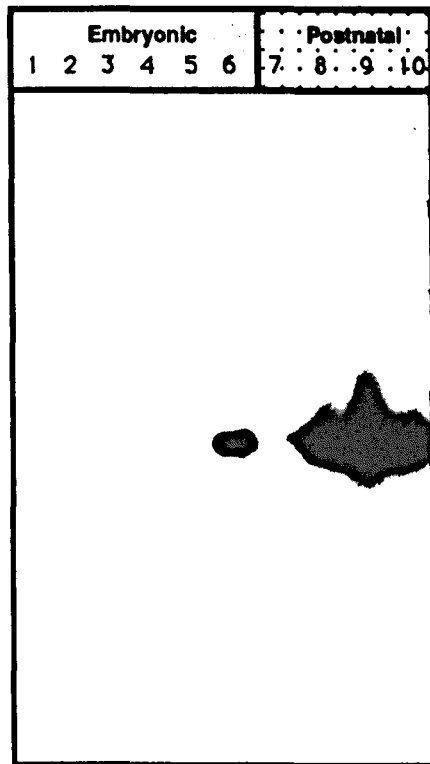
**Figure 3.5 Southern blot analysis of PCR products with GABA<sub>A</sub> receptor subunit specific oligonucleotide probes.** Each lane of a 0.8% agarose gel was loaded with 10 µl of a PCR reaction containing degenerate oligonucleotide primers designed to the conserved regions of transmembranes 2 and 4 for GABA<sub>A</sub> receptor β1, 2 and 3 subunits. First strand cDNA from embryonic and postnatal stages of development were used in the PCR reactions. Prior to hybridization with <sup>32</sup>P-labeled GABA<sub>A</sub> receptor subunit specific oligonucleotide probes, PCR products electrophoresed through a 0.8% agarose gel were immobilized onto Hybond N membranes. PCR products from embryonic stages, lanes 1-6: 1) E7.5; 2) E8.5; 3) E9.5; 4) E12; 5) E12 poly A+ mRNA; 6) E14. PCR products from postnatal developmental stages, lanes 7-10: 7) P0; 8) P7; 9) P14; 10) adult. There was no signal observed in control lanes that represent PCR reactions without a DNA template (results not shown). DNA size markers 0.51, 0.64, 0.76, 1.2 kb.



$\beta 1$



$\beta 2$

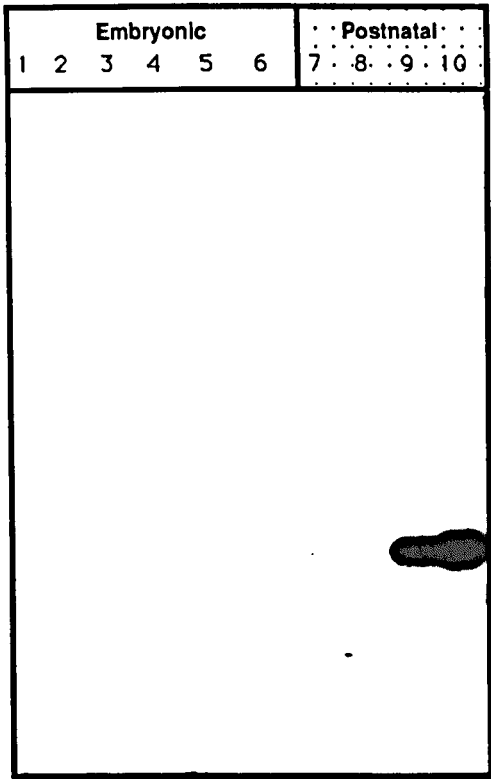


$\beta 3$

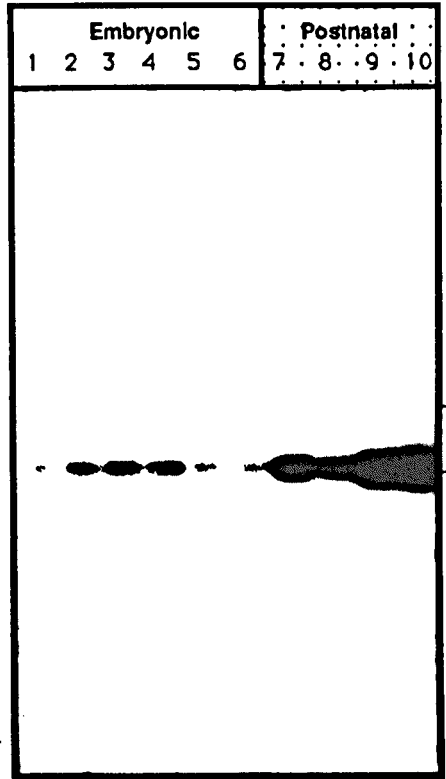
**Figure 3.6**

**Figure 3.6 Southern blot analysis of PCR products with GABA<sub>A</sub> receptor subunit specific oligonucleotide probes.** Each lane of a 0.8% agarose gel was loaded with 10 µl of a PCR reaction containing degenerate oligonucleotide primers designed to the conserved regions of transmembranes 2 and 4 for GABA<sub>A</sub> receptor γ1, 2 and 3 subunits. First strand cDNA from embryonic and postnatal stages of development were used in the PCR reactions. Prior to hybridization with <sup>32</sup>P-labeled GABA<sub>A</sub> receptor subunit specific oligonucleotide probes, PCR products electrophoresed through a 0.8% agarose gel were immobilized onto Hybond N membranes. PCR products from embryonic stages, lanes 1-6: 1) E7.5; 2) E8.5; 3) E9.5; 4) E12; 5) E12 poly A+ mRNA; 6) E14. PCR products from postnatal developmental stages, lanes 7-10: 7) P0; 8) P7; 9) P14; 10) adult. There was no signal observed in control lanes that represent PCR reactions without a DNA template (results not shown). DNA size markers 0.50, 0.59, 0.98 kb

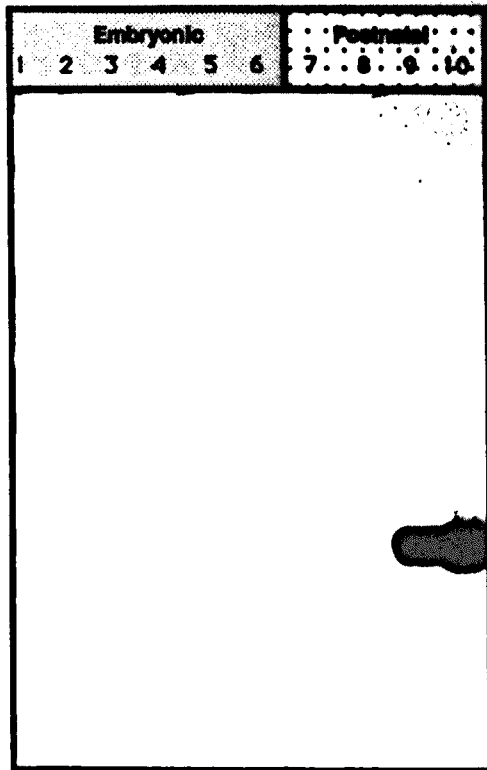




$\gamma 1$



$\gamma 2$



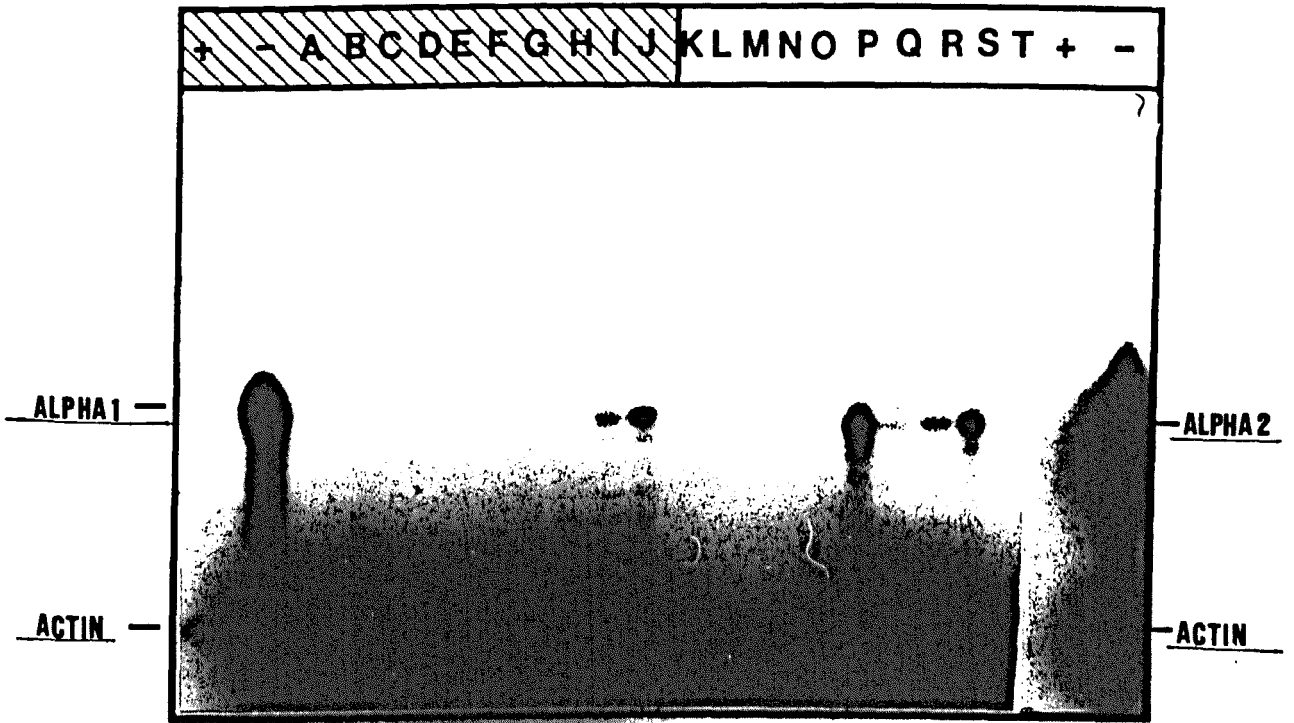
$\gamma 3$

**Figure 3.8**

**Figure 3.8 S1 nuclease protection analysis of GABA<sub>A</sub> receptor subunit mRNA abundance during embryonic and postnatal development.** <sup>32</sup>P-labeled GABA<sub>A</sub> receptor  $\alpha$ 1, 2, 3 and 6 subunit-specific antisense oligonucleotide probes (45mers) were hybridized with postnatal whole brain or embryonic total RNA in the presence of a <sup>32</sup>P-labeled  $\beta$  actin-specific antisense oligonucleotide probe (24mer). S1 nuclease was used to remove excess <sup>32</sup>P-labeled oligonucleotide prior to electrophoresis on 12% denaturing polyacrylamide gels.  $\alpha$ 1: 5  $\mu$ g of total RNA (A,B,C,D,E); 30  $\mu$ g of total RNA (F,G,H,I,J).  $\alpha$ 2: 5  $\mu$ g of total RNA (K,L,M,N,O); 30  $\mu$ g of total RNA (P,Q,R,S,T).  $\alpha$ 3: 30  $\mu$ g of total RNA (A,B,C,D,E).  $\alpha$ 6: 5  $\mu$ g of total RNA (F,G,H,I,J); 30  $\mu$ g of total RNA (K, L, M, N, O). Developmental stages:  $\alpha$ 1 and  $\alpha$ 2 E14 (A,F,K,P), P0 (B,G,L,Q), P7 (C,H,M,R), P14 (D,I,N,S), Adult (E,J,O,T);  $\alpha$ 3 and  $\alpha$ 6 E14 (A,F,K), P0 (B,G,L), P7 (C,H,M), P14 (D,I,N), Adult (E,J,O). +, oligonucleotide in the absence of template with S1 nuclease treatment. -, oligonucleotide in the absence of template without S1 nuclease treatment.

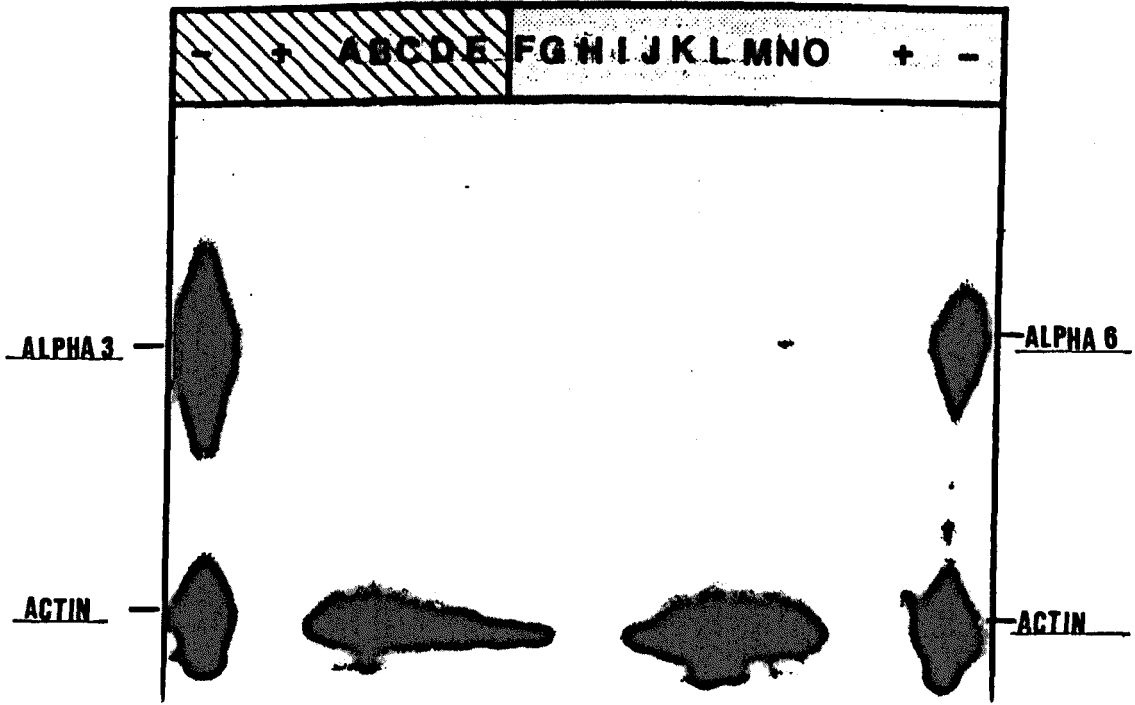
$\alpha 1$

$\alpha 2$



$\alpha 3$

$\alpha 6$

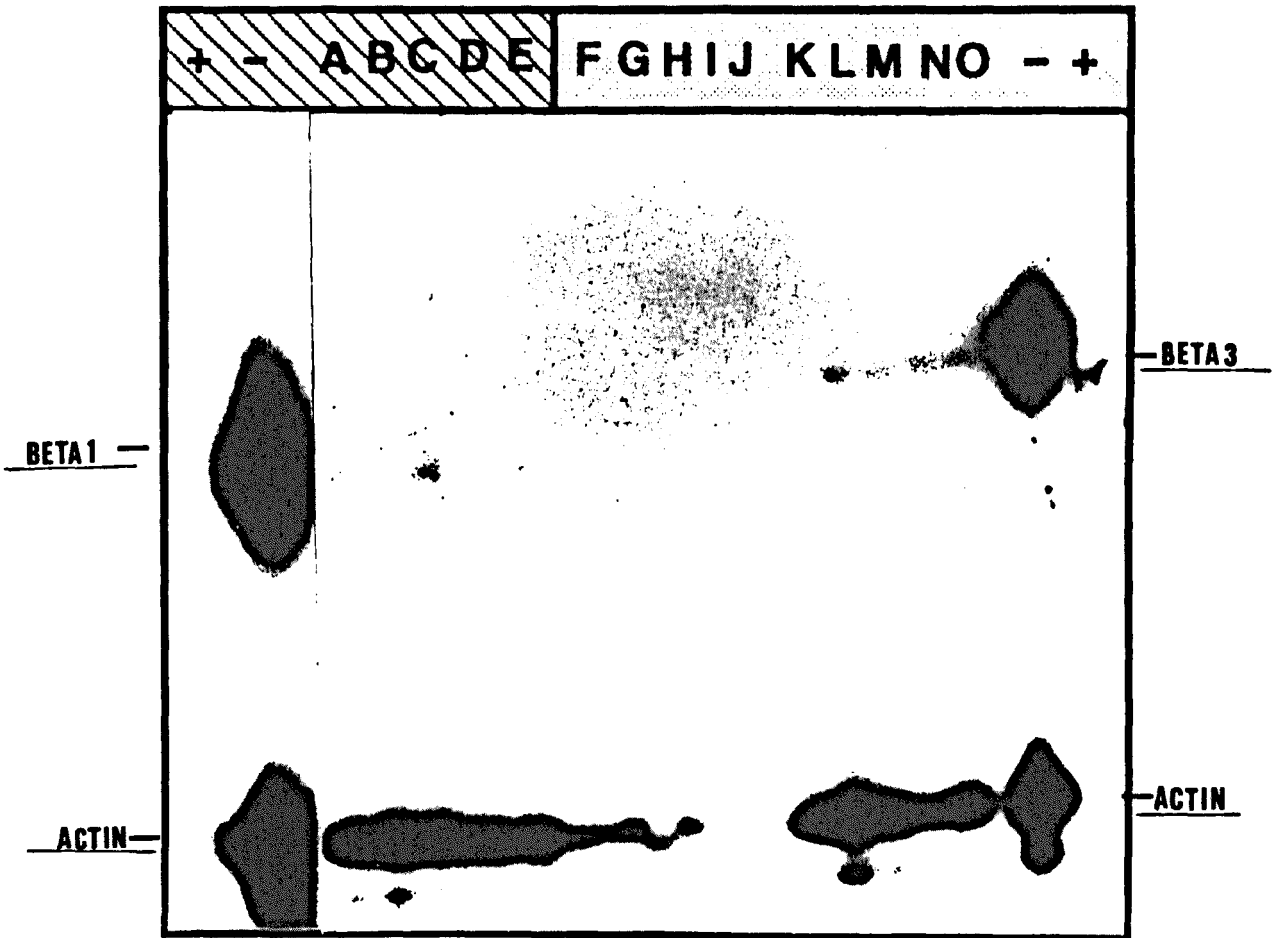


**Figure 3.9**

**Figure 3.9** *S1 nuclease protection analysis of GABA<sub>A</sub> receptor subunit mRNA abundance during embryonic and postnatal development.* <sup>32</sup>P-labeled GABA<sub>A</sub> receptor β1 and 3 subunit-specific antisense oligonucleotide probes (45mers) were hybridized with postnatal whole brain or embryonic total RNA in the presence of a <sup>32</sup>P-labeled β actin-specific antisense oligonucleotide probe (24mer). S1 nuclease was used to remove excess <sup>32</sup>P-labeled oligonucleotide prior to electrophoresis on 12% denaturing polyacrylamide gels. β1: 30 μg of total RNA (A,B,C,D,E). β3: 5 μg of total RNA (F,G,H,I,J); 30 μg of total RNA (K,L,M,N,O). Developmental stages: β1 and β3 E14 (A,F,K), P0 (B,G,L), P7 (C,H,M), P14 (D,I,N), Adult (E,J,O). +, oligonucleotide in the absence of template with S1 nuclease treatment. -, oligonucleotide in the absence of template without S1 nuclease treatment.

$\beta 1$

$\beta 3$

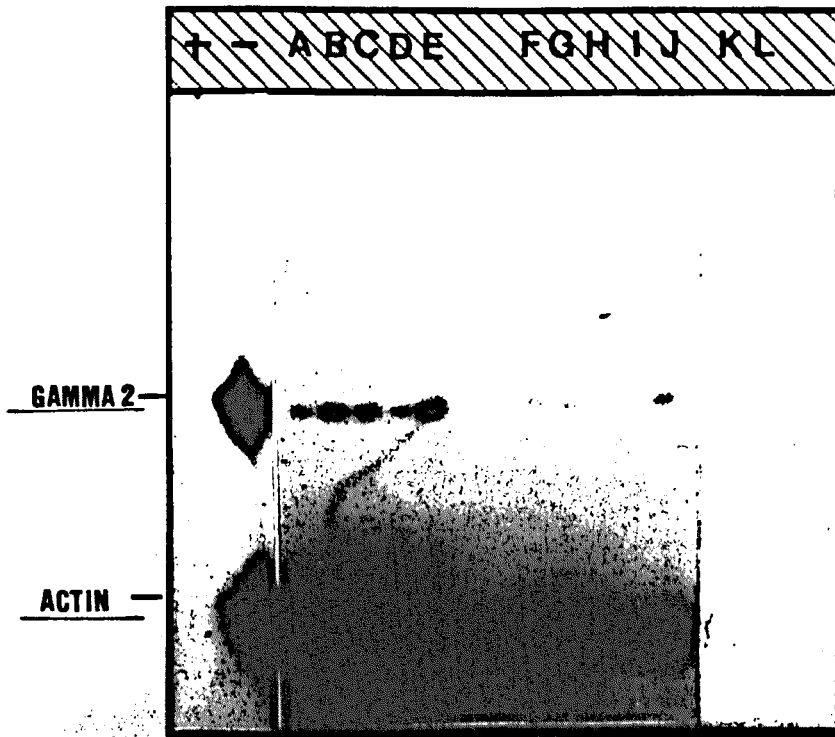


**Figure 3.10**



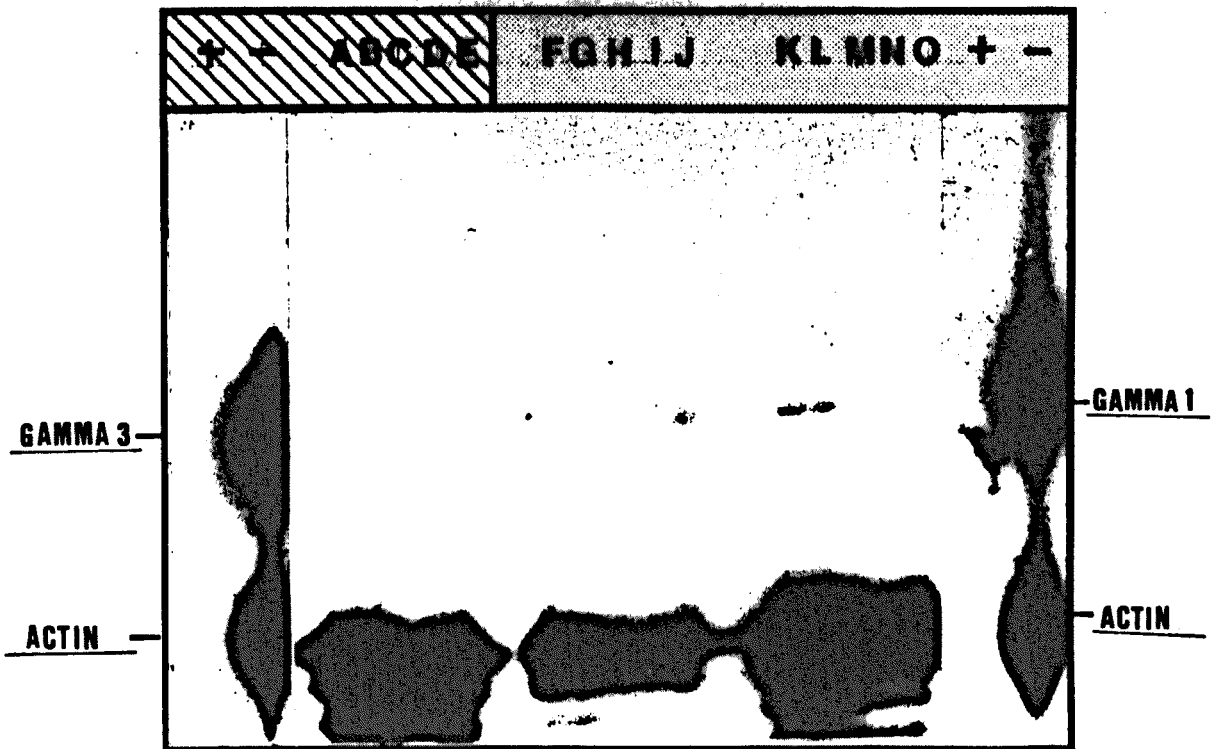
**Figure 3.10 S1 nuclease protection analysis of GABA<sub>A</sub> receptor subunit mRNA abundance during embryonic and postnatal development.** <sup>32</sup>P-labeled GABA<sub>A</sub> receptor  $\gamma$ 1, 2 and 3 subunit-specific antisense oligonucleotide probes (45mers) were hybridized with postnatal whole brain or embryonic total RNA in the presence of a <sup>32</sup>P-labeled  $\beta$  actin-specific antisense oligonucleotide probe (24mer). S1 nuclease was used to remove excess <sup>32</sup>P-labeled oligonucleotide prior to electrophoresis on 12% denaturing polyacrylamide gels.  $\gamma$ 1: 5  $\mu$ g of total RNA (F,G,H,I,J); 30  $\mu$ g of total RNA (K,L,M,N,O).  $\gamma$ 2: 5  $\mu$ g of total RNA (F,G,H,I,J); 30  $\mu$ g of total RNA (A,B,C,D,E).  $\gamma$ 3: 30  $\mu$ g of total RNA (A,B,C,D,E). Developmental stages:  $\gamma$ 2 E14 (A,F), P0 (B,G), P7 (C,H), P14 (D,I,N,S), Adult (E,J) Blank lanes (K,L);  $\gamma$ 1 and  $\gamma$ 3 E14 (A,F,K), P0 (B,G,L), P7 (C,H,M), P14 (D,I,N), Adult (E,J,O). +, oligonucleotide in the absence of template with S1 nuclease treatment. -, oligonucleotide in the absence of template without S1 nuclease treatment.

$\gamma_2$



$\gamma_3$

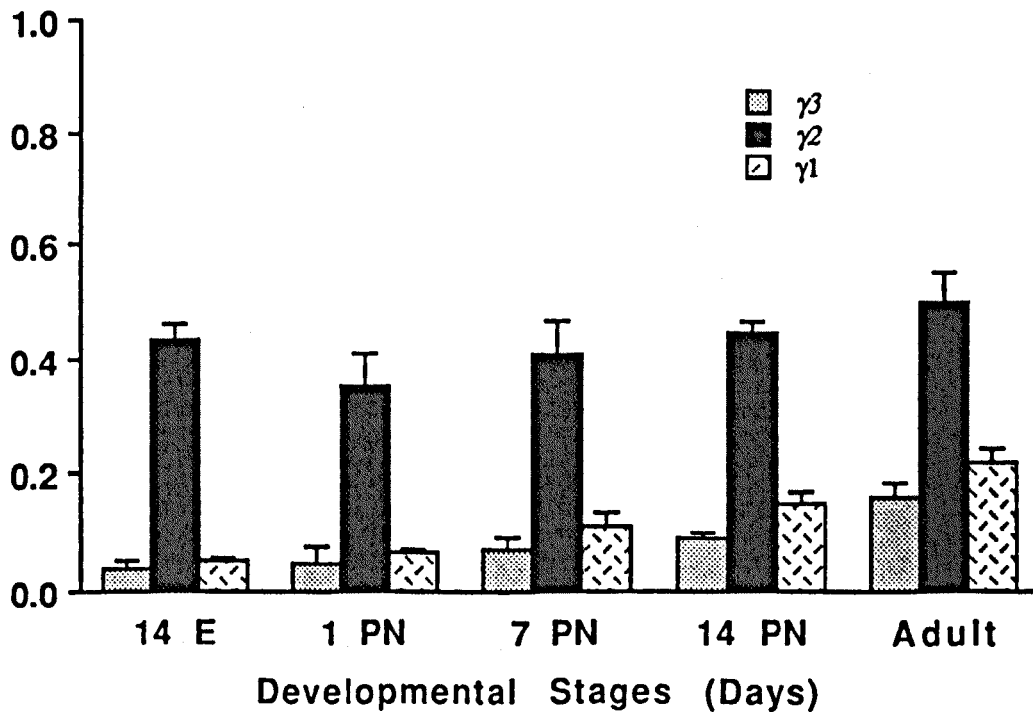
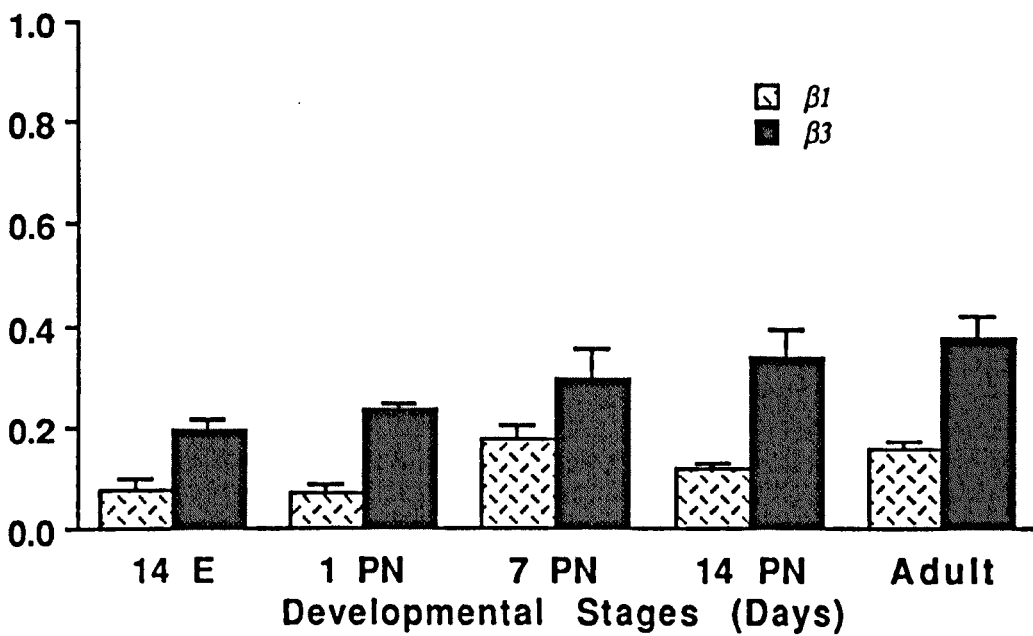
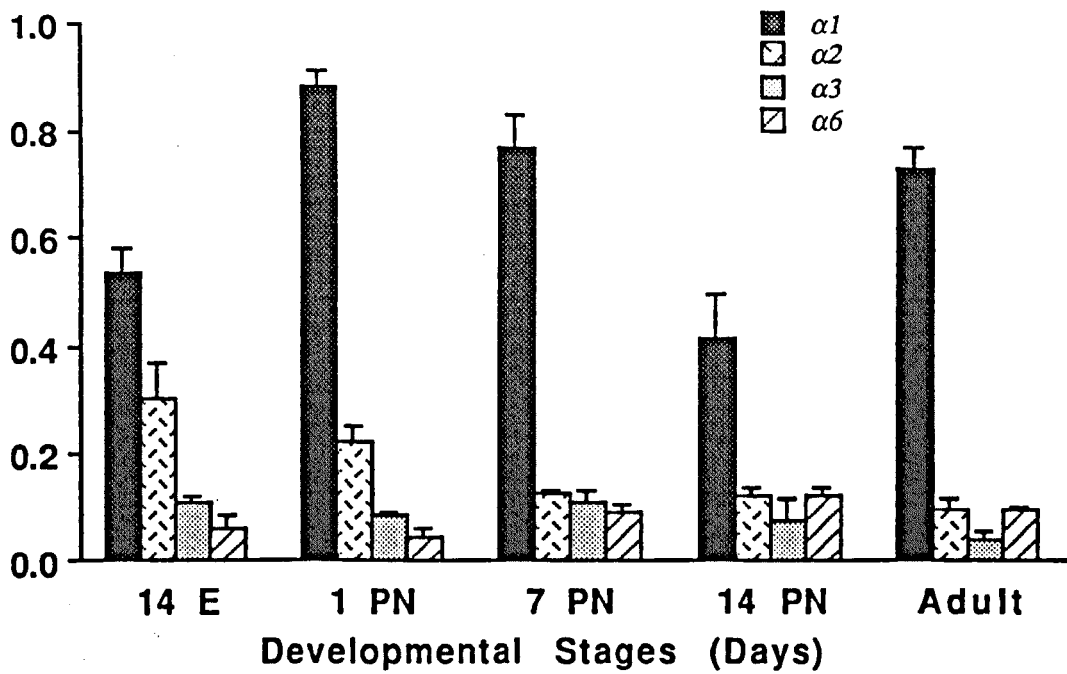
$\gamma_1$



**Figure 3.11**

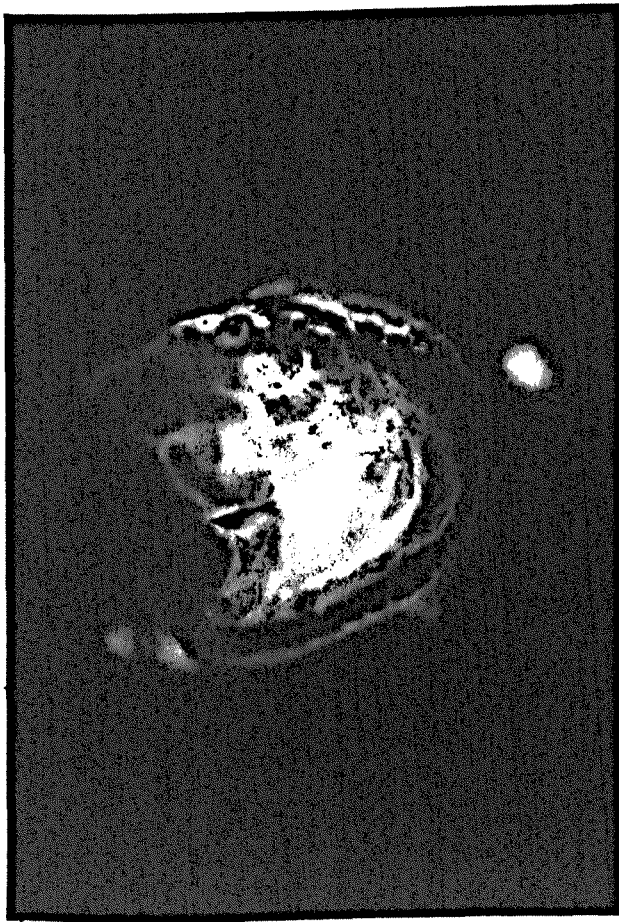
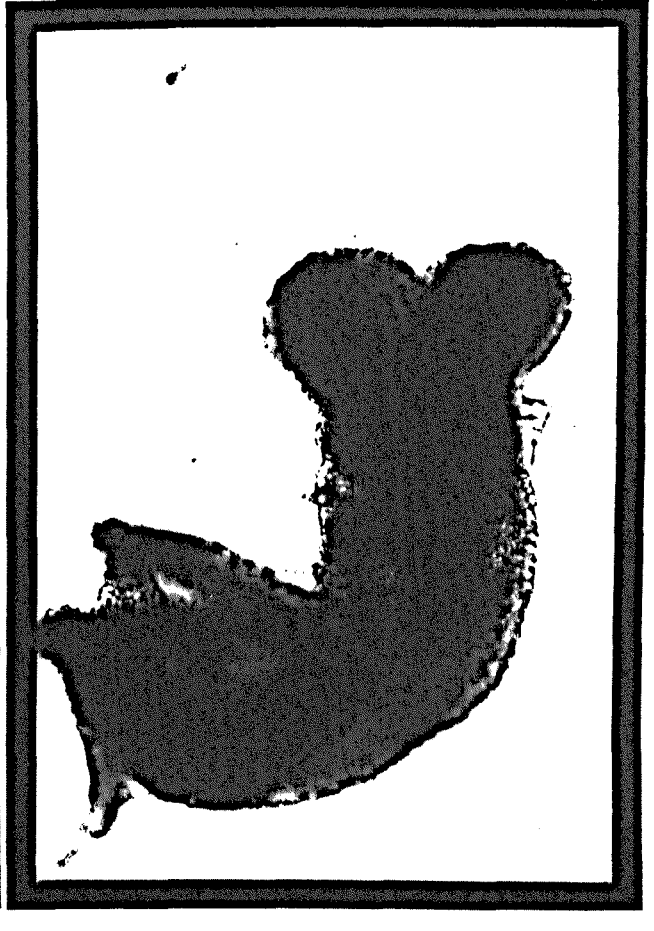
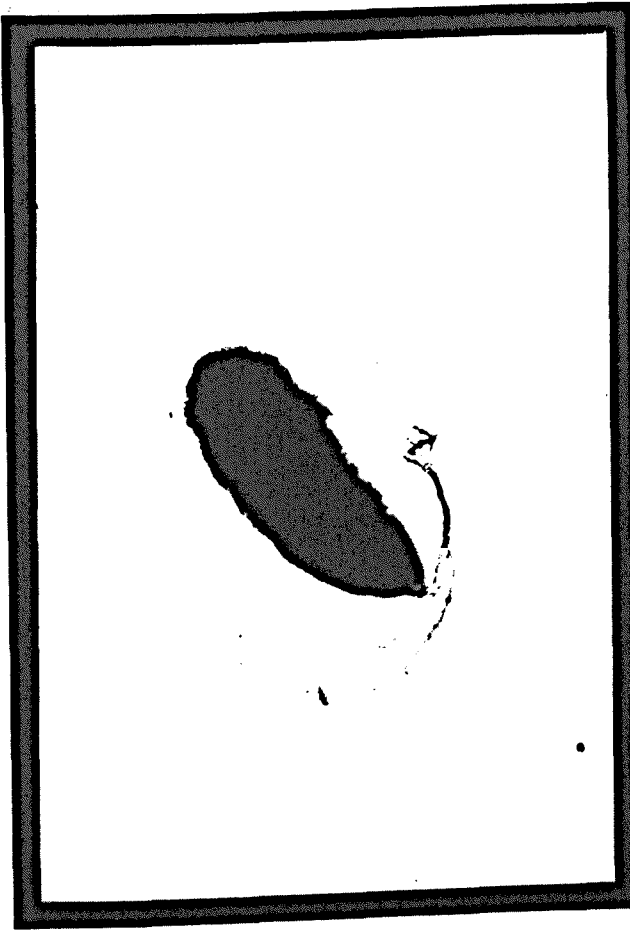
***Figure 3.11 Developmental analysis of the ratio of GABA<sub>A</sub> receptor subunit mRNA expression to  $\beta$  actin mRNA expression.*** S1 nuclease reactions were run on a 12% denaturing polyacrylamide gel, dried under vacuum onto Whatman 3mm paper and exposed to preflashed Fuji X-ray film for 2 to 24 hours. Densitometry was used to quantitate the signal intensity of the GABA<sub>A</sub> receptor subunit and  $\beta$  actin S1 nuclease protection products for developmental stages: embryonic day 14 (14 E), postnatal day 1 (1 PN), postnatal day 7 (7 PN), postnatal day 14 (14 PN) and adult.

Ratio of GABA/Actin mRNA Expression



**Figure 3.12**

**Figure 3.12 Four Stages of Murine Embryogenesis.** Rapid developmental and morphogenic changes occur during the second week of murine embryogenesis. **A:** 7.5 day mouse embryo photographed under bright-field conditions. **B:** 8.5 day mouse embryo photographed under bright-field conditions. **C:** 9.5 day mouse embryo stained with thionin and photographed using a dark-field condenser. **D:** 14.5 day mouse embryo photographed using a dark -field condenser.





# **Chapter 4**

## **Developmental Expression II**

## 4.1 Introduction

Diversity of response to chemical signals within the central nervous system is enhanced through several mechanisms including; the cotransmission of neurotransmitters with neuropeptides, the interaction of a neurotransmitter with different receptor systems, such as GABA<sub>A</sub> and GABA<sub>B</sub> receptors and the interaction of a neurotransmitter with subtypes of receptor. Many channels and ligand-gated ion receptors of the mammalian central nervous system are able to form either homo-oligomeric or hetero-oligomeric complexes that account in large part for the variation in rates of activation and inactivation, toxin sensitivity, differences in ligand affinity, and disparate pharmacologies.

As outlined in Chapter 1, at least fifteen different GABA<sub>A</sub> receptor subunit genes have been cloned in the rodent, and are known to play various roles in a receptor complex. Studies of combinations of GABA<sub>A</sub> receptor subunit cDNAs in transient expression systems have aided in the elucidation of the role of individual subunits and subunit combinations in the resultant pharmacologies. For example, the  $\gamma$  subunit class confers a robust benzodiazepine responsiveness on any  $\alpha/\beta$  subunit combination (Pritchett, Lüddens and Seeburg, 1989; Pritchett *et al.*, 1989), such that a minimum requirement for conventional GABA<sub>A</sub> receptor pharmacology would be an  $\alpha x \beta x \gamma x$  combination, where x is any isoform variant. When the  $\gamma$  subunit becomes  $\gamma 2$  and the combinations are then  $\alpha x \beta x \gamma 2$ , transient expression studies have revealed that it is the  $\alpha$  subunit that dictates the type of benzodiazepine interaction and associated allosteric modulation of the GABA<sub>A</sub> receptor complex (Pritchett *et al.*, 1989; Pritchett, Lüddens and Seeburg, 1989; Pritchett and Seeburg, 1990; Lüddens *et al.*, 1990; Seeburg *et al.*, 1990; Lüddens and Wisden, 1991).

Type I and II benzodiazepine (BZ) receptors have been identified in terms of their relative sensitivity to  $\beta$ -carbolines and the triazolopyridazine CL 218872 (Braestrup and Nielsen, 1981; Lippa *et al.*, 1981). Benzodiazepine binding to the cerebellar receptor is sensitive to these agents and is designated type I. The pharmacological and electrophysiological properties of GABA<sub>A</sub>/BZ type I receptors can only be mimicked by recombinant receptors of the  $\alpha 1 \beta x \gamma 2$  type. BZ II receptors assembled in HEK293 cells from the  $\alpha 2$ ,  $\alpha 3$  or  $\alpha 5$  variants together with the  $\beta x \gamma 2$  combination display the characteristics of the "classical" BZ type II receptors. BZ I and BZ II receptors distribute unevenly over the rat brain. BZ I receptors predominate in the cerebellum, but are rare in the hippocampus (Faull and Villiger, 1988; Faull, Villiger and Holford, 1987, Olsen, McCabe and Wamsley 1990). In contrast, BZ II receptors are strongly expressed in the hippocampus and nearly absent from the cerebellum. Both receptor subtypes are equally expressed in the cortical layers (Faull and Villiger, 1988; Faull, Villiger and Holford 1987; Olsen, McCabe and Wamsley 1990). BZ type II receptors are also enriched in the striatum and spinal cord (Lo, Strittmatter and Snyder, 1983; Olsen and Tobin, 1990, Sieghart *et al.*, 1985).  $\alpha 5 \beta 2 \gamma 2$  receptors have similarly low affinities for CL 218,872 and 2-oxoquazepam as  $\alpha 2 \beta x \gamma 2$  and

$\alpha 3\beta x\gamma 2$  receptors. However, the binding properties for imidazopyridines like zolpidem and alpidem differ from conventional GABA<sub>A</sub>/BZ II receptors (Pritchett and Seeburg, 1990), and hence sensitivity to these imidazopyridines constitutes a subtype of the BZ II receptors. Recombinant receptors expressing  $\alpha 6\beta 2\gamma 2$  in HEK293 cells bind with high affinity muscimol and the imidazo-1,4-benzodiazepine Ro-15-4513. Other benzodiazepines and  $\beta$ -carbolines are either not recognized by this receptor or at an affinity two to three orders of magnitude lower than that of  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  or  $\alpha 5$ -containing receptors.

During development of the CNS, changes in receptor composition play a role in the developmental modifications of glycine, nicotinic acetylcholine, and glutamate receptor function. As discussed in Chapter 3, several differences also exist between adult and neonatal GABA responses. These differences which are characteristics of neonatal responses include: 1) the depolarizing effects of GABA on neonatal neurons (Ben-Ari *et al.*, 1989; Cherubini *et al.*, 1990); 2) reduced desensitization of neonatal GABA currents (Cherubini *et al.*, 1990); 3) limited outward rectification of neonatal GABA currents at membrane potentials more depolarized than -40 mV (Ito and Cherubini, 1991); and 4) the potentiation of neonatal GABA currents by barbiturates but not benzodiazepines (Rovira and Ben-Ari, 1991).

In this chapter, *in situ* hybridization analysis has been used to investigate the onset of expression and the localisation of ten GABA<sub>A</sub> receptor subunit mRNAs during postnatal development of the murine central nervous system in order to determine if changes in GABA<sub>A</sub> receptor subunit mRNA expression and hence corresponding changes in GABA<sub>A</sub> receptor composition may account for the differences in neonatal versus adult GABA responses.

## 4.2 Results

GABA<sub>A</sub> receptor subunit gene expression was studied during murine postnatal CNS development through *in situ* hybridization with antisense subunit-specific oligonucleotide probes. Probes were designed either to sequences for GABA<sub>A</sub> receptor subunits  $\alpha 1$ ,  $\alpha 3$ ,  $\beta 1$ ,  $\beta 3$ , and  $\gamma 1$  that were isolated as outlined in Chapter 3 or to mouse GABA<sub>A</sub> receptor subunit sequences  $\alpha 2$ ,  $\alpha 6$ ,  $\gamma 2$ ,  $\gamma 3$  and  $\delta 1$  retrieved from the Genebank database. The regional distribution patterns of the GABA<sub>A</sub> receptor  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 6$ ,  $\beta 1$ ,  $\beta 3$ ,  $\gamma 1$ ,  $\gamma 2$ ,  $\gamma 3$  and  $\delta 1$  subunit mRNAs in postnatal 1 day (P0), 7 day (P7), 14 day (P14) and adult (four to five month old) mouse brains are shown in Figures 4.1 to 4.17 and summarized in Tables 4.1 to 4.7. After exposure to X-ray film, slides were dipped in emulsion and stored in the dark for 60 to 120 days at 4 °C. After exposure the slides were developed, counterstained with thionin and viewed using a Zeiss microscope under bright-field and dark-field optics.

All cellular localisation of the various GABA<sub>A</sub> receptor subunit probes was determined using emulsion-dipped slides.

Although the experiments were standardized such that each contained the same amount of radioactively labeled probe, no attempt was made to accurately quantitate the results. It is clear, however from these results that the levels of transcript detected, in general, correspond well with the relative abundance of mRNA for the various GABA<sub>A</sub> receptor subunits detected by S1 nuclease analysis in the previous chapter. Images were virtually devoid of signal on parallel control sections, where each of the ten antisense oligonucleotide probes were competed out by a thirty-fold molar excess of the appropriate unlabelled oligonucleotide (Figures 4.18 and 4.19), thus confirming the specificity of the individual hybridization signals.

## 4.2.1 GABA<sub>A</sub> Receptor $\alpha$ Subunit mRNA Distributions

### 4.2.1.1 Cortex

During postnatal development of the CNS, the  $\alpha$  subunits of the GABA<sub>A</sub> receptor were expressed differentially among neocortical layers. At birth, for instance, distribution of the GABA<sub>A</sub> receptor  $\alpha$ 1 subunit was restricted to the outermost layers (I and II) of the cerebral cortex (Figures 4.1A, 4.2A). However, by P7, expression of the GABA<sub>A</sub> receptor  $\alpha$ 1 subunit transcript was abundant throughout the neocortex and then declined sometime after P14 to adult levels. Emulsion-dipped slides showed silver grain distribution for the  $\alpha$ 1 subunit mRNA in the P14 cortex was distributed over large pyramidal cell bodies (Figure 21B, 21D). The GABA<sub>A</sub> receptor  $\alpha$ 2 subunit mRNA was expressed in cerebral cortical layers I, II and III of the P0 murine brain (Figures 4.3A, 4.4A). At P7 (Figures 4.3B, 4.4B) and P14 (Figures 4.3C, 4.4C), the  $\alpha$ 2 subunit transcript was moderately expressed in cerebral cortical layers I and II and weakly expressed throughout the remaining cerebral cortical layers. In the adult CNS, the  $\alpha$ 2 subunit mRNA of the GABA<sub>A</sub> receptor was only weakly expressed throughout the neocortex (Figures 4.3D and 4.4D). Of the four GABA<sub>A</sub> receptor  $\alpha$  subunits studied, the  $\alpha$ 3 mRNA was the most abundant transcript in the P0 neocortex (Figures 4.5A, 4.6A) and this level of  $\alpha$ 3 subunit mRNA expression in the cerebral cortex was maintained throughout postnatal development as well as in the adult brain (Figures 4.5, 4.6). There was no expression of the  $\alpha$ 6 GABA<sub>A</sub> receptor subunit transcript in the cerebral cortex during postnatal development or in the adult brain.

### 4.2.1.2 Hippocampus

The  $\alpha$ 2 subunit of the GABA<sub>A</sub> receptor was the predominant  $\alpha$  isoform of the four studied in the P0 hippocampus (Figures 4.3A, 4.4A).  $\alpha$ 2 mRNA was present throughout the stratum pyramidale and weakly in the granule cells of the dentate gyrus. The  $\alpha$ 1 isoform

was weakly expressed in the P0 CA1 (Figures 4.1A, 4.2A), while the transcript for the  $\alpha 3$  subunit variant was weakly expressed throughout the P0 hippocampal pyramidal cell layer (Figures 4.5A, 4.6A). At P7 (4.1B, 4.2B, 4.3B, 4.4B) and P14 (4.1C, 4.2C, 4.3C, 4.4C), mRNAs for the  $\alpha 1$  (Figures 4.1, 4.2) and  $\alpha 2$  (Figures 4.3, 4.4) GABA<sub>A</sub> receptor subunit isoforms were abundantly expressed in the CA1 pyramidal cell layer. In the P7 CA3,  $\alpha 2$  was again the predominant isoform (Figures 4.3B and 4.4B), while the  $\alpha 3$  mRNA was moderately expressed (Figures 4.5B and 4.6B) and the  $\alpha 1$  transcript was weakly expressed (Figures 4.1B and 4.2B).  $\alpha 1$  and  $\alpha 2$  mRNAs were moderately expressed in the P7 (Figures 4.1B, 4.2B, 4.3B, 4.4B) dentate gyrus, whereas by P14 both transcripts were abundantly expressed in the dentate granule cells (Figures 4.1C, 4.2C, 4.3C, 4.4C). Emulsion-dipped slides demonstrated that the silver grain distribution for the  $\alpha 1$  subunit mRNA was present over granule cell bodies of the P14 dentate gyrus and over a subset of cells in the hilus (4.21F, 4.21H, 4.22F). The transcript for  $\alpha 3$  was not detected in the dentate gyrus during postnatal development or in the adult brain (Figures 4.5, 4.6).  $\alpha 1$  (Figures 4.1D, 4.2D) and  $\alpha 2$  (Figures 4.3D, 4.4D) transcripts were moderately expressed in the adult CA1. In contrast, in adult CA3 pyramidal cells, the  $\alpha 1$  transcript (Figures 4.1D, 4.2D) was weakly expressed, while the  $\alpha 2$  subunit mRNA (Figures 4.3D, 4.4D) was detected at moderate levels.

#### 4.2.1.3 Thalamus

In the newborn thalamus,  $\alpha 1$  mRNA expression was absent (Figure 4.2B, Table 4.1), whereas the  $\alpha 2$  and  $\alpha 3$  GABA<sub>A</sub> receptor subunit mRNAs were differentially expressed among thalamic nuclei (Figures 4.4A, 4.6A). For example, in the P0 thalamus,  $\alpha 2$  mRNA was weakly expressed in the ventrolateral, anteromedial, dorsal lateral genicular, ventral posterior and lateral dorsal thalamic nuclei (Figure 4.4A, Table 4.1), whereas the  $\alpha 3$  subunit mRNA was weakly expressed in the anteroventral, paraventricular, anteromedial, ventrolateral, dorsal lateral genicular, and ventroposterior lateral thalamic nuclei (Figure 4.6A, Table 4.1). At P0, the  $\alpha 3$  subunit mRNA was abundantly expressed in the reticular thalamic nucleus and  $\alpha 2$  mRNA was absent (Figures 4.6A and 4.4A, respectively). By P7, the  $\alpha 1$  transcript was moderately expressed in the anteromedial, dorsal lateral geniculate, laterodorsal and lateroposterior thalamic nuclei (Figure 4.2B). The  $\alpha 2$  transcript was present in the anteroventral, paraventricular, ventrolateral, dorsal lateral genicular, medial genicular, laterodorsal and lateral posterior P7 thalamic nuclei (Figure 4.4B, Table 4.1), whereas the  $\alpha 3$  transcript was present in the same nuclei as the  $\alpha 2$  subunit mRNA as well as the anteromedial, reticular and ventroposterior lateral thalamic nuclei (Figure 4.6B, Table 4.1). By P14,  $\alpha 1$  and  $\alpha 3$  subunit mRNAs were present at different levels of expression in all thalamic nuclei listed in Table 4.1, except in the anteroventral thalamic nucleus where the  $\alpha 3$  subunit mRNA was absent (Figures 4.2C and 4.6C). The  $\alpha 2$  mRNA was present at

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P14 in the anteroventral, paraventricular, anteromedial and medial genicular thalamic nuclei (Figure 4.4C, Table 4.1). Adult thalamic levels of  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  GABA<sub>A</sub> receptor subunit mRNA expression were significantly reduced from the levels observed at P7 and P14 (Table 4.1). In the adult,  $\alpha 2$  mRNA expression was restricted to the anteromedial, paraventricular and anteromedial thalamic nuclei (Figure 4.4D, Table 4.1).  $\alpha 1$  and  $\alpha 3$  transcripts were weakly co-expressed in the adult anteroventral, paraventricular, anteromedial, reticular, laterodorsal, and lateral posterior thalamic nuclei (Figures 4.2D and 4.6D). In addition, the  $\alpha 1$  transcript was weakly expressed in the adult dorsal lateral genicular nucleus (Figure 4.2D, Table 4.1), whereas the  $\alpha 3$  mRNA was present in the adult ventrolateral thalamic nucleus (Figure 4.4D, Table 4.1).

#### 4.2.1.4 Cerebellum

Expression of  $\alpha$  subunit mRNAs in the P0 cerebellum included weak expression of  $\alpha 1$ ,  $\alpha 3$  and  $\alpha 6$  GABA<sub>A</sub> receptor subunit mRNAs in the external granule cell layer (Figures 4.1A, 4.2A, 4.5A, 4.6A, 4.7A). By P7, the  $\alpha 1$  subunit mRNA of the GABA<sub>A</sub> receptor was abundantly expressed in Purkinje cells and the internal granule cell layer, and moderately expressed in basket cells of the molecular layer and in the external granule cell layer (Figures 4.1B, 4.2B and 4.20E, 4.20F). Weak levels of mRNA expression were apparent for the  $\alpha 2$  subunit in the P7 Purkinje cell layer and for the  $\alpha 2$  and  $\alpha 3$  subunits in the internal and external granule cell layers (Figures 4.3B, 4.4B, 4.5 B, 4.6B). The  $\alpha 6$  subunit mRNA was uniquely and abundantly expressed in the internal granular cell layer of the P7 (Figures 4.20G, 4.20H) and P14 (Figures 4.20K, 4.20L) cerebellum and moderately expressed in the internal cerebellar granule cell layer of the adult (Figures 4.20O, 4.20P). Emulsion-dipped slides of the P14 cerebellum demonstrated that silver grains for the  $\alpha 1$  transcript were abundantly distributed over the Purkinje cells and the internal and external granule cells (Figures 4.20). In the adult cerebellum, the  $\alpha 1$  subunit mRNA was moderately expressed in the Purkinje cells and internal and external granule cell layers (Figures 4.1D, 4.1D, 4.20M, 4.20N). The  $\alpha 1$  subunit mRNA was also weakly expressed in the molecular layer of the P14 (Figures 4.20I, 4.20J) and adult cerebellum (Figures 4.20M, 4.20N).  $\alpha 2$  mRNA was weakly expressed in the P14 (Figures 4.3C, 4.4C) and adult (Figures 4.3D and 4.4D) Purkinje cell layer of the cerebellum. In the P14 cerebellum (Figures 4.5C, 4.6C), the  $\alpha 3$  transcript was weakly in the molecular and internal and external granule cell layers, whereas in the adult cerebellum  $\alpha 3$  mRNA expression was absent (Figures 4.5D and 4.6D).

## 4.2.2 GABA<sub>A</sub> Receptor $\beta$ Subunit mRNA Distributions

### 4.2.2.1 Cortex

At birth,  $\beta 1$  and  $\beta 3$  GABA<sub>A</sub> receptor subunit mRNAs were differentially distributed among the cerebral cortical cell layers. For example  $\beta 1$  (Figure 4.8A) and  $\beta 3$  (Figures 4.9A, 4.10A) subunit mRNAs were moderately expressed in cerebral cortical layers I and II and weakly expressed in layers V and VI. In contrast to this similar signal intensity of  $\beta 1$  and  $\beta 3$  subunit mRNAs in cortical layers I, II, V and VI, the  $\beta 1$  transcript was also weakly expressed in cerebral cortical layers III and IV (Figure 4.8A), whereas the  $\beta 3$  subunit mRNA was moderately expressed in cortical layers III and IV (Figures 4.9A and 4.10A). In the P7 cerebral cortex, the  $\beta 1$  transcript was expressed weakly in all layers (Figure 4.8B), while the  $\beta 3$  subunit was expressed moderately in layers I, II, III, IV, and V (Figures 4.9B and 4.10B). The  $\beta 3$  transcript localisation and levels of expression observed in the P7 cerebral cortex were also present in the P14 and adult cerebral cortex (Figures 4.9 and 4.10). Emulsion-dipped slides revealed that the silver grain distribution for the  $\beta 3$  subunit mRNA was localised over pyramidal cell somata in P14 cerebral cortical layers II, III and IV (Figure 4.21A, 4.21C) In contrast, the  $\beta 1$  transcript was expressed moderately throughout the P14 (Figure 4.8C) cerebral cortex and weakly in all adult cortical layers (Figure 4.8D).

### 4.2.2.2 Hippocampus

In the P0 pyramidal cell layer, both the  $\beta 1$  (Figure 4.8A) and  $\beta 3$  (Figures 4.9A, 4.10A) GABA<sub>A</sub> receptor subunit mRNAs were moderately expressed (Figures 4.8, 4.9 and 4.10). In addition to the pyramidal cell distribution, the signal for the  $\beta 3$  subunit transcript was also present at a moderate level in the P0 dentate gyrus granule cell layer (Figures 4.9A and 4.10A). In the P7 (Figures 4.9B, 4.10B) and P14 (Figures 4.9C, 4.10C) hippocampus, the  $\beta 3$  transcript was abundantly expressed throughout the pyramidal cell layer and in the dentate gyrus (Figures 4.9B,C and 4.10B,C), whereas the  $\beta 1$  subunit mRNA was expressed at moderate levels in the P7 (Figure 4.8B) and P14 (Figure 4.8C) pyramidal cell layer and increased from weak to moderate expression levels in the P7 (Figure 4.8B) and P14 (Figure 4.8C) dentate gyrus granule cells, respectively. Silver grain distribution for the  $\beta 3$  subunit mRNA in the P14 dentate gyrus was observed over granule cell bodies, and only weakly over a small subset of hilar interneurons (Figures 4.21E, 4.21G) In the adult hippocampus,  $\beta 1$  (Figure 4.8D) and  $\beta 3$  (Figures 4.9D, 4.10D) transcripts were moderately expressed in the pyramidal cell layer. The  $\beta 1$  transcript (Figure 4.8D) was weakly expressed in the adult dentate gyrus, while the  $\beta 3$  mRNA (Figures 4.9D, 4.10D) was expressed moderately in the adult dentate granule cells.

### 4.2.2.3 Thalamus

Throughout postnatal development and in the adult brain, the  $\beta 3$  subunit mRNA of the GABA<sub>A</sub> receptor was expressed weakly in the anteroventral, paraventricular, anteromedial, ventrolateral, dorsal lateral genicular, medial lateral genicular, ventral posterior, laterodorsal, and lateral posterior thalamic nuclei (Figures 4.9, 4.10). In the P0 (Figure 4.8A), P7 (Figure 4.8B) and adult (Figure 4.8D) thalamus,  $\beta 1$  mRNA was expressed at weak levels in the anteroventral, paraventricular, anteromedial, ventrolateral, ventral posterior, laterodorsal and lateral posterior thalamic nuclei. The  $\beta 1$  subunit mRNA was expressed at moderate levels in the laterodorsal and lateral posterior P14 thalamic nuclei (Figure 4.8C, Table 4.2) and at weak levels in the anteroventral, paraventricular, anteromedial, ventrolateral and ventral posterior P14 (Figure 4.8C, Table 4.2) thalamic nuclei.

### 4.2.2.4 Cerebellum

At birth, of the two  $\beta$  subunit mRNAs studied, only the  $\beta 3$  subunit transcript (Figures 4.9A, 4.10A) was expressed in the molecular and internal granular cell layers of the newborn cerebellum. By P7, the  $\beta 1$  transcript (Figure 4.8B and Table 4.2) was expressed weakly in the stellate and basket cells of the molecular layer and in the internal and external granule cell layers. In contrast, the  $\beta 3$  subunit mRNA (Figures 4.9B, 4.10B) was expressed moderately in the molecular layer and abundantly in the internal and external granule cell layers. There was a moderate level of  $\beta 1$  mRNA expression (Figure 4.8C) in the molecular and internal and external granule cell layers of the P14 cerebellum, whereas silver grain distribution for the  $\beta 3$  transcript (Figures 4.9C, 4.10C), at P14 was distributed weakly over basket cell somata of the molecular layer and abundantly over internal and external granule cell somata of the cerebellum (Figures 4.21I, 4.21J). In the adult cerebellum both the  $\beta 1$  (Figure 4.8D) and  $\beta 3$  (Figures 4.9D and 4.10D) subunit mRNAs were expressed weakly in the molecular and internal and external granule cell layers.

## 4.2.3 GABA<sub>A</sub> Receptor $\gamma$ and $\delta$ Subunit mRNA Distributions

### 4.2.3.1 Cortex

At birth, the mRNAs for the  $\gamma 1$  (Figures 4.11A, 4.12A) and  $\gamma 3$  (Figure 4.15A) subunits of the GABA<sub>A</sub> receptor were expressed weakly throughout all cerebral cortical layers. The  $\gamma 2$  subunit probe ( $\gamma 2T$ ), which identifies both the  $\gamma 2L$  and  $\gamma 2S$  alternatively-spliced variants, was intensely distributed in cerebral cortical layers I, II, III, and V and showed a moderate signal intensity in cortical layers IV and V (Figures 4.13A, 4.14A). Throughout postnatal development and in the adult brain, the  $\gamma 3$  subunit mRNA of the GABA<sub>A</sub> receptor was expressed weakly throughout the neocortex (Figure 4.15). In the P7 (Figures 4.11B, 4.12B) and P14 (Figures 4.11C, 4.12C) cerebral cortex, the  $\gamma 1$  transcript was moderately

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expressed in all cortical layers, whereas in the adult (Figures 4.11D, 4.12D) the level of  $\gamma 1$  mRNA was reduced to weak levels of expression in all cortical layers. The signal for the  $\gamma 2T$  mRNA was abundantly expressed in cortical layers III and V and was expressed at moderate levels in layers I, II, IV, and VI of the P7 (Figures 4.13B, 4.14B) and adult (Figures 4.13D, 4.14D) cerebral cortex. In contrast to the differential levels of  $\gamma 2T$  subunit mRNA expression in the P7 and adult cerebral cortex, at P14, the  $\gamma 2T$  transcript was abundantly expressed in all cortical layers (Figures 4.13C, 4.14C). Expression of the transcript for the  $\delta 1$  subunit of the GABA<sub>A</sub> receptor was not detected in the P0 brain (Figures 4.16A, 4.17A), however in the P7 (Figures 4.16B, 4.17B) and P14 (Figures 4.16C, 4.17C) neocortex, mRNA for the  $\delta 1$  subunit was moderately expressed in layers I, II, III, and IV and was weakly expressed in layers V and VI. In the adult a weak signal for the  $\delta 1$  GABA<sub>A</sub> receptor subunit mRNA was found in all layers of the cerebral cortex (Figures 4.16D, 4.17D).

#### 4.2.3.2 Hippocampus

The  $\gamma 2T$  subunit mRNA was the only  $\gamma$  subunit mRNA of the three studied to be expressed at birth in the hippocampus. Expression of the  $\gamma 2T$  transcript was abundant in P0 CA1 pyramidal cells and moderately expressed in the CA3, in the hilus and in the granule cells of the dentate gyrus (Figures 4.13A, 4.14A). In the P7 (Figures 4.13B, 4.14B) and P14 (Figures 4.13C, 4.14C) hippocampus, the  $\gamma 2T$  subunit mRNA was expressed abundantly throughout the pyramidal cell layer as well as in the hilus and dentate gyrus. Silver grain distribution for the  $\gamma 2T$  subunit mRNA was present over P14 dentate granule cell bodies and hilar neurons (Figure 4.22E). The  $\gamma 3$  subunit transcript was expressed weakly in the CA1, CA3 and dentate gyrus of the P7 (Figure 4.15B), P14 (Figure 4.15C) and adult (Figure 4.15D) hippocampus. The  $\gamma 1$  subunit transcript was expressed weakly in the pyramidal cell layer, in hilar cells and in dentate granule cells of the P7 (Figures 4.11B, 4.12B) and adult (Figures 4.11D, 4.12D) hippocampus. In the P14 hippocampus, the  $\gamma 1$  transcript was expressed moderately in the CA1 through CA3 pyramidal cells, in hilar interneurons and in dentate granule cells (Figures 4.11C, 4.12C). Expression of the  $\delta 1$  subunit mRNA of the GABA<sub>A</sub> receptor was restricted to the dentate granule cells of the P7 (Figures 4.16B, 4.17B), P14 (Figures 4.16C, 4.17C) and adult (Figures 4.16D, 4.17D).

#### 4.2.3.3 Thalamus

Expression of the  $\gamma 3$  (Figure 15A, Table 4.3) and  $\delta 1$  (Figure 4.17A) subunit mRNAs of the GABA<sub>A</sub> receptor was absent in the P0 thalamus. In the P0 thalamus, the  $\gamma 1$  (Figure 4.12A) subunit mRNA was expressed at moderate levels in the anteroventricular, paraventricular, anteromedial, and ventrolateral thalamic nuclei and was expressed abundantly in the medial geniculate, lateral dorsal and lateroposterior thalamic nuclei. At P0,

the signal for the  $\gamma 2T$  (Figure 4.14A, Table 4.3) subunit mRNA was present at moderate levels in the paraventricular, ventrolateral, medial geniculate, reticular, laterodorsal and lateral posterior thalamic nuclei and at weak levels in the anteroventral, anteromedial, dorsal lateral geniculate, and ventral posterior thalamic nuclei. In the P7 thalamus, moderate levels of the  $\gamma 1$  mRNA were present in the paraventricular, anteromedial, dorsal lateral geniculate, laterodorsal and lateral posterior nuclei, while an abundant level of the  $\gamma 1$  transcript was present in the medial geniculate thalamic nucleus (Figure 4.12B, Table 4.3). The  $\gamma 2T$  subunit mRNA, at P7 was expressed abundantly in the anteroventral and medial geniculate thalamic nuclei, moderately in the paraventricular, anteromedial, dorsal lateral geniculate and reticular thalamic nuclei and finally at weak levels in the ventrolateral, ventral posterior, laterodorsal and later posterior thalamic nuclei (Figure 4.14B, Table 4.3). The  $\gamma 3$  subunit mRNA was absent in the thalamus throughout postnatal development, as well as in the adult brain (Figure 4.15). At P7, the  $\delta 1$  subunit mRNA was expressed weakly in the paraventricular, ventrolateral, dorsal lateral geniculate, medial geniculate, ventral posterior and laterodorsal thalamic nuclei, and was moderately expressed in the anteroventral and anteromedial thalamic nuclei (Figure 4.17B, Table 4.3). In the P14 thalamus, the  $\gamma 2T$  subunit mRNA was expressed abundantly in the antroventral, paraventricular, dorsal lateral geniculate and medial geniculate nuclei, and at moderate levels in the anteromedial, ventrolateral, reticular, ventral posterior, laterodorsal, and lateral posterior (Figure 4.14C, Table 4.3). The  $\gamma 1$  subunit mRNA was expressed at moderate levels in the anteroventral, ventral lateral, dorsal lateral geniculate, medial geniculate, ventral posterior, laterodorsal and lateral posterior P14 thalamic nuclei, and at abundant levels in the P14 paraventricular nucleus (Figure 4.12C, Table 4.3). At P14, the  $\delta 1$  subunit mRNA was expressed weakly in the anteroventral, anteromedial, ventrolateral, dorsal lateral geniculate, and medial geniculate thalamic nuclei and at moderate levels in the the ventral posterior and laterodorsal thalamic nuclei (Figure 4.17C, Table 4.3). In the adult thalamus  $\gamma 1$  (Figure 4.12D, Table 4.3) and  $\gamma 2T$  (Figure 4.14D) GABA<sub>A</sub> receptor subunit mRNAs were expressed weakly in the anteromedial, ventrolateral, ventral posterior, lateral dorsal and lateral posterior thalamic nuclei and moderately in the paraventricular, dorsal lateral geniculate and medial geniculate thalamic nuclei. The  $\delta 1$  GABA<sub>A</sub> receptor subunit mRNA was expressed weakly in the anteroventricular, dorsal lateral geniculate and medial geniculate thalamic nuclei, and was moderately expressed in the anteromedial, ventrolateral, and ventral posterior adult thalamic nuclei (Figure 4.17D, Table 4.3).

#### 4.2.3.4 Cerebellum

In the newborn cerebellum,  $\gamma 2T$  (Figures 4.13A, 4.14A) and  $\gamma 3$  (Figure 4.15A) subunit mRNAs were expressed at moderate levels in the internal granule cell layer, while the mRNA for the  $\gamma 1$  (Figures 4.11A, 4.12A) subunit of the GABA<sub>A</sub> receptor was expressed

weakly and the  $\delta 1$  (Figures 4.16A, 4.17A) subunit mRNA was absent from all areas of the P0 cerebellar cortex. The  $\gamma 2T$  subunit mRNA was also expressed moderately in the Purkinje cell and molecular layers of the P0 cerebellar cortex (Figure 4.13A, 4.14A). In the P7 cerebellum, the  $\gamma 2T$  subunit mRNA was abundantly expressed in the Purkinje cell layer and the internal and external granule cell layers (Figures 4.13B, 4.14B). The  $\gamma 3$  (Figure 4.15B) and  $\delta 1$  (Figures 4.16B, 4.17B) subunit mRNAs were weakly expressed in the P7 basket cells of the cerebellar molecular layer and were moderately expressed in the P7 internal and external granule cell layers. The  $\gamma 1$  subunit mRNA was expressed weakly in the P7 molecular layer and internal and external granule cell layers. The P7 pattern of  $\gamma 2T$  mRNA expression was also present in the P14 cerebellar cortex. Silver grain distribution for the  $\gamma 2T$  transcript was abundantly distributed over P14 cerebellar Purkinje cell somas and granule cell bodies (Figure 4.22B). At P14, the  $\gamma 1$  subunit mRNA was expressed moderately in all layers of the cerebellar cortex (Figures 4.11C, 4.12C), whereas the  $\gamma 3$  subunit mRNA was expressed weakly in the molecular layer and abundantly in the internal and external granular cell layers (Figure 4.15C). The  $\delta 1$  subunit mRNA is also present in the cerebellum at P14 in the molecular and internal and external granule cells layers (Figures 4.16C, 4.17C). In the adult cerebellum the  $\gamma 2T$  subunit transcript was abundantly expressed in the Purkinje cell layer, weakly in the molecular layer and moderately in the internal and external granule cell layers (Figures 4.13D, 4.14D). The  $\gamma 1$  (Figures 4.11D, 4.12D),  $\gamma 3$  (Figure 4.15) and  $\delta 1$  (Figures 4.16D, 4.17D) subunit mRNAs of the GABA<sub>A</sub> receptor were expressed weakly in the adult molecular layer and moderately in the adult internal and external granule cell layers.

#### 4.2.4 Co-Expression of GABA<sub>A</sub> Receptor Subunit mRNAs during Postnatal Development

Serial sections were used to study the co-distribution of GABA<sub>A</sub> receptor mRNAs in the cerebellum and hippocampus of the developing murine CNS. In parallel P14 sections, both the  $\gamma 2T$  and  $\alpha 1$  subunit transcripts were co-localised to Purkinje cells and granule cells of the cerebellum, as illustrated by the arrowheads in Figure 4.22B and 4.22C. In hippocampal parallel P14 sections, cells within the internal granular cell layer of the dentate gyrus that uniquely expressed the  $\gamma 2T$  subunit mRNA were devoid of  $\alpha 1$  subunit mRNA expression as illustrated by the small arrowheads in Figures 4.22E and 4.22F. However, co-expression of the  $\alpha 1$  and  $\gamma 2T$  subunit mRNAs was present over hilar neurons, as indicated by the large arrowhead in Figures 4.22E and 4.22F. In both the P14 hippocampus and cerebellum, serial sections demonstrated that the mRNAs for the  $\beta 3$  and  $\alpha 1$  subunits of the GABA<sub>A</sub> receptor were co-localised in the dentate granular cell somata and cerebellar granular cell bodies, respectively. The  $\beta 3$  (Figures 4.21A, 4.21C) and  $\alpha 1$  (Figures 4.21B, 4.21D) subunit transcript were also co-expressed in pyramidal cell somata in layers II, III,

and IV of the P14 cerebral cortex. Similar to the  $\alpha 1$  and  $\gamma 2T$  localisation, there were also differences in the presence or absence of  $\beta 3$  and  $\alpha 1$  subunit mRNAs in specific cell types. For example, the  $\beta 3$  subunit mRNA was not expressed in P14 cerebellar Purkinje cells, whereas the  $\alpha 1$  subunit transcript was abundantly expressed in the P14 Purkinje cell type.

### 4.3 Discussion

This chapter describes the regional and cellular localisation of 10 GABA<sub>A</sub> receptor subunit mRNAs during murine postnatal CNS development. The results presented in this chapter demonstrate that during postnatal development and in the adult brain, each GABA<sub>A</sub> receptor subunit mRNA has a unique, but overlapping pattern of mRNA distribution. The number of GABA<sub>A</sub> receptor subunit transcripts and the abundance of GABA<sub>A</sub> receptor subunit mRNA expression increases with the completion of cell migration and differentiation and the coincidence of synapse development. In the adult murine CNS, the level of GABA<sub>A</sub> receptor subunit transcript expression had decreased from postnatal levels, yet the number of subunit mRNAs expressed was similar to the number reported for the later stages of postnatal development.

At birth, Table 4.4 indicates that of the four  $\alpha$  isoform mRNAs studied, the  $\alpha 2$  and  $\alpha 3$  subunits are the predominant  $\alpha$  isoforms, while  $\beta 3$  is the predominant  $\beta$  isoform and the  $\gamma 1$  and  $\gamma 2$  are the predominant  $\gamma$  isoforms of the GABA<sub>A</sub> receptor in the P0 CNS. The possible type and number of GABA<sub>A</sub> receptor complexes in the newborn is dependent on the regional distribution of the various GABA<sub>A</sub> receptor subunit mRNAs. For example in the P0 cerebral cortex although the  $\alpha 2$  and  $\alpha 1$  subunit mRNAs are expressed in layers I ( $\alpha 1$ ,  $\alpha 2$ ) or II ( $\alpha 2$ ), only the  $\alpha 3$  subunit of the  $\alpha$  isoforms studied is expressed in cerebral cortical layers III through VI. Hence the most abundant receptor complex in P0 cerebral cortical layers III through VI would be  $\alpha 3/\beta 3/\gamma 2T$ . In contrast, in the P0 hippocampus the most abundant GABA<sub>A</sub> receptor complex would be  $\alpha 2/\beta 3/\gamma 2T$ . Other studies on the ontogenic expression of GABA<sub>A</sub> receptor subunit mRNAs in rat indicate that at P0, the  $\alpha 5$ ,  $\alpha 4$ , and  $\beta 2$  subtype mRNAs are also present at moderate levels (Poulter *et al.*, 1993; Wisden *et al.*, 1992, Laurie, Wisden and Seeburg, 1992b) in the developing cerebral cortex.

As this study has demonstrated, the pronounced cortical expression of the  $\alpha$ ,  $\beta$ , and  $\gamma$  subunit genes by birth predicts the early formation of functional GABA<sub>A</sub> receptors. Paradoxically, although cortical BZ binding is already substantial at birth (60% of adult) (Candy and Martin, 1979; Lippa *et al.*, 1981; Chisholm, Kellogg and Lippa, 1983) and is coupled to GABA<sub>A</sub> receptors (Palacios, Niehoff and Kuhar, 1979; Eichinger and Sieghart, 1986; Kellogg and Pflieger, 1989), the number of rat cortical GABA and muscimol binding sites is approximately 25% of adult levels. The impedance of substantial GABA<sub>A</sub> receptor binding in the newborn may be due to either early subunit composition or post-translational subunit modifications. In the adult rat brain, such mismatches in the densities of GABA<sub>A</sub>

and BZ binding sites also occur in regions such as the cerebellum, thalamus and hippocampus (Unnerstall *et al.*, 1981; Olsen, McCabe and Wamsley, 1990).

At P0, in the medial and triangular septal regions, very few GABA<sub>A</sub> receptor subunit mRNAs are expressed and hence the possible receptor complexes at this stage in development would consist of only  $\alpha 2$  and  $\gamma 1$  or  $\gamma 2$  subunits. From transient expression studies in L929 fibroblasts,  $\alpha 1\beta 1$ , but not  $\alpha 1\gamma 2$  or  $\beta 1\gamma 2$ , subtypes assemble to produce benzodiazepine insensitive GABA<sub>A</sub> receptor channels (Angelotti, Uhler and Macdonald, 1993). Hence at this stage in postnatal development either the GABA<sub>A</sub> receptor complexes assembled are non-functional or another  $\beta$  subunit, such as  $\beta 2$  may be the primary  $\beta$  transcript in this region of the developing CNS. In fact, in a study of ontogenic expression of GABA<sub>A</sub> receptor subunit mRNAs in the rat, the  $\beta 2$  subunit mRNA was also detected in the P0 medial septum (Wisden *et al.*, 1992).

In the P0 thalamic nuclei, results from *in situ* hybridisation analysis would predict GABA<sub>A</sub> receptor complexes with subunit compositions of  $\alpha 2/\beta 1/\gamma 1$ ,  $\alpha 2/\beta 3/\gamma 1$ ,  $\alpha 2/\beta 1/\gamma 2T$ ,  $\alpha 2/\beta 3/\gamma 2T$ ,  $\alpha 3/\beta 1/\gamma 1$ ,  $\alpha 3/\beta 1/\gamma 2T$ ,  $\alpha 3/\beta 3/\gamma 1$  and  $\alpha 3/\beta 3/\gamma 2T$ . Although  $\alpha 4$  and  $\alpha 5$  subunit mRNAs are also expressed in the rat P0 thalamus (Laurie, Wisden and Seeburg, 1992b; MacLennan *et al.*, 1991), the levels of transcript expression for both  $\alpha$  variants are weak and therefore  $\alpha 4$  and  $\alpha 5$  containing receptor complexes would be a minor component of the GABA<sub>A</sub> receptor response of the P0 thalamus. The  $\beta 2$  transcript was not detected in the thalamus at this stage of CNS development (Laurie, Wisden and Seeburg, 1992b).

Transcripts for  $\alpha 1$ ,  $\alpha 3$ ,  $\alpha 6$ ,  $\beta 1$ ,  $\beta 3$ ,  $\gamma 1$ ,  $\gamma 2$ , and  $\gamma 3$  subunits of the GABA<sub>A</sub> receptor are present in the thick P0 external granule cell layer and in granule cells migrating through the molecular layer to the internal granule cell layer.  $\alpha 6$  mRNA expression at this stage in development is very weak. In fact, results of this study indicate that the  $\alpha 6$  transcript is abundantly expressed only after the completion of granule cell migration from the external to the internal granule cell layer, which occurs between P2 and P21 (Altman, 1972; Meinecke and Rakic, 1990). After migration, granule cells within the internal granule cell layer extend dendrites which will become postsynaptic to the axons of the Golgi II cells and it is at this time that the receptor proteins can be detected by immunocytochemistry and electron microscopy (Meinecke and Rakic, 1990). In contrast, both GAD and GABA immunoreactivities can be detected in the very early stages (P2-P7) of postnatal cerebellar development.

Although this study and others have concluded that  $\alpha 6$  expression is limited to cerebellar granule cells, Varecka *et al.*, (1994) have also observed murine  $\alpha 6$  GABA<sub>A</sub> receptor subunit expression in the cochlear granule cells. Both cerebellar granule cells and cochlear granule cells are thought to arise from a single precursor pool. Immunocytochemical analysis further supports the localisation of the  $\alpha 6$  polypeptide in granule cells of the dorsal cochlear nucleus, as well as in axons of the olfactory nerve including the glomerular endings, layer II

of the dorsal horn of the spinal cord and in the retinal synaptic layers, particularly the inner plexiform layer (Gutierrez, Khan and De Blas, 1996).

Although cerebellar granule cells receive GABAergic input from only a single cell type, the Golgi cell, two types of GABA<sub>A</sub> receptor-mediated inhibition (phasic and tonic) have been described. In adult rats, granule cells express six GABA<sub>A</sub> receptor subunits abundantly ( $\alpha 1$ ,  $\alpha 6$ ,  $\beta 2$ ,  $\beta 3$ ,  $\gamma 2$ , and  $\delta$ ). This relative abundance of rat GABA<sub>A</sub> receptor subunits differs from data presented in this chapter, where of the subunits studied in the adult mouse,  $\alpha 1$ ,  $\alpha 6$ ,  $\beta 3$ ,  $\gamma 1$ ,  $\gamma 2$  and  $\delta$  were expressed abundantly in cerebellar granule cells. Co-assembly of these subunits results in at least four to six distinct GABA<sub>A</sub> receptor subtypes. With the use of electron microscopy Nusser, Sieghart and Somogyi (1998) have demonstrated that the subcellular localisations of  $\alpha 1$ ,  $\alpha 6$ ,  $\beta 2/3$  and  $\gamma 2$  subunits are concentrated in GABAergic Golgi synapses (Nusser et al., 1996) and at lower concentrations in the extrasynaptic membrane. In contrast, immunoparticles for the  $\delta$  subunit could not be found in synaptic junctions, although they were abundantly present in the extrasynaptic dendritic and somatic membranes. Gold particles for the  $\alpha 6$ ,  $\gamma 2$  and  $\beta 2/3$ , but not the  $\alpha 1$  and  $\delta$  subunits were also concentrated in some glutamatergic mossy fiber synapses, where their co-localisation with AMPA-type glutamate receptors was demonstrated. The authors conclude that the exclusive extrasynaptic presence of the  $\delta$  subunit-containing receptors, together with their kinetic properties, suggests that tonic inhibition could be mediated mainly by extrasynaptic  $\alpha 6\beta 2/3\delta$  receptors, whereas phasic inhibition is attributable to the activation of synaptic  $\alpha 1\beta 2/3\gamma 2$ ,  $\alpha 6\beta 2/3\gamma 2$  and  $\alpha 1\alpha 6\beta 2/3\gamma 2$  receptors (Nusser, Sieghart and Somogyi, 1998). Jones et al., (1997) have recently demonstrated that in an  $\alpha 6$ -deficient mouse, the levels of the  $\delta$  polypeptide were drastically reduced, suggesting that an  $\alpha 6\delta$ -containing receptor complex is required for proper membrane targeting.

Recently, Jechlinger et al., (1998) have utilised a new method to quantitate the subtypes of  $\alpha 6$ -containing GABA<sub>A</sub> receptors present in the adult rat cerebellum. Results obtained from these studies indicated that  $\alpha 6$  receptors in the cerebellum are composed predominantly of  $\alpha 6\beta x\gamma 2$  (32%),  $\alpha 1\alpha 6\beta x\gamma 2$  (37%),  $\alpha 6\beta x\delta$  (14%) or  $\alpha 1\alpha 6\beta x\delta$  (15%) subunits. Experiments to determine the relative abundance of the  $\beta$  subunits indicated that 10%, 51% and 21% of  $\alpha 6$  receptors contained homogeneous  $\beta 1$ ,  $\beta 2$  or  $\beta 3$  subunits, respectively, whereas two different  $\beta$  subunits were present in 18% of all  $\alpha 6$  receptors. This method can be used to resolve the total number, subunit composition and abundance of GABA<sub>A</sub> receptor subtypes in other brain regions.

In the cerebellum, Wisden *et al.* (1992) described the detection of GABA<sub>A</sub> receptor subunit mRNAs in rat Purkinje cells at birth, while McKernan *et al.*, (1991a) described the postnatal increase in cerebellar  $\alpha 1$ -like immunoreactivity on Western blots. Results from Wisden *et al.*, 1992 indicate that increases in postnatal expression of GABA<sub>A</sub> transcripts in

both rat Purkinje and postmigratory granule cells ( $\alpha 1$ ,  $\beta 2$ ,  $\beta 3$ ,  $\gamma 2$ ,  $\delta$ ) would be paralleled by increases in GABA<sub>A</sub> and type I BZ binding after P8 (Coyle and Enna, 1976; Candy and Martin, 1979; Palacios and Kuhar, 1981; Chisholm, Kellogg and Lippa, 1983; Zdilar, Rotter and Frostholm, 1991). An acceleration of BZ binding mainly in the molecular layer between P14 and P28 is probably due to the coincident development of the Purkinje cell dendritic tree (Jacobson, 1978) and extensive production of receptors containing  $\alpha 1/\beta 1, \beta 3/\gamma 2/\delta$ . In cultured rat hippocampal neurons, the suppression of  $\alpha 6$  expression can be over-ridden temporarily (Killisch *et al.*, 1991). Whereas moderate BZ binding is present in rat neonatal cerebellum, [3H]-muscimol and [3H]-GABA binding only begins 1 to 2 weeks after birth (Coyle and Enna, 1976; Palacios and Kuhar, 1981). Diazepam-resistant [3H] Ro15-4513 binding, that is a feature of the  $\alpha 6\beta x\gamma 2$  GABA<sub>A</sub> receptors appears in the rat cerebellar granule cell layer after P6 (Uusi-Oukari *et al.*, 1991). Exchange of  $\alpha 1$  for  $\alpha 2$  or  $\alpha 3$  and  $\gamma 1$  for  $\gamma 2$  in recombinant  $\alpha\beta\gamma$  receptors drastically alters their responses to GABA and BZ agonists and inverse agonists (Ymer *et al.*, 1990; Puia *et al.*, 1991). Until P8-P12, GABA apparently mediates neuronal depolarisation through GABA<sub>A</sub> receptors rather than hyperpolarisation as found in the adult brain. This difference is thought to be due to opposite electrochemical chloride gradients.

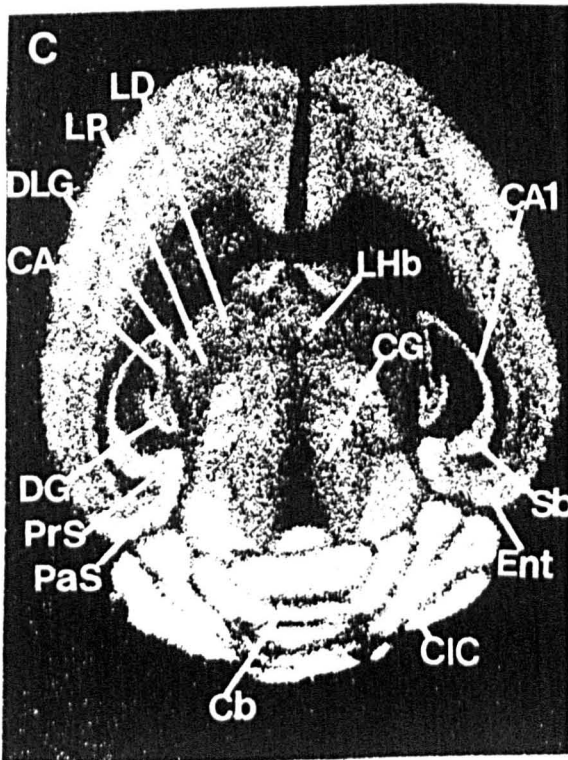
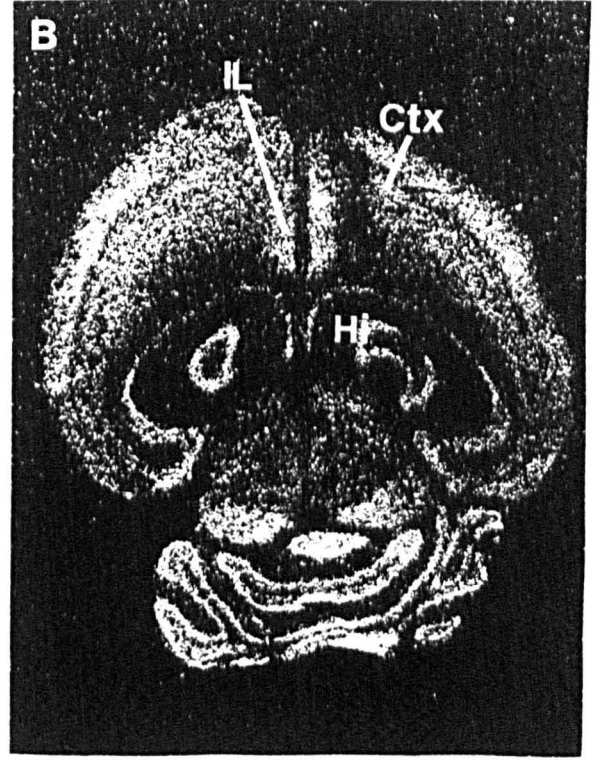
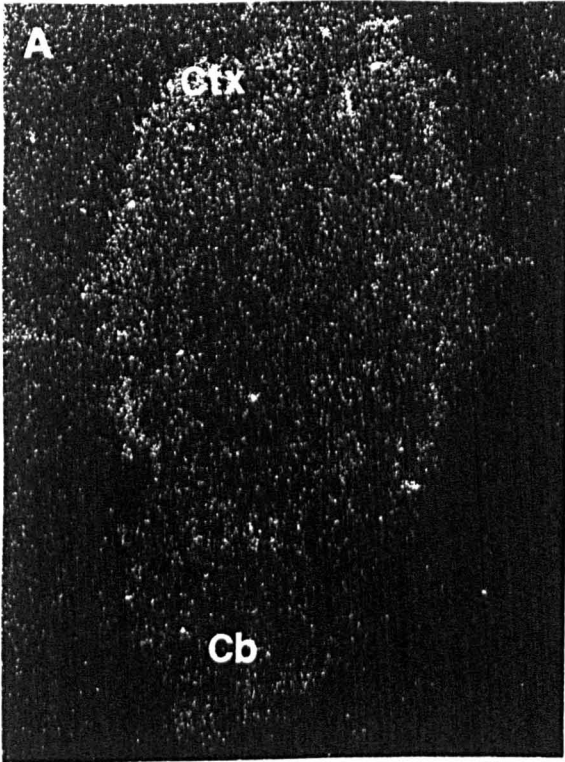
One exception of this switch from the depolarising to the hyperpolarising effect of GABA-activated chloride currents in the adult occurs in the hypothalamic suprachiasmatic nucleus (SCN) where circadian behaviour is manifest by an endogenous clock. In the SCN, immunoreactivity for  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ , and  $\gamma 2$  GABA<sub>A</sub> receptor subunit polypeptides have been identified, while immunoreactivity for  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\beta 2/3$  and  $\gamma 2$  GABA<sub>A</sub> receptor subunit polypeptides has been demonstrated in areas that receive SCN input including the intergeniculate leaflet, subparaventricular zone, paraventricular hypothalamic nucleus, the retrochiasmatic area and the paraventricular nucleus of the thalamus (Gao, Fritschy and Moore, 1995). Within the circadian timing system there is also a differential distribution of GABA<sub>A</sub> receptor polypeptides at the cellular level. Immunoreactivities for the  $\alpha 2$  and  $\beta 2/3$  subunit polypeptides were predominantly found in neuropil, while immunoreactive products for  $\alpha 3$ ,  $\alpha 5$  and  $\gamma 2$  subunits were predominantly localised over perikarya. Immunoreactive localisation of the  $\alpha 1$  subunit polypeptide occurred over both neuropil and perikarya thalamus (Gao, Fritschy and Moore, 1995). Wagner *et al.*, (1997) used an *in vitro* brain-slice technique to study the effect of bath-applied GABA on adult SCN neurons at various times of the day and discovered that GABA acted as an inhibitory neurotransmitter at night, decreasing the firing frequency, but during the day GABA acted as an excitatory neurotransmitter, increasing the firing frequency. This dual effect was mediated through GABA<sub>A</sub> receptors and may be attributed to an oscillation in intracellular chloride concentration.

**Figure 4.1**



**Figure 4.1** *Regional distribution of the GABA<sub>A</sub> receptor  $\alpha$ 1-subunit transcript in the brains of C57BL/6J mice during postnatal development.*

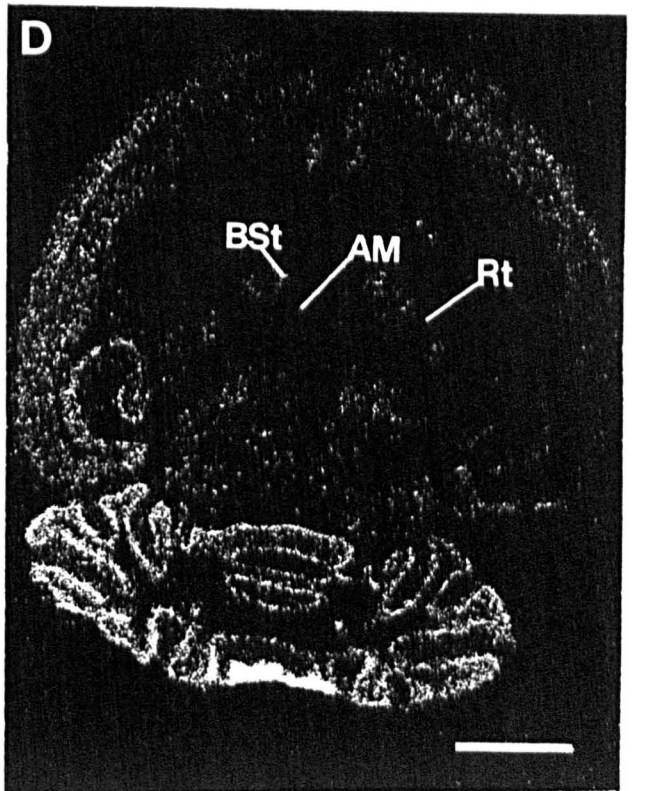
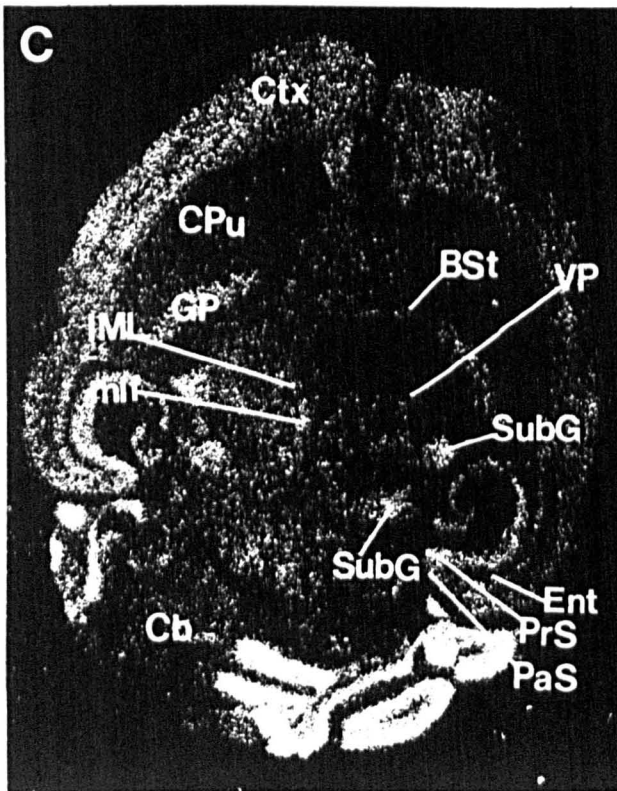
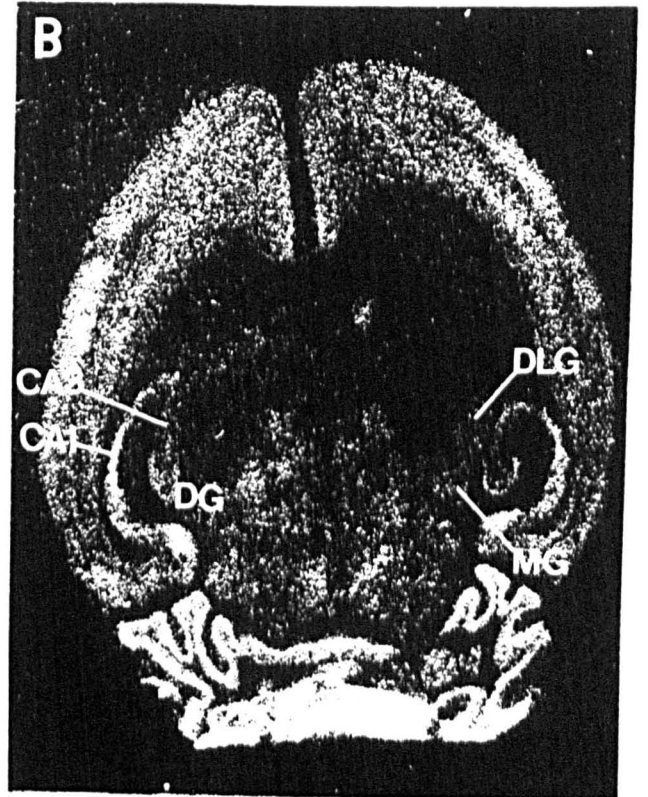
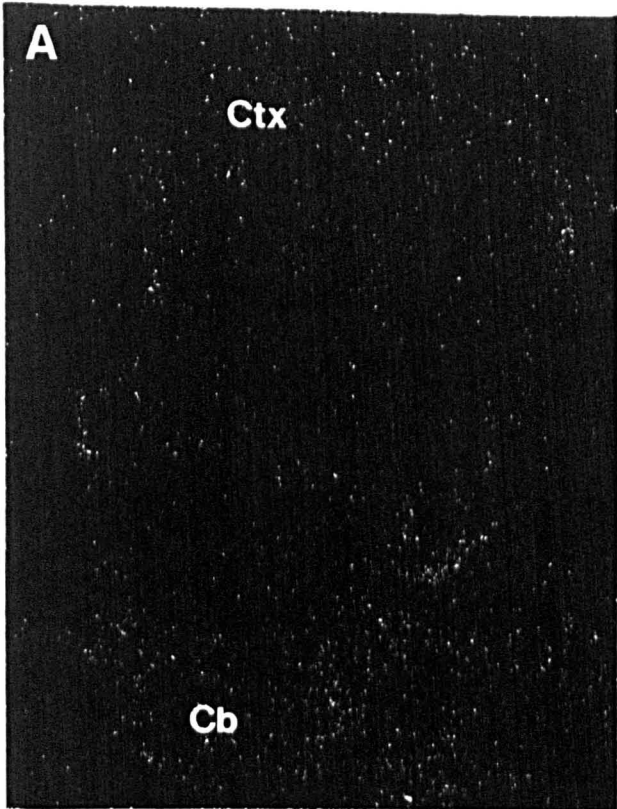
1-day old (A), 7-day-old (B), 14 day-old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\alpha$ 1-subunit mRNA of the GABA<sub>A</sub> receptor. CA1-3, field CA1-3 of Ammon's horn; Cb, cerebellum; CG, central grey; CIC, central nucleus, inferior colliculus; Ctx, cortex; DG, dentate gyrus; DLG, dorsal lateral geniculate nucleus; Ent, entorhinal cortex; Hi, hippocampus; Ic, inferior colliculus, IL, infralimbic cortex; LD, laterodorsal thalamic nucleus; LHb, lateral habenular nucleus; LP, lateral posterior thalamic nucleus; PaS, parasubiculum; PrS, presubiculum; Sb, subiculum. Scale bar represents 0.3 mm. Exposure time was 25 days on Kodak XAR-5 film.



**Figure 4.2**

**Figure 4.2** *Regional distribution of the GABA<sub>A</sub> receptor  $\alpha$ 1-subunit transcript in the brains of C57BL/6J mice during postnatal development.*

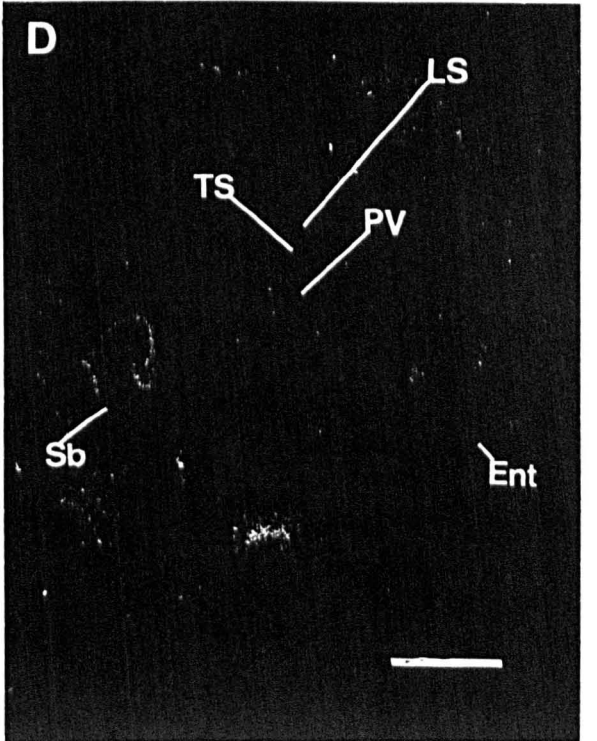
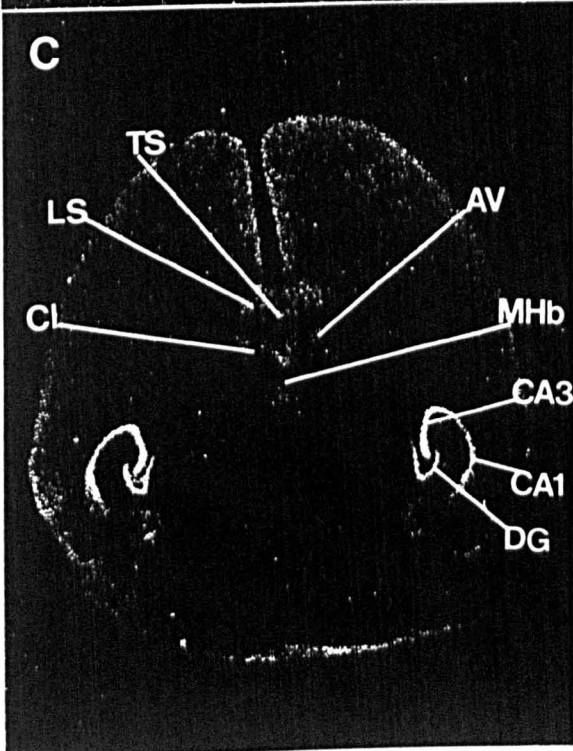
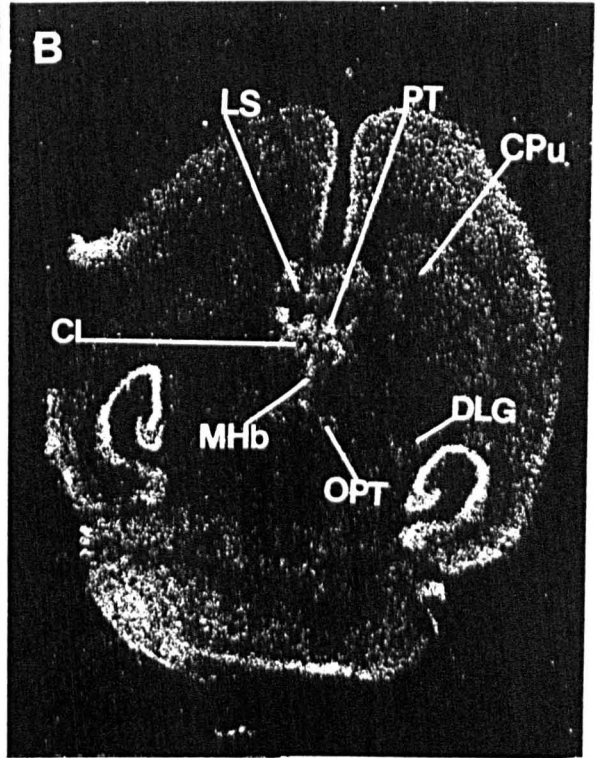
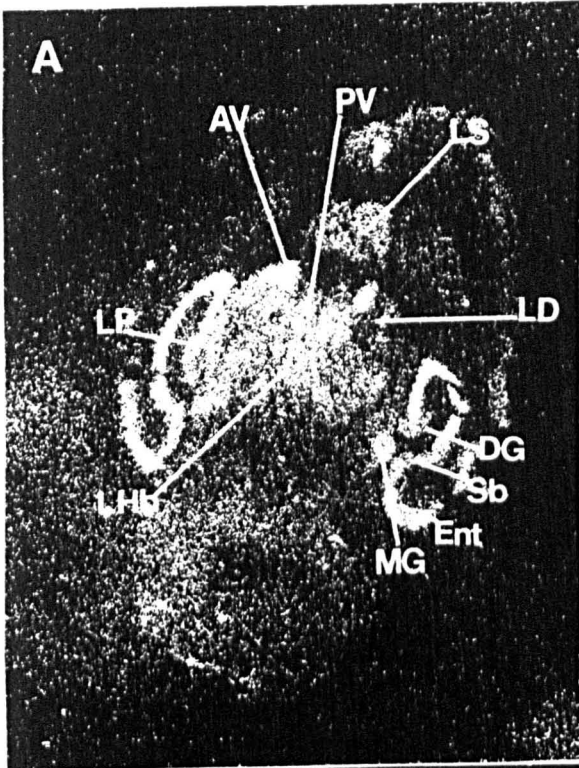
1-day old (A), 7-day-old (B), 14 day-old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\alpha$ 1-subunit mRNA of the GABA<sub>A</sub> receptor. AM, anteromedial thalamic nucleus; BSt, bed nucleus stria terminalis; CA1-3, field CA1-3 of Ammon's horn; Cb, cerebellum; CPu, caudate putamen; Ctx, cortex; DG, dentate gyrus; DLG, dorsal lateral geniculate nucleus; Ent, entorhinal cortex; GP, globus pallidus; IML, interstitial nucleus mlf; MG, medial geniculate nucleus; mlf, medial longitudinal fasciculus; PaS, parasubiculum; PrS, presubiculum; Rt, reticular thalamic nucleus; Sb, subiculum; SubG, subgeniculate nucleus; VP, ventral posterior thalamic nucleus. Scale bar represents 0.3 mm. Exposure time was 25 days on Kodak XAR-5 film.



**Figure 4.3**

**Figure 4.3** *Regional distribution of the GABA<sub>A</sub> receptor  $\alpha$ 2-subunit transcript in the brains of C57BL/6J mice during postnatal development.*

1-day old (A), 7-day-old (B), 14 day-old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\alpha$ 2-subunit mRNA of the GABA<sub>A</sub> receptor. Av, anteroventral thalamic nucleus; CA1-3, field CA1-3 of Ammon's horn; Cl, centrolateral thalamic nucleus; CPu, caudate putamen; DG, dentate gyrus; DLG, dorsal lateral geniculate nucleus; Ent, entorhinal cortex; LD, laterodorsal thalamic nucleus; LHb, lateral habenular nucleus; LP, lateral posterior thalamic nucleus; LS, lateral septal nucleus; MHb, medial habenular nucleus; MG, medial geniculate nucleus; OPT, olivary pretectal nucleus; PT, paratenial thalamic nucleus; PV, paraventricular thalamic nucleus; Sb, subiculum; TS, triangular septal nucleus. Scale bar represents 0.3 mm. Exposure time was 25 days on Kodak XAR-5 film.

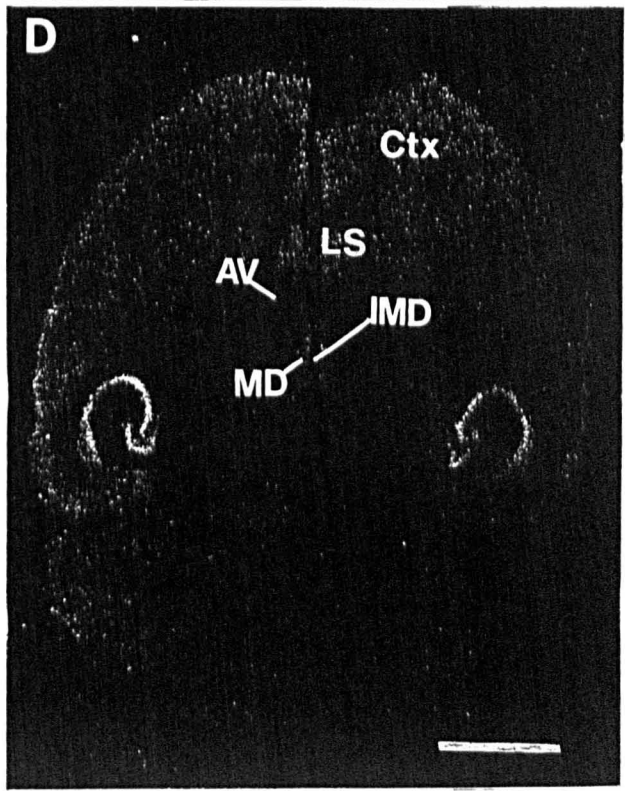
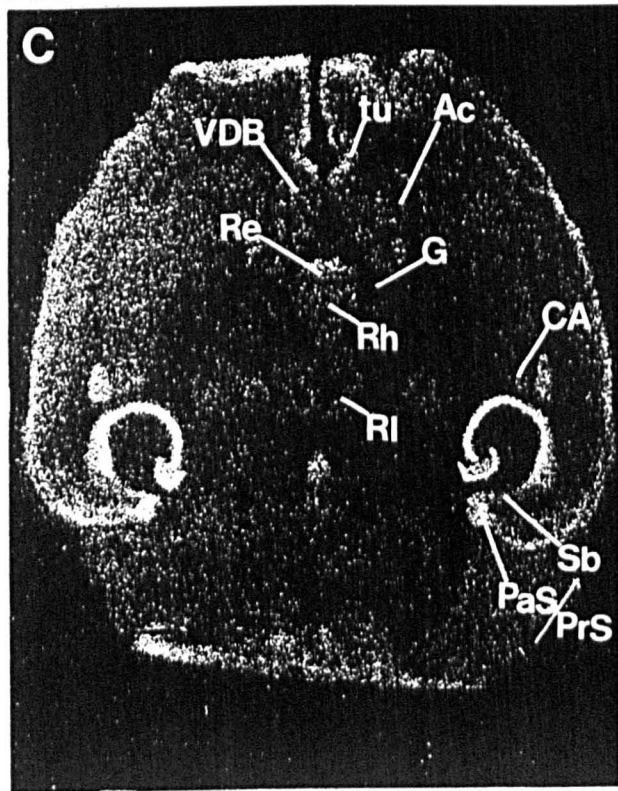
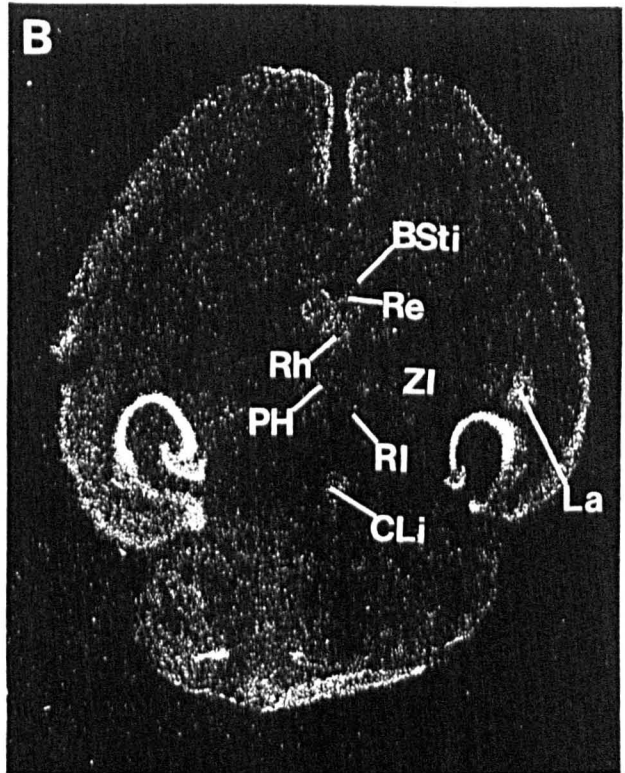
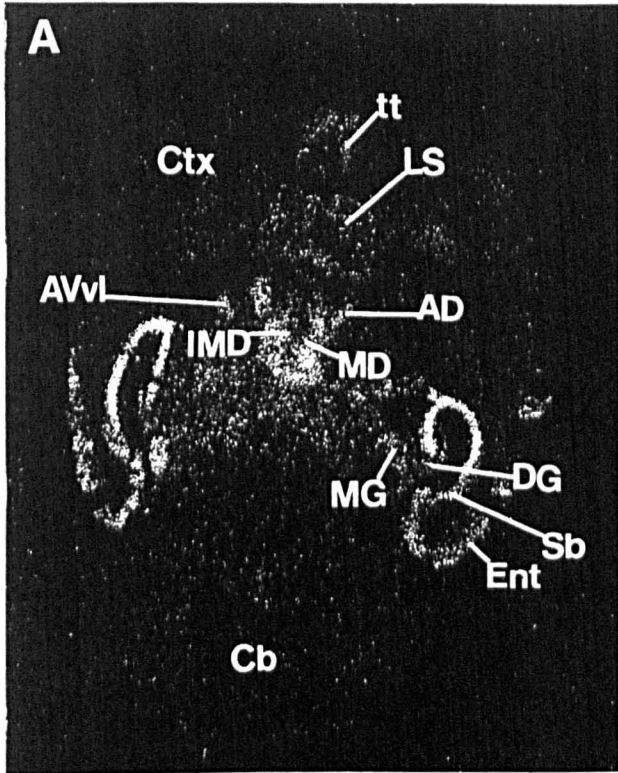




**Figure 4.4**

**Figure 4.4** *Regional distribution of the GABA<sub>A</sub> receptor  $\alpha$ 2-subunit transcript in the brains of C57BL/6J mice during postnatal development.*

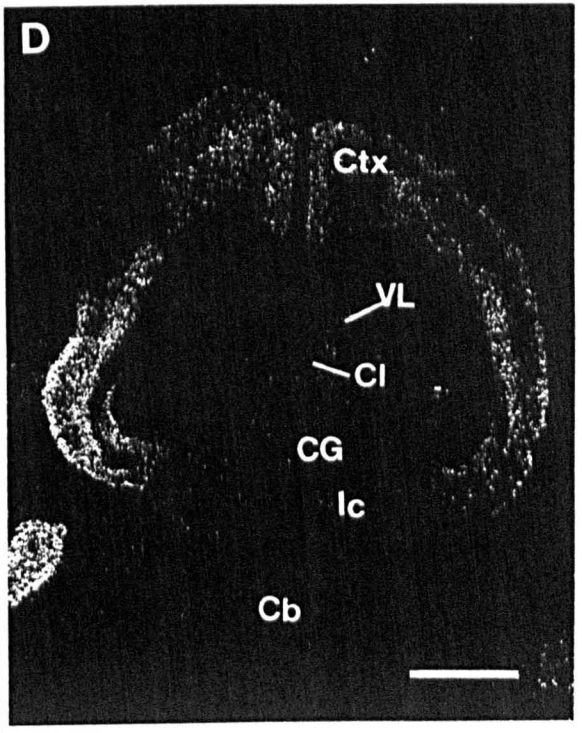
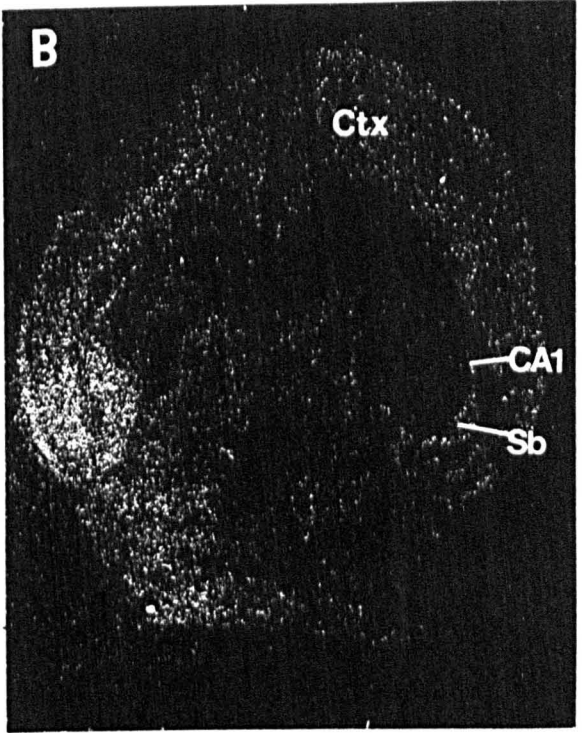
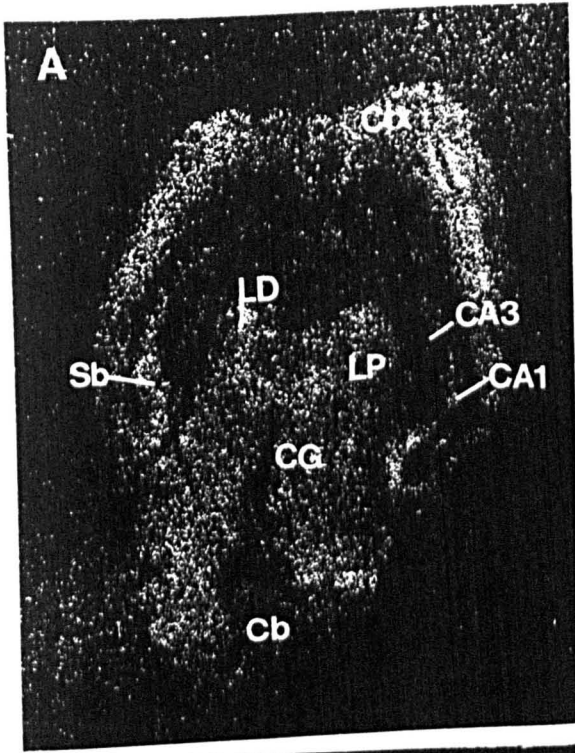
1-day old (A), 7-day-old (B), 14 day-old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\alpha$ 2-subunit mRNA of the GABA<sub>A</sub> receptor. Ac, accumbens nucleus; AD, anterodorsal thalamic nucleus; AV, anteroventral thalamic nucleus; AVvl, anteroventral thalamic nucleus, ventrolat; BSti, bed nucleus stria terminalis, intermed div; CA, central amygdaloid nucleus; Cb, cerebellum; CLi, caudal linear nucleus raphe; Ctx, cortex; DG, dentate gyrus; Ent, entorhinal cortex; G, gelatinosus thalamic nucleus; IMD, intermediodorsal thalamic nucleus; La, lateral amygdaloid nucleus; LS, lateral septal nucleus; MD, mediodorsal thalamic nucleus; MG, medial geniculate nucleus; PaS, parasubiculum; PH, posterior hypothalamic area; PrS, presubiculum; Re, reuniens thalamic nucleus; Rh, rhomboid thalamic nucleus; RI, rostral interstitial nucleus mlf; Sb, subiculum; tt, tenia tecta; tu, olfactory tubercle; VDB, nucleus vertical limb diagonal band; ZI, Zona incerta . Scale bar represents 0.3 mm. Exposure time was 25 days on Kodak XAR-5 film.



**Figure 4.5**

**Figure 4.5** *Regional distribution of the GABA<sub>A</sub> receptor  $\alpha$ 3-subunit transcript in the brains of C57BL/6J mice during postnatal development.*

1-day old (A), 7-day-old (B), 14 day-old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\alpha$ 3-subunit mRNA of the GABA<sub>A</sub> receptor. CA1-3, field CA1-3 of Ammon's horn; Cb, cerebellum; CG, central grey; Cl, centrolateral thalamic nucleus; Ctx, cortex; Ic, inferior colliculus; LD, laterodorsal thalamic nucleus; LP, lateral posterior thalamic nucleus; Sb, subiculum; VL, ventrolateral thalamic nucleus. Scale bar represents 0.3mm. Exposure time was 25 days on Kodak XAR-5 film.

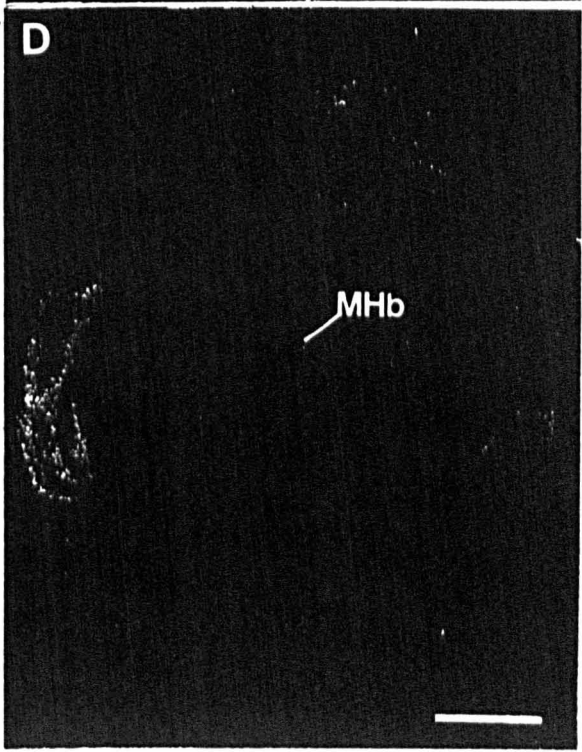
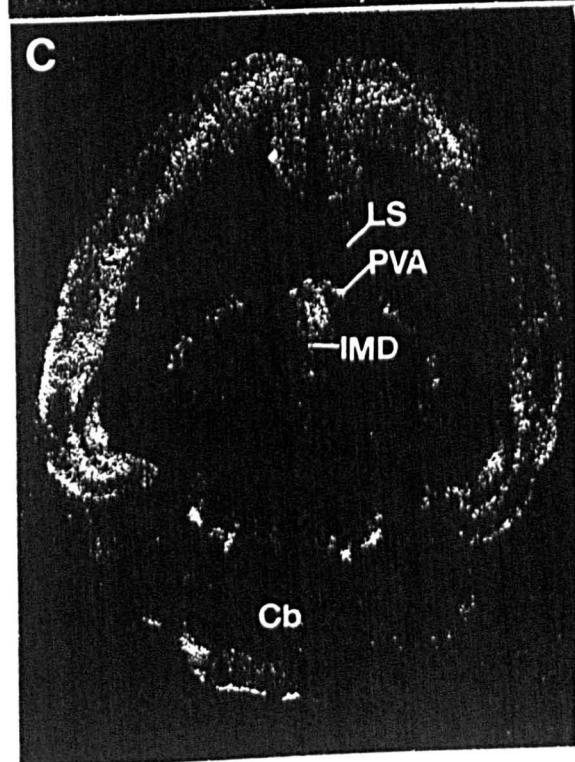
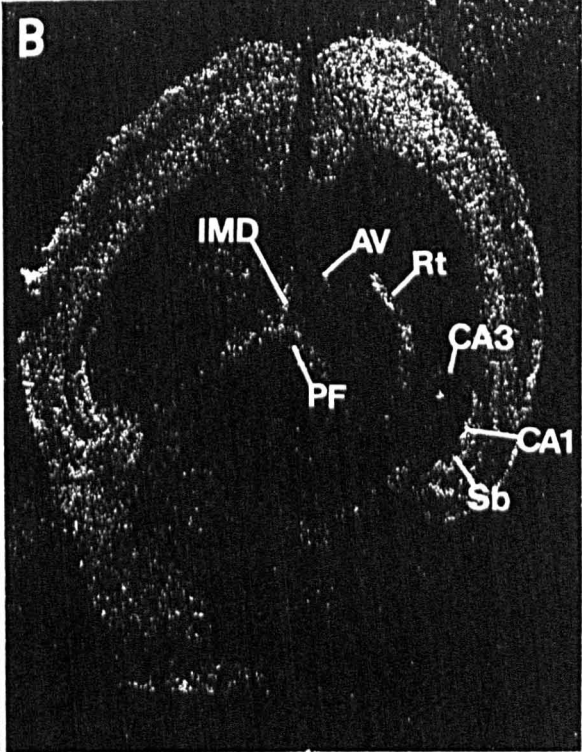
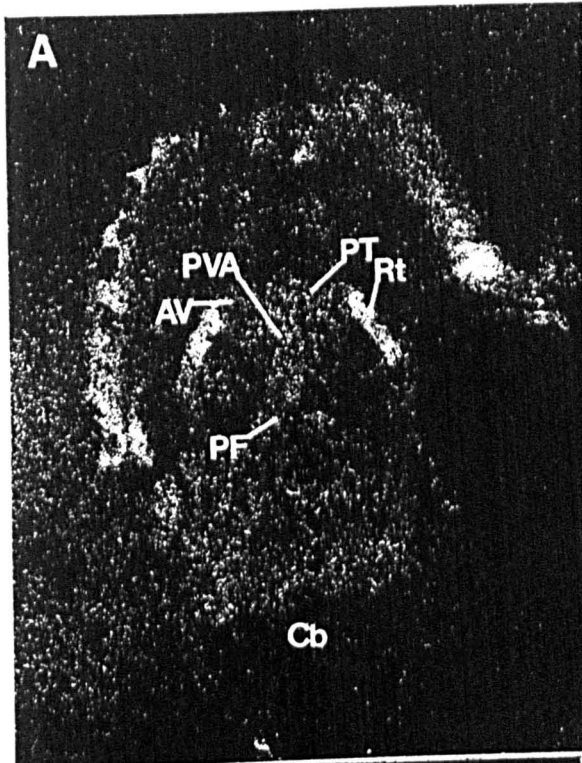


**Figure 4.6**

**Figure 4.6** *Regional distribution of the GABA<sub>A</sub> receptor  $\alpha$ 3-subunit transcript in the brains of C57BL/6J mice during postnatal development.*

1-day old (A), 7-day-old (B), 14 day-old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\alpha$ 3-subunit mRNA of the GABA<sub>A</sub> receptor. AV, anteroventral thalamic nucleus; CA1-3, field CA1-3 of Ammon's horn; Cb, cerebellum; IMD, intermediodorsal thalamic nucleus; LS, lateral septal nucleus; MHb, medial habenular nucleus; PF, parafascicular thalamic nucleus; PT, paratenial thalamic nucleus; PVA, paraventricular thalamic nucleus, anterior; Rt, reticular thalamic nucleus; Sb, subiculum;. Scale bar represents 0.3 mm. Exposure time was 25 days on Kodak XAR-5 film.

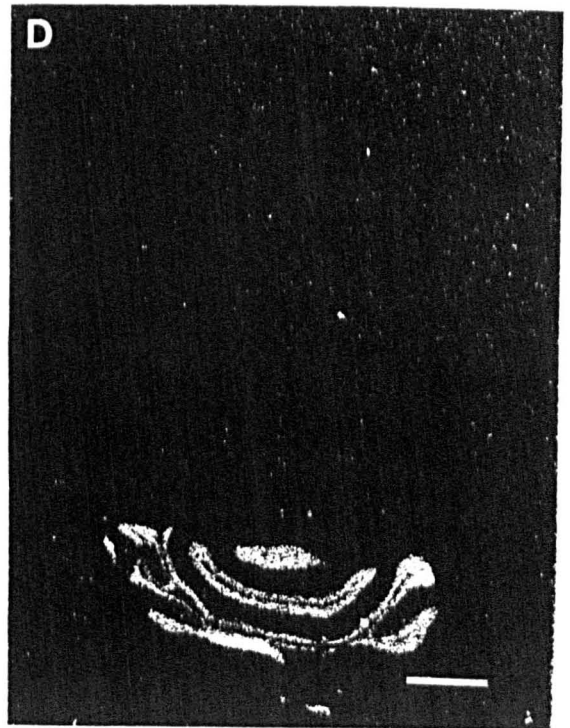
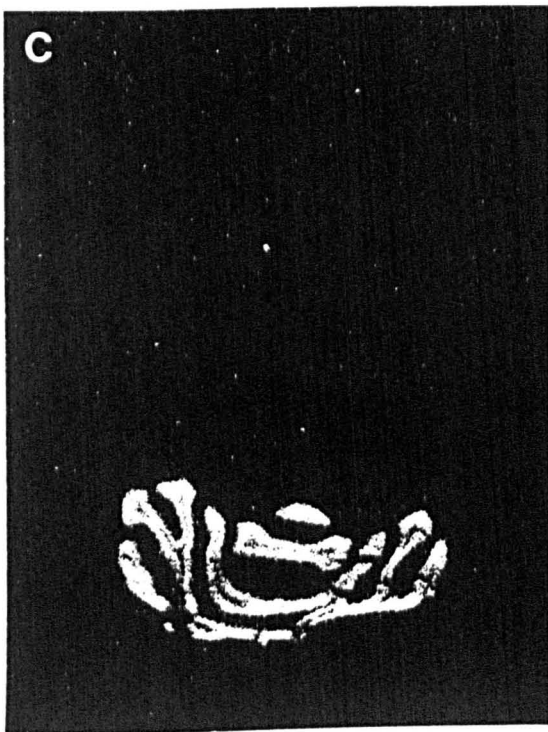
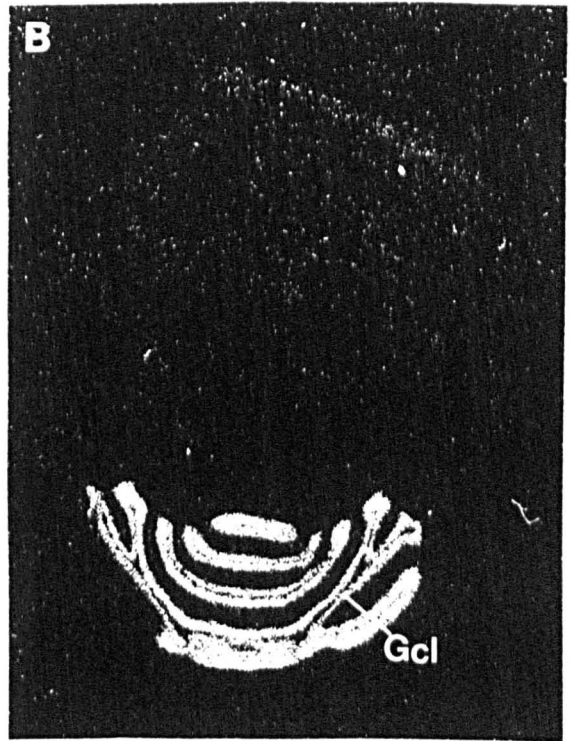
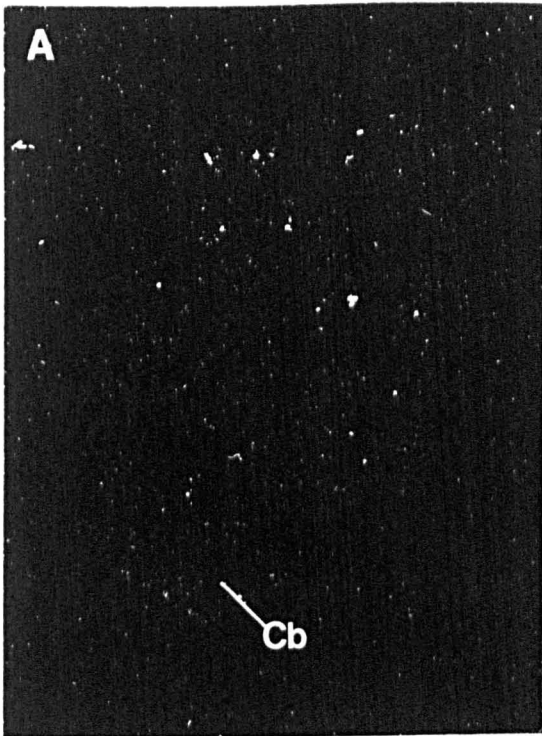




**Figure 4.7**

**Figure 4.7** *Regional distribution of the GABA<sub>A</sub> receptor  $\alpha$ 6-subunit transcript in the brains of C57BL/6J mice during postnatal development.*

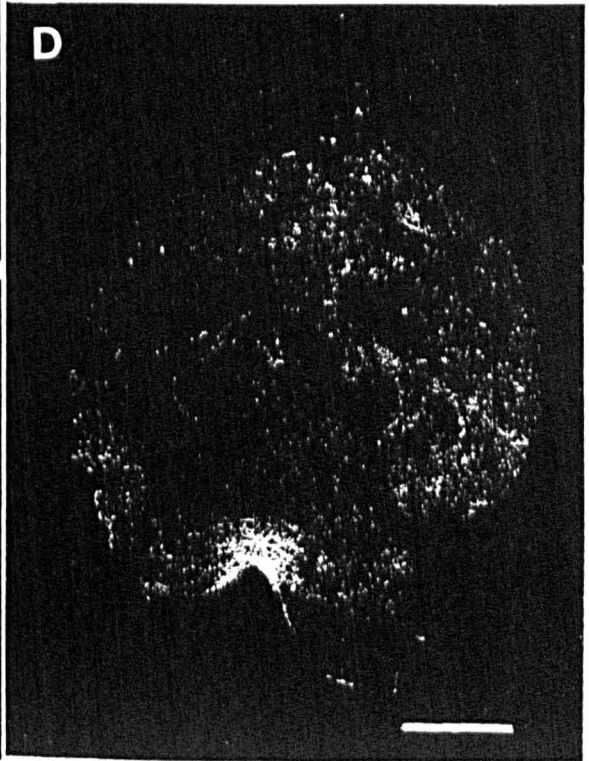
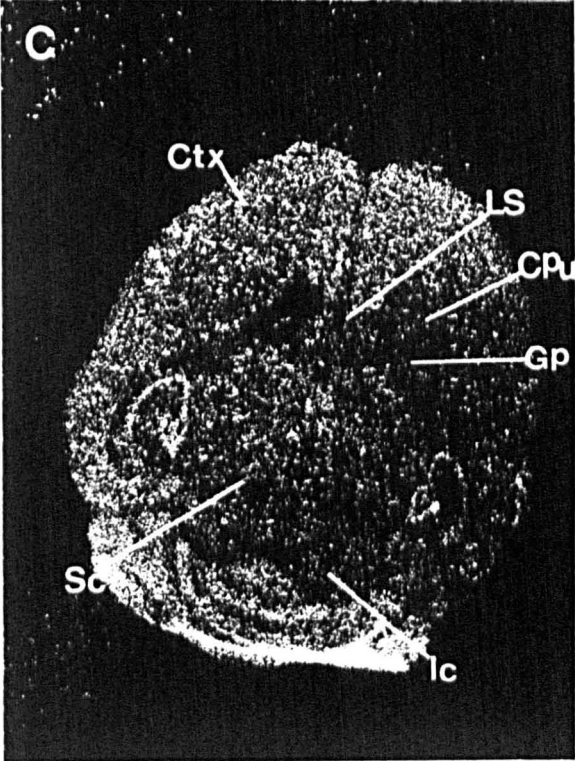
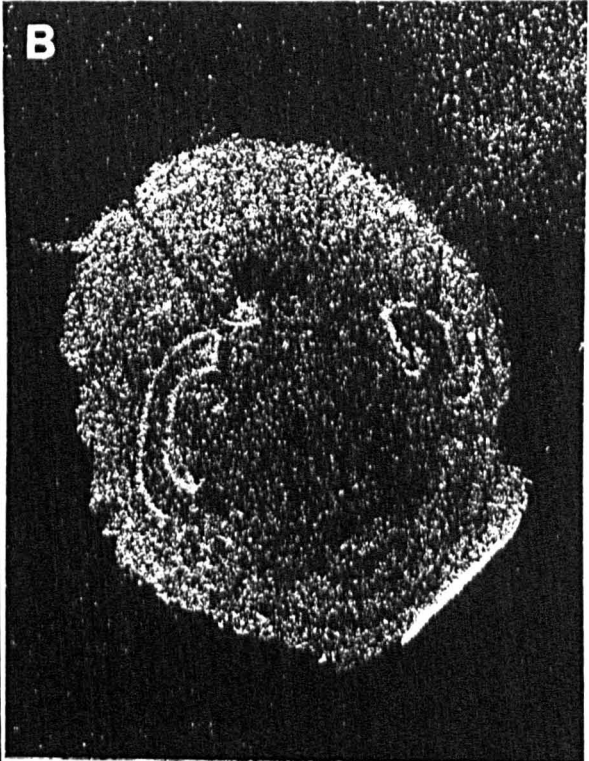
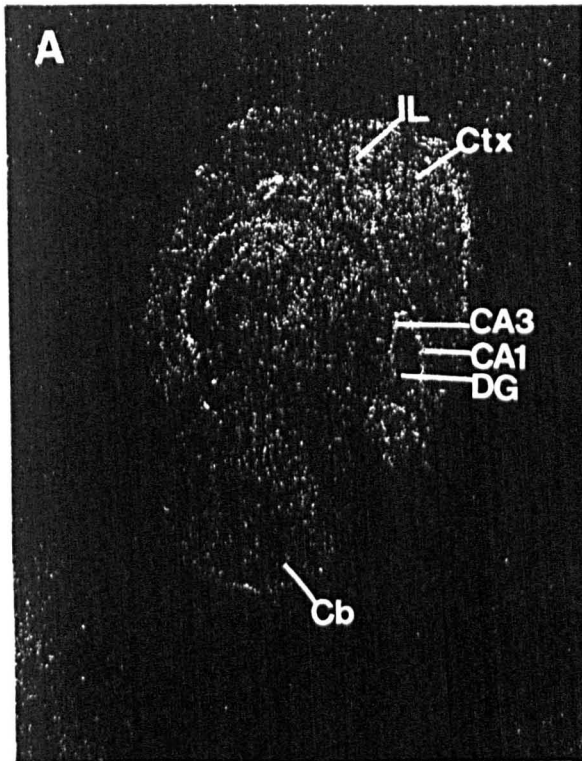
1-day old (A), 7-day-old (B), 14 day-old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\alpha$ 6-subunit mRNA of the GABA<sub>A</sub> receptor. Cb, cerebellum; Gcl, granule cell layer. Scale bar represents 0.3 mm. Exposure time was 25 days on Kodak XAR-5 film.



**Figure 4.8**

**Figure 4.8** *Regional distribution of the GABA<sub>A</sub> receptor  $\beta$ 1-subunit transcript in the brains of C57BL/6J mice during postnatal development.*

1-day old (A), 7-day-old (B), 14 day-old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\beta$ 1-subunit mRNA of the GABA<sub>A</sub> receptor. CA1-3, field CA1-3 of Ammon's horn; Cb, cerebellum; CPu, caudate putamen; Ctx, cortex; DG, dentate gyrus; GP, globus pallidus; Ic, inferior colliculus, IL, infralimbic cortex; LS, lateral septum; Sc, superior colliculus. Scale bar represents 0.3 mm. Exposure time was 25 days on Kodak XAR-5 film.

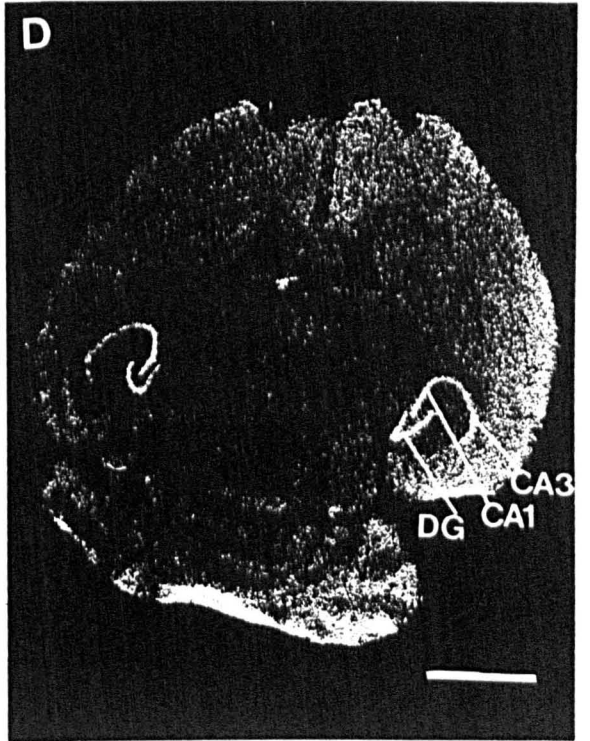
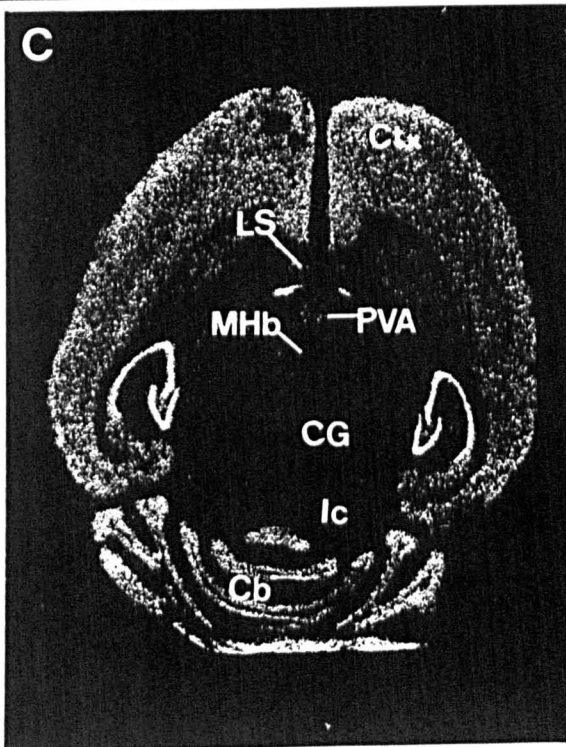
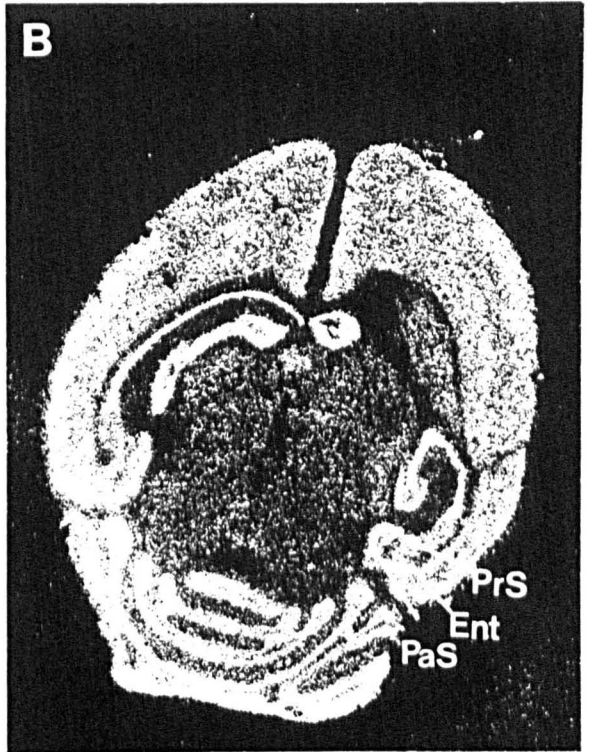
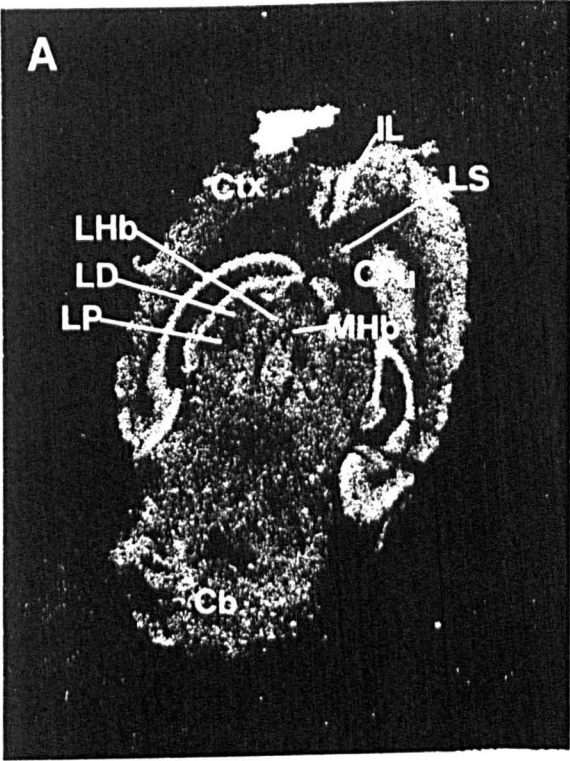


**Figure 4.9**



***Figure 4.9 Regional distribution of the GABA<sub>A</sub> receptor  $\beta$ 3-subunit transcript in the brains of C57BL/6J mice during postnatal development.***

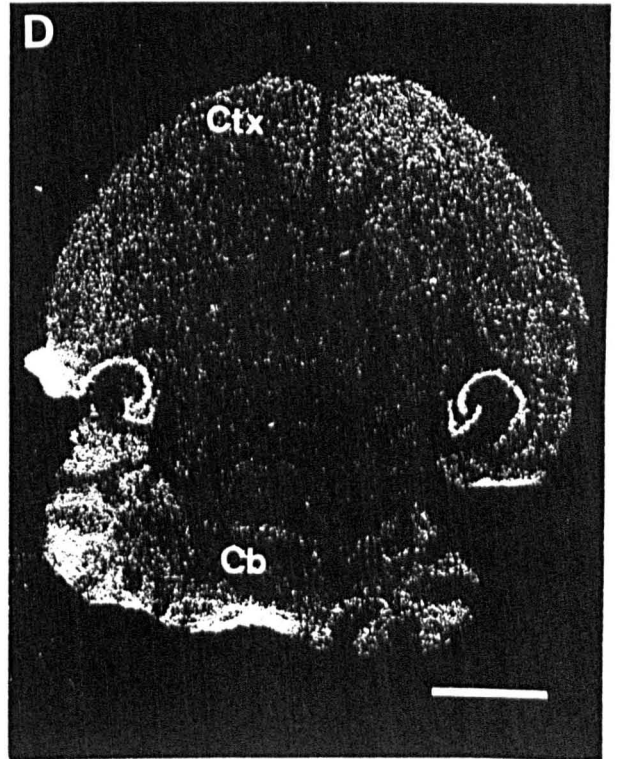
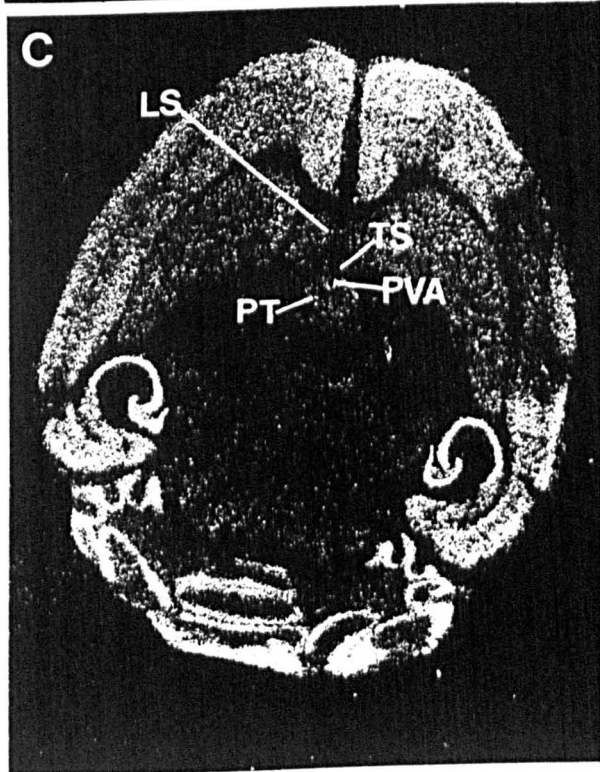
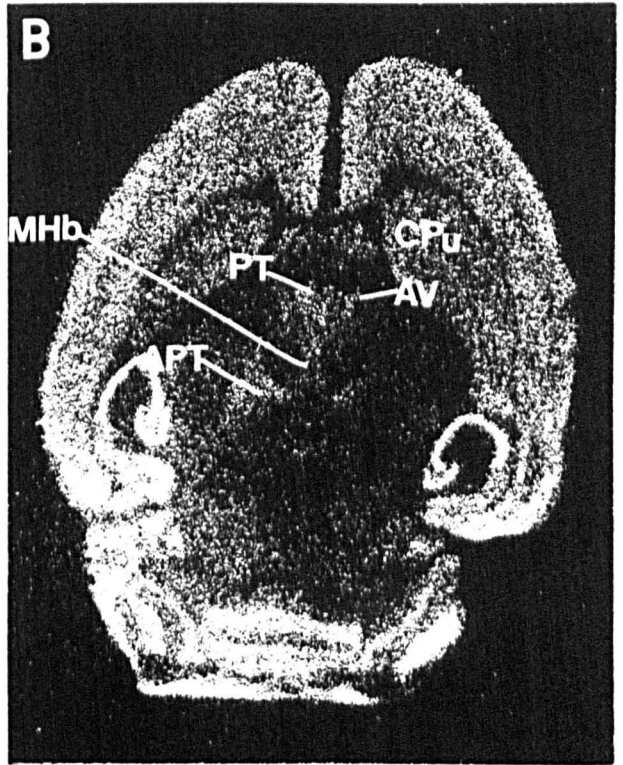
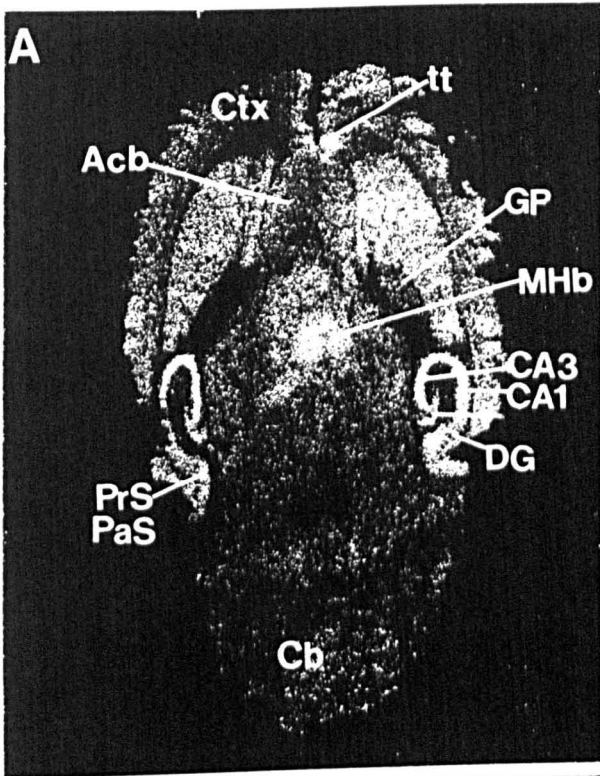
1-day old (A), 7-day-old (B), 14 day-old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\beta$ 3-subunit mRNA of the GABA<sub>A</sub> receptor. CA1-3, field CA1-3 of Ammon's horn; Cb, cerebellum; CG, Central gray; Cpu, caudate putamen; Ctx, cortex; DG, dentate gyrus; Ent, entorhinal cortex; Ic, inferior colliculus; IL, infralimbic cortex; LD, laterodorsal thalamic nucleus; LHb, lateral habenular nucleus; LP, lateral posterior nucleus; LS, lateral septum; MHb, medial habenular nucleus, PVA, paraventricular nucleus. Scale bar represents 0.3 mm. Exposure time was 25 days on Kodak XAR-5 film.



**Figure 4.10**

**Figure 4.10** *Regional distribution of the GABA<sub>A</sub> receptor  $\beta$ 3-subunit transcript in the brains of C57BL/6J mice during postnatal development.*

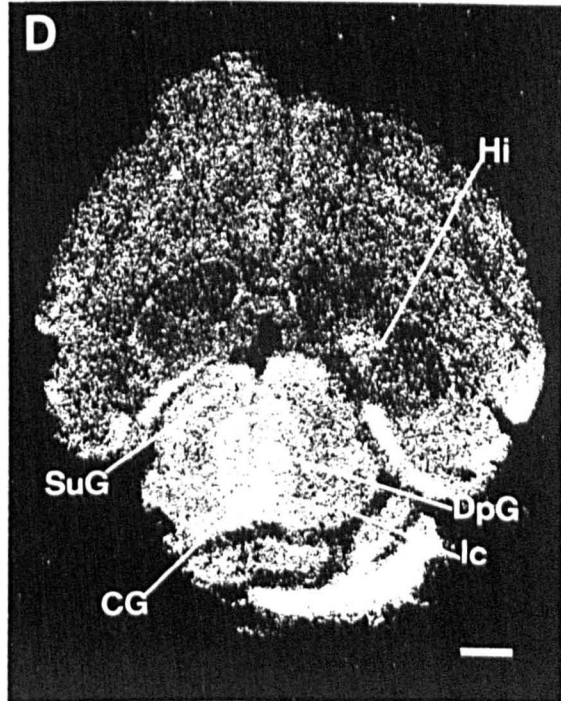
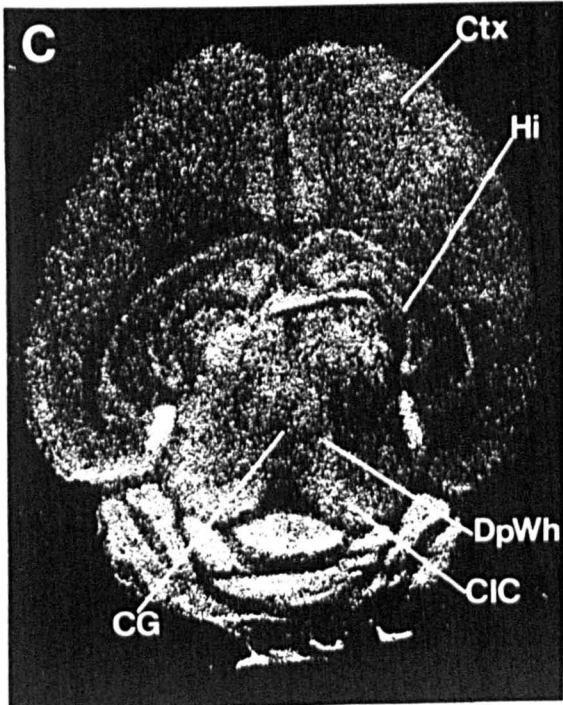
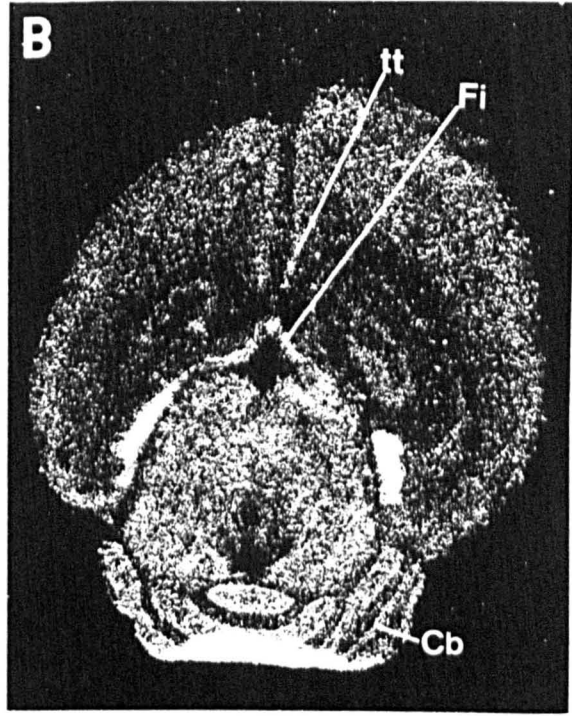
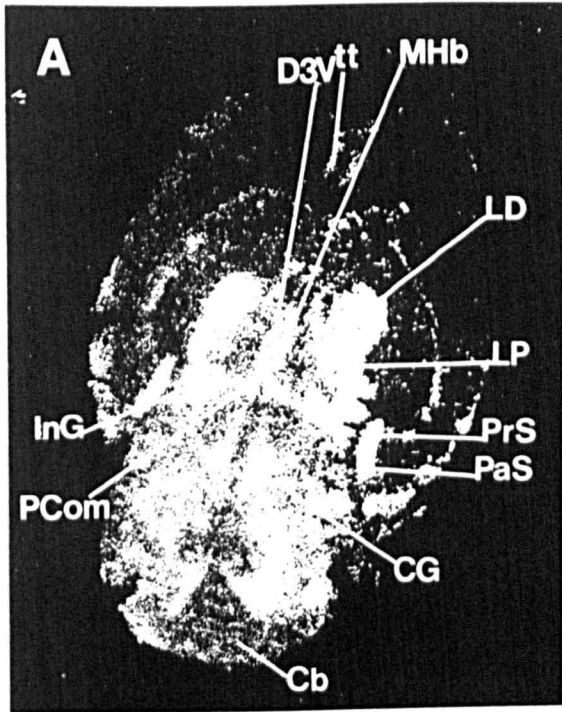
1-day old (A), 7-day-old (B), 14 day-old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\beta$ 3-subunit mRNA of the GABA<sub>A</sub> receptor. Acb, accumbens; APT, anterior pretecal nucleus; AV, anteroventral nucleus; CA1-3, field CA1-3 of Ammon's horn; Cb, cerebellum; CPu, caudate putamen; Ctx, cortex; DG, dentate gyrus; GP, globus pallidus; MHb, medial habenular nucleus; PaS, parasubiculum; PrS, presubiculum; PT, paratenial thalamic nucleus; PVA, paraventricular thalamic nucleus; TS, triangular septal nucleus; tt, tenia tecta. Scale bar represents 0.3 mm. Exposure time was 25 days on Kodak XAR-5 film.



**Figure 4.11**

***Figure 4.11 Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 1-subunit transcript in the brains of C57BL/6J mice during postnatal development.***

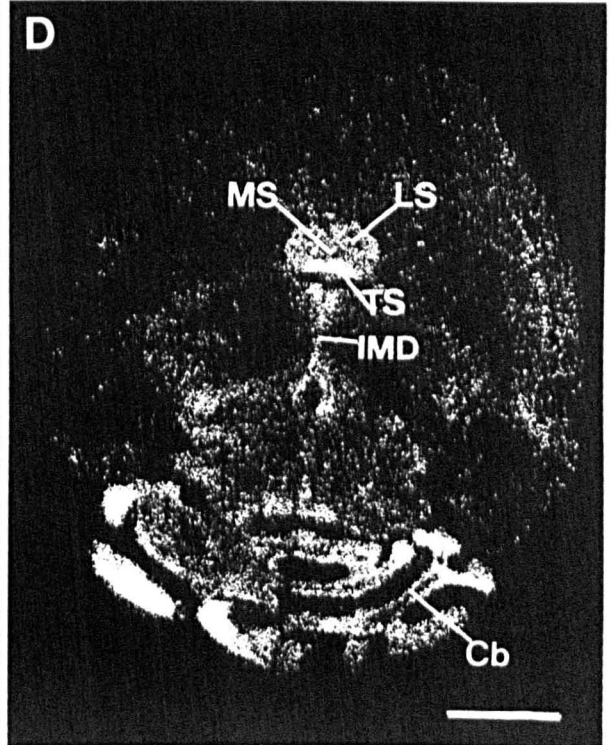
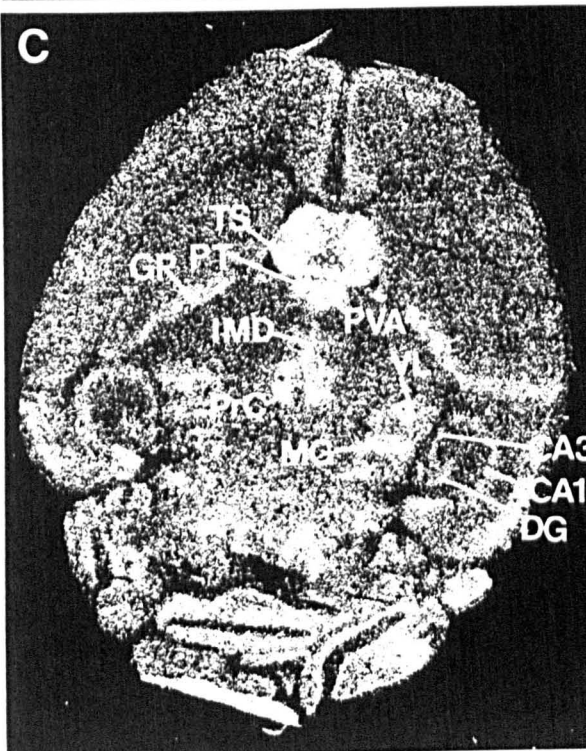
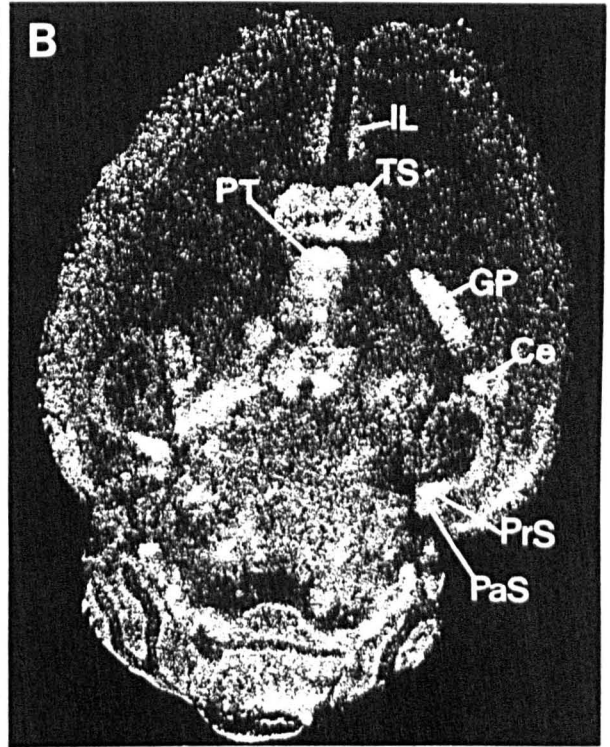
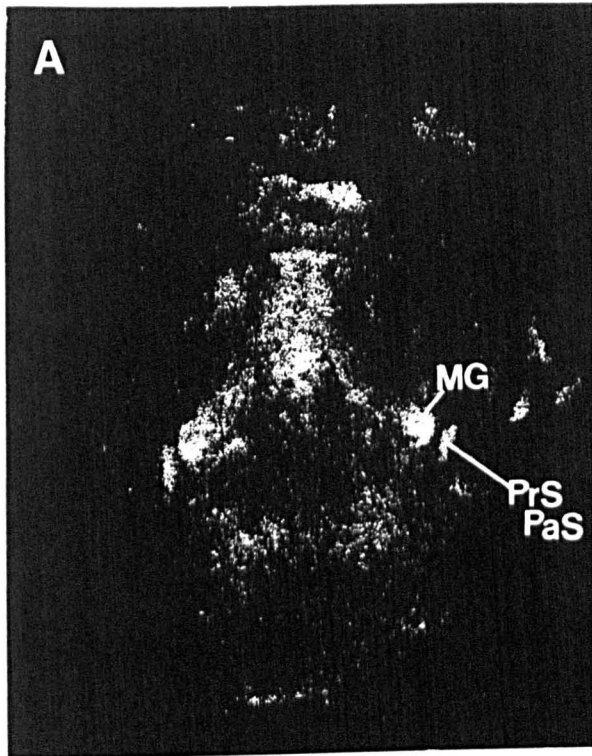
1-day old (A), 7-day-old (B), 14 day-old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\gamma$ 1-subunit mRNA of the GABA<sub>A</sub> receptor. Cb, cerebellum; CG, central gray; CIC, central nucleus inferior colliculus; Ctx, cortex; DpG, deep gray layer of superior colliculus; DpWh, deep white layer of superior colliculus; D3V, dorsal 3rd ventricle; Fi, fimbria hippocampus; Hi, hippocampus; Ic, inferior colliculus; InG, intermediate gray layer superior colliculus; LD, laterodorsal thalamic nucleus; LP, lateral posterior thalamic nucleus; MHb, medial habenular nucleus; PaS, parasubiculum; Pcom, nucleus posterior commissure; PrS, presubiculum; SuG, superficial gray layer of superior colliculus; tt, tenia tecta. Scale bar represents 0.3 mm. Exposure time was 25 days on Kodak XAR-5 film.





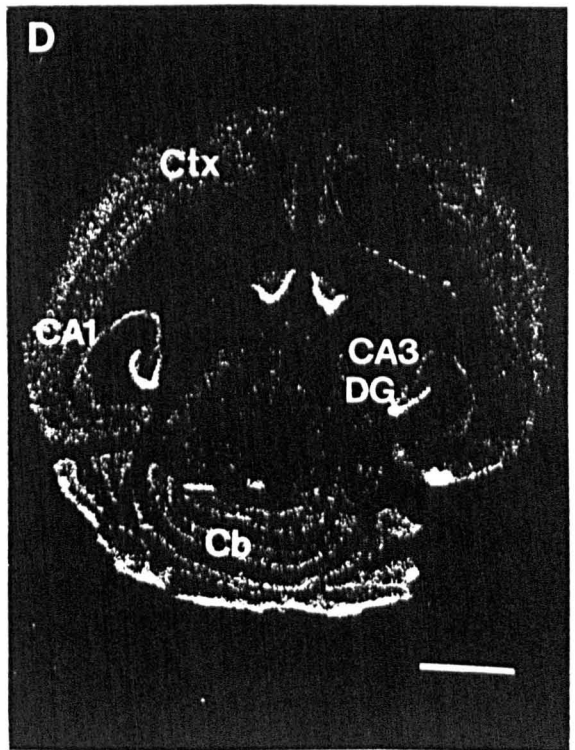
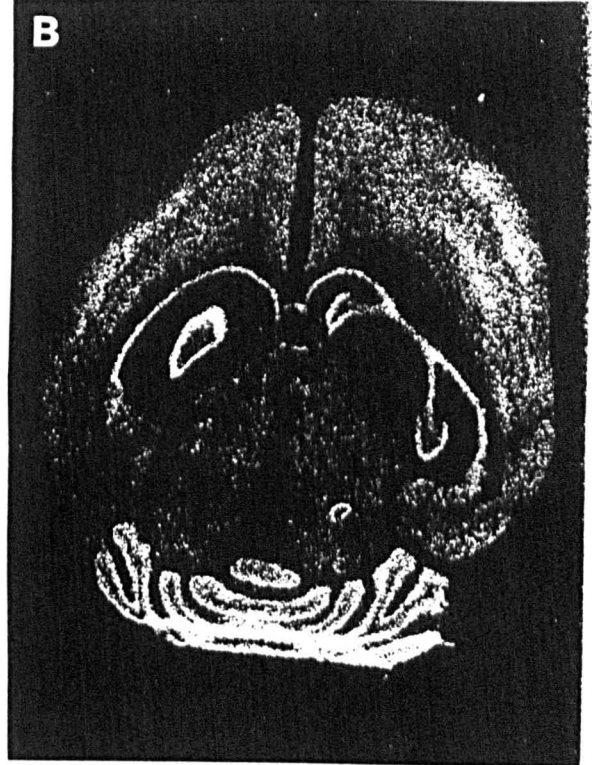
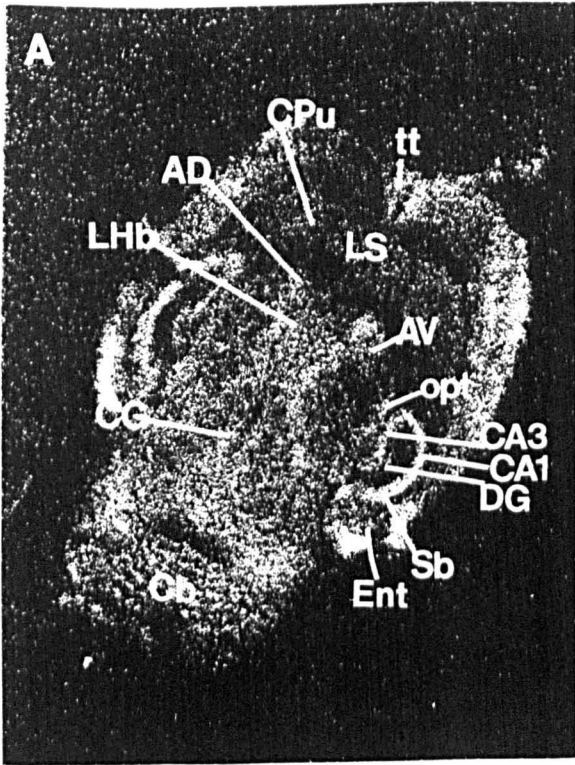
**Figure 4.12**

**Figure 4.12** *Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 1-subunit transcript in the brains of C57BL/6J mice during postnatal development:* A: 1-day old (A), 7-day old (B), 14-day old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\gamma$ -1 subunit mRNA of the GABA<sub>A</sub> receptor. CA1-3, field CA1-3 of Ammon's horn; Cb cerebellum; Ce, central amygdaloid nucleus; DG, dentate gyrus; GP, globus pallidus; IL, infralimbic cortex; IMD, intermediodorsal thalamic nucleus; MG, medial geniculate nucleus; MS, medial septal nucleus; PaS, parasubiculum; PrC precommissural nucleus; PrS, presubiculum; PT, paratenial thalamic nucleus; PVA, paraventricular thalamic nucleus; TS, triangular septal nucleus; VL, venterolateral thalamic nucleus. Scale bar represents 0.3mm. Exposure time was 25 days on Kodak XAR-5 film.



**Figure 4.13**

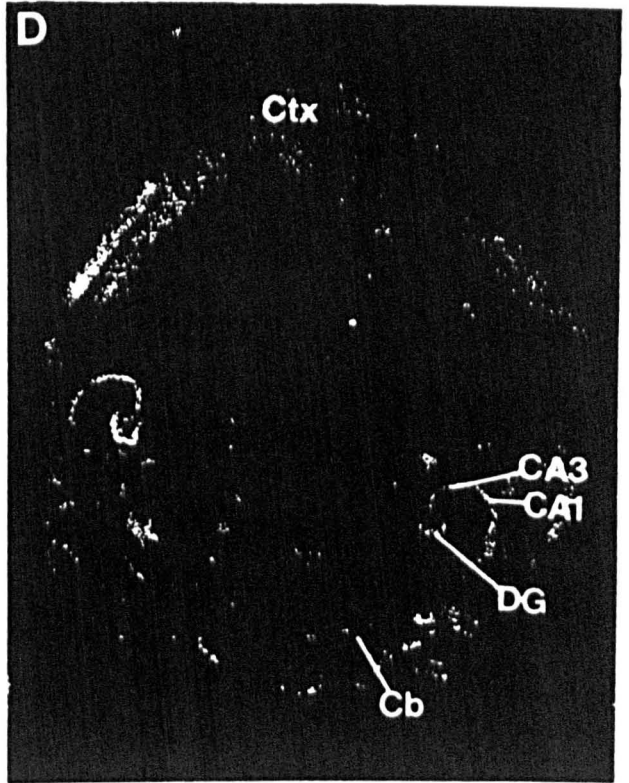
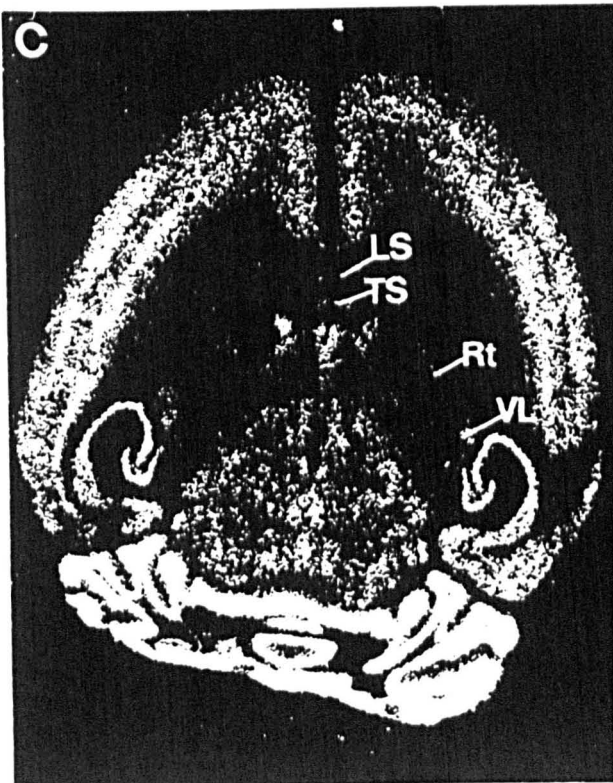
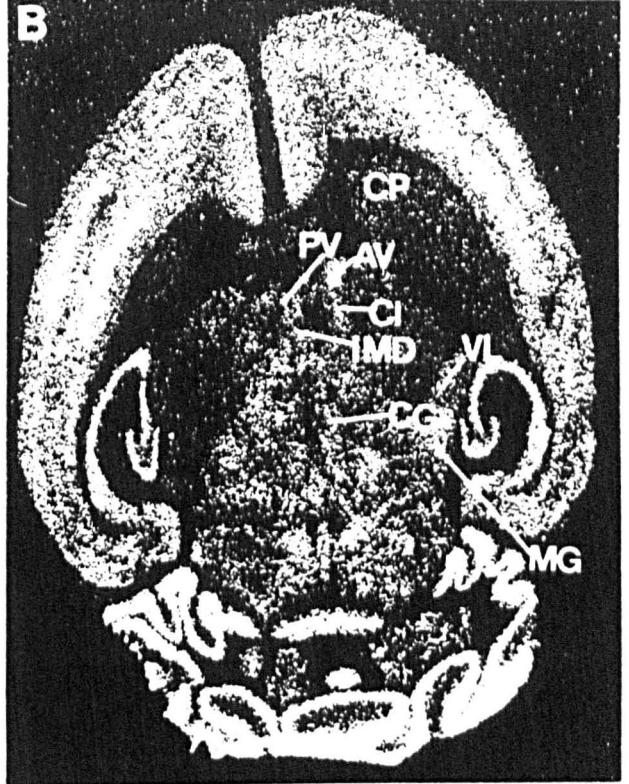
**Figure 4.13** *Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 2T -subunit transcript in the brains of C57BL/6J mice during postnatal development.* A: 1-day old (A), 7-day old (B), 14-day old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\gamma$ 2T-subunit mRNA of the GABA<sub>A</sub> receptor. AD, anterodorsal thalamic nucleus; AV, anteroventral thalamic nucleus; CA1-3, fields CA 1-3 of Ammon's horn; Cb, cerebellum; CG, central gray; Cpu, caudate putamen; Ctx, cortex; DG, dentate gyrus; Ent, entorhinal cortex; Lhb, lateral habenular nucleus; LS, lateral septum; opt, optic tract; tt, tenia tecta. Scale bar represents 0.3mm. Exposure time was 25 days on Kodak XAR-5 film.



**Figure 4.14**

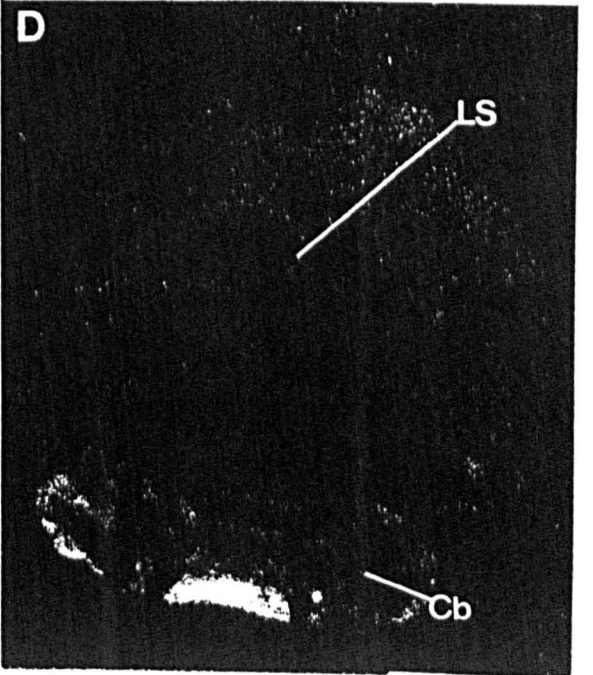
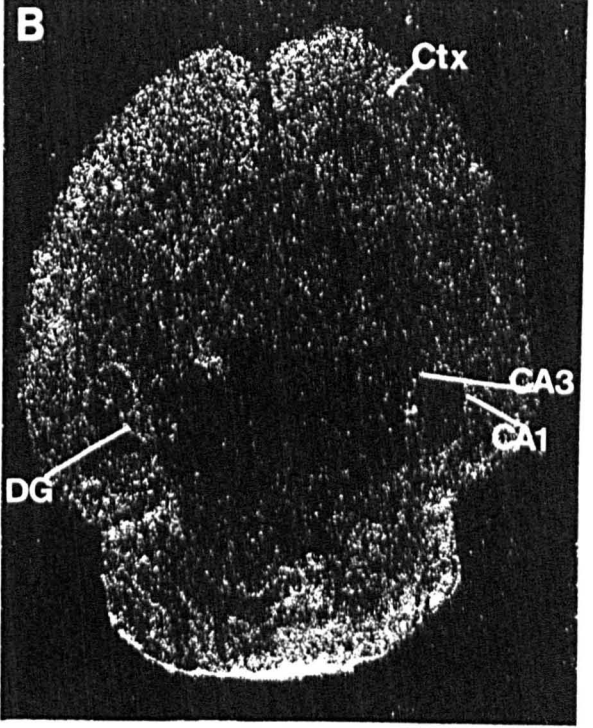
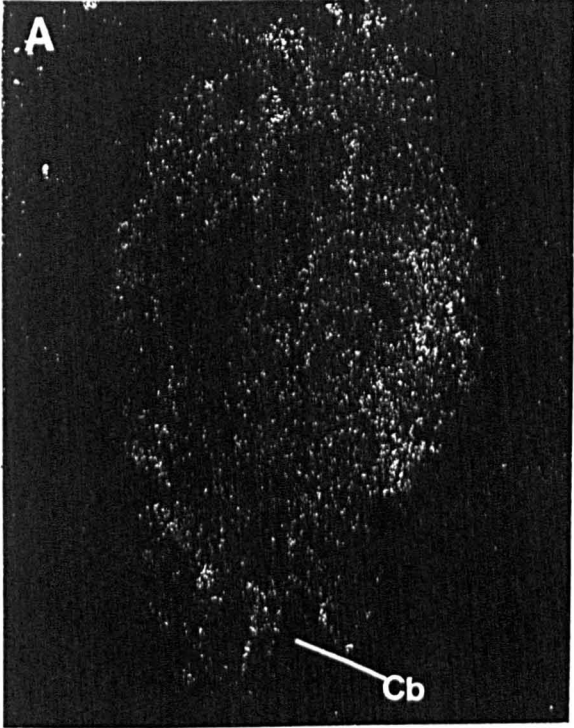
**Figure 4.14** *Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 2T-subunit transcript in the brains of C57BL/6J mice during postnatal development.* A: 1-day old (A), 7-day old (B), 14-day old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\gamma$ 2T-subunit mRNA of the GABA<sub>A</sub> receptor. AV, anteroventral thalamic nucleus; CA1-3, fields CA 1-3 of Ammon's horn; Cb, cerebellum; CG, central gray; Cl, claustrum; Cpu, caudate putamen; Ctx, cortex; DG, dentate gyrus; IMD, intermediodorsal thalamic nucleus; LS, lateral septum; MG, medial geniculate nucleus; PV, paraventricular thalamic nucleus; Rt reticular thalamic nucleus; TS, triangular septal nucleus; VL, ventrolateral thalamic nucleus. Scale bar represents 0.3mm. Exposure time was 25 days on Kodak XAR-5 film.





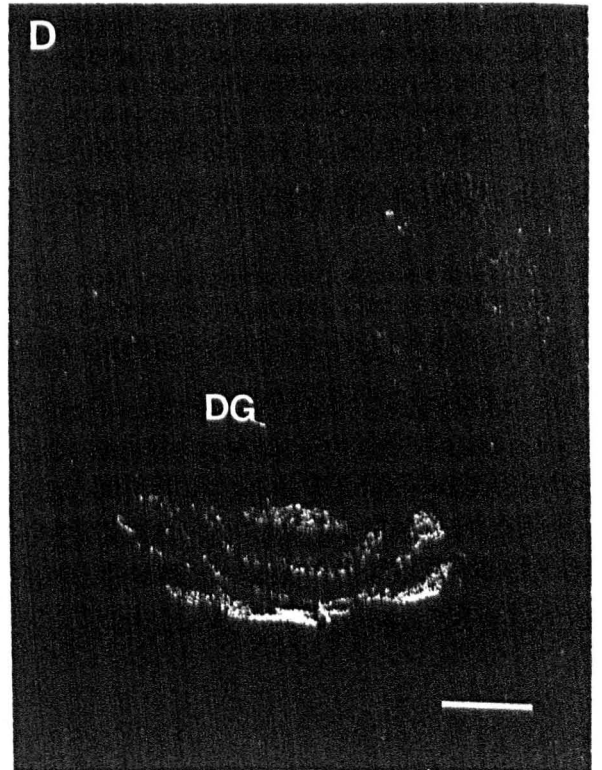
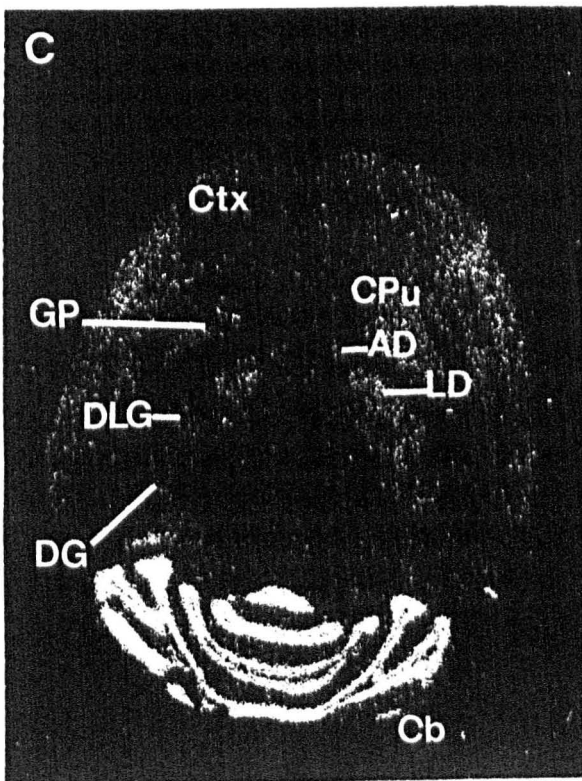
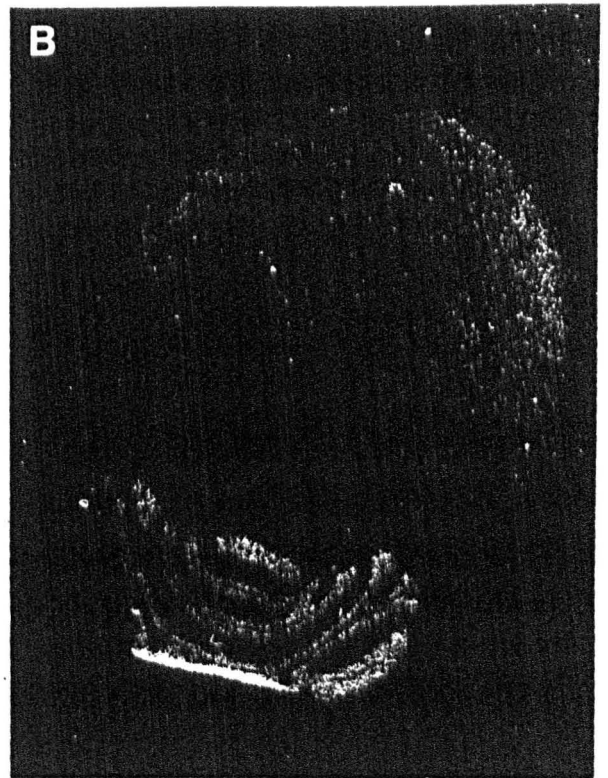
**Figure 4.15**

**Figure 4.15** *Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ <sub>3</sub>-subunit transcript in the brains of C57BL/6J mice during postnatal development. A: 1-day old (A), 7-day old (B), 14-day old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\gamma$ <sub>3</sub>-subunit mRNA of the GABA<sub>A</sub> receptor. Cb, cerebellum; CA1-3, fields CA1-3 of Ammon's horn; Ctx, cortex; DG, dentate gyrus; LS, lateral septum. Scale bar represents 0.3mm. Exposure time was 25 days on Kodak XAR-5 film.*



**Figure 4.16**

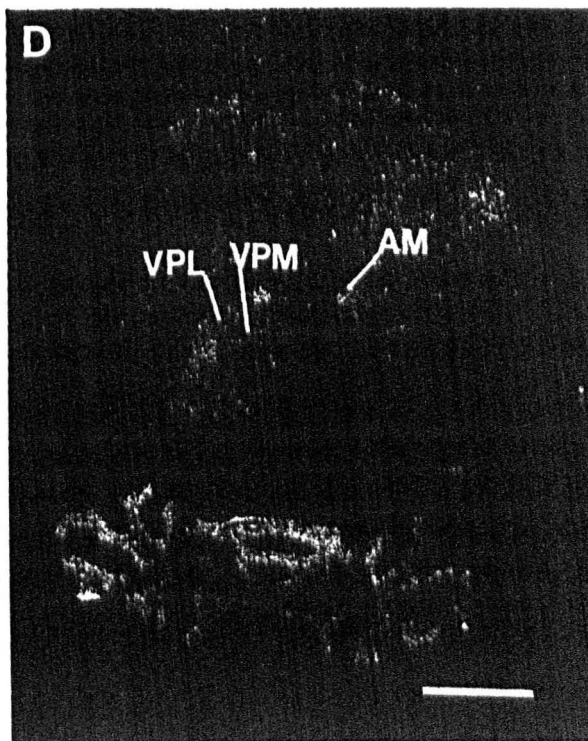
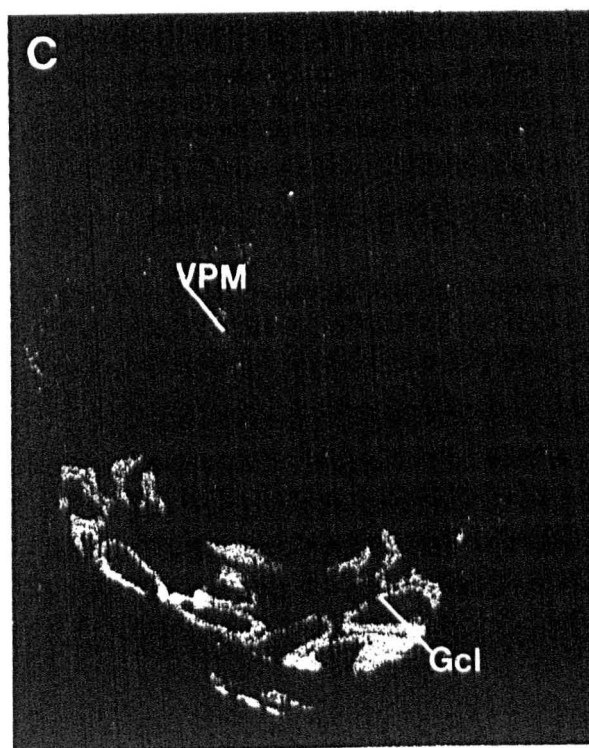
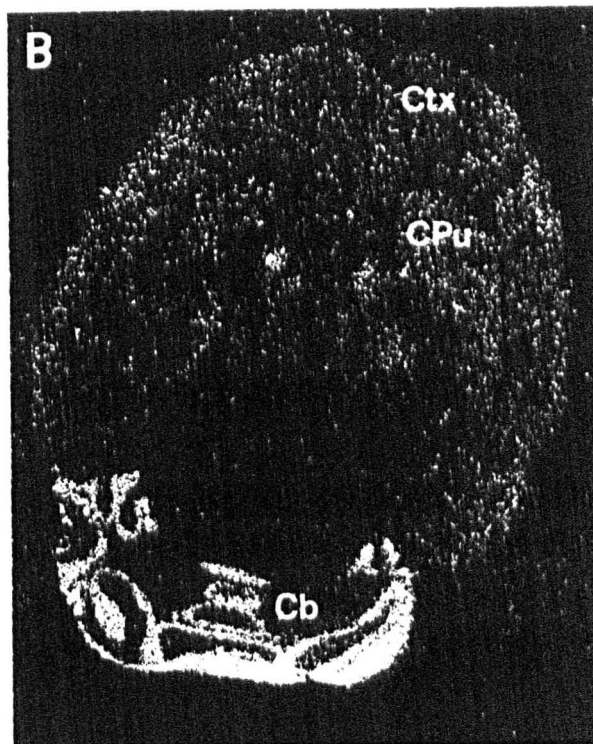
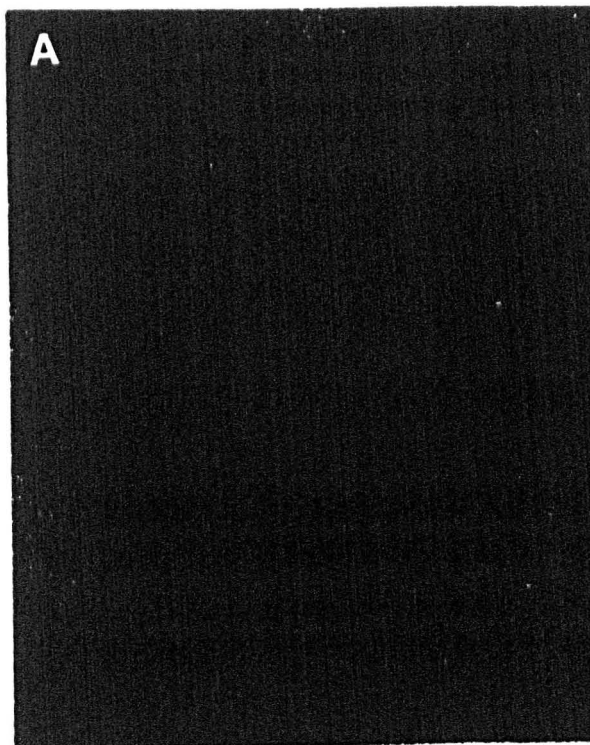
**Figure 4.16** *Regional distribution of the GABA<sub>A</sub> receptor  $\delta$ 1-subunit transcript in the brains of C57BL/6J mice during postnatal development.* A: 1-day old (A), 7-day old (B), 14-day old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\delta$ 1-subunit mRNA of the GABA<sub>A</sub> receptor. Av, anteroventral thalamic nucleus; Cb, cerebellum; Cpu, caudate putamen; Ctx, cortex; DG, dentate gyrus; DLG, dorsal lateral geniculate nucleus; GP, globus pallidus; LD, laterodorsal thalamic nucleus. Scale bar represents 0.3mm. Exposure time was 25 days on Kodak XAR-5 film.



**Figure 4.17**

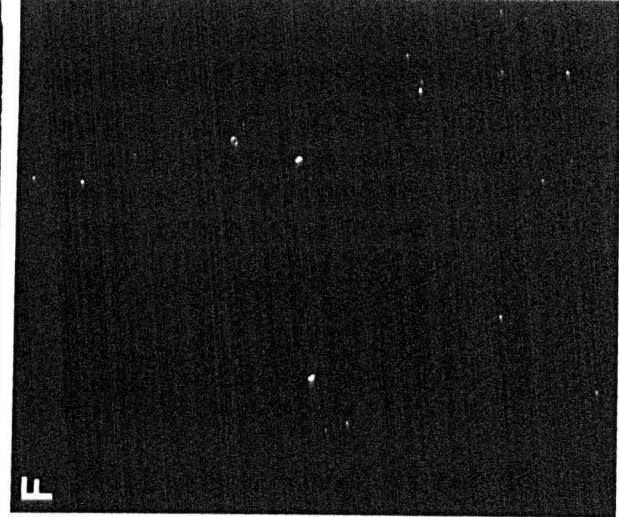
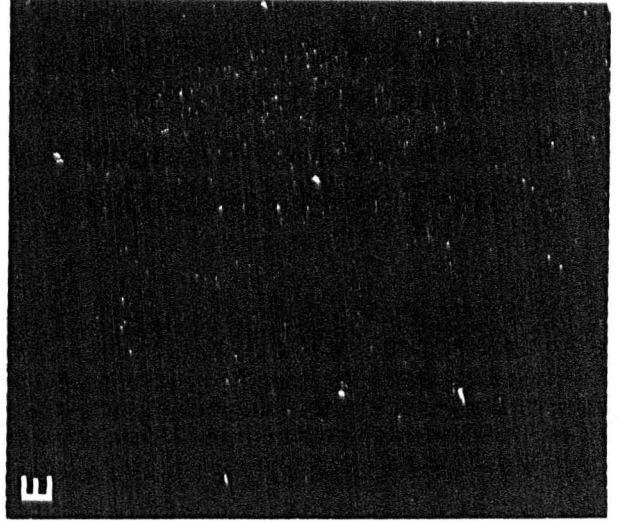
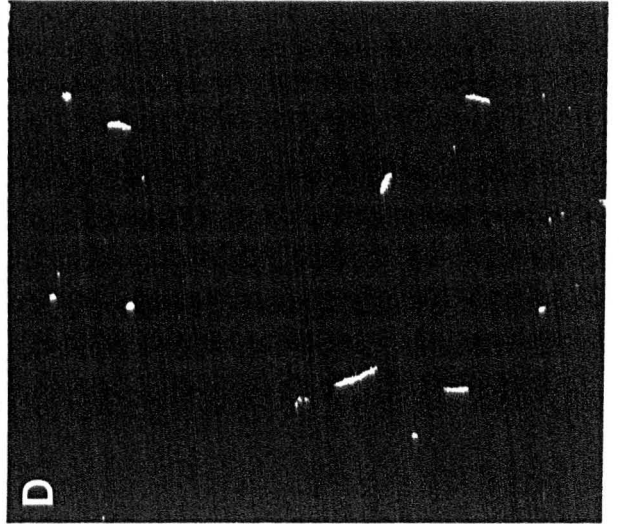
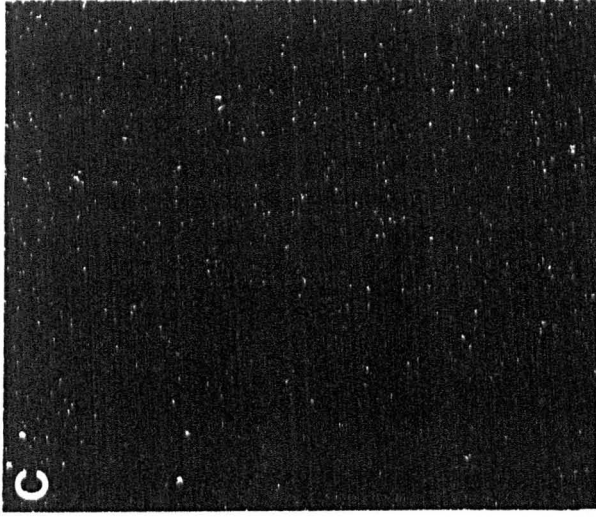
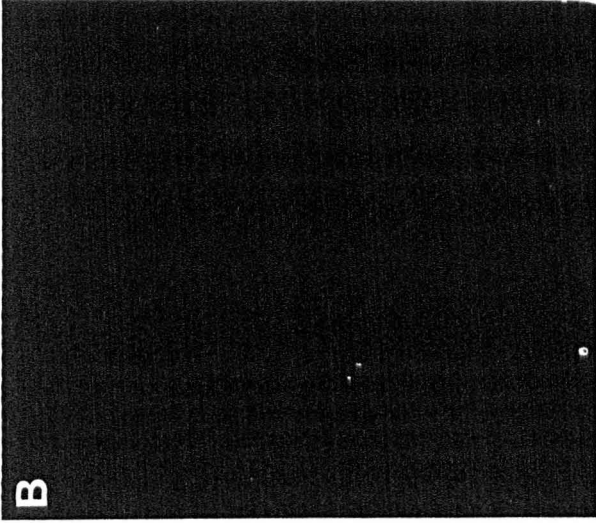
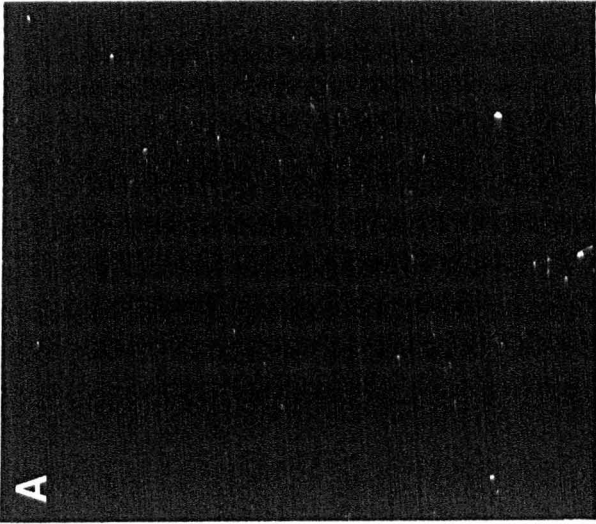


**Figure 4.17** *Regional distribution of the GABA<sub>A</sub> receptor  $\delta$ 1-subunit transcript in the brains of C57BL/6J mice during postnatal development.* A: 1-day old (A), 7-day old (B), 14-day old (C) and adult (D) mouse horizontal brain sections hybridized with an oligonucleotide specific for the  $\delta$ 1-subunit mRNA of the GABA<sub>A</sub> receptor. Am, anteromedial thalamic nucleus; Cb, cerebellum; Cpu, caudate putamen; Ctx, cortex; Gcl, granule cell layer; VPL, ventral posterior lateral thalamic nucleus; VPM, ventral posterior medial thalamic nucleus. Scale bar represents 0.3mm. Exposure time was 25 days on Kodak XAR-5 film.



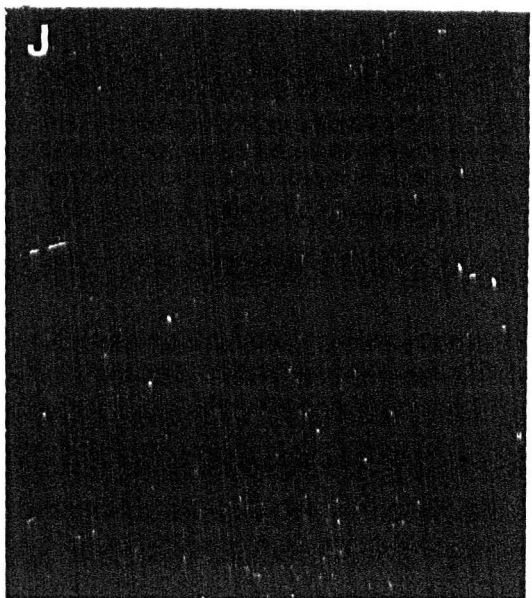
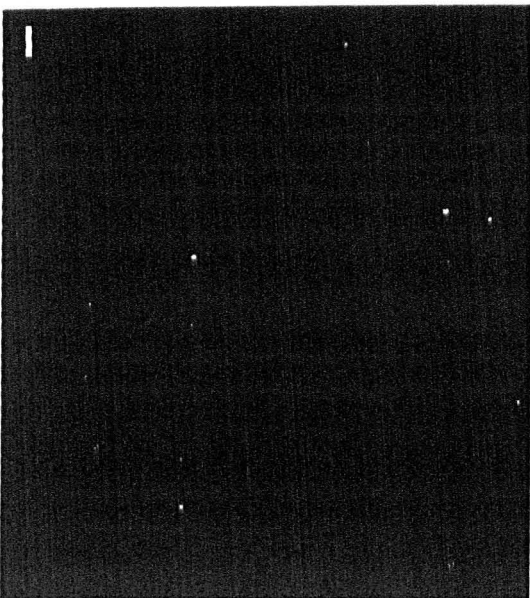
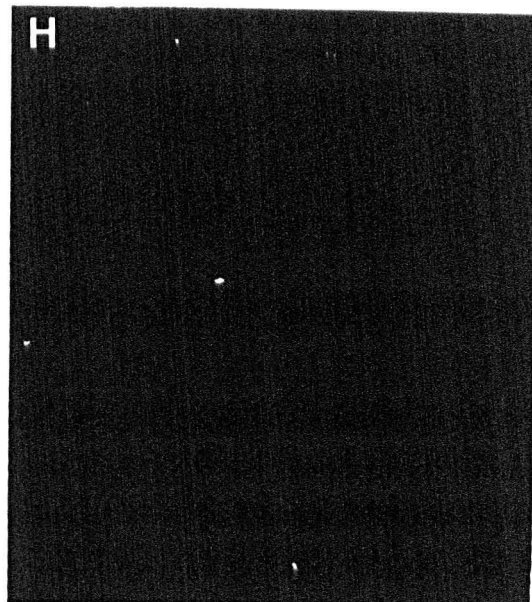
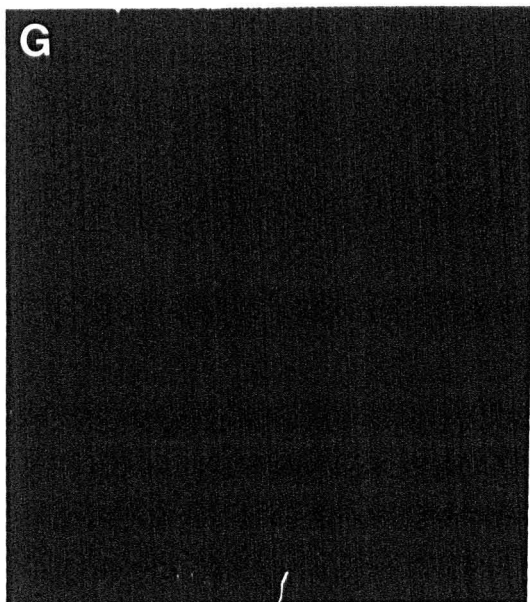
**Figure 4.18**

**Figure 4.18 Control sections for the GABA<sub>A</sub> receptor  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 6$ ,  $\beta 1$ ,  $\beta 3$ -subunit probes.** Horizontal sections were hybridized with a probe labeled to a specific activity of  $10^9$  dpm/ $\mu$ g in the presence of a 30-fold excess of unlabeled oligonucleotide. All controls shown are virtually devoid of signal demonstrating the specificity of the probe and hybridization conditions. (A),  $\alpha 1$  probe, (B)  $\alpha 2$  probe, (C)  $\alpha 3$  probe, (D)  $\alpha 6$  probe, (E)  $\beta 1$  probe, (F)  $\beta 3$  probe. Exposure time was 25 days on Kodak XAR-5 film.



**Figure 4.19**

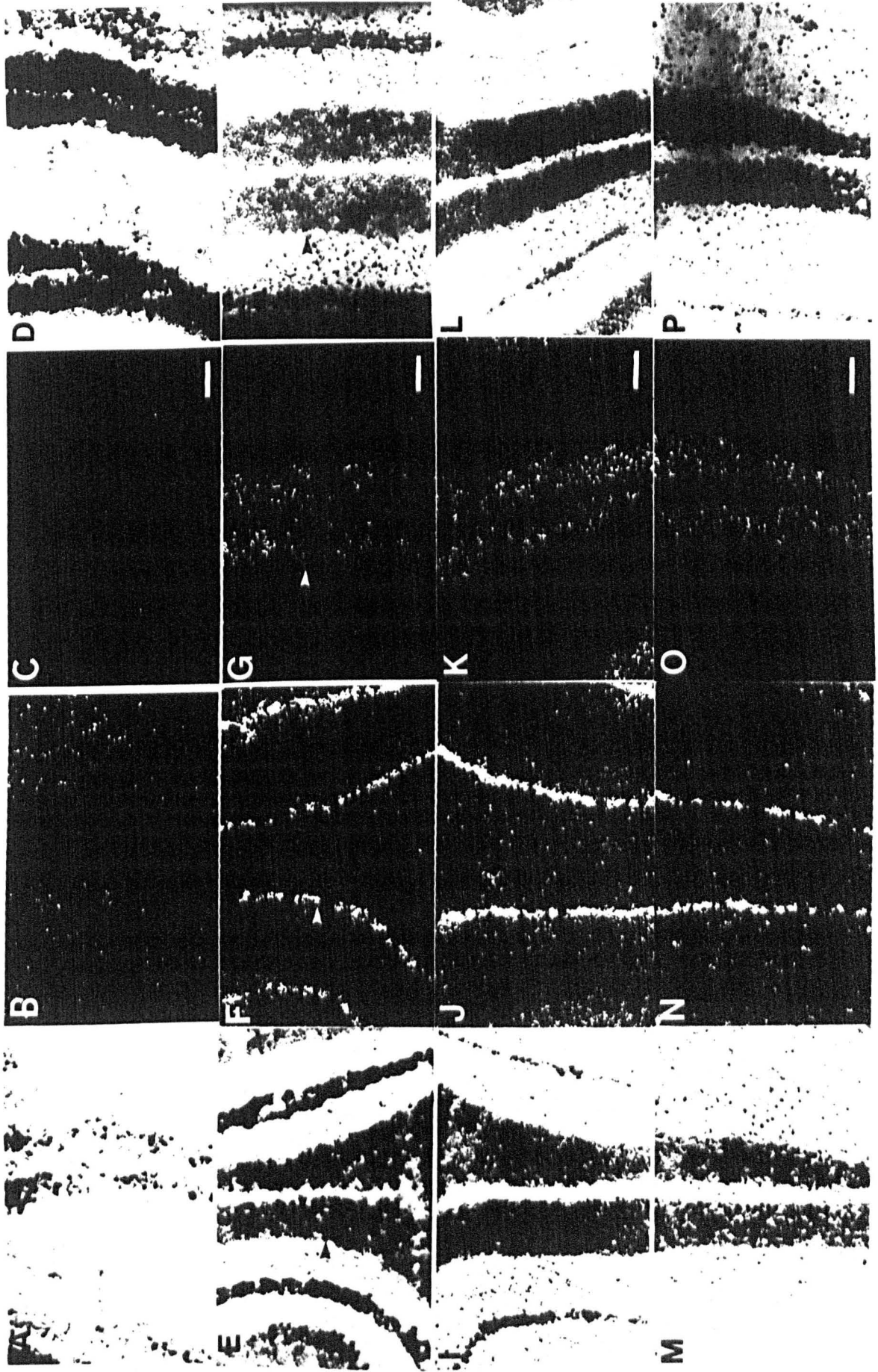
**Figure 4.19 Control sections for the GABA<sub>A</sub> receptor  $\gamma 1$ ,  $\gamma 2T$ ,  $\gamma 3$ ,  $\delta 1$ -subunit probes.** Horizontal sections were hybridized with a probe labeled to a specific activity of  $10^9$  dpm/ $\mu$ g in the presence of a 30-fold excess of unlabeled oligonucleotide. All controls shown are virtually devoid of signal demonstrating the specificity of the probe and hybridization conditions. (A),  $\delta 1$  probe, (B)  $\gamma 2T$  probe, (C)  $\gamma 3$  probe, (D)  $\delta 1$  probe. Exposure time was 25 days on Kodak XAR-5 film.





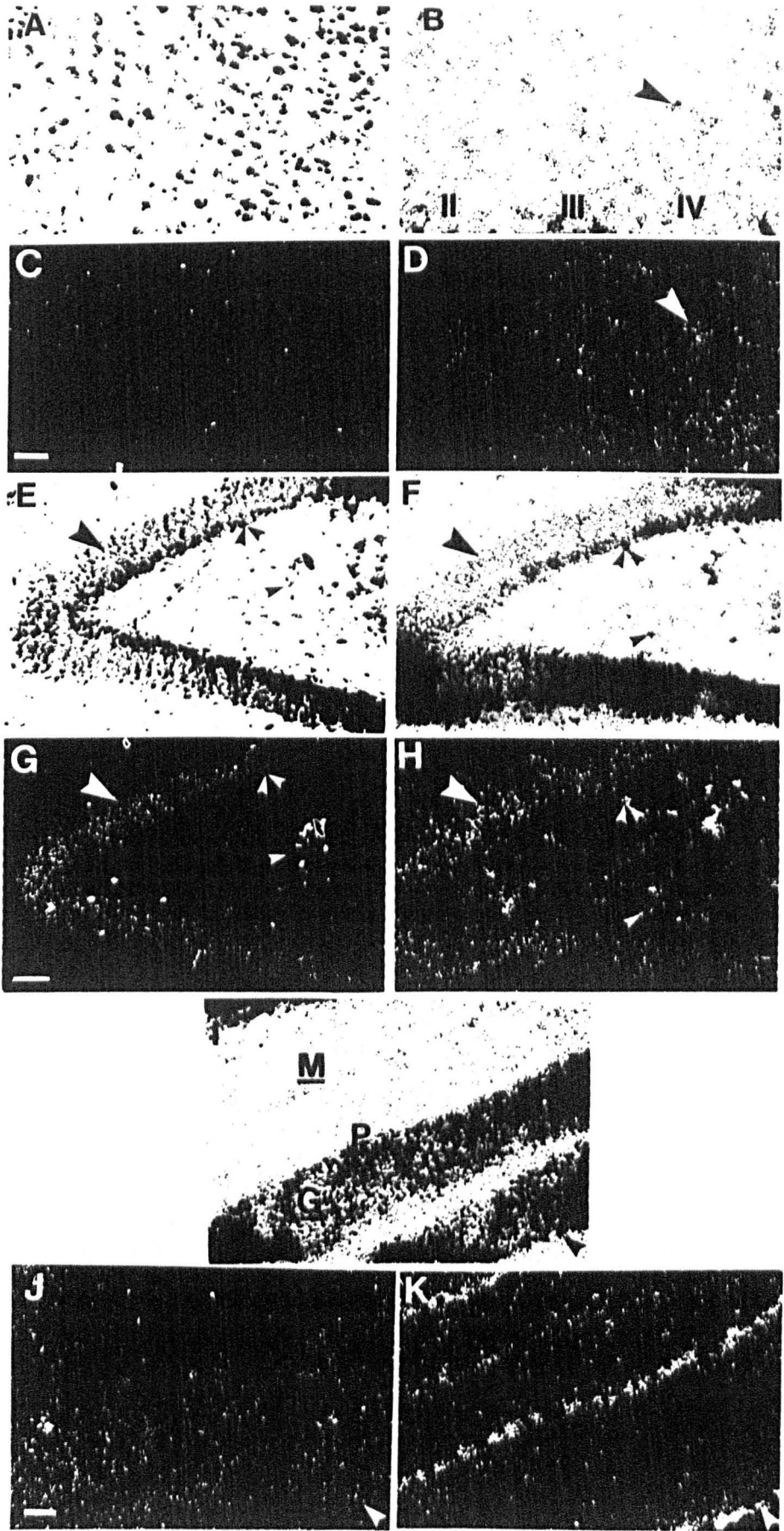
**Figure 4.20**

**Figure 4.20 Cellular localization of the  $\alpha 1$  and  $\alpha 6$  subunit mRNAs of the GABA<sub>A</sub> receptor during postnatal development and in the adult brain.**  $\alpha 1$  probe (A, B, E, F, I, J, M, N) and  $\alpha 6$  probe (C, D, G, H, K, L, O, P). Bright-field (A, E, I, M) and dark-field (B, F, J, N) views of the  $\alpha 1$  GABA<sub>A</sub> receptor subunit mRNA in the C57BL/6J cerebellum at P0 (A, B), P7 (E, F), P14 (I, J) and adult (M, N) cerebellum. Bright-field (C, G, K, O) and dark-field (D, H, L, P) views of the  $\alpha 6$  GABA<sub>A</sub> receptor subunit mRNA in the C57BL/6J cerebellum at P0 (C, D), P7 (G, H), P14 (K, L) and adult (O, P) cerebellum. Scale bar represents 6  $\mu\text{m}$ .



**Figure 4.21**

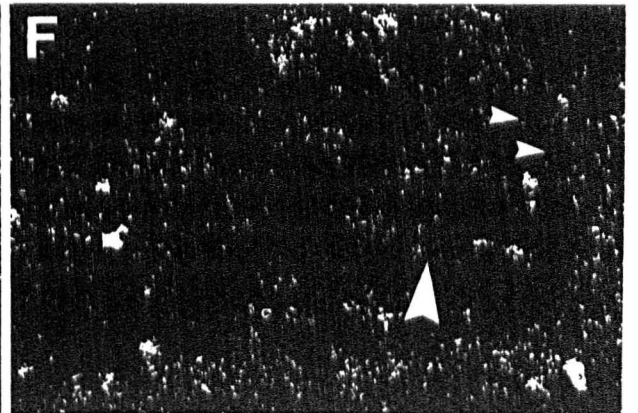
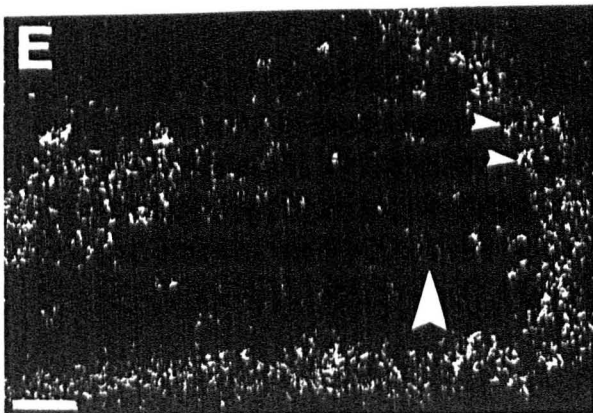
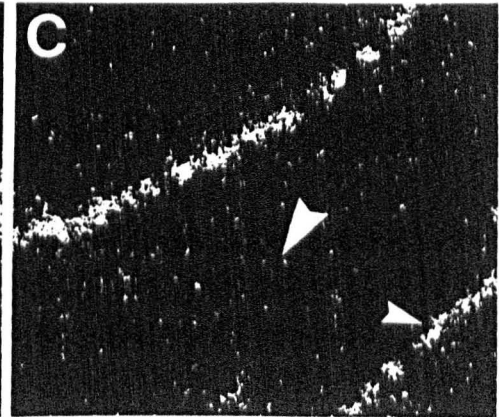
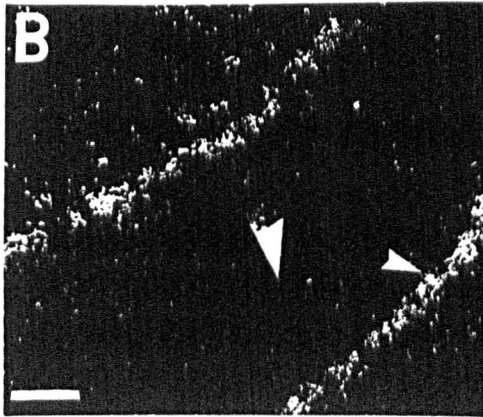
**Figure 4.21 Cellular localization of the  $\alpha 1$  and  $\beta 3$  subunit mRNAs of the GABA<sub>A</sub> receptor during postnatal development and in the adult brain.**  $\alpha 1$  probe (B, D, F, H, I, K) and  $\beta 3$  probe (A, C, E, G, J). Bright-field (B) and dark-field (D) views of the  $\alpha 1$  GABA<sub>A</sub> receptor subunit mRNA in the C57BL/6J cerebral cortex at P14. Bright-field (F) and dark-field (H) views of the  $\alpha 1$  GABA<sub>A</sub> receptor subunit mRNA in the C57BL/6J hippocampus at P14. Bright-field (M) and dark-field (K) views of the  $\alpha 1$  GABA<sub>A</sub> receptor subunit mRNA in the C57BL/6J cerebellum at P14. Bright-field (A) and dark-field (C) views of the  $\beta 3$  GABA<sub>A</sub> receptor subunit mRNA in the C57BL/6J cerebral cortex at P14. Bright-field (E) and dark-field (G) views of the  $\beta 3$  GABA<sub>A</sub> receptor subunit mRNA in the C57BL/6J hippocampus at P14. Dark-field (J) view of the  $\beta 3$  GABA<sub>A</sub> receptor subunit mRNA in the C57BL/6J cerebellar cortex at P14. The large arrowheads indicate the co-localisation of  $\alpha 1$  and  $\beta 3$  subunit mRNAs in cerebral cortical pyramidal cells (B and D) and dentate gyrus granule cells (E, F, G, H). Small arrowheads indicate the absence of  $\alpha 1$  and  $\beta 3$  co-localisation in a population of dentate granule cells in the inner layer of the dentate gyrus, in hippocampal hilar cells (E, F, G, H) and in cerebellar Purkinje cells (I, J, K). Scale bar represents 6  $\mu\text{m}$ .



**Figure 4.22**

**Figure 4.22 Cellular localization of the  $\alpha 1$  and  $\gamma 2T$ -subunit mRNAs of the  $GABA_A$  receptor during postnatal development and in the adult brain.**  $\alpha 1$  probe (A, C, F) and  $\gamma 2$  probe (B, D, E). Dark-field (C, F) views of the  $\alpha 1$   $GABA_A$  receptor subunit mRNA in the P14 cerebellum (C) and P14 hippocampus (F). Bright-field (D) and dark-field (B, E) views of the  $\beta 3$   $GABA_A$  receptor subunit mRNA in the C57BL/6J P14 cerebellum. The large arrowhead (A, B, C) indicates co-localisation of the  $\gamma 2T$  and  $\alpha 1$  subunit mRNA in granule cell somas and the small arrowhead (B, C) indicates co-localization of the  $\gamma 2T$  and  $\alpha 1$  subunit in the Purkinje cell soma. The large arrowhead (E, F) indicates the co-localization of both the  $\alpha 1$  and  $\gamma 2T$  mRNAs in hilar interneurons, while the small arrowhead indicates that only the  $\gamma 2T$  mRNA is present in cells in the inner layer of the dentate gyrus. Scale bar (E) represents 26  $\mu m$ .





**Table 4.1**

**Table 4.1** *Regional distribution of the GABA<sub>A</sub> receptor  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3- and  $\alpha$ 6-subunit transcripts in the brains of C57BL/6J mice during postnatal development.*

Hybridization signals, obtained with oligonucleotides specific for the  $\alpha$ 1-subunit mRNA, the  $\alpha$ 2-subunit mRNA, the  $\alpha$ 3-subunit mRNA, or the  $\alpha$ 6-subunit mRNA were arbitrarily scored as either high, +++; moderate, ++; low, +; weak, (+); or not detectable, -. Nd indicates not determined. The results are derived from Figures 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7 and from data not shown. 1-day, 7-day and 14-day correspond to the days after birth and adult refers to a 50 to 60-day old mouse.

Region/ Nucleus	1 DAY				7 DAY				14 DAY				ADULT			
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 6$	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 6$	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 6$	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 6$
<b>Cortex</b>																
Layer I-II	+	+	+	-	+++	++	++	-	+++	+++	+++	-	++	+	++	-
Layer III	-	+	++	-	+	+	+	-	++	+	++	-	+	+	+	-
Layer IV	-	-	++	-	+++	+	++	-	+++	+	++	-	++	+	++	-
Layer V	-	-	++	-	++	+	++	-	+++	+	+++	-	++	+	++	-
Layer VI	-	-	++	-	++	+	++	-	++	+	++	-	+	+	++	-
Entorhineal Ctx	-	+	++	-	+++	+	+	-	+++	+	+++	-	++	+	++	-
<b>Hippo- campus</b>																
CA1	+	+++	+	-	+++	+++	++	-	+++	+++	+	-	++	++	+	-
CA3	-	+++	+	-	+	+++	++	-	++	+++	-	-	+	++	-	-
DG	-	+	-	-	++	++	-	-	+++	+++	-	-	++	++	-	-
<b>Basal Nuclei</b>																
CPu	+	+	-	-	+	+	-	-	+	+	-	-	-	+	-	-
Acb	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-
GP	-	-	-	-	-	+	-	-	+++	+	-	-	-	+	-	-
<b>Amygdala</b>																
La	-	-	-	-	-	++	-	-	-	++	-	-	-	-	-	-
<b>Septum</b>																
LS	-	+	+	-	-	+	-	-	+	++	+	-	-	+	-	-
MS	-	+	-	-	-	+	-	-	+	++	+	-	-	+	-	-
TS	-	+	-	-	-	+	-	-	+	++	+	-	-	-	-	-
<b>Medial Habenula</b>																
<b>Thalamus</b>																
AV	-	++	+	-	-	+	+	-	+	++	-	-	+	+	+	-
PV	-	++	+	-	-	++	+	-	+	++	+++	-	+	+	+	-
AM	-	+	+	-	++	-	+	-	+	++	++	-	+	+	+	-
VL	-	+	+	-	-	+	+	-	+	-	+	-	-	-	+	-
DLG	-	+	+	-	++	+	+	-	++	+	++	-	+	-	-	-
MG	-	++	+	-	++	-	+	-	++	-	++	-	-	-	-	-
Rt	-	-	+++	-	-	-	++	-	+	-	+++	-	+	-	++	-
VPL	-	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
LD	-	+	++	-	++	-	+	-	++	-	+	-	+	-	+	-
LP	-	++	++	-	++	-	+	-	++	-	++	-	+	-	+	-
<b>Inferior colliculi</b>																
Central nu.	-	-	+	-	+++	-	+	-	+++	-	+	-	++	-	++	-
<b>Superior colliculi</b>																
Central grey	-	-	+	-	++	-	+	-	++	-	+	-	+	-	++	-
DpWh	-	-	+	-	++	-	+	-	++	-	+	-	+	-	++	-
<b>Subi- culum</b>																
Para R. Sub	+	++	+	-	+++	++	++	-	+++	++	++	-	++	+	++	-
Para A. Sub	+	++	+	-	+++	++	++	-	+++	++	++	-	++	+	++	-
<b>Cere- bellum</b>																
Purkinje	-	-	-	-	+++	+	-	-	+++	+	-	-	++	+	-	-
Stellate/ basket cells	-	-	-	-	++	-	-	-	+	-	+	-	+	-	-	-
I.G. cells	+	-	+	+	+++	+	+	+++	+++	-	+	+++	++	-	-	+++
E.G. cells	-	-	-	-	+++	+	+	+++	+++	-	+	+++	++	-	-	+++

**Table 4.2**

**Table 4.2** *Regional distribution of the GABA<sub>A</sub> receptor  $\beta$ 1- and  $\beta$ 3-subunit transcripts in the brains of C57BL/6J mice during postnatal development.*

Hybridization signals, obtained with oligonucleotides specific for the  $\beta$ 1-subunit mRNA, and the  $\beta$ 3-subunit mRNA were arbitrarily scored as either high, +++; moderate, ++; low, +; weak, (+); or not detectable, -. Nd indicates not determined. The results are derived from Figures 4.8, 4.9, 4.10 and from data not shown. 1-day, 7-day and 14-day correspond to the days after birth and adult refers to a 50 to 60-day old mouse.

Region/ Nucleus	1 DAY		7 DAY		14 DAY		ADULT	
	$\beta 1$	$\beta 3$	$\beta 1$	$\beta 3$	$\beta 1$	$\beta 3$	$\beta 1$	$\beta 3$
<b>Cortex</b>								
Layer I-II	++	++	+	++	++	++	+	++
Layer III	+	++	+	++	++	++	+	++
Layer IV	+	++	+	++	++	++	+	++
Layer V	+	+	+	++	++	++	+	++
Layer VI	+	+	+	+	++	+	+	+
Entorhineal Ctx	+		+	++	++	++	+	++
<b>Hippocampus</b>								
CA1	++	++	++	+++	++	+++	++	++
CA3	++	++	++	+++	++	+++	++	++
Dentate Gyrus	-	++	+	+++	++	+++	+	++
<b>Basal Nuclei</b>								
Caudate-putamen	+	++	+	+	+	+	+	+
Nu. accumbens	+	-	+	-	+	+	+	+
Globus pallidus	+	-	+	-	+	+	+	+
<b>Amygdala</b>								
Lateral amygdaloid	-	-	-	-	-	-	-	-
<b>Septum</b>								
Bed nucleus s.t.	-	-	-	-	-	-	-	-
Lateral septum	+	+	+	+	+	+	+	+
Medial septum	-	-	-	-	-	-	-	+
Triangular septum	-	-	-	-	-	-	-	-
<b>Medial Habenula</b>	-	+	-	+	-	+	-	+
<b>Thalamus</b>								
Anteroventral thal.	+	+	+	+	+	+	+	+
Paraventricular nu.	+	+	+	+	+	+	+	+
Reuniens thal. nu.	+	+	+	+	+	+	+	+
Anteromedial thal.	+	+	+	+	+	+	+	+
Ventrolateral thal.	+	+	+	+	+	+	+	+
Dorsal lateral genicul.	-	+	-	+	-	+	-	+
Medial lateral genicul.	-	+	-	+	-	+	-	+
Reticular nucleus	-	-	-	-	-	-	-	-
Ventr. posterior nu.	+	+	+	+	+	+	+	+
Laterodorsal thal.	+	+	+	+	++	+	+	+
Lateral posterior thal.	+	+	+	+	++	+	+	+
<b>Inferior colliculi</b>								
Central nucleus.	-	+	+	+	++	+	+	+
<b>Superior colliculi</b>								
Central grey	-	+	+	+	++	+	+	+
Deep white layer	-	+	+	+	++	+	+	+
<b>Subiculum</b>								
Para R. Sub	+	++	+	+++	+	++	+	+
Para A. Sub	+	++	+	+++	+	++	+	+
<b>Cerebellum</b>								
Purkinje layer	-	-	-	-	-	-	-	-
Stellate/basket cells	-	+	+	++	++	+	+	+
Interior granule. cells	-	-	+	+++	++	+++	+	+
Exterior granule cells	-	-	+	+++	++	+++	+	+

**Table 4.3**



**Table 4.3** *Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 1-,  $\gamma$ 2-,  $\gamma$ 3- and  $\delta$ 1-subunit transcripts in the brains of C57BL/6J mice during postnatal development.*

Hybridization signals, obtained with oligonucleotides specific for the  $\gamma$ 1-subunit mRNA, the  $\gamma$ 2-subunit mRNA, the  $\gamma$ 3-subunit mRNA, or the  $\delta$ 1-subunit mRNA were arbitrarily scored as either high, +++; moderate, ++; low, +; weak, (+); or not detectable, -. Nd indicates not determined. The results are derived from Figures 4.11, 4.12, 4.13, 4.14, 4.15, 4.16, 4.17 and from data not shown. 1-day, 7-day and 14-day correspond to the days after birth and adult refers to 50 to 60-day old mice.

Region/ Nucleus	1 DAY				7 DAY				14 DAY				ADULT			
	$\gamma$ 1	$\gamma$ 2T	$\gamma$ 3	$\delta$ 1	$\gamma$ 1	$\gamma$ 2T	$\gamma$ 3	$\delta$ 1	$\gamma$ 1	$\gamma$ 2T	$\gamma$ 3	$\delta$ 1	$\gamma$ 1	$\gamma$ 2T	$\gamma$ 3	$\delta$ 1
<b>Cortex</b>																
Layer I-II	+	+++	+	-	++	++	+	++	++	+++	+	+	+	++	+	+
Layer III	+	+++	+	-	++	+++	+	++	++	+++	+	+	+	+++	+	+
Layer IV	+	++	+	-	++	++	+	++	++	+++	+	+	+	++	+	+
Layer V	+	+++	+	-	++	+++	+	+	++	+++	+	+	+	+++	+	+
Layer VI	+	++	+	-	++	++	+	+	++	+++	+	+	+	++	+	+
Entorhineal Ctx	+	++	+	-	++	++	+	+	++	+++	+	+	+	++	+	+
<b>Hippo- campus</b>																
CA1	-	+++	-	-	+	+++	+	-	++	+++	+	-	+	++	+	-
CA3	-	++	-	-	+	+++	+	-	++	+++	+	-	+	++	+	-
DG	-	++	-	-	+	+++	+	+	++	+++	+	+	+	+++	+	+
<b>Basal Nuclei</b>																
CPu	+	+	+	-	+	+	+	+	++	+	+	+	+	+	+	+
Acb	+	+	-	-	+	+	+	-	++	+	+	-	+	+	+	-
GP	+	+	+	-	+	+++	+	+	+++	+	+	+	++	+	+	+
<b>Amygdala</b>																
La	-	+	-	-	-	+	-	-	-	++	-	-	-	+++	-	-
<b>Septum</b>																
Lateral	++	+	-	-	+++	+	+	-	+++	+	+	-	+++	+	+	-
Medial	++	+	-	-	++	+	+	-	++	+	+	-	++	+	+	-
Triangular	++	-	-	-	+++	+	-	-	+++	++	+	-	+++	+	+	-
<b>Medial Habenula</b>	+++	++	+	-	+++	++	-	-	+++	+++	-	-	++	++	-	-
<b>Thalamus</b>																
AV	++	+	-	-	+	+++	-	++	++	+++	-	+	+	++	-	+
PVA	++	++	-	-	++	++	-	+	+++	+++	-	-	++	++	-	-
AM	++	+	-	-	++	++	-	++	+	++	-	+	+	+	-	++
VL	++	++	-	-	+	+	-	+	++	++	-	+	+	+	-	++
DLG	-	+	-	-	++	++	-	+	++	+++	-	+	++	++	-	+
MG	+++	++	-	-	+++	+++	-	+	++	+++	-	+	++	++	-	+
Rt	-	++	-	-	-	++	-	-	-	++	-	-	-	+	-	-
VPL	+	+	-	-	+	+	-	+	++	++	-	++	+	+	-	++
LD	+++	++	-	-	++	+	-	+	++	++	-	++	+	+	-	-
LP	+++	++	-	-	++	+	-	-	++	++	-	-	+	+	-	-
<b>Colliculi</b>																
I.F. C	++	+++	-	-	++	++	-	-	++	+++	-	+	++	++	-	-
Central grey	++	++	-	-	++	++	-	-	++	+++	-	-	++	++	-	-
DpWh	++	++	-	-	++	++	-	-	++	+++	-	-	++	++	-	-
<b>Subi- culum</b>																
Para R. Sub	++	+++	+	-	+++	++	+	-	+++	+++	+	-	+++	+++	+	-
Para A. Sub	++	+++	+	-	+++	++	+	-	+++	+++	+	-	+++	+++	+	-
<b>Cere- bellum</b>																
Purkinje	-	++	-	-	-	+++	-	-	-	+++	-	-	-	+++	-	-
Stellate/ basket cells	-	++	+	-	+	++	+	+	++	++	+	+	+	+	+	+
I.G. cells	-	-	-	-	+	+++	++	++	++	+++	+++	++	++	++	++	++
E.G. cells	+	++	++	-	+	+++	++	++	++	+++	+++	++	++	++	++	++

**Table 4.4**

**Table 4.4** *Relative expression levels of the GABA<sub>A</sub> receptor  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3-,  $\beta$ 1-,  $\beta$ 3-,  $\gamma$ 1-,  $\gamma$ 2-,  $\gamma$ 3- and  $\delta$ 1-subunit transcripts in serial horizontal sections of 1 day-old C57BL/6J mouse brains.*

Hybridization signals, obtained with oligonucleotides specific for these murine GABA<sub>A</sub> receptor subunit transcripts were arbitrarily scored as either high, +++; moderate, ++; low, +; weak, (+); or not detectable, -. Nd indicates not determined. The results are derived from Figures 4.1 - 4.17 and from data not shown. Note that these results take into account differences in the relative autoradiographic and photographic exposure times (approximately 5-fold) of the  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3-,  $\alpha$ 6-,  $\beta$ 1-,  $\beta$ 3-, and  $\gamma$ 2-subunit transcripts compared with the  $\gamma$ 1-,  $\gamma$ 3- and  $\delta$ 1-subunit transcripts.

Region/ Nucleus	1 DAY									
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 6$	$\beta 1$	$\beta 3$	$\gamma 1$	$\gamma 2T$	$\gamma 3$	$\delta 1$
<b>Cortex</b>										
Layer I-II	+	+	+	-	++	+++	+	+++	-	-
Layer III	-	+	++	-	+	+++	+	+++	+	-
Layer IV	-	-	++	-	+	+++	+	++	+	-
Layer V	-	-	++	-	+	++	+	+++	+	-
Layer VI	-	-	++	-	+	++	+	++	+	-
<b>Hippocampus</b>										
CA1	+	+++	+	-	++	+++	-	+++	-	-
CA3	-	+++	+	-	++	+++	-	++	-	-
Dentate gyrus	-	++	-	-	+	++	-	++	-	-
<b>Basal Nuclei</b>										
Caudate-putamen	+	+	-	-	+	+++	+	+	+	-
Nu. accumbens	-	+	-	-	+	++	+	+	-	-
Globus pallidus	-	+	-	-	+	-	+	+	+	-
<b>Septum</b>										
Lateral septum	-	++	+	-	+	+	++	+	-	-
Medial septum	-	++	-	-	-	-	+	+	-	-
Triangular septum	-	++	-	-	-	-	++	-	-	-
<b>Medial Habenula</b>	-	++	++	-	+	++		++	+	-
<b>Thalamus</b>										
Anteroventral thal.	-	+++	++	-	+	+	++	+	-	-
Paraventricular nu.	-	++	+	-	+	++	++	++	-	-
Anteromedial thal.	-	+	+	-	+	++	++	+	-	-
Ventrolateral thal.	-	+	+	-	+	++	+	++	-	-
Dorsal lateral genicul	-	+	+	-	+	+	++	+	-	-
Medial genicul	-	+++	++	-	+	+	+++	++	-	-
Reticular nucleus	-	-	+++	-	-	-	-	++	-	-
Ventr. posterior nu.	-	+	+	-	+	+	++	+	-	-
Laterodorsal thal.	-	+	++	-	+	+	+++	++	-	-
Lateral posterior thal.	-	++	++	-	+	+	+++	++	-	-
<b>Colliculi</b>										
I.C. Central nucleus.	-	-	++	-	-	+	++	+++	-	-
Central grey	-	-	++	-	-	+	+++	++	-	-
Deep white layer	-	-	++	-	-	+	++	+++	-	-
<b>Subiculum</b>										
Para R. Sub	+	+++	++	-	+	+++	+++	+++	+	-
Para A. Sub	+	+++	++	-	+	+++	+++	+++	+	-
<b>Cerebellum</b>										
Purkinje layer	-	+	-	-	-	-	-	+	-	-
Stellate/basket cells	-	-	-	-	+	++	-	+	+	-
Interior granule cells	-	-	-	-	-	-	-	-	-	-
Exterior granule cells	++	-	+	+	+	++	+	++	+	-

**Table 4.5**

**Table 4.5** *Relative expression levels of the GABA<sub>A</sub> receptor  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3-,  $\beta$ 1-,  $\beta$ 3-,  $\gamma$ 1-,  $\gamma$ 2-,  $\gamma$ 3- and  $\delta$ 1-subunit transcripts in serial horizontal sections of 7 day-old C57BL/6J mouse brains.*

Hybridization signals, obtained with oligonucleotides specific for these murine GABA<sub>A</sub> receptor subunit transcripts were arbitrarily scored as either high, +++; moderate, ++; low, +; weak, (+); or not detectable, -. Nd indicates not determined. The results are derived from Figures 4.1 - 4.17 and from data not shown. Note that these results take into account differences in the relative autoradiographic and photographic exposure times (approximately 5-fold) of the  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3-,  $\alpha$ 6-,  $\beta$ 1-,  $\beta$ 3-, and  $\gamma$ 2-subunit transcripts compared with the  $\gamma$ 1-,  $\gamma$ 3- and  $\delta$ 1-subunit transcripts.

Region/ Nucleus	7 DAY									
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 6$	$\beta 1$	$\beta 3$	$\gamma 1$	$\gamma 2T$	$\gamma 3$	$\delta 1$
<b>Cortex</b>										
Layer I-II	+++	++	++	-	+	+++	++	++	+	+
Layer III	++	+	+	-	+	+++	++	+++	+	+
Layer IV	+++	+	++	-	+	+++	++	++	+	+
Layer V	++	+	++	-	+	+++	++	+++	+	+
Layer VI	++	+	++	-	+	+++	++	++	+	+
Entorhineal Ctx		+	++	-	+	+++	++	++	+	+
<b>Hippocampus</b>										
CA1	++	+++	++	-	++	+++	++	+++	+	-
CA3	+	+++	++	-	++	+++	++	+++	+	-
Dentate gyrus	++	++	-	-	++	+++	+	+++	+	+
<b>Basal Nuclei</b>										
Caudate-putamen	+	+	-	-	+	+	+	+	+	+
Nu. accumbens	-	+	-	-	+	+	+	+	+	+
Globus pallidus	-	+	-	-	+	-	+++	+	+	+
<b>Septum</b>										
Lateral septum	-	+	-	-	+	-	+++	+	+	-
Medial septum	-	+	-	-	+	-	+++	+	+	-
Triangular septum	-	+	-	-	+	+	+++	+	+	-
<b>Medial Habenula</b>	++	-	++	-	+	+	+++	+	-	-
<b>Thalamus</b>										
Anteroventral thal.	-	+	+	-	+	-	+	+++	-	++
Paraventricular nu.	-	++	++	-	+	++	+++	++	-	+
Anteromedial thal.	++	-	+	-	+	-	+	+	-	++
Ventrolateral thal.	-	+	+	-	+	+	+	+	-	+
Dorsal lateral genicul	++	+	+	-	+	+	++	++	-	++
Medial lateral genicul	++	-	+	-	+	+	++	+++	-	+
Reticular nucleus	-	-	+++	-	+	-	-	+	-	-
Ventr. posterior nu.	-	-	-	-	+	+	+	+	-	++
Laterodorsal thal.	++	-	+	-	+	+	+	+	-	+
Lateral posterior thal.	++	-	+	-	+	+	++	+	-	-
<b>Inferior colliculi</b>	+++	-	+	-	+	+	++	++	-	-
<b>Superior colliculi</b>										
Central grey	++	-	+	-	+	+	++	++	-	-
Deep white layer	++	-	+	-	+	+	++	++	-	-
<b>Subiculum</b>										
Para R. Sub	+++	++	++	-	+	+++	+++	++	+	-
Para A. Sub	+++	++	++	-	+	+++	+++	++	+	-
<b>Cerebellum</b>										
Purkinje	+++	+	-	-	-	-	-	+++	-	-
Stellate/basket cells	++	-	-	-	+	++	+	++	+	+
Interior granule cells	+++	+	+	+++	+	+++	++	+++	++	++
Exterior granule cells	+++	+	+	+++	+	+++	++	+++	++	++



**Table 4.6**

**Table 4.6** *Relative expression levels of the GABA<sub>A</sub> receptor  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3-,  $\beta$ 1-,  $\beta$ 3-,  $\gamma$ 1-,  $\gamma$ 2-,  $\gamma$ 3- and  $\delta$ 1-subunit transcripts in serial horizontal sections of 14 day-old C57BL/6J mouse brains.*

Hybridization signals, obtained with oligonucleotides specific for these murine GABA<sub>A</sub> receptor subunit transcripts were arbitrarily scored as either high, +++; moderate, ++; low, +; weak, (+); or not detectable, -. Nd indicates not determined. The results are derived from Figures 4.1 - 4.17 and from data not shown. Note that these results take into account differences in the relative autoradiographic and photographic exposure times (approximately 5-fold) of the  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3-,  $\alpha$ 6-,  $\beta$ 1-,  $\beta$ 3-, and  $\gamma$ 2-subunit transcripts compared with the  $\gamma$ 1-,  $\gamma$ 3- and  $\delta$ 1-subunit transcripts.

Region/ Nucleus	14 DAY									
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 6$	$\beta 1$	$\beta 3$	$\gamma 1$	$\gamma 2T$	$\gamma 3$	$\delta 1$
<b>Cortex</b>										
Layer I-II	+++	+++	+++	-	++	++	++	+++	+	++
Layer III	++	+	++	-	++	++	++	+++	+	++
Layer IV	+++	+	++	-	++	++	++	+++	+	++
Layer V	+++	+	+++	-	++	++	++	+++	+	+
Layer VI	++	+	++	-	+	++	++	+++	+	+
Entorhineal Ctx	+++	+	+++	-	++	++	++	+++	+	+
<b>Hippocampus</b>										
CA1	+++	+++	++	-	++	+++	++	+++	++	-
CA3	+	+++	-	-	++	+++	++	+++	++	-
Dentate gyrus	++	+++	-	-	++	+++	++	+++	++	+
<b>Basal Nuclei</b>										
Caudate-putamen	+	+	-	-	+	+	++	+	+	+
Nu. accumbens	-	+	-	-	+	+	++	+	+	-
Globus pallidus	+++	+	-	-	+	+	+++	+	+	+
<b>Septum</b>										
Lateral septum	+	++	+	-	++	+	+++	+	+	-
Medial septum	+	++	+	-	++	-	++	+	+	-
Triangular septum	+	++	+	-	++	-	+++	++	+	-
<b>Medial Habenula</b>										
	++	++	+++	-	++	+	+++	++	+	-
<b>Thalamus</b>										
Anteroventral thal.	+	++	-	-	++	+	++	+++	-	++
Paraventricular nu.	+	++	+++	-	+	++	+++	+++	-	-
Anteromedial thal.	+	++	-	-	+	+	+++	++	-	+
Ventrolateral thal.	+	+	-	-	+	+	++	++	-	+
Dorsal lateral genicul	+++	+	++	-	+	+	++	+++	-	+
Medial lateral genicul	+++	-	++	-	+	+	++	+	-	+
Reticular nucleus	-	-	+++	-	-	-	-	-	-	-
Ventr. posterior nu.	++	+	+	-	+	+	++	+	-	++
Laterodorsal thal.	++	-	+	-	++	+	++	+	-	++
Lateral posterior thal.	++	-	+	-	++	+	++	+	-	++
<b>Inferior colliculi</b>										
	+++	-	+	-	++	++	++	+++	-	+
<b>Superior colliculi</b>										
Central grey	++	-	+	-	++	++	++	+++	+	-
Deep white layer	+++	-	+	-	++	++	++	+++	+	-
<b>Subiculum</b>										
Para R. Sub	+++	+++	+	-	+	++	+++	+++	+	-
Para A. Sub	+++	+++	+	-	+	++	+++	+++	+	-
<b>Cerebellum</b>										
Purkinje	+++	+	-	-	-	-	++	+++	-	-
Stellate/basket cells	++	+	+	-	++	+	++	++	+	+
Interior granule cells	+++	+	+	+++	++	+++	++	+++	+++	++
Exterior granule cells	+++	+	+	+++	++	+++	++	+++	+++	++

**Table 4.7**

**Table 4.7** *Relative expression levels of the GABA<sub>A</sub> receptor  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3-,  $\beta$ 1-,  $\beta$ 3-,  $\gamma$ 1-,  $\gamma$ 2-,  $\gamma$ 3- and  $\delta$ 1-subunit transcripts in serial horizontal sections of adult C57BL/6J mouse brains.*

Hybridization signals, obtained with oligonucleotides specific for these murine GABA<sub>A</sub> receptor subunit transcripts were arbitrarily scored as either high, +++; moderate, ++; low, +; weak, (+); or not detectable, -. Nd indicates not determined. The results are derived from Figures 4.1 - 4.17 and from data not shown. Note that these results take into account differences in the relative autoradiographic and photographic exposure times (approximately 5-fold) of the  $\alpha$ 1-,  $\alpha$ 2-,  $\alpha$ 3-,  $\alpha$ 6-,  $\beta$ 1-,  $\beta$ 3-, and  $\gamma$ 2-subunit transcripts compared with the  $\gamma$ 1-,  $\gamma$ 3- and  $\delta$ 1-subunit transcripts. Adult mice were 50 to 60 days old.

Nucleus	Adult									
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 6$	$\beta 1$	$\beta 3$	$\gamma 1$	$\gamma T 2$	$\gamma 3$	$\delta 1$
<b>Cortex</b>										
Layer I-II	++	++	++	-	+	++	+	+	+	+
Layer III	++	+	+	-	+	++	++	++	+	+
Layer IV	++	+	++	-	+	++	++	+	+	+
Layer V	++	+	++	-	+	++	++	++	+	+
Layer VI	++	+	++	-	+	++	++	++	+	+
<b>Hippocampus</b>										
CA1	++	++	+	-	+	+++	+	++	-	-
CA3	+	++	-	-	+	+++	+	++	-	-
Dentate gyrus	++	++	-	-	+	+++	+	+++	-	-
<b>Basal Nuclei</b>										
Caudate-putamen	-	+	-	-	+	+	+	-	+	+
Nu. accumbens	-	+	-	-	+	+	+	-	-	-
Globus pallidus	-	+	-	-	+	+	++	-	-	-
<b>Septum</b>										
Lateral septum	-	+	-	-	+	+	+++	+	+	-
Medial septum	-	+	-	-	+	+	+++	+	+	-
Triangular septum	-	-	-	-	+	+	+++	+	+	-
<b>Medial Habenula</b>	-	++	++	-	-	+	++	-	-	-
<b>Thalamus</b>										
Anteroventral thal.	+	+	+	-	+	-	+	+	-	-
Paraventricular nu.	+	+	+	-	+	++	++	+	-	-
Anteromedial thal.	+	+	+	-	+	-	++	+	-	++
Ventrolateral thal.	-	-	+	-	+	-	+	+	-	+
Dorsal lateral genicul.	+	-	-	-	+	-	+	++	-	-
Medial geniculate	++	-	-	-	+	-	++	++	-	++
Reticular nucleus	+	-	++	-	+	-	-	++	-	-
Laterodorsal thal.	+	-	-	-	+	-	++	+	-	-
Lateral posterior thal.	+	-	-	-	+	-	+	+	-	-
<b>Colliculi</b>										
I.C. Central nucleus.	++	-	++	-	+	+	++	+	-	-
Central grey	+	-	++	-	+	+	+++	+	-	-
Deep white layer	+	-	++	-	+	+	++	+	-	-
<b>Subiculum</b>										
Para R. Sub	++	++	++	-	+	+	+	+	-	-
Para A. Sub	++	++	++	-	+	+	+	+	-	-
<b>Cerebellum</b>										
Purkinje cell layer	+++	+	-	-	-	-	-	++	-	-
Stellate/basket cells	+	-	-	-	+	+	+	+	-	+
Interior granular cells	++	-	-	+++	+	+	++	+	++	++
Exterior granular cells	++	-	-	+++	+	+	++	+	++	++

# **Chapter 5**

## **Alternative-Splicing**

## 5.1 Introduction

Heterogeneity within a receptor class, as demonstrated for the GABA<sub>A</sub> receptor in Chapter 4 is due predominantly to the developmental and differential localization of large numbers of receptor subunit mRNAs. Translation of these mRNAs and the assembly of their products into hetero-oligomeric receptors creates numerous subtype variations. Further synaptic diversification may also occur, however through molecular alterations to either nucleic acid or protein and include: 1) RNA editing; 2) alternative-splicing of a mRNA; 3) allelic variation as seen with D4 dopamine receptor variants in the human population (Vantol *et al.*, 1992); and post-translational modifications, (protein phosphorylation).

Protein phosphorylation is a common mechanism for regulating the function of a wide variety of proteins, including ligand-gated ion channels. A variety of data now indicate that protein phosphorylation within the CNS regulates the efficacy of synaptic transmission, both by modulating the release of neurotransmitter from the presynaptic nerve terminal and by modulating the sensitivity of receptors in the postsynaptic membrane (Greengard, 1978; Kandel and Schwartz, 1982; Huganir and Greengard, 1990; Nicoll *et al.*, 1988). The desensitization of neurotransmitter receptors could play a role in the modulation of synaptic transmission during high-frequency firing of the presynaptic neuron or under conditions in which there are significant resting levels of neurotransmitter in the synaptic cleft.

All GABA<sub>A</sub> receptor  $\beta$  subunits have a consensus site for PKA phosphorylation, corresponding to serine 409 in  $\beta$ 1 (Swope *et al.*, 1992; Ymer *et al.*, 1989), and this residue can be phosphorylated by PKA and PKC (Moss, Doherty and Huganir, 1992). PKA or PKC activation in HEK-293 cells transiently expressing GABA<sub>A</sub> receptors decreases the sensitivity to GABA (Moss *et al.*, 1992; Krishek *et al.*, 1994). The GABA<sub>A</sub> receptor  $\gamma$ 2 subunit has two serine phosphorylation sites, one of which is missing in the  $\gamma$ 2 splice variant (Moss, Doherty and Huganir, 1992; Whiting, McKernan and Iversen, 1991). Mutation of the 409 ( $\beta$ 1), 327 ( $\gamma$ 2) and 343 ( $\gamma$ 2) serine sites abolishes the effect of PCK.

The molecular alteration of the AMPA GluR-B(Q) and GluR-B(R) subtypes of the glutamate receptor was the first reported example of variation in a mRNA arising from RNA editing (Sommer *et al.*, 1991). The editing process is highly selective for GluR-B subunits only, and is developmentally regulated (Burnashev *et al.*, 1992). The conversion of a glutamine to arginine amino acid in the putative M2 domain of the GluR-B subunit results in hetero-oligomeric AMPA receptors containing GluR-B subunits and low divalent ion (Ca<sup>2+</sup>) permeabilities (Hume, Dingledine and Heinemann, 1991; Burnashev *et al.*, 1992).



Alternative-RNA splicing, as a mechanism for creating diversity within the CNS has been reported for several receptor systems. The D2 dopamine receptor (Dal Toso *et al.*, 1989; Giros *et al.*, 1989; Monsma *et al.*, 1989; Chio *et al.*, 1990) and the  $\alpha$  subunit of the acetylcholine receptor (Beeson *et al.*, 1990) are alternatively-spliced transcripts with 29 and 25 amino acid insertions, respectively. The function of these insertions remains unknown.

As described in section 1.4.6, alternative-splicing of primary transcripts of the GABA<sub>A</sub> receptor  $\gamma$ 2 subunit in human, bovine, chick, rat and mouse (Whiting, McKernan and Iverson, 1990; Kofuji *et al.*, 1991; Glencorse, Bateson and Darlison, 1992; Bateson *et al.*, 1991) results in two mRNAs that differ by the presence or absence of a 24 base pair (8 amino acids) insertion in the cytoplasmic loop between the third and fourth putative transmembrane-spanning domains. The insertion contains a site for protein kinase C (PKC) phosphorylation and may be involved in the potentiating effect of EtOH on the GABA response of the GABA<sub>A</sub> receptor (Wafford, *et al.*, 1990). The 24 base insertion may also play a role in the inhibitory modulation by the benzodiazepine inverse agonist, Ro15-4513 on ethanol potentiation (Wafford, McKernan and Iversen, 1990).

To date, the  $\gamma$ 2 GABA<sub>A</sub> receptor subunit is the only example within the ligand-gated ion superfamily of an alternatively-spliced transcript with a possible role in synaptic plasticity. The determination of the temporal expression of the  $\gamma$ 2 subunit mRNA variants will provide an insight into the patterns of heterogeneity expressed during the development of the CNS. This chapter describes the embryonic and postnatal expression of the alternatively-spliced  $\gamma$ 2 GABA<sub>A</sub> receptor subunit mRNAs in the C57BL/6J mouse brain.

## 5.2 Results

### 5.2.1 Regional and Cellular *in situ* hybridisation

Oligonucleotide probes specific for either the  $\gamma$ 2S subunit mRNA or the  $\gamma$ 2L subunit mRNA, and an oligonucleotide ( $\gamma$ 2T) that recognizes both of these transcripts, were used for *in situ* hybridisation studies. The expression patterns of the  $\gamma$ 2L and  $\gamma$ 2S transcripts during late murine embryogenesis (embryonic days 14.5 to 19.5) are shown in Figure 5.1. The regional distribution patterns of the  $\gamma$ 2-subunit-variant transcripts in 1-day-old, 7-day-old, 14-day-old and adult mouse brains are shown in Figures 5.2 to 5.5. The signals on parallel control sections with each of the three oligonucleotide probes were competed with a thirty-fold molar excess of the appropriate unlabelled oligonucleotide (Figure 5.6), thus confirming the specificity of the hybridisations. The  $\gamma$ 2S subunit and the  $\gamma$ 2L subunit mRNAs are distributed throughout the mouse neuraxis. They are developmentally

regulated with the  $\gamma 2S$  subunit mRNA being the predominant  $\gamma 2$  splice variant expression in the one-day old murine brain. The abundance of the  $\gamma 2S$  transcript decreases at postnatal day 7 but peaks again at postnatal day 14 (Figures 5.2 D, 5.3 D, 5.4 A and 5.5 A). The  $\gamma 2L$  subunit mRNA is the predominant  $\gamma 2$  transcript in the adult CNS (Figures 5.3 D and E, 5.4 D and E). The mRNA distribution patterns resulting from *in situ* hybridisation experiments using 7-day-old, 14-day-old and adult brains reveal that the  $\gamma 2S$ - and  $\gamma 2L$ -subunit mRNAs are co-expressed in the cortex, hippocampus, and cerebellum. The *in situ* hybridisation results are summarized in Tables 5.1 and 5.2.

### 5.2.2 Embryonic Expression

The  $\gamma 2S$  subunit mRNA was the only splice variant of the two  $\gamma 2$  alternatively-spliced mRNAs studied, present in the CNS during late embryogenesis (Figure 5.1, Table 5.1). In the 14.5 day embryo, the  $\gamma 2L$  transcript is absent (Figure 5.1F), while the  $\gamma 2S$  mRNA is strongly expressed in the medulla oblongata, midbrain, dorsal root ganglia and weakly expressed in the cerebellum primordium, cortical plate, and diencephalon (Figure 5.1E). By embryonic day 19.5, the  $\gamma 2S$  transcript is strongly expressed in the cortical plate, olfactory lobe, diencephalon and midbrain, and weakly expressed in the cerebellum primordium, hippocampus, spinal cord and medulla oblongata (Figure 5.1A). The  $\gamma 2L$  transcript is not detectable in the 19.5 day embryo (Figure 5.1B). Expression of the  $\gamma 2S$  transcript is CNS specific during later stages of embryogenesis.

### 5.2.3 Postnatal Expression - Cortex

The  $\gamma 2S$ -subunit mRNA is expressed in neuronal cells in layers 1 through 6 of the 1-day old mouse isocortex (Figure 5.7 A and B). In 7-day old, and 14-day old mouse brains, the  $\gamma 2S$ -subunit mRNA predominates, although both subunit variants are expressed (Figures 5.2 D, 5.3 D, 5.4 A, 5.5 A) and appear in some instances to co-localize within subpopulations of granule and pyramidal cells (data not shown). In the adult brain, the level of  $\gamma 2S$ -subunit mRNA within the isocortex has been greatly reduced in comparison with the expression of  $\gamma 2L$ -subunit mRNA (Figures 5.4 D, 5.5 D). Both  $\gamma 2$  subunit mRNAs are detected in layers II to VI, in the adult mouse brain (data not shown).

### 5.2.4 Postnatal Expression - Basal Nuclei

Again, as seen in the cortex only the  $\gamma 2S$ -subunit variant is expressed in the caudate-putamen and nucleus accumbens of a 1-day old mouse brain. Both subunit variants are expressed in the 1-day old globus pallidus and within this structure the  $\gamma 2L$ -subunit mRNA is the dominant isoform (Figures 5.2 A and B, 5.3 A and B). By day 7, the  $\gamma 2L$ -subunit mRNA is the only isoform expressed in the three basal nuclei (Figures 5.2 D and

E, 5.3 D and E) and the expression of this transcript peaks by postnatal day 14 (Figures 5.4 A and 5.5 A), whereby the level of mRNA for the  $\gamma 2L$  subunit decreases to adult values (Figures 5.4 B and 5.5 B). Expression of the  $\gamma 2S$ -subunit mRNA is present in the caudate putamen, nucleus accumbens and globus pallidus at postnatal day 14, but is absent in the basal nuclei of the adult mouse brain (Figures 5.4 D and 5.5 D).

### 5.2.5 Postnatal Expression - Septum

In the 1-day old brain,  $\gamma 2S$ -subunit mRNA is expressed in the lateral and medial septal nuclei as well as in the nucleus of the diagonal band (Figures 5.2 A, 5.3 A).  $\gamma 2L$ -subunit mRNA expression within these region is first detected in the 7-day postnatal brain (Figure 5.3 E). In the adult brain, only the the  $\gamma 2L$ -subunit mRNA signal is detectable in the lateral and medial septal nuclei and in the nucleus of the diagonal band (Figure 5.5 E). In the ventral septum of 1-day old mouse brain, a strong hybridization signal for the  $\gamma 2S$  subunit variant is detected in the bed nucleus of the stria terminalis (Figure 5.3 A)

### 5.2.6 Postnatal Expression - Colliculi

In the 1-day postnatal mouse brain,  $\gamma 2S$  is the only  $\gamma 2$  transcript expressed in the midbrain inferior and superior colliculi (Figure 5.2 A and B). By postnatal day 7, the  $\gamma 2L$  transcript is the predominant  $\gamma 2$  mRNA expressed in both the inferior and superior colliculi (Figure 5.2 D and E).  $\gamma 2S$  mRNA expression in the colliculi was weak in the 7-day old brain while the hybridization signal intensity increased slightly at postnatal day 14 (Figures 5.2 D and 5.3 D). In the adult mouse brain the  $\gamma 2L$  transcript is the only  $\gamma 2$  mRNA expressed in the inferior and superior colliculi (Figures 5.4 D and E).  $\gamma 2L$  mRNA is strongly expressed in the central nucleus and deep layers of the adult superior colliculus (Figures 5.4 E).

### 5.2.7 Postnatal Expression - Hippocampus

$\gamma 2S$  transcript expression, in the 1-day postnatal mouse brain is detected in the CA1-3 and faintly in the dentate gyrus regions of the hippocampus (Figures 5.2 A and 5.3A). At day 7 of postnatal development, the  $\gamma 2L$ -subunit mRNA signal is detected in the CA1-3 regions of the inferior horn and very faintly in the dentate gyrus, while  $\gamma 2S$  mRNA expression is strong in the dentate gyrus and pyramidal cell regions (Figures 5.2 D and E, 5.3 D and E). In the adult mouse brain the hybridisation signal for the  $\gamma 2S$  transcript is decreased from the 14-day postnatal values, however, the pattern of  $\gamma 2S$  mRNA expression remained the same as that seen in the 7-day old mouse brain. The signal for the  $\gamma 2L$  and  $\gamma 2S$  mRNAs is strongest at postnatal day 14, although only a weak level of  $\gamma 2L$  mRNA expression was detected in the dentate gyrus (Figures. 5.4 A and B, 5.5 A and B) In the

adult brain  $\gamma 2L$  is the dominant form of the  $\gamma 2$  transcripts expressed and a strong signal for the  $\gamma 2L$  mRNA is now found in both the CA1-3 and dentate gyrus regions (Figures 5.4 E and 5.5 E). The expression of the  $\gamma 2$  variants was further studied at the cellular level using liquid emulsion autoradiography. Dark field analysis of signal distribution within the molecular cell layer of the 1-day old mouse dentate gyrus detected a strong hybridization pattern for the  $\gamma 2S$  transcript.  $\gamma 2S$  mRNA is weakly expressed in the dentate granule cell layer of the postnatal day 1 brain (Figure 5.7 C and D). The  $\gamma 2L$  mRNA was not present in the dentate gyrus at this stage of CNS development (data not shown). A punctate distribution of signal for the  $\gamma 2L$  mRNA, in the hilus suggests that the expression of the  $\gamma 2L$  transcript is localized to interneurons, in the hilus of 7-day, 14-day and adult mouse brain (Figure 5.8 F and H and data not shown).  $\gamma 2L$  mRNA is detected as a weak signal in the granule and molecular cell layers of the 7-day and 14-day dentate gyrus (Figures 5.2 E, 5.3 E, 5.4 B and 5.5 B). In the adult brain the hybridisation signal for the  $\gamma 2L$  transcript is strong in the granule cell layer of the dentate gyrus and appears to overlap the expression of the  $\gamma 2S$  transcript in subsets of dentate cells (Figure 5.8 B, D, F and H). Overlapping patterns of expression for the  $\gamma 2S$  and  $\gamma 2L$  transcripts are present in the pyramidal cells of the adult hippocampus (Figure 5.8 A, C, E, and G). In postnatal day 1 brain only the  $\gamma 2S$  subunit is expressed in the pyramidal cells of CA1-3 (Figure 5.7 C and D).

### 5.2.8 Postnatal Expression - Thalamus

The  $\gamma 2S$  mRNA is widely distributed among the thalamic nuclei of the 1 day postnatal brain (Figures 5.2 A and 5.3 A). The rhomboid, anteroventral, mediodorsal, medial geniculate and paraventricular thalamic nuclei of the postnatal 1-day brain express the only one splice variant, the  $\gamma 2S$  transcript, of the  $\gamma$  subunit of the GABA<sub>A</sub> receptor. Overlapping patterns of expression for the two alternatively spliced mRNAs are found in the ventral posterolateral and ventral posteromedial thalamic nuclei and in the deep mesencephalic nucleus of the 1-day old brain (Figures 5.2 A, B and 5.3 A, B). Within these nuclei the  $\gamma 2S$  is the predominant splice variant.

At postnatal day 7 a hybridisation signal for the  $\gamma 2L$  mRNA is found in the ventrolateral and rostral interstitial thalamic nuclei and in the red nucleus of the midbrain (Figure 5.2 E and 5.3 E). Both the  $\gamma 2L$  and  $\gamma 2S$  transcripts are expressed in the anteroventral and paraventricular thalamic nuclei. The  $\gamma 2L$  transcript is the predominant splice variant in the anteroventral 7-day thalamic nucleus whereas the  $\gamma 2s$  mRNA is the most abundant  $\gamma 2$  transcript in the paraventricular 7-day thalamic nucleus.

By day 14, the hybridisation signal for the  $\gamma 2S$  mRNA has increased relative to the levels expressed in the 7-day old brain. The  $\gamma 2S$  transcript is the only  $\gamma$  splice variant found in the paraventricular, anteroventral and medial thalamic nuclei. The  $\gamma 2L$  mRNA

expression is unique to the ventral lateral geniculate and rostral interstitial thalamic nuclei and the midbrain red nucleus. In the 14-day brain, overlapping patterns of expression for the two splice variants in the 14-day postnatal brain are detected in the posterior thalamic nucleus, where the  $\gamma 2S$  transcript is the predominant mRNA expressed.

### 5.2.9 Postnatal Expression - Cerebellum

Hybridisation signals indicate that the  $\gamma 2S$  mRNA is the major splice variant expressed in the external cerebellar granule cell layer at P1, while  $\gamma 2L$  mRNA levels at this stage of development are very low (Figure 5.7 E and F and data not shown). The  $\gamma 2S$  splice variant is never found in Purkinje cells at any developmental stage, nor in the adult brain. In the cerebellar granule cell and molecular layers, the  $\gamma 2S$  transcript decreases from P1 to P7, whereby the level of  $\gamma 2S$  mRNA expression remains constant through P14 before expression decreases yet further in the granule cell and molecular layers of the adult cerebellum. In the adult cerebellum the transcript for  $\gamma 2S$  is barely detectable (Figures 5.4 D, 5.5 D, and 5.9 B). The  $\gamma 2L$  transcript is present in the cerebellar Purkinje cell layer, where its expression increases during postnatal development. The hybridisation signal for the  $\gamma 2L$  transcript is strongest in Purkinje cells compared to other cell types in the adult cerebellum (Figure 5.9 A).

## 5.3 Discussion

The results from *in situ* hybridisation studies using oligonucleotide probes specific for the two alternatively-spliced forms of the  $\gamma 2$  GABA<sub>A</sub> receptor subunit are summarized in Tables 5.1 and 5.2 and demonstrate that the expression of the splice variants is developmentally regulated. The  $\gamma 2S$  mRNA is widely distributed along the mouse neuraxis and is the predominant transcript during late embryogenesis and in the 1-day postnatal mouse brain. In contrast, in the adult brain,  $\gamma 2L$  is the major  $\gamma 2$  mRNA splice variant, with widespread distribution in the cortex, hippocampus, basal nuclei, cerebellum, thalamus and midbrain.

The predominance of the  $\gamma 2S$  mRNA splice variant during early postnatal development is in agreement with results from RNase protection analysis of C57Bl/6 mouse whole brain total RNA isolated at various postnatal stages ranging from the day of birth to six weeks of age (Wang and Burt, 1991). This observation is also supported by quantitative PCR analysis of  $\gamma 2$  splice variant expression in cerebellar and cortical postnatal P0 to P21 total RNA preparations (Bovolin *et al.*, 1992).

The studies by Wang and Burt, (1991) and Bovolin *et al.*, (1992) also support observations described in this chapter that the  $\gamma 2L$  mRNA splice variant is the predominant form of  $\gamma 2$  mRNA in the adult CNS. This contrasts with the chick (Glencorse, Bateson

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and Darlison, 1991) and the rat (Miralles *et al.*, 1994), where such a clear distinction between the two  $\gamma 2$  mRNA isoforms has not been observed. This difference may reflect species variations, although the design of the oligonucleotides and hybridisation conditions used in the rat *in situ* analysis (Miralles *et al.*, 1994) is such that the  $\gamma 2S$  probe is very likely to have hybridised to the  $\gamma 2L$  mRNA obscuring any drop in  $\gamma 2S$  mRNA level.

Expression of the GABA<sub>A</sub> receptor  $\gamma 2$  subunit gene is subject to clear transcriptional regulation (considering  $\gamma 2S$  and  $\gamma 2L$  mRNAs together) between various developmental timepoints and between the cell types of different brain regions. Although the  $\gamma 2$  gene is expressed virtually everywhere in the brain at some level throughout development, a variety of regulation patterns can be discerned. Cells of hippocampal CA1 and CA2 regions, and of cerebral cortex layers II-V, show consistently high  $\gamma 2$  gene expression from P1 through to the adult, while cells of the central nuclei of the superior and inferior colliculi also show consistent postnatal  $\gamma 2$  gene expression although at a somewhat lower level than is seen in the hippocampus and cortex. Initial high postnatal expression at P1, followed by a progressive decrease to low adult levels is a pattern of GABA<sub>A</sub> receptor  $\gamma 2$  subunit gene expression found in cells of the caudate-putamen and nucleus accumbens of the basal ganglia, the lateral and medial septum and most thalamic nuclei. The reverse expression pattern, with low levels of  $\gamma 2$  gene transcription at P1 increasing to high levels thereafter, is found in cells of the dentate gyrus, the entorhinal cortex and in Purkinje cells of the cerebellum. More moderate increases in  $\gamma 2$  expression during postnatal development are found in cells of the medial habenula. Some regions such as the colliculi and the granule cell layer of the cerebellum also show observably higher  $\gamma 2$  expression at P14 than at any other developmental time point. All these observations point to a precise developmental programme of regulation of GABA<sub>A</sub> receptor  $\gamma 2$  subunit gene expression, with each cell type showing a characteristic temporal pattern of expression level.

Alternative splicing of the  $\gamma 2$  mRNA precursor transcript is also clearly subject to temporal regulation during development. The alternative splicing mechanism is switched on only after birth. The earliest appearance of the  $\gamma 2L$  splice variant mRNA is in cells of the globus pallidus at P1, but by P7 significant levels of  $\gamma 2L$  mRNA are seen in virtually all brain regions (Table 5.2). There does not seem to be a general correlation between the onset of alternative splicing and stages of cellular brain development such as proliferation, migration or innervation, since the various regions of the mouse brain are at quite different stages in their development at birth, yet most cells turn on alternative splicing of the  $\gamma 2$  mRNA precursor in a similar time frame. Myelination appears to be the one developmental CNS process that does correlate with the onset of  $\gamma 2$  alternative splicing, however it is just as likely that alternative splicing of the  $\gamma 2$  precursor is activated by

signaling events triggered by the environmental and physiological alterations associated with birth.

In most cell types of the developing mouse brain, alternative splicing leading to insertion of the exon encoding an extra PKC phosphorylation site is superimposed upon pre-existing  $\gamma 2$  gene expression with the  $\gamma 2S$  mRNA as the only splice variant. As alternative splicing is activated, the  $\gamma 2L$  mRNA splice variant appears progressively and eventually predominates. The increase in  $\gamma 2L$  levels relative to  $\gamma 2S$  as postnatal development progresses arises from a combination of the gradual activation of alternative splicing in increasing numbers of cells with time, and the increasing level of alternative splicing within each cell. This *in situ* hybridization analysis used adjacent sections for  $\gamma 2L$  and  $\gamma 2S$  assessment, but it is not possible to prove that a particular cell gives both  $\gamma 2S$  and  $\gamma 2L$  signals. It is therefore not yet clear whether the activation of alternative splicing leads to a rapid all or nothing switch from  $\gamma 2S$  to  $\gamma 2L$  mRNA in each cell. In areas such as the subiculum, cortex and hippocampus, where high levels of  $\gamma 2L$  mRNA and lower but significant levels of  $\gamma 2S$  mRNA are found together in the adult brain, in regions with well-defined cell types, it is possible that alternative splicing only acts on a portion of the mRNA precursor population of each cell. This is an important point to establish, since such a situation would make the alternative splicing process itself a site for regulation of the relative phosphorylation state of GABA<sub>A</sub> receptors in adult hippocampal and cortical cells.

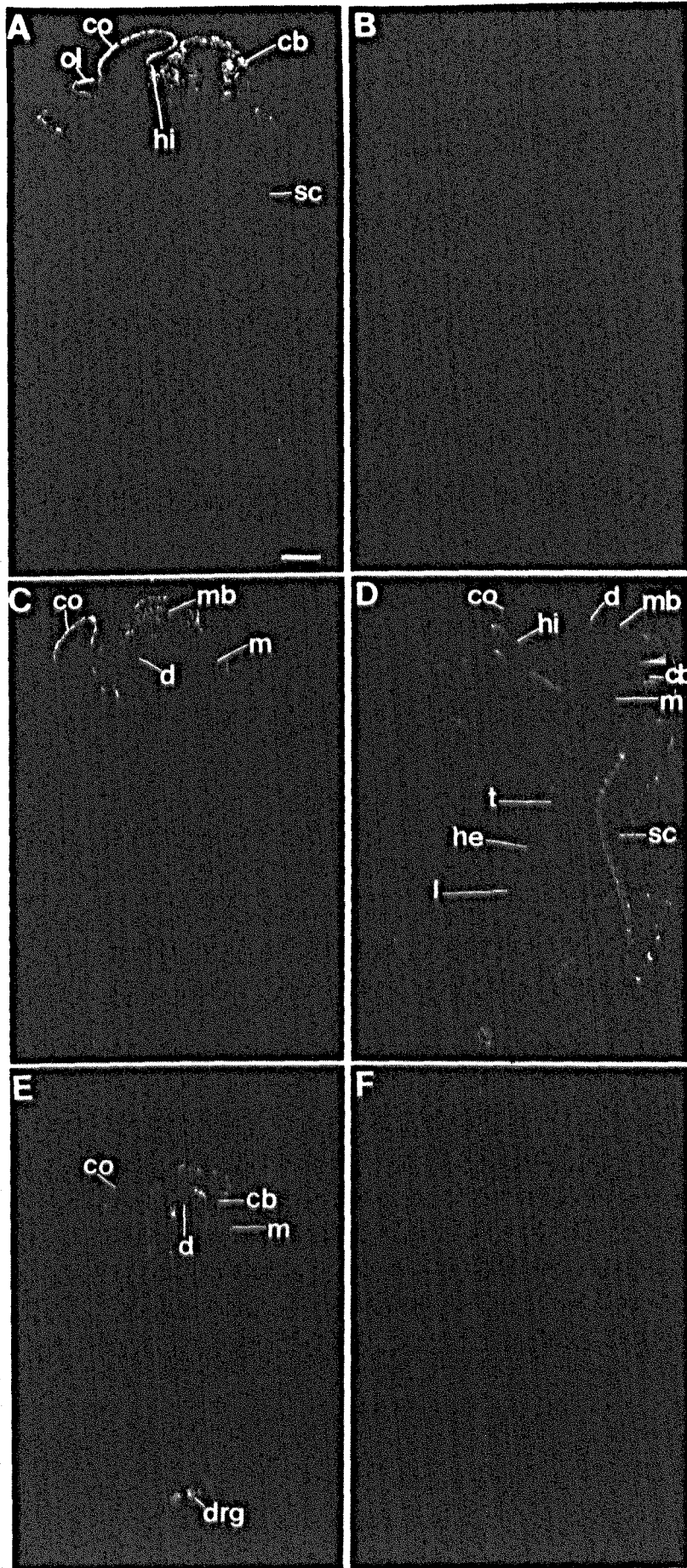
Purkinje cells of the cerebellum show a unique developmental expression pattern, in which the onset of  $\gamma 2$  gene transcription and alternative splicing are correlated, so that only  $\gamma 2L$  mRNA is ever formed. It is possible that the alternative splicing mechanism is constitutive, or is at least switched on earlier in development, in Purkinje cells, but this would be an exception to the general rule. An intriguing alternative possibility is that the switch that activates alternative splicing also, directly or indirectly, activates  $\gamma 2$  gene transcription in Purkinje cells.

Recently a null mutation in the  $\gamma 2$  gene demonstrated that loss of the  $\gamma 2$  subunit results in postnatal lethality, retarded growth, and sensorimotor dysfunction (Günther et al., 1995). These findings further the studies demonstrating that GABA<sub>A</sub> receptors are necessary for normal CNS development. Our results demonstrate a temporal and cellular regulation of  $\gamma 2$  splice variants during late embryogenesis and postnatal CNS development. Although a number of *in vitro* studies have demonstrated the regulation of GABA<sub>A</sub> receptors by protein phosphorylation (Kirkness et al., 1989; Browning et al., 1990; Moss et al., 1992; Lin et al., 1994; Krishek et al., 1994), it is yet to be elucidated what role phosphorylation of the receptor  $\gamma 2$  subunit plays *in vivo*.

**Figure 5.1**

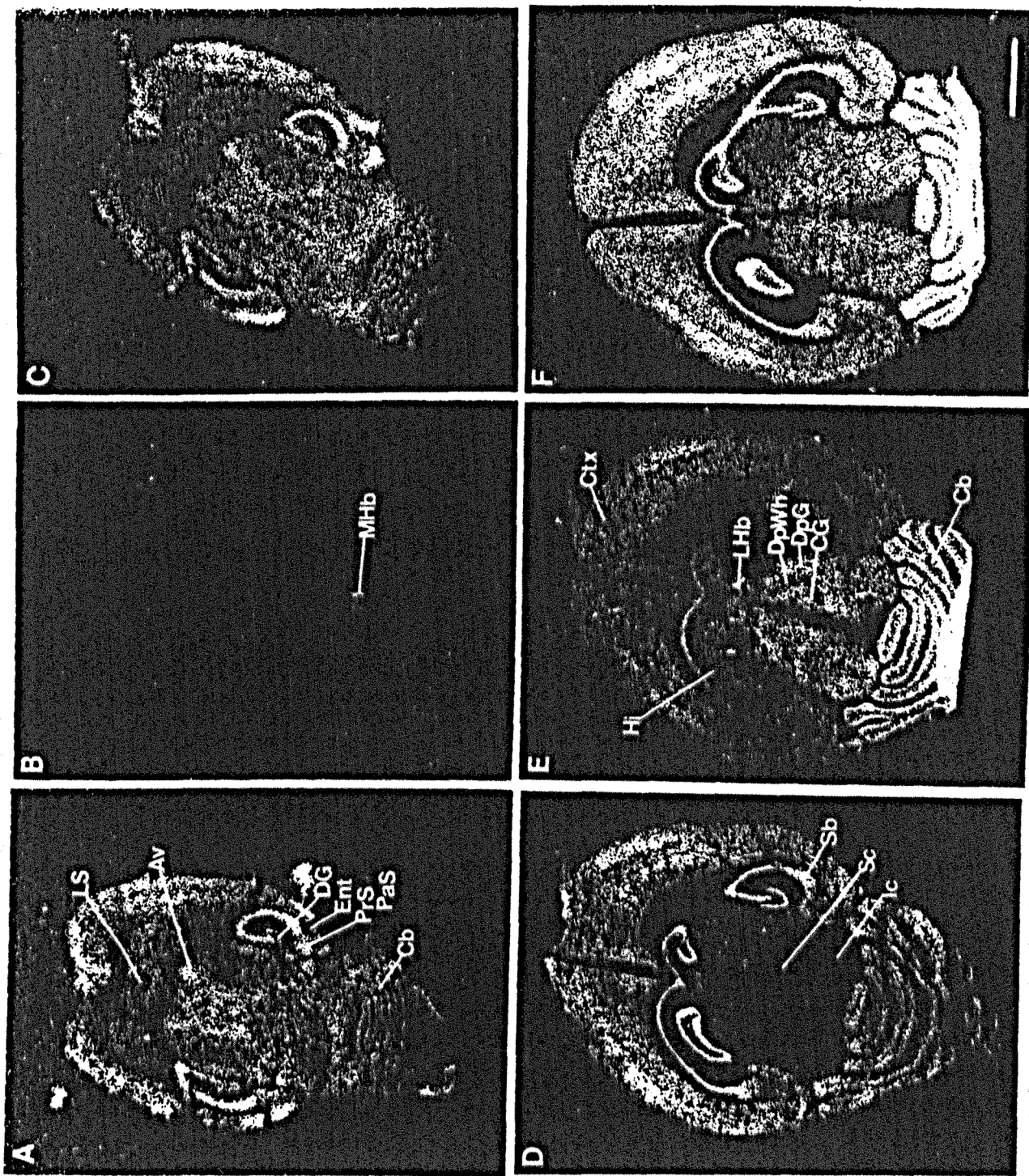


**Figure 5.1**      *Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 2S- and  $\gamma$ 2L-subunit transcripts in 14.5, 17.5 and 19.5 day old C57Bl/6 embryos. A: embryonic 19.5 day saggital section hybridised with an oligonucleotide specific for the  $\gamma$ 2 short ( $\gamma$ 2S)-subunit mRNA of the GABA<sub>A</sub> receptor; B: embryonic 19.5 day saggital section probed with an oligonucleotide specific for the  $\gamma$ 2 long ( $\gamma$ 2L)-subunit mRNA of the GABA<sub>A</sub> receptor; C: embryonic 17.5 day sagital section hybridised with an oligonucleotide specific for the  $\gamma$ 2S-subunit mRNA of the GABA<sub>A</sub> receptor; D: sagital section of a 17.5 day embryo stained with thionin; E: embryonic 14.5 day saggital section hybridised with an oligonucleotide specific for the  $\gamma$ 2 short ( $\gamma$ 2S)-subunit mRNA of the GABA<sub>A</sub> receptor and F: embryonic 14.5 day sagital section probed with an oligonucleotide specific for the  $\gamma$ 2 long ( $\gamma$ 2L)-subunit mRNA of the GABA<sub>A</sub> receptor. cb, cerebellar primordium; co, cortical plate; d, diencephalon; drg, dorsal root ganglia; he, heart; hi, embryonic hippocampus; l, liver; m, medulla oblongata; mb, midbrain; ol, olfactory lobe; sc, embryonic spinal cord; t, thymus. Scale bar represents 1.5 mm. Exposure time was 15 days on Kodak XAR-5 film.*



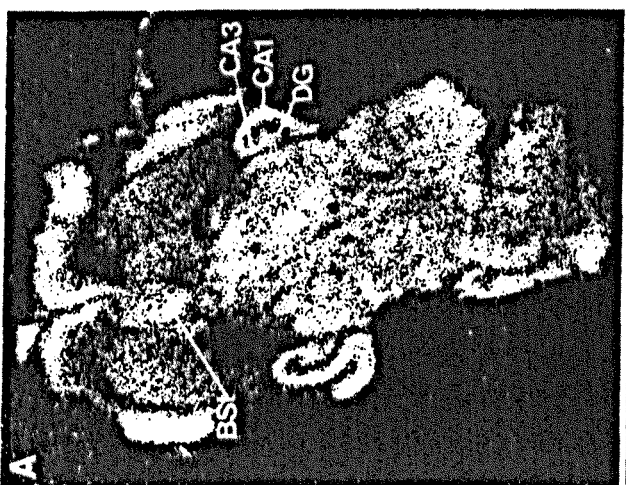
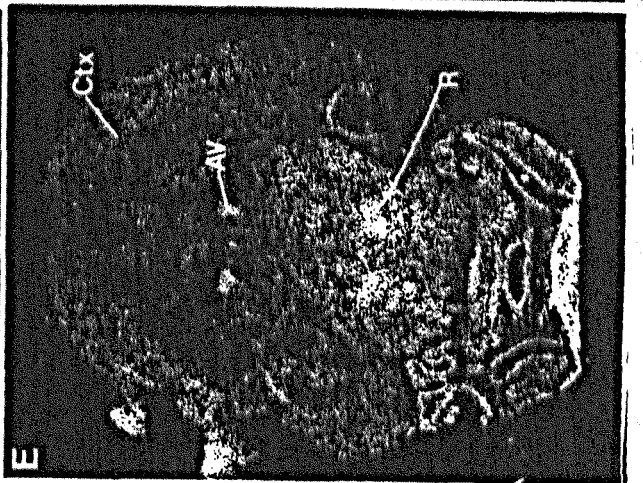
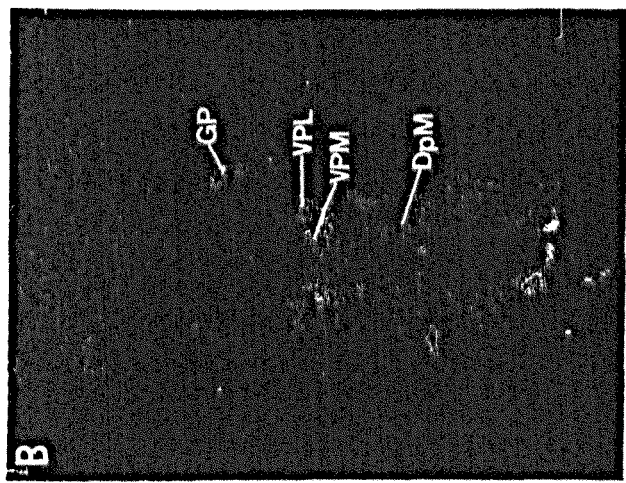
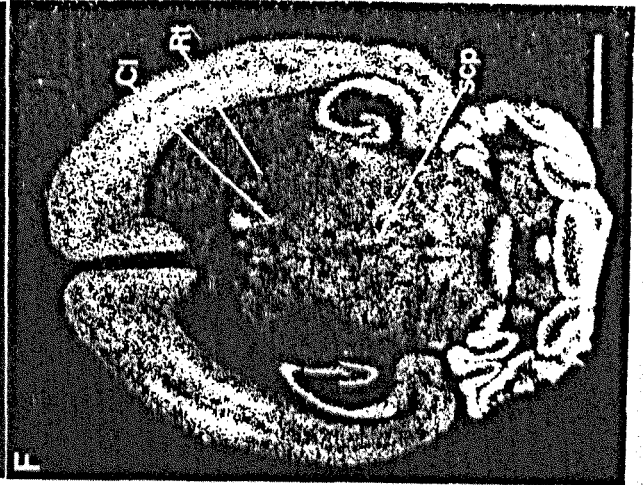
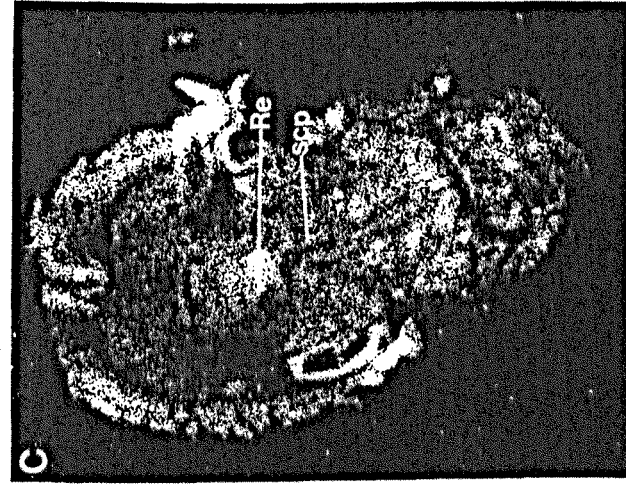
**Figure 5.2**

**Figure 5.2 Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 2S-,  $\gamma$ 2L- and  $\gamma$ 2T-subunit transcripts in the brains of C57BL/6J mice during postnatal development.** A: 1-day old mouse horizontal brain section hybridised with an oligonucleotide specific for the  $\gamma$ 2 short ( $\gamma$ 2S)-subunit mRNA of the GABA<sub>A</sub> receptor; B: 1-day old mouse brain section probed with an oligonucleotide specific for the  $\gamma$ 2 long ( $\gamma$ 2L)-subunit mRNA of the GABA<sub>A</sub> receptor; C: 1 day-old mouse brain section hybridised with an oligonucleotide designed to recognize both the  $\gamma$ 2L- and  $\gamma$ 2S-alternatively-spliced forms of the  $\gamma$ 2 GABA<sub>A</sub> receptor subunit mRNA; D: 7 day-old mouse brain section hybridised with an oligonucleotide specific for the  $\gamma$ 2S-subunit mRNA of the GABA<sub>A</sub> receptor; E: 7 day-old mouse horizontal brain section probed with an oligonucleotide specific for the  $\gamma$ 2L-subunit mRNA of the GABA<sub>A</sub> receptor and F: 7 day-old mouse brain section hybridised with an oligonucleotide designed to recognize both the  $\gamma$ 2L- and  $\gamma$ 2S-alternatively-spliced forms of the  $\gamma$ 2 GABA<sub>A</sub> receptor subunit mRNA. Av, anteroventral thalamic nucleus; Cb, cerebellum; CG, central grey; Ctx, cortex; DG, dentate gyrus; DpG, deep grey layer; DpWh, deep white layer; Ent, entorhinal cortex; Hi, hippocampus; Ic, inferior colliculus; LHb, lateral habenular nucleus; LS, lateral septum; MHb, medial habenal nucleus; PaS, parasubiculum ; PrS, presubiculum ; Sb, subiculum; Sc, superior colliculus. Scale bar represents 2.2 mm. Exposure time was 15 days on Kodak XAR-5 film.



**Figure 5.3**

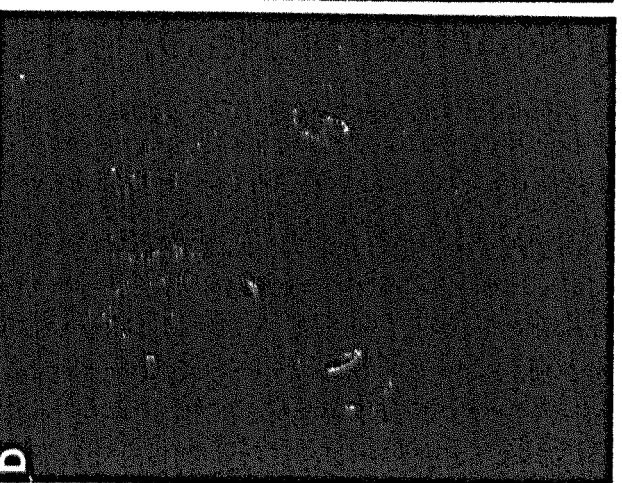
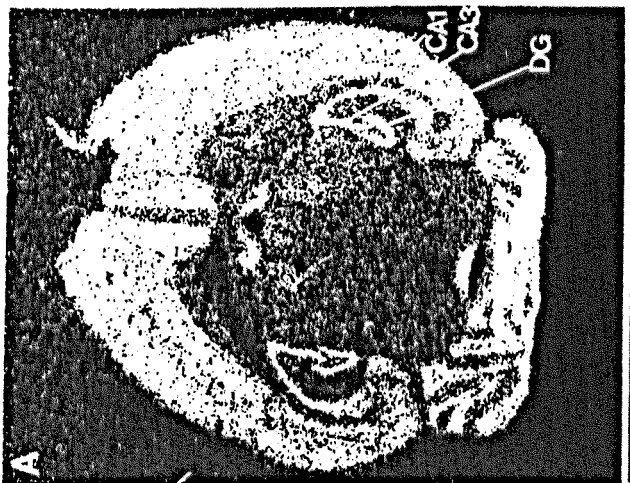
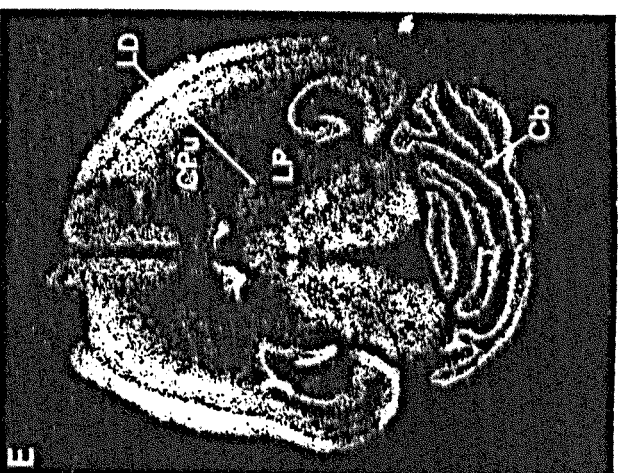
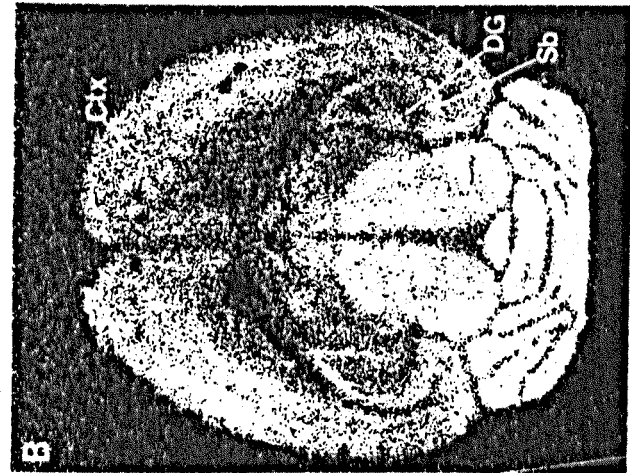
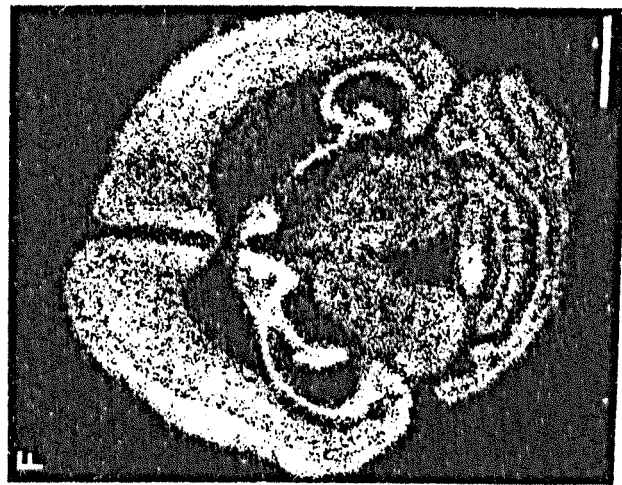
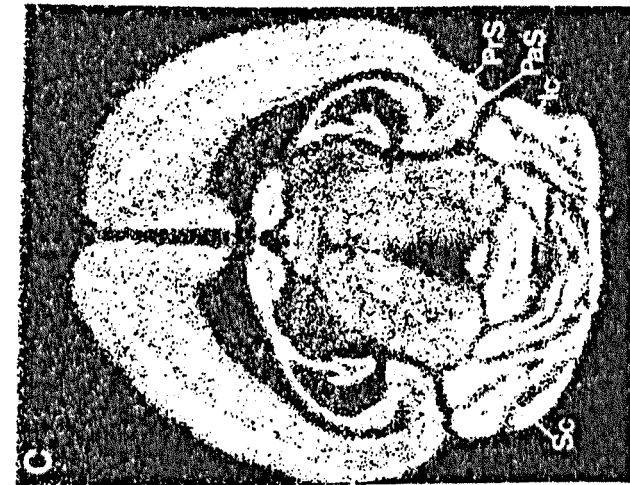
**Figure 5.3 Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 2S-,  $\gamma$ 2L- and  $\gamma$ 2T-subunit transcripts in the brains of C57BL/6J mice during postnatal development.** A: 1-day old mouse horizontal brain section hybridised with an oligonucleotide specific for the  $\gamma$ 2S-subunit mRNA of the GABA<sub>A</sub> receptor; B: 1-day old mouse brain section probed with an oligonucleotide specific for the  $\gamma$ 2L-subunit mRNA of the GABA<sub>A</sub> receptor; C: 1 day-old mouse brain section hybridised with an oligonucleotide designed to recognize both the  $\gamma$ 2L- and  $\gamma$ 2S-alternatively-spliced forms of the  $\gamma$ 2 GABA<sub>A</sub> receptor subunit mRNA; D: 7 day-old mouse horizontal brain section hybridised with an oligonucleotide specific for the  $\gamma$ 2S-subunit mRNA of the GABA<sub>A</sub> receptor; E: 7 day-old mouse brain section probed with an oligonucleotide specific for the  $\gamma$ 2L-subunit mRNA of the GABA<sub>A</sub> receptor and F: 7 day-old mouse brain section hybridised with an oligonucleotide designed to recognize both the  $\gamma$ 2L- and  $\gamma$ 2S-alternatively-spliced forms of the  $\gamma$ 2 GABA<sub>A</sub> receptor subunit mRNA. AM, anteromedial thalamic nucleus ; AV, anteroventral thalamic nucleus; BSt; bed nucleus stria terminalis; CA1-3, fields CA1-3 of Ammon's horn; Cl, centrolateral thalamic nucleus; Ctx, cortex; DG, dentate gyrus; DpM, deep mesencephalic nucleus; GP, globus pallidus; IA, interanteromedial thalamic nucleus; PVA, parventricular thalamic nucleus, anterior; R, red nucleus; Re, reuniens thalamic nucleus; Rt, reticular thalamic nucleus; scp, superior cerebellar peduncle; VPL, ventral posterolateral thalamic nucleus; VPM, ventral posteromedial thalamic nucleus. Scale bar represents 2.2 mm. Exposure time was 15 days on Kodak XAR-5 film.





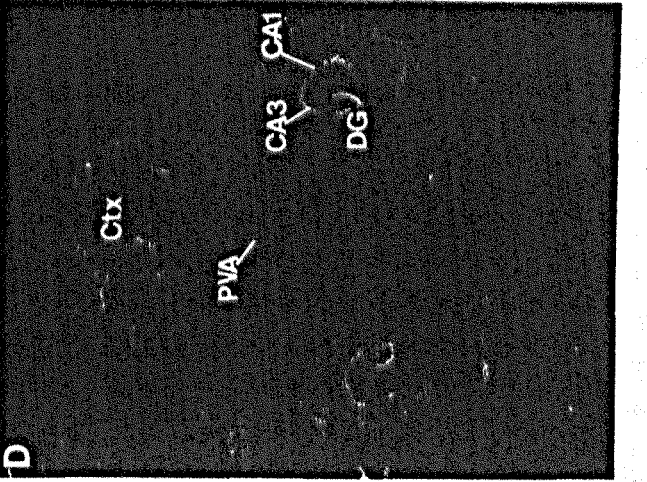
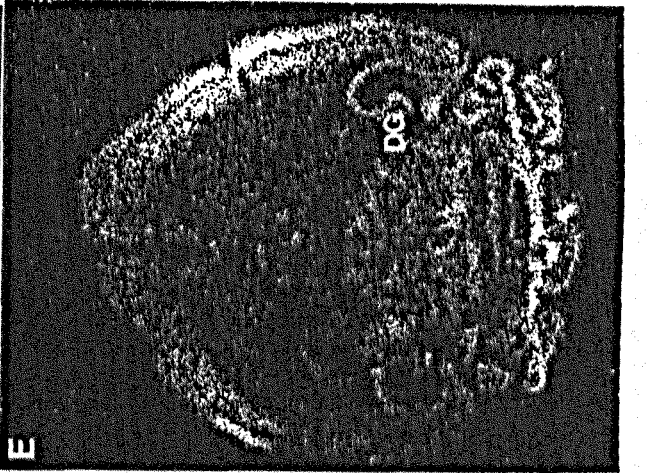
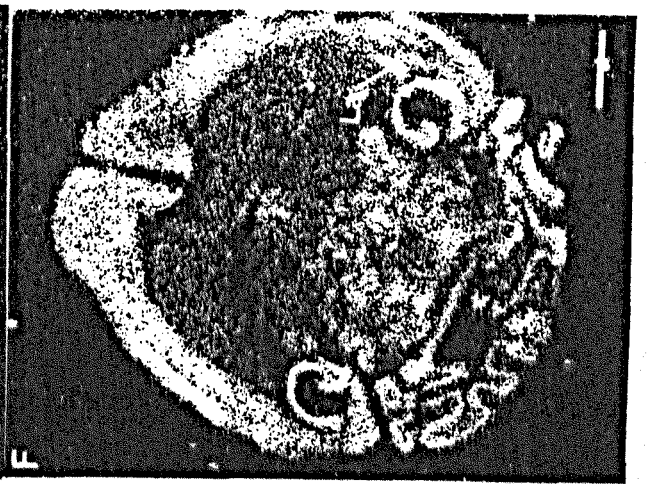
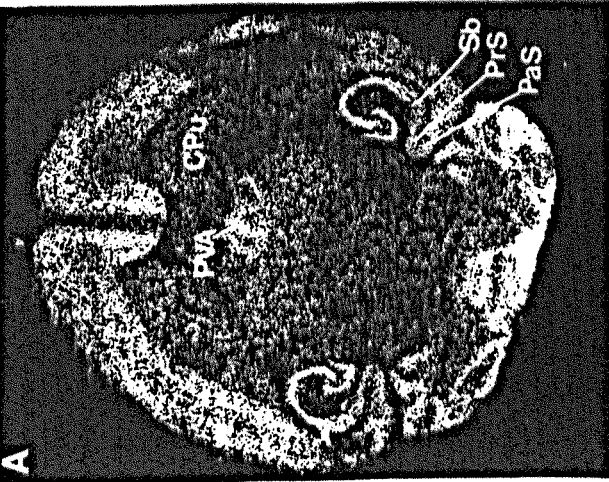
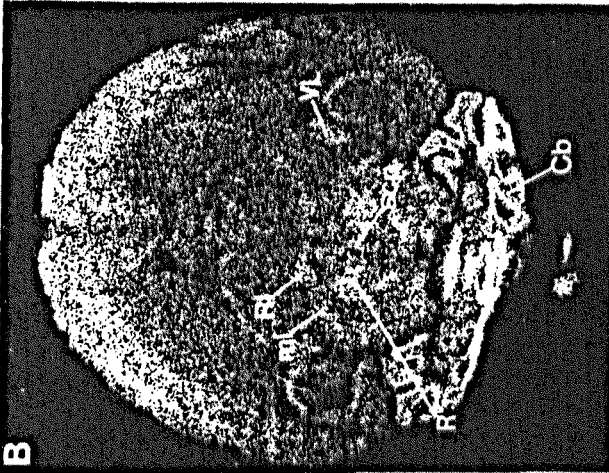
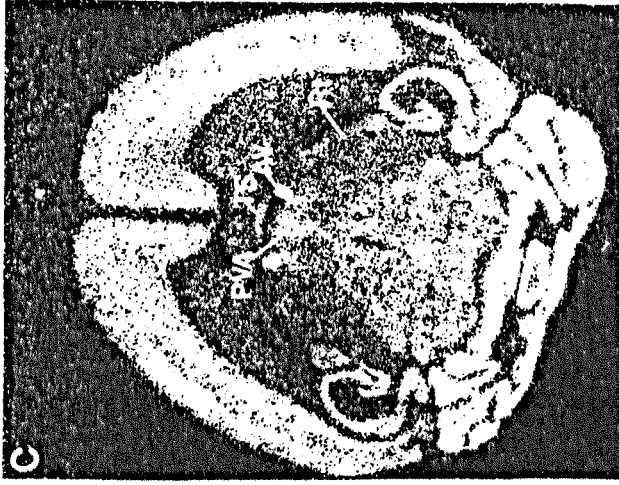
**Figure 5.4**

**Figure 5.4 Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 2S-,  $\gamma$ 2L- and  $\gamma$ 2T-subunit transcripts in the brains of C57BL/6J mice during postnatal development.** **A:** 14-day old mouse horizontal brain section hybridised with an oligonucleotide specific for the  $\gamma$ 2S-subunit mRNA of the GABA<sub>A</sub> receptor; **B:** 14-day old mouse brain section probed with an oligonucleotide specific for the  $\gamma$ 2L-subunit mRNA of the GABA<sub>A</sub> receptor; **C:** 14 day-old mouse brain section hybridised with an oligonucleotide designed to recognize both the  $\gamma$ 2L- and  $\gamma$ 2S-alternatively-spliced forms of the  $\gamma$ 2 GABA<sub>A</sub> receptor subunit mRNA; **D:** Adult mouse brain section hybridised with an oligonucleotide specific for the  $\gamma$ 2S-subunit mRNA of the GABA<sub>A</sub> receptor; **E:** Adult mouse horizontal brain section probed with an oligonucleotide specific for the  $\gamma$ 2L-subunit mRNA of the GABA<sub>A</sub> receptor and **F:** adult mouse brain section hybridised with an oligonucleotide designed to recognize both the  $\gamma$ 2L- and  $\gamma$ 2S-alternatively-spliced forms of the  $\gamma$ 2 GABA<sub>A</sub> receptor subunit mRNA. CA1-3, fields CA1-3 of Ammon's horn; Cb, cerebellum; CPu, caudate putamen; Ctx, cortex; DG, dentate gyrus; Ic, inferior colliculus; LD, laterodorsal thalamic nucleus; LP, lateral posterior thalamic nucleus; PaS, parasubiculum; PrS, presubiculum; Sb, subiculum; Sc, superior colliculus. Scale bar represents 2.0 mm. Exposure time was 15 days on Kodak XAR-5 film.



**Figure 5.5**

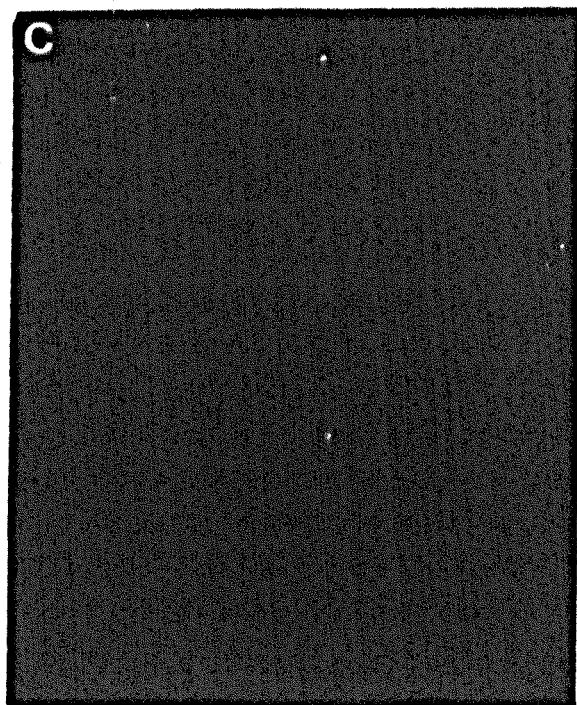
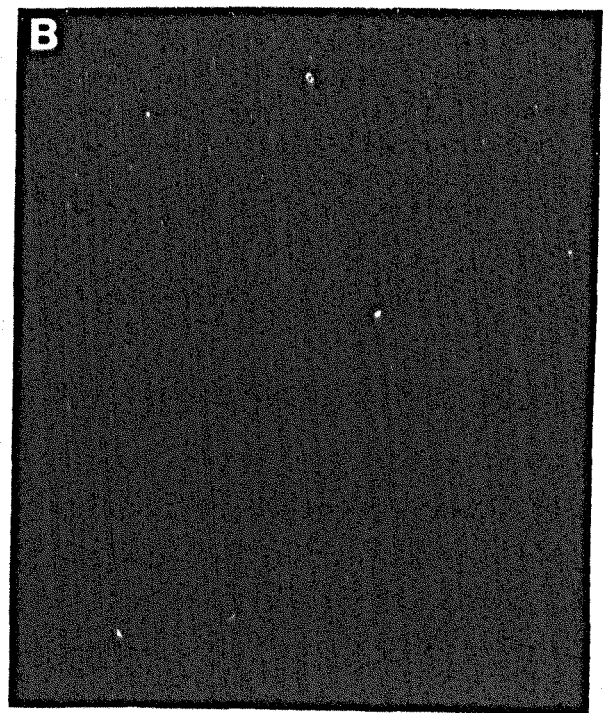
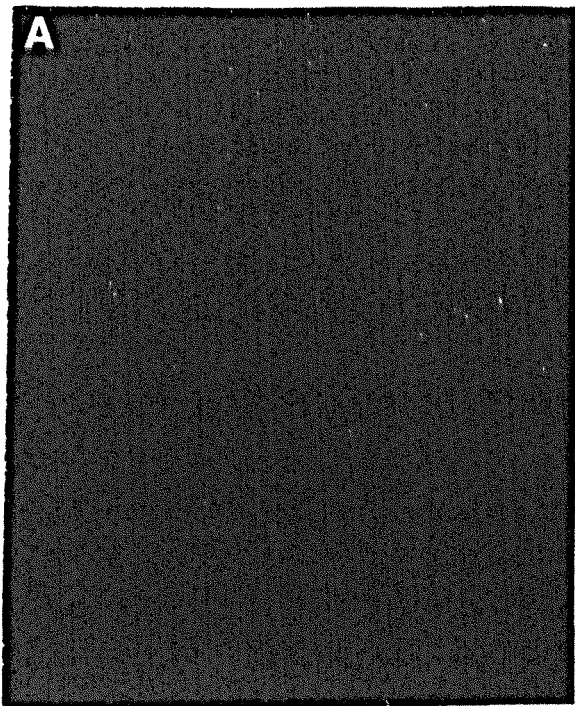
**Figure 5.5 Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 2S-,  $\gamma$ 2L- and  $\gamma$ 2T-subunit transcripts in the brains of C57BL/6J mice during postnatal development.** A: 14-day old mouse horizontal brain section hybridised with an oligonucleotide specific for the  $\gamma$ 2S-subunit mRNA of the GABA<sub>A</sub> receptor; B: 14-day old mouse brain section probed with an oligonucleotide specific for the  $\gamma$ 2L-subunit mRNA of the GABA<sub>A</sub> receptor; C: 14 day-old mouse brain section hybridised with an oligonucleotide designed to recognize both the  $\gamma$ 2L- and  $\gamma$ 2S-alternatively-spliced forms of the  $\gamma$ 2 GABA<sub>A</sub> receptor subunit mRNA; D: Adult mouse horizontal brain section hybridised with an oligonucleotide specific for the  $\gamma$ 2S-subunit mRNA of the GABA<sub>A</sub> receptor; E: Adult mouse brain section probed with an oligonucleotide specific for the  $\gamma$ 2L-subunit mRNA of the GABA<sub>A</sub> receptor and F: adult mouse brain section hybridised with an oligonucleotide designed to recognize both the  $\gamma$ 2L- and  $\gamma$ 2S-alternatively-spliced forms of the  $\gamma$ 2 GABA<sub>A</sub> receptor subunit mRNA. AV, anteroventral thalamic nucleus; CA1-3, fields CA1-3 of Ammon's horn; Cb, cerebellum; CPu, caudate putamen; Ctx, cortex; DG, dentate gyrus; La, lateral amygdaloid nucleus; ml, medial lemniscus; PaS, parasubiculum; PrS, presubiculum; PVA, paraventricular thalamic nucleus, anterior; R, red nucleus; RI, rostral interstitial nucleus mlf, medial longitudinal fasciculus; Rt, reticular thalamic nucleus; Sb, subiculum; scp, superior cerebellar peduncle; TS, triangular septal nucleus; VL, ventrolateral thalamic nucleus. Scale bar represents 1.7 mm. Exposure time was 15 days on Kodak XAR-5 film.



**Figure 5.6**

**Figure 5.6** *Control hybridisations.* Parallel control sections were hybridised in the presence of a 30-fold molar excess of the appropriate unlabelled oligonucleotide in all experiments. These resulted in the generation of blank autoradiographs. Control sections were hybridised with the  $\gamma 2(S)$ -subunit specific (A), the  $\gamma 2(L)$ -subunit specific (B) and the  $\gamma 2T$ -subunit specific (C) oligonucleotide probes. Exposure time was for 15 days on Kodak XAR-5 film.

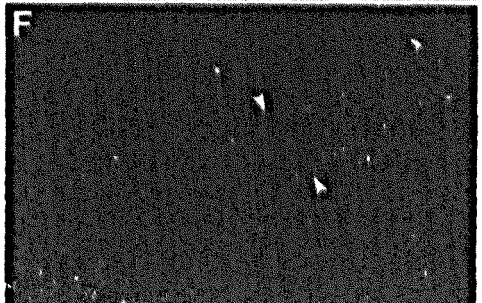
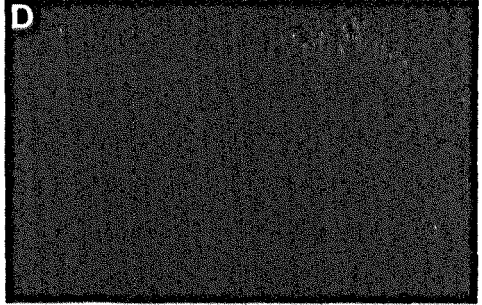
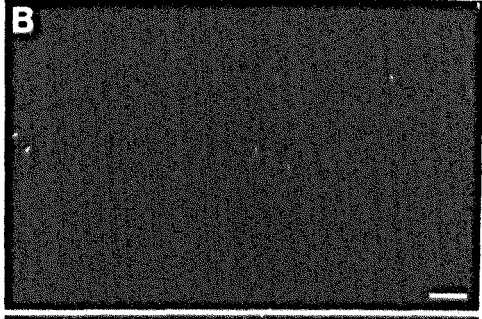
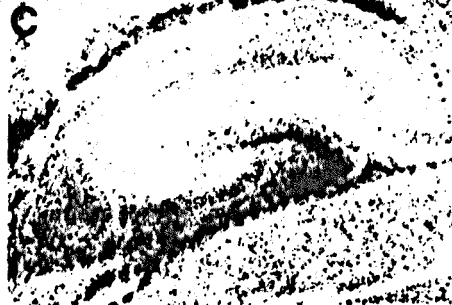
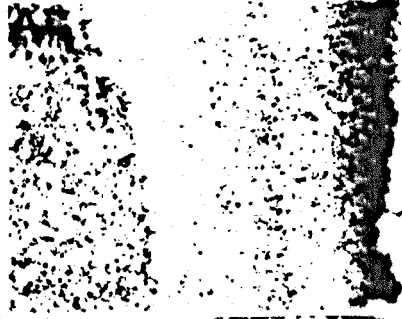




**Figure 5.7**

***Figure 5.7 Cellular localisation of the GABA<sub>A</sub> receptor  $\gamma$ 2S- subunit transcript in the cortex, hippocampus and cerebellum of one day old C57BL/6J mice.***

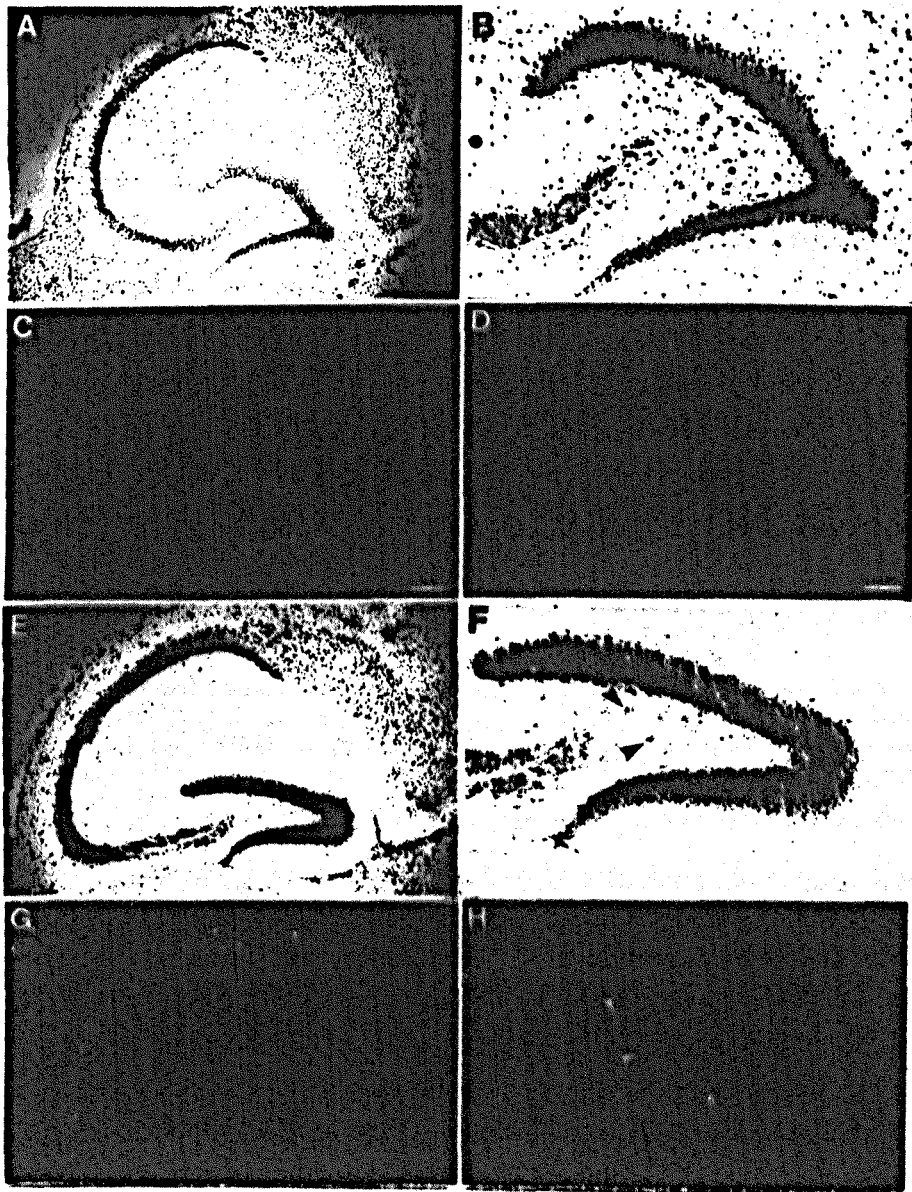
Bright-field (A) and dark-field (B) views of the  $\gamma$ 2S GABA<sub>A</sub> receptor subunit mRNA in the C57BL/6J cortex at P0. Bright-field (C) and dark-field (D) views of the  $\gamma$ 2S GABA<sub>A</sub> receptor subunit mRNA in the C57BL/6J hippocampus at P0. Bright-field (E) and dark-field (F) views of the  $\gamma$ 2S GABA<sub>A</sub> receptor subunit mRNA in the C57BL/6J cerebellum at P0. Arrow heads illustrate the labeling of cerebellar granule cells by the  $\gamma$ 2S probe. Scale bar represents 21  $\mu$ m.



**Figure 5.8**

***Figure 5.8 Cellular localisation of the GABA<sub>A</sub> receptor  $\gamma$ 2S- and  $\gamma$ 2L-subunit transcripts in the hippocampus of adult C57BL/6J mice.***

$\gamma$ 2S probe (A, B, C, D) and  $\gamma$ 2L probe (E,F,G,H). Bright-field (A, B) and dark-field (C,D) views of the  $\gamma$ 2S GABA<sub>A</sub> receptor subunit mRNA in the adult C57BL/6J. Bright-field (E, F) and dark-field (G, H) views of the  $\gamma$ 2L GABA<sub>A</sub> receptor subunit mRNA in the adult C57BL/6J hippocampus. Arrowheads illustrate labeling of hilar cells by the  $\gamma$ 2L GABA<sub>A</sub> receptor subunit probe. (C) Scale bar represents 27  $\mu$ m, (D) Scale bar presents 15  $\mu$ m.

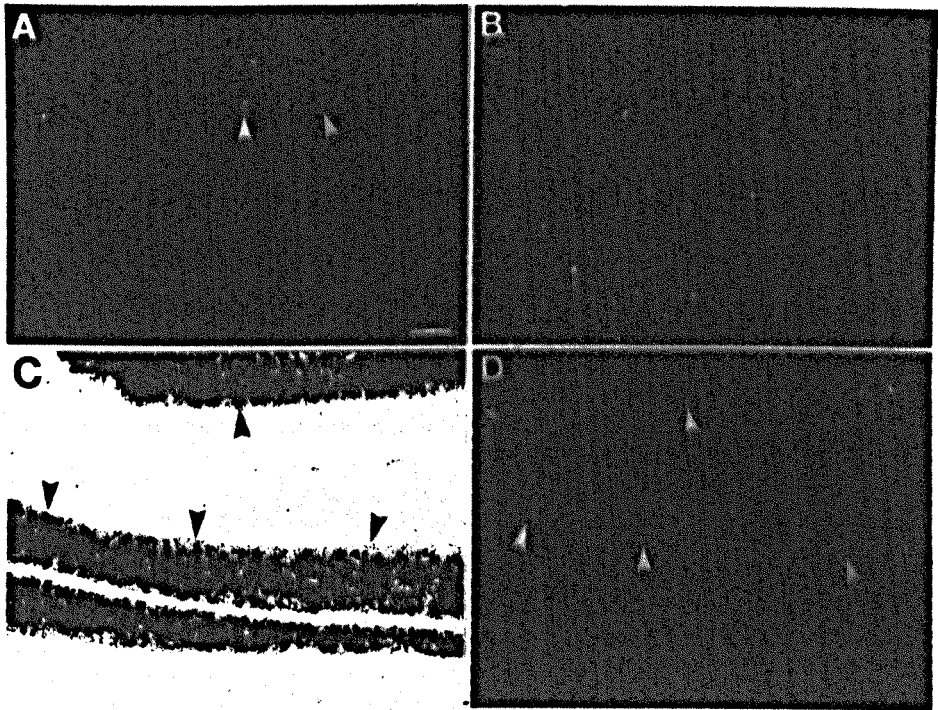


**Figure 5.9**



***Figure 5.9 Cellular localisation of the GABA<sub>A</sub> receptor  $\gamma$ 2S-,  $\gamma$ 2L- and  $\gamma$ 2T-subunit transcripts in the cerebellar cortex of adult C57BL/6J mice.***

$\gamma$ S probe (B),  $\gamma$ 2L probe (A) and  $\gamma$ 2T probe (C, D). Bright-field (C) and dark-field (A,B,D) views of the  $\gamma$ 2 GABA<sub>A</sub> receptor subunit mRNA in the adult C57BL/6J cerebellum. Arrowheads indicate the labeling of Purkinje cells by the  $\gamma$ 2L and  $\gamma$ 2T probes. Scale bar represents 6  $\mu$ m.



**Table 5.1**

**Table 5.1**      **Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 2S- and  $\gamma$ 2L-subunit transcripts in 14.5, 17.5 and 19.5 day old C57Bl/6 embryos.** Hybridisation signals, obtained with oligonucleotides specific for the  $\gamma$ 2S-subunit mRNA and the  $\gamma$ 2L-subunit mRNA were arbitrarily scored as either high, +++; moderate, ++; low, +; weak, (+); or not detectable, -. The results are derived from Figure 5.1 and data not shown. The day a vaginal plug is visible is recorded as day 0.5.

Region	E14.5		E17.5		E19.5	
	$\gamma$ 2S	$\gamma$ 2L	$\gamma$ 2S	$\gamma$ 2L	$\gamma$ 2S	$\gamma$ 2L
Neopallial cortex	+	-	+++	-	+++	-
Olfactory lobe	-	-	++	-	+++	-
Midbrain	++	-	++	-	++	-
Hypothalamus	+	-	++	-	++	-
Thalamus	+	-	++	-	++	-
Hippocampus	-	-	+	-	+	-
Spinal cord	+	-	+	-	++	-
Dorsal Root Ganglia	++	-	++	-	+	-
Medulla oblongata	+	-	+	-	++	-
Pons	+	-	+	-	++	-
Cerebellar primordium	+	-	+	-	++	-

**Table 5.2**

**Table 5.2** *Regional distribution of the GABA<sub>A</sub> receptor  $\gamma$ 2S-,  $\gamma$ 2L- and  $\gamma$ 2T-subunit transcripts in the brains of C57BL/6J mice during postnatal development.* Hybridisation signals, obtained with oligonucleotides specific for the  $\gamma$ 2S-subunit mRNA, the  $\gamma$ 2L-subunit mRNA or the  $\gamma$ 2T-subunit mRNA, which recognizes both the  $\gamma$ 2S- and  $\gamma$ 2L-subunit transcripts, were arbitrarily scored as either high, +++; moderate, ++; low, +; weak, (+); or not detectable, -. The results are derived from Figures 5.2, 5.3, 5.4, 5.5 and from data not shown. 1-day, 7-day and 14-day correspond to the days after birth and adult refers to a 50 to 60-day old mouse.

Region/ Nucleus	1 DAY			7 DAY			14 DAY			ADULT		
	γ2S	γ2L	γ2T	γ2S	γ2L	γ2T	γ2S	γ2L	γ2T	γ2S	γ2L	γ2T
<b>Cortex</b>												
Layer I-II	+	-	+	+	+	++	+	+	++	(+)	++	++
Layer III-IV	+++	-	+++	+	+	++	+	+	++	+	++	+++
Layer IV-VI	++	-	++	+	+	++	+	+	++	+	++	+++
Entorhineal Ctx	+	-	+	++	+	+++	++	+	+++	+	++	+++
<b>Hippocampus</b>												
CA1	+++	-	+++	++	+	+++	++	+	+++	+	++	+++
CA3	+++	-	+++	++	+	+++	++	+	+++	+	++	+++
DG	+	-	+	++	(+)	++	++	+	+++	+	++	+++
Tenia Tecta	++	-	++	+	+	++	+	+	++	+	++	+++
<b>Basal Nuclei</b>												
Caudate-putamen	+++	-	+++	-	+	+	(+)	(+)	+	-	+	+
Nu. accumbens	+++	-	+++	-	+	+	(+)	(+)	+	-	+	+
Globus pallidus	+	++	+++	-	+	+	(+)	(+)	+	-	+	+
<b>Amygdala</b>												
Lateral amygdaloid nu.	-	-	-	-	-	-	-	-	-	(+)	(+)	+
<b>Septum</b>												
Bed nucleus s.t.	++	-	++	(+)	+	+	(+)	+	+	(+)	+	+
Lateral septum	++	-	++	-	+	+	(+)	+	+	-	+	+
Medial septum	++	-	++	-	+	+	(+)	+	+	-	+	+
Triangular septum	+	-	+	-	+	+	(+)	++	++	-	+	+
<b>Medial Habenula</b>	+	+	++	(+)	++	++	(+)	++	++	-	++	++
<b>Thalamus</b>												
Anteroventral thal.	++	-	++	+	+	++	+	+	++	-	+	+
Paraventricular nu.	++	-	++	+	+	++	+	(+)	+	-	+	+
Reuniens thal. nucleus	+++	-	+++	+	(+)	+	++	+	+++	-	+	+
Anteromedial thal.	+	-	+	(+)	+	+	(+)	+	+	-	+	+
Ventrolateral thal.	++	-	++	(+)	+	+	(+)	+	+	(+)	+	+
Dorsal lateral genicul	++	-	++	-	+	+	-	+	+	-	+	+
Medial lateral genicul	++	-	++	-	(+)	(+)	-	(+)	(+)	-	++	++
Reticular nucleus	++	-	++	-	-	-	-	+	+	-	+	+
Red nucleus	++	-	++	-	+	+	-	+	+	-	+	+
Ventr. posterior nu.	++	-	++	-	+	+	(+)	+	+	-	+	+
Laterodorsal thal.	++	-	++	-	+	+	-	++	++	-	++	++
Lateral posterior thal.	++	-	++	-	+	+	-	++	++	-	++	++
<b>Colliculi</b>												
Central nucleus	++	(+)	++	(+)	++	++	+	++	+++	-	++	++
Central grey	+	(+)	+	(+)	++	++	+	++	+++	-	++	++
Deep white layer	+	(+)	+	(+)	+	+	(+)	+	+	-	+	+
<b>Subiculum</b>	++	-	++	++	+	+++	++	+	+++	+	++	+++
Para R. Sub	+	-	+	+	(+)	+	++	+	+++	(+)	++	++
Para A. Sub	+	-	+	+	(+)	+	++	+	+++	(+)	++	++
<b>Cerebellum</b>												
Purkinje	-	+	+	-	++	++	-	++	++	-	+++	+++
Stellate/basket cells	+	+	+	+	+	+	+	++	+++	(+)	++	++
Interior granule cells	++	(+)	++	+	++	+++	+	++	+++	(+)	++	++
Exterior granule cells	++	(+)	++	+	++	+++	+	++	+++	(+)	+	+



# **Chapter 6**

## **Mutagenesis**

## 6.1 Introduction

Substitution mutagenesis and site-directed mutagenesis are important tools for the determination of the structural components of ligand-gated ion channels. With this methodology, primary sequences essential for channel gating, conduction and selectivity, pharmacological specification, ligand interactions, sites of allosteric drug interactions and sites required for subunit assembly and subcellular localization have been identified. Within the ligand-gated ion channel superfamily, structure-function analysis has been most extensively carried out for the muscle and neuronal nicotinic acetylcholine receptors.

The pore region (M2), of the muscle nicotinic acetylcholine receptor was the first structural feature of a receptor identified through chimaera assembly and site-directed mutagenesis (Imoto *et al.*, 1986; 1988, Galzi *et al.*, 1991a). Mutational analysis demonstrated that M2 and its vicinity contain important determinants of ion flux through the AChR channel and contribute binding sites for noncompetitive antagonists (Imoto *et al.*, 1986; Giraudat *et al.*, 1986; Hucho, Oberthur, Lottspeich, 1986). The importance of the muscle nicotinic AChR M2 sequence for ion flux was further illustrated using an alternative approach which demonstrated that a highly charged peptide corresponding to the M2 segment of the nAChR could form stable ion channels with two discrete conductance states (Ghosh and Stroud, 1991). The peptides which had the potential to form an amphipathic  $\alpha$ -helix with 4 negatively charged and 3 positively charged residues on one side of the  $\alpha$ -helix did not however, sufficiently mimic the electrophysiological properties of the native AChR.

Site-directed mutagenesis allows discrete nucleotide changes in the coding sequence for a targeted protein. This mutagenesis method was used to further the characterization of the AChR M2 domain, through the introduction of point mutations that altered the net charge of the charged or glutamine residues around the proposed transmembrane segments. Analysis of these mutants demonstrated that three clusters of negatively charged and glutamine residues neighbouring segment M2 of the  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -subunits form anionic (cytoplasmic, intermediate and extracellular) rings that act as major determinants of ion flux (Imoto *et al.*, 1988). Mutations altering the size and polarity of uncharged polar amino acid residues between the extracellular and cytoplasmic rings, determined that a ring-like cluster of uncharged polar residues adjacent to the intermediate ring also play a critical role in determining the rate of ion flux (Imoto *et al.*, 1991) and the residence time and equilibrium binding affinity of QX-222, a quaternary ammonium anesthetic derivative that acts as an open channel blocker (Leonard *et al.*, 1988).

As discussed in the introduction (Section 1.4.5), the anion-selective glycine and GABA<sub>A</sub> receptors display significant amino acid identities with the cation-selective

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nAChRs. In particular, in the M2 transmembrane domain, several of the structural moieties such as the cytoplasmic ring and polar rings of threonines or serines are critical for the determination of ion flux, and therefore these amino acids are highly conserved across anionic and cationic ligand-gated ion channel sequences.

One difference between the anionic and cationic channels occurs at the N-terminal region of M2, where an additional amino acid in the short segment linking M1 and M2 is included in anionic channel sequences. Insertion of an uncharged residue at the N-terminus of the nicotinic  $\alpha 7$  M2 converts the cationic-selective channel into an anion-selective channel gated by acetylcholine (Galzi *et al.*, 1992).

In this chapter we describe the use of single amino acid changes in GABA<sub>A</sub>  $\alpha 1$  and  $\beta 1$  receptor subunits to define the structure-function relationships of two highly conserved amino acids for agonist (muscimol) and antagonist (Ro15-1788) binding. The two conserved amino acids are located in the putative cysteine loop and M1 transmembrane domain of ligand-gated ion channel receptors. Transient expression of mutant GABA<sub>A</sub> receptor subunits in HEK293 cells was used to analyze the effect of the single amino acid substitutions on [<sup>3</sup>H] muscimol and [<sup>3</sup>H] Ro15-1788 binding of  $\alpha 1, \beta 1 \gamma 2 L$  GABA<sub>A</sub> receptor complexes

## 6.2 Results

### 6.2.1 Mutation Strategy

Ligand-gated ion channels belong to a superfamily of membrane proteins that mediate fast neuronal signaling. As outlined in Chapter 1, the glycine receptor  $\alpha 1$  subunit (Grenningloh *et al.*, 1987), and the nicotinic AChR  $\alpha 1$  subunit (Noda *et al.*, 1983), exhibit approximately 34% and 20% identity, respectively, to the GABA<sub>A</sub> receptor subunits. Hydropathy analysis of acetylcholine, GABA, glycine and serotonin 5HT<sub>3</sub> ligand-gated ion channel sequences predicts four transmembrane spanning regions, with a large extracellular N-terminus containing consensus sites for N-linked glycosylation, an extracellular C-terminus and an intracellular loop between putative transmembranes 3 and 4. Within the extracellular loop of acetylcholine, GABA, glycine and serotonin 5HT<sub>3</sub> ligand-gated ion channels, are two cysteine residues (bovine GABA<sub>A</sub> receptor  $\alpha 1$  subunit numbering, C139 and C153) positioned 13 amino acids apart. This putative cysteine loop contains two amino acids at position P147 (proline) and D149 (aspartic acid) that are invariant across species and subunits of ligand-gated ion channels (Figure 6.1). Another site of 100 percent amino acid conservation is within the predicted M1 transmembrane domain, where as shown in Figure 6.2, the proline at position 243 in the bovine  $\alpha 1$  GABA<sub>A</sub> receptor subunit is present in all species and subunits of ligand-gated ion channel receptors.

### 6.2.2 Mutant Preparation

To investigate the role of these highly conserved amino acids, 31 to 33 base oligonucleotides were designed to mutate the conserved sites from aspartic acid (Asp) to asparagine (Asn) or glutamine (Glu) in the putative cysteine loop and from proline (Pro) to alanine (Ala) in the predicted M1 transmembrane spanning domain. Bovine  $\alpha 1$  and  $\beta 2$  GABA<sub>A</sub> receptor subunit cDNAs were subcloned into the bacteriophage vector, M13mp19 for production of single-stranded templates used in oligonucleotide-directed mutagenesis as outlined by Kunkel, Roberts, and Zakour, 1987. This mutagenesis method (Figure 6.3) increases the efficiency of mutagenesis, by utilizing a wild-type template in which uracils have been incorporated and therefore can be selected against in wild-type *E. coli* which synthesize uracil-N-glycosylase, an enzyme that removes uracil residues incorporated into DNA. Removal of the uracils results in the blockade of DNA synthesis and an increase in susceptibility of the template to cleavage by specific nucleases. Since the strand of DNA containing the mutation is synthesized *in vitro*, it therefore lacks uracil incorporation and remains intact in the wild-type *E. coli*.

	139	144	149	153
Bovine $\alpha 1$ GABA <sub>A</sub> R	RAECPMHLEDFPMDAHACPLK			
Bovine $\alpha 2$ GABA <sub>A</sub> R	QAECPMHLEDFPMDAHSCPLK			
Bovine $\alpha 3$ GABA <sub>A</sub> R	SAECRMHLEDFPMDVHACPLK			
Bovine $\beta 1$ GABA <sub>A</sub> R	TAACMMDLRRYPLDEQNCTLE			
Bovine $\gamma 2$ GABA <sub>A</sub> R	DAECQLQLHNFPMDEHSCPLE			
Bovine $\alpha$ AchR	KSYCEIIVTHFFPFDEQNCSMK			
Bovine $\beta$ AchR	RSSCSIQVTYFFPFDWQNCTMV			
Bovine $\delta$ AchR	RSSCPISVTYFFPFDWQNCSLK			
Bovine $\gamma$ AchR	RSSCPVSVTFFFPFDWQNCSLI			
Bovine $\epsilon$ AchR	RSTCAVEVTYFFPFDWQNCSLT			
Rat $\alpha 1$ GlycineR	TLACPMDLKNFPMDVQTCIMQ			

**Figure 6.1 Amino Acid Sequence Alignment of the Cys-Loop Domains of Several Ligand-gated Ion Channel Receptor Subunits.** The proline (P) at position 147 in the bovine GABA<sub>A</sub>  $\alpha 1$  subunit cysteine (C) loop, as well as the aspartic acid at position 149 of the bovine GABA<sub>A</sub>  $\alpha 1$  subunit represent amino acids which are conserved across species and subunits of ligand-gated ion channel receptors.

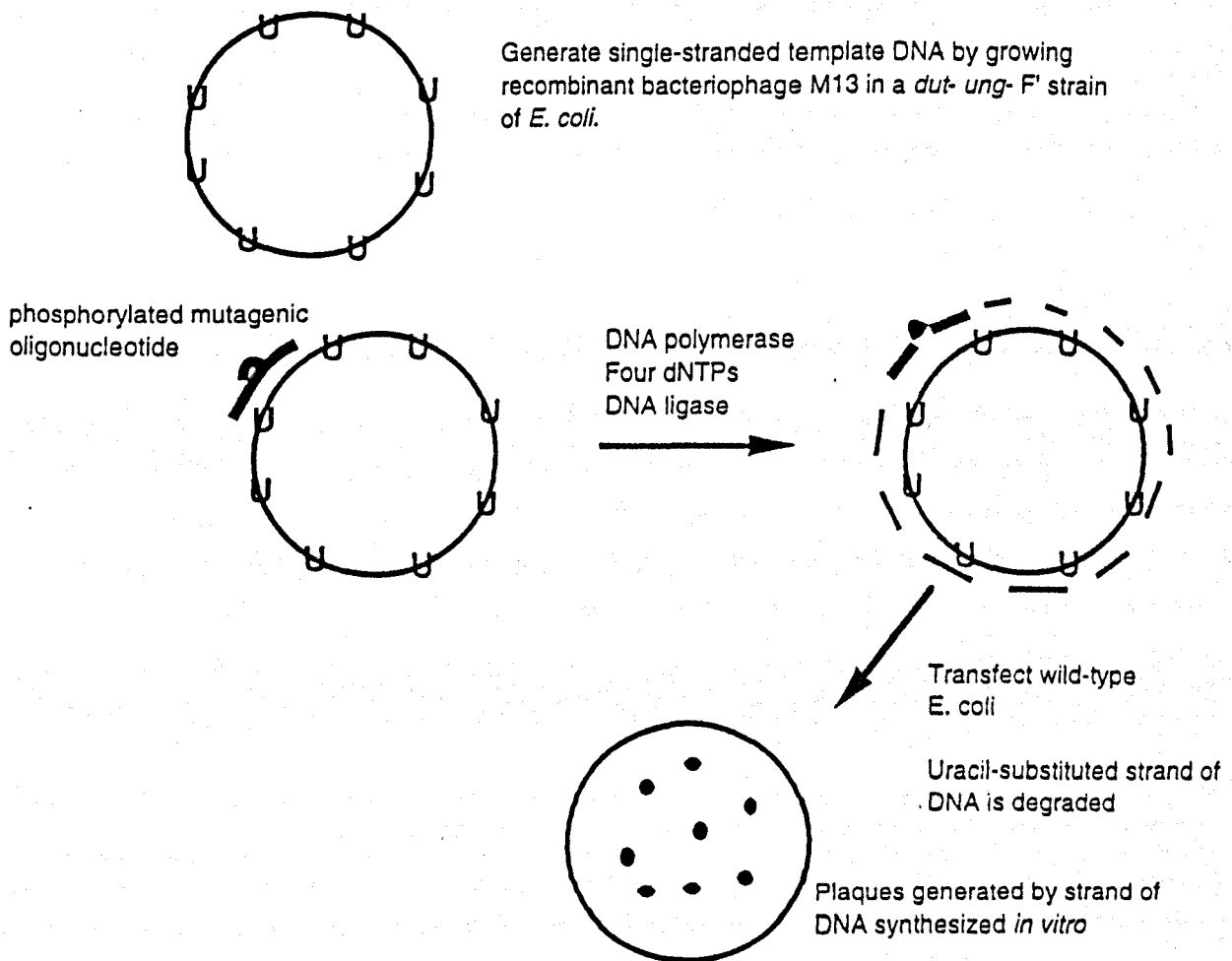
	235	243	249	256
Bovine $\alpha 1$ GABA <sub>A</sub> R	YFVIQTYLPC	IMTVILS	QVVSFWL	
Bovine $\alpha 2$ GABA <sub>A</sub> R	YFVIQTYLPC	IMTVILS	QVVSFWL	
Bovine $\alpha 3$ GABA <sub>A</sub> R	YFVIQTYLPC	IMTVILS	QVVSFWL	
Bovine $\beta 1$ GABA <sub>A</sub> R	YFILQTYMP	STLITIL	SWVSFWI	
Bovine $\gamma 2$ GABA <sub>A</sub> R	YFTIQTYIP	CTLIVVL	SWVSFWI	
Bovine $\alpha$ AchR	YFIVNVIIP	CLLFSFL	TGLVFYL	
Bovine $\beta$ AchR	FYLVNVIAP	CILITLL	AIFVFYL	
Bovine $\delta$ AchR	FYVINILVP	CVLISFM	INLVFYL	
Bovine $\gamma$ AchR	FYVINIIAP	CVLISSV	AILIYFL	
Bovine $\epsilon$ AchR	FYVINIIVP	CVLISGL	VLLAYFL	
Rat $\alpha 1$ GlycineR	YYLIQMYIP	SLLIVIL	SWISFWI	

**Figure 6.2 Amino Acid Sequence Alignment of the M1 Transmembrane Domains of Several Ligand-gated Ion Channel Receptor Subunits.** At position 243 in the bovine  $\alpha 1$  GABA<sub>A</sub> receptor subunit M1 transmembrane domain, the proline (P) residue is conserved across all subunits of ligand-gated ion channel receptors.

For generation of single amino acid changes in GABA<sub>A</sub> receptor subunit cDNAs, single-stranded  $\alpha 1$  and  $\beta 1$  templates were prepared from the CJ236 *E. coli* bacterial strain, which carries both *dut*- and *ung*- mutations. Mutation in the *dut*- gene leads to deficiency in dUTPase, an enzyme which normally converts dUTP to dUMP and hence in the CJ236 bacterial strain results in increase intracellular pools of dUTP. This increase in intracellular dUTP concentrations leads to incorporation of dUTP into DNA at sites normally occupied by thymine. A mutation in the *ung*- gene leads to the inability of the CJ236 *E. coli* strain to remove incorporated uracils from DNA and hence a small fraction of thymidine residues in the bacteriophage DNA are replaced by uracil (Sambrook, Fritsch and Maniatis, 1989).

After annealing of the phosphorylated mutagenic oligonucleotide to the uracil-containing template, the complementary strand was synthesized *in vitro* as outlined in Materials and Methods 2.3.3. The mutagenesis reaction was transfected into JM109 cells and plaques were selected and grown overnight at 37°C in 2TY for single-stranded template preparation prior to dideoxy sequence analysis (Methods 2.3.2 and 2.3.4). Sixty to eighty percent of the templates screened carried the mutation. Figure 6.4 demonstrates the dideoxy sequence analysis of the Asp to Asn mutation in the cysteine loop of the bovine GABA<sub>A</sub> receptor  $\alpha 1$  subunit and the dideoxy sequence analysis of the Asp to Glu and Asp to Asn in the cysteine loop of the GABA<sub>A</sub> receptor  $\beta 1$  subunit. A second base change in the Asp to Asn mutant maintains the arginine at position 145 in the bovine GABA<sub>A</sub>  $\beta 1$  subunit. Figure 6.5 illustrates the dideoxy sequence analysis of the Pro to Ala mutations in the putative M1 transmembrane domain of the bovine GABA<sub>A</sub> receptor

$\alpha 1$  and  $\beta 1$  subunits. One mutant template for each mutation generated was then sequenced in its entirety to confirm that no other changes had been made during the mutagenesis and subcloning reactions.



**Figure 6.3:** Oligonucleotide-mediated, site-directed mutagenesis using the method of Kunkel, Roberts and Zakour, (1987). Selection against uracil substituted wild-type template in wild-type *E. coli*. This figure was modified from Sambrook, Fritsch and Maniatis, 1989.

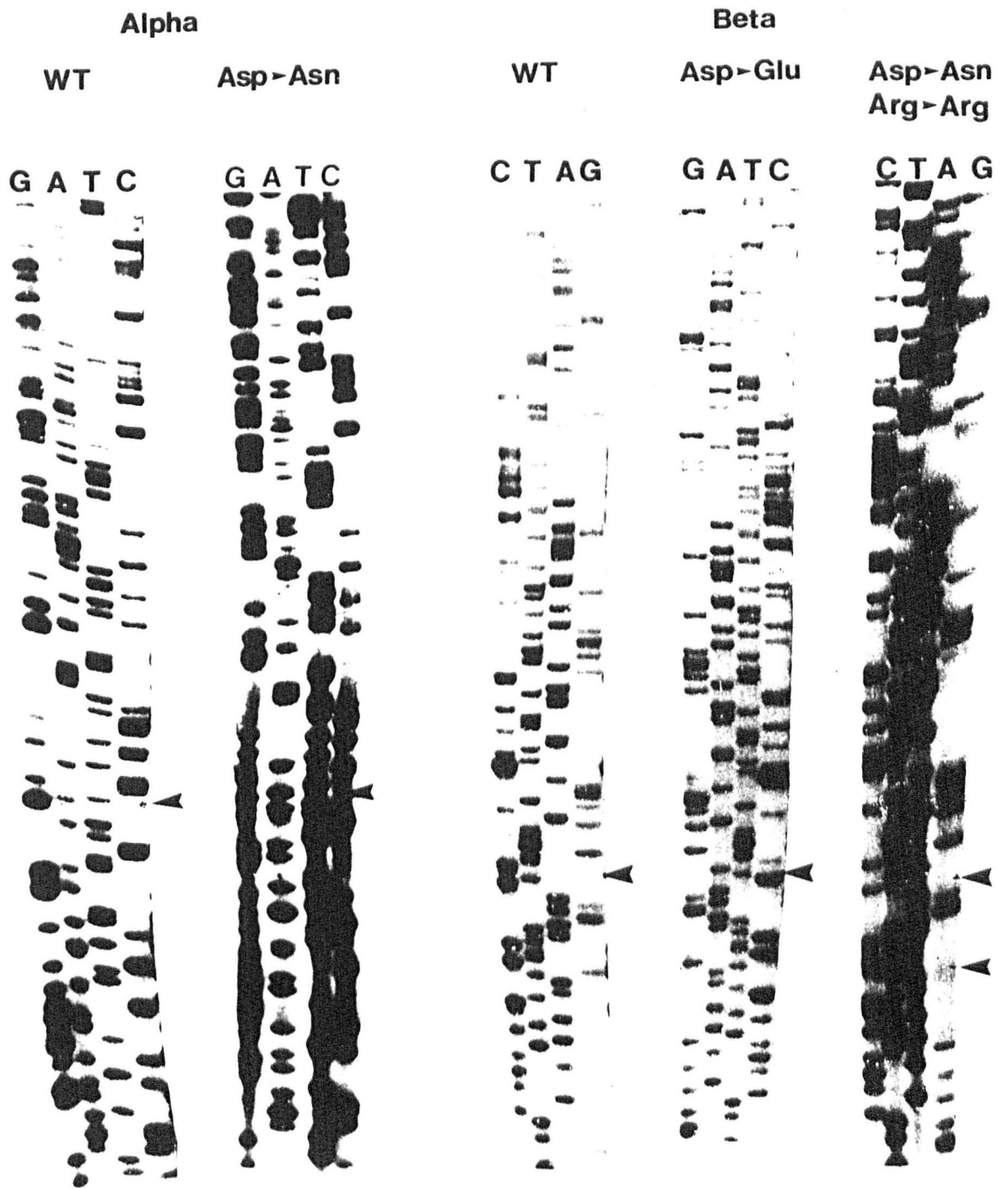
Mutant  $\alpha 1$  and  $\beta 1$  bovine GABA<sub>A</sub> receptor subunit cDNAs were then subcloned into the eukaryotic expression vector pcDNA8 for transient expression in HEK293 cells. In a separate set of experiments mutant GABA<sub>A</sub> receptor  $\alpha 1$  or  $\beta 1$  subunit cDNAs or cRNAs transcribed from the Bluescript constructs with T7 RNA polymerase were co-injected with either wild-type  $\alpha 1$  or  $\beta 1$  and  $\gamma 2L$  subunit cDNAs or cRNAs into *Xenopus* oocytes and two electrode voltage clamp was used to record from the oocytes two days post injection. The oocyte experiments were done by Dr. Shahid Zaman and are described in his thesis

**Figure 6.4**

***Figure 6.4*** Sequence analysis of the nucleotide changes resulting in amino acid changes in the predicted cysteine loop of the bovine  $\alpha 1$  and  $\beta 1$  GABA<sub>A</sub> receptor subunits.

Amino acid changes and receptor subunit are shown above the sequence. Site-directed changes in the wild-type sequence are indicated by arrows.

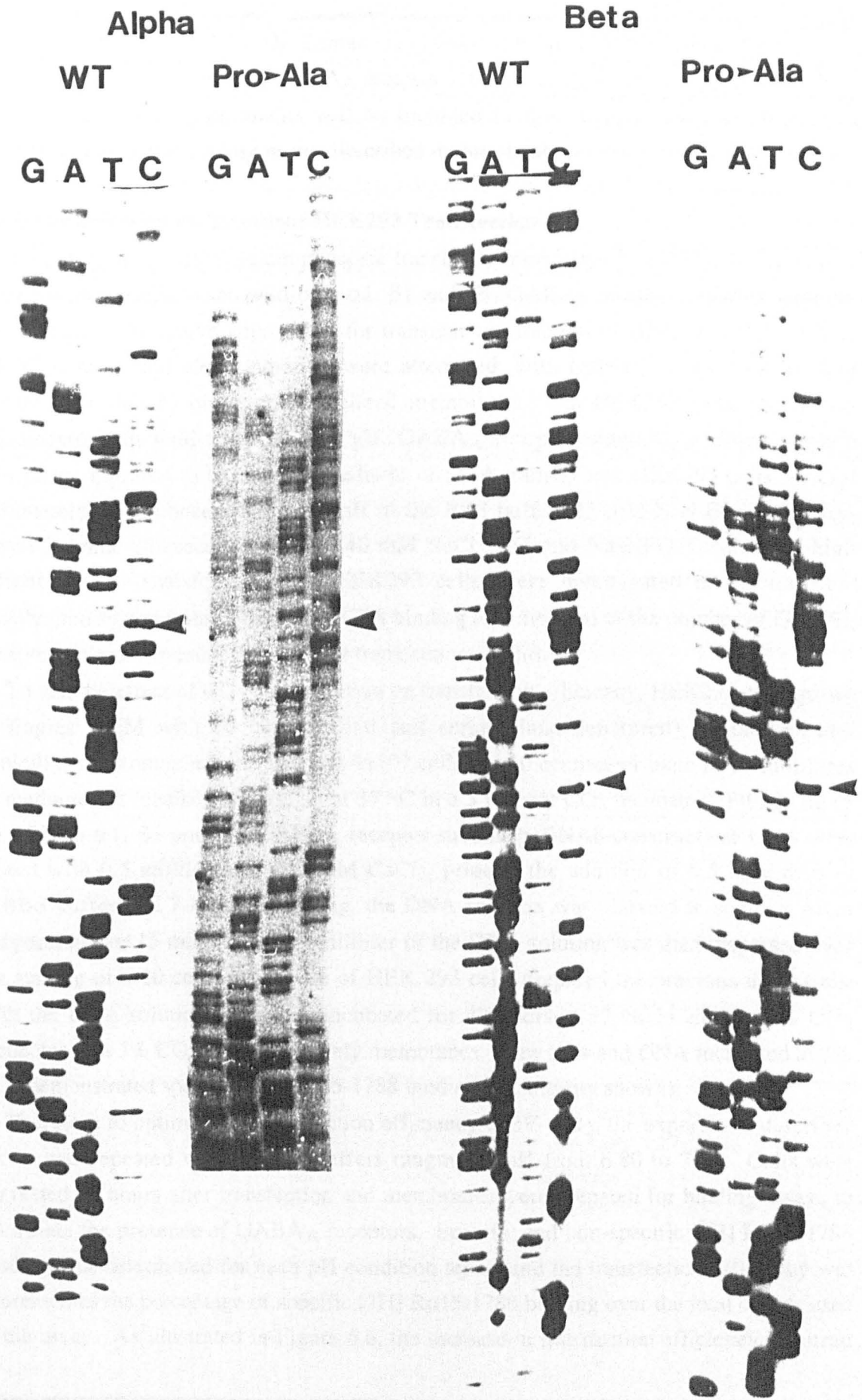




**Figure 6.5**

***Figure 6.5 Sequence analysis of the nucleotide changes resulting in amino acid changes in the putative M1 transmembrane spanning domain of the bovine  $\alpha 1$  and  $\beta 1$  GABA<sub>A</sub> receptor subunits.***

Amino acid changes and receptor subunit are shown above the sequence. Site-directed changes in the wild-type sequence are indicated by arrows.



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dissertation (Zaman, 1993). Dr. Zaman also recorded from HEK293 cells expressing  $\alpha 1$ ,  $\beta 1$ ,  $\gamma 2L$  wild-type and mutant GABA<sub>A</sub> receptor subunits using whole cell patch recording. Results from these experiments will be included in this chapter's discussion only in relation to the ligand binding results described in this chapter.

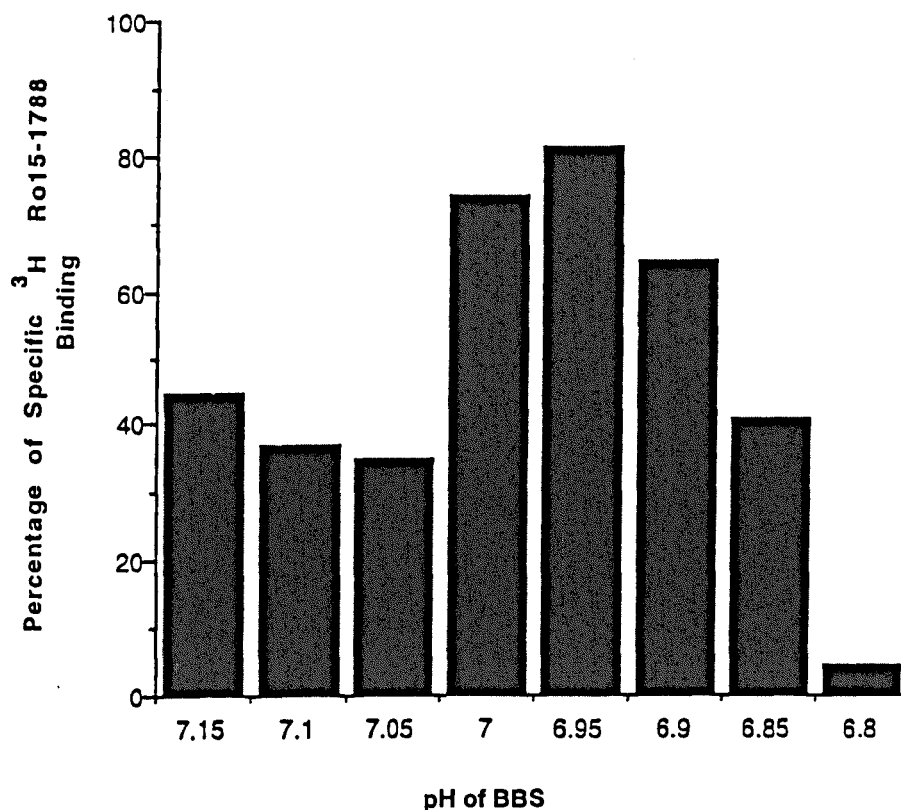
### 6.2.3 Optimization of Transient HEK293 Transfection

Initial experiments with calcium phosphate transiently transfected HEK293 cells gave very poor binding results, when wild-type  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2L$  GABA<sub>A</sub> receptor subunits were co-transfected. Alternative approaches for transient transfection of HEK293 cells, such as DEAE-dextran and electroporation were attempted, with little or no specific binding (results not shown) observed in isolated membranes from HEK293 cells transiently transfected with wild-type  $\alpha 1$ ,  $\beta 1$ ,  $\gamma 2L$  GABA<sub>A</sub> receptor subunits. Since calcium phosphate appeared to be the best mediator of DNA transfer into HEK293 cells, several parameters (CO<sub>2</sub> concentration and pH of the BBS buffer (25 mM N-N Bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid, 140 mM NaCl, 0.75 mM Na<sub>2</sub>HPO<sub>4</sub>) critical for high efficiency stable transformation of HEK293 cells, were investigated in the transient transfection system using [<sup>3</sup>H] Ro15-1788 binding as a measure of the number of GABA<sub>A</sub> receptors expressed under the different transfection conditions.

To test the effect of CO<sub>2</sub> concentration on transfection efficiency, HEK293 cells grown in Eagles MEM with 10 percent fetal calf serum (heat denatured), glutamine, and penicillin/streptomycin were plated at  $4 \times 10^6$  cells per 10 centimeter plate in 10 millilitres of medium and incubated overnight at 37 °C in a 5 percent CO<sub>2</sub> incubator. Fifteen  $\mu$ g (5  $\mu$ g of each  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2L$  GABA<sub>A</sub> receptor subunit pcDNA8 construct) of DNA were mixed with 0.5 millilitres of 250 mM CaCl<sub>2</sub>, prior to the addition of 0.5 millilitres of 2xBBS buffer, pH 7.1. After mixing, the DNA solution was allowed to stand at room temperature for 15 minutes. One milliliter of the DNA solution was then dispersed over the surface of a 10 centimetre plate of HEK 293 cells prepared the previous day. Cells with the DNA solution were then incubated for 48 hours at 37 °C in either a 3% CO<sub>2</sub> incubator or a 5% CO<sub>2</sub> incubator. Only membranes, from cells and DNA incubated at 3% CO<sub>2</sub> demonstrated specific [<sup>3</sup>H] Ro15-1788 binding ( results not shown).

Therefore to optimize the transfection efficiency at 3% CO<sub>2</sub>, the experiment described above was repeated with 2xBBS buffers ranging in pH from 6.80 to 7.15. Cells were harvested 48 hours after transfection and membranes were prepared for binding assays to determine the presence of GABA<sub>A</sub> receptors. Specific and non-specific [<sup>3</sup>H] Ro15-1788 binding was determined for each pH condition tested and the transfection efficiency was expressed as the percentage of specific [<sup>3</sup>H] Ro15-1788 binding over the total counts used in the assay. As illustrated in Figure 6.6, the increase in transfection efficiency occurred

over a very narrow range of pH values (pH 7.00 to 6.90). Based on these results, all future transfection experiments were performed using BBS buffer at pH 6.95 and a 3 percent CO<sub>2</sub> incubator.

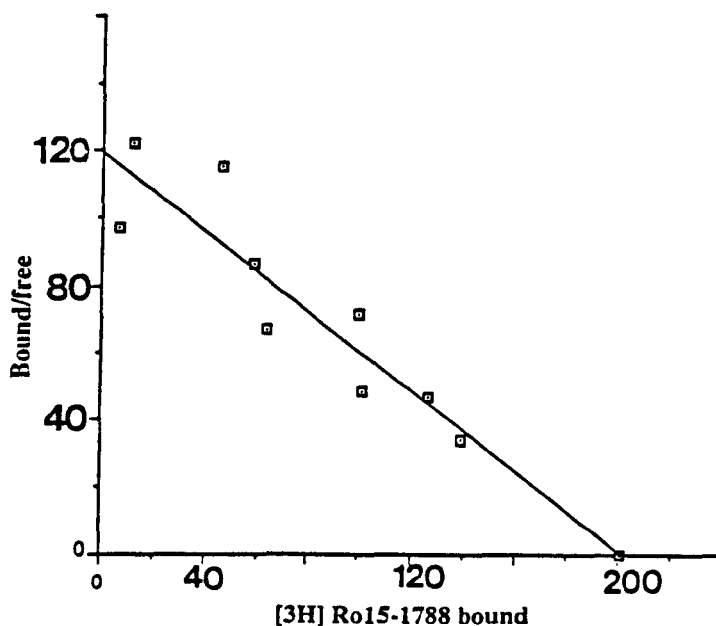


**Figure 6.6: Optimization of calcium phosphate transfection of HEK293 cells.** Wild-type bovine GABA<sub>A</sub> receptor  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2L$  subunits subcloned into the mammalian expression vector pCDNA8 were transiently transfected into HEK 293 cells using 250 mM calcium chloride and a series of BBS (25 mM N-Bis(2-hydroxy-ethyl)-2-aminoethanesulfonic acid, 140 mM NaCl, 0.75 mM Na<sub>2</sub>HPO<sub>4</sub>) buffers (pH 6.80 to 7.15). Cells were harvested 48 hours post transfection, membranes were prepared and [<sup>3</sup>H]-Ro15-1788 binding assays were used to determine the extent of GABA<sub>A</sub> receptor subunit expression. The percentage of specific [<sup>3</sup>H]-Ro15-1788 binding was determined by dividing the specific counts [total counts (dpm) - nonspecific counts (dpm)] by the total counts (dpm).

#### 6.2.4 Mutant Analysis

To study the  $\alpha 1$  and  $\beta 1$  D149N, D149Q, and P243A mutations, ten 90 centimetre plates were prepared for each wild-type and mutant combination of an  $\alpha 1$ ,  $\beta 1$ , and  $\gamma 2L$  GABA<sub>A</sub> receptor subunit. Forty-eight hours post transfection cells were harvested at 4°C, by scraping into 5 millilitres of TBS (Tris buffered saline), pH 7.4. The cell suspension was

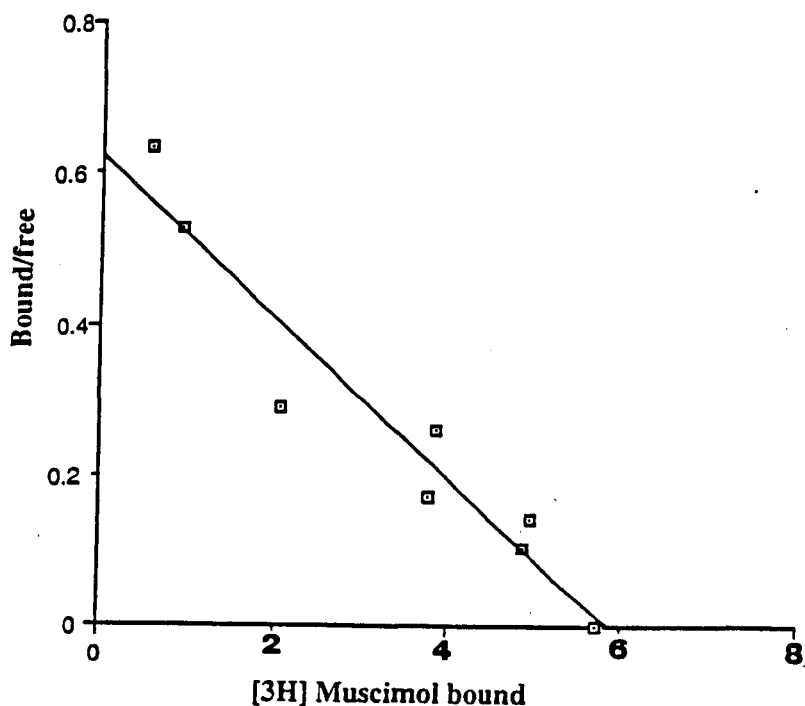
centrifuged at 5,000 rpm for 5 minutes at 4°C and the pellet was resuspended in 20 millilitres of ice cold 50 mM Tris pH 7.3 prior to homogenization at 4°C. The homogenate was centrifuged at 20K rpm for 30 minutes at 4°C in a Sorval SS34 rotor. The pellet was resuspended in 20 millilitres of ice cold 50 mM Tris, pH 7.4, homogenized and centrifuged at 20K rpm for 30 minutes at 4°C in a Sorval SS34 rotor. The membrane pellet was resuspended in 5 millilitres of TBS, pH 7.4. Binding assays with [<sup>3</sup>H] muscimol or [<sup>3</sup>H] Ro15-1788 were set up in triplicate and incubated on ice for 90 minutes. In assays using [<sup>3</sup>H] Ro15-1788, 10 μM flunitrazepam was used to determine non-specific binding in triplicate, at each concentration of Ro15-1788, while in assays using [<sup>3</sup>H] muscimol, 10 mM KCl was added to the binding reactions and 1 μM GABA was used to determine non-specific binding, in triplicate, at each concentration of muscimol. Assays were harvested onto presoaked GF/B filters using 5 mM Tris, 1 mM EDTA, pH 7.4 at 4°C. Protein concentrations for each membrane homogenate were determined by Lowry assays.



**Figure 6.7** Scatchard plot of [<sup>3</sup>H] Ro15-1788 binding to C57Bl/6 adult mouse cortical membranes. Specific binding was determined by subtracting the binding in the presence of 10 μM flunitrazepam. The results shown are from a single binding assay and were determined by regression analysis of the data. Each dilution of ligand is done in triplicate for the determination of both specific and non-specific binding. As observed from three separate experiments,  $K_d$  values were  $1.67 \pm 0.10$  nM and  $B_{max}$  values were  $200 \pm 20$  pmol/mg and were determined by regression analysis using the EBDA-LIGAND program.

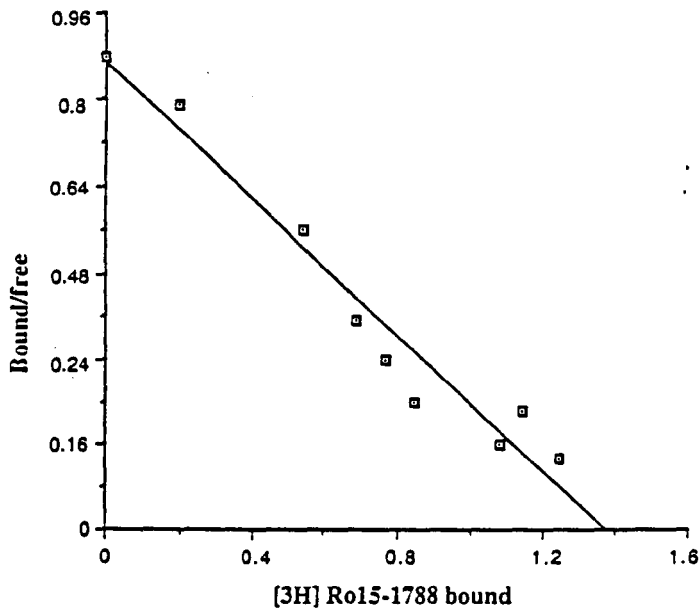
The results of binding assays were expressed either as a saturation analysis or Scatchard plot. A saturation analysis was used when binding results indicated that there was no discernable specific binding from the membranes tested and in general is used to illustrate a saturation curve for a ligand that binds to a homogeneous receptor population. In a saturation curve, the total binding includes a component of nonspecific binding, which is nonsaturable, and a component of specific binding, which saturates at  $B_{\max}$ . The  $B_{\max}$  is the maximum amount of drug (usually expressed in picomoles or femtomoles per mg protein) which can bind specifically to the receptors in a membrane preparation. If one drug molecule binds to each receptor, the  $B_{\max}$  also indicates the concentration of receptors in the membrane preparation. The dissociation constant for a radiolabelled drug ( $K_d$ ) or ligand is the concentration of ligand or drug, at equilibrium, that occupies 50% of the receptors and can be calculated from a Scatchard plot.

The Scatchard plot is a graph of the amount of radioligand bound divided by the amount of radioligand free in the solution (Y axis) versus the amount of radioligand bound (x axis). The intercept on the X axis is equal to the  $B_{\max}$  and the slope ( $-1/K_d$ ) of the Scatchard plot, is determined by regression analysis using an EBDA /Ligand program.

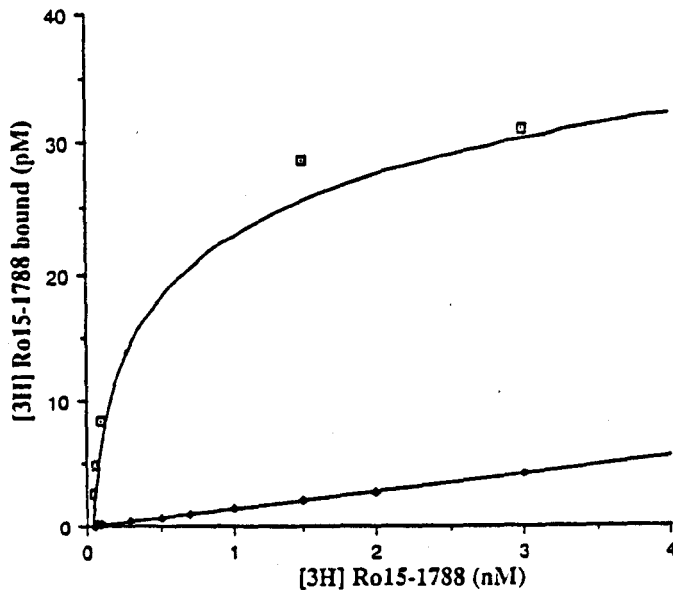


**Figure 6.8** Scatchard plot of [ $^3\text{H}$ ] muscimol binding to C57Bl/6 adult mouse cortical membranes. Specific binding was determined by subtracting the binding in the presence of  $1\ \mu\text{M}$  GABA. The results shown are from a single binding assay. Each dilution of ligand is done in triplicate for the determination of both specific and non-specific binding. As observed from three separate experiments,  $K_d$  values were  $3.2 \pm 0.3\ \text{nM}$  and  $B_{\max}$  values were  $6.4 \pm 1.7\ \text{pmol/mg}$  and were determined by regression analysis of the data.

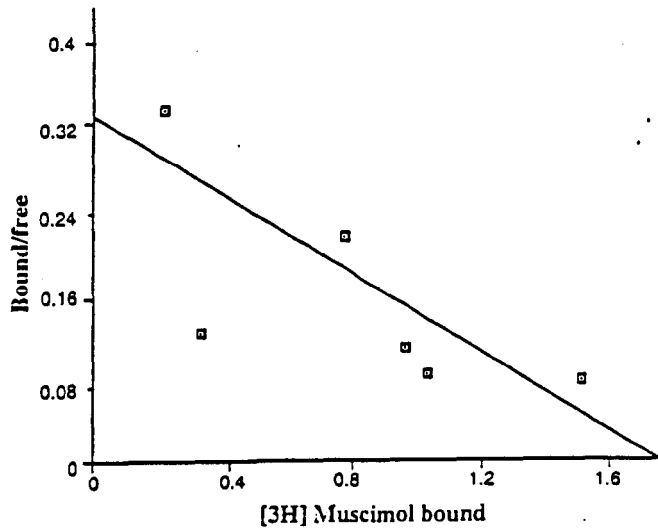




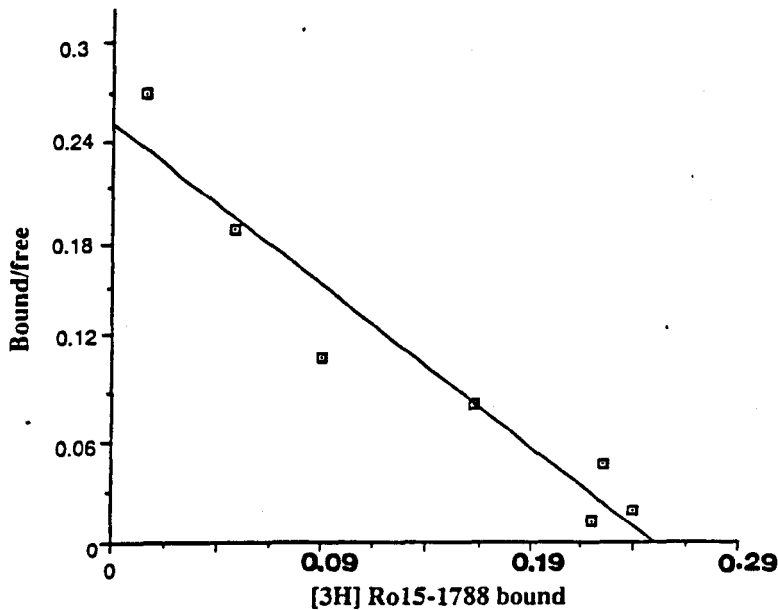
**Figure 6.9** Scatchard plot of [ $^3\text{H}$ ] Ro15-1788 binding to membranes isolated from HEK293 cells transfected with wildtype  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2\text{L}$ . Specific binding was determined by subtracting the binding in the presence of  $10\ \mu\text{M}$  flunitrazepam. The results shown are from a single binding assay and were determined by regression analysis of the data. Each dilution of ligand is done in triplicate for the determination of both specific and non-specific binding. The  $K_d$ ,  $1.59\ \text{nM}$  and the  $B_{\text{max}}$ ,  $1.38\ \text{pmol/mg}$  were determined using the EBDA-LIGAND program.



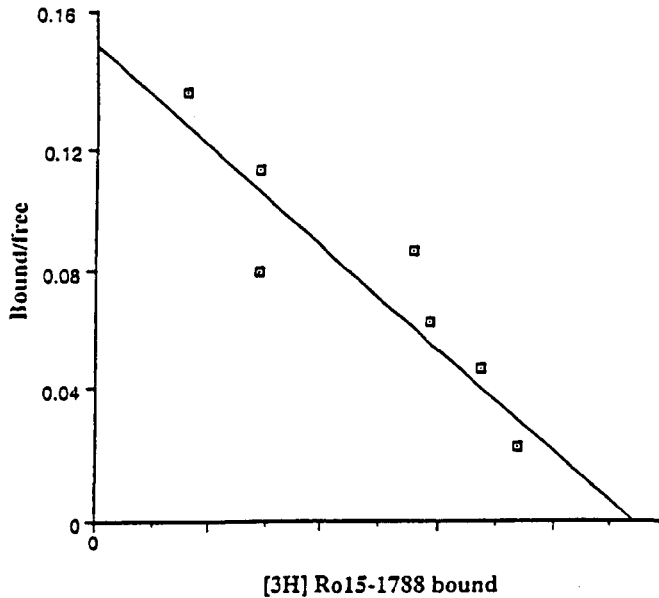
**Figure 6.10** Binding isotherm of [ $^3\text{H}$ ] Ro15-1788 binding to membranes isolated from HEK293 cells transfected with wildtype  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2\text{L}$ . Specific binding was determined by subtracting the binding in the presence of  $10\ \mu\text{M}$  flunitrazepam. Untransfected cells showed no specific binding.



**Figure 6.11** Scatchard plot of [ $^3\text{H}$ ] muscimol binding to membranes isolated from HEK293 cells transfected with wildtype  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2\text{L}$ . Specific binding was determined by subtracting the binding in the presence of  $10\ \mu\text{M}$  flunitrazepam. The results shown are from a single binding assay and were determined by regression analysis of the data. Each dilution of ligand is done in triplicate for the determination of both specific and non-specific binding. The  $K_d$ ,  $5.67\ \text{nM}$  and the  $B_{\text{max}}$ ,  $1.69\ \text{pmol/mg}$  were determined by the EBDA-LIGAND program.



**Figure 6.12** Scatchard plot of [ $^3\text{H}$ ] Ro15-1788 binding to HEK 293 membranes transiently transfected with wt  $\alpha 1$ ,  $\beta 1$  (149Asp-Glu), wt  $\gamma 2\text{L}$  GABA<sub>A</sub> receptor subunits. Specific binding was determined by subtracting the binding in the presence of  $10\ \mu\text{M}$  flunitrazepam. The results shown are from a single binding assay and were determined by regression analysis of the data using the EBDA-LIGAND program. Each dilution of ligand is done in triplicate for the determination of both specific and non-specific binding.  $K_d$   $1.02\ \text{nM}$  and  $B_{\text{max}}$   $0.25\ \text{pmol/mg}$ .



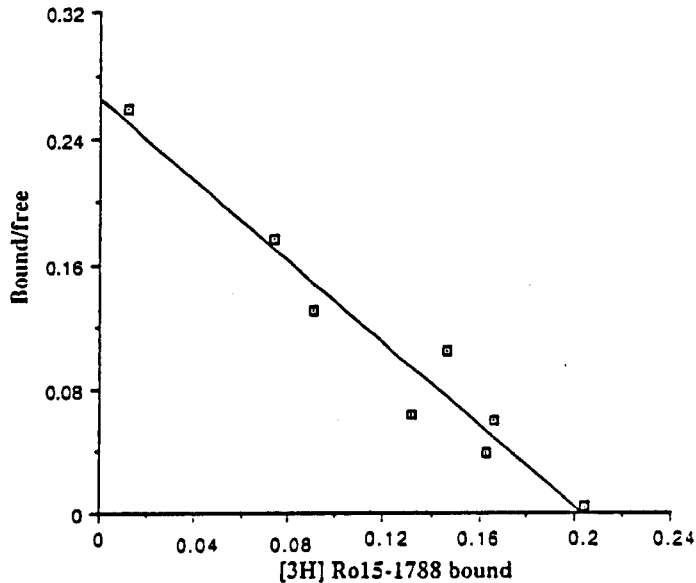
**Figure 6.13** Scatchard plot of [ $^3\text{H}$ ] Ro15-1788 binding to HEK 293 membranes transiently transfected with wt  $\alpha 1$ ,  $\beta 1$  (149Asp-Asn), wt  $\gamma 2\text{L}$  GABA $_A$  receptor subunits. Specific binding was determined by subtracting the binding in the presence of 10  $\mu\text{M}$  flunitrazepam. The results shown are from a single binding assay and were determined by regression analysis of the data using the EBDA-LIGAND program. Each dilution of ligand is done in triplicate for the determination of both specific and non-specific binding.  $K_d$  0.82 nM and  $B_{max}$  0.15 pmol/mg.

Subunit combination expressed	[ $^3\text{H}$ ]Ro15-1788 binding: $K_d$ , $B_{max}$	[ $^3\text{H}$ ]Muscimol binding: $K_d$ , $B_{max}$
wt $\alpha 1$ , wt $\beta 1$ , wt $\gamma 2\text{L}$	1.35 $\pm$ 0.7 nM, 1.46 $\pm$ 0.1 pmol/mg	5.89 $\pm$ 0.3nM, 1.69 $\pm$ 0.3 pmol/mg
$\alpha 1_{\text{D-N}}$ , wt $\beta 1$ , wt $\gamma 2\text{L}$	ND	ND
$\alpha 1$ , $\beta 1_{\text{D-E}}$ , wt $\gamma 2\text{L}$	1.1 + 0.1 nM, 0.31 + 0.1 pmol/mg	ND
$\alpha 1$ , $\beta 1_{\text{D-N}}$ , wt $\gamma 2\text{L}$	0.82 + .08 nM, 0.20 + 0.07 pmol/mg	ND

**Table 6.1:** Summary of  $K_d$  and  $B_{max}$  values for receptors formed from wild-type subunits only or with wild-type subunits and GABA $_A$   $\alpha$  or  $\beta$  subunits with point mutations in the cysteine loop.  $K_d$  values are expressed in nM, and  $B_{max}$  values in pmol/mg. ND: No specific binding detected. Values were averaged from three separate transfection experiments in HEK293 cells.

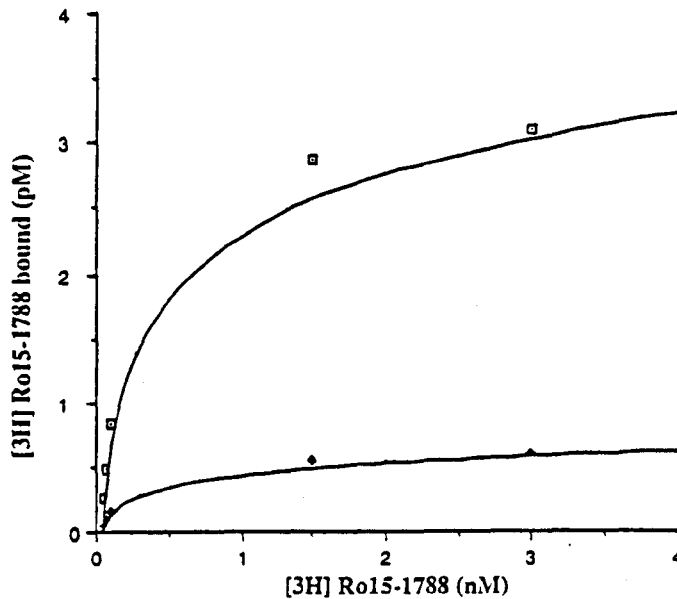
No expression was detected using either [ $^3\text{H}$ ]Ro15-1788 or [ $^3\text{H}$ ]muscimol with the receptor combination containing the  $\alpha 1_{\text{D-N}}$  mutation. Successful transfection was determined by the presence of mRNA for all three subunits, in HEK293 cells transiently transfected with the wild-type  $\beta 1$ ,  $\gamma 2\text{L}$  and mutant  $\alpha 1$  subunits (data not shown). Although

[<sup>3</sup>H]Ro15-1788 binding was detected in receptor combinations with a mutant  $\beta 1$  subunit, no [<sup>3</sup>H]muscimol binding was detected. mRNA for all three GABA<sub>A</sub> subunits transfected were detected in the HEK293 cells, 48 hours after transfection (data not shown).

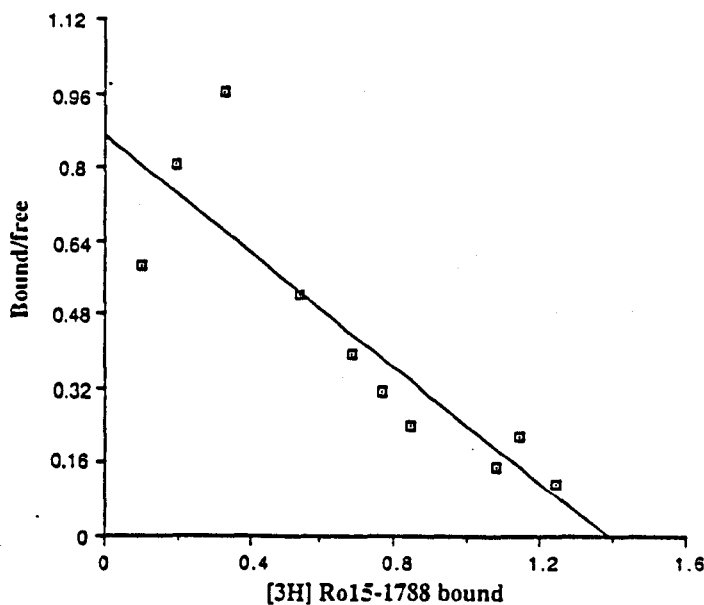


**Figure 6.14** Scatchard plot of [<sup>3</sup>H] Ro15-1788 binding to HEK 293 membranes transiently transfected with mutant  $\alpha 1$  pro-ala, wt  $\beta 1$  and wt  $\gamma 2L$  GABA<sub>A</sub> receptor subunits. Specific binding was determined by subtracting the binding in the presence of 10  $\mu$ M flunitrazepam. The results shown are from a single binding assay and were determined by regression analysis using the EBDA program. Each dilution of ligand is done in triplicate for the determination of both specific and non-specific binding.  $K_d$  0.73 nM and  $B_{max}$  0.19 pmol/mg.

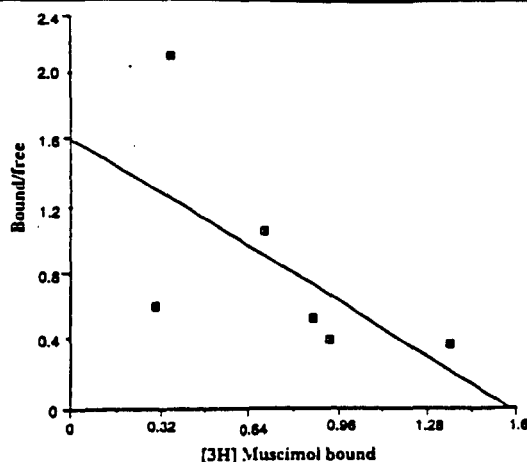
The  $K_d$  for [<sup>3</sup>H] Ro15-1788 binding was decreased for the receptor combination containing the mutant  $\beta 1_{149D-E}$  subunit when compared with the  $K_d$  for [<sup>3</sup>H] Ro15-1788 binding. This decrease in the  $K_d$  was not statistically different as determined by a Student's t-test, ( $p=0.065$ ). The decrease in the  $B_{max}$  for the receptor combination containing the mutant  $\beta 1_{149D-E}$  subunit when compared with the  $B_{max}$  for the wild-type receptor combination was statistically significant ( $p<0.0001$ ). The difference in the  $K_d$ s of the receptor combination containing the  $\beta 1_{149D-N}$  mutation compared with the receptor combination containing wildtype subunits only, was statistically different as determined by a Student's t-test ( $p=0.008$ ). As seen with the  $\beta 1_{D-E}$  mutant subunit, the  $B_{max}$  for the receptor combination containing mutant  $\beta 1_{D-N}$  mutation was significantly different when compared with the  $B_{max}$  of the wild-type receptor combination ( $p<0.0001$ ).



**Figure 6.15** Binding isotherm of [ $^3\text{H}$ ] Ro15-1788 binding to membranes isolated from HEK293 cells transfected with wild-type  $\alpha 1$ ,  $\beta 1$ pro-ala and wild-type  $\gamma 2\text{L}$ . Specific binding was determined by subtracting the binding in the presence of  $10\ \mu\text{M}$  flunitrazepam. Untransfected cells show no specific binding.



**Figure 6.16** Scatchard plot of [ $^3\text{H}$ ] Ro15-1788 binding to HEK 293 membranes transiently transfected with wt  $\alpha$ , mutant  $\beta 1$  MI pro-ala, and wt  $\gamma 2\text{L}$  GABA<sub>A</sub> receptor subunits. Specific binding was determined by subtracting the binding in the presence of  $10\ \mu\text{M}$  flunitrazepam. The results shown are from a single binding assay and were determined by least-squares analysis of the data. Each dilution of ligand is done in triplicate for the determination of both specific and non-specific binding. As observed from three different experiments,  $K_d$   $1.47\ \text{nM}$  and  $B_{\text{max}}$   $1.39\ \text{pmol/mg}$  and were determined by the EBDA-LIGAND program.



**Figure 6.17** Scatchard plot of [<sup>3</sup>H] muscimol binding to HEK 293 membranes transiently transfected with wt  $\alpha$ , mutant  $\beta$ 1 M1 pro-ala, and wt  $\gamma$ 2L GABA<sub>A</sub> receptor subunits. Specific binding was determined by subtracting the binding in the presence of 10  $\mu$ M flunitrazepam. The results shown are from a single binding assay. Each dilution of ligand is done in triplicate for the determination of both specific and non-specific binding.  $K_d$  0.98 nM and  $B_{max}$  1.53 pmol/mg and were determined using the EBDA-LIGAND program.

Subunit combination expressed	[ <sup>3</sup> H]Ro15-1788 binding: $K_d$ , $B_{max}$	[ <sup>3</sup> H]Muscimol binding: $K_d$ , $B_{max}$
wt $\alpha$ 1, wt $\beta$ 1, wt $\gamma$ 2L	1.35 $\pm$ 0.1 nM, 1.5 $\pm$ 0.2 pmol/mg	5.89 $\pm$ 0.3nM, 1.7 $\pm$ 0.1 pmol/mg
wt $\beta$ 1, wt $\gamma$ 2L	0.51 $\pm$ 0.03 nM, 0.16 $\pm$ 0.03 pmol/mg	ND
$\alpha$ 1 <sub>P-A</sub> , wt $\beta$ 1, wt $\gamma$ 2L	0.87 $\pm$ 0.09 nM, 0.19 $\pm$ 0.04 pmol/mg	ND
$\alpha$ 1, $\beta$ 1 <sub>P-A</sub> , wt $\gamma$ 2L	1.47 $\pm$ 0.1 nM, 1.5 $\pm$ 0.3 pmol/mg	0.89 $\pm$ 0.09nM, 1.6 $\pm$ 0.4 pmol/mg

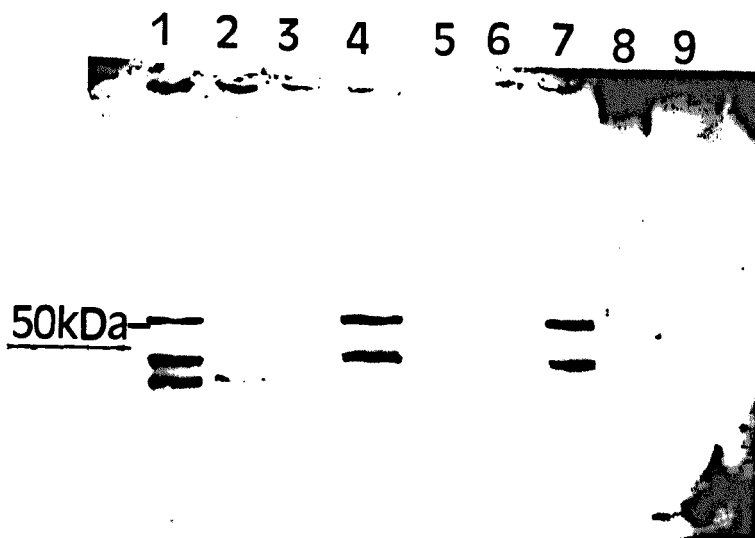
**Table 6.2:** Summary of  $K_d$  and  $B_{max}$  values for receptors containing wildtype subunits, only or with wildtype subunits and GABA<sub>A</sub>  $\alpha$  or  $\beta$  subunits with point mutations in the putative M1 transmembrane domain.

$K_D$  values are expressed in nM, and  $B_{max}$  values in pmol/mg. ND: No specific binding detected. Values were averaged from three separate transfection experiments.

The difference in the  $K_d$ s of [<sup>3</sup>H]Ro15-1788 binding for the wild-type  $\beta$ 1,  $\gamma$ 2L receptor combination only, compared with the wild-type  $\alpha$ 1,  $\beta$ 1,  $\gamma$ 2L receptor combination was statistically significant as determined by a Student's t-test ( $p < 0.0001$ ). Comparison of the  $K_d$  of [<sup>3</sup>H]Ro15-1788 binding for receptor combinations including mutant  $\alpha$ 1<sub>243pro-ala</sub> subunits with the  $K_d$  of [<sup>3</sup>H]Ro15-1788 binding for the wild-type  $\alpha$ 1,  $\beta$ 1,  $\gamma$ 2L receptor combination indicated that there was a statistical difference between the two  $K_d$ s ( $p = 0.0016$ ). A Student's t-test comparing the  $K_d$ s of [<sup>3</sup>H]Ro15-1788 binding for receptor

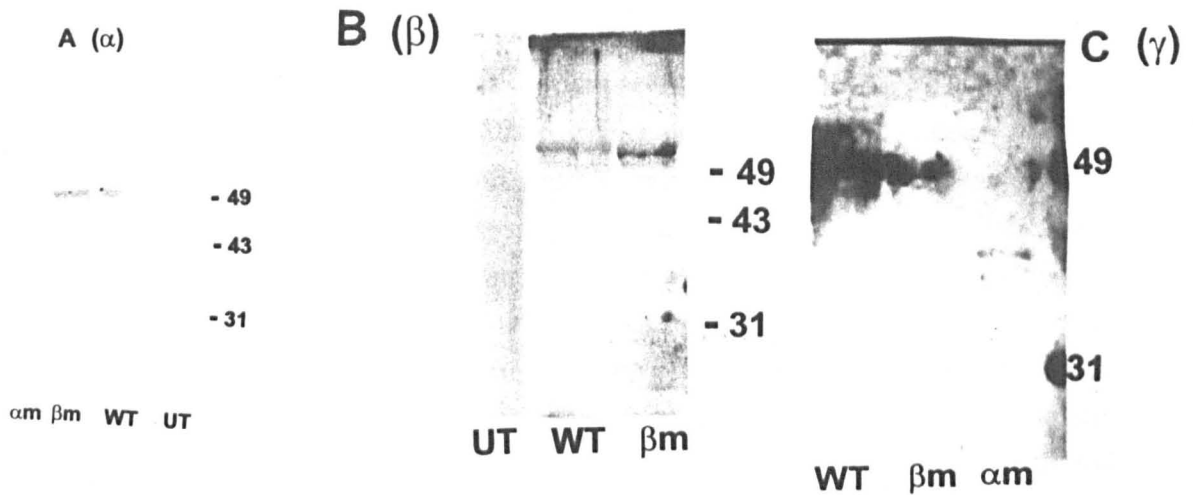
a one minute incubation in Amersham detection buffers 1 and 2. Following the one minute incubation, the detection buffer was removed and the membrane exposed to Hyperfilm-ECL for 10 minutes.

Western blot analysis, of membrane preparations from transiently transfected HEK293 cells using a polyclonal anti- $\alpha 1$  GABA<sub>A</sub> receptor antibody demonstrates that although an  $\alpha 1$  subunit protein is present in the membranes of cells transfected with; 1) wild-type  $\alpha 1$ ,  $\beta 1$ ,  $\gamma 2L$  subunits, 2) wild-type  $\alpha 1$ ,  $\gamma 2L$  and mutant  $\beta 1_{149D-N}$  subunits, and 3) mouse cortical membranes, no immunoreactive product was present for the  $\alpha 1$  subunit protein in the membranes of cells transfected with wild-type  $\beta 1$ ,  $\gamma 2L$  and mutant  $\alpha 1_{243pro-ala}$  subunits (Figures 6.18 and 6.19A). Western blot analysis of membrane preparations from transiently transfected HEK293 cells using a polyclonal anti- $\beta 1$  GABA<sub>A</sub> receptor antibody demonstrated that the  $\beta 1$  protein was abundantly represented in membranes of cells transfected with; 1) wild-type  $\alpha 1$ ,  $\beta 1$   $\gamma 2L$  subunits and 2) wild-type  $\alpha 1$ ,  $\gamma 2L$  and mutant  $\beta 1_{243P-A}$  subunits, and weakly expressed in membranes of cells transfected with; 3) wild-type  $\beta 1$ ,  $\gamma 2L$  and mutant  $\alpha 1_{243P-A}$ , subunits (Figure 6.19B). Western blot analysis of membrane preparations from transiently transfected HEK293 cells using a polyclonal anti- $\gamma 2$  GABA<sub>A</sub> receptor antibody (recognizes both the L and S splice variants) demonstrated that the  $\gamma 2$  protein was abundantly represented in membranes of cells transfected with; 1) wild-type  $\alpha 1$ ,  $\beta 1$   $\gamma 2L$  subunits and 2) wild-type  $\alpha 1$ ,  $\gamma 2L$  and mutant  $\beta 1_{243P-A}$  subunits, and weakly expressed in membranes of cells transfected with; 3) wild-type  $\beta 1$ ,  $\gamma 2L$  and mutant  $\alpha 1_{243P-A}$ , subunits (Figure 6.19C).



**Figure 6.18** Western blot analysis of membrane preparations from transiently transfected HEK293 cells using a polyclonal anti- $\alpha 1$  GABA<sub>A</sub> receptor antibody. HEK293 cells were transfected with 1) wild-type  $\beta 1$ ,  $\gamma 2L$  and mutant  $\alpha 1_{149D-N}$  {transfection #1}, 2) wild-type  $\beta 1$ ,  $\gamma 2L$  and mutant  $\alpha 1_{149D-N}$  {transfection #2}, 3) wild-type  $\beta 1$ ,  $\gamma 2L$  and mutant  $\alpha 1_{243P-A}$  {transfection #1}, 4) wild-type  $\alpha 1$ ,  $\beta 1$ ,  $\gamma 2L$  {transfection #1}, 5) wild-type  $\beta 1$ ,  $\gamma 2L$  and mutant  $\alpha 1_{149D-N}$  {transfection #3}, 6) wild-type  $\beta 1$ ,  $\gamma 2L$  and

mutant  $\alpha 1_{243P-A}$  {transfection #2}, 7) wild-type  $\alpha 1$ ,  $\beta 1$ ,  $\gamma 2L$  {transfection #2} GABA<sub>A</sub> receptor subunits. Controls for anti- $\alpha 1$  GABA<sub>A</sub> receptor antibody specificity included: 8) mouse cortical membranes and 9) untransfected HEK293 membranes.



**Figure 6.19** Western blot analysis of membrane preparations from transiently transfected HEK293 cells using a polyclonal anti- $\alpha 1$  subunit, anti- $\beta 1$  subunit or anti- $\gamma 2$  subunit GABA<sub>A</sub> receptor antibody.

Western blots of membrane proteins isolated from transfected HEK293 cells were probed with anti- $\alpha 1$  subunit (A), anti- $\beta 1$  subunit (B) or anti- $\gamma 2$  subunit (C) antibodies. In the lanes are samples from HEK293 cells transfected with the cDNA combinations: (1) wild-type  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2L$  subunits; (2) wild-type  $\beta 1$ ,  $\gamma 2L$  and mutant  $\alpha 1_{243P-A}$  subunits; (3) wild-type  $\alpha 1$ ,  $\gamma 2L$  and mutant  $\beta 1_{243P-A}$  subunits: UT, untransfected HEK293 cell membranes. Molecular marker sizes (kDa) are marked (and two of the marker proteins are shown in C). The  $\alpha 1$  subunit is the band at 50 kDa in A. In B, the heavy band at 52 kDa corresponds to the  $\beta 1$  subunit and in C, the heavy band at 49 kDa corresponds to the  $\gamma 2$  subunit.

### 6.3 Discussion

In this chapter, site-directed mutagenesis was used to mutate two amino acids that are highly conserved among members of the ligand-gated ion channel superfamily. The aspartic acid which is at position 149 in the bovine  $\alpha 1$  amino acid sequence was mutated to asparagine and the aspartic acid in the comparable position in the bovine  $\beta 1$  subunit was mutated to either asparagine or glutamic acid. In the putative M1 transmembrane domain, the proline was mutated to alanine in both the  $\alpha 1$  and  $\beta 1$  subunits. The effects of mutations within the  $\alpha 1$  and  $\beta 1$  GABA<sub>A</sub> receptor subunits were studied through [<sup>3</sup>H]Ro15-1788 and [<sup>3</sup>H]muscimol binding analysis of membrane preparations isolated from transiently transfected HEK293 cells.



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### 6.3.1 Amino acid exchange at residue 149 (wild-type D) within the putative cysteine loop

Is there an agonist protobinding site for ligand-gated ion channels? Within the ligand-gated ion superfamily, a highly conserved sequence of 15 amino acids (cys loop) in the N-terminal extracellular region, has been modeled as a rigid amphiphilic  $\beta$ -hairpin and postulated to act as a major determinant of a conserved structural binding cleft for an agonist. Aspartic acid is one of only two invariant acidic residues present in the extracellular region of ligand-gated ion channel subunits and available for interaction with the positively charged amine group of agonists such as GABA. In the Cockcroft model (Cockcroft *et al.*, 1990) of the binding complex of ligand-gated ion channel receptors, the invariant aspartate residue at position 11 of the cys-loop is postulated as the anionic site that interacts with the positively charged amine group of agonists and a local dipole within the  $\pi$  electron system of agonists is modeled in a favorable orientation in the electrostatic field of the invariant aspartate. In this model, a marked two residue periodicity in the average hydrophobicity predicted a  $\beta$ -strand to occur over positions 1 through 7 and over positions 10 through 14. A type VIa  $\beta$ -turn is predicted to start at position 7 and is consistent with invariance of the proline residue at position 9 of the cys-loop (Cockcroft *et al.*, 1990). In this model, the invariant aspartate residue appears on the hydrophilic face. This model predicts that when residues within the cys loop are substituted for alanine, there is little change in the main chain conformation.

In this chapter, we described mutagenesis of the aspartic acid in the cysteine loop of the bovine  $\alpha 1$  and  $\beta 1$  GABA<sub>A</sub> receptor subunits which resulted in either a nonconservative change leading to loss of a negative charge (asparagine) or a conservative change in which a negatively charged residue was maintained at position 11 (glutamic acid). Transient expression of these mutated subunits with wild-type subunits in an  $\alpha 1$ ,  $\beta 1$   $\gamma 2L$  configuration, in HEK293 cells, resulted in: 1) loss of GABA<sub>A</sub> receptor Ro15-1788 binding ( $\alpha 1_{D-N}$ , wt $\beta 1$ , wt $\gamma 2L$ ), 2) no change in Ro15-1788 binding (wt $\alpha 1$ ,  $\beta 1_{D-E}$ , wt $\gamma 2L$ ), and 3) a statistically significant increase in GABA<sub>A</sub> receptor Ro15-1788 binding affinity (wt $\alpha 1$ ,  $\beta 1_{D-N}$ , wt $\gamma 2L$ ). No muscimol binding was detected for any of the mutant receptor combinations expressed in HEK293 cells. Since muscimol has both a lower specific activity and a lower binding affinity than Ro15-1788, reduction of the  $B_{max}$  for receptor combinations including either a mutated  $\alpha 1$  or  $\beta 1$  subunit in the aspartic acid of the cysteine loop obviously had a dramatic effect on the ability to detect the membrane-localized GABA<sub>A</sub> receptor complexes through muscimol binding. In the experiment including a mutant  $\alpha 1_{D-N}$  and wt $\beta 1$  and wt $\gamma 2L$  subunits, neither Ro15-1788 or muscimol binding was detected. Western blot analysis of membrane preparations used in the receptor binding analysis, demonstrated that immunoreactive products were present for

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both the  $\alpha 1$  and  $\beta 1$  subunits, and mRNA analysis confirmed the presence of all three subunit ( $\alpha 1$ ,  $\beta 1$ , and  $\gamma 2L$ ) mRNAs in the transiently transfected cells. The presence of GABA<sub>A</sub> receptor protein in the membrane preparations, but a lack of Ro15-1788 and muscimol binding suggest that the aspartic acid residue in the cysteine loop can play a role in both agonist and antagonist binding. Conservation of the negative charge at the aspartic acid site in the cysteine loop of the  $\beta 1$  subunit resulted in receptors that were identical to the wild-type receptors in Ro15-1788 binding affinity. In further comparison of wild-type and mutant receptors containing the  $\beta 1_{D-E}$  subunit, there was a significant decrease in the  $B_{max}$ , and lack of muscimol binding for the mutant receptor membrane preparations. As discussed earlier, a decrease in the  $B_{max}$  suggests that the assembly or efficient trafficking of the GABA<sub>A</sub> receptor complex was impaired. An increase in affinity for Ro15-1788 in the mutant receptor containing the  $\beta 1_{D-N}$  subunit further suggests that the aspartic acid site in the cysteine loop plays a role in antagonist binding.

In comparison to our results, Amin, Dickerson and Weiss, (1994) also found that mutation of  $\alpha 1$  and  $\gamma 2$  GABA<sub>A</sub> receptor subunits to glutamic acid (conservative change) at the aspartic acid position in the cysteine loop resulted in either no impairment of GABA-mediated activation or a slight decrease in the  $EC_{50}$ , when  $\alpha 1$ ,  $\beta 2$  and  $\gamma 2$  subunit cRNAs were co-expressed in *Xenopus* oocytes. Similar to our results with the  $\beta 1_{D-N}$  mutation, Amin, Dickerson and Weiss, (1994) found that substitution of the aspartic acid position in the cysteine loop with asparagine in the  $\beta 2$  subunit resulted in no GABA-activated currents. In *Xenopus* oocyte experiments, substitution of aspartic acid for glutamic acid (conservation of charge) in the  $\beta 2$  subunit resulted in GABA-activated currents, where there was no effect on the  $EC_{50}$ , a dramatic decrease in the amplitude of the current and a decrease in the Hill coefficient, suggesting a decrease in the cooperativity of channel activation (Amin, Dickerson and Weiss, 1994). Mutations involving changes to amino acids at positions 5 (leucine) and position 9 (proline) in the cysteine loop of the  $\beta 2$  GABA<sub>A</sub> receptor subunit, when co-expressed in *Xenopus* oocytes with wild-type  $\alpha 1$ ,  $\gamma 2$  subunits generally resulted in only a slight impairment in activation of the GABA channel, suggesting that this impairment may occur indirectly through structural alterations of the protein (Amin, Dickerson and Weiss, 1994).

Mutation of the cysteines in the cysteine loop to serine resulted in little or no expression of the GABA<sub>A</sub>  $\gamma$  subunit in the receptor complexes, as determined by blockade of the GABA induced current by 10  $\mu M$  Zn<sup>2+</sup>, as well as a lack of potentiation by 1  $\mu M$  diazepam (Amin, Dickerson and Weiss, 1994). Lack of functional expression of subunits with a mutation of cysteine to serine in the cysteine loop suggests that the cysteine loop may be required for proper folding or assembly of the subunits in the plasma membrane.

Conservative substitutions of amino acids forming the binding sites of ACh-, glycine-, and GABA-operated ion channels typically yield  $EC_{50}$  increases of at least 10-fold, whereas less conservative substitutions typically abolish agonist-dependent gating (Galzi *et al.*, 1991a; O'Leary and White, 1992; Tomaselli *et al.*, 1991; Vandenberg, Handford, and Schofield, 1992; Amin and Weiss, 1993). Interestingly, mutation of aspartic acid in the cysteine loop of the homomeric glycine receptor did not impair sensitivity to action by glycine (Vandenberg *et al.*, 1993) and monoclonal antibodies directed against the cysteine loop of the nACh channel indicated that the cysteine loop was not part of the agonist binding site (Lennon *et al.*, 1985; Criado *et al.*, 1986). Cysteine to serine mutations at the conserved cysteines in the extracellular domain of the glycine receptor abolished expression of functional glycine receptors in *Xenopus* oocytes (Akagi, Hirai and Hishinuma, 1991). Disruption of the disulfide loop structure in the nACh channel  $\alpha$  or  $\beta$  subunits led to intracellular retention of the assembled receptor complexes, suggesting that the cysteine loop may be required for efficient transport of the receptors to the plasma membrane (Sumikawa and Gehle, 1992). These data indicate that these 2 cysteines are critical for AChR and glycine receptor function but do not define the precise role they play.

### 6.3.2 Amino acid exchange at residue 243 (wild-type P) in putative transmembrane domain 1 (M1)

Within M1, an internal proline (Pro243) and cysteine (Cys244) are strongly conserved for all subunits and species of GABA<sub>A</sub>, nicotinic acetylcholine and glycine receptors. The residues immediately preceding and following Pro-Cys are almost always aliphatic but are not strictly conserved. The cysteine residue is invariant among vertebrate AChR and is replaced only by serine or threonine in some invertebrate AChR subunits (Hermans-Borgmeyer *et al.*, 1986) and in the  $\beta$  subunits of GABA<sub>A</sub> and glycine receptors. Thus, the M1 cysteine appears to be a critical residue that can, however, tolerate conservative substitutions. In comparison the proline residue at position 243 is invariant across all species and subunits of GABA<sub>A</sub>, nicotinic acetylcholine and glycine receptor subunits. To determine the role of this invariant proline in agonist and antagonist binding, we mutated pro243 in the  $\alpha 1$  and  $\beta 1$  GABA<sub>A</sub> receptor subunits to alanine.

In HEK293 cells, transient expression of wild-type  $\beta 1$ ,  $\gamma 2L$  and mutant  $\alpha 1_{243\text{pro-ala}}$  subunits resulted in receptors with a statistically significant increase in Ro15-1788 binding affinity, a significant decrease in the  $B_{\text{max}}$  and no detectable muscimol binding when compared with  $K_{\text{ds}}$  and  $B_{\text{max}}$ s for muscimol and Ro15-1788 binding of wild-type  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2L$  GABA<sub>A</sub> receptors. Examination of the membrane preparations from transiently transfected HEK293 cells expressing the mutant  $\alpha 1_{243\text{pro-ala}}$  subunit, by Western blot

analysis with an  $\alpha 1$  subtype-specific antibody, revealed the absence of the  $\alpha 1_{243\text{pro-ala}}$  subunit protein. Western blot analysis with a  $\beta 1$  subunit-specific antibody demonstrated however, the presence of the  $\beta 1$  GABA<sub>A</sub> subunit protein in the same membrane preparation used to determine the presence or absence of the  $\alpha 1_{243\text{pro-ala}}$  subunit protein. A Student's t-test also demonstrated that there was no significant difference between the Ro15-1788 binding affinity for  $\beta 1$  and  $\gamma 2L$  only receptor subunits expressed in HEK293 cells and the Ro15-1788 binding affinity for  $\alpha 1_{243\text{pro-ala}}$ ,  $\beta 1$ ,  $\gamma 2L$  subunits expressed in HEK293 cells. The inability to detect the  $\alpha 1_{243\text{pro-ala}}$  subunit in the HEK293 membranes and the lack of statistical significance between the  $K_{ds}$  and  $B_{max}$ s for the  $\beta 1, \gamma 2L$  and  $\alpha 1_{243\text{pro-ala}}$ ,  $\beta 1$ ,  $\gamma 2L$  subunit combinations indicates that transient expression of  $\alpha 1_{243\text{pro-ala}}$ ,  $\beta 1$ ,  $\gamma 2L$  subunit in HEK293 cells results in receptors with  $\beta 1$ ,  $\gamma 2L$  subunits only.

Further evidence of the absence of the  $\alpha 1_{243\text{pro-ala}}$  mutant subunit from the HEK293 membrane preparations was demonstrated in whole cell patch recordings from the transiently transfected HEK293 cells. As reported by Zamin (Cambridge thesis, 1993), the response elicited by 100  $\mu\text{M}$  GABA from HEK 293 cells expressing the  $\alpha 1_{\text{pro}243\text{ala}}, \beta 1, \gamma 2L$  receptor combination, in separate experiments was blocked by 100  $\mu\text{M}$  bicuculline and inhibited by 100  $\mu\text{M}$   $\text{Zn}^{2+}$ . In the HEK293 cells transiently expressing the mutant  $\alpha 1$  subunit, there was also no potentiation by 50  $\mu\text{M}$  diazepam and an increase in the rate of repolarization following 10  $\mu\text{M}$   $\beta$ -CCM (methyl  $\beta$ -carboline 3-carboxylate) application (Zamin, Cambridge thesis, 1993). As seen with the  $\alpha 1_{243\text{pro-ala}}, \beta 1, \gamma 2L$  combination, when  $\beta 1, \gamma 2L$  subunits only were expressed in HEK293 cells, currents elicited by 100  $\mu\text{M}$  GABA, in separate experiments were inhibited by 100  $\mu\text{M}$   $\text{Zn}^{2+}$ , potentiated by 50  $\mu\text{M}$  diazepam yet were unaffected by 10  $\mu\text{M}$   $\beta$ -CCM (Zamin, Cambridge thesis, 1993). In all experiments reported above voltages were clamped at -60 mV. For zinc sensitivity, there was no statistical difference between mutant and  $\beta 1, \gamma 2L$  combinations expressed in HEK 293 cells ( $p=0.3$ ) (Zamin, Cambridge thesis, 1993). The ability of  $\text{Zn}^{2+}$  to block GABA responses was used as an indicator for the presence of the  $\gamma 2L$  subunit in the receptor complex and modulation of the GABA response to the  $\beta$ -carboline,  $\beta$ -CCM was used to differentiate the expression of a  $\beta 1, \gamma 2L$  combination from the expression of an  $\alpha 1, \beta 1, \gamma 2L$  combination, since it has been reported that the GABA response elicited by an expressed  $\beta 1, \gamma 2S$  subunit combination is antagonised by DMCM (Knoflach *et al.*, 1991). These results suggest that the  $\alpha 1$  subunit-specific antibody did not detect the presence of the  $\alpha 1_{243\text{pro-ala}}$  mutant subunit in the HEK293 membrane preparations, because either the  $\alpha 1$  mutant subunit was present at concentrations below detection by the antibody or because the mutation in the  $\alpha 1$  subunit had decreased the affinity of the protein for the antibody. Further analysis will be necessary to determine the stoichiometry of the mutant receptor complex.

In HEK293 cells, transient expression of wild-type  $\alpha 1$ ,  $\gamma 2L$  and mutant  $\beta 1_{243\text{pro-ala}}$  subunits resulted in receptors with a statistically significant increase in muscimol binding affinity, no significant change in the  $B_{\text{max}}$  and no detectable alteration in Ro15-1788 binding kinetics, when compared with  $K_{\text{d}}$ s and  $B_{\text{max}}$ s for muscimol and Ro15-1788 binding of wild-type  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2L$  GABA<sub>A</sub> receptors. Examination of the membrane preparations from transiently transfected HEK293 cells expressing the mutant  $\beta 1_{243\text{pro-ala}}$  subunit, by Western blot analysis with a  $\beta 1$  subtype-specific antibody, confirmed the presence of the  $\beta 1_{\text{pro-ala}}$  subunit protein. In HEK 293 cells transiently expressing the  $\alpha 1$ ,  $\beta 1_{243\text{pro-ala}}$ ,  $\gamma 2L$  GABA<sub>A</sub> receptor subunits, whole cell patch recording demonstrated that currents elicited by application of 100  $\mu\text{M}$  GABA were blocked by 100  $\mu\text{M}$  bicuculline and potentiated by 50  $\mu\text{M}$  diazepam (Zaman, Cambridge thesis, 1993). Expression of the  $\alpha 1$ ,  $\beta 1_{243\text{pro-ala}}$ ,  $\gamma 2L$  combination of subunits in HEK293 cells resulted in a decrease in the  $\text{EC}_{50}$  for GABA which was statistically greater than wildtype (Zamin, Cambridge thesis, 1993), an observation which is in agreement with the binding results reported in this chapter. In L929 fibroblasts, the GABA<sub>A</sub> receptor  $\alpha 1\beta 1_{243\text{pro-ala}}\gamma 2L$  subtype had increased GABA affinity (Greenfield *et al.*, unpublished results), as was reported in this chapter for [<sup>3</sup>H]muscimol binding of the  $\alpha 1\beta 1_{243\text{pro-ala}}\gamma 2L$  subtype in HEK293 cells.

Barbiturates have long been known to enhance postsynaptic GABA<sub>A</sub> currents (Schulz and Macdonald, 1981; Ransom and Barker, 1976; Macdonald and Barker, 1978) both by modulation of GABA-induced currents and by direct activation in the absence of GABA, and these effects are thought to be responsible for the anesthetic, sedative and anticonvulsant actions of the barbiturates (Schulz and Macdonald, 1981; Macdonald and Barker, 1978). In acutely transfected L929 cells the  $\alpha 1\beta 1_{243\text{pro-ala}}\gamma 2L$  subtype demonstrated selectively reduced sensitivity to barbiturates, suggesting a role for the  $\beta$  subunit in barbiturate-induced enhancement of GABA<sub>A</sub> receptor currents. The  $\beta 1_{243\text{pro-ala}}$  mutation may affect the barbiturate binding site directly, but it is more likely that it changes the allosteric coupling between agonist binding and channel gating.

As evidence against the possibility that there are shared or overlapping sites of action for GABA and barbiturates, there are several mutations which provide a precedence for separate sites of binding for GABA and its modulators. For example, in two regions between the cysteine loop and the M1 transmembrane domain, threonine (T160, T202) and tyrosine (Y157, Y205) residues, through site-directed mutagenesis have been identified as critical sites for the activation of currents by GABA but not by pentobarbital.

In conclusion, we have shown that mutations of the highly conserved amino acids in the putative cysteine loop and transmembrane domain 1 of ligand-gated ion channel can have significant effects on either agonist or antagonist binding and on subunit trafficking or assembly. In particular: 1) amino acid exchange of aspartic acid to asparagine in the

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putative cysteine loop of  $\alpha 1$  GABA<sub>A</sub> receptor subunit results in loss of agonist binding, probably due to an impairment in the assembly or processing of the mutant subunit; 2) amino acid exchange of aspartic acid to asparagine in the putative cysteine loop of the  $\beta 1$  GABA<sub>A</sub> receptor subunit results in a significant increase in antagonist (Ro15-1788) binding; 3) amino acid exchange of aspartic acid to glutamic acid (conservative change) in the putative cysteine loop of the  $\beta 1$  GABA<sub>A</sub> receptor subunit results in a reduction in the B<sub>max</sub> for flunitrazepam suggesting that the assembly or efficient trafficking of the GABA<sub>A</sub> receptor complex is impaired. Mutation in the highly conserved proline residue of the  $\beta 1$  GABA<sub>A</sub> receptor subunit putative M1 transmembrane domain results in a significant decrease in the binding affinity of muscimol, while the same mutation in the  $\alpha 1$  subunit of the GABA<sub>A</sub> receptor results in no detectable binding by muscimol or flunitrazepam and an absence of  $\alpha 1$  immunoreactivity in membrane preparations from transiently transfected HEK293 cells. The inability of the  $\alpha\beta\gamma$  ternary complex to assemble when the M1 mutation is in the  $\alpha$  subunit but not the  $\beta$  subunit suggests that the proline in M1 is important for oligomeric assembly in the  $\alpha$  subunit but not the  $\beta$  subunit. An alternative interpretation of this difference includes a consideration of the composition of the ternary structure. If the pentameric GABA<sub>A</sub> receptor assembly is composed of  $(\alpha 1)_2(\beta 1)_1(\gamma 2L)_2$ , then a mutation in an  $\alpha$  subunit could have a greater distortion on the structure of the receptor complex.

# **Chapter 7**

## **Discussion**

## 7.1 Introduction

Molecular diversity within the GABA<sub>A</sub> receptor ion channel gene family arises through subunit multiplicity, temporal and spatial regulation of gene expression and alternative splicing of subunit mRNAs. At the protein level, the sensitivity of the GABA<sub>A</sub> receptor complex to agonists and modulators of the GABA response, occurs through the heteromultimerization of GABA<sub>A</sub> subunits which thereby generates vast combinatorial possibilities in terms of GABA<sub>A</sub> receptor composition. Further diversification of GABA<sub>A</sub> receptor responses occur through subunit trafficking and post translational modifications, such as phosphorylation. In this thesis, I have described the developmental onset of GABA<sub>A</sub> receptor gene expression, the distribution and temporal expression of GABA<sub>A</sub> receptor subunit genes and splice variants within the developing and adult murine central nervous system, and the role of highly conserved amino acids within the cysteine loop and M1 transmembrane domains in structural characteristics associated with binding of agonist and antagonists at these sites. These results must be considered in the context of similiar studies involving voltage-gated ion channels and other members of the ligand-gated ion channel receptor superfamily.

## 7.2 Diversity at the gene level

A considerable amount of ion channel molecular diversity stems from the existence of multiple gene members within a family. Typical ion channel families have six or more genes coding an  $\alpha$  subunit protein, as well as several auxiliary subunits ( $\gamma$ ,  $\delta$ ,  $\epsilon$ ) that act to modulate ion channel function. The  $\beta$  subunits can be integral parts of the ion channel (ACh and GABA) or act as modulators of channel activity (Kv $\beta$ 1) (Isom, De Jongh and Catterall 1994). Although NMDA receptors lack a  $\beta$  subunit, they share a similiar complexity to ACh and GABA receptors, in that the NMDA receptor complex is composed of the essential subunit NMDAR1, which when combined with various NMDAR2 subunits (A-D) yields functional diversity (Monyer *et al.*, 1992). So far, only one type of subunit has been detected for AMPA/KA receptors based on a 70 to 85 percent amino acid identity among identified sequences, however as is the case with GABA<sub>A</sub> receptor subunits, formation of tetrameric or pentameric structures with varying subunit composition clearly leads to an enormous potential diversity of AMPA/KA ion channel variants.

Receptor complexity becomes even greater if the ordering of subunits is important and therefore a receptor consisting of just two subunits  $\alpha$  and  $\beta$ , with a stoichiometry of  $\alpha_2\beta_3$ , could exist for example as  $\alpha\alpha\beta\beta\beta$ , or  $\alpha\beta\alpha\beta\beta$ . If the specific temporal and spatial expression patterns of gene products are carefully controlled, for example, by tissue specific promoters, then precise functional variations are possible. Expression of nAChR at the neuromuscular junction is one example of this process. During embryonic development,



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nAChR channels consist of  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits. This channel complex ( $\alpha\beta\delta\gamma$ ) has the properties of; 1) a more rapid turnover, 2) longer channel open time and 3) smaller conductance, than the adult nACh receptor  $\alpha\beta\delta\varepsilon$  subunit combination. The adult channels also tend to be clustered at subsynaptic sites while the embryonic form is found over the entire muscle surface. Thus the presence of the  $\gamma$  versus  $\varepsilon$  subunit drastically alters the properties of the channel. An exception to the restricted temporal expression of the  $\gamma$  subunit occurs in response to denervation.

Another member of the ligand-gated ion channel receptor superfamily, the glycine receptor also demonstrates developmentally-restricted subunit expression. In this case, the glycine receptor  $\alpha 1$  subunit is found almost exclusively in the adult central nervous system, while  $\alpha 2$  expression is confined to the embryo and the first three weeks of life (Becker, Hoch and Betz 1988; Kuhse, Schmieden and Betz 1990). The predominantly embryonic form of the glycine receptor ( $\alpha 2\beta$ ) has a single channel conductance that is similar to the adult form ( $\alpha 1\beta$ ), but differs from the adult form in that  $\alpha 2\beta$  receptors have a much longer mean open time (Takahashi *et al.*, 1992). Consistent with these glycine receptor properties, is the observation that dorsal horn neurons have more rapid IPSPs in adult cells (Takahashi *et al.*, 1992).

Other ligand-gated ion channels, however, do not have specific embryonic subunits, yet do demonstrate developmental changes in expression. One example of this developmental regulation occurs in cerebellar granule cells, where changes in NMDA receptor subunit composition may play a role in cell migration during early postnatal development (Komuro and Rakic 1993). Single-channel analysis of developing granule cells indicates the expression of a high conductance channel with a long open time, in migrating neurons is replaced by a lower conductance channel with a brief open time in the adult (Farrant *et al.*, 1994). These properties correspond well with the properties of heterologously expressed NMDA receptors of NR1 and NR2A/B (high conductance), and NR1 and NR2C (low conductance) respectively (Monyer *et al.*, 1992; Stern *et al.*, 1992). mRNA for the NR2C subunit appears postnatally and is predominantly localized to cerebellum (Monyer *et al.*, 1992). Other areas of the adult CNS retain the high conductance NR2A/B type of channel.

Assuming a pentameric structure, with no constraints on assembly, there are 151,887 possible subunit combinations of the GABA receptor (Burt and Kamatchi, 1991), excluding splice variants. More realistically, if one assumes a stoichiometry of  $\alpha_2\beta_1\gamma_2$  there are 137 variants. Although this is still an impressive number, its relevance is limited unless at least some of these variants exhibit different biological activities. Distribution patterns described in Chapter 4 of this thesis support the hypothesis that diversity is achieved in part, through differential subunit composition. Differences in the temporal regulation of GABA<sub>A</sub> receptor genes leads to distinct functional properties. This is particularly evident during

embryogenesis and early postnatal development where activation of hippocampal GABA<sub>A</sub> receptors leads to marked membrane depolarization (Ben-Ari *et al.*, 1989), rather than hyperpolarization.

### 7.3 Developmental regulation of GABA<sub>A</sub> receptor subunit expression

#### 7.3.1 Embryonic differences in the expression of GABA<sub>A</sub> receptor subunit mRNAs between rat and mouse.

In Chapter 3, RT-PCR analysis of embryonic, postnatal and adult total RNA isolated from the entire embryo or from whole brain indicated that in the mouse, GABA<sub>A</sub> receptor  $\alpha 4$ ,  $\alpha 5$ ,  $\beta 2$ ,  $\gamma 1$ , and  $\gamma 3$  subunit expression begins during the later stages of embryogenesis, while GABA<sub>A</sub> receptor  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 6$ ,  $\beta 1$  and  $\gamma 2$  subunit mRNAs are expressed throughout murine development and in the adult murine CNS. This pattern of murine GABA<sub>A</sub> receptor subunit expression differs from *in situ* hybridization studies characterizing GABA<sub>A</sub> receptor subunit mRNA distribution during rat embryogenesis (Laurie *et al.*, 1992). Laurie *et al.*, (1992) found abundant  $\alpha 3$  expression in the 14 day rat embryo, but very little expression of  $\alpha 4$  GABA<sub>A</sub> receptor subunit mRNA and an absence of  $\alpha 6$  GABA<sub>A</sub> receptor mRNA at rat embryonic day 14. These results differ from the RT-PCR results presented in Chapter 3, where, for example, the  $\alpha 6$  GABA<sub>A</sub> receptor mRNA was detectable at E14 in the mouse. These differences in detection of GABA<sub>A</sub> receptor subunit mRNAs may be a reflection of the greater assay sensitivity of RT-PCR over *in situ* hybridization analysis.

In the study by Laurie *et al.*, (1992) the  $\beta 1$  subunit transcript was present in the E14 rat embryo, but the  $\beta 2$  and  $\beta 3$  GABA<sub>A</sub> receptor mRNAs were rare or absent at embryonic day 14 in rat development. These rat developmental results for GABA<sub>A</sub> receptor  $\beta$  subunit mRNA expression are similar to the results for the mouse E14 day embryo, reported in Chapter 3 of this thesis. At embryonic day 14 in the rat, both  $\gamma 1$  and  $\gamma 2$  GABA<sub>A</sub> receptor subunit transcripts were detectable (Laurie *et al.*, 1992), while the  $\gamma 3$  transcript was expressed at very low levels. In contrast, murine transcripts for  $\gamma 1$  and  $\gamma 3$  were not detected by RT-PCR analysis, while the  $\gamma 2$  transcript was present in all developmental stages studied.

Although embryonic day 14.5 in the rat corresponds to embryonic day 13 in the mouse, the differences observed between rat and murine GABA<sub>A</sub> receptor subunit expression cannot be explained by this offset in rat and murine prenatal development. As mentioned above, RT-PCR analysis is a more sensitive detection assay for identifying the presence of a transcript and may account for the presence of subunit mRNAs, which are not observed through *in situ* hybridization studies.

It should also be noted that the RT-PCR described in Chapter 3 of this thesis was used as

a descriptive tool for determining the type of GABA<sub>A</sub> receptor subunit transcripts at early and later stages of murine embryogenesis and was not used to quantitate the levels of the various transcripts present at each developmental stage. The importance of determining what GABA<sub>A</sub> receptor subunit transcripts are present during embryonic development is manifest in the important role GABA<sub>A</sub> receptors play in such processes as neurite extension and synaptogenesis during neonatal development (Hansen *et al.*, 1987; Meier *et al.*, 1987; Wolff *et al.*, 1987) and in the observation that administration of benzodiazepines during pregnancy causes biochemical and behavioral manifestations in the progeny that can persist into adulthood (Simmons *et al.*, 1984a,b; Kellogg, 1988). Ma *et al.*, 1993 used a hybrid approach that involved RT-PCR and *in situ* hybridization analysis to identify all GABA<sub>A</sub> receptor subunit mRNAs  $\alpha 6$  which was not detectable by either method and  $\delta$ , which was weakly detected only by RT-PCR. Two anatomically distinctive sets of subunit mRNAs were found by *in situ* hybridisation within the ventricular zone and mantle zone of the E19 day rat. The mRNA expressions declined during postnatal development, by region-selective depletion, with  $\alpha 4$ ,  $\alpha 5$ ,  $\beta 1$ ,  $\beta 2$  and  $\gamma 3$  subunit mRNAs becoming barely detectable (Ma *et al.*, 1993).

### 7.3.2 Postnatal Expression of GABA<sub>A</sub> receptor subunit mRNAs in murine CNS during postnatal development

#### 7.3.2.1 GABA<sub>A</sub> ion channel receptor pharmacology

Comparative levels of GABA<sub>A</sub> receptor subunit mRNAs were described in Chapter 4 of this thesis, where *in situ* hybridization was used to determine the distribution of the transcripts in the postnatal and adult murine CNS. RT-PCR demonstrates the presence, in the P0 CNS, of all GABA<sub>A</sub> receptor subunit mRNAs studied and this is in agreement with the *in situ* hybridization studies described in Chapter 4. The significance of which GABA<sub>A</sub> receptor subunit transcripts are the predominant isoforms during various stages of postnatal development is evident in the changes in GABA<sub>A</sub> receptor pharmacology during development. As has been observed in ligand binding studies, benzodiazepine, BZ type II receptors, are the predominant form of GABA<sub>A</sub> receptor subtype found at birth and in the early stages of postnatal development. When  $\alpha$  subunit variants are transiently co-expressed with  $\beta 1-3$  and  $\gamma 2$  GABA<sub>A</sub> receptor subunits in HEK-293 cells, the BZ type II subtype of GABA<sub>A</sub> receptor is observed. BZ I and BZ II receptors distribute unevenly over the rat brain. BZ I receptors predominate in the cerebellum, but are rare in the hippocampus (Faull and Villiger, 1988, Faull, Villiger and Holford, 1987, Olsen, McCabe and Wamsley 1990). In contrast, BZ II receptors are strongly expressed in the hippocampus and nearly absent from the cerebellum. Both receptor subtypes are equally expressed in the cortical layers

(Faull and Villiger, 1988; Faull, Villiger and Holford 1987; Olsen, McCabe and Wamsley 1990). BZ type II receptors are also enriched in the striatum and spinal cord (Lo, Strittmatter and Snyder, 1983; Olsen and Tobin, 1990, Sieghart *et al.*, 1985).  $\alpha 5\beta 2\gamma 2$  receptors have similarly low affinities for CL 218,872 and 2-oxoquazepam as  $\alpha 2\beta x\gamma 2$  and  $\alpha 3\beta x\gamma 2$  receptors. However, the binding properties for imidazopyridines like zolpidem and alpidem differ from conventional GABA<sub>A</sub>/BZ II receptors (Pritchett and Seeburg, 1990), and hence sensitivity to these imidazopyridines constitutes a unique subtype of the BZ II receptors. Recombinant receptors expressing  $\alpha 6\beta 2\gamma 2$  in HEK293 cells bind with high affinity muscimol and the imidazo-1,4-benzodiazepin, Ro-15-4513. Other benzodiazepines and  $\beta$ -carbolines are either not recognized by this receptor or at an affinity two to three orders of magnitude lower than that of  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  or  $\alpha 5$ -containing receptors.

To add further complexity, Nusser *et al.*, (1996) have recently demonstrated that some subunits of the GABA<sub>A</sub> receptor expressed in the hippocampus are selectively localized at subsets of GABAergic synapses. The  $\alpha 1$  subunit has been found in most GABAergic synapses on all postsynaptic domains of pyramidal cells. In contrast, the  $\alpha 2$  subunit was located only in the subset of synapses on the somata and dendrites, but in most synapses on axon initial segments innervated by axo-axonic cells.

### 7.3.3 Differences in the expression of GABA<sub>A</sub> receptor subunit mRNAs between rat and mouse at P0.

One striking difference between  $\alpha$  GABA<sub>A</sub> receptor subunit mRNA expression at P0 in the rat and mouse CNS, is the detection, in the mouse of the  $\alpha 6$  subunit transcript in the external granule cell layer of the cerebellum. Another example of differences between rat and mouse expression of GABA<sub>A</sub> receptor subunit mRNAs during postnatal development is found in the hippocampus, where the  $\gamma 1$  subunit transcript is abundantly expressed in both pyramidal cells and dentate granule cells in the P0 rat brain, but is absent from all regions of the hippocampus in the P0 mouse brain. The  $\gamma 2T$  subunit mRNA was expressed at high levels in the dentate granule cells of the P0 mouse brain, but was only weakly present in the P0 rat dentate granule cells. The  $\gamma 3$  subunit mRNA was present in the P0 mouse cerebellum, but was absent from the P0 rat cerebellum. Changes in  $\gamma 2$  subunit expression during development are believed to play a key role in the change from zinc sensitive embryonic GABA<sub>A</sub> receptor responses to zinc insensitive responses in the adult (Smart *et al.*, 1991; Xie and Smart, 1991).

### 7.3.4 Differences in the expression of GABA<sub>A</sub> receptor subunit mRNAs between rat and mouse at P6 and P7.

By postnatal days 6 and 7, the GABA<sub>A</sub> receptor  $\alpha 1$  subunit transcript was abundantly

expressed in the murine CNS, while the same transcript was only weakly expressed in the P6 rat CNS.  $\alpha 2$  transcript expression also differed between the two species. In the P6 rat CNS,  $\alpha 2$  expression was abundant in the hippocampus, septum, cortex, and various thalamic nuclei. In contrast, in the murine P7 CNS,  $\alpha 2$  mRNA expression was abundant in hippocampal pyramidal cells, but only moderately expressed in dentate gyrus granule cells. In the P7 mouse, the  $\alpha 2$  transcript was weakly expressed in the cortex and several thalamic nuclei. In the P7 mouse CNS, the  $\alpha 6$  transcript was also abundantly expressed throughout the internal and external granule cell layers, which is in contrast to expression of the  $\alpha 6$  transcript in the P6 rat cerebellum, where the transcript is found in only the outer granule cell layer.

One of the major differences between rat and mouse GABA<sub>A</sub> receptor ontogeny occurs around postnatal days 6 and 7. At this period in postnatal development, GABA<sub>A</sub> receptor  $\beta$  and  $\gamma$  subunit mRNA expression occurs in the cerebellum, where there is an absence of  $\beta 1$ ,  $\beta 3$ ,  $\gamma 1$ , and  $\gamma 3$  expression in the rat. There is also virtually no expression of the  $\delta$  subunit in the rat P6 CNS, whereas in the mouse CNS, the  $\delta$  subunit mRNA can be found in cerebellar granule cells, throughout all layers of the cortex and in some thalamic nuclei.

### 7.3.5 Differences in the expression of GABA<sub>A</sub> receptor subunit mRNAs between rat and mouse at P12 and P14.

By P12, the  $\alpha 2$  transcript is found in both the rat and mouse cerebellar granule cells. While in contrast to the distribution of the  $\alpha 3$  subunit in rat (Laurie *et al.*, 1992), the  $\alpha 3$  transcript is absent from mouse CA3 pyramidal cells and dentate gyrus granule cells. By P12, the  $\beta 1$  subunit is absent from the rat cerebellum, but is expressed at moderate levels in mouse P14 cerebellum. This discrepancy is also true for the  $\beta 3$  subunit mRNA, where the  $\beta 3$  is abundantly expressed in the mouse P14 cerebellum, but is absent or weakly expressed in the rat P12 cerebellum. The  $\delta$  subunit mRNA is present in the same distribution and at similar levels in the P12 rat and P14 mouse CNS.

The differences that exist between the development of GABA<sub>A</sub> receptor subunit mRNAs in the rat and mouse central nervous systems will have significant influences on subunit combinations and hence GABA<sub>A</sub> receptor composition. Differences in channel gating and desensitization rates are likely to be more important physiologically and have been described for several combinations of subunits (Levitan *et al.*, 1988a and b; Saxen and MacDonald 1994). Recombinantly expressed GABA<sub>A</sub> receptors consisting of  $\alpha 1$ ,  $\alpha 3$  or a mixed  $\alpha 1\alpha 3$ , coinjected with a  $\beta$  and  $\gamma$  subunit, had up to a five fold range in GABA sensitivity, differing sensitivity to diazepam, and different kinetics (Verdoorn 1994). Different subunit combinations also have differential sensitivity to endogenous allosteric modulators such as zinc and the neurosteroids (Draguhn *et al.*, 1990; Puia *et al.*, 1991; Smart *et al.*, 1991), and

differential phosphorylation by protein kinases (Krishek *et al.*, 1994).

#### 7.4 Post-transcriptional Modifications

The majority of voltage- and ligand-gated channel genes undergo alternative splicing of mRNA (a notable exception is the mammalian *Shaker* potassium channel gene  $\alpha$  subunit). Extensive splicing occurs in AMPA/kainate receptors, where AMPA receptors (Glu-R A-D) contain a functional module of 38 amino acids in one of two versions, flip or flop. (Sommer *et al.*, 1990b). AMPA/kainate receptor subunits can also have alternate C-terminal sequences (Kohler *et al.*, 1994). The physiological effects of the flip versus flop versions have been extensively characterized with *in vitro* translation systems and also in recordings from mammalian brain slices. The overall level of flip versus flop is very similar, but considerable regional variations are observed, for instance, CA3 pyramidal cells exhibit exclusive flip expression while dentate granule cells have more flop than flip (Sommer *et al.*, 1990b). Flip/flop versions are also expressed differentially during development, the flip form being expressed at a relatively constant level, and the flop variant increasing during development (Monyer, Seeburg and Wisden, 1991). The pharmacological properties of these receptors is very similar, except for sensitivity to cyclothiazide, a drug that reduces desensitization (Partin, Patneau and Mayer, 1994). The flip form of the receptor yields larger maximal responses to glutamate than flop homomultimers, and subunit mixtures exhibit intermediate values (Sommer *et al.*, 1990b). These differences are attributable to differences in rates of desensitization, GluR-C and GluR-D receptors showing faster desensitization in the flop form than in the flip form (Mossbacher *et al.*, 1994; Partin, Patneau and Mayer, 1994). Since flop containing receptors can desensitize with a time constant in the region of 1 millisecond, it has been proposed that such rapid desensitization might shape the decay time course of excitatory synaptic interactions (see review by Trussell, Raman and Zhang 1994). Measurements of the time course of synaptic currents at different synapses, and using single cell PCR analysis, supports the hypothesis that the decay of synaptic currents does depend on subunit combination, splice variants and RNA editing (Geiger *et al.*, 1995). Another splice form identified for the AMPA receptor is a C-terminal variant of GluR-D which is shorter than the originally described receptor (Gallo *et al.*, 1992). Recently a similar phenomenon was described for GluR-B (Kohler, Kornau and Seeburg 1994). The functional consequences of these variants is unclear at present.

A rather novel type of mRNA processing has been described for the AMPA/KA receptor family, in which specific nucleotide bases are subject to modification by RNA editing (see Seeburg 1996 for review). Single amino acid substitutions can dramatically alter the kinetic and calcium permeability of the receptor (see below). AMPA/kainate and serotonin receptors are the only CNS proteins that have been shown to undergo RNA editing so far although it

has been suggested that sodium channels may undergo a similar process (Simpson and Emerson 1996).

#### 7.4.1 Alternative splice variants of GABA<sub>A</sub> receptor subunit gene transcripts

Alternative splice variants appear to be less extensive for GABA receptors, but have been observed for mammalian  $\gamma 2$ ,  $\beta 3$  and  $\alpha 6$ , and in the chick  $\beta 2$  and  $\beta 4$  (Stephenson 1995). An exception to this rule is the invertebrate *Rdl* GABA receptor which shows extensive alternative splicing (French-Constant and Rocheleau, 1993). This is rather reminiscent of the situation for the potassium channel gene *Shaker* which is encoded by numerous distinct genes in the mammal, but does not give rise to splice variants, and the *Drosophila Shaker* which is a single gene that undergoes multiple splicing events to generate channel diversity (Pongs 1994).

Alternative splicing for  $\beta 3$  occurs at the N-terminal end where there is an alternate exon 1, called exon 1a. (Kirkness and Fraser, 1993). The transcripts are of equal length, and while they do show different spatial and developmental patterns of expression, a functional difference has not yet been observed between these receptors. Among  $\alpha 6$  subunit gene transcripts, a short form of the mRNA is found which lacked nucleotides 226-255 (Korpi *et al.*, 1994). Interestingly this 10 amino acid deletion results in a non functional receptor, thought to result from the inability of the subunit to assemble. Alternate splicing of the  $\gamma 2$  and chick  $\beta 2$  and  $\beta 4$  subunits affect a putative intracellular loop between domains III and IV (Bateson *et al.*, 1991; Harvey *et al.*, 1994; Kofuji *et al.*, 1991; Whiting *et al.*, 1990). No functional consequences have been reported for the  $\beta 4$  subunit, but  $\gamma 2$  and  $\beta 2$  subunits contain consensus phosphorylation sites within this domain (Harvey, Chinchetru and Darlison, 1994; Kofuji *et al.*, 1991; Whiting, McKernan and Iversen 1990).

The results presented in Chapter 5 of this thesis, describe the temporal regulation of the  $\gamma 2$  splice variants in the murine CNS. The  $\gamma 2S$  splice variant is the only  $\gamma 2$  transcript expressed during murine embryonic development. The  $\gamma 2S$  splice variant is expressed as early as E11 in the neocortex, cerebellum primordium and spinal cord of the developing CNS. At postnatal day 1, the  $\gamma 2S$  transcript remains the only  $\gamma 2$  mRNA expressed in the cerebral cortex, septum and hippocampus. Expression of the  $\gamma 2L$  transcript was detected only in the day 1 globus pallidus, yet by day 7, the  $\gamma 2L$  transcript was detected in many other regions of the murine CNS including the inferior and superior colliculi, cerebral cortical layers 1 through 6, basal nuclei, septum, hippocampal CA1-3 pyramidal cells. In the adult murine CNS, the  $\gamma 2L$  mRNA is the predominant transcript, throughout the murine CNS, although co-localization of the two alternatively-spliced  $\gamma 2$  transcripts does occur, in several regions including the hippocampus, thalamus, colliculi, and cerebral cortex.

The ontogeny of  $\gamma 2$  splice variant expression reported in Chapter 5 of this thesis furthers

a study of  $\gamma 2L$  and  $\gamma 2S$  GABA<sub>A</sub> receptor subunit mRNA expression in the rat brain reported by Gutierrez *et al.*, 1996. In the Gutierrez *et al.*, (1996) study, *in situ* hybridization, dot blot analysis and quantitative immunocytochemistry were used to study the expression of  $\gamma 2S$  and  $\gamma 2L$  in two month old and 30 month old Sprague-Dawley and Fischer 344 rats. Using splice variant specific- $\gamma 2$  antibodies, they demonstrated a small but significant increase in anti- $\gamma 2L$  immunoreactivity in cerebral cortical layers III-V and in the subiculum, as well as an accompanying age-related decrease in  $\gamma 2S$  protein expression in these regions. In contrast to the changes seen in  $\gamma 2L$  protein abundance, there was no increase in  $\gamma 2L$  mRNA expression. The probe used in the *in situ* hybridization studies (Gutierrez *et al.*, 1996) was not splice-variant specific and therefore only detects changes in overall  $\gamma 2$  gene expression. Dot blot analysis, however indicated that there were significant decreases in  $\gamma 2S$  mRNA expression, therefore suggesting an increase in  $\gamma 2L$  mRNA abundance. Reconstitution of GABA<sub>A</sub>R in heterologous systems has shown that  $\gamma 2S$  and  $\gamma 2L$  subunits can associate with both  $\alpha$  and  $\beta$  subunits to produce GABA<sub>A</sub> receptors with high affinity for benzodiazepines in which GABA-induced opening of the Cl<sup>-</sup> channel is potentiated by benzodiazepines (Angelotti *et al.*, 1993; Hadingham *et al.*, 1992; Horne, *et al.*, 1992; Moss *et al.*, 1991). Age-related changes in GABA<sub>A</sub> receptor Cl<sup>-</sup> channel properties as well as allosteric interactions among subunits have been described (Concas *et al.*, 1988; Erdo and Wolff, 1989; Mhatre *et al.*, 1991) and may in part be accounted for changes in GABA<sub>A</sub> receptor subunit composition during aging.

The role of phosphorylation in the ethanol sensitivity of GABA receptor subunits is an interesting, though controversial story. Ethanol sensitivity maybe related to GABA<sub>A</sub> receptor subunit composition and this hypothesis has been tested in oocytes expressing: 1) total RNA from long-sleep (LS) and short-sleep (SS) mice, which differ in the response of GABA receptors to ethanol, as well as in the sensitivity of the GABA receptor to benzodiazepines (Wafford *et al.*, 1990); and 2) cRNAs encoding mouse  $\alpha 1$ ,  $\beta 1$  and  $\gamma 2L$  subunits (Wafford *et al.*, 1991). Ethanol sensitivity further depended on the PKC consensus site within the splice insert suggesting that phosphorylation was necessary for the effects of ethanol (Wafford and Whiting, 1992). Other groups, however, have suggested that the  $\gamma 2L$  subunit is not necessary (Kurata *et al.*, 1993; Sigel, Baur and Malherbe, 1993) for ethanol stimulation. In fact, Sigel, Baur and Maherbe, (1993) failed to repeat the results of Wafford *et al.*, (1991) using *Xenopus* oocytes to measure the ability of ethanol to stimulate, directly or indirectly, the response of GABA<sub>A</sub>  $\alpha 1\beta 1\gamma 2L$  receptors to GABA application. Recently, Zhai *et al.*, (1998) demonstrated that there was no affect of ethanol on GABA-evoked currents in dissected rat dorsal root ganglion neurons and further demonstrated through RT-PCR analysis that these neurons contain mRNAs for the  $\gamma 2L$  and other GABA<sub>A</sub> receptor subunits. Mutagenesis studies with chimeras of the rho and glycine  $\alpha 1$  subunit identified



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two amino acids in transmembranes 2 and 3 that were sufficient for the enhancement of the GABA-activated current via ethanol and volatile anaesthetics (Mihic *et al.*, 1997).

The issue of the involvement of the  $\gamma 2$  GABA<sub>A</sub> subunit and phosphorylation in ethanol sensitivity remains to be resolved, but interestingly a recent study of a PKC  $\gamma$  null mutant mouse has suggested that PKC  $\gamma$  is required for the action of ethanol in cerebral cortex and cerebellum, at least for some measures of GABA function (Harris *et al.*, 1995).

### 7.5 Post-translational processes

As outlined above, it is during the post-translational period that a great number of diverse processes occur that can affect channel function. The channel must be assembled from the correct constituents to form an appropriate complex, the channel must be transported to an appropriate subcellular compartment, the channel must be inserted into the plasma membrane, and appropriate glycosylation events must occur. While these events are critical, very little is known concerning how these processes occur, how they are controlled and whether they can be modulated. Once channel assembly has occurred, however, a form of modulation, phosphorylation, can occur and has been extensively studied (Raymond, Blackstone and Huganir 1993; Swope *et al.*, 1992).

Ligand-gated ion channel superfamily members and members of the glutamate receptor family have canonical phosphorylation sites. For instance, all GABA<sub>A</sub> receptor  $\beta$  subunits have a consensus site for PKA phosphorylation, corresponding to serine 409 in  $\beta_1$  (Swope *et al.*, 1992; Ymer *et al.*, 1989), and this residue can be phosphorylated by PKA or PKC (Moss, Doherty and Huganir, 1992). The GABA<sub>A</sub> receptor  $\gamma 2$  subunit, as discussed above, has two serine phosphorylation sites, one of which is missing in the  $\gamma 2S$  splice variant (Moss, Doherty and Huganir, 1992; Whiting, McKernan and Iversen, 1991). These sites are found on the intracellular loop between M3 and M4. Phosphorylation sites for tyrosine kinases and CaMK II have also been identified (Pritchett *et al.*, 1989; McDonald and Moss, 1994). There are also potential sites for PKC phosphorylation on  $\alpha 4$ ,  $\alpha 6$ ,  $\delta$ ,  $\rho 1$  and  $\rho 2$  (Macdonald 1995). The action of PKA activation, in cells transiently expressing GABA<sub>A</sub> receptors, was to decrease the sensitivity to GABA (Moss *et al.*, 1992), an effect similar to that seen *in vivo* for cultured mouse spinal neurons (Porter *et al.*, 1990). Phosphorylation by PKC of  $\alpha 1\beta 1$  or  $\alpha 1\beta 1\gamma 2$  receptor combinations expressed in oocytes or HEK293 cells also led to a decrease in GABA response, although these effects were dependent on the level of endogenous kinase activity (Krishek *et al.*, 1994). The effects could be abolished by mutagenesis of the serine sites 409 ( $\beta_1$ ), 327( $\gamma 2$ ) and 343( $\gamma 2$ ). The most potent site for phosphorylation was contained in the 8 amino acid segment unique to  $\gamma 2L$ . Similar effects were seen in acutely dissociated hippocampal neurons on application of phorbol esters (Stelzer, 1992). In contrast to these studies, recombinant  $\alpha 1\beta 1\gamma 2$  receptors transiently

expressed in L929 cells are potentiated by activation of PKA or PKC (Angelotti, Uhler and Macdonald, 1993; Lin *et al.*, 1994). The effect of phosphorylation on GABA receptor function is therefore quite dependent on the expression system employed, and suggests that kinases can achieve bidirectional modulation of GABA<sub>A</sub> receptors. Recently Moss *et al.*, (1995) have shown that a tyrosine kinase (vSRC) can enhance GABA whole-cell currents in HEK293 cells. Tyrosine residues in both  $\beta$  and  $\gamma 2L$  were phosphorylated, but the  $\gamma 2L$  subunit was critical for the enhancement of GABA currents. Similar effects were seen in cultured ganglionic neurons, and the tyrosine kinase inhibitor, genistein, reduced native GABA currents. These data suggest that constitutive phosphorylation of GABA<sub>A</sub> receptors is physiologically important, at least in some cell types. In Chapter 5, *in situ* hybridisation results indicate that during later stages of postnatal development and in the adult murine CNS, the  $\gamma 2L$  alternative-splice variant of the  $\gamma 2$  GABA<sub>A</sub> receptor subunit mRNA is the predominant splice variant and therefore suggests that in a cell type specific manner GABA<sub>A</sub> responses are subject to modulation by phosphorylation through PKA or tyrosine kinase pathways.

The effects of phosphorylation on ligand-gated ion channel receptor responses have also been reported for other members of the ligand-gated ion channel superfamily. For example, the nAChR has phosphorylation sites for protein kinase A (PKA), protein kinase C (PKC), and tyrosine kinases. Phosphorylation can accelerate the rate of receptor desensitization (Steinbach and Zempel, 1987), although this effect may not be universal (Cachelin and Colquhoun 1989). Phosphorylation may also play a part in receptor clustering at synaptic sites (Swope *et al.*, 1992). Phosphorylation of the glutamate receptors is quite widespread and has been proposed to play an important function as a possible mediator of synaptic plasticity (Roche, Tingley and Haganir, 1994). Phosphorylation of NMDA receptors via tyrosine kinases increases channel activity while phosphatase mediated dephosphorylation reduces activity (Lieberman and Mody, 1994; Wang *et al.*, 1994; Wang and Salter, 1994). PKC activity can also potentiate NMDA responses, but in this case by reducing Mg<sup>++</sup> block (Chen and Huang 1992). Interestingly some of the consensus phosphorylation sites lie within regions that are alternatively spliced (Tingley *et al.*, 1993). AMPA receptors are potentiated by PKA (Greengard *et al.*, 1991), and synaptic transmission can be potentiated by activators of PKA (Chavez-Noriega and Stevens, 1994). PKC and Ca<sup>++</sup>/calmodulin-dependent protein kinase II (CaMK II) have also been shown to phosphorylate AMPA receptors, leading to enhanced activity (Tan, Wenthold and Soderling, 1994). Hence phosphorylation can play key roles in desensitization, distribution and function.

## 7.6 Molecular basis of subunit diversity

Members of the ligand-gated ion channel superfamily are thought to be derived from a

common ancestral protein (Hille 1992) which may have arisen around 2,500 million years ago (Ortells and Lunt, 1995). The idea of common ancestry has given rise to the expectation that structure-function concepts might be generally applied to all members of the superfamily, and possibly to the closely related glutamate receptor family. Two key areas have been the focus of extensive study and they include: 1) what amino acid sequences and structures determine the properties of the ion channel pore and; 2) which amino acid residues are involved in forming the ligand binding site. The initial predicted secondary structure of the GABA<sub>A</sub> receptor is presented in Chapter 1, Figure 1.3.

### 7.6.1 Ion selectivity in the ligand-gated ion channel superfamily

The substituted-cysteine accessibility method (SCAM) has been used to probe the structure of ion channel pores. Individual residues are mutated to cysteine and a sulphhydryl specific reagent is used to label the cysteine. If the residue is located within the ion channel pore then addition of the sulphhydryl agent will alter agonist induced responses. Using this method Akabas *et al.*, (1994) have extensively investigated the M2 region of the nAChR  $\alpha$  subunit. Examining residues 241-262 they found that Glu<sub>241</sub>, Thr<sub>244</sub>, Leu<sub>245</sub>, Ser<sub>248</sub>, Leu<sub>250</sub>, Leu<sub>251</sub>, Ser<sub>252</sub>, Val<sub>255</sub>, Leu<sub>258</sub> and Glu<sub>262</sub> were all exposed in the channel lumen. These data are compatible with the M2 segment forming a helix (Numa, 1989), but with three amino acids in an extended conformation. These data are consistent with an  $\alpha$  helical structure for M2, that has been proposed from diffraction X-ray crystallography for the acetylcholine receptor (Unwin, 1995). A similar though much less extensive analysis has been attempted with the GABA<sub>A</sub> receptor (Xu and Akabas, 1993). Residues 268-271 were mutated to cysteine and labelled with a charged sulphhydryl reagent. Although these data are limited they are consistent with an  $\alpha$  helical structure and inconsistent with a  $\beta$  sheet conformation. Using sulphhydryl reagents of varying sizes these authors have also suggested that the GABA channel is narrower than the nAChR channel (Xu and Akabas, 1993; Akabas *et al.*, 1992).

The results reported in Chapter 6 of this thesis suggest that the conserved proline in M1 of GABA<sub>A</sub> receptor subunits can play a pivotal role in channel gating. Mutation of the  $\alpha$ <sub>1</sub><sup>243pro-ala</sup> residue in M1 of the GABA<sub>A</sub> receptor  $\alpha$ <sub>1</sub> subunit and co-expression with wild-type  $\beta$ <sub>1</sub> and  $\gamma$ <sub>2L</sub> subunits in transiently transfected HE293 cells resulted in a  $K_d$  for Ro15-1788 binding that was not statistically different from receptors containing wild-type  $\beta$ <sub>1</sub>,  $\gamma$ <sub>2L</sub> subunits only. Western blot analysis of  $\alpha$ <sub>1</sub><sup>243pro-ala</sup>,  $\beta$ <sub>1</sub>,  $\gamma$ <sub>2L</sub> HEK membrane preparations demonstrated that the mutated  $\alpha$ <sub>1</sub> protein was absent from these preparations and suggests that the  $\alpha$ <sub>1</sub> subunit in a GABA<sub>A</sub> receptor complex is essential for efficient membrane integration. Mutation of the conserved M1 proline in the  $\beta$ <sub>1</sub> subunit resulted in a  $K_d$  for Ro15-1788 binding similar to that for a wild-type  $\alpha$ <sub>1</sub>,  $\beta$ <sub>1</sub>,  $\gamma$ <sub>2L</sub> subunit combination, but the

$K_d$  for muscimol binding of receptors containing the mutated  $\beta_{1243\text{pro-ala}}$  subunit was significantly different from the  $K_d$  for wild-type GABA<sub>A</sub> receptor muscimol binding. The  $B_{\text{max}}$  for muscimol receptor binding did not differ between the mutant  $\beta_{1243\text{pro-ala}}$  and wild-type receptor combinations indicating a true change in agonist affinity for the mutant receptor complex. Single channel recordings of GABA<sub>A</sub>  $\alpha_1\beta_1(243\text{pro-ala})\gamma_2\text{L}$  receptors acutely expressed in L929 fibroblasts demonstrated increases; 1) in channel conductance, 2) in the mean burst duration, and 3) in the number of openings per burst compared with the wildtype receptor combination (Greenfield *et al.*, unpublished results). Acutely transfected L929 cells expressing the  $\alpha_1\beta_1(243\text{pro-ala})\gamma_2\text{L}$  subtype demonstrated selectively reduced sensitivity to barbiturates, suggesting a role for the  $\beta$  subunit in barbiturate-induced enhancement of GABA<sub>A</sub> receptor currents. Whole cell patch recordings from HEK293 cells expressing the  $\alpha_1$ ,  $\beta_{1243\text{pro-ala}}$  and  $\gamma_2\text{L}$  subunits demonstrated that currents elicited by application of 100  $\mu\text{M}$  GABA were blocked by 100  $\mu\text{M}$  bicuculline and potentiated by 50  $\mu\text{M}$  diazepam (Zaman, 1993) and the  $\text{EC}_{50}$  for GABA was statistically greater than wild-type (Zamin, 1993), an observation which is in agreement with the binding results reported in Chapter 6 of this thesis.

In contrast to the possibility that there are shared or overlapping sites of action for GABA and barbiturates, Amin and Weiss (1993) created several mutations which provide evidence suggesting separate sites of binding for GABA and its modulators. For example two regions between the cysteine loop and the M1 transmembrane domain of the  $\beta_2$  subunit, threonine (T160, T202) and tyrosine (Y157, Y205) residues, through site-directed mutagenesis have been identified as critical sites for the activation of currents by GABA but not by pentobarbital. Mutation of either tyrosine led to a 50-fold decrease in GABA binding, while mutating either threonine decreased binding by 20 fold. Similar mutations in  $\alpha$  or  $\gamma$  were ineffectual. Since there was no effect on the maximal GABA activated current the authors concluded that gating efficiency was not impaired, and this was further supported by the observation that barbiturates could activate the mutant channel as effectively as wild type. These findings imply that the pentobarbital site is physically distinct from the GABA binding site. Our results demonstrate that mutation of  $\beta_{1243\text{pro-ala}}$  affects the GABA binding site, as well as receptor assembly when the identical mutation is in the  $\alpha_1$  subunit. The mutation in the  $\beta_1$  subunit does not affect the ability of the receptor complex to integrate into the plasma membrane, as there was no change in the  $B_{\text{max}}$  between mutant and wild-type  $\alpha_1$ ,  $\beta_1$ ,  $\gamma_2\text{L}$  GABA<sub>A</sub> receptors. The effect of the proline mutation in the  $\beta$  subunit on both agonist and barbiturate binding suggests that the proline residue provides a pivotal point in the receptor complex which facilitates an allosteric interaction and the subsequent reorientation of the pore region into an open state.

### 7.6.2 Molecular determinants of the ligand binding site

As illustrated above, analysis of GABA receptor ligand binding domains is rather more complex because of the diverse nature of interactions that have been characterized for these receptors. In addition to the expected agonist and antagonist binding domains one must also take into account the benzodiazepine site, barbiturate site and the steroid site. Initial studies used photoaffinity labeling and suggested that muscimol labeled the  $\beta$ -subunit, while flunitrazepam binding was localized to the  $\alpha$ -subunit (Casalotti, Stephenson and Barnard, 1986). A number of questions have been raised, however, concerning the validity of these findings, based mainly on difficulties in resolving individual components of the GABA receptor on the basis of molecular weight. Heterologous expression studies indicate that co-expression of  $\alpha$  and  $\beta$  subunits forms functional channels, but modulation by benzodiazepines is not seen unless  $\gamma$  is also present (Pritchett, Luddens and Seeburg, 1990; Knoflach *et al.*, 1991). These data suggest that the  $\alpha$  subunit alone does not confer benzodiazepine responsiveness. Sigel *et al.*, (1992) have shown that residue Phe<sub>64</sub> in the  $\alpha 1$  subunit strongly affects both agonist and antagonist binding. This is interesting because this residue is located considerably closer to the N-terminus than those previously suggested to be involved in ligand interaction for nAChR or glycine receptors. A homologous mutation in the  $\beta$  subunit had a similar but smaller effect on GABA binding (30 fold versus 200 fold), while the effect in the  $\gamma$  subunit was small (6 fold change). This site can also be photoaffinity labeled by [<sup>3</sup>H]muscimol (Smith and Olsen, 1994). In contrast to these data, however, Amin and Weiss (1993) suggest that the  $\beta$  subunit is key to agonist binding, thus supporting previous photoaffinity labeling studies.

The benzodiazepine binding site is quite complex in that not only does it show a normal spectrum of agonist, partial agonist and antagonist binding, it also is a site for *inverse* agonists i.e ligands that decrease the effectiveness of GABA (Macdonald and Olsen, 1994; Schofield *et al.*, 1987). Early evidence from photoaffinity labelling indicated that the  $\alpha$  subunit was the site of [<sup>3</sup>H]-flunitrazepam binding (Casalotti, Stephenson and Barnard 1986; Sieghart and Karobath, 1980), but the observation that  $\alpha\beta$  subunits expressed in oocytes showed benzodiazepine insensitive GABA responses led to a reevaluation of these data. Use of subunit specific antibodies, however, has confirmed these earlier observations, with mapping experiments indicating a region between residues 59-148 in the  $\alpha$  subunit (Stephenson and Duggan, 1989; Stephenson, Duggan and Pollard, 1990). Furthermore the benzodiazepine pharmacology of heterologously expressed  $\alpha\beta\gamma 2$  subunit combinations depends on which of the  $\alpha$  subunits is present (Benke *et al.*, 1991b; Pritchett *et al.*, 1990). One of these  $\alpha$  subunits  $\alpha 6$  only binds benzodiazepine antagonists and inverse agonists (Luddens *et al.*, 1990). Exploiting this interesting property the structural determinants of benzodiazepine binding were probed (Wieland, Luddens and Seeburg, 1992). It was found

that His<sub>101</sub> was a critical determinant of binding. Replacing His with Arg (the equivalent  $\alpha 6$  residue) abolished diazepam binding. The effect was not dependent on pH indicating that charge properties were less important than steric factors. This mutation also determines whether GABA activated ion currents are potentiated by diazepam (Kleingoor *et al.*, 1993). The reverse mutation in  $\alpha 6$  Arg<sub>100</sub> to His generated benzodiazepine sensitive receptors. Similar effects were seen with the antagonist flumazenil and with the type I specific ligand CL 218872. Zolpidem, which binds to  $\alpha 1$  with high affinity,  $\alpha 2$  and  $\alpha 3$  with intermediate affinity, and  $\alpha 4$ ,  $\alpha 5$ , and  $\alpha 6$  with negligible affinity, did not bind to the mutant  $\alpha 1$ , but also did not bind to  $\alpha 6$  with the His residue substitution. This indicates that other residues are important for zolpidem binding. In support of a role for other residues Pritchett and Seeburg (1991) found that mutating Glu-225 to Gly<sub>225</sub> in  $\alpha 3$  significantly increased the affinity for CL 218872. Mutation in the  $\gamma 2$  subunit (Thr<sub>142</sub> to Ser) can also affect benzodiazepine interactions, but in this case the effect appears to be to alter the efficiency of coupling binding to allosteric modulation (Mihic *et al.*, 1994). This mutation did not affect barbiturate or steroid modulation of GABA action.

In Chapter 6 of this thesis we found that mutation of the conserved aspartic acid residue in the cysteine loop of  $\alpha 1$  and  $\beta 1$  subunits to asparagine could have an effect on benzodiazepine binding and the ability of the receptor complex to integrate into the plasma membrane. In the experiment including a mutant  $\alpha 1_{D-N}$  and wt $\beta 1$  and wt $\gamma 2L$  subunits, neither Ro15-1788 or muscimol binding was detected. Western blot analysis of membrane preparations used in the receptor binding analysis, demonstrated that immunoreactive products were present for both the  $\alpha 1$  and  $\beta 1$  subunits, and mRNA analysis confirmed the presence of all three subunit ( $\alpha 1$ ,  $\beta 1$ , and  $\gamma 2L$ ) mRNAs in the transiently transfected cells. The presence of GABA<sub>A</sub> receptor protein in the membrane preparations, but a lack of Ro15-1788 and muscimol binding suggests that this site in the cysteine loop can play a role in both agonist and antagonist binding.

Conservation of the negative charge at the aspartic acid site in the cysteine loop of the  $\beta 1$  subunit (149D-E) resulted in receptors that were identical to the wild-type receptors in Ro15-1788 binding affinity suggesting that charge rather than steric hindrance plays a key role in determining the affinity of benzodiazepines at that site. In further comparison of wild-type and mutant receptors containing the  $\beta 1_{D-E}$  subunit, there was a significant decrease in the  $B_{max}$ , and lack of muscimol binding for the mutant receptor membrane preparations. As discussed earlier, a decrease in the  $B_{max}$  suggests that the assembly or efficient trafficking of the GABA<sub>A</sub> receptor complex was impaired. An increase in affinity for Ro15-1788 in the mutant receptor containing the  $\beta 1_{D-N}$  subunit further suggests that the aspartic acid site in the cysteine loop plays a role in antagonist binding.

In comparison to our results, Amin *et al.*, (1994) also found that mutation of  $\alpha 1$  and  $\gamma 2$

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GABA<sub>A</sub> receptor subunits to glutamic acid (conservative change) at the aspartic acid position in the cysteine loop resulted in either no impairment of GABA-mediated activation or a slight decrease in the EC<sub>50</sub>, when  $\alpha 1$ ,  $\beta 2$  and  $\gamma 2$  subunit cRNAs were co-expressed in *Xenopus* oocytes. Similar to our results with the  $\beta 1_{D-N}$  mutation, Amin *et al.*, (1994) found that substitution of the aspartic acid position in the cysteine loop with asparagine in the  $\beta 2$  subunit resulted in no GABA-activated currents. In *Xenopus* oocyte experiments, substitution of aspartic acid for glutamic acid (conservation of charge) in the  $\beta 2$  subunit resulted in GABA-activated currents, where there was no effect on the EC<sub>50</sub>, a dramatic decrease in the amplitude of the current and a decrease in the Hill coefficient, suggesting a decrease in the cooperativity of channel activation (Amin *et al.*, 1994). Mutations involving changes to amino acids at positions 5 (leucine) and position 9 (proline) in the cysteine loop of the  $\beta 2$  GABA<sub>A</sub> receptor subunit, when co-expressed in *Xenopus* oocytes with wild-type  $\alpha 1$ ,  $\gamma 2$  subunits generally resulted in only a slight impairment in activation of the GABA channel, suggesting that this impairment may occur indirectly through structural alterations of the protein (Amin *et al.*, 1994). Our studies further the concept that multiple residues in both  $\alpha$  and  $\beta$  subunits play key roles in determining the benzodiazepine binding site of GABA<sub>A</sub> receptors.

In this thesis we have described the ontogeny of GABA<sub>A</sub> receptor subunits in the embryonic and postnatal murine CNS using RT-PCR and *in situ* hybridization analysis and compared these results with those reported for rat GABA<sub>A</sub> receptor subunit ontogeny. To further our understanding of the role conserved amino acids play in the binding sites for agonists and antagonists of GABA<sub>A</sub> receptors we employed site-directed mutagenesis coupled with heterologous expression of mutant and wild-type subunits in HEK293 cells to determine binding affinities as well as GABA<sub>A</sub> receptor subunit mRNA and protein expression.

# **Chapter 8**

## **References**



## References

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