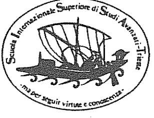

**ISAS - INTERNATIONAL SCHOOL FOR ADVANCED STUDIES**

# EXPRESSION OF GABA$_A$ RECEPTOR SUBUNITS IN THE DEVELOPING RAT HIPPOCAMPUS

Thesis submitted for the degree of "Doctor Philosophiae"

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**I dedicate this thesis to my wife Katia.**

## Declaration

The work described in this dissertation was carried out at the International School for Advanced Studies, Trieste, between December 1995 and July 1999. All work reported, with the exceptions listed below, arise solely from my own experiments and this work has not been submitted in whole or in part to any other University.

The construction and screening of the cDNA library form the rat neonatal hippocampus (sections 4.2 and 4.3) were carried out in collaboration with Miranda Mladinic.

The electrophysiological part of the single-cell RT-PCR experiments (section 4.7) were carried out by Natasha Savic and Marina Sciancalepore, with my assistance.

Frédéric Didelon
29 th August 1999

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# ABBREVIATIONS

ACh = acetylcholine
ACSF = artificial cerebrospinal fluid
AMPA = $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMV = avian myeloblastosis virus
ATP = adenosine triphosphate
bp = base pair
BSF1 = brain specific factor 1
CA1 and CA3 = Ammon's horn regions 1 and 3
CACA = cis-4-aminocrotonic acid
$Cl^-$ = chloride ions
ClC-2 = chloride channel-2
cDNA = complementary DNA
CNS = central nervous system
dATP, dCTP and dUTP = deoxyadenosine, deoxycytidine and deoxyuridine triphosphate
dbpB = DNA-binding protein B
DIG = digoxigenin
DNA = deoxyribonucleic acid
dNTPs = deoxyribonucleotides triphosphate
DTT = dithiothreitol
$E_{Cl}$ = chloride equilibrium potential
$E_{HCO3^-}$ = carbonate equilibrium potential
EDTA = ethylene diamine tetraacetate
$EF1_A$ = enhancer factor 1 subunit A
GABA = $\gamma$-aminobutyric acid ($\gamma$-aminobutyrate)
GABARAP = $GABA_A$-receptor-associated protein
5-HT = serotonine
$5\text{-}HT_3$ = serotonine-gated receptor channel
LTP = long term potentiation
MAP-1B = microtubule-asociated protein-1B
mGluR = metabotropic receptor for L-glutamate
MMLV = Moloney murine leukemia virus
mRNA = messenger ribonucleic acid
P = postnatal day
PBS = phosphate buffered saline
PCR = polymerase chain reaction
PFA = paraformaldehyde
PKA = protein kinase A
PKC = protein kinase C
RACE = rapid amplification of the cDNA ends
RNA = ribonucleic acid
RNase = ribonuclease
RT-PCR = reverse transcription-polymerase chain reaction
SSC = standard saline solution
TBE = tris-borate/EDTA electrophoresis buffer
THDOC= $3\alpha,5\alpha$ tetrahydrodeoxycorticosterone
TPMPA = 1,2,5,6-tetrahydropyridine-4-yl methylphosphinic acid
YB-1 = Y-box transcription factor
$Zn^{2+}$ = zinc ions

# ABSTRACT

In the neonatal rat hippocampus, GABA elicits chloride-mediated, bicuculline sensitive and insensitive responses. The latter closely resembles GABAergic currents well characterized in the retina, supposed to be mediated by receptors formed by the ρ subunits. In order to identify the subunits responsible for these responses, a cDNA library from P0-P2 rat hippocampus was constructed and screened with probes derived from ρ subunits sequences. This strategy however did not permit the identification of ρ subunits or related members of the $GABA_A$ receptor subunit gene family. A degenerate oligonucleotide probe derived from the highly conserved M2 domain of $GABA_A$ receptor subunits was then chosen to screen the cDNA library. With this approach, most $GABA_A$ receptor subunits were isolated. Thus, the α5 subunit was by far the most abundant, followed by the γ2, α2 and α4 subunits. The expression of the β2, α1, γ1, β1 and β3 subunits was moderate, while that of the α3 and δ was weak. However, the γ3 as well as the ρ subunits were not cloned. In contrast to previous reports, *in situ* hybridization experiments using digoxigenin labeled cRNA probes clearly showed that δ subunit was expressed from birth in the hippocampus, while the γ3 subunit was absent. Using RT-PCR, low levels of the ρ1-3 and ε subunits transcripts in both neonatal and adult hippocampus were detected, indicating that these subunits would be expressed at very low levels, below the detection threshold of the cDNA library screening process. Furthermore, in order to correlate $GABA_A$ receptor subunits gene expression pattern with the physiological responses observed in neonatal hippocampus, single-cell RT-PCR was performed on P3-P4 CA3 pyramidal cells for which responses to GABA (in the presence of bicuculline) were electrophysiologically recorded. Interestingly, the ρ subunits were expressed in cells exhibiting bicuculline-insensitive responses to GABA. This suggests that the ρ subunits could be at the origin of the bicuculline-insensitive responses observed, probably in combination with other known $GABA_A$ receptor subunits, possibly through heteromeric receptors. Further experiments however will be necessary to validate this hypothesis and understand the function of the ρ subunits in the hippocampus.

# 1. INTRODUCTION: NEUROBIOLOGY OF THE $GABA_A$ RECEPTORS

γ-Aminobutyric acid (γ-aminobutyrate or GABA), is a very simple molecule of the following formula: $NH_2$-$CH_2$-$CH_2$-$CH_2$-COOH. It is known to be the most widely distributed and important inhibitory neurotransmitter in the central nervous system (CNS) of vertebrates. Virtually all neurons that have been tested have been found to be sensitive to it. GABA inhibits neuronal firing by activating $Cl^-$ ($GABA_A$) and cationic ($GABA_B$) conductances that stabilize the membrane potential below the threshold for action potential generation. It acts through two main different receptor types: $GABA_A$ and $GABA_B$. $GABA_A$ receptors are integral ion channels that belong to the ligand-gated ion-channel superfamily. Recently a novel class of structurally defined receptors, composed by the newly discovered ρ subunits and named $GABA_C$, has been described in the retina (Bormann & Feigenspan, 1995). Both $GABA_A$ and $GABA_C$ receptors conduct chloride ions but exhibit different pharmacological characteristics. However, $GABA_C$ receptors formed by subunits having a common origin and structural features, therefore I shall refer in the rest of the thesis to $GABA_A$ and $GABA_A$-ρ receptors, to indicate $GABA_A$ and $GABA_C$ receptors, respectively. A similar classification was proposed by the International Union of Pharmacology (Barnard *et al.*, 1998) and allows the rationalization of the diversity of $GABA_A$ receptors.

$GABA_B$ receptors are coupled to cationic $K^+$ and $Ca^{2+}$ channels through GTP binding proteins and second messengers and belong to the G-protein-linked receptor family (Bettler *et al.*, 1998).

The modulation of the responses to GABA has profound implications for brain function. GABA is involved in the control of many essential physiological functions such as vigilance and circadian rhythms (Wagner *et al.*, 1997). It also has a role in Long Term Potentiation (Bear *et al.*, 1994), a candidate cellular mechanism for the storage of memories in the brain. $GABA_A$ receptors possess binding sites for several therapeutically active drugs such as benzodiazepines (the most widely prescribed neuroactive drug), barbiturates, general anaesthetics, and alcohol as well as for endogenous modulators such as zinc ions ($Zn^{2+}$) and neurosteroids.

GABA also seems to play an important role in various neuropsychiatric disorders and syndromes, such as epilepsy and schizophrenia. Anxiety, sleep disorders, and convulsive disorders have been effectively treated with therapeutic agents that enhance the action of GABA at the $GABA_A$ receptors or increase the concentration of GABA in the nervous system. Moreover, GABA acts not only as a neurotransmitter, but also as a neurotrophic factor during development of the CNS.

Recent advances in molecular biology, pharmacology, biochemistry, electrophysiology, anatomy, cell biology and behavioral studies have enlarged our knowledge and understanding of the structure, function, regulation and evolution of GABA neurotransmission and in particular its fast-acting $GABA_A$ receptors.
Because of the complexity and breadth of this subject as well as for the comprehension of the present thesis, I will focus on the following aspects in this introduction:

I. A brief history of research into GABA
II. The two classes of GABA receptors: $GABA_A$, $GABA_B$
III. The $GABA_A$ receptors
1) The structure of the $GABA_A$ receptors
2) The functional and pharmacological properties conferred by the different subunits
3) The subunit composition of the native $GABA_A$ receptors:
a) The expression of the different $GABA_A$ receptor subunits revealed by
- *In situ* hybridization and immunocytochemical studies
- Immunobiochemical studies
- Single-cell RT-PCR
b) The information gained from recombinant studies
4) Post-translational modifications of the $GABA_A$ receptors
5) The mechanisms of receptor localization and clustering
6) The regulation of the expression of different $GABA_A$ receptor subunits
7) The genomic organization of $GABA_A$ receptor subunit genes

IV. The developmental role of GABA:
1) GABA acting as a neurotrophic factor
2) GABA: an excitatory neurotransmitter in the neonatal hippocampus
3) $GABA_A$-ρ like response in the hippocampus during early postnatal development.

## 1.1 BRIEF HISTORY OF GABA RESEARCH

Despite the functional importance of GABA, it is only relatively recently that its role has been established. The presence of GABA in plants has been known for more than half a century, but it is only in 1950 that Roberts and Frankel, and Awapara *et al.* first described its existence in brain tissue.

A brief chronological record of the events associated with the history of GABA research is shown in **Table 1**. Some years after GABA was identified as a common constituent of brain tissue, it was found to have a marked depressant effect on cortical neurons (Hayashi, 1956). The earliest evidence that GABA could be a transmitter was reported in 1957 by Bazemore *et al.* who found GABA to be a constituent of Factor I - an extract of brain tissue found to have a strong inhibitory action on certain isolated neurons of Crustacea. The possibility was therefore considered that it might act as a chemical transmitter at synapses, or as a controller of neuron excitability. Many studies ensued in attempts to establish its significance, such that by the end of the fifties it had been both proposed and rejected as an inhibitory neurotransmitter. The inhibition of crayfish neurons and muscle proved to be particularly significant as it became clear that GABA was the inhibitory transmitter of crustacean neurons (Kuffler *et al.*, 1963) and was only found in the inhibitory axons of lobster nerves (Kravitz *et al.*, 1963). However, the hypothesis that GABA might be the main inhibitory transmitter in the CNS was considered incompatible with certain experimental observations. The most consistent and serious objections were those raised by Curtis and his associates (1959), who claimed that the action of GABA in the spinal cord differs significantly from that of the inhibitory transmitter. Their experiments with co-axial pipettes failed to detect any hyperpolarization of motoneurones during the administration of GABA. As there was no other proof that GABA had a hyperpolarizing action in other parts of the CNS, they concluded that GABA could not be the main central inhibitory transmitter. GABA was finally identified as a neurotransmitter at the crustacean neuromuscular junction in 1963 (Dudel *et al.*, 1963; Kravitz *et al.* 1963a; Kravitz *et al.*, 1963b) and in the vertebrate nervous system in 1966 by Krnjevic & Schwartz who obtained firm electrophysiological evidence to implicate GABA as an inhibitory transmitter in the cerebral cortex. They proved that GABA imitates the action of the endogenous cortical inhibitory transmitter: it hyperpolarizes the membrane potential and raises the membrane conductance of cat cortical neurons, just like activation of inhibitory synapses, and when the latter inhibitory effect is reversed, the action of GABA is reversed in a similar way. This provided a major impetus for further studies in neurochemistry and pharmacology as well as electrophysiology, which together have now substantiated its important physiological role. Recent molecular biology studies

have provided valuable information about the sequences and structure of the receptor complexes and indicated the positions of recognition sites for the modulators.
They have also revealed a great and unexpected complexity and diversity in $GABA_A$ receptor structure, both at the primary sequence level as well as in the heterogeneity of the spatio-temporal distribution of different $GABA_A$ receptor subunits.

---

**TABLE 1.** **BRIEF HISTORY OF GABA RESEARCH**

**Presence in the brain**
1950 Roberts & Frankel, J. Biol. Chem., 187: 55-63
1950 Awapara, Landau, Furest & Seale, J. Biol. Chem., 187: 35-39

**Action on brain neurons**
1956 Hayashi, Chemical physiology of excitation in muscle and nerve, Tokyo: Nakayama-Shoten,Ltd.

**Proposed as inhibitory neurotransmitter**
1957 Bazemore, Elliott & Florey, J. Neurochem., 1: 334-339

**Rejected as inhibitory neurotransmitter**
1959 McLennan, J. Physiol., 146: 358-368
1959 Curtis, Phillis & Watkins, J. Physiol., 146: 185-203

**Evidence for role as inhibitory neurotransmitter**
-at the crustacean neuromuscular junction:
1963 Dudel, Gryder, Kaji, Kuffler & Potter, J. Neurophysiol., 26: 721-728
1963 Kuffler, Potter & van Gelder, J. Neurophysiol., 26: 729-738
1963 Kravitz, Kuffer & Potter, J. Neurophysiol.,26: 739-751

-in the cerebral cortex:
1966 Krnjevic & Schwartz, Nature, 211: 1372-1374

**Selective $GABA_A$ receptor antagonism**
-picrotoxin
1969 Takeuchi & Takeuchi, J. Physiol., 205: 377-391

-bicuculline
1971 Curtis, Duggan, Felix & Johnston, Brain Res., 32: 69-96

**Evoked release in vivo**

1971 Iversen, Mitchell & Srinivasan, J. Physiol., 212: 519-534

**GABA as excitatory neurotransmitter**

1974 Obata, Brain Res., 73: 71-88

**Benzodiazepine facilitation of GABA synaptic activity**

1977 Haefely, Agents Actions, 3: 353-359

**Evidence for $GABA_B$ receptors**

1981 Hill & Bowery, Nature, 290: 149-152

**Evidence for $GABA_C$ receptors**

1986 Johnston, Multiplicity of GABA receptors, New York, Vol.b: 67-71

**First sequence of $GABA_A$ receptor**

1987 Schofield, *et al.*, Nature, 328: 221-227

**First sequence of $GABA_B$ receptor**

1997 Kaupmann, *et al.*, Nature, 386: 239-246

---

## 1.2 THE DIFFERENT CLASSES OF GABA RECEPTORS: $GABA_A$ AND $GABA_B$

GABA mediates its effects through a specific interaction with integral membrane proteins: the GABA receptors. Prior to the introduction of molecular biological techniques, the taxonomy of GABA receptors was based mainly on their pharmacological properties. With the cloning of GABA receptors a structurally based classification was finally made possible.

GABA, like other neurotransmitters, including L-glutamate, acetylcholine (ACh) and serotonin (5-hydroxytryptamine or 5-HT), activates both ionotropic and metabotropic receptors. The ionotropic receptors are ligand-gated ion channels that convey fast synaptic transmission. In contrast, metabotropic receptors are coupled to G proteins (guanine-nucleotide-binding proteins) and mediate slow synaptic transmission through intracellular effector systems. It is presumed that the duality between these two receptor types arises from a need to produce both immediate and slower actions from the same small amount of transmitter released from nerve endings (Barnard, 1992).

The fast-acting ligand-gated ion channels constitute a group that encompasses nicotinic ACh, $5\text{-}HT_3$, P2X purinergic, $GABA_A$ and glycine receptors. The different subunits from which they are composed all share significant sequence similarities, suggesting that they are homologous and derived from a common evolutionary ancestor gene. All the subunit members of this group of ligand-gated ion channels are therefore considered to form a gene superfamily.

The molecular identity of the metabotropic glutamate receptors (mGluRs) has been recently revealed, showing that this series of genes belong to a new family which differs structurally from other 7TM G-protein coupled neurotransmitter receptors (Pin & Duvoisin, 1995). Recently, the elusive molecular structure of metabotropic $GABA_B$ receptors, functionally described in 1981, has been elucidated by functional cloning. This strategy allowed the isolation of cDNAs encoding two $GABA_B$ receptor proteins designated $GABA_BR1a$ and $GABA_BR1b$, two splicing forms derived from the same gene (Kaupmann *et al.*, 1997) that share the structural organization of mGluRs, suggesting that mGluRs and $GABA_B$ receptors both form a separate gene family. Next, a similar cDNA was identified as $GABA_BR2$ subtype, which assembles with $GABA_BR1$ to form heteromeric complexes (Kaupmann *et al.*, 1998). Such heteromeric receptor complexes probably represent the predominant native $GABA_B$ receptor.

## 1.3 THE $GABA_A$ RECEPTORS

As previously stated, $GABA_A$ receptors are the main site of action of GABA in the central nervous system, and the target of many endogenous modulators and neuroactive drugs. In this section, the structure, functional and pharmacological properties, subunit composition, assembly, localization, regulation, genomic organization and developmental role of the $GABA_A$ receptors are derived.

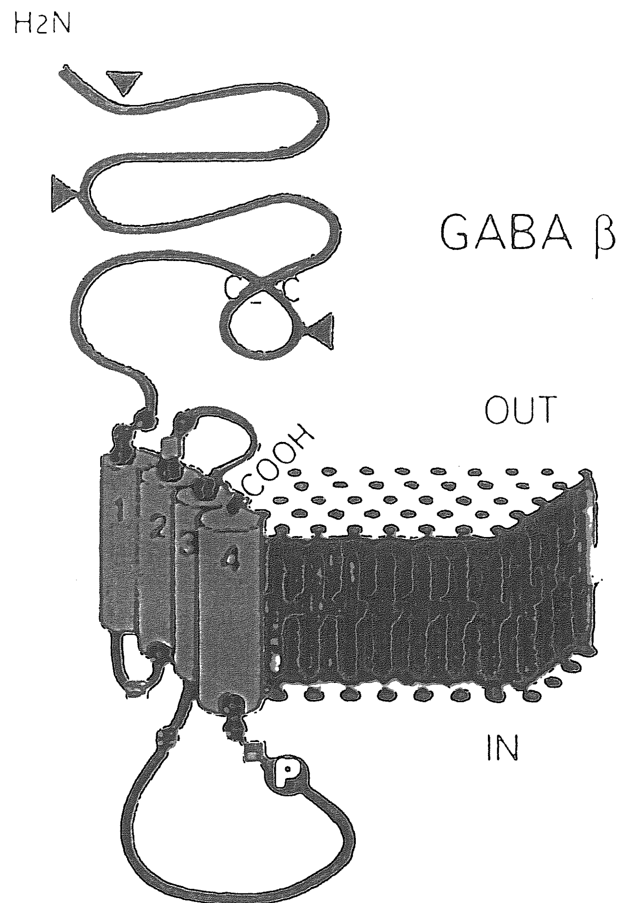
### 1.3.1 STRUCTURE OF THE $GABA_A$ RECEPTORS

#### 1.3.1.1 $GABA_A$ RECEPTOR SUBUNIT POLYPEPTIDE STRUCTURE

As a member of a superfamily of ligand-gated ion channels, the $GABA_A$ receptor is a heterooligomeric glycoprotein, probably pentameric (Barnard *et al.*, 1987), that spans the membrane to form an ion channel.
Analysis of the primary amino acid sequences and derived hydropathy profiles of all known $GABA_A$ receptor subunits (see **Table 2** in the next section) suggest that each subunit contains (**Figure 1**):

1) a large extracellular N-terminus domain (half of the protein) containing :
   a) a short signal peptide,
   b) a cysteine bridge,
   c) consensus sites for N-linked glycosylation,
2) four highly hydrophobic transmembrane domains (M1-M4) (the presence of positive charges at both ends of the transmembrane regions may determine ion selectivity),
3) an intracellular loop between M3 and M4 which is variable in length and in composition between the different subunits (in some subunits this loop contains the consensus sequence for phosphorylation by protein kinase A and C and tyrosine kinase),
4) a short extracellular C-terminus (not present in all subunits).

**FIGURE 1.** **Schematic presentation of the putative $GABA_A$ receptor β subunit polypeptide** (source: Barnard, 1992)




In red are represented the four transmembrane domains (M1-M4). The violet line is the N-terminal domain. On the extracellular side are located the small extracellular loop between M2 and M3 and when present the short C-terminus of the polypeptide. The small loop between M1 and M2 and the large loop between M3 and M4 are located on the intracellular side. In the extracellular N-terminal domain there are potential sites for N-glycosylation (indicated by triangles), and a Cys-Cys loop (indicated by C-C). A site for cAMP-dependent serine phosphorylation in the large intracellular loop is indicated by an encircled P. The charged residues are located close to the ends of the membrane-spanning domains and are shown as blue circles for positive charges, or as red squares for negative charges. The subunit polypeptide is anchored in the cell membrane (in blue).

The transmembrane domains and their immediate flanking regions are the most conserved parts, the M2 domain being the most conserved of all because it is thought to line the channel pore and thus crucial for receptor gating and selectivity. The least conserved region is the cytoplasmic loop between M3 and M4, as well as the N-terminal domain. Variation in the N-terminal extracellular region may allow for diversity of ligand binding while variation in the intracellular loop may allow for separate regulation via second messengers and phosphorylation (Tyndale *et al.*, 1995) (see section 1.3.4).

#### 1.3.1.2 CLONING OF THE VARIOUS $GABA_A$ RECEPTOR SUBUNITS

Molecular biological studies have revealed that $GABA_A$ receptors display an unexpectedly high degree of diversity at the subunit level (Table 2). In vertebrates, $GABA_A$ receptors consist of at least 20 different receptor subunits, which can be divided into seven subfamilies ($\alpha$1-6, $\beta$1-4, $\gamma$1-4, $\delta$, $\varepsilon$, $\pi$ and $\rho$1-3). Most of the $GABA_A$ subtypes are made up of the GABA $\alpha$, $\beta$, ($\gamma$, $\delta$, $\varepsilon$, or $\pi$) receptor subunits, while the GABA receptor $\rho$ subunits are thought to be assembled in homomeric receptors with relatively different pharmacological profiles with respect to the previously mentioned subtypes. The component subunits of the different $GABA_A$ receptors share a high percentage of homology and have a similar predicted polypeptide structure.

**TABLE 2. Cloning of the subunits of the $GABA_A$ receptors from vertebrate and invertebrate species**

| SUBUNIT | VERTEBRATES | | | | |
|---|---|---|---|---|---|
| | MAMMALS | | | | NON-MAMMALS |
| | RAT (16) | MOUSE (10) | HUMAN (14) | COW (10) | CHICKEN (8) |
| α1 | Lolait *et al.*, 1989; Khrestchatisky *et al.*, 1989 | Keir *et al.*, 1991 | Schofield *et al.*, 1989a | Schofield *et al.*, 1987 | Bateson *et al.*, 1991a |
| α2 | Khrestchatisky *et al.*, 1991 | Wang *et al.*, 1992 | Hadingham *et al.*, 1993 | Levitan *et al.*, 1988 | |
| α3 | Malherbe *et al.*, 1990 | Wang *et al.*, 1992 | Hadingham *et al.*, 1993 | Levitan *et al.*, 1988 | |
| α4 | Wisden *et al.*, 1991 | | McLean *et al.*, 1995; Yang *et al.*, 1995 | Ymer *et al.*, 1989b | |
| α5 | Khrestchatisky *et al.* (as α4) 1989; Malherbe *et al.*, 1990 | | Knoll *et al.*, 1993 | | |
| α6 | Lüddens *et al.*, 1990* | Kato, 1990 | Hadingham *et al.*, 1996 | Lüddens *et al.*, 1990 | |
| β1 | Ymer *et al.*, 1989a | Kamatchi *et al.*, 1995 | Schofield et al., 1989 | Schofield et al., 1987 | |
| β2 | Ymer et al., 1989a | Kamatchi et al., 1995 | Wagstaff et al., 1991a* | Ymer et al., 1989 | Harvey et al., 1991* |
| β3 | Lolait et al., 1989; Ymer et al., 1989a | Kamatchi et al., 1995 | Wagstaff et al., 1991b | Ymer et al., 1989 | Bateson et al., 1990 |
| β4 | | | | | Bateson et al., 1991b* |
| γ1 | Ymer et al., 1990 | | Ymer et al., 1990 | Ymer et al., 1990 | |
| γ2 | Shivers et al., 1989 * | Kofuji *et al.*, 1991* | Pritchett *et al.*, 1989* | Whiting *et al.*, 1990* | Glencourse *et al.*, 1990 |
| γ3 | Knoflach *et al.*, 1991; Herb *et al.*, 1992 | Wilson-Shaw *et al.*, 1991 | | | |
| γ4 | | | | | Harvey *et al.*, 1993 |
| δ | Shivers *et al.*, 1989; Zhao *et al.*, 1990 | Sommer *et al.*, 1990 | | | |
| ε | Hanna *et al.*, unpublished, Genbank accession U92284 | | Davies *et al.*, 1997 Whiting *et al.*, 1997 | | |
| θ | | | Bonnert *et al.*, 1999 | | |
| π | | | Hedblom & Kirkness, 1997 | | |
| ρ1 | Zhang *et al.*, 1995 | Greka *et al.*, 1998 | Cutting *et al.*, 1991 | | Albrecht and Darlison, 1995 |
| ρ2 | Ogurusu *et al.*, 1995 | Greka *et al.*, 1998 | Cutting *et al.*, 1992 | | Albrecht and Darlison, 1995 |
| ρ3 | Ogurusu and Shingai, 1995 | | | | |

| | INVERTEBRATES | | | |
|---|---|---|---|---|
| SUBUNIT | SNAIL (2) *Lymnea stagnalis* | NEMATODE (3) *Caenorhabditis elegans* | FRUIT FLY (1) *Drosophila melanogaster* | MOSQUITO (1) *Aedes aegypti* |
| β | Harvey *et al.*, 1991 | | | |
| ζ | Hutton *et al.*, 1993 | | | |
| Rdl | | | ffrench-Constant *et al.*, 1991* | Thompson *et al.*, 1993 |
| UNC-49A | | Bamber *et al.*, 1999 | | |
| UNC-49B | | Bamber *et al.*, 1999 | | |
| UNC-49C | | Bamber *et al.*, 1999 | | |

*subunits with alternatively spliced forms (S and L)

The different $GABA_A$ receptor subunits share a high degree of homology at the predicted amino acid level. It is interesting that $GABA_A$ receptor genes are highly conserved during evolution, with the same identifiable subunits (e.g. α1) being found in a wide range of different species (see **Table 2**). However, even a slight difference in the amino acid sequence, such as point mutations, can cause a tremendous difference in the physiological and pharmacological characteristics of the related receptor. In order to distinguish them as α, β, γ etc., not only sequence homology or divergence is used, but the functionality of the different subunits also serves as a criterion for classification. For example, the initial distinction between α1 and β1 subunits (Schofield *et al.*, 1987) was also provided by their respective properties of benzodiazepine and GABA binding.

The $GABA_A$ receptor subunits have molecular masses between 48,000 (γ2) and 64,000 (α4) Dalton. The subunits that belong to the same subunit class contain 60-80% overall amino acid identity (or 88% conservative substitutions), while there is about 20-40% of amino acid sequence identity (or 50% conservative substitutions) between different subunit classes (Olsen & Tobin, 1990). The ρ1 subunit displays a 30-38% amino acid sequence similarity to the α–δ subunits and exhibits the greatest divergence from the other subtypes (Cutting *et al.*, 1991). ρ2 displays 74% sequence similarity to ρ1 (Cutting *et al.*, 1992). The β subfamily appears to contain the highest degree of similarity with about 80% amino acid identity between members and less than 67% with subunits of the other subfamilies. The α subfamily shows the major diversity, containing members with as little as 71% amino acid similarity between them, while also having similarities to members of different subunit subfamilies as high as 56-68% (Tyndale *et al.*, 1995). Subunits are very well conserved across vertebrate species, showing more than 90% amino acid sequence conservation, while invertebrate counterparts of $GABA_A$ receptors share about 28% identities with vertebrate $GABA_A$ receptor genes and are most closely related to the β subunits. In the chick a γ4 subunit has been reported but to date no mammalian homologue has been found.

#### 1.3.1.3 SPLICING VARIANTS OF $GABA_A$ RECEPTOR SUBUNITS

An additional mechanism of further increasing $GABA_A$ receptor subunit diversity is that of alternative splicing. This has been found in a number of $GABA_A$ receptor subunits, with the alternatively spliced isoforms modified in the promoter region (α5), in the leader sequence (β3), in the N-terminal region (α6, ρ1), in the intracellular domain between M3 and M4 (γ2, β4, β2), or even regulating the brain-specific expression of the subunit concerned (ε).

1) Three alternative first exons 1A, 1B and 1C were revealed for the α5 subunit by Kim et al. (1997) using a combination of cDNA library screening and 5' RACE (Rapid Amplification of cDNA Ends). These exons were then proved to be located next to promoter elements, which drive the expression of the α5 subunit in a tissue and region-specific manner. Thus, the usage of differential exon 1 appears to arise from different promoter activation.

2) The β3 subunit gene was also shown to bear an alternative exon 1a which encodes for a variant leader sequence (Kirkness & Fraser, 1993). The transcription of the β3 mRNA initiates at different sites within the region bearing this exon according to the developmental stage (adult or fetal) and the brain regions. The differential usage of the transcriptional start site therefore determines which of the exon 1 or 1a will be used.

3) Two forms of the γ2 subunit cDNAs, termed γ2S and γ2L, were isolated from mouse and bovine tissue. The γ2L form contains a 24 base pair (8 amino acids) insertion in the cytoplasmic domain between the M3 and M4 domains. This insertion includes a consensus sequence for protein kinase C phosphorylation (Pritchett *et al.*, 1989, Whiting *et al.*, 1990). Both forms of the γ2 subunit can also be found in human and rat brain, and it has been shown that the two forms can coexist in the same receptor (Khan *et al.*, 1994).

4) In the chicken two forms, termed β4 and β4', were isolated and found to differ in the insertion of 12 bp between the M3 and M4 domains (Bateson *et al.*, 1991a; Lasham *et al.*, 1991).

5) There are also two possible forms of the chicken β2 subunit. The insertion of 17 additional amino acids distinguishes the β2L variant from the shorter β2S form (Harvey *et al.*, 1994) and has a site for protein kinase C phosphorylation. In human brain, a longer form of the β2 subunit (hβ2L) was also identified and bears a 114 bp insertion (38 amino acids), again in the sequence corresponding to the large intracellular loop between M3 and M4 and has a consensus sequence for calmodulin-dependent protein kinase II (McKinley *et al.*, 1995).

The additional exon in the γ2 subunit gene in several vertebrate species and those in the chicken and human β2 genes are found in the same relative position. A possible explanation is that this phenomenon originates from the ancestral gene common for all

subunits - or an early descendant - contained 10 but not 9 exons and therefore in some cases this exon has been retained and is still used (Harvey *et al.*, 1994).

6) In the rat there is a short form of the α6 subunit in cerebellar granule cells, termed α6S, that lacks a 10 amino acid sequence in a portion of the extracellular domain and constitutes 20% of the total α6 population (Korpi *et al.*, 1994). The α6S variant appears to be nonfunctional.

7) In addition to the originally described ρ1 subunit, two new variants were identified in the human retina. These variants contain large deletions in the putative extracellular domain of the receptor. These deletions extended from a common 5' site to different 3' sites. The first of these isoforms contains a large 450 nucleotide deletion (150 amino acids) and does not form homomeric channels, but could be associated with other subunits, thus modifying the properties of the resulting heteromeric channels. The second isoform, however, shows a 51 base pair deletion but its functional properties do not seem to be significantly altered with respect to the non-truncated subunit (Martinez-Torres *et al.*, 1998).

8) It has been shown that the recently cloned ε subunit is expressed as two major polyadenylated transcripts: the larger transcript is expressed in a variety of peripheral transcripts, with barely detectable levels in the brain, while the smaller transcript is expressed in the brain. cDNA cloning revealed that the smaller transcript is the appropriately spliced mature mRNA, whereas the larger transcript contains an unspliced intron between putative exons 6 and 7, suggesting a regulation of expression of the ε subunit *via* neuronally restricted RNA splicing (Whiting *et al.*, 1997).

This variegation of different $GABA_A$ receptor subunits isoforms suggests that a large number of possible $GABA_A$ receptor complexes can theoretically be made, combining the full-length isoforms with the splicing variants, explaining the rich spectrum of different actions on brain neurons mediated through $GABA_A$ receptors (Schofield *et al.*, 1989b).

#### 1.3.1.4 STOICHIOMETRY OF $GABA_A$ RECEPTORS

After an initial tetrameric prediction of the receptor structure, the pentameric organization of the $GABA_A$ receptors has been subsequently favored. This was first demonstrated by the molecular mass of a heterogeneous population of isolated $GABA_A$ receptors which was 230,000 – 240,000 Dalton. This molecular weight is consistent with the coassembly of five glycosylated $GABA_A$ receptor subunits. Subsequently, the quaternary structure was verified in electron microscopic image analysis of $GABA_A$ receptors isolated from pig brain, which have a rotational symmetry of 5 (Nayeem *et al.*, 1994). These findings are also supported by the results of Unwin and colleagues, who applied electron microscopy to the related nicotinic acetylcholine receptors of *Torpedo*, which are naturally found at very high

concentration in its electrical organs, also revealing a five-fold pseudosymmetry (Unwin *et al.*, 1993).

With regard to the stoichiometry of the different $GABA_A$ receptor subunits within the receptors, only indirect (and controversial) evidence was first available. In an electrophysiological study on the recombinant receptor subtype $\alpha3\beta2\gamma2$, the most likely subunit stoichiometry (based on a correlation between changes in rectification and the mutation of charged amino acid residues near the channel) was found to be $2\alpha$, $1\beta$ and $2\gamma$ subunits (Backus *et al.*, 1993). Some other studies showed results regarding the assembly of the subunits in native receptors. In particular, Quirk *et al.* (1994a) and Khan *et al.* (1994) showed by Western blot analysis combined with immunoprecipitation experiments that $\gamma2$ and $\gamma3$ on one hand, and $\gamma2S$ and $\gamma2L$ on the other hand can coexist in the same receptor. It is possible that the stoichiometry of $GABA_A$ receptors depends on the particular $\alpha$, $\beta$, and $\gamma/\delta$ subunit isoforms.

On the contrary, however, a study of the $\alpha1\beta2\gamma2$ recombinant receptor (based on correlation of the changes in the GABA sensitivity in relative proportion to the number of subunits carrying a mutation in the putative M2 domain) revealed that these receptors are pentamers composed of $2\alpha$, $2\beta$ and $1\gamma$ subunit (Chang *et al.*, 1996). The research of Im *et al.* (1995) with a tandem construct of $\alpha6$ and $\beta2$ subunit cDNAs (where the carboxyl-terminal of $\alpha6$ was linked to the amino-terminal of $\beta2$ via a linker encoding 10 glutamine residues) support a pentameric structure of $GABA_A$ receptors consisting of two $\alpha6$, two $\beta2$ and one $\gamma2$ for the $\alpha6\beta2\gamma2$ and three $\alpha6$ and two $\beta2$ for the $\alpha6\beta2$ subtype.

More recent studies shed light with more evidence on the stoichiometry of the $GABA_A$ receptors. In a study based on the use of subunit specific antibodies and subunits chimeras to analyse the assembly of recombinant $\alpha1\beta3\gamma2$ receptors (Tretter *et al.*, 1997) showed that the ratio of subunits is $2\alpha$, $2\beta$ and $1\gamma$.

A very recent study performed by Farrar *et al.* (1999) took advantage of the fluorescence resonance energy transfer (FRET) on recombinant $\alpha1\beta2\gamma2$ receptors. They showed that when coupling one recombinant c-myc tagged subunit to a fluorescence acceptor and a fluorescent donor, energy transfer occurred significantly only for the $\alpha$– and $\beta$-tagged subunits, but not for the $\gamma$-tagged subunit, suggesting a stoichiometry of $2\alpha$, $2\beta$ and $1\gamma$.

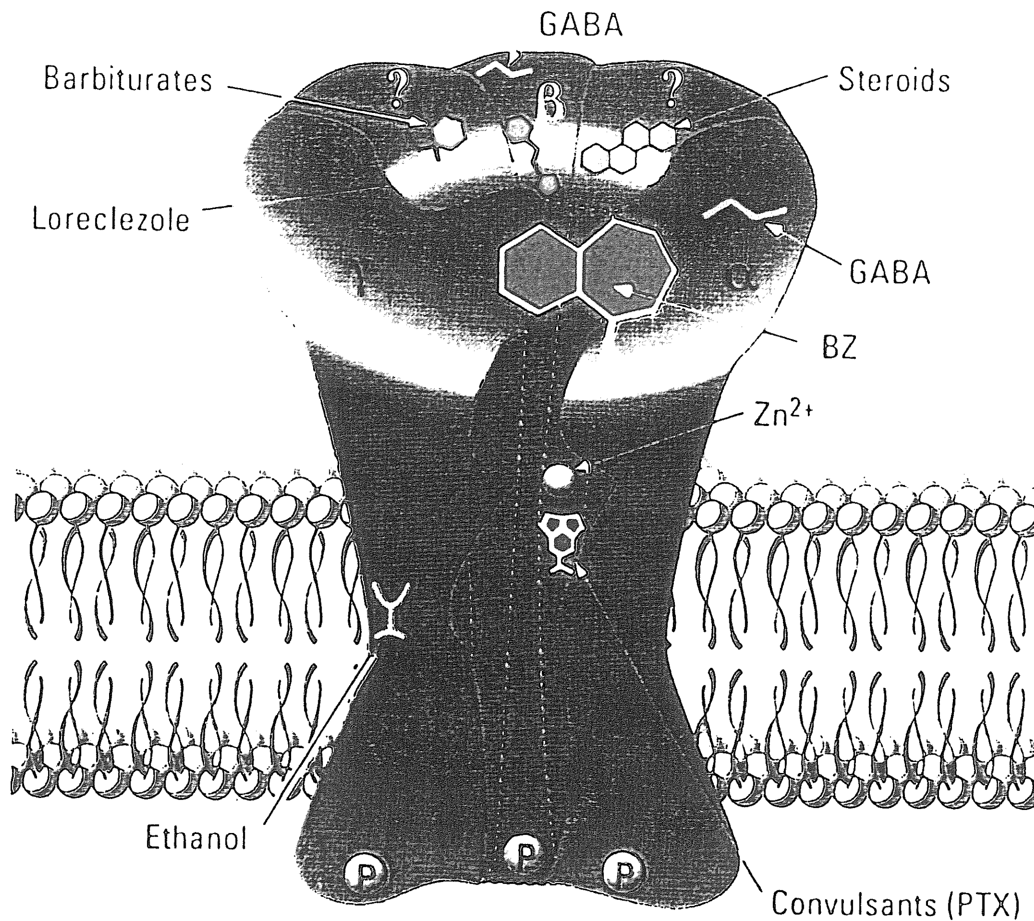
Taken together, these numerous studies strongly suggest that most of the $GABA_A$ receptors subtypes are likely to be formed of $2\alpha$, $2\beta$ and 1 $\gamma/\delta/\varepsilon$ subunits, although some exceptions could occur, depending on the $\gamma$ subunit isoforms (see **Figure 2** below).

### 1.3.2 FUNCTIONAL AND PHARMACOLOGICAL PROPERTIES CONFERRED BY THE DIFFERENT SUBUNITS

Molecular cloning has revealed the great diversity of $GABA_A$ receptor subunits. Presumably this multiplicity of subunits is likely to have crucial consequences for the function and the biophysical properties of the receptors assembled from these subunits. The recent development of heterologous expression systems such as *Xenopus* oocytes or mammalian cells (e.g., human embryonic kidney cells 293 or HEK 293), combined with patch-clamp analysis or ligand-binding assays, represents a precious tool to better understand and predict the properties of subunits and receptors (Levitan *et al.*, 1988; Sigel *et al.*, 1990; Verdoorn *et al.*, 1990). Numerous studies showed that the GABA receptors are the site of action of many drugs such as barbiturates, benzodiazepines, alcohol, picrotoxin, as well as endogenous ligands, like GABA, zinc, and neurosteroids. In addition, molecular biology has brought a significant contribution with the routine use of mutant and chimeras that permitted the identification of target motifs and regions for the different ligands acting on different subunit polypeptides (for review see Smith & Olsen, 1995). A schematic representation of $GABA_A$ receptor binding sites found thanks to these studies is shown in **Figure 2.**

In the following paragraphs, I will try to summarize and account for the functional, pharmacological and kinetic properties that the different subunit subtypes are able to produce in the many receptors they potentially form.

**FIGURE 2. Schematic representation of the $GABA_A$ receptor structure and binding sites** (source: McKernan & Whiting, 1996)




Protein subunits form a pore that when opened will selectively admit chloride ions trapped in the vestibule of the channel lumen. The β subunit is depicted between two other subunits which are most likely an α and a γ subunit, although the arrangement of the five subunits around the pore is unknown. The natural agonist GABA and its antagonist, the plant alkaloid bicuculline are believed to share structural determinants. There are extracellular sites for neurosteroids, barbiturates, benzodiazepines and loreclezole. Picrotoxin (PTX) and $Zn^{2+}$ are binding to sites located within the channel pore. There is also a possible ethanol binding site. The receptor function is also regulated by phosphorylation of cytoplasmic residues (marked P).

1.3.2.1 ALPHA SUBUNITS

Among all the subtypes, the $\alpha$ subunits are the more numerous and include six members, namely $\alpha1$, $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$ and $\alpha6$. This high number of subtypes (unique to the family) is likely to have an important physiological meaning.
Indeed, recombinant studies have revealed the properties of the $\alpha$ subunits regarding ligand binding as well as kinetics. Some of the properties are common to the $\alpha$ subtype, while others are radically different from one $\alpha$ subunit to the other.
First of all, the natural ligand and agonist of the receptors, namely GABA, has been shown to have part of its binding site located on the $\alpha$ subunit. A single point mutation of the rat $\alpha1$ subunit at Phe64, in the putative N-terminal domain produced a marked decrease in agonist and antagonist affinities when coexpressed with $\beta2$ and $\gamma2$ subunits (Sigel *et al.*, 1992). In addition, this residue Phe64 was also shown to be covalently modified by the GABA site photoaffinity agonist [$^3$H]-muscimol. Such a residue is present in all $\alpha$ subunits (as well as in $\gamma$ and $\delta$ subunits) but not in $\beta$ subunits (Smith & Olsen, 1995).
Benzodiazepines, which are the most widely used neuroactive drugs, allosterically modulate the actions of agonists on $GABA_A$ receptor function. Briefly, benzodiazepines act by increasing the probability of opening in the presence of GABA, thus potentiating the response, but are unable to open the channel themselves. The benzodiazepine site is the target of many molecules, ranging from agonists, partial agonists, antagonists, partial inverse agonists to inverse agonists. Mutagenesis experiments have identified some residues involved in the binding of benzodiazepines ligands. The site of action of flunitrazepam, for example, has been shown by several groups to be His102 (bovine $\alpha1$ subunit) (Wieland *et al.*, 1992).
This amino acid has been shown to interact either with the nitrogen present in the core structure of most benzodiazepines ligands (Duncalfe *et al.*, 1996), or with a phenyl moiety close to the core strucutre of these ligands (McKernan *et al.*, 1998).
Interestingly, $\alpha4$ and $\alpha6$ subunits do not have this histidine residue and were shown to be insensitive to diazepam but have high affinity for the inverse agonist Ro-15-4513 (Turner *et al.*, 1991). These two subunits have indeed very similar pharmacological properties, also explained by their high degree of homology in amino acid sequence. Although the His100 residue is missing in the ligand-binding domain, Hauser *et al.* (1997) found that flunitrazepam (a full benzodiazepine agonist like diazepam) acts as an inverse agonist on $\alpha6$-containing receptors. In addition to that, the non-competitive antagonist furosemide (which is also a diuretic compound) acts similarly on $\alpha6$ and $\alpha4$ containing receptors, although its selectivity is far higher for $\alpha6$ (Wafford *et al.*, 1996). More recently the amino acid Ile228 has been found to confer furosemide selectivity to the $\alpha6$ subunit. (Thompson *et al.*, 1999).
The $\alpha5$ subunit, on the contrary to $\alpha1$, is relatively insensitive to the imidazopyridines ligands like zolpidem, and recently, three amino acids found in $\alpha1$ were shown to

confer sensitivity to zolpidem to the corresponding mutant α5 subunit (bearing P162T, E200G and T204S). In particular, amino acid Ser204 from α1 is involved in the binding pocket of zolpidem (Renard *et al.*, 1999).

In line with that, one of the residues described above and located between the disulphide loop and TM1, namely Gly200 of the α1 subunit, also affects binding affinity for benzodiazepine ligands (Pritchett & Seeburg, 1991). This residue is Glu in all other α subunits and conversion to Gly in α3 subunit produces, for example, high affinity for some ligands (CL218872, β-carbolines and zolpidem).

Several other amino acids were found to surround the benzodiazepine binding site. For instance, α1Y159S and α1Y209S substitutions affect diazepam-mediated potentiation (Amin *et al.*, 1997). Finally, the amino acids α1Y161 and α1T206 seem to play a role in diazepam modulation (Buhr *et al.*, 1997).

The α subtype has been shown to be important for the modulation of other ligands such as $Zn^{2+}$ (Fisher *et al.*, 1998), neuroactive steroids (Maitra *et al.*, 1999), general anesthetics (Mihic *et al.*, 1997) and barbiturates (Thompson *et al.*, 1996).

Although the functional differences between the different α subunits starts to be understood for many ligand binding sites, and in particular the benzodiazepine site, only a few studies have focused on the physiological and kinetic characteristics of the various α subunits. Activation and deactivation rates of recombinant receptors are dependent on the α1 to α2 isoform. The α2 subunit seems to confer rapid rise-time but slow deactivation with respect to α1 (Lavoie *et al.*, 1997; McClellan & Twyman, 1999). Receptors containing α6 also have a slower deactivation than those with α1, but lack desensitization (Tia *et al.*, 1996). In addition, the α3 subunit tends to slow activation, desensitization and deactivation in recombinant α3β2γ2S with respect to α1β2γ2S (Gingrich *et al.*, 1995).

More systematic studies comparing different α subtypes-containing receptors using recombinant expression and detailed patch-clamp analysis and ultra-fast agonist perfusion are required to understand the functional and physiological significance of the existence of the six different α subunits found in native receptors.

#### 1.3.2.2 BETA SUBUNITS

The β subunits, together with the α, are necessary for a $GABA_A$ receptor to assemble and gate $Cl^-$ ions in response to GABA. These subunits have been indeed shown to be essential for the binding of GABA by site-directed mutagenesis experiments by Amin & Weiss (1993). They showed that two homologous domains located in the distal N-terminal part of the β2 subunit, Tyr157-Gly158-Tyr159-Thr160 and Thr202-Gly203-Ser204-Tyr205 are necessary for functional activation by GABA. Mutation of Tyr157, Thr160, Thr202 or Tyr205 significantly reduced binding affinity for both agonists and antagonists at the GABA site.

In addition to their importance at this site, the β subunits also bear a number of other binding sites. First, loreclezole is an antielpileptic drug that acts specifically at a novel allosteric site to enhance the activity of $GABA_A$ receptors. Its action occurs through a site present on the β2 and β3 subtypes, the amino acid Asn290 (for β3) or 289 (for β2). The β1 subtype contains a serine residue at that position, and loreclezole is much less effective at $GABA_A$ receptors having this subunit (Wafford *et al.*, 1994; Wingrove *et al.*, 1994). Second, a high affinity binding site for the endogenous modulator $Zn^{2+}$ has been located at His292 of the murine β3 subunit (His267 in rat β1). This amino acid, conserved in all β subunits (but in no other) is responsible for the submicromolar affinity of $Zn^{2+}$ with α1β1 $GABA_A$ receptors. Remarkably, when mutated to the α counterpart, affinity to $Zn^{2+}$ drops by about 300-fold (Wooltorton *et al.*, 1997; Horenstein & Akabas, 1998). The nature of the β subunit also affects channel properties (Verdoorn *et al.*, 1990), benzodiazepine efficacy (Sigel *et al.*, 1990; von Blankenfeld *et al.*, 1990), affinity for the GABA analogue taurine as well as allosteric modulation by barbiturates and steroids (Bureau & Olsen, 1991, 1993; Birnir *et al.*, 1997).

Furthermore, the β subunits are essential for the correct assembly of the $GABA_A$ receptors at the neuronal plasma membrane (see section 1.3.5). They also represent a target for the regulation of the receptor activity by phosphorylation through different classes of protein kinases. In particular, the β subtype was shown to be crucial for inhibiting or potentiating $GABA_A$ receptor currents upon phosphorylation of subtype-specific serine residues and therefore represent an important mechanism for the modulation of $GABA_A$ receptor activity in the CNS (see section 1.3.4).

#### 1.3.2.3 GAMMA SUBUNITS

$GABA_A$ receptor channels formed by α and β subunits have a single-channel conductance of about 15 pS, while the conductance rises to about 29 pS when a γ subunit is present (Angelotti & Macdonald, 1993). This value is reminiscent of the native receptors, suggesting that the γ subunits are intrinsic elements of the $GABA_A$ receptors. More recently, Haas & Macdonald (1999) showed that receptors with a γ2 subunit activate very rapidly, deactivate slowly and desensitize almost completely. They also proposed a kinetic model that could help in assessing the functional significance of different $GABA_A$ receptor isoforms.

Not only is the presence of the γ subunits important for conductance and gating, but this type of subunit confers unique pharmacological properties to the receptors. Of particular importance is the fact that the γ subunits are essential for complete benzodiazepine pharmacology. Several residues have been identified to interact with these ligands.

Firstly the γ2 subunit Thr142, when conservatively mutated to Ser, improved the efficacy of some benzodiazepines agonists and reduced that of others; it also changed

the action of antagonists to partial agonist (Mihic *et al.*, 1995). In addition to this residue, the $\gamma 2$ subunit Phe77 has been also shown to be important for increased stimulatory effects on $\alpha 1\beta 2\gamma 2$ receptor channels expressed in *Xenopus* oocytes (Buhr *et al.*, 1996). More recently this amino acid has been changed to Tyr, Leu or Trp and each of these changes produced recombinant $\alpha 1\beta 2\gamma 2$ with differently altered benzodiazepine pharmacology. These observations confirm the prime role of this position and suggest that because it interacts with most benzodiazepine ligands, it defines part of the benzodiazepine binding pocket (Buhr *et al.*, 1997).

The presence of the different $\gamma 1$, $\gamma 2$ and $\gamma 3$ subtypes also specifically confers properties to the $GABA_A$ receptors. Little is known about the glial $\gamma 1$ subunit, but the antagonists flumazenil and Ro 15-4513 do not act or have very low affinity for the $\gamma 1$-containing receptors (Ymer *et al.*, 1990). More significantly, $\gamma 2$ and $\gamma 3$ subtypes differ in their effect on the benzodiazepine site with regard to agonist binding specificity. Indeed, while zolpidem has a high affinity for $\alpha 1\beta x\gamma 2$ receptors, the replacement of $\gamma 2$ by $\gamma 3$ result in 1000-fold loss in affinity. However such a drastic effect is not observed for all benzodiazepine ligands, and furthermore, the responsiveness to these ligands depends also very much on the $\alpha$ subtype, confirming the importance of the side chains interactions of both $\alpha$ and $\gamma$ in forming the benzodiazepine pharmacophore (Lüddens *et al.*, 1994; Hadingham *et al.*, 1995).

As we have seen in the former section, the divalent cation $Zn^{2+}$ acts on an histidine residue unique to the $\beta$ subunits in the M2 domain. Remarkably, $Zn^{2+}$ inhibits GABA-mediated current when an $\alpha\beta$ combination is expressed in heterologous cells ($IC_{50}$ = 0.11 μM). However, the addition of $\gamma 2$ subunit suppresses the inhibition by $Zn^{2+}$ ions ($IC_{50}$ = 639 μM) (Krishek *et al.*, 1998). This modulation by $Zn^{2+}$ ions on $GABA_A$ receptor is probably of physiological importance since $Zn^{2+}$ ions have been shown to be released from nerve terminals following stimulation (Smart & Constanti, 1990).

The modulation of $GABA_A$ receptors by ethanol was shown in an early study to be dependent on the 8 amino acids insertion in the $\gamma 2L$ isoform. The receptors containing this isoform are enhanced by ethanol, while the ones with $\gamma 2S$ isoform do not respond to ethanol modulation (Wafford *et al.*, 1991). The $\gamma$ subunit subtype was also recently found to be involved in the modulation of $GABA_A$ receptor activity by neuroactive steroids (Maitra *et al.*, 1999) and the gaseous messenger nitric oxide (NO) on the $\gamma 2$ subunit (Fukami *et al.*, 1998). Since the $\gamma 2$ subunit is the most widely diffused subunit in the brain, its modulation by many endogenous ligands as well as pharmaceutically important drugs is likely to arise from well-defined binding domains that in some cases have yet to be identified.

1.3.2.4 DELTA SUBUNIT

The $\delta$ subunit has been first cloned in 1989 from rat brain, but its functional and pharmacological roles were poorly understood until recently. The first characteristic assigned to the $\delta$ subunit is the benzodiazepine insensitivity of the $\alpha\beta\delta$ receptors, which is a marked difference with receptors assembled from $\alpha\beta\gamma$ (Shivers *et al.*, 1989).

More attention has been paid recently to the properties of the receptors bearing the $\delta$ subunit and some useful information have been deduced. First, neurosteroid allosteric modulation was analysed in HEK 293 cells containing $GABA_A$ receptor with $\gamma2$ or $\delta$ subunit. The neurosteroids THDOC ($3\alpha$, $5\alpha$-tetrahydrodeoxycortisone) and pregnenolone sulfate significantly potentiated GABA-gated chloride currents in cells transfected with combinations of $\alpha1$, $\alpha6$, $\beta3$ and $\gamma2$ subunits while cotransfection with the $\delta$ subunit inhibited this potentiation. This effect was also found in cerebellar cells in culture, where the $\delta$ subunit is known to be expressed (Zhu *et al.*, 1996).

Second, the effect on $Zn^{2+}$ ions on recombinant receptors $\alpha1\beta1\delta$ was studied by Krishek *et al.* (1998). They showed that these receptors were more sensitive to $Zn^{2+}$ ($IC_{50}$= 16 $\mu$M) than the $\alpha1\beta1\gamma2$ receptors ($IC_{50}$ = 639 $\mu$M), although they are all much less sensitive than $\alpha1\beta1$ receptors ($IC_{50}$ = 1.2 $\mu$M). The different sensitivity to $Zn^{2+}$ between the different receptor combinations probably originates in a change in quaternary structure of the receptor complex (when a $\gamma/\delta$ subunit is added). It could well also be that different binding sites may exist on the $\delta$ subunit or that the affinity of a common binding site for $\alpha1\beta1$ and $\alpha1\beta1\delta$ may be reduced in the latter. Construction of $\gamma2/\delta$ chimeras and site-directed mutagenesis experiments on the $\delta$ subunit might help to resolve this issue.

Finally, kinetics properties of the $\delta$ subunit were compared to the ones of the $\gamma2$ subunit by Haas *et al.* (1999). Briefly, with respect to $\alpha1\beta3\gamma2L$ currents, $\alpha1\beta3\delta$ GABA-currents activate less rapidly, deactivate rapidly (but similarly to $\alpha1\beta3$), and remarkably, desensitize much less and with a slower mean rate. This study could provide a framework to analyse at the level of macroscopic curents and steady-state single-channels the behaviour of different receptors to better understand their intrinsic characteristics.

1.3.2.5 EPSILON SUBUNIT

The $\varepsilon$ subunit is a new member of $GABA_A$ receptor subunits and was first cloned in 1997 by Davies *et al.*. After a first characterization suggesting that this subunit assembled together with $\alpha$ and $\beta$ and produced receptors insensitive to barbiturates such as pentobarbital, extended studies in recombinant systems showed that the $\alpha1\beta1\varepsilon$ receptors, like $\alpha1\beta1\gamma2S$ receptors are modulated by pentobarbital and neurosteroids. Similarly to the $\delta$ and to the $\gamma2$ subunit, the incorporation of the $\varepsilon$

subunit into a ternary receptor reduces the inhibition of GABA-currents by $Zn^{2+}$ (Whiting *et al.*, 1997). Another very recent study by Neelands *et al.* (1999) on $\alpha 1\beta 3\varepsilon$ receptors indicates that ε-containing receptors are also sensitive to loreclezole, lanthanum and furosemide. Interestingly this receptor combination gives rise to spontaneously active but also GABA-activated channels with a conductance of about 24 pS. These data extend the pharmacological and functional characterization of ε-containing $GABA_A$ receptors which still need to be understood in more detail.

#### 1.3.2.6 PI SUBUNIT

This newly identified subunit is not present in the central nervous system but is expressed in the reproductive system, and in particular in the uterus. It is forming receptors through its assembly with an α and a β subunit, in a similar manner to γ, δ or ε subunits described above. Pharmacological characterization in recombinant system suggests that the π subunit is insensitive to benzodiazepine ligands but sensitive to pentobarbital. Uniquely, the presence of π subunit in a receptor significantly reduced sensitivity to the endogenous steroid pregnanolone, but the physiological role of this altered sensitivity is still unclear (Hedblom *et al.*, 1998).

#### 1.3.2.7 RHO SUBUNITS

Last but not least, the ρ subunits, cloned in the early 1990s, were soon shown to form the so-called $GABA_C$ receptors. The first lines of evidence was that their distribution was mainly retinal and that in recombinant system the properties of the receptors formed by these subunits were similar to the native $GABA_C$ receptors. In fact $GABA_C$ receptors or $GABA_A$-ρ receptors show distinct electrophysiological properties with respect to $GABA_A$ receptors. They have a higher sensitivity for GABA, their currents are smaller and do not desensitize. On the single-channel level, these receptors are characterized by longer mean open times and smaller chloride conductance (about 8 pS) (Feigenspan *et al.*, 1993). Pharmacologically, their profile also differ from other $GABA_A$ receptors because they are insensitive to many $GABA_A$ modulators like barbiturates, benzodiazepines as well as the typical $GABA_A$ antagonist bicuculline (reviewed by Bormann & Feigenspan, 1995).
The ρ subunits have therefore been a source of interest in identifying molecular motifs and domains responsible for their unusual pharmacology, and also represent a precious comparative model to better understand the "classical" $GABA_A$ receptors formed by α, β, γ/δ/ε/π combinations. I will here summarize the main observations and conclusions drawn from numerous studies.
A prominent characteristic of ρ subunits is their ability to form functional homooligomeric channels, which stands in sharp contrast to other $GABA_A$ receptors.

Specific parts of the ρ subunit polypeptide have been involved in this phenomenon; the N-terminal domain and in particular 100 amino acids on the ρ1 subunit being key elements in the formation of homomers (Hackam *et al.*, 1997a, 1997b). It has been also shown that both ρ1 and ρ2 are able to form homo- as well as hetero-oligomeric receptors, a view which has been recently confirmed by Enz & Cutting (1999). They indeed demonstrated that human ρ1 and ρ2 can form both types of channels by a careful study using recombinant receptors.

These interesting results also raise the question whether ρ subunits are able to assemble with other $GABA_A$ receptor subunits. This issue has been addressed but so far, it seems that ρ subunits do not co-assemble with other related subunits, as shown by negative results using either transfected wild-type or chimeric subunits coupled with patch-clamp analysis or co-immunoprecipitation (Shimada *et al.*, 1992; Hackam *et al.*, 1998).

Several binding sites and functional domains have also been identified on the ρ subunits, and because of their homomeric structure, the task has been facilitated compared to other $GABA_A$ receptors.

The agonist binding-domain, similarly to the β subunits, has been shown by site-directed mutagenesis to be essentially composed by two motifs: Y198, Y200 on the one hand and Y241, T244, Y247 on the other hand (Amin & Weiss, 1994).

Additionally, a high-affinity binding-site for divalent cations and in particular $Zn^{2+}$ ions involves an histidine residue (His156) on the extracellular N-terminal domain of all ρ subunits. Site-directed mutagenesis was again used to reveal a site responsible for picrotoxin inhibition on the ρ1 subunit, namely the amino acid Thr314, which is replaced by a methionine on ρ2 (Zhang *et al.*, 1995).

Recently, a domain responsible for anesthetics and alcohol sensitivity on GABAA and glycine receptors has been identified by comparison with the ρ1 receptors which, on the contrary, are not modulated by these agents. This 45 residues region lies in the M2-M3 domain, and in particular two amino acids on GABAA or glycine receptors ($GABA_A$ α1 S270 and glycine α1 A288) found by mutating them to the ρ1 counterpart seem to be critical (Mihic et al., 1997).

In a similar way, a site for barbiturate insensitivity has been characterized on the ρ subunits. Indeed, the residue 328 (Trp in ρ1) located in M3 domain, when mutated into a hydrophobic residue is able to confer barbiturate modulation to the mutant receptors, and interestingly also reveals mechanisms of barbiturate modulation on classical $GABA_A$ receptors (Amin, 1999).

Thus, the ρ subunits by their unique assembly characteristics and pharmacology represent a valuable model for deciphering both GABAA receptor assembly mechanisms as well as structural domains important for physiological and therapeutical functions.

### 1.3.3 SUBUNIT COMPOSITION OF THE NATIVE $GABA_A$ RECEPTORS

In the previous section, we have seen that there is a direct relationship between the nature of the subunits and the pharmacological, functional and kinetics properties that one particular subunit gives to the receptor. Therefore, it is of very high relevance to know the subunit composition of the native receptors to better understand their properties. The task assigned is at present very difficult to achieve, because of the multiplicity of $GABA_A$ reeceptor subunits and their potential myriad of combinations.

Assuming that the receptor is pentameric and that most of the subtypes are composed of $2\alpha$, $2\beta$ and $1(\gamma, \delta, \varepsilon)$, one can infer that approximately 300 combinations can occur (Tyndale *et al.*, 1995). Practically, it has been demonstrated that some subtypes such as $\alpha1\beta2\gamma2$ exist predominanly (Fritschy *et al.*, 1992), thus reducing the actual number of subtypes present throughout the brain that however have yet to be clearly identified.

In the past ten years, the unresolved composition of the native receptors has been a major challenge in the field of GABA research and several different approaches have been employed in trying to understand this issue:

1) the spatial and temporal distribution of the respective $GABA_A$ receptor subunit mRNAs or polypeptides were determined by *in situ* hybridization or immunohistological techniques,

2) $GABA_A$ receptor subunit-specific antibodies were used to determine the co-assembly of different $GABA_A$ receptor subunits in the same receptor,

3) single-cell RT-PCR was used to identify the subunit composition of the $GABA_A$ receptors within single cells.

4) recombinant receptor studies allowed the electrophysiological and pharmacological characterization of receptors formed by defined subunits and their comparison with the properties of native $GABA_A$ receptors.

#### 1.3.3.1 EXPRESSION OF THE DIFFERENT $GABA_A$ RECEPTOR SUBUNITS IN THE BRAIN USING *IN SITU* HYBRIDIZATION AND IMMUNOCYTOCHEMISTRY

Most information about the temporal and spatial distribution of the different $GABA_A$ receptor subunits has come from *in situ* hybridization experiments. It has been shown that each subunit mRNA has a distinct spatio-temporal pattern of expression, with each mRNA also undergoing developmental changes of expression.

The development of subunit-specific antibodies has also been a determining progress in analysing the pattern of expression of the $GABA_A$ receptor subunits. The antibodies have been primarily employed in immunohistological experiments, mostly confirming the results first obtained by *in situ* hybridization.

*1.3.3.1.1 SPATIAL DISTRIBUTION*

The results of *in situ* hybridization experiments have shown that, with the exception of the α6 subunit mRNA which is located almost exclusively in a single cell type, the mature cerebellar granule cell, different $GABA_A$ receptor subunit mRNAs have a heterogeneous distribution across many different cell types in adult brain. The results of the complex *in situ* hybridization experiments conducted by Wisden *et al.* (1992) are summarized in **Table 3**. The most ubiquitously expressed subunits in the adult rat brain are the α1, α2, α3, β2, β3 and γ2 subunits, while messenger RNAs for the α5, α6 and β1 subunits exhibit a highly tissue-specific distribution. Recently, the expression profile in the brain of the ρ subunits, thought to be mainly retinal, has been analysed by *in situ* hybridisation (as well as by RT-PCR) and are likely to increase the diversity of $GABA_A$ receptors in some brain areas (see section 5.2 for more details).
It is clear from these different studies that some cell types express more than five different $GABA_A$ receptor subunit transcripts, thus implying the presence of more than one receptor subtype per cell. Neocortex, hippocampus and caudate-putamen are the areas displaying the most complex expression patterns of $GABA_A$ receptor subunit transcripts. This idea was recently confirmed by Brooks-Kayal *et al.*, (1998) who showed using the method of single-cell mRNA amplification (Eberwine *et al.*, 1995), that in acutely dissociated hippocampal cells (P5) as well as in hippocampal neurons in culture, multiple subunits (in most cases α1, α2, α5, β1 and γ2) are expressed simultaneously.

## TABLE 3. Distribution of $\alpha1$-$\alpha6$, $\beta1$-$\beta3$, $\gamma1$-$\gamma3$ and $\delta$ mRNAs of $GABA_A$ receptors in the rat CNS (source: Wisden *et al.*, 1992)

| | $\alpha1$ | $\alpha2$ | $\alpha3$ | $\alpha4$ | $\alpha5$ | $\alpha6$ | $\beta1$ | $\beta2$ | $\beta3$ | $\gamma1$ | $\gamma2$ | $\gamma3$ | $\delta$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Olfactory bulb** | | | | | | | | | | | | | |
| Periglomerular | 0 | + | (+) | (+) | 0 | 0 | 0 | ++ | + | (+) | + | (+) | (+) |
| Tufted cells | ++ | (+) | + | 0 | 0 | 0 | 0 | +++ | ++ | 0 | ++ | 0 | 0 |
| Mitral cells | +++ | 0 | + | 0 | 0 | 0 | ++ | +++ | +++ | 0 | +++ | 0 | 0 |
| Granule cells | 0 | +++ | (+) | ++ | ++ | 0 | 0 | 0 | +++ | (+) | (+) | (+) | + |
| **Neocortex** | | | | | | | | | | | | | |
| layer II/III | ++ | ++ | + | ++ | (+) | 0 | (+) | ++ | ++ | (+) | ++ | + | + |
| layer IV | + | + | + | + | (+) | 0 | (+) | + | + | (+) | + | + | (+) |
| layer V/VI | ++ | + | ++ | + | + | 0 | + | ++ | ++ | (+) | ++ | + | (+) |
| Pyriform cortex | +++ | +++ | ++ | ++ | 0 | 0 | + | +++ | +++ | + | +++ | + | + |
| **Hippocampus** | | | | | | | | | | | | | |
| CA1 str. pyramidalis | ++ | +++ | (+) | ++ | +++ | 0 | +++ | + | +++ | + | +++ | (+) | (+) |
| CA3 str. pyramidalis | + | +++ | (+) | ++ | +++ | 0 | +++ | + | +++ | + | +++ | (+) | (+) |
| DG granule cells | ++ | +++ | + | +++ | + | 0 | +++ | ++ | +++ | + | +++ | (+) | ++ |
| Tenia tecta | + | +++ | 0 | +++ | ++ | 0 | +++ | 0 | +++ | 0 | 0 | 0 | 0 |
| **Basal nuclei** | | | | | | | | | | | | | |
| Caudate -putamen | (+) | ++ | (+) | ++ | 0 | 0 | (+) | (+) | ++ | (+) | + | + | + |
| Nucleus accumbens | (+) | ++ | (+) | ++ | 0 | 0 | (+) | (+) | ++ | (+) | + | + | + |
| Globus pallidus | +++ | + | (+) | 0 | 0 | 0 | (+) | +++ | (+) | ++ | + | 0 | 0 |
| Endopeduncular n. | +++ | (+) | 0 | 0 | 0 | 0 | 0 | +++ | 0 | 0 | + | 0 | 0 |
| Claustrum | ++ | ++ | +++ | + | + | 0 | ++ | ++ | ++ | (+) | + | ++ | (+) |
| Subthalamic nucleus | ++ | 0 | 0 | 0 | 0 | 0 | 0 | ++ | (+) | 0 | ++ | (+) | 0 |
| **Amygdala** | | | | | | | | | | | | | |
| central amygdaloid n. | (+) | ++ | (+) | (+) | 0 | 0 | + | + | + | ++ | + | (+) | 0 |
| med. amygdaloid n. | (+) | +++ | + | + | 0 | 0 | + | ++ | ++ | +++ | + | 0 | 0 |
| lateral amygdaloid n. | ++ | +++ | + | + | (+) | 0 | + | + | ++ | (+) | ++ | (+) | 0 |
| **Septum** | | | | | | | | | | | | | |
| bed nucleus s. t. | + | +++ | ++ | + | (+) | 0 | ++ | + | ++ | +++ | + | (+) | 0 |
| lateral septum | + | +++ | ++ | + | 0 | 0 | + | (+) | + | ++ | + | (+) | 0 |
| medial septum | +++ | 0 | (+) | 0 | 0 | 0 | (+) | +++ | 0 | 0 | ++ | 0 | 0 |
| diagonal band | +++ | + | + | (+) | 0 | 0 | (+) | +++ | + | (+) | ++ | + | 0 |
| **Medial Habenula** | (+) | ++ | (+) | 0 | 0 | 0 | (+) | (+) | ++ | + | ++ | (+) | 0 |
| **Thalamus** | | | | | | | | | | | | | |
| Medio dorsal | ++ | 0 | 0 | +++ | 0 | 0 | 0 | +++ | 0 | (+) | (+) | (+) | + |
| Paraventricular n. | + | ++ | + | +++ | (+) | 0 | (+) | +++ | (+) | (+) | ++ | (+) | 0 |
| Rhomboid nucleus | ++ | ++ | ++ | +++ | 0 | 0 | + | +++ | (+) | + | + | (+) | 0 |
| Dorsolat. geniculate | ++ | 0 | 0 | +++ | 0 | 0 | 0 | +++ | 0 | (+) | (+) | (+) | + |
| Ventrolat. geniculate | ++ | 0 | 0 | +++ | 0 | 0 | 0 | +++ | 0 | (+) | (+) | (+) | ++ |
| Medial geniculate | ++ | 0 | 0 | +++ | 0 | 0 | 0 | +++ | 0 | 0 | + | ++ | ++ |
| Parafascicular n. | +++ | + | (+) | +++ | 0 | 0 | (+) | +++ | (+) | (+) | + | (+) | 0 |
| Reticular nucl. | + | (+) | + | (+) | 0 | 0 | 0 | (+) | (+) | (+) | + | (+) | 0 |
| Ventr. posterior n. | + | 0 | 0 | +++ | 0 | 0 | 0 | +++ | 0 | 0 | (+) | (+) | ++ |
| Zona incerta | ++ | 0 | (+) | 0 | 0 | 0 | 0 | +++ | 0 | 0 | + | (+) | 0 |
| **Hypothalamus** | | | | | | | | | | | | | |
| Medial preoptic area | + | +++ | + | (+) | + | 0 | + | (+) | ++ | +++ | + | (+) | 0 |
| Arcuate nucl. | (+) | ++ | 0 | 0 | + | 0 | 0 | 0 | ++ | + | + | 0 | 0 |
| Dorsomedial nucl. | (+) | + | (+) | (+) | 0 | 0 | 0 | 0 | + | (+) | + | 0 | 0 |
| Ventromedial nucl. | (+) | ++ | + | 0 | + | 0 | + | 0 | ++ | + | ++ | 0 | 0 |
| **Midbrain** | | | | | | | | | | | | | |
| Red nucleus | +++ | (+) | 0 | 0 | 0 | 0 | 0 | ++ | (+) | (+) | ++ | (+) | 0 |
| **Inferior colliculi** | | | | | | | | | | | | | |
| Central nucleus | +++ | + | (+) | 0 | 0 | 0 | 0 | ++ | 0 | (+) | ++ | (+) | 0 |
| **Substantia nigra** | | | | | | | | | | | | | |
| Pars reticulata | +++ | (+) | (+) | 0 | 0 | 0 | 0 | ++ | (+) | + | + | (+) | 0 |
| Pars compacta | (+) | 0 | + | + | 0 | 0 | (+) | (+) | + | (+) | + | (+) | 0 |
| **Cerebellum** | | | | | | | | | | | | | |
| Stellate/Basket cells | +++ | 0 | (+) | 0 | 0 | 0 | 0 | (+) | 0 | 0 | ++ | 0 | 0 |
| Purkinje | +++ | 0 | 0 | 0 | 0 | 0 | 0 | +++ | +++ | 0 | +++ | 0 | 0 |
| Bergmann glia | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ++ | 0 | 0 | 0 |
| Granule cells | +++ | 0 | 0 | (+) | 0 | +++ | (+) | +++ | +++ | 0 | ++ | (+) | +++ |

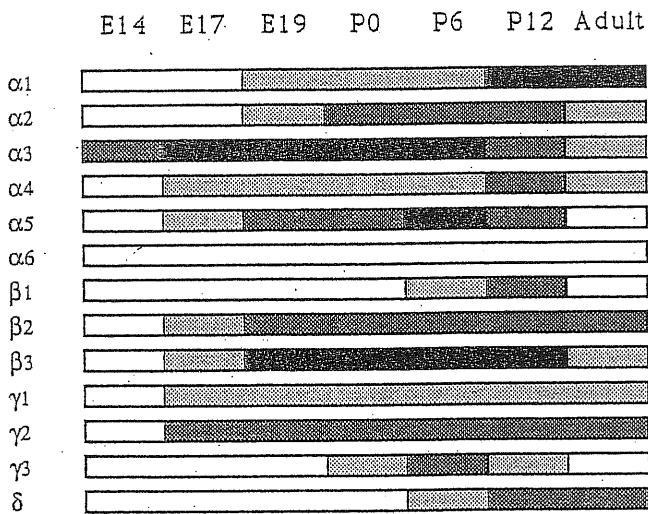
*In situ* hybridization signals obtained with $^{35}S$-labeled oligonucleotide probes on serial sections were assessed as intense, +++; strongly positive, ++; positive, +; weakly positive, (+); or not detectable, 0.

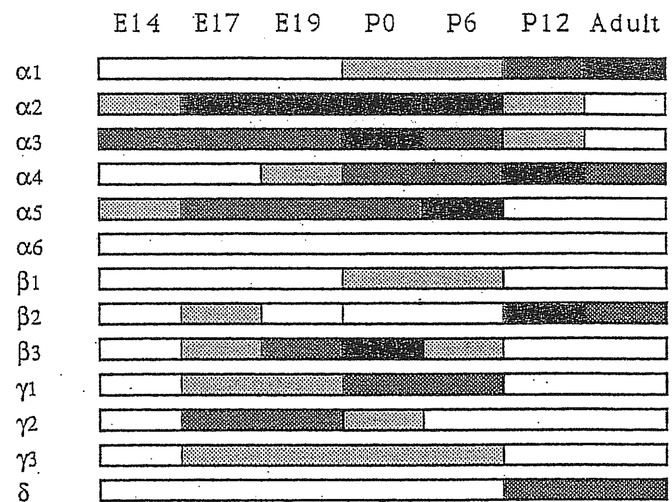
*1.3.3.1.2 TEMPORAL DISTRIBUTION*

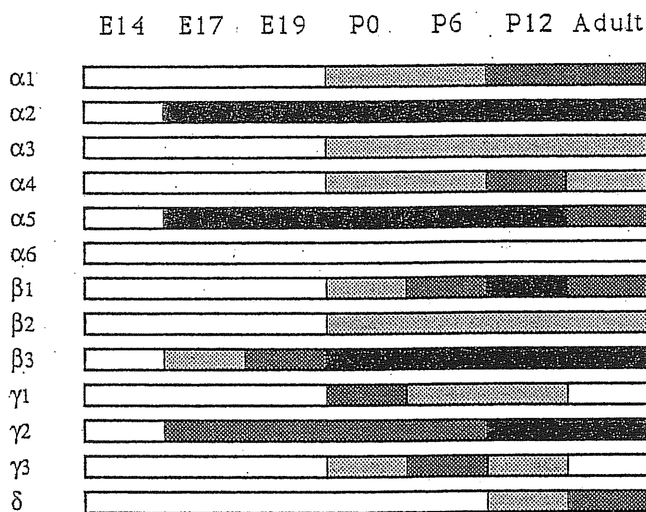
Although the role of GABA during ontogeny is still to be fully understood, the subunit expression of the $GABA_A$ receptor is certainly very dynamic. In fact, the major changes in receptor distribution and subunit mRNA expression patterns occur during development. Numerous $GABA_A$ receptor subunits are expressed before birth in a region and age specific manner (Gutierrez *et al.*, 1994). During postnatal development, there is a switch in the subunit composition of $GABA_A$ receptors, suggesting the existence of molecularly distinct immature and adult forms of $GABA_A$ receptors in the rat CNS (Fritschy *et al.*, 1994; Laurie *et al.*, 1992b). All $GABA_A$ subunit genes exhibit different developmental expression patterns in the rat brain (Laurie *et al.*, 1992b). $GABA_A$ receptors in the perinatal brain are proposed to contain combinations of $\alpha2$, $\alpha3$, $\alpha5$, $\beta1$, $\beta2$, $\beta3$, $\gamma1$, $\gamma2$ and $\gamma3$ subunits. These receptors are often replaced in the adult by others containing $\alpha1$, $\alpha4$, $\alpha6$, $\beta2$, $\gamma2$ and $\delta$ subunits, except in some regions such as the hippocampus that in maturity express both neonatal and adult receptor forms (Laurie *et al.*, 1992b) (**Figure 3**). The neurotrophic actions of GABA may therefore be mediated through $GABA_A$ receptors constructed from a group of subunits expressed in the perinatal brain (e.g. $\alpha2$, $\alpha3$, $\alpha5$, $\beta3$, $\gamma2$) (Laurie *et al.*, 1992b), which are then replaced by other subunits in adulthood. However, once established, $GABA_A$ receptor composition in the adult brain is not maintained unchanged throughout life, but the plasticity in $GABA_A$ receptor subunit expression is retained in adulthood (Gutiérrez *et al.*, 1994, 1996).

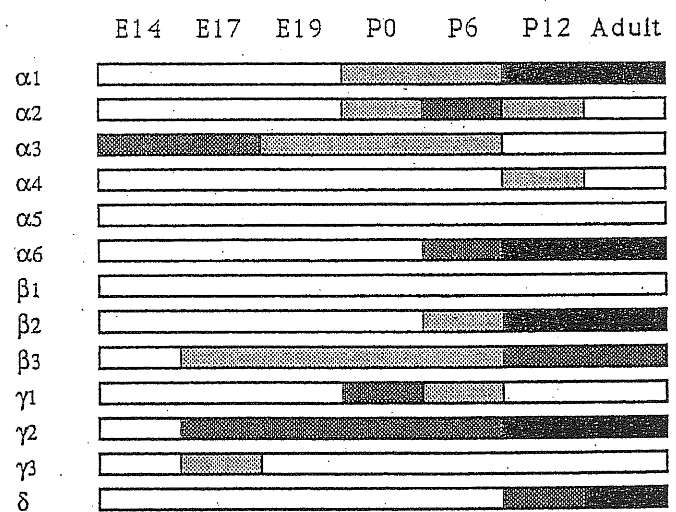
In line with that, Brooks-Kayal *et al.* (1998) showed by *in situ* hybridization and single-cell mRNA amplification that the expression of $GABA_A$ receptor subunits in embryonic hippocampal cells in culture follows a similar pattern, but somewhat delayed, with respect to the *in vivo* situation. These cultures could therefore represent a useful model for understanding the developmental regulation of $GABA_A$ receptor expression.

**FIGURE 3. Schematic representation of the expression of the $GABA_A$ receptors α1-α6, β1-β3, γ1-γ3 and δ subunit mRNAs in selected regions of the embryonic and postnatal rat brain** (source: Laurie *et al.*, 1992b)




Black, strong signal; dark gray, moderate signal; light gray, weak signal; white, very weak or undetectable signal.

### 1.3.3.2 IMMUNOBIOCHEMICAL STUDIES TO REVEAL THE ASSEMBLY OF DIFFERENT SUBUNITS WITHIN RECEPTORS

*In situ* hybridization, and classical immunohistological techniques have begun to address the open question of the subunits forming the $GABA_A$ receptors in the brain. However these approaches are mainly descriptive and cannot give detailed information about the physical assembly of different subunit polypeptides within given receptor subtypes. A further step in the determination of the subunits that co-assemble in native $GABA_A$ receptors has been the use of subunit specific antibodies in immunoaffinity purification, immunoprecipitation or multiple immunofluorescence studies. As for immunohistology, the antigens used for the production of subunit-specific antibodies are N-terminal and C-terminal regions and cytoplasmic loops of putative $GABA_A$ receptor proteins produced recombinantly.

The summary of the subunits co-assembling in native $GABA_A$ receptors is shown in **Table 4.**

**Table 4:** Primary antibodies against the various subunits shown in the first column were used for either immunoaffinity purification or immunoprecipitation studies. The co-assembled subunits were then identified by immunoblotting with specific antibodies as indicated. Positive results only are shown, since each group does not have the full repertoire of $GABA_A$ receptor subunit antibodies, not all polypeptides were screened in each case. ND: not detectable; parentheses stand for low amount.

**TABLE 4. A summary of the subunits co-assembling in native $GABA_A$ receptors.** (source: Stephenson, 1995)

| PRIMARY ANTIBODY | EXPERIMENTAL PARADIGM | CO-ASSEMBLED SUBUNIT | REFERENCE |
|---|---|---|---|
| α1 | Purification | α1,α2,α3,β3,γ2 | Duggan *et al.*, 1991<br>Pollard *et al.*, 1991 |
| | Purification | α1,α3 | Zezula & Sieghard, 1991 |
| | Precipitation | α1,α3 | Luddens *et al.*, 1991 |
| | Precipitation | α1,α3,α5,β2/3,γ2,δ | Mertens *et al.*, 1993 |
| | Precipitation | α1,β2/3,$\gamma2_L$,$\gamma2_S$,α6 | Khan *et al.*, 1994 |
| α2 | Purification | α1,α2,α3,β3,γ2 | Duggan *et al.*, 1991<br>Pollard *et al.*, 1991 |
| α3 | Purification | α1,α2,α3,β3,γ2 | Duggan *et al.*, 1991<br>Pollard *et al.*, 1991 |
| | Purification | α1,α3,(α5),β2/3,γ2,δ | Mertens *et al.*, 1993 |
| | Precipitation | α1,α3 | McKernan *et al.*, 1991 |
| α4 | Purification | α1,α4,β2/3,γ2 | Kern & Sieghart, 1994 |
| α6 | Purification | α1,α6,β2/3,γ2<br>ND α2,α3 | Pollard *et al.*, 1993 |
| | Precipitation | α6,γ2,δ<br>ND α1,α2,α3 | Quirk *et al.*, 1994 |
| β2 | Purification | α1,(α2),β2,γ2<br>ND α3,α5,β1 | Benke *et al.*, 1994 |
| γ1 | Purification | α2,γ1<br>ND α1,α3,α6,γ2,γ3,δ | Quirk *et al.*, 1994 |
| | Purification | α1,α2,α3,α5,β2/3,γ1<br>ND γ2,γ3 | Mossier *et al.*, 1994 |
| γ2 | Purification | α1,α3,(α5),β2/3,γ2,δ<br>ND δ | Mertens *et al.*, 1993<br>Araujo *et al.*, 1998 |
| γ3 | Purification | α1,α2,α3,α4,α6,β2/3,γ3<br>ND α5,γ2 | Togel *et al.*, 1994 |
| | Purification | γ2,γ3<br>ND γ1 | Quirk *et al.*, 1994 |
| δ | Purification | α1,α3,β2/3,γ2,δ<br>ND γ2 | Mertens *et al.*, 1993<br>Araujo *et al.*, 1998 |

A striking fact shown in **Table 4**, is the inconsistency of the results. Different groups found different subunits co-assembled; Quirk *et al.* (1994), for example, were unable to detect $\alpha 1$ or $\alpha 3$ associated with $\gamma 1$, whereas Mossier *et al.* (1994), found both of these subunits associated. Part of the discrepancy of these results may be due to the differing quality of the antisera used, and indicates the need for well characterised monoclonal antibodies which could be widely available. A further limitation of immunoprecipitation and immunoaffinity studies is that detergent-insoluble $GABA_A$ receptors, anchored in the cell membrane (probably linked to the cytoskeleton), which represent more than 50% of all $GABA_A$ receptors are inaccessible to this kind of study and so the composition of these receptors remains unknown (Sigel & Kannenberg, 1996).

However some conclusions can be drawn from the different studies using immunobiochemical techniques. In particular, the subunit composition of cerebellar receptors have been extensively studied because they represent a "simpler" model of subunit assembly, because less subunits are coexpressed in that tissue. Immunoaffinity chromatography experiments have revealed that these receptors seem to be composed of $\alpha 1 \alpha 6 \beta x$, $\alpha 6 \beta x$, combined with $\gamma 2$ (in most cases) or $\delta$ subunits (Jechlinger *et al.*, 1998). In addition, knock-out mice for the $\alpha 6$ subunit recently helped to better understand its contribution to the cerebellar receptors, showing that together with the disappearance of this subunit from the cerebellum, the amount of $\beta 2$, $\beta 3$ and $\gamma 2$ subunits was also significantly reduced (Nusser *et al.*, 1999). Furthermore the $\delta$ subunit polypeptide (but not mRNA) virtually disappeared from cerebellar granule cells, suggesting a post-translational loss of the $\delta$ subunit as well as a specific association between $\alpha 6$ and $\delta$ subunit (Jones *et al.*, 1997). The further use of transgenic mice deficient for a given subunit will probably constitute a valuable mean to evaluate the role of the different subunits in receptor assembly.

Other results obtained using quantitative immunoprecipitation also demonstrated that interestingly two beta subunits can co-exist in a same receptor, which was not clear until then (Li *et al.*, 1997).

In addition to the immunoprecipitation and immunopurification-based approaches, progresses in understanding the structural diversity of subunits forming native $GABA_A$ receptors has been made by *in situ* identification of the receptor subtypes and subunits visualised by double and triple immunofluorescence staining using polyclonal or monoclonal antibodies. This approach allowed to detrmine the location of different $GABA_A$ receptor subunits. at both cellular and subcellular levels by confocal laser misroscopy.

First, Fritschy *et al.* (1992) using antibodies against $\alpha 1$, $\alpha 3$, $\gamma 2$ and $\beta 2/3$ identified five different patterns of subunit colocalization, but with most neurons expressing a single subtype. Immunocytochemical analysis of the $GABA_A$-$\rho$ receptors in the retina has been made using polyclonal antibodies, and showed that the $\rho$ subunits coassemble

between them, but not with other $GABA_A$ or glycine receptor subunits (Koulen *et al.*, 1998). This example strongly suggests that specific interaction are required between subunits for their assembly into some determined receptor subtypes, which are therefore likely to be less numerous than the predicted combinatorial diversity.

#### 1.3.3.3 SINGLE-CELL RT-PCR: CORRELATION BETWEEN RECEPTOR PROPERTIES AND SUBUNIT GENE EXPRESSION

A few years ago, a powerful new approach to analyse ion channel gene expression in patch-clamped cells from brain slices was developed by several laboratories (Lambolez *et al.*, 1992, Eberwine *et al.*, 1992). The protocol combines electrophysiology and molecular biology on the same cell and allows a direct correlation between the biophysical properties of the receptors and the subunits from which they are assembled. Recently, this approach was applied to the analysis of $GABA_A$ receptor subunit mRNAs and their splicing variants at the single-cell level (Ruano *et al.*, 1997). First the $GABA_A$-mediated currents are recorded using the whole-cell patch-clamp technique and then the mRNAs from the same cell are reversibly transcribed and cDNA products are amplified by multiplex polymerase chain reaction, using $GABA_A$ receptor subunit specific (or degenerate) primers. This study showed that, in Purkinje cells, $\alpha 1$, $\beta 2$, $\beta 3$, $\gamma 2S$ and $\gamma 2L$ subunit mRNAs were detected within a single cell. In layer V cortical pyramidal cells, a total of ten $GABA_A$ receptor subunit mRNAs could be detected, with a mean of seven subunit mRNAs per cell.

Another study used the same approach to basket-cells of the dentate gyrus and tried to link the kinetics as well as benzodiazepines and $Zn^{2+}$ sensitivity to subunit gene expression. As expected, $\gamma$ subunits were found to be consistently present, and are the major determinants of $Zn^{2+}$ and flunitrazepam sensitivity. They also found that in some cells, up to four different $\alpha$, all three $\beta$ or all three $\gamma$ subunits could be detected (Berger *et al.*, 1998).

In general, the results obtained with this technique agree well with the results obtained by *in situ* hybridization experiments. This technique represents a very resolutive tool for the potential identification of the composition of the $GABA_A$ receptor subtypes in neurons. However, due to the high number of subunits coexpressed in a same cell and to the weak differences in terms of channel properties between different subtypes, prediction of the exact nature of the $GABA_A$ receptors turns out to be difficult to achieve. The use of this technique would become extremely advantageous when more data will be available from single-channel studies in recombinant systems for the definition of specific kinetic signatures for the various possible $GABA_A$ receptor subtypes.

### 1.3.3.4 INFORMATION GAINED FROM RECOMBINANT RECEPTORS STUDIES

Pharmacological or endogenous substances have been tools of choice in trying to decipher the subunit composition of the receptors that are occurring in the brain. However, they are often not selective enough to identify the subunit(s) upon which they act, and their diversity is not high enough to cover all $GABA_A$ receptor subtypes. Patch-clamp analysis and binding assays on native receptors have revealed some valuable characteritics of the receptors found *in vivo*, but the exact subunit composition of these can be only certified when the channel behaviour is reproduced in a recombinant system.

To date, there are few reports describing a correspondance between native and recombinant receptors.

In the case of cerebellar receptors, where the complexity of subunits expressed is the lowest, prediction of the main cerebellar receptor subtypes made by Quirk *et al.* (1994) using immunoprecipitation of radioligands binding sites led to the analysis of corresponding receptors. Indeed, as they concluded that the main subunits occurring together were $\alpha6\gamma2$, $\alpha6\delta$ and $\alpha1\gamma2$, Saxena & Mcdonald (1996), analysed the properties of the $\alpha6\beta3\gamma2L$, $\alpha6\beta3\delta$ and $\alpha1\beta3\gamma2L$ receptors in transfected cells because they are very likely to be found *in vivo*. They then found that these receptors display differences in sensitivity to $Zn^{2+}$, β-carboline and in enhancement by diazepam.

Another more recent example by Sur *et al.* (1998) showed that the pharmacological characteristics of $\alpha5\beta3\gamma2$ in recombinant cells were also found and reproduced in benzodiazepine binding sites in rat and human hippocampi, suggesting that this receptor subtype is the main one in the hippocampus.

Because the benzodiazepine site is the target of many substances ranging from full agonist, partial agonist, antagonist, partial inverse agonist and inverse agonist, it is now possible to better discriminate between receptor combinations. Most of these combinations have been tested in recombinant cells, and as described above, some do occur in native $GABA_A$ receptors.

The results of these attempts to characterize and determine subunit composition are summarized in **Table 5**, which is now a recommended classification of the most common $GABA_A$ receptors (Barnard *et al.*, 1998)

**TABLE 5. A summary of the characteristics of $GABA_A$ receptors from recombinant expression studies** (modified from Barnard *et al.*, 1998)

| RECEPTOR COMBINATION | CHARACTERISTIC PROPERTIES |
|---|---|
| α1βnγ2 | High affinities and efficacies for classical BZ agonists, CL 218872, zolpidem |
| α1βnγ3 | Similar to α1βnγ2, but ~400-fold less sensitive to zolpidem, lower affinity for classical BZ ligands[1] |
| α1βnγ1 | Similar to α1βnγ3 but lower affinity for flumazenil and Ro 15-4513 which act as low-potency positive agonists [2] |
| α2βnγ2 | Similar to α3βnγ2 receptors for ligands noted there, other properties not yet defined |
| α2βnγ1 | BZ agonists have 2- to 20-fold lower potency than on α2βnγ2. Affinity for zolpidem 5-fold higher but very low efficacy. Insensitive to antagonists flumazenil and Ro 15-4513 [3] |
| α3βnγ2 | High affinities and potencies for classical BZ agonists, similar to those of α1-containing receptors, but intermediate for zolpidem [4] |
| α4βnγ2 | Insensitive to classical BZ agonists, zolpidem and many other agonists. A notable exception is bretazenil. Flumazenil and Ro 15-4513 are agonists [5] |
| α5β1/3γ2 | α5-containing receptors: high affinity for classical BZ agonists but insensitive to imidazopyridines |
| α5β3γ3 | Affinities of α5β3γ3 are as for α5β1/3γ2 but triazolam and β-carbolines are 10 to 30- fold weaker and CL 218872 is 10-fold stronger |
| α5β2γ2 | α5β1/3γ2 differs from α5β2γ2 in its outward rectification and its slower desensitization at depolarized voltages [6] |
| α6β1γ2 | Insensitive to all BZ ligands except bretazenil and some other partial agonists. Flumazenil and Ro 15-4513 become partial agonists and DMCM an antagonist |
| α6β2/3γ2 | Same as α6β1γ2 but antagonized selectively by furosemide |
| α6α1β2/3γ2 | Combines the binding sites of α1βnγ2 and α6β2/3γ2 [7] |
| α1βnδ | Insensitive to all BZ ligands, sensitive to bicuculline, and highly sensitive to zinc |
| α4βnδ | Generally similar to α1βnδ |
| α6βnδ | Same as α1βnδ ; in cerebellar granule cells only [8] |
| α1βnε | Generally similar to α1βnδ |

| | |
|---|---|
| ρ1 | Insensitive to all BZ ligands, but also bicuculline and pentobarbital; not activated by isoguvacine. Strong currents |
| ρ2 | Similar to ρ1, but usually lower currents |
| ρ1ρ2 | Similar to ρ1 but very low sensitivity to picrotoxin (in the rat) |
| ρ3 | Similar to ρ1 |

n = any of β1, β2 or β3 subunit.

[1] Herb *et al.* (1992); Lüddens *et al.* (1994); Hadingham *et al.* (1995).

[2] Ymer *et al.* (1990); Puia (1991); Giusti *et al.* (1993).

[3] Wisden & Seeburg (1992); Hadingham *et al.* (1993); Wafford *et al.* (1993).

[4] Faure-Halley *et al.* (1993); Lüddens *et al.* (1994).

[5] Yang *et al.* (1995); Huh *et al.* (1996); Knoflach *et al.* (1996); Scholze *et al.* (1996); Wafford *et al.* (1996).

[6] α5: Pritchett & Seeburg (1990); Faure-Halley *et al.* (1993); Hadingham *et al.* (1993). α5β1/3γ2: Burgard *et al.* (1996). α5β3γ3: Lüddens *et al.* (1994); Hadingham *et al.* (1995). α5β2γ2: Burgard *et al.* (1996).

[7] α6β1γ2, α6β2/3γ2: Lüddens *et al.* (1990); Korpi *et al.* (1995); Yang *et al.* (1995); Hadingham *et al.* (1996); Huh *et al.* (1996); Nusser *et al.* (1996); Wafford *et al.* (1996). α6α1β2/3γ2: Pollard *et al.* (1995); Khan *et al.* (1996).

[8] α1βnδ: Saxena & Mcdonald (1994); Ducic *et al.* (1995). α6βnδ: Quirk *et al.* (1995); Saxena & Macdonald (1996); Jones *et al.* (1997).

## 1.3.4 POST-TRANSLATION MODULATION OF THE GABA$_A$ RECEPTOR

The GABA$_A$ receptor complex, after assembly and insertion in the neuronal plasma membrane, is functional and becomes available for regulation of its activity by phosphorylation. This mechanism occurs via the action of protein kinase A (PKA), protein kinase C (PKC) or tyrosine kinase on the intracellular domain of the different target subunits. The number and location of the phosphorylation sites vary between different types of subunits but also within the same subunit in different species. All β subunits possess protein kinase A and protein kinase C consensus sequences. The γ2 subunit bears sites for protein kinase C and tyrosine kinase, while the γ2L spliced form contains an additional phosphorylation site for protein kinase C, which has been suggested to be essential for the ethanol sensitivity in recombinant receptors (Wafford *et al.*, 1991). The ρ2 and ρ3 subunits also contain consensus sequences for phosphorylation by protein kinase C. Many α subunits also contain a protein kinase C consensus sequence (e.g. human α5 contains four protein kinase C consensus sites as shown by Wingrove *et al.*, 1991, by computer search performed at the NCBI, using the BLAST network), although no one has succeeded in demonstrating their

functional significance (Sigel *et al.*, 1991). Interestingly, the δ subunit is devoid of these consensus sequences.

Much effort has been directed towards elucidating a role for phosphorylation in $GABA_A$ receptor function. First, evidence has been obtained for PKA involvement in $GABA_A$ receptor desensitization (Tehrani *et al.*, 1989). Phosphorylation has also been postulated to play a role in the phenomenon of rundown by Gyenes *et al.* (1988) (rundown is a decrease in receptor response that occurs over the time scale of several to many minutes and may require periodic agonist application). However, these phosphorylation studies have been surrounded by controversy. There have been numerous reports of apparent PKA and PKC effects but also many studies where the effects of PKA and PKC modulators could be explained by phosphorylation-independent mechanisms. Different effects are thought to be due to different receptor subunit combinations in the various preparations or to the indirect effects of phosphorylation on other regulatory proteins.

Recently, this matter has been partially resolved and it turns out that the differences observed on distinct neuronal preparations are likely to be due to subunit composition of the $GABA_A$ receptor. Indeed, a study published by McDonald *et al.* (1998) led to the conclusion that the β subunit isoform is crucial for modulation of $GABA_A$ receptor activity by cAMP-dependent protein kinase (PKA). In particular they found that two adjacent Ser residues (Ser408 and Ser409) influence the direct modulation of β3-subunit containing $GABA_A$ receptors. These two consecutive Ser residues are not found on the β1 or β2 subunits, Ser408 being replaced by an Ala residue. Essentially, the phosphorylation of only Ser409 (like in β1 and β2 subunits) produces inhibition, while phosphorylation on both S408 and S409 (β3-specific sequence) potentiates GABA-activated responses.

This suggests a novel mechanism for the differential modulation of $GABA_A$ receptor function acting specifically on the β subtype. Given the role of $GABA_A$ receptors in the mediation of synaptic inhibition, regulation by phosphorylation could have profound effects on neuronal excitability by affecting post-synaptic efficacy of GABAergic transmission.

### 1.3.5 MECHANISMS OF RECEPTOR LOCALIZATION AND CLUSTERING

The assembly of the subunits to different $GABA_A$ receptor subtypes is likely to be a regulated phenomenon since some receptor combinations are preferentially occurring, as emphasized in an earlier section. The association of different subunits has been shown to follow particular routes involving interactions of the subunits with proteins of

the endoplasmic reticulum (Connolly *et al.*, 1996a). In addition to that, the β subunits have been shown to have a predominant role in the correct assembly of the $GABA_A$ receptors as well as in their subcellular targeting to the plasma membrane (Connolly *et al.*, 1996b).
Recently, a growing number of studies have focused on the localization and clustering of the receptors mainly using immunocytochemical stainings of the various subunits polypeptides for their colocalization. Most of the subunits turned out to be located at the post-synaptic densities but also at extra-synaptic sites (Nusser *et al.*, 1995, 1998) or even non-functional synapses (Kannenberg *et al.*, 1999).
However the specific mechanisms underlying the differential targeting of $GABA_A$ receptors remained unanswered for a long time but have been partially clarified in the very last months. Among the family of ligand-gated channels, specific proteins interacting with the receptors have been already found. For the acetylcholine receptors, the protein rapsyn has been identified as an anchor to the cytoskeleton (Frail *et al.*, 1987). Similarly, the tubulin-binding protein gephyrin has been shown to be the link between glycine receptors and the cytoskeleton (Kirsch *et al.*, 1993).
Postsynaptic gephyrin clusters are not only colocalized with glycine receptors, but are also present at GABAergic synapses in various cell types and tissues, and these clusters are in close association with $GABA_A$ receptor subunits. Essrich *et al.* (1998) using a γ2 knock-out model demonstrated elegantly that gephyrin is indeed interacting with the γ2 subunit. Briefly, they showed that loss of $GABA_A$ receptor clusters in γ2 deficient mice is parallel to the loss of gephyrin and synaptic GABAergic function, and that conversely, inhibiting gephyrin expression causes loss of $GABA_A$ receptor clusters. Some other proteins have followed in the comprehension of the mechanisms of $GABA_A$ receptor clustering and targeting. Wang *et al.* and Hanley *et al.* (1999) have brought to light two proteins, GABARAP (stands for $GABA_A$-receptor-associated protein) and MAP-1B (microtubule-asociated protein-1B) specifically interacting with γ2 and ρ1 subunits, respectively. They both used a yeast-two hybrid screening to show that each of these two proteins are able to interact with a portion of the intracellular loop of the corresponding subunits to anchor them to the cytoskeleton. Interestingly, these proteins seem to discriminate with the receptor subtype, since for example GABARAP does not bind to β1 and several α subunits, and MAP-1B does not interact with ρ2 or other $GABA_A$ receptor subunits.
These works have now paved the way to the search of possible other players in the clustering of $GABA_A$ receptor subtypes. Understanding the clustering determinants will probably allow us to get insights into receptor regulation, targeting, degradation, internalization, influence of phosphorylation on synaptic plasticity as well as interactions with the cytoskeleton.

## 1.3.6 REGULATION OF THE EXPRESSION OF $GABA_A$ RECEPTOR SUBUNITS

Neuronal cells are able to respond and adapt to changes of their environment by modifying their metabolism and physiology. The $GABA_A$ receptor system, with its ability to switch the expression of different subunits and therefore to modify the function of the receptors, is an important part of that phenomenon. However, the mechanisms regulating the expression of different $GABA_A$ receptor subunits are still poorly understood. It is thought that intrinsic, genetic determinants are prevalent in the regulation of receptor expression but that epigenetic factors may also be involved. It is known, for example, that very tiny changes in GABA concentration (Roca *et al.*, 1990; Kim *et al.*, 1993; Mhatre & Ticku, 1994; Fenelon & Herbison, 1996), in $GABA_A$ receptor activity (Poulter *et al.*, 1997) as well as exposure to different drugs, especially GABA agonists and modulators (Kang & Miller, 1991; Montpied *et al.*, 1991; Hirouchi *et al.*, 1992; O'Donnovan *et al.*, 1992) can cause an imbalance or change in subunit expression. Distinct $GABA_A$ receptor subunits may exhibit specific partnership in gene expression. For instance, it has been shown that inactivation of one of the $\alpha 6$ or $\delta$ subunit genes in cerebellar granule cells (Jones *et al.*, 1997) inhibits the expression of the other one. Pathological states induce changes in the level of mRNA expression of the different $GABA_A$ receptor subunits (Rice *et al.*, 1996; Huntsman *et al.*, 1998). Electroconvulsive shock (Pratt *et al.*,1993), swim stress (Montpied *et al.*, 1993) and the seizures induced by rapid kindling stimulation (Kokaia *et al.*, 1994) change the levels of different $GABA_A$ receptor subunit mRNAs. The neurotransmitter glutamate has been also shown to regulate expression of $GABA_A$ receptor genes (Memo *et al.*, 1991).

The change of expression of different $GABA_A$ receptor subunits is evident at both mRNA and protein levels (Huntsman *et al.*, 1994), suggesting a transcriptional control and regulation of $GABA_A$ receptor expression. Until now, only one transcriptional factor that regulates subunit expression, termed brain-specific factor 1 (BSF1), has been identified (Motejlek *et al.*, 1994). BSF1 is a sequence-specific DNA-binding protein which selectively interacts with a conserved purine element in the 5'-flanking region of the $GABA_A$ receptor $\delta$ subunit gene. The expression of this brain specific protein correlates with the regional expression pattern of the $\delta$ subunit mRNA in the brain and thus has been proposed as its transcriptional regulator.

Recently it has been suggested that regulation of expression of the $\varepsilon$ GABA receptor subunit is regulated via neuronally restricted RNA splicing (Whiting *et al.*, 1997). Namely, Northern analysis revealed that the $\varepsilon$ subunit is expressed as two major polyadenylated transcripts: the larger transcript is expressed in a variety of peripheral tissues, while the smaller transcript is expressed in the brain and spinal cord. The smaller transcript represents the spliced mature mRNA, whereas the larger transcript contains an unspliced intron between putative exons 6 and 7. Because there is

abundant expression of the unspliced transcript in a number of peripheral tissues, it has been suggested that the neuronal-specific expression of mature ε subunit transcripts is regulated by a neuronally restricted RNA splicing mechanism. This finding is important, as it indicates that the presence of mRNA does not necessarily indicate that functional protein is also present.

Studies on the mechanisms of $GABA_A$ receptor gene expression regulation will undoubtedly help in the understanding of the physiology of receptor adaptation, but also the pathophysiology of diseases in which the $GABA_A$ receptor expression is altered, such as in Huntington's Chorea, epilepsy and possibly schizophrenia (Faull *et al.*, 1993; Kokaia *et al.*, 1994, Poulter *et al.*, 1999; Huntsman *et al.*, 1998).

### 1.3.7 GENOMIC ORGANIZATION OF $GABA_A$ RECEPTOR SUBUNITS GENES

It is interesting that $GABA_A$ receptor genes lie in clusters within human and mouse chromosomes. Especially striking is the occurrence of individual α, β and γ subunits together on single chromosomes. For example, human α2, α4, β1 and γ1 subunit genes are found together on the short arm of chromosome 4 (Buckle *et al.*, 1989; Kirkness *et al.*, 1991; Dean *et al.*, 1991; Wilcox *et al.*, 1992; McLean *et al.*, 1995), the α1, α6, β2 and γ2 on chromosome 5 (5q34-q35) (Buckle *et al.*, 1989; Johnston *et al.*, 1992; Wilcox *et al.*, 1992; Russek & Farb, 1994; Hicks *et al.*, 1994) and α5, β3 and γ3 on human chromosome 15 (15q11-q13) (Wagstaff *et al.*, 1991a,b; Knoll *et al.*, 1993; Nakatsu *et al.*, 1993). The human ρ subunit genes, ρ1 and ρ2, are also found together on chromosome 6q14-q21 (Cutting *et al.*, 1992). Likewise, α5, β3 and γ3 subunit genes are close to one another on mouse chromosome 7 (Wagstaff *et al.*, 1991a; Nakatsu *et al.*, 1993, Danciger *et al.*, 1993). Recent data from Russek (1999) show the existence of beta-alpha-alpha-gamma gene clusters on human chromosomes 4 and 5. Phylogenetic tree analysis has predicted the existence of beta-alpha-gamma ancestral gene cluster in which internal duplication of an ancestral alpha gene was followed by cluster duplication, resulting in the relative chromosomal positions in the human genome. The close proximity of $GABA_A$ receptor genes on individual chromosomes has been hypothesized to be related to coordinate temporal and spatial expression of the subunits. It is possible that clustered genes are expressed together and that related $GABA_A$ receptor subunits are combined together *in vivo* to form functional channels, although data supporting this view have not yet been found (see **Figure 3**).

## 1.4 DEVELOPMENTAL ROLE OF GABA

At early stages of postnatal development, GABA, by acting on $GABA_A$ receptors, depolarizes cell membranes through an outward flux of chloride, thus allowing the activation of voltage-dependent calcium channels, leading to an increase in intracellular $Ca^{2+}$ concentration. Calcium signaling is required for stabilization of synaptic contacts and growth of the adult neuronal network. As a result, GABA exerts a trophic action during this stage of development.

In addition, in neonatal hippocampal neurons, GABA elicits two distinct chloride dependent responses: the predominant classical $GABA_A$ mediated response defined by its conventional inhibition by the competitive antagonist bicuculline and a novel one, which is insensitive to bicuculline and baclofen but is sensitive to picrotoxin (Strata & Cherubini, 1994; Martina *et al.*, 1995). The latter represents 5-10% of the total GABA responses. These novel responses to GABA are similar in some aspects to those found in the retina and described as $GABA_A$-ρ. The $GABA_A$-ρ-like currents in the hippocampus are transiently expressed during a critical period of postnatal development between P0 and P12. In spite of their physiological and pharmacological characterization, the subunits responsible for $GABA_A$-ρ-like responses remain to be determined.

In contrast to adult neurons, both $GABA_A$ and $GABA_A$-ρ-like responses present in the neonatal hippocampus are depolarizing. This depolarization reaches threshold for action potential generation and GABA, therefore, acts during this period as an excitatory neurotransmitter.

The trophic action of GABA, the various responses to GABA from neonatal hippocampal neurons as well as the depolarizing and excitatory role of GABA during the early period of postnatal development are important phenomena to understand. Certainly the nature of the $GABA_A$ receptors together with other factors involved in these GABA-mediated effects are determinant in their genesis.

### 1.4.1 GABA AS A NEUROTROPHIC FACTOR

During development there are myriads of external stimuli, some of them poorly understood, that influence cell migration, differentiation, and synapse formation. Among these, various neurotransmitters may influence the development of the CNS by stimulating neurotransmitter receptors (Lauder, 1993; Lipton & Kater, 1989).

GABA has been shown to influence cell migration, to stimulate chemokinesis (Behar *et al.*, 1994) and neurite sprouting (Barbin *et al.*, 1993). GABA, as well as glutamate, have been implicated in arresting DNA synthesis and the cell cycle in progenitor cells during neurogenesis (Lo Turco *et al.*, 1995).
In the hippocampus, at the time when the excitatory glutamatergic connections are still silent, the GABAergic system is already present and functioning. It is established that GABA is produced and $GABA_A$ receptor subunits are expressed in neuronal cells before they migrate to their final positions, and before mature synapses are found in significant numbers. In the developing hippocampus, at this stage when synapses are still immature, there are numerous GABA and glutamic acid decarboxylase immunoreactive interneurons present in the vicinity of pyramidal cells (Ben-Ari *et al.*, 1989). It is thought that non-synaptic GABA release, possibly originating from growth cones, may precede GABAergic synaptogenesis (Gordon-Weeks *et al.*, 1984; Balcar *et al.*, 1986). It is supposed that at this early period of postnatal development GABA exerts mainly a trophic action through membrane depolarization and rise in intracellular calcium (Cherubini *et al.*, 1991, Leinekugel *et al.*, 1995).

### 1.4.2 GABA: AN EXCITATORY NEUROTRANSMITTER IN THE NEONATAL HIPPOCAMPUS

In neonatal hippocampal neurones GABA depolarizes and excites neuronal membranes, instead of hyperpolarizing and damping cell excitability, as in adulthood. At the time when GABA acts as an excitatory neurotransmitter, the glutamatergic synapses, which carry most of the excitatory drive in the adult CNS, are still poorly developed (Cherubini *et al.*, 1991; Hosokawa *et al.*, 1994).
The depolarizing and excitatory action of GABA is noticeable in both CA1 and CA3 hippocampal subfields as well as in interneurons between P0 and P7 (Ben-Ari *et al.*, 1989; Cherubini *et al.*, 1991; Strata *et al.*, 1997). During the first postnatal week bicuculline fully blocks spontaneous and evoked excitatory synaptic activity and slightly hyperpolarizes the membrane. These results suggest that most of the excitatory synaptic drive at this early postnatal period is mediated by $GABA_A$ receptors. Until P5-P7, the spontaneous activity of CA1 and CA3 pyramidal neurons and interneurons is characterized by the presence of network-driven giant depolarizing potentials (GDPs), which are mediated by GABA acting on $GABA_A$ receptors (Ben-Ari *et al.*, 1989; Cherubini *et al.*, 1991; Strata *et al.*, 1997). At the end of the first week of life the responses to GABA shift from the depolarizing to the hyperpolarizing direction in concomitance with a progressive reduction of spontaneous GDPs and with the

appearance of spontaneous hyperpolarizing potentials (inverted GDPs) (Ben-Ari *et al.*, 1989). The time when GABA is proposed to become an inhibitory transmitter (after P12), perfectly correlates with the dramatic acceleration of the genesis of mature-type synapses (Aghajanian & Bloom, 1967). The CA1 region of the hippocampus also shows the slow maturation of the GABAergic inhibitory system in early postnatal life (Muller *et al.*, 1989). The excitatory action of GABA is not only limited to rat neonatal hippocampus and is also found in other parts of the nervous system such as the spinal cord (Wu *et al.*, 1992; Rohrbough & Spitzer, 1996), the olfactory bulb (Serafini *et al.*, 1995), the hypothalamus (Chen *et al.*, 1996), and the cortex (Owens *et al.*, 1996).

GABA depolarization in neonatal neurons is chloride dependent, therefore this phenomenon may be due to a modified chloride gradient. This idea is also supported by the fact that in neonatal neurons the $Cl^-$ reversal potential ($E_{Cl^-}$) is more positive than the resting membrane potential (about -50mV in neonates and about -70mV in adults; Cherubini *et al.*, 1991).

The mechanisms controlling chloride gradients were poorly understood until recently, when Rivera *et al.* (1999) early this year found a molecular basis for the modified chloride homeostasis in neonatal neurons. They showed that the ontogenic change in $GABA_A$-mediated responses from depolarizing to hyperpolarizing is associated with the developmental induction of the neuronal $Cl^-$ extruding $K^+/Cl^-$ co-transporter KCC2. This co-transporter is not expressed in embryonic or early postnatal neurons; its expression starts around P5, and attains adult levels after the first two post-natal weeks.

Another channel which contributes to maintain chloride homeostasis is the chloride ClC-2 channel. Therefore the lack or low expression of this channel in neonatal neurons could also be involved in maintaining a high intracellular $Cl^-$ concentration. Indeed, we have recently cloned an alternative splice variant of the ClC-2 chloride channel which lacks exon 2 in the N-terminal part and turns out to be out of frame, leading to a non-functional protein. In the neonatal- but not adult- hippocampus, the specific mRNA expression of this splice variant is higher with respect to the wild-type which therefore shows a decreased expression compared to adulthood. The low expression of ClC-2 channels in neonatal hippocampal neurons may contribute to the high intracellular $Cl^-$ concentration and to the depolarizing action of GABA (Mladinic *et al.*, 1999). This mechanism however has to be verified at the protein level, using antibodies against the ClC-2 channel. Furthermore, in order to verify this hypothetical mechanism, the expression of the channel could also be altered by transiently overexpressing the full-length form (for example using recombinant adenoviruses) in neonatal neurons, or conversely inhibiting ClC-2 expression in adult neurons by an antisense strategy.

### 1.4.3 GABA$_A$-ρ LIKE RESPONSE IN THE HIPPOCAMPUS DURING EARLY POSTNATAL DEVELOPMENT

In the CA3 hippocampal neurons, during an early period of postnatal development, exogenous application of GABA induces responses mediated by $Cl^-$ permeable receptor channels that are bicuculline and baclofen insensitive (Strata & Cherubini, 1994; Martina *et al.*, 1995). In that respect, they closely resembles those described in the visual system, mediated by GABA$_A$–ρ receptors, composed of the ρ subunits. The pharmacology of this novel response however differs from the classical GABA$_A$-ρ response from visual pathways. Therefore I will refer to the new response as a GABA$_A$-ρ-like response. The novel GABA$_A$-ρ-like response is detectable only during the first two weeks of postnatal development (P0-P12), during the time period critical for the maturation of the CNS. This bicuculline insensitive depolarization can favour calcium entry through voltage activated channels and this calcium signal may be essential for synaptogenesis during this early period of postnatal development.

Even though the GABA$_A$-ρ responses are prominent along visual pathways, they were first observed in the spinal cord by Johnston and colleagues (1975). This early study indicated that the partially folded GABA analog cis-4-aminocrotonic acid (CACA) was able to selectively activate a third class of GABA receptor, later designed as GABA$_C$ (Drew *et al.*, 1984). These receptors are insensitive to both bicuculline (the classical competitive antagonist for the GABA$_A$ receptor) and baclofen (selective GABA$_B$ agonist) (Bormann and Feigenspan, 1995). GABA$_C$ or GABA$_A$-ρ have been reported to be present in *Xenopus* oocytes expressing mRNA from bovine retina (Polenzani *et al.*, 1991), in subpopulations of neurons in rat (Feigenspan *et al.*, 1993; Feigenspan & Bormann, 1994), fish (Qian & Dowling, 1993, 1994, 1995; Dong *et al.*, 1994) and tiger salamander retinae (Lukasiewicz *et al.*, 1994; Zhang & Slaughter, 1995). After the cloning of the ρ subunits in 1991 and 1992 by Cutting *et al.*, it became evident that these subunits were forming GABA$_C$ receptors from the retina.
The ρ subunits are now believed to be expressed in a number of brain tissues (Cutting *et al.*, 1991; Enz *et al.*, 1995; Boue-Grabot *et al.*, 1998, Wegelius *et al.*, 1998), and are therefore likely to have a physiological role in these brain structures. In addition to these anatomical studies, an increasing number of reports suggest a possible functional role of the ρ subunits outside the retina. The first evidence was by Ault & Nadler (1983) whose electrophysiological data showed a bicuculline-baclofen resistant action of bath-applied GABA on synaptic responses in rat hippocampal slices. Bicuculline-insensitive responses have also been reported in frog tectal neurons (Nistri & Sivilotti, 1985, Sivilotti & Nistri, 1989), and in guinea pig superior colliculus (Arakawa & Okada, 1988; Platt & Whitington, 1998). In view of recent findings demonstrating ρ subunit expression in the rat superior colliculus (Boue-Grabot *et al.*, 1998; Wegelius *et*

*al.*, 1998), it is conceivable that these bicuculline-resistant GABA responses could be mediated by $GABA_A$-ρ receptors. In addition to that, the cerebellum has been found by immunocytochemistry to express ρ1 subunit (Boue-Grabot *et al.*, 1998), which strikingly correlates with the observation of bicuculline-baclofen insensitive binding of GABA on cerebellar membranes (Drew & Johnston, 1992), except that a seasonal dependence was reported in this latter study. Finally, $GABA_A$-ρ receptors have been recently described in the spinal cord of neonatal rats (Rozzo *et al.*, 1999) using the new specific antagonist (1,2,5,6- tetrahydropyridine-4-yl) methylphosphinic acid (TPMPA) (Ragozzino et al. (1996).
These reports probably represent the tip of an iceberg concerning the action of GABA on the $GABA_A$-ρ receptors in the CNS. More studies involving molecular biology and gene expression, like single-cell RT-PCR, will help in definitively knowing whether these bicuculline-resistant responses to GABA could be attributed to the presence of the ρ subunits. These subunits, when expressed in recombinant systems, form receptors which exhibit a distinct pharmacology with respect to other $GABA_A$ receptors.

Briefly, the main differences between classical GABAA and $GABA_A$-ρ responses from the visual pathways are:
1) The $GABA_A$-ρ receptor is more sensitive to GABA than the classical $GABA_A$ receptor:
-the $EC_{50}$ (concentration of GABA producing half-maximal response) at $GABA_A$-ρ receptors is 1-4 μM, and at $GABA_A$ receptors is 10-100 μM,
2) desensitization of $GABA_A$-ρ and $GABA_A$ response is markedly different:
the amplitude of $GABA_A$ receptor-mediated currents decreases notably in the presence of agonist, while the time-course of $GABA_A$-ρ-like responses is more sustained,
3) the conductance states of $GABA_A$ and $GABA_A$-ρ receptors differ:
single-channel recordings from $GABA_A$-ρ receptors reveal small currents of 0.5 pA at -70 mV, corresponding to a conductance of 7 pS; this value is in contrast with the 27-30 pS main-state conductance of $GABA_A$ receptors,
4) the mean open time of $GABA_A$ and $GABA_A$-ρ receptors differs:
analysis of the gating behaviour showed that $GABA_A$-ρ receptors display a mean open time in the order of 150 ms, which is about five times longer than that of $GABA_A$ receptors,
5) $GABA_A$ and $GABA_A$-ρ receptors show a different pharmacology:
- the partially folded compound CACA, preferentially activates $GABA_A$-ρ-subtypes and is considered as a selective $GABA_A$-ρ receptor agonist, while the new compound TPMPA is considered as a $GABA_A$-ρ antagonist.
- $GABA_A$-ρ-receptors are insensitive to potent $GABA_A$ receptor modulators, such as benzodiazepines, barbiturates and neurosteroids.

Even though the response from the neonatal CA3 hippocampal neurones resembles those designated as $GABA_A$-ρ in the retina, there are some differences that suggest the presence of a new type of receptor. The $GABA_A$-ρ-like response from rat neonatal hippocampus resembles those of the classical $GABA_A$-ρ responses in that both responses are :

1) bicuculline and baclofen insensitive,
2) chloride dependent,
3) sensitive to picrotoxin (at least 5-10 times more sensitive than those reported in perch and rat retinal bipolar and horizontal cells by Feigenspan *et al.*, (1993) and Qian & Dowling, (1993). In this respect, the novel responses found in the neonatal hippocampus are reminiscent of those obtained by expressing the rat ρ1 or ρ3, but not ρ2 subunit in *Xenopus* oocytes (Zhang *et al.*, 1995; Shingai *et al.*, 1996). The higher resistance of ρ2 subunit to picrotoxin is probably due to a single amino acid in the M2 domain - a methionine in ρ2 instead of a threonine in ρ1 - as suggested by site-directed mutagenesis (Zhang *et al.*, 1995)],
4) both responses do not desensitize and show a fast recovery from desensitization.

The novel responses in neonatal hippocampus differ from $GABA_A$-ρ responses in the retina because:

1) they have single channel conductance states of 14, 22 and 31 pS similar to those obtained for $GABA_A$ receptors, while in rat retinal bipolar cells GABA activates only a low conductance channel (7 pS), clearly different from those obtained for $GABA_A$ receptors from the same preparation,
2) they are less sensitive to GABA (the $EC_{50}$ of the novel response is 30-100 μM GABA, similar to the 10-100 μM for the classical $GABA_A$ response and very different to the 1-4 μM of the $GABA_A$-ρ response (Bormann and Feigenspan, 1995),
3) they are sensistive to CACA (300-1000 μM) in the same way as bicuculline sensitive responses (therefore CACA is not a selective agonist for this novel response).

Furthermore, the novel bicuculline resistant responses from neonatal hippocampus are either potentiated by low concentrations or inhibited by high concentrations of $Zn^{2+}$, while bicuculline sensitive $GABA_A$ responses are strongly depressed by this divalent cation.

This novel GABA response may be due to a novel type of GABA receptor subunit, to the existence of modified versions of already cloned subunits (alternative splicing, RNA editing) or the existence of new, unexpected combinations of already known subunits. The novel subunit could be of the ρ type, since the response resembles that of $GABA_A$-ρ receptors, or of the α type, since the novel response to GABA from neonatal hippocampus is reminiscent of that obtained in *Xenopus* oocytes expressing an α1 subunit in which position 64 was occupied by Leu instead of Phe (Sigel *et al.*, 1992). This single amino acid change has a profound influence on the gating by

GABA and on affinity of the competitive antagonist bicuculline: in the receptor containing the mutated $\alpha 1$ subunit the apparent affinity for GABA dependent channel gating was decreased from 6 μM to 1260 μM and the apparent affinity to bicuculline was decreased 60- to 200-fold. It is also possible that in this early period of the postnatal development, when changes in subunit expression are very likely, the short co-existence of certain subunits may result in the creation of unusual receptor combinations.

The diversity of GABA receptors in the vertebrate retina as well as in developing hippocampus suggests that the physiological function of $GABA_A$-ρ and $GABA_A$-ρ-like receptors respectively is different from that of $GABA_A$ receptors. From $GABA_A$-ρ receptor localization on bipolar and horizontal cells, these receptors are expected to mediate the lateral inhibition of light responses in the vertebrate retina, through presynaptic regulation of calcium entry and transmitter release (Bormann & Feigenspan, 1995). The function of the $GABA_A$-ρ-like responses in developing hippocampus is not known. No synaptic responses to GABA can be detected in the presence of relatively low concentrations (3-10 μM) of bicuculline. It could be possible, however, that spillover of GABA during intense nerve activation activates extrasynaptic receptors with characteristics similar to those described for $GABA_A$-ρ. Moreover, localization of $GABA_A$-ρ-like receptors on presynaptic nerve terminals can not be excluded.

# 2. THE AIM OF THE RESEARCH

The aim of the research undertaken in this thesis is to gain insight into the molecular mechanisms that underly the action of GABA on the $GABA_A$ receptors in the neonatal rat hippocampus during the immediate postnatal period.

A novel response to GABA, transiently expressed in the neonatal hippocampus during a critical period of postnatal development, has been described in our laboratory. This response which is bicuculline and baclofen insensitive is reversibly blocked by picrotoxin and appears to be mediated by $Cl^-$-permeable receptor channels. Functionally, this response clearly resembles those obtained in visual pathways, which are thought to be mediated by $GABA_A$-ρ receptors made up of the recently cloned ρ subunits, but also shares common features with classical $GABA_A$ receptors.

In relation to that, $GABA_A$ receptor subunit gene expression has been mainly studied by oligonucleotide-based *in situ* hybridisation in the early 90s and only the study of Laurie *et al.* (1992) was exhaustive. Since then a number of new subunits, including the ρ subunits, have been found, and for some of these their presence in the brain has yet to be investigated, in particular during the early postnatal period.

With this in mind, the aims of the research were:

1) to clone potential new gene(s) encoding for the $GABA_A$ receptor subunits that form the $GABA_A$-ρ-like receptor in the neonatal rat hippocampus during the critical period of the postnatal development.

2) to assess the expression of all $GABA_A$ receptor subunits and their eventual isoforms present in the developing rat hippocampus by cDNA library screening as an alternative to *in situ* hybridisation.

3) to carefully look at the expression of the ρ subunits in the hippocampus using the RT-PCR methodology as well as at their potential contribution to the physiological responses to GABA observed at early developmental stages thanks to the single-cell RT-PCR technique.

These issues if solved should allow the identification of the $GABA_A$ receptor subunits responsible for the unusual postnatal $GABA_A$ pharmacology. They should also provide a complete picture of the subunits present in the neonatal hippocampus as well as their potential contribution to the native receptors.

# 3. MATERIALS AND METHODS

## 3.1 CHEMICALS AND REAGENTS

Enzymes for modification of DNA or RNA were obtained from Boehringer Mannheim (Mannheim, Germany), Gibco BRL (Rockville, MD, USA), New England Biolabs (Beverly, MA, USA), Pharmacia (Uppsala, Sweden), Promega (Madison, WI, USA), or Stratagene (La Jolla, CA, USA).
Plasmid preparations were made using the Qiagen mini- or midi-prep plasmid kits (Qiagen, Hilden, Germany) or Talent mini-prep plasmid kit (Talent s.r.l., Trieste, Italy).
All other routine chemicals and salts for molecular biology or electrophysiology were from Sigma (St Louis, MO, USA), Merck (Darmstadt, Germany), Calbiochem (La Jolla, CA, USA) or Carlo Erba (Milan, Italy).

## 3.2 CONSTRUCTION OF THE cDNA LIBRARY FROM NEONATAL RAT HIPPOCAMPUS

### 3.2.1 ISOLATION OF THE mRNA FROM NEONATAL RAT HIPPOCAMPUS

One hundred Wistar rats of age P0-P2 were killed by decapitation and the dissected hippocampi were immediately frozen in liquid nitrogen. About 0.4 g of neonatal hippocampal tissue was isolated. Frozen tissue was thawed and homogenized in the presence of RNase inhibitors. Four micrograms of poly(A)$^+$ selected RNA was extracted using Fast-Track™ 2.0 mRNA extraction kit (Invitrogen, San Diego, CA, USA) in RNAse free conditions, according to the manufacturer's instructions. The quality of the RNA extracted from the neonatal rat hippocampi was checked on a 1% denaturating formaldehyde RNA agarose gel and RNA was quantitated by measuring the optical density of a diluted RNA solution.
The unidirectional cDNA library was then constructed using the ZAP Express™ cDNA Synthesis and Gigapack II Gold® Cloning Kits (Stratagene).

### 3.2.2 cDNA SYNTHESIS AND CLONING

First-strand cDNA synthesis was performed using the 50-base oligonucleotide linker-primer (**Figure 4A & 4B**). The oligonucleotide was designed with a "GAGA" sequence to protect the *Xho* I restriction enzyme recognition site and an 18-base poly(dT) sequence. The restriction site allowed the completed cDNA to be cloned into the ZAP Express vector (**Figure 4D**) in a sense orientation (using *Eco*R I and *Xho* I) with respect to the lacZ promoter.

Briefly, the poly(dT) region hybridized to the 3' poly(A) region of the mRNA template, and first-strand cDNA was synthesized using Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT) and 5-methyl dCTP. The use of 5-methyl dCTP during first-strand synthesis hemimethylated the cDNA, protecting it from the subsequent digestion with *Xho* I restriction endonuclease.

During second-strand synthesis, RNase H nicked the RNA bound to the first-strand cDNA to produce a multitude of fragments, which served as primers for DNA polymerase I. DNA polymerase I "nick-translated" these RNA fragments into second-strand cDNA. The second-strand nucleotide mixture was supplemented with dCTP to reduce the probability of 5-methyl dCTP becoming incorporated in the second-strand and ensuring that the restriction sites in the linker-primer were susceptible to restriction with *Xho* I. The uneven termini of the double-stranded cDNA were nibbled back or filled with *Pfu* DNA polymerase, and *Eco*R I adapters (**Figure 4A & 4C**) were ligated to the blunt ends. These adapters were composed of 9- and 13-mer oligonucleotides, which were complementary to each other and formed *Eco*R I cohesive ends following annealing. The 9-mer oligonucleotide was treated with kinase (to allow it to ligate to other blunt termini available in the form of cDNA and other adapters) and the 13-mer was kept dephosphorylated to prevent it from ligating to other cohesive ends. After adapter ligation, the ligase was heat inactivated, and the 13-mer oligonucleotide was treated with kinase to enable its ligation into the dephosphorylated vector arms.

## <u>FIGURE 4.</u> Construction of the $\lambda$ ZAP Express cDNA library.

**Panel A.** cDNA synthesis flow chart.

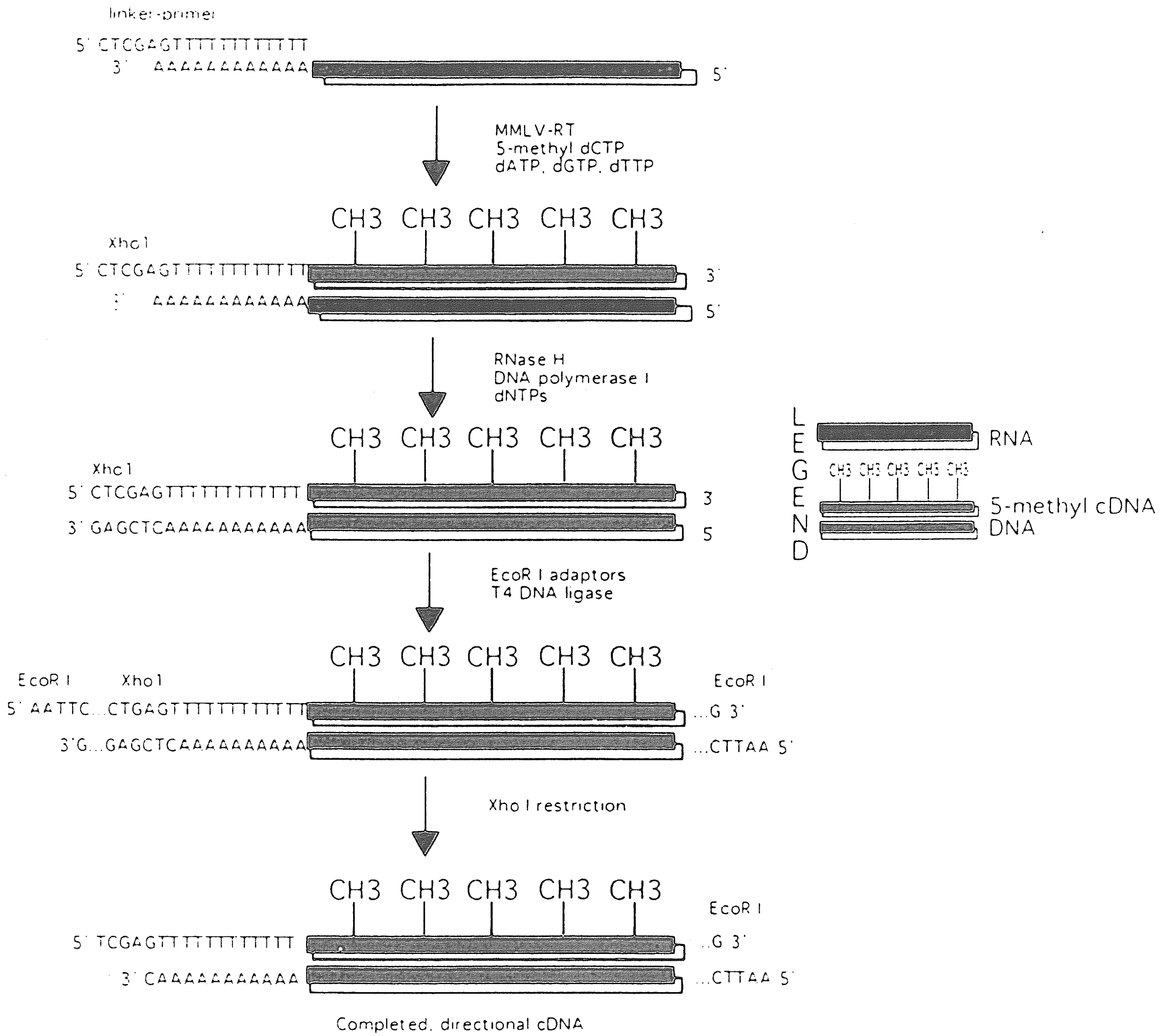
**Panels B. and C.** The sequences of the oligonucleotide linker-primer and adapters.

**Panel D.** Expression cassette in ZAP Express and pBK vectors.

The Express vector excision is shown in **E.**
Individual lambda phage or an amplified library are allowed to infect *E. coli* cells which are co-infected with filamentous helper phage. Inside the cell, trans-acting proteins from helper phage recognize initiator (I) and terminator (T) domains within the ZAP Express vector arms. Both of these signals are recognized by the helper phage gene II protein and a new DNA strand is synthesized, displacing the existing strand. The displaced strand is circularized by the helper phage proteins, and secreted from the cell as a phagemid. pBK plasmids are recovered by infecting an F' strain and growing in the presence of kanamycin.

**Figure 4.**

A. cDNA synthesis flow chart



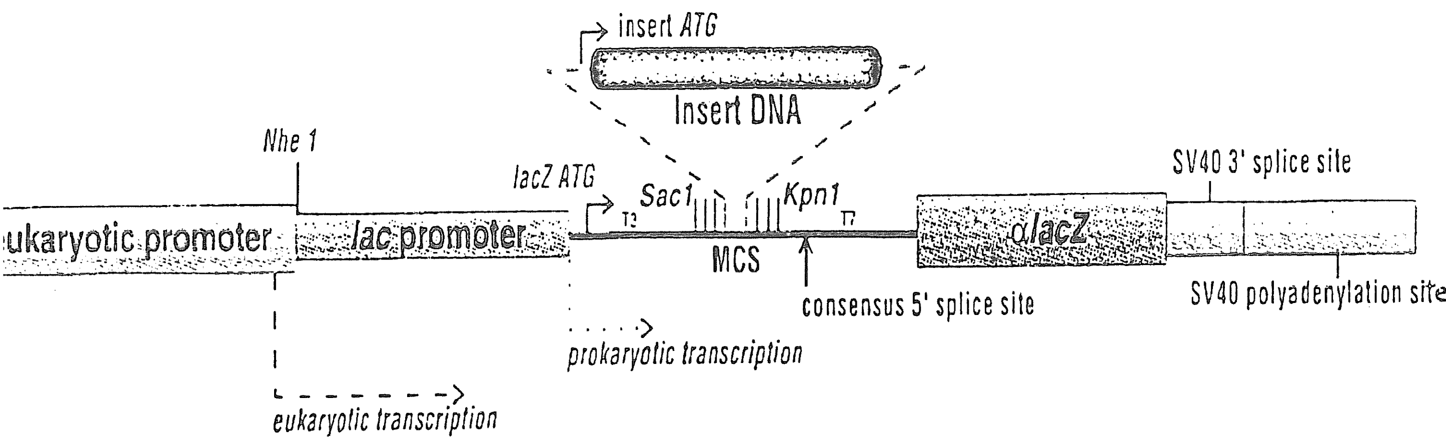

B. Oligonucleotide linker-primer

5' GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTTT 3'

"GAGA" Sequence Xho I Poly(dT)

C. Adapters

5' AATTCGGCACGAG 3'
3' GCCGTGCTC 5'




E.

T3 Sac I Sal I Spe I BamH I EcoR I Hind III Xho I Xba I Not I Apa I Sma I Kpn I T7

lac P

MCS

A J bla αlacZ CMV P ColE1 Neo r

1. Construct DNA library
2. Isolate positive clone

T3 DNA insert T7

lac P

A J bla CMV P ColE1 Neo r

3. Excise pBK-CMV phagemid containing cloned DNA insert by co-infection with helper phage

bla P f1⁻ origin (I + T)

SV40 ori SV40 polyA Neo r lacZ T7 DNA insert T3 TK polyA CMV ColE1 ori

**pBK-CMV**
phagemid vector

The subsequent *Xho* I digestion released the *Eco*R I adapter and residual linker-primers from the 3' end of the cDNA. These two fragments as well as cDNA smaller than 500 bp were separated on a Sephacryl S-500 spin columns. The size-fractionated cDNA was then precipitated and ligated to the ZAP Express vector arms.

### 3.2.3 PACKAGING, TITERING AND AMPLIFICATION OF THE LIBRARY

The lambda ZAP Express vector library was packaged using a high-efficiency in vitro packaging system (Gigapack II Gold® packaging extract kit, Stratagene) and infected into *E. coli* XL1-Blue MRF' cells (which are $McrA^-McrB^-$ strain and do not digest hemimethylated DNA).
The final library from the rat neonatal hippocampus contained 2.56 x $10^6$ p.f.u. and was amplified once to a titer of 2.13 x $10^9$ p.f.u./ml (2.13 x $10^6$ p.f.u./µl). The insert size was determined by PCR with the flanking T3 and T7 primers and ranged between 500 bp and 4 kb, with an average of about 1 kb.

## 3.3 SCREENING OF THE cDNA LIBRARY

### 3.3.1 MEDIA AND SOLUTIONS USED IN SCREENING OF cDNA LIBRARY

NZY agar (1 l): 5 g of NaCl, 2 g of $MgSO_4$, $7H_2O$, 5 g of yeast extract, 10 g of NZ amine (casein hydrolysate), 15 g of agar in $H_2O$, pH 7.5.
NZY broth (1 l): 5 g of NaCl, 2 g of $MgSO_4$, $7H_2O$, 5 g of yeast extract, 10 g of NZ amine (casein hydrolysate) in $H_2O$, pH 7.5.
LB-Kanamycin agar (1 l): 10 g of NaCl, 10 g of tryptone, 5 g of yeast extract, 20 g of agar in $H_2O$, pH 7.0 + 50 mg of filter-sterilized kanamycin.
SM buffer (1 l): 5.8 g of NaCl, 2 g of $MgSO_4$, $7H_2O$, 50 ml of 1 M Tris-HCl (pH 7.5), 5 ml of 2% (w/v) gelatin in $H_2O$.

Hybridization solution: 5 x SSC, 5 x Denhardt's solution, 0.5 %SDS, 100 mg/ml of acid-alkali cleaved salmon sperm DNA (Sambrook *et al.*, 1989).
Denaturation solution: 1.5 M NaCl, 0.5 M NaOH, in $H_2O$.
Neutralization solution: 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5, in $H_2O$.

### 3.3.2 PRIMARY SCREENING OF THE cDNA LIBRARY

Approximately 1.2 x $10^6$ p.f.u. from the rat neonatal cDNA library were plated on each of 25 NZY agar plates (137 mm, round) with a XL1-Blue MRF' bacterial lawn (600 µl of cells at an $OD_{600}$ of 0.5). The plates were incubated at 37°C for 8-10 hours. The plates were chilled for 2 hours at 4°C to prevent the NZY top agar from sticking to the nylon membranes (Hybond™-N membranes, Amersham Life Science). The plaques were transferred for 1 min to the first and 2 min to the second duplicate membranes. The membranes were then treated following the manufacturer's instructions (soaked for 1 min in 10% SDS, for 3 min in denaturation solution, twice for 3 min in neutralization solution followed by rinsing in 2 x SSC). The dried membranes were fixed by exposure to ultraviolet (UV) light (120,000 µJ of UV energy for 30 seconds), using a Stratalinker™ UV crosslinker (Stratagene).
The membranes were then prehybridized for at least 1 hour in hybridization solution without added probe, at the same temperature as hybridization.
The probes were radioactively labelled using T4 polynucleotide kinase (New England Biolabs) and [$\gamma$-$^{32}$P]ATP (10 mCi/ml; 3000-6000 Ci/mmol) for oligonucleotide probes. For longer double-stranded cDNA probes, Prime-It® II random-priming labelling kit, (Stratagene) or alternatively Klenow DNA polymerase (New England Biolabs or Boehringer Mannheim), random hexanucleotides primers (Boehringer Mannheim) with [$\alpha$-$^{32}$P]-dATP or [$\alpha$-$^{32}$P]-dCTP (10 mCi/ml; 3000-6000 Ci/mmol) were used. Radiolabelled probes were separated from unincorporated nucleotides using NAP™-5 or -10 columns with DNA grade Sephadex G-25 medium (Pharmacia).
The hybridization was performed in hybridization solution with the radioactive probe added to a final concentration of 1-15 ng/ml or $>10^6$ cpm/ml of hybridization solution, for 24 to 65 hours. The temperature of hybridization depended on the type of probe: oligonucleotide probes were usually hybridized at 42°C and cDNA probes at 65°C. The temperature was adjusted for each probe individually, with regard to probe degeneracy and the desired stringency of hybridization required.
After hybridization, membranes were washed until the background radioactivity was monitored at about 10 counts/sec, with washing starting in 2 x SSC/0.1% SDS at the temperature of hybridization and slowly increasing the temperature and decreasing the salt concentration in the washing solution. The last washes were performed in 0.2 or 0.1 x SSC/0.1%SDS at 55 to 65°C.
The membranes were exposed to Kodak BioMax MS-1 films for 24-48 hours and the primary positive clones were identified if found on both membranes.

### 3.3.3 SECONDARY AND TERTIARY SCREENING

A square centimeter "window" around clones identified as primary "putative" positives was taken from the primary plates and dissolved in SM buffer with chloroform (2% v/v). The phages from this primary phage stock were titered, diluted and plated on secondary NZY plates (with the same host cells as in the primary screening) to obtain about 500 well isolated plaques. The screening was then performed as before. The real positive clones were picked up from secondary plates, or the whole procedure was repeated until the individual positive clones were isolated.

### 3.3.4 *IN VIVO* EXCISION

The ZAP Express vector has been designed to allow simple, efficient *in vivo* excision and recircularization of any insert cloned within the lambda ZAP Express vector. Following *in vivo* excision, the insert is found within a phagemid pBK, which permits both eukaryotic and prokaryotic expression (Short *et al.*, 1988). The protocol included use of the ExAssist helper phage with XLOLR *E. coli* strain, to increase the efficiency of excision. The ExAssist helper phage contains an amber mutation that prevents replication of the phage genome in a nonsuppressing E. coli strain such as XLOLR cells. This allows only the excised phagemid to replicate in the host, removing the possibility of productive co-infection from the ExAssist helper phage. Since the ExAssist helper phage cannot replicate in the XLOLR strain, single-stranded rescue cannot be performed in this strain using this helper phage. XLOLR cells are also resistant to lambda infection, preventing lambda DNA contamination after excision. The excision was performed following the manufacturer's instructions. Briefly, the plaque of interest was picked up from the agar plate and incubated in 500 μl of SM buffer and 20 μl of chloroform overnight at 4°C. A 250 μl aliquot of the phage stock was mixed with 200μl of XL1-Blue MRF' cells at $OD_{600}$ of 1.0 and 1 μl of the ExAssist helper phage (>1 x $10^6$ pfu/μl) and incubated at 37°C for 15 min. Then 3 ml of NZY broth were added and incubated for further 2-3 hours at 37°C with shaking. The mixture was heated at 65-70 °C for 20 min and spin at 1000 x g for 15 min. The supernatant was stored at 4°C. To plate the excised phagemids, 200 μl of XLOLR cells ($OD_{600}$ of 1.0) was added to 100 μl of the phage supernatant and incubated at 37°C for 15 min. 300 μl of NZY broth was added and incubated for further 45 min to allow expression of kanamycin resistance. XLOLR cells containing pBK-CMV double-stranded phagemid vector with cloned DNA insert were plated on LB-kanamycin agar plates and single clones could be further treated.

## 3.4 ANALYSIS OF POSITIVE CLONES

### 3.4.1 SOUTHERN BLOT ANALYSIS

PCR reaction (10 ng to 1 μg of DNA) or plasmid DNA were electrophoretically separated on 1% TBE (Tris-borate 90 mM / EDTA 2 mM electrophoresis buffer) agarose gels and transferred to nylon Hybond-N membranes by classical capillary transfer. After drying, the membranes were fixed by exposure to UV light (120,000 μJ of UV energy for 30 seconds), using Stratalinker™ UV crosslinker. The blots were prehybridized in hybridization solution (5 x SSC, 5 x Denhardt's solution, 0.5%SDS, 100 mg/ml of acid-alkali cleaved salmon sperm DNA) for 1 hour and subsequently hybridized with a radioactive oligonucleotide or cDNA probe. Labelling was made as for the screening and the labelled probes were purified on NAP-5™ columns (Pharmacia). The final probe concentration was $>10^5$ cpm/ml of hybridization solution. After hybridization at 42°C, the membranes were washed to a final stringency of 0.1 x SSC/ 0.1% SDS at 50-55°C and exposed to Kodak X-OMAT AR films at -80°C for times varying from less than 1 hour to 3 days.

### 3.4.2 PCR ANALYSIS

Known $GABA_A$ receptor subunits were identified by PCR from 1 μl of primary phage stock using subunit specific primers derived from the variable intracellular loop located between M3 and M4 domains (**Table 6**). PCR amplifications were performed with *Taq* polymerase (Promega, USA) using the following conditions: 95°C for 5 min followed by 40 cycles at 95°C for 40 sec, 55°C for 1 min, 72°C for 30 sec and elongation at 72°C for 10 min. The negative clones after PCR analysis were submitted to secondary and tertiary screening, *in vivo* excision and partial sequence analysis.

**TABLE 6.** **Primers used for the identification of $GABA_A$ receptor subunits clones using PCR.**

| Subunit | Forward primer 5'->3' | Reverse primer 5'->3' | EMBL Accession n° | Product length (bp) |
|---|---|---|---|---|
| α1 | 1162 -CCTACAGCAACCAGCTATACC- 1182 | 1416 -GGGTGTGGGGGCTTTTAGCTG- 1396 | L08490 | 255 |
| α2 | 1012 -AAAGGCTCCGTCATGATACAG- 1032 | 1293 -GACTAACCCCTAATACAGGC- 1274 | L08491 | 282 |
| α3 | 1316 -GTACCAGAGGCCCTGGAGATG- 1296 | 1667 -GGATCATGCCCTTGATAGCGG- 1647 | L08492 | 351 |
| α4 | 1223 -CAGAATACACATGCTAATTTG- 1243 | 1747 -CATTAGACTTTCTGATTTCTCC- 1726 | L08493 | 525 |
| α5 | 1346 -GGCCTTGGAAGCAGCTAAAATC-1325 | 1541 -CTCTCAGAAGTCTTCTCCTCA- 1521 | L08494 | 196 |
| β1 | 1095 -GGAGCGAGCAAACAAGACCAG- 1115 | 1358 -ACCCTTGCCGGGCACCCCGTG- 1338 | X15466 | 264 |
| β2 | 1100 -GCTGAGAAAGCTGCTAATGCC- 1120 | 1345 -TGCCACATGTCGTTCCAGGGC- 1325 | X15467 | 246 |
| β3 | 1132 -AAGAGTGAAATCAACCGGGTG- 1152 | 1334 -GCTTCTGTCTCCCATGTACCG- 1314 | X15468 | 202 |
| γ1 | 1177 -AACAATAAAGGAAAAACC- 1195 | 1300 -CATCTTCCCCTTGAGGCATAG- 1280 | X57514 | 124 |
| γ2 | 1092 -GTGGAGTATGGTACCCTGCACTATTT TGTG- 1121 | 1402-CAGAAGGCGGTAGGGAAGAAGATCC GAGCA- 1373 | L08497 | 310 |
| γ3 | 1203 -TGTCGAAAGCCAACCATCAGG- 1223 | 1453 -GACTTGCACTCCTCATAGCAG- 1433 | X63324 | 251 |
| ρ1 | 1279 -CTGCCCCAGCCTCGAGGGGTG- 1299 | 1461 -CCTCATGCTCACATAGCTGCC- 1441 | X95579 | 183 |
| ρ2 | 1327 -AACAGCCTGGCTGGCTACCC- 1346 | 1566 -CTAGGAAAACACTGACCAATA- 1546 | D38494 | 240 |
| ρ3 | 1312 -GATGCAGTTCAAGCCATGGCC- 1332 | 1587 -CACATATATACCCCAGTAAAAC- 1566 | D50671 | 277 |

### 3.4.3 SEQUENCING

Sequencing was initially performed using a double-stranded plasmid DNA template and the T7 Sequencing™ Kit (Pharmacia). Plasmid DNA was prepared by the standard alkaline lysis method (Sambrook *et al.*, 1989). Double-stranded plasmid DNA was denatured by incubating in 0.4 M NaOH for 10 min at room temperature and purified through the MicroSpin™ S-400 HR Columns (Pharmacia). T3 and T7 primers were used since they flanked cDNA inserts on pBK-CMV vector excised from library clones. Because 5' UTR regions of full-length clones were first encountered, additional internal primers specific for each clone were also designed until identification of the clones. The sequencing reaction was performed starting from 1-2 μg of denaturated

and purified plasmid DNA, following the manufacturer instructions. The radioactive sequencing mixture was separated electrophoretically on 6% buffer-gradient polyacrylamide gel, which was then post-fixated in 10% methanol/10% acetic acid, vacuum-dried and exposed to X-ray film for 12 hours.

Sequencing was also performed with the automated LI-COR DNA sequencer 4000L (LI-COR Inc, Lincoln, NE, USA) and the Sequitherm EXCEL II Long-Read DNA Sequencing Kit-LC (Epicentre Technologies, Madison, WI, USA) with 1 pmol of IRD-800 modified oligonucleotide primer to allow laser detection at 800 nm. DNA was prepared with Qiagen mini-, midi-prep or Talent mini-prep kits for reproducible results, and in general 1 μg of DNA was used to perform the sequencing reactions. Primers were designed to fully sequence the coding region of the different $GABA_A$ receptor subunit clones of interest.

## 3.5 NON-RADIOACTIVE *IN SITU* HYBRIDIZATION

### 3.5.1 MATERIALS

All glassware, plasticware, solutions and chemicals used were RNAse free.

### 3.5.2 PREPARATION AND FIXATION OF TISSUE

Adult and P2 rats were anaesthetised with 10.5% chloral hydrate in physiological solution (0.1 ml/100 g of rat) and perfused with buffered 4% paraformaldehyde (PFA). The dissected brains were fixed for at least 1-2 h in 4% PFA at 4°C and cryoprotected in buffered 4% PFA with 15% sucrose at 4°C for at least 12 h. Forty micrometers sections were cut with a slicing microtome frozen at -20°C and slices were immediately immersed in buffered 4% PFA and stored at 4°C until used in *in situ* hybridisation experiments.

### 3.5.3 PREPARATION AND LABELLING OF THE cRNA PROBES

cDNA probes used in *in situ* hybridisation experiments were cloned by PCR from first strand cDNA synthesised from total brain for the γ3 subunit, and from full-length cDNA clones isolated from the cDNA library screening for β3 and δ subunits. The three probes were derived from the very variable intracellular domain of the corresponding subunits to ensure specificity. The primers used to clone these cDNA fragments were (restriction sites used for cloning in pBluescript KS- are underlined): for β3, β3 Pst-F 5'-AAA ACT GCA GGC CAA GGC CAA GAA TGA TCG ATC CA-3', β3 IS-R 5'-CGC GGA TCC TAG GTG CGT CTT CTT GTG CGG GAT-3' (nucleotide 1109 to 1354, EMBL accession n° X15468, cDNA size 246 bp); for γ3, γ3 IS-F 5'-CGG AAT TCC TCA ACT ACT ATT CGA GCT GTC GAA-3', γ3 IS-R 5'-CGC GGA TCC GAG TCC AGC TCA GAG ACG TCA ATG T-3' (nucleotide 1185 to 1504, EMBL accession n° X63324, cDNA size 320 bp); for δ, δ IS-F 5'-CGG AAT TCC ACT TCA ATG CTG ACT ACA GGA AGA- 3', δ IS-R 5'-CGC GGA TCC GTC GAT GGT GTC TGC ATC GAT GGG- 3' (nucleotide 1024 to 1305, EMBL accession n° L08496, cDNA size 282 bp). The cDNA fragments were subcloned into pBluescript KS- and checked by double-strand DNA automatic sequencing as described above. Antisense or sense cRNA probes were transcribed from linearized plasmids pBluescript KS- containing the desired cDNA fragment using T7 and T3 RNA polymerases respectively and a labeling mixture containing DIG-11-dUTP (Roche Molecular Biochemicals). Riboprobes were checked on a 1% denaturing formaldehyde RNA agarose gel, transferred to nylon Hybond-N membranes (Amersham, UK) and visualized using anti-DIG F(ab)$_2$ fragments conjugated with alkaline phosphatase (Roche Molecular Biochemicals) and colorimetric staining.

### 3.5.4 *IN SITU* HYBRIDIZATION

Prior to hybridization the brain sections were washed twice for 5 min each in PBST (PBS + 0.1% Tween 20) and then treated with 2.3% (w/v) sodium-m-periodate for 5 min, followed by buffered (in 0.1% Tris-HCl pH 7.5) 1% sodium-borohydrate for 10 min. After the washing in PBST, the brain sections of adult rats only were treated with Proteinase K (0.8 μg/ml) for about 15 min to permeabilize the cell membranes.
The sections were prehybridized for a few hours at 55°C in prehybridization solution (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, pH 8.0, 1 x Denhardt's solution, 0.33 M NaCl, 0.1 M DTT, 50% deionized formamide, 500 μg/ml acid-alkali cleaved salmon sperm DNA, 500 μg/ml long chain polyadenylic acid, in DEPC-treated $H_2O$).

The hybridizations were performed at a final probe concentration of 100 ng/ml in a hybridization solution (prehybridization solution + 1% dextran sulfate), at 60-65°C for 12h. After hybridisation, sections were washed twice for 30 min each in 2 x SSCT (SSC + 0.1% Tween 20)/50% formamide at 55°C, 2 x SSCT at 55°C for 20 min and twice in 0.1 x SSCT at 60°C for 30 min (Tongiorgi *et al.*, 1998).
Immunodetection of digoxigenin was performed according to the instructions in the non-radioactive DIG DNA labelling and detection kit (Roche Molecular Biochemicals), using alkaline phosphatase-conjugated antibodies and colorimetric development of staining. A blue-black precipitate forms in positively stained cells. The development time varied from 2h to 12h to allow detection of less abundant transcripts.
Representative sections were photographed with a microscope equipped with Zeiss (Germany) optics and automated film exposure using black and white Ilford films.

## 3.6 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

### 3.6.1 mRNA ISOLATION AND cDNA SYNTHESIS

Poly(A)+ RNA was prepared from dissected hippocampi from P2 and adult rats using Fast-Track™ mRNA extraction kit (Invitrogen, San Diego, CA, USA). First-strand cDNA was synthesized from 150 ng of poly(A)+ RNA in a total volume of 20 μl using 100 ng of $p(dN)_6$ (Roche Molecular Biochemicals), 26 Units RNAguard RNase inhibitor (Pharmacia) and 200 Units SuperscriptII $RNaseH^-$ reverse transcriptase (Life Technologies) following manufacturer's protocol. "Sham" reactions, containing all of the above components except Superscript enzyme were set up in parallel.

### 3.6.2 PCR AMPLIFICATION

Reactions (50 μl) contained 2.5 units of RedTaq DNA polymerase (Sigma) in the buffer provided by the manufacturer, 0.2 mM of each dNTP, 5 pmol of each primer and 1 μl of first-strand cDNA or sham reactions. In addition a "no DNA" PCR was performed where no template was present. Primers used for gene expression analysis were derived from the 5' UTR and the variable N-terminal region among β3, γ3, ρ1-3

and ε $GABA_A$ receptor subunits (**Table 7**). For these subunits, reactions consisted of 94°C for 3 min followed by 35 cycles of 94°C for 40 s, 60°C for 30 s, 72°C for 50 s (except for γ3 for which the annealing temperature was 55°C). In the case of ρ3, 1 μl of a first PCR reaction was reamplified to generate reasonable amounts of PCR products to perform subsequent restriction analysis. Twenty microlitres of each PCR-product were run on 2% agarose gel stained with ethidium bromide and digested with an appropriate restriction enzyme to confirm their identity: for ρ1, *Ava* II; for ρ2, *Rsa* I, for ρ3, *Eco*R V. The RT-PCR experiments were repeated 2-3 times on independent mRNA samples.

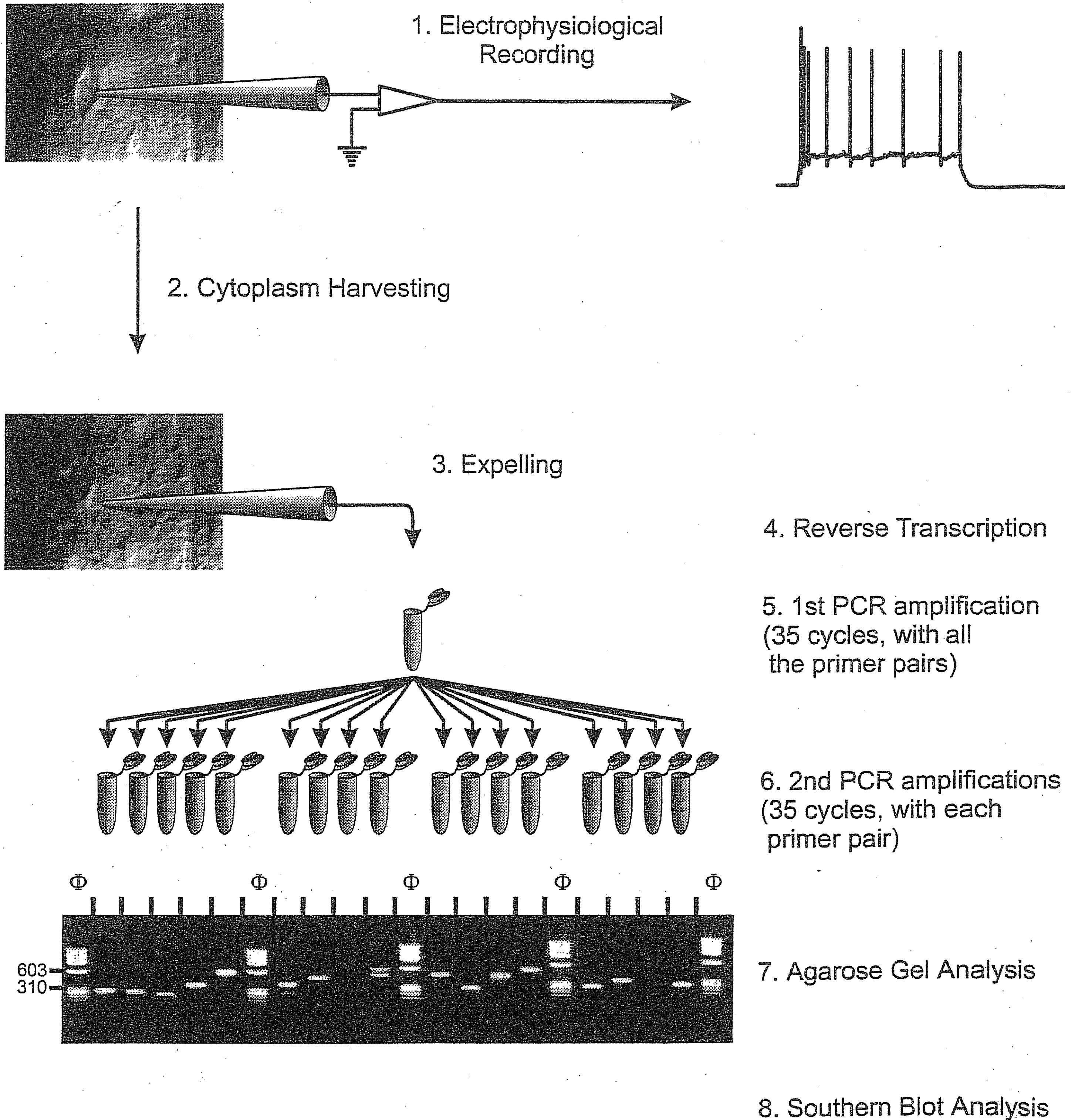
**TABLE 7. Primers used for the RT-PCR analysis of β3, ρ1-3 and ε $GABA_A$ receptor subunits expression in the rat hippocampus.**

| Subunit | Forward primer 5'->3' | Reverse primer 5'->3' | EMBL Accession n° | Product length (bp) |
|---|---|---|---|---|
| β3 | 71 -ATG TGG GGC TTT GCG GGA GGA AGG- 94 | 740 -CAA CAT TCC TGG AGA CCA GAC GGT- 717 | X15468 | 670 |
| ρ1 | 72 -TCT CAC GGC TTC TCG GGA GAA CTG- 95 | 657 -CTG TAC CCG CAA CAT GAC GTT GTC- 634 | X95579 | 586 |
| ρ2 | 141 -TGC CTG TTG CGT CAT AGA CGT TTG- 164 | 662 -TGG CCA TCG GGA AAC ACT CGC AGC- 639 | D38494 | 522 |
| ρ3 | 211 -TGG TTG GCC TTC TTC ACC TAC ACC- 234 | 770- GTG TCC AGA GGA AAC CTG CTG AAG- 747 | D50671 | 560 |
| ε | 908 -GCA AGA TGC TCA CTC CAC ATG CTC- 931 | 6235 -TGT GGC CAT GGT GAG CAC AGA AC- 6213 | U92284 | 392 |

## 3.7 SINGLE-CELL RT-PCR

**A simplified scheme of the single-cell RT-PCR protocol is shown below (Figure 5).**

FIGURE 5. Simplified scheme of the single-cell RT-PCR protocol.



### 3.7.1 REAGENTS AND SOLUTIONS FOR ELECTROPHYSIOLOGY

Artificial cerebrospinal fluid (ACSF) (in mM): NaCl, 126; KCl, 3.5; $CaCl_2$, 2; Na $H_2PO_4$, 1.2; $MgCl_2$, 1.3; $NaHCO_3$, 25; and glucose, 11; gassed with 95% $O_2$-5% $CO_2$ (pH 7.3).
Intracellular solution was (in mM): KCl, 140; EGTA, 1; $MgCl_2$, 1; sterile $Na_2ATP$, 2; sucrose, 10; HEPES, 5, pH adjusted to 7.3 with KOH.
Stock solutions of TTX (1 mM), GABA (10 mM), bicuculline (100 mM) were prepared in water and diluted to the final concentration (TTX, 1 μM; GABA, 10 μM; bicuculline, 100 μM) in ACSF.

### 3.7.2 REAGENTS AND SOLUTIONS FOR MOLECULAR BIOLOGY

- 5 x random primer/dNTPs mix containing 25 μM hexamer random primers (Boehringer Mannheim) and 2.5 mM each dNTP (Pharmacia) in 10 mM Tris-HCl, pH 8.0
- 200 mM DTT (Calbiochem) dissolved in autoclaved water and filtered (0.2 μm pore size).
- 26 U/ml RNAguard RNAse inhibitor (Pharmacia)
- 200 U/ml Superscript RNAse H- revese transcriptase (Gibco B.R.L.)
- 5 U/μl Taq polymerase (Qiagen).
- 10 x PCR buffer ( Qiagen) containing 15 mM $MgCl_2$ (1.5 mM final concentration)
- 25 mM each dNTPs stock solution, diluted before use in 5 mM each dNTP solution (100 x dNTPs solution)

The reagents were prepared in advance and stored in 50 μl aliquots. DTT was stored at -80°C. Random primers/dNTPs mix (discarded at the end of the day), dNTPs stock and 100 x solutions were stored at -20°C. Enzymes were stored at -20°C in 10 μl aliquots.

### 3.7.3 HIPPOCAMPAL SLICE PREPARATION

Transverse hippocampal slices were prepared from hippocampi of P3-P4 or adult Wistar rats according to the method of Edwards et al. (1989). Rats were decapitated under anaesthesia (5% urethane intra peritoneally) and their brains were rapidly removed and placed in ice cold artificial cerebrospinal fluid (ACSF). After bisecting the brain, the tissue was immersed in low temperature (2-4°C), oxygenated ACSF solution. Slices (250 μm thick) were cut with a vibrating microslicer (Vibracut, FTB,

Weinheim, Germany) and incubated 1 h at 32°C before use. Individual slices were transferred to a recording chamber where they were continuously superfused at room temperature (22-24°C) with oxygenated ACSF at a rate of 3 ml/min.

### 3.7.4 ELECTROPHYSIOLOGICAL RECORDINGS

Patch pipettes had resistances comprised between 1.5-2.3 MΩ when filled with intracellular solution. The glass tubing was autoclaved before used and dried at 250°C for 4 hours before use . The silver wire connected to the patch electrode was chlorided before each experiment. The whole cell configuration of the patch-clamp technique was used to record currents from CA3 pyramidal hippocampal neurons which were identified by infrared differential interference contrast (IR-DIC) videomicroscopy, as well as by their action potentials firing frequency after sustained membrane depolarization in the current-clamp mode. Currents induced by pressure application of GABA (10 μM) were recorded (in voltage-clamp configuration) in the presence and in the absence of bicuculline methiodide (100 μM), from a holding potential of -50/-60 mV. Tetrodotoxin (1 μM) was routinely applied in the bath to block fast sodium spikes. Application of 10 μM GABA (10 sec, 8 psi) was made using a Picospritzer II (General Valve, Fairfield, NJ, USA) from a pipette (resistance: 2 MΩ) whose tip was about 50 μm away from the recorded cell. Intervals of 3 min were observed after application of either bicuculline or GABA before another drug application was made to allow wash of the compound.

### 3.7.5 HARVESTING PROCEDURE

After recording of the cell, its cytoplasm and nucleus were harvested into the patch pipette by suction under visual control on the monitor. Cells were used only when the seal remained intact until the very end of the aspiration and rejected whenever debris remained attached to the outside or entered inside the patch pipette. The contents of the patch pipettes (ca. 6 μl) were expelled into 0.5 ml Eppendorf tubes by first slighly breaking the pipette tip and then applying a positive pressure using a valve-controlled pressure system (N2, 4 bar).

## 3.7.6 REVERSE TRANSCRIPTION

During the course of the electrophysiological experiments, the aliquots of intacrellular solution, 5 x primers/dNTPS mix and 0.2 M DTT are kept on ice and RNAguard and Superscript enzymes are kept at -20°C. In the Eppendorf tubes used for collection of the harvested material, 2 µl DTT 0.2 M and 2 µl dNTPs/random primers mix were preliminary pipetted, and the tubes were kept on ice until expelling. After expelling, a flash-spin centrifugation (Picofuge, Stratagene) was applied and 0.5 µl Superscript reverse transcriptase (200 U/µl) and 0.5 µl RNAguard RNase inhibitor (26 U/µl) were added and again the tube was centrifugated. This reaction mix was then put at 37°C for 1h to overnight.

## 3.7.7 PCR AMPLIFICATIONS

### 3.7.7.1 1ST ROUND

Coamplification of $GABA_A$ receptor α1-5 and ρ1-3 subunits was performed with primers derived from the variable N-terminal region of these subunits. The α6 subunit was not tested because of its selective cerebellar location. For the first round of PCR amplification, two degenerate primers were used for all α subunits: α5', 5'-TGG ACY CCW GAY ACH TTY TT-3' and α3', 5'-GCN ATR AAC CAR TCC ATG-3' (primers designed by Berger *et al.*, 1998). These primers were shown to amplify with a comparable efficiency the different $GABA_A$ receptor α subunits. For the ρ subunits, the same pairs of primers as for the RT-PCR experiments were used (see **Table 7**) and tested for their ability to detect low amounts of transcripts. The forward and reverse primer locations span several predicted introns and are distant of about 500-600 bp, thus preventing amplification of genomic DNA. As a control these primers were tested with genomic DNA and never led to PCR products of the corresponding sizes (not shown).

Hot start always gave better results and was routinely used in the multiplex PCR experiments. In this option, primers are added in the pre-heated thin-wall PCR tube (Stratagene) since primer dimers are mainly due to the reverse transcriptase present in the 10 µl of reverse transcription reaction. For multiplex PCR, two solutions are prepared :

- Solution I: 70 µl x number of cell tubes. It contains per tube: 10 µl of the 10 x PCR Buffer, 0.5 µl *Taq* polymerase (2.5 U) and water to 70 µl.
- Solution II: 20 µl x number of cell tubes. Content per tube: 1 µl each primer and H2O to 20 µl.

For α5' and α3' degenerate primers, 40 pmol were used, while in the case of the non-degenerate primers for the ρ subunits, 10 pmol were added in solution II.
To the 10 μl of RT reaction , 70 μl of Solution I and 3 drops mineral oil were added; the PCR machine was pre-heated at 94°C and the PCR tubes were put inside after 1 minute. After another minute, solution II was added (total volume: 100 μl), and after pipetting the PCR cycles were started. The programme used was the following: denaturation step : 94°C , from 3 to 10 min. (depending on the number of tubes) then 35 cycles at 94°C, 30s.; $T_{annealing}$, 30 sec; 72°C, 30 to 40 sec. (1 kb polymerisation per minute); followed by an extension step at 72°C for 10 minutes. For the analysis of the α subunits, the annealing temperature was 53°C, as well as when combined with the ρ subunits. However, when the ρ subunits primers were used alone, annealing tempearature was 55°C.

#### 3.7.7.2 2ND ROUND

From the first round of amplification, 2 μl of the reaction were reamplified for each individual subunit analysed using semi-nested PCR (same forward primer but nested backward primer). The nested backward primers were for α1, α1-SB 5'-TGA GCG GGC TGG CTC CCT TG-3'; for α2, α2-SB 5'-AGA GTC AGA AGC ATT GTA AG-3'; for α3, α3-SB 5'-AGA TTT GTT CTT CCC AAG AG-3'; for α4, α4-SB 5'-TGA CTT CTC AGG GCC TTT GG-3'; for α5, α5-SB 5'-AGA CTT GGT GGA ACC ATT GG-3' (Berger et al., 1998). The annealing tempeature used was also 53°C except for α1-SB and α3-SB, annealed at 50°C instead. The PCR product size was 252 bp in all cases.
For the ρ subunits, three nested backward primers were designed : for ρ1, ρ1-scR 5'-TGC TGA AGT CGT GGT CAT CTA TCC-3' (304 bp product with forward primer 1st round); for ρ2, ρ2-scR 5'-ACC TCG GAG ATG CTG TCC AGA CTC-3' (317 bp product with forward primer 1st round); for ρ3, ρ3-scR 5'-CTG GAC ATC TAT ACC CAC TGG CAC-3' (246 bp product with forward primer 1st round). The annealing temperature used was 55°C.
Again, two solutions are prepared :
Solution I: 10 μl 10 x Taq Buffer, 1 μl 100 x dNTPs solution, 0.5 μl Taq (2.5 U), 66.5 μl $H_2O$ (total 78 μl).
Solution II: prepared as for the first round except that only one pair of primers was added.
Briefly, 78 μl of Solution I, 2 μl of 1st round reaction and 3 drops mineral oil were assembled in a thin-wall tube; the PCR machine was pre-heated at 94°C and the PCR tubes were put inside after 1 minute. After another minute, solution II was added (total reaction volume: 100 μl), and after at least an additional minute the PCR cycles were

started. The PCR programme was the same as for the first round, the annealing temperature depending on the subunits analysed.

### 3.7.8 SOUTHERN BLOT ANALYSIS

20 μl of a second round PCR reaction (10 ng to 1 μg of DNA) was electrophoretically separated on 1% TBE (Tris-borate 90 mM / EDTA 2 mM electrophoresis buffer) agarose gels and transferred to nylon Hybond-N membranes by classical capillary transfer. Treatment of the membranes, solutions and pre-hybridization were as described before (see cDNA library screening section). The following probes were designed within the amplified PCR product of the corresponding subunit transcripts: for ρ1, ρ1-5' 5'-TGG CAT ATT CCT TTT GTG GTG GGG-3'; for ρ2, ρ2-5' 5'-TGC CTG ATG GCT CTT GTG GAG AGC-3'; for ρ3, ρ3-5' 5'-CTG CCG TCA AGG AAC CCC ACC AG-3'. Labelling was made as for the screening, i.e., [$\gamma$-$^{32}$P]-ATP was kinased to the oligonucleotide with the T4 polynucleotide kinase (Boehringer) and the labelled probe was then purified on NAP-5™ columns (Pharmacia). The final probe concentration was >$10^5$ cpm/ml of hybridization solution. After hybridization at 42°C, the membranes were washed to a final stringency of 0.1 x SSC/ 0.1% SDS at 50-55°C and exposed to Kodak X-OMAT AR films at -80°C for times varying from less than 1 hour to 24 hours.

# 4. RESULTS

The research described in this thesis is an attempt to decipher the exact nature of $GABA_A$ receptor subunits present in the neonatal hippocampus. Of particular relevance is the search for subunits that could be responsible for the peculiar response to GABA observed during the first postnatal week. In this respect the ρ subunits represent good candidates since, as already mentioned, these subunits have been shown to be involved in the response to GABA observed in the rat and fish retina which bear many similarities with those obtained in the immature hippocampus.

In order to address this issue, several experimental strategies were applied.
First, a cDNA library from neonatal rat hipocampus was constructed and screened with several probes judged suitable for the cloning of new as well as already known $GABA_A$ receptor subunits. Variants of existing subunits (mutations, alternatively spliced or edited isoforms) were sought by analysing clones identified as $GABA_A$ receptor subunits by sequencing or PCR. The cDNA library screening strategy has been combined with *in situ* hybridization for confirmation of the data obtained.
Since the cDNA library screening experiments did not reveal the ρ subunits, RT-PCR experiments were performed in order to determine whether the ρ subunits are present in the hippocampus. Finally, the expression of the ρ subunits was analysed in single pyramidal hippocampal neurons to assess their potential contribution to the formation of $GABA_A$ receptor in the hippocampus, and in particular to assess their possible role regarding the bicuculline-insensitive responses found during the early postnatal period.

### 4.1 STATE-OF-THE-ART OF THE PROJECT

The novel GABA response found in the neonatal hippocampus resembles that first described as $GABA_C$, thought to be mediated by ρ subunits which have a prominent retinal expression. At the time this response was discovered, there was no unequivocal data about the presence of the ρ subunits in the brain. Northern blotting (Cutting *et al.*, 1991) and RT-PCR (Enz *et al.*, 1995) analysis of poly(A)$^+$ RNA extracted from several brain regions have demonstrated the presence of ρ1 and ρ2 mRNAs in bovine cerebellum (Cutting *et al.*, 1991) and different rat brain regions (highest mRNA concentrations were found in the hippocampus and in the cortex) (Enz *et al.*, 1995).

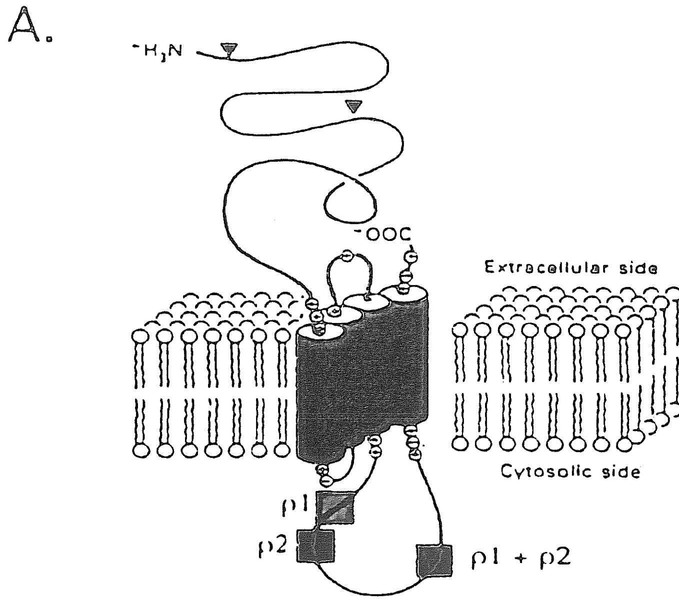
However, the expression of the ρ subunits in the brain was not yet demonstrated by *in situ* hybridization or immunohistochemical studies at the time the project started (see Discussion). These data suggest that ρ1 and ρ2 mRNAs might be expressed at least in some brains areas and hopefully in the hippocampus.
The first goal in the realization of the project was to determine whether ρ-like $GABA_A$ receptor subunits were present or absent in the rat brain, especially in the neonatal hippocampus. At the time these experiments were carried out, the sequences of the rat ρ1 and ρ2 subunits were not known. For this reason oligonucleotide probes based on the human receptors were designed using regions of the genes (intracellular loop) which were specific for the ρ1 and ρ2 genes and were unlikely to cross-hybridize with other $GABA_A$ receptors (**Figure 6**). When the sequence of the rat genes were published, it became clear that the 65% sequence homology between the ρ2 probe and the corresponding region of the rat gene was unlikely to provide meaningful results. Therefore the experiments with this particular probe have not been considered. The ρ1 probe however seemed to be useful since the homology with the corresponding rat sequence was 92% (**Figure 6D**). A common probe for both subunits named ρ1+ρ2 was also synthesized. This probe bears two mismatches with respect to the human ρ1 and two mismatches with respect to the human ρ2 sequences but should be able to recognize both ρ1 (5 mismatches out of 45 bases, 89% homology) and ρ2 (9 mismatches out of 45 bases, 80% homology) subunits from rat. (**Figure 6D**).

**FIGURE 6. Oligonucleotide probes derived from the $GABA_A$ receptor ρ subunits used in *in situ* hybridisation, RACE or cDNA library screening experiments.**

**Panel A.** A putative $GABA_A$ receptor ρ subunit protein is shown. The red, blue and violet squares represent the parts of the intracellular loop from which the ρ1, ρ2 and ρ1+ρ2 probes are derived.
**Panel B.** Amino acid sequences of different human $GABA_A$ receptor subunits derived from the same region as the ρ1+ρ2 probe.
**Panel C.** Nucleotide sequences of the ρ1, ρ2 and ρ1+ρ2 probes used are shown. The GC content is given for each probe.
**Panel D.** The homology between the ρ1, ρ2 and ρ1+ρ2 probes and the corresponding sequences of the rat ρ1 and ρ2 genes is shown.

FIGURE 6.

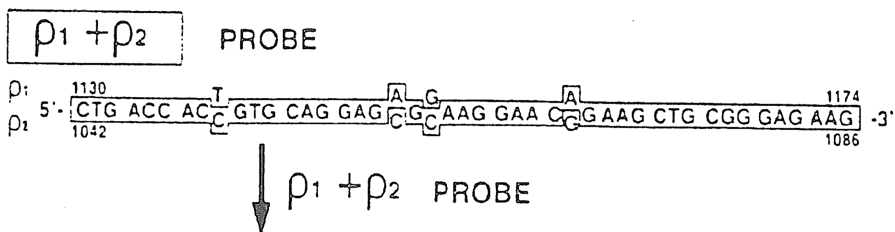
A.




B.

```
α1 ... F T K R G Y A W D G K S V V P E K P K K V K D P ... 336
β1 ... I F F G K G P Q K K G A S K Q D Q S A N E K N K ... 331
γ1 ... F V S N R K P S K D K D K K K K N P A P T T D I ... 346
δ  ... ————————————————————— N A D Y R K K R K A K V K ... 333
         362
ρ1 ... L ——————————————————— T T V Q E R K E [Q] K L R E K ... 376
         348
ρ2 ... L ——————————————————— T T V Q E R K E [R] K L R E K ... 362
```

C.




5'- CTG ACC ACC GTG CAG GAG AGC AAG GAA CAG AAG CTG CGG GAG AAG -3'

27 GC / 18 AT ⇒ 60 % GC

ρ1 PROBE

```
  427                                                        442
...R   S   S   P   Q   R   K   S   Q   R   S   S   Y   V   S   M...
5'- AGG AGC TCC CCA CAG AGG AAA AGT CAG AGA AGG AGC TAT GTG AGC ATG -3'
    1325                                                     1372
```

25 GC / 23 AT ⇒ 52,8 % GC

ρ2 PROBE

```
  417                                                        432
...A   N   A   A   R   K   K   G   L   L   K   G   Q   T   G   F...
5'- GCC AAC GCT GCC AGA AAG AAG GGG CTT CTG AAG GGC CAG ACG GGT TTT -3'
    1249                                                     1296
```

## D.

| ρ1+ρ2 probe | CTG | ACC | ACC | GTG | CAG | GAG | AGC | AAG | GAA | CAG | AAG | CTG | CGG | GAG | AAG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rat ρ1 sequence | CTG | ACC | AC**A** | GTG | CAG | GAG | **C**G**G** | AAG | GAA | C**G**G | AAG | CT**T** | CGG | GAG | AAG |
| rat ρ2 sequence | CTG | ACC | AC**A** | GTG | CAG | GA**A** | **CAG** | AAG | GAA | C**G**G | AAG | CT**T** | CGG | GA**C** | AA**A** |

Homology between ρ1+ρ2 probe and:

- ρ1 rat gene is 89% (5 mismatches out of 45 bases)
- ρ2 rat gene is 80% (9 mismatches out of 45 bases)

| ρ1 probe | AGG | AGC | TCC | CCA | CAG | AGG | AAA | AGT | CAG | AGA | AGG | AGC | TAT | GTG | AGC | ATG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rat ρ1 seq. | AGG | **G**GC | TCC | CCA | CAG | AGG | AAA | AG**C** | CAG | AGA | **G**G**C** | AGC | TAT | GTG | AGC | ATG |

Homology between ρ1 probe and rat ρ1 gene sequence is 92% (4 mismatches out of 48 bases).

| ρ2 probe | GCC | AAC | GCT | GCC | AGA | AAG | AAG | GGG | CTT | CTG | AAG | GGC | CAG | ACG | GGT | TTT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rat ρ2 seq. | **TTG** | A**CT** | **T**C**A** | **T**CC | AGA | AAG | AA**A** | GG**C** | CTT | CT**C** | AA**A** | GGC | CA**A** | A**T**G | GG**A** | **C**T**C** |

Homology between ρ2 probe and rat ρ2 gene is 65% (17 mismatches out of 48 bases).

Radioactive *in situ* hybridisation with these probes has been performed on brain slices. The autoradiographic signals obtained with ρ1 specific oligonucleotide probe were low in both neonatal and adult rat brains. However, the signals obtained with the ρ1+ρ2 probe were surprisingly strong in the neonatal rat brain, especially in the hippocampus and cortex. In the adult rat brain a very low signal was present in the cerebellum. Both probes gave a strong signal in the rat neonatal retinal sections, as expected, especially in the inner nuclear layer. The β3 probe used as a control gave high signals in almost all brain regions in neonatal, as well as in adult brain sections.

The next step was to clone the gene that gave the high signal in the *in situ* hybridization experiments in the neonatal rat brain sections, using the ρ1+ρ2 specific oligonucleotide probe. This gene could hopefully be a new ρ–like $GABA_A$ receptor subunit present exclusively in the neonatal rat hippocampus during the critical period of postnatal development, or another novel gene, unrelated to the $GABA_A$ receptor gene, which shared homology with the ρ1+ρ2 oligonucleotide probe. Since the only available information about the gene of interest was the short stretch of sequence comprised in the ρ1+ρ2 oligonucleotide probe, the RACE (Rapid Amplification of cDNA Ends) (Frohman, 1994) cloning strategy was used to isolate this new gene. Briefly, the RACE protocol generates cDNA by using PCR to amplify copies of the region between a single point in the transcript and 3' and 5' ends. Thus, the minimum information required for this amplification is a single short stretch of sequence within the mRNA to be cloned, and in this case it was the sequence of the oligonucleotide probe ρ1+ρ2. From this region, primers oriented in the 3' and 5' direction were chosen that would produce overlapping cDNA when fully extended.

However, the fragments obtained with this protocol were mostly non-specific, revealing only house-keeping genes with no homology to the ρ1+ρ2 oligonucleotide probe. The most interesting result was a 450 bp fragment, obtained as a 3' RACE product. The product was of the expected length for a ρ subunit sequence, and the sequence obtained was novel and not found in the databases. It was most similar to the expressed sequence tag HFBEL69 from fetal human brain, but with no apparent homology with the $GABA_A$ receptor genes (for details on these experiments, see Miranda Mladinic, Ph.D. Thesis, SISSA, 1997).

These discouraging results suggested that the ρ1+ρ2 probe was not completely (or absolutely) homologous to the part of the gene of interest. In particular, it was reasoned that if the homology of the sequence is comprised only in the middle part and is not preserved at the ends of the sequence, the primers oriented in either the 3' or 5' directions, derived from the ρ1+ρ2 sequence, would never be able to give specific PCR products.

For these reasons, the cloning strategy was changed to a cDNA library screening strategy proved in the past decade to be the tool of choice in cloning $GABA_A$ receptor subunits genes.

## 4.2 CONSTRUCTION OF A cDNA LIBRARY FROM THE NEONATAL (P0-P2) RAT HIPPOCAMPUS

Since the RACE cloning technique did not give the desired result, a cDNA library from the neonatal hippocampus was constructed. Such a library is an extremely valuable source of genes expressed in the neonatal hippocampus, among which are expressed the $GABA_A$ receptor genes in the early period of postnatal development.

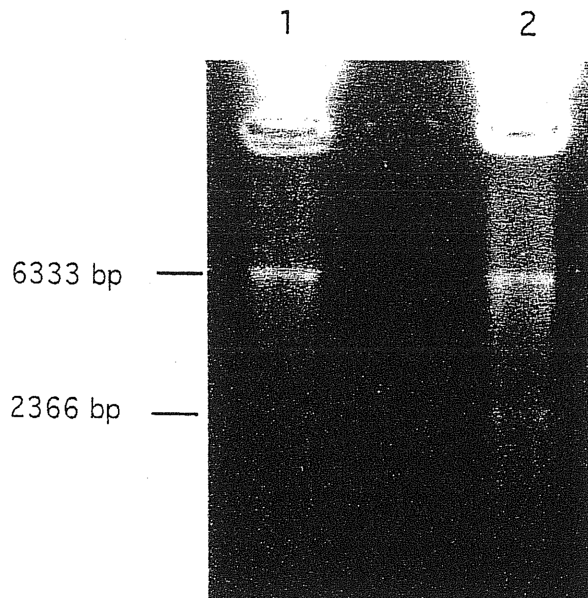
An unidirectional cDNA library was constructed using the ZAP Express™ cDNA Synthesis and Gigapack II® Gold Cloning Kits (Stratagene), as described in the Materials & Methods section. A quality control and evaluation procedure was performed at each step of the cDNA library construction, to obtain a cDNA library of high quality, with the following characteristics:

1) representative, containing all sequences present in the initial poly(A)+ RNA population in the same relative frequencies,
2) unidirectionally cloned so that the orientation of each cDNA is known, facilitating subsequent sequence analysis, and expression,
3) composed of a high proportion of long or full-length inserts,
4) not contaminated with genomic, mitochondrial or ribosomal RNA inserts and
5) composed of a large proportions of inserts with short poly (A) tails.

The quality of the RNA extracted from the neonatal rat hippocampus was checked on a denaturating formaldehyde agarose gel (**Figure 7**) and RNA was quantified by measuring the optical density of a diluted RNA solution. The starting quantity of poly(A)+ RNA used for the cDNA synthesis was 4 μg.

The efficiency of the cDNA synthesis reaction was monitored with incorporation of radioactive nucleotides and by performing the same reactions on the control sample of RNA of a known length. A portion of each reaction product was separated electrophoretically on alkaline agarose gels and exposed to autoradiographic film, to determine the size range of the first- and second-strand cDNA (**Figure 8**).

**FIGURE 7. RNA extracted from neonatal hippocampal tissue used for the construction of the cDNA library.**



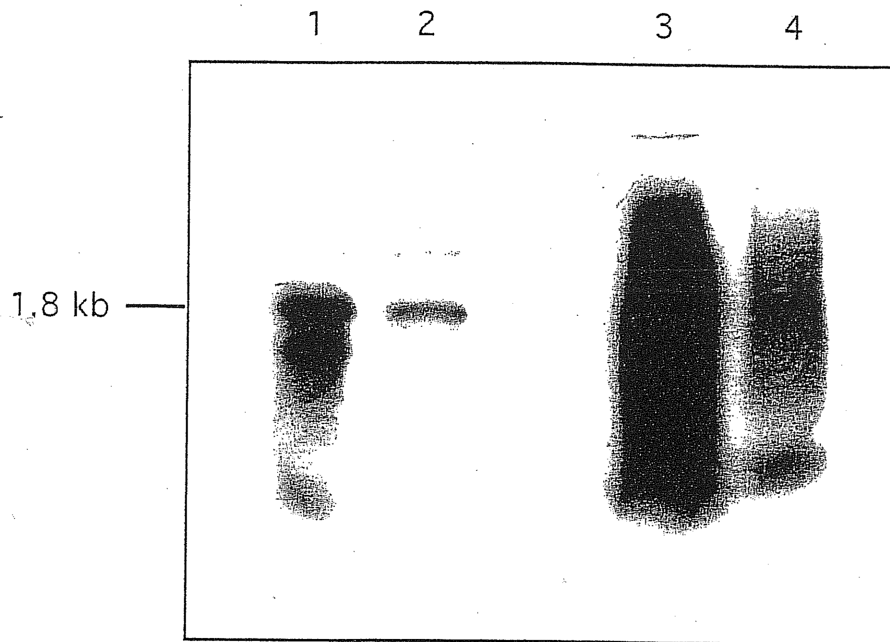

Denaturing formaldehyde RNA agarose gel to check the quality of the RNA extracted.

**lane 1**: sample of the RNA extracted from 1g of neonatal rat cortex.

**lane 2**: sample of the RNA extracted from 4g of neonatal rat hippocampal tissue.

In each lane, about 20% of the total amount of the extracted RNA was loaded. The traces of 28S and 18S rRNA (migrating at 6333 bp and 2366 bp respectively) in both samples indicate the presence of long RNA molecules and thus good RNA quality.

**FIGURE 8. Control of the size range of the first-strand and second-strand cDNA synthesised.**




The efficiency of cDNA reaction was monitored with the incorporation of radioactive nucleotides and by performing the same reactions on the control RNA sample of a defined length (1.8 kb). 10% of each reaction was separated electrophoretically on an alkaline agarose gel and exposed to autoradiographic film to determine the size range of the first- and second-strand cDNA.
**lane 1**: 1st strand cDNA from control RNA; **lane 2**: 2nd strand cDNA from control RNA;
**lane 3**: 1st strand cDNA from neonatal hippocampal mRNA; **lane 4**: 2nd strand cDNA from neonatal hippocampal mRNA. The average size of the cDNA obtained is > 1kb.

A Sephacryl S-500 spin column was used to size select the cDNA and remove excess adapters. The 1/10 of the volume of each fraction collected was analysed electrophoretically on a 5% non denaturating polyacrylamide gel and exposed to autoradiographic film to check sizes of cDNA in each fraction. cDNA greater than 500 bp in size was pooled, precipitated, quantitated and ligated to the Lambda ZAP Express vector arms and subsequently packaged with Gigapack II Gold packaging extract (Stratagene).

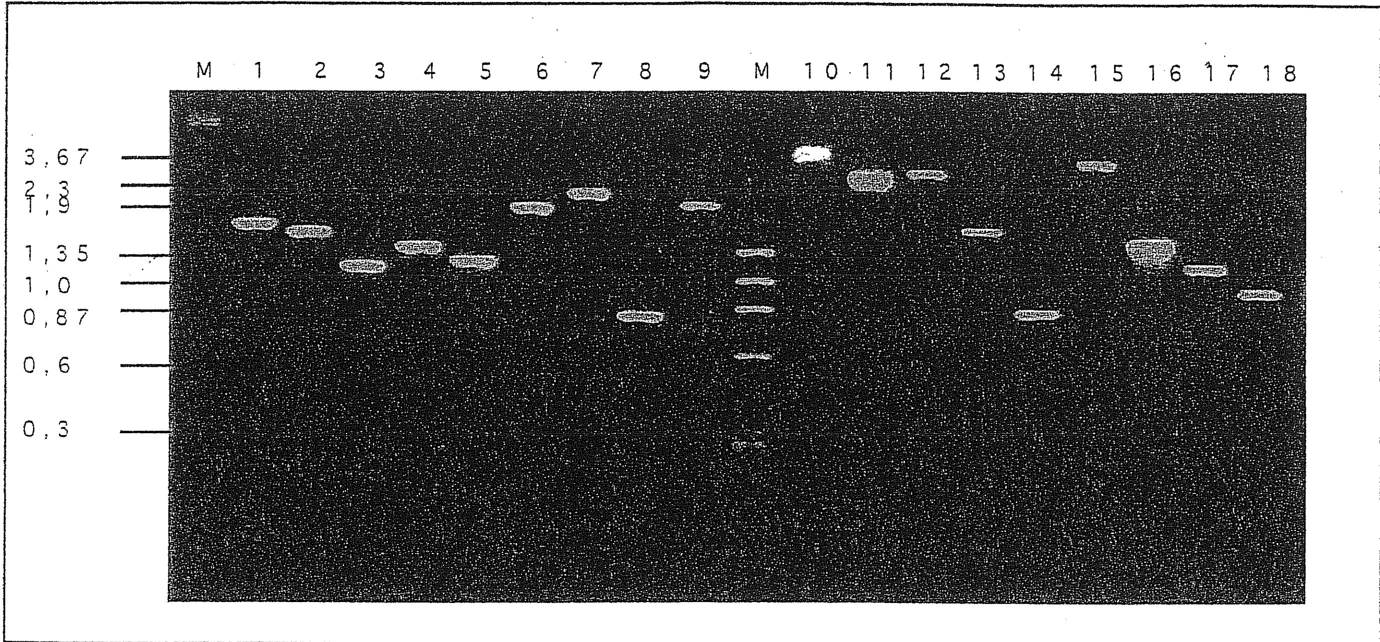
After construction and amplification, the quality and characteristics of the cDNA library were evaluated by determining the efficiency of the cloning and packaging steps, the ratio of recombinant to non-recombinant clones, the total number of clones (**Table 8**) and the average size of the cDNA inserts using PCR analysis of approximately 20 individual clones (**Figure 9**).

Regarding the parameters shown in **Table 8**, the neonatal hippocampal cDNA library can be considered to be of high quality.

**TABLE 8. Parameters showing the quality of the constructed cDNA library from the neonatal rat hippocampus**

| | EXPECTED VALUES | OBTAINED VALUES |
|---|---|---|
| EFFICIENCY OF cDNA CLONING (p.f.u./ng of cDNA) | $> 2 \times 10^4$ | $2.56 \times 10^4$ |
| EFFICIENCY OF PACKAGING (p.f.u./μg of vector DNA) | $\sim 5 \times 10^6$ | $1.03 \times 10^6$ |
| NON-RECOMBINANT CLONES (%) | 1-10% | 1.37% |
| TOTAL NUMBER OF RECOMBINANT PLAQUES (p.f.u.) | $> 1 \times 10^6$ | $2.56 \times 10^6$ |
| TITER OF AMPLIFIED LIBRARY (p.f.u./ml) | $> 1 \times 10^9$ | $2.13 \times 10^9$ |
| AVERAGE INSERT SIZE | > 500 bp | > 1 kb |

**FIGURE 9. Evaluation of cDNA insert size of recombinant clones from the cDNA library by PCR.**




18 recombinant clones from the rat neonatal hippocampal cDNA library were chosen randomly, purified and excised. PCR amplification has been performed on these clones using T3 and T7 primers to reveal the cDNA insert size.
**M**: λ *Bst*E II DNA size marker; **lanes 1-9**: PCR products from single excised recombinant clones; **M**: ϕx174 *Hae* III DNA size marker; **lanes 10-18**: PCR products from single excised recombinant clones.

## 4.3 ATTEMPTS TO CLONE ρ-LIKE SUBUNITS FROM THE NEONATAL RAT HIPPOCAMPUS cDNA LIBRARY

### 4.3.1 SCREENING OF THE cDNA LIBRARY WITH THE ρ1+ρ2 PROBE

Since RACE cloning of the gene from the neonatal rat brain that gave high signals in *in situ* hybridization experiments using the ρ1+ρ2 oligonucleotide probe was unsuccessful, the new cloning approach using the cDNA library from the rat neonatal hippocampus was implemented.
About $1.2 \times 10^6$ p.f.u. from the rat neonatal cDNA hippocampal library were screened with the ρ1+ρ2 oligonucleotide probe. The conditions of hybridization and following washings were as described in Materials & Methods. A total of 18 putative primary positive clones were isolated and submitted to further analysis (secondary and tertiary screening, excision of the pBK-CMV phagemid vector and sequencing). Three clones remained real positives. Partial sequencing of the clones from both ends revealed that one clone had an interesting sequence that could correspond to the gene that gave a high signal in the *in situ* hybridization with the ρ1+ρ2 oligonucleotide probe. Namely, that clone (internally named 4B) turned out to be a novel gene, probably a transcription factor. The novel sequence (**Figure 10**) is similar to a group of genes comprising:
-Rat mRNA for DNA-binding protein B (dbpB) (dbj/D13309/RATRDBPB),
-Rat enhancer factor I subunit A gene ($EF1_A$) (gb/M57299/RATEFIA),
-Rat mRNA (gb/M69138/RATCDS), and
-Mouse Y-box transcription factor (MSY-1).

**<u>FIGURE 10.</u> Novel partial sequence of the EFI$_A$/dbpB/YB-1-like gene, cloned from the neonatal hippocampal cDNA library using ρ1+ρ2 oligonucleotide probe.**

from the 5' end:
GGAGCGGAGAG**CAAC**CCCAGAGAGCCCTGAGAGCCCACCGCCGCCGCCGG
CC**A**AGTCACCATCACACCCGGGAGGAGCCGCAGCCGTCGCCGCCGG**T**CCAG
TCACCATCACCGCAACCATGAGCAGCGAGGCCGAGACCCAGCAGCCGCCC
GCCGCCCCCGCCGCCGCCCTC**GAGC**CCGCCGACACCAAGCCCGGCTCCACG
GC···etc.

from the 3' end:
CTGAGTAAATGCCGGCTTACCATCTCTACCATCATCCGGTTTGGTCATCCAA
CAAGAAGAAATGAATATGAAATTCCAGCAATAAGAAATGAACAAAGATTG
GAGCTGAAGACCTTAAGTGCTTGCTTTTTGCCCGTTGACCAGATCCACTAGA
ACTGTCTGCATTATCTATGCAGCATGGGGTTTTTATTATTTTTACCTAAAGAT
GTCTCTTTTTGGTAATGACAAACGTGTTTTTTAAAAAAAAAA (poly A tail)

The sequences represent a few hundred bp from 5' and 3' ends of the cloned cDNA insert. In bold are bases that differ from the sequence of the rat Enhancer Factor I subunit A (EFI$_A$) gene.

A cDNA for EFI$_A$ has been isolated from a rat liver cDNA expression library (Ozer *et al.*, 1990). EFI$_A$ is a trans-acting factor which binds to the Rous sarcoma virus long terminal repeat enhancer and promoter at two inverted CCAAT-box motifs. EFI$_A$ cDNA is nearly identical (95-97% of homology) to two human DNA binding proteins. These are dbpB, a DNA binding protein of unknown specificity which binds to the epidermal growth factor receptor enhancer and the c-erbB-2 gene promoter (Sakura *et al.*, 1988), and YB-1, a protein which recognizes the Y-box (inverted CCAAT motif) of the HLA-DR α chain gene (Didier *et al.*, 1988). EFI$_A$/dbpB/YB-1 shares a highly conserved region of 100 amino acids with dbpA, another protein identified by Sakura *et al.* (1988) which binds to the epidermal growth factor receptor enhancer and the c-erbB-2 gene promoter, and with two *Xenopus* CCAAT binding proteins, FRG Y1 and FRG Y2 (Tafuri & Wolffe, 1990). This highly conserved domain among all six proteins is presumed to represent or contain a DNA binding domain for the CCAAT motif. Since the novel sequence from the neonatal hippocampus shares more than 95% homology with the genes quoted before, it is likely that this clone encodes for a related protein.

All these genes appeared as a result of the initial FASTA and BLAST computer

searches performed on the ρ1+ρ2 sequence. The sequence similarity was 53.3% (24 out of 45 bases), and was found in the middle of the oligonucleotide, but not at either end of the oligonucleotide sequence. This was probably the reason for the lack of success of the RACE cloning strategy.

It is very difficult to speculate about a possible function of the novel protein or about its connection with the $GABA_A$ receptor genes. However, to confirm that this novel $EFI_A$/dbpB/YB-1-like gene was really the gene that gave a high signal in the *in situ* hybridization experiments with the ρ1+ρ2 probe, a novel *in situ* hybridization on the neonatal and adult brain sections, using the cDNA of clone 4B as a probe was performed (data not shown). The results of the *in situ* hybridization showed that the mRNA corresponding to the 4B clone is expressed only in neonatal rat brain until P8 and not after that age. The spatio-temporal expression of this gene overlaps with that of the $GABA_A$-ρ-like response, as well as that of the ρ1+ρ2 probe.

The conclusions that could be drawn at that point were dual:

1) Unfortunately, it has been shown that the high signals from neonatal hippocampus in *in situ* hybridization experiments using a ρ1+ρ2 oligonucleotide as a probe, were not due to a novel ρ–like GABA receptor subunit gene, but from a novel $EFI_A$/dbpB/YB-1-like gene which is unrelated to the $GABA_A$ receptor genes, since the expression pattern of the novel $EFI_A$/dbpB/YB-1-like gene overlaps with the appearance of the $GABA_A$-ρ-like response.

2) Since the novel gene belongs to a family of DNA-binding proteins and transcription factors, there is a possibility that this gene is involved in the control of the expression of certain $GABA_A$ receptor genes. However, this hypothesis is purely speculative and it seems more likely that the similar expression patterns observed are a mere coincidence.

### 4.3.2 SCREENING OF THE cDNA LIBRARY WITH A ρ1 cDNA PROBE

The results of the initial screening experiments using the ρ–like $GABA_A$ receptor subunit probe did not lead to the cloning of related $GABA_A$ receptor ρ subunits and constituted a misleading working hypothesis. Even though the present results were not in favour of the idea of novel ρ–like GABA receptor subunits in the neonatal brain, there are good reasons why this strategy may have failed: such novel ρ–like GABA receptor subunits may not share sufficient homology with the ρ subunits in the region of the ρ1+ρ2 probe.

So, the cDNA library from the neonatal hippocampus was screened with a complete cDNA for the human ρ1 GABA receptor subunit (kindly provided by Dr. G.R. Uhl, NIH, Bethesda, USA), as a probe. The $1.6 \times 10^6$ p.f.u. were screened with a radioactive ρ1 full-length cDNA probe to the final concentration of 15 ng/ml or $> 10^6$

cpm/ml of hybridization solution. The hybridization was performed at 42°C overnight. The conditions of the washing and exposure were as described in the Materials & Methods section. Nineteen putative primary positive clones were obtained. Further analysis of the putative positive clones revealed that none of them was a gene related to the $GABA_A$ receptor ρ subunit genes (data not shown). This probe however seemed suitable to clone ρ subunits from rat, because the sequence homologies between human ρ1 and rat ρ1 are quite high (88 %), and the ρ2 and ρ3 subunits share about 75% homology with ρ1. In addition, the use of cDNAs from a given species to clone genes in other species is of routine use in molecular cloning. Moreover, since the ρ subunits are relatively distinct from other $GABA_A$ receptor subunits at the amino acid level (30-40% similarity) and therefore even more at the DNA sequence level, it is not surprising that no other $GABA_A$ receptor subunit was identified using the human ρ1 cDNA probe. These results, together with the data from the literature, strongly suggest that $GABA_A$ receptor ρ subunits or analogue genes, if present in the neonatal hippocampus, might not be in sufficient amounts to be detected with the present techniques.

## 4.4 CLONING THE $GABA_A$ RECEPTOR GENES EXPRESSED IN DEVELOPING HIPPOCAMPUS

Until now, the cloning of the new $GABA_A$ receptor subunit, transiently expressed in the neonatal hippocampus, was unsuccessful. The novel subunit, if it exists, may not be exclusively of the ρ type, but also of α type. Indeed, the novel $GABA_A$-ρ-like response from the neonatal hippocampus is reminiscent of that obtained in *Xenopus* oocytes injected with an α1 subunit in which position 64 was occupied by Leu instead of Phe (Sigel *et al.*, 1992) and the conductance states of the current were similar to those obtained for $GABA_A$ receptors (Martina *et al.*, 1995). It is also possible that new alternatively spliced forms or new RNA edited forms of the subunits exist. The new receptor could also be the result of a different, unexpected subunit combination. To test all these hypotheses simultaneously, the neonatal hippocampal cDNA library was screened with probes that would be able to reveal all $GABA_A$ receptor subunits from the neonatal hippocampus, including the putative novel one. For this purpose oligonucleotide probes derived from the most conserved region of all currently cloned $GABA_A$ receptor subunits, the M2 domain (second transmembrane region) of the putative $GABA_A$ receptor protein, were used. Such probes have been proven to be successful in the past to clone many of the $GABA_A$ and glycine receptor subunits (Ymer *et al.*, 1989). The designed oligonucleotide probes are shown in **Table 9**.

A group of seven oligonucleotide probes chosen recognised:
α1 and α3 subunits
α2 and α6 subunits
α4 and α5 subunits
β1 and β3 subunits
β2 and γ2 subunits
γ3 and δ subunits
all three ρ subunits (ρ1, ρ2 and ρ3).

All probes sequences refer to rat $GABA_A$ receptor subunit genes sequences. The probes were 24-36 nucleotides in length, with 6.25-15.38% of degeneracy with respect to the original rat sequences. The probe that recognized the γ1 subunit was intentionally omitted. The reason for this choice was that this subunit is known to be present in the neonatal hippocampus (see **Figure 3**) and has a M2 domain DNA sequence that is similar, but not 100% identical, to the sequences of the probes used to recognise other subunit sequences. We reasoned that if we were able to select γ1 subunit cDNAs, other (novel) subunits with similar degrees of homology could also be identified. The success or failure of the cloning of the γ1 subunit, could be taken as a measure of the quality of the screening. For example, if a new $GABA_A$ receptor subunit was not cloned, but if the γ1 subunit was also not cloned after screening the library with the selected M2 probes under the chosen conditions of the hybridization and washing, then it could not be categorically claimed that the new subunit does not exist in the neonatal hippocampus. It is clear, however, that conditions of screening under which the γ1 subunit is cloned, but other putative subunits are not, does not conclusively prove that such novel subunits do not exist.
Since the ε subunit was described after the initiation of this work, a corresponding probe could not be included in this screening. An analysis of its sequence showed that it could be recognised by the γ3δ probe which shares a stretch of 22 bases with 2 mismatches with the rat ε subunit M2 domain. Later analysis of non-$GABA_A$ receptor subunit clones showed that such a homology is enough to give a positive signal in the screening. Therefore if present the ε subunit should be isolated with the γ3δ probe used.
The rationale for choosing seven probes was that we wished to avoid picking probes which were too specific for each individual subunit, since we wanted these to hybridize not only to known subunits, but also closely related genes. Simultaneously, we also wished to avoid using a probe which was so degenerate that it was able to recognise all subunits at a time, as we felt that this would give too many false positives. Seven probes with a moderate degree of degeneracy (48 different oligos in total compared to at least 96-fold degeneracy used in the early 90s for the cloning of the $GABA_A$ receptor subunits) seemed a good compromise.

**TABLE 9.** **Oligonucleotide probes designed from M2 domains of the rat $GABA_A$ receptor subunits.**

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| α1 | TTT | GGA | GTG | ACG | ACC | GTT | CTG | ACC | ATG | ACA | ACC | TTG | AGT | |
| α3 | TTT | GGT | GTC | ACC | ACT | GTT | CTC | ACC | ATG | ACC | ACC | TTG | AGT | |
| α1+α3 | | | | | | **GTT** | **CTS** | **ACC** | **ATG** | **ACM** | **ACC** | **TTG** | **AGT** | 4/24 |
| α2 | TTT | GGA | GTA | ACA | ACT | GTT | TTG | ACA | ATG | ACC | ACA | TTA | AGC | |
| α6 | TTT | GGA | ATC | ACC | ACG | GTT | TTA | ACC | ATG | ACC | ACC | TTA | AGC | |
| α2+α6 | | | | | | **GTT** | **TTR** | **ACM** | **ATG** | **ACC** | **ACM** | **TTA** | **AGC** | 8/24 |
| α4 | TTT | GGA | ATA | ACC | ACA | GTC | CTC | ACG | ATG | ACC | ACC | CTA | AGC | |
| α5 | TTT | GGA | GTG | ACC | ACA | GTG | CTG | ACC | ATG | ACA | ACC | CTC | AGC | |
| α4+α5 | | | | **ACC** | **ACA** | **GTS** | **CTS** | **ACS** | **ATG** | **ACM** | **ACC** | **CT** | | 16/26 |
| β1 | CTA | GGA | ATC | ACC | ACG | GTG | CTG | ACC | ATG | ACA | ACC | ATC | AGT | |
| β3 | CTA | GGG | ATT | ACC | ACC | GTG | CTC | ACC | ATG | ACA | ACC | ATC | ACC | |
| β1+β3 | | | | **ACC** | **ACS** | **GTG** | **CTS** | **ACC** | **ATG** | **ACA** | **ACC** | **ATC** | **A** | 4/28 |
| β2 | TTA | GGA | ATT | ACA | ACT | GTC | CTG | ACG | ATG | ACC | ACA | ATC | AAT | |
| γ2 | TTA | GGA | ATC | ACG | ACT | GTC | CTG | ACG | ATG | ACC | ACT | CTC | AGC | |
| β2+γ2 | **TTA** | **GGA** | **ATY** | **ACR** | **ACT** | **GTC** | **CTG** | **ACG** | **ATG** | **ACC** | **AC** | | | 4/32 |
| γ3 | TTA | GGC | ATC | ACC | ACG | GTG | CTA | ACC | ATG | ACC | ACA | CTC | AGC | |
| δ | CTA | GGC | ATC | ACC | ACT | GTG | CTG | ACA | ATG | ACC | ACA | CTC | ATG | |
| γ3+δ | **TA** | **GGC** | **ATC** | **ACC** | **ACK** | **GTG** | **CTR** | **ACM** | **ATG** | **ACC** | **ACA** | **CTC** | **A** | 8/36 |
| ρ1 | TTA | GGC | ATC | ACC | ACG | GTG | CTG | ACC | ATG | TCC | ACC | ATC | ATC | |
| ρ2 | CTG | GGC | ATC | ATG | ACG | GTG | CTG | ACC | ATG | TCC | ACC | ATC | ATC | |
| ρ3 | CTG | GGA | ATC | ACC | ACG | GTG | CTC | ACC | ATG | TCC | ACA | ATC | GTC | |
| ρ1+ρ2+ρ3 | | | | | **ACG** | **GTG** | **CTS** | **ACC** | **ATG** | **TCC** | **ACM** | **ATC** | | 4/24 |
| γ1 | CTG | GGT | ATC | ACT | ACG | GTG | TTG | ACT | ATG | ACA | ACC | CTC | AGT | |

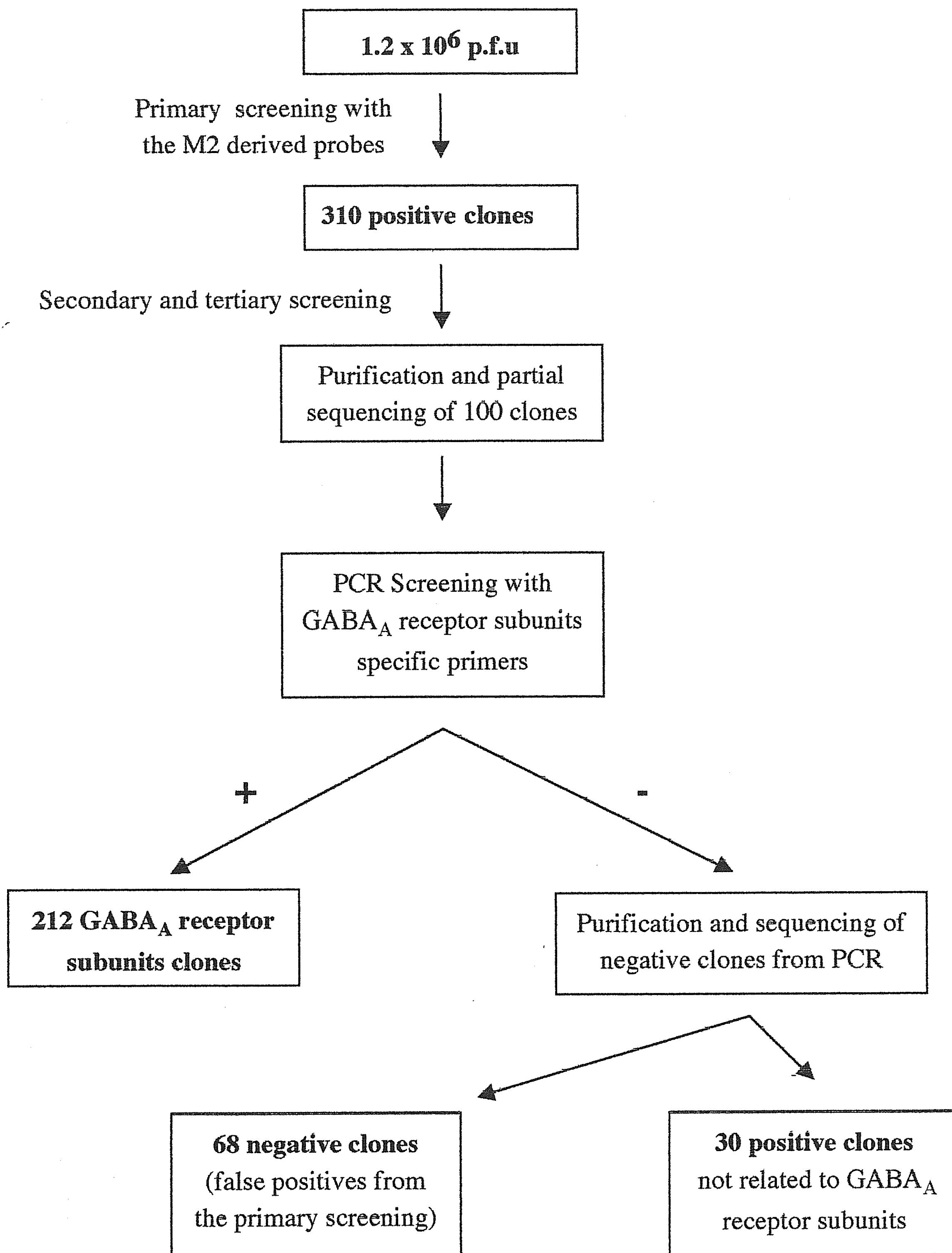
In the table the nucleotide sequences of the M2 domain of all cloned rat $GABA_A$ receptor subunits (α1-6, β1-3, γ1-3, δ and ρ1-3) and the sequences of the 7 derived oligonucleotide probes (α1+α3, α2+α6, α4+α5, β1+β3, β2+γ2, γ3+δ and ρ1+ρ2+ρ3) are shown in bold. The numbers in the last column represent the total degeneracy and the length of each oligonucleotide probe.

The 1.6 x $10^6$ p.f.u. were screened with the collection of M2 oligonucleotide probes, radioactively labeled to a final concentration of 1 pmol/ml or > $10^6$ cpm/ml of hybridization solution. The molar ratio between the different oligonucleotides present in the probe mixture was proportional to the length and degeneracy of the probe. The primary screening was performed at 50°C for 64 hours, followed by washings at 42°C, two times in 2 x SSC / 0.1%SDS for 15 min, two times in 0.5 x SSC / 0.1% SDS for 15 min, one time in 0.2xSSC / 0.1%SDS for 15 min, once at 50°C in 0.2 x SSC / 0.1%SDS for 15 min and two times in 0.2 x SSC / 0.1%SDS for 30 min at 55°C. The other conditions of the screening were as described in section 3.3.
A flow chart summarizing the screening with the degenerate probes and the subsequent analysis of the positive clones is shown in **Figure 11**.
310 putative positive clones were obtained after the primary screening. The first 100 clones were submitted to secondary and tertiary screening (the same hybridization and washing conditions, as for the primary screening), and were excised and partially sequenced as described in sections 3.3 and 3.4.

The results of the first analyses were:
- 20 clones of $\alpha 5$ $GABA_A$ receptor subunit (4 full-length clones)
- 9 clones of $\alpha 2$ (2 full-length clones)
- 9 clones of $\gamma 2$ (1 full-length clone)
- 8 clones of $\alpha 4$ (2 full-length clones)
- 6 clones of $\beta 2$ (2 full-length clones)
- 4 clones of $\alpha 1$
- 3 clones of $\beta 1$ (1 full-length clone)
- 2 clones of $\beta 3$ (1 full-length clone)
- 1 clone of $\gamma 1$
- 1 clone with a novel sequence, not present in the data bases (> 500bp)
- 1 clone encoding mitochondrial RNA protein.
- 36 clones that turned out to be false positives (negative in secondary screening).

These preliminary results indicate that the cDNA library used was of good quality (many full-length clones), and also that the conditions of hybridization and washing used were of a good stringency. Indeed, only 2 clones were not related to $GABA_A$ receptor subunits and a sequence which was not 100% homologous, namely one $\gamma 1$ $GABA_A$ receptor subunit clone, was identified. These conditions could therefore hopefully permit the cloning of a putative new related $GABA_A$ receptor subunit. Some of the sequences were novel and not present in the nucleotide data banks. One clone (initially named as 76) exhibited a novel sequence (>700 bp). The other novel sequences were the 5' untranslated regions of known subunits ($\alpha 4$, $\alpha 5$, $\beta 2$).

**FIGURE 11. Flow chart of the screening of the cDNA library with the M2 derived oligonucleotide probes and analysis of the clones.**



Since several subunits were abundantly found, a PCR analysis of the primary positive clones using subunit-specific primer pairs was used as a more rapid way to identify known subunit clones with respect to the classic and time-consuming secondary and tertiary screening procedure. The phage stocks from the primary positive clones were submitted to PCR using subunit specific primers derived from the intracellular loop between M3 and M4 domains (see **Table 6**). This is the most variable region of the $GABA_A$ receptor subunits, and provides the highest specificity. In total, 212 clones were identified as $GABA_A$ clones, representing 11 different subunits. The remaining clones, negative by PCR analysis, were subsequently re-screened, excised and partially sequenced. This analysis showed that 30 clones were true positives but unrelated to any $GABA_A$ receptor subunit, representing 18 known genes and 12 unknown cDNA sequences (not shown). The remaining 68 clones were false positives, negative at the secondary screening (22% of the total primary positive clones). The final results of the screening are shown in **Table 10**.

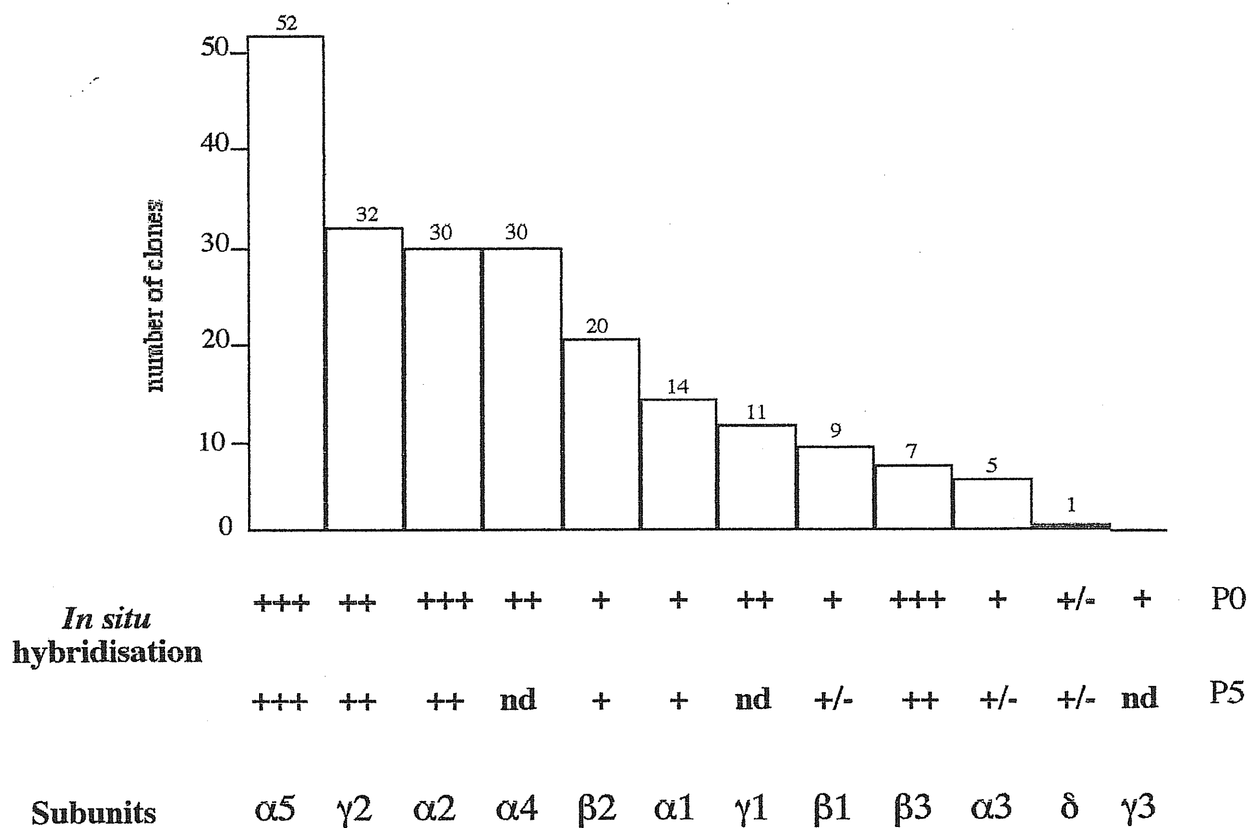
These results show that the $\alpha5$ subunit is by far the most highly expressed, along with the $\gamma2$, $\alpha2$ and $\alpha4$ subunits. The expression of the $\beta2$, $\alpha1$, $\gamma1$, $\beta1$ and $\beta3$ subunits is moderate, while that of the $\alpha3$ and $\delta$ subunits is weak. Interestingly, two new splice variants of the $\gamma2$ subunit, in which exon 2 on one hand and exon 2 and 3 on the other hand were missing (as shown by sequence comparison to the homologous murine $\delta$ subunit gene, EMBL accession n° M60587 to M60596, data not shown), were also found. Although these two clones led to early truncated gene products which were non-functional (in electrophysiological experiments on transfected HEK 293 cells, not shown), nevertheless they confirmed that the technical approach used was appropriate for isolating rare forms of already known subunits. The $\alpha6$ subunit which is known to be expressed only in the cerebellum (Lüddens *et al.*, 1990) was not found. Similarly, the $\gamma3$ subunit, which has been previously detected by oligonucleotide directed *in situ* hybridisation experiments (Laurie *et al.*, 1992) was not revealed. cDNA clones for the $\rho1$, $\rho2$ and $\rho3$ subunits as well as for the recently described $\varepsilon$ subunit (Davies *et al.*, 1997) were not detected. The absence of isolated clones for $\gamma3$, $\rho$ or $\varepsilon$ subunits indicate that, if present, their level of expression is below the sensitivity of this technique.

Most of the screening results were in good agreement with previous *in situ* hybridisation studies (Gambarana *et al.*, 1991; Laurie *et al.* 1992; Poulter *et al.*, 1992, 1993, **Figure 12**). However a few discrepancies were found. In particular, the $\gamma3$ subunit, previously described by Laurie *et al.* (1992) in the neonatal hippocampus was not detected by the screening experiments, and the $\delta$ subunit which was not clearly revealed in previous *in situ* hybridisation experiments was observed here, although at a low level of expression.

**TABLE 10. Distribution of the $GABA_A$ receptor subunits clones from the cDNA library screening.**

| Subunit | Number of clones | % total $GABA_A$ clones | Number of full-length clones |
|---|---|---|---|
| α5 | 53 | 25 | 19 |
| γ2 | 32 | 15 | 17 |
| α2 | 30 | 14 | 18 |
| α4 | 30 | 14 | 4 |
| β2 | 20 | 9.5 | 9 |
| α1 | 14 | 6.5 | 9 |
| γ1 | 11 | 5 | 4 |
| β1 | 9 | 4 | 6 |
| β3 | 7 | 3 | 3 |
| α3 | 5 | 2.5 | 0 |
| δ | 1 | 0.5 | 1 |

**FIGURE 12. Expression of $GABA_A$ receptor subunits in ther neonatal hippocampus revealed by cDNA library screening compared to previous *in situ* hybridisation studies.**

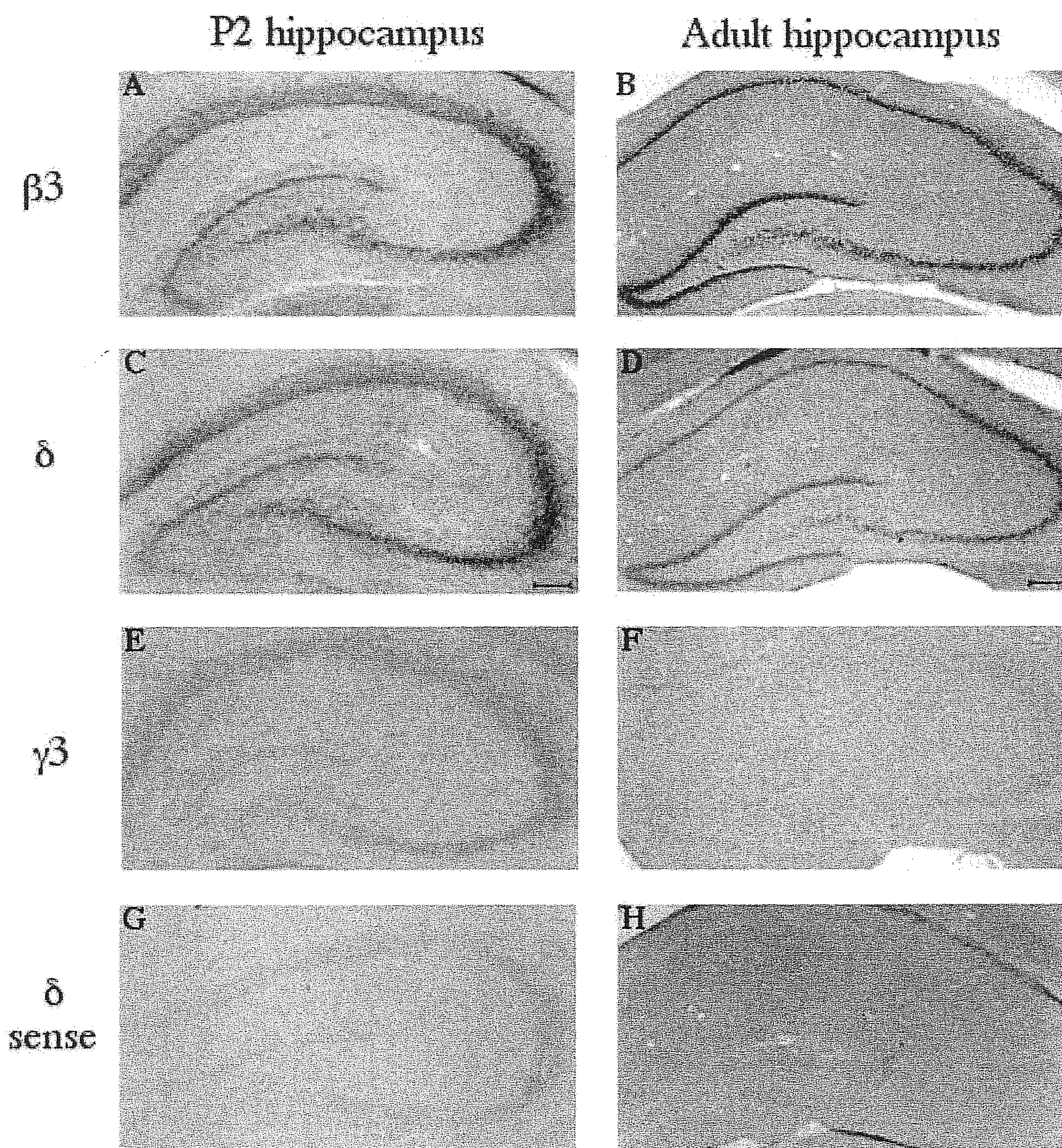



The number of clones found for each subunit is indicated above histogram bars.
+++, strong signal; ++, moderate signal; +, weak signal; +/-, very weak or undetectable signal; nd, non determined.
P0 : *in situ* hybridisation data from Laurie *et al.*,1992.
P5 : *in situ* hybridisation data from Poulter *et al.* 1992 & 1993.

## 4.5 *IN SITU* HYBRIDIZATION

In order to confirm the presence of the δ subunit, *in situ* hybridisation experiments on P2 as well as on adult hippocampal slices were performed, using δ antisense and sense digoxigenin-labeled cRNA probes (in contrast to previously used oligonucleotide probes). As controls, cRNA probes for the β3 subunit, known to be present in both neonatal and adult hippocampus (Laurie *et al.*, 1992; Wisden *et al.*, 1992; Poulter *et al.*, 1993) were utilized. Moreover, since, in contrast to a previous *in situ* hybridisation study (Laurie *et al.*, 1992), the γ3 subunit was not found in the screening experiments, neonatal and adult hippocampal slices were also hybridised with γ3 antisense and sense digoxigenin-labeled cRNA probes. As shown in **Figure 13**, signals obtained on hippocampal slices hybridised with the γ3 antisense probe were extremely weak at P2 and completely negative in adult animals. In contrast to previous observations (Laurie *et al.*, 1992; Poulter *et al.*, 1993), a very clear signal for the δ subunit was consistently found *in* both neonatal and adult hippocampus. This subunit was mainly distributed at the level of the CA1 and CA3 pyramidal layers (**Figure 13**). No signals were detected with the corresponding sense probes in adult tissue, while a light background consistently appeared on P2 slices hybridised with the sense probes. As expected, the expression of the β3 subunit was relatively strong at both ages. Similar results were obtained from three independent experiments. These data further support the findings of the screening experiments that the δ subunit is definitively expressed whereas the γ3 subunit is barely present in the neonatal hippocampus

**FIGURE 13.** *In situ* **hybridisation using digoxigenin-labelled cRNA probes of the β3, γ3 and δ subunits on hippocampal slices.**

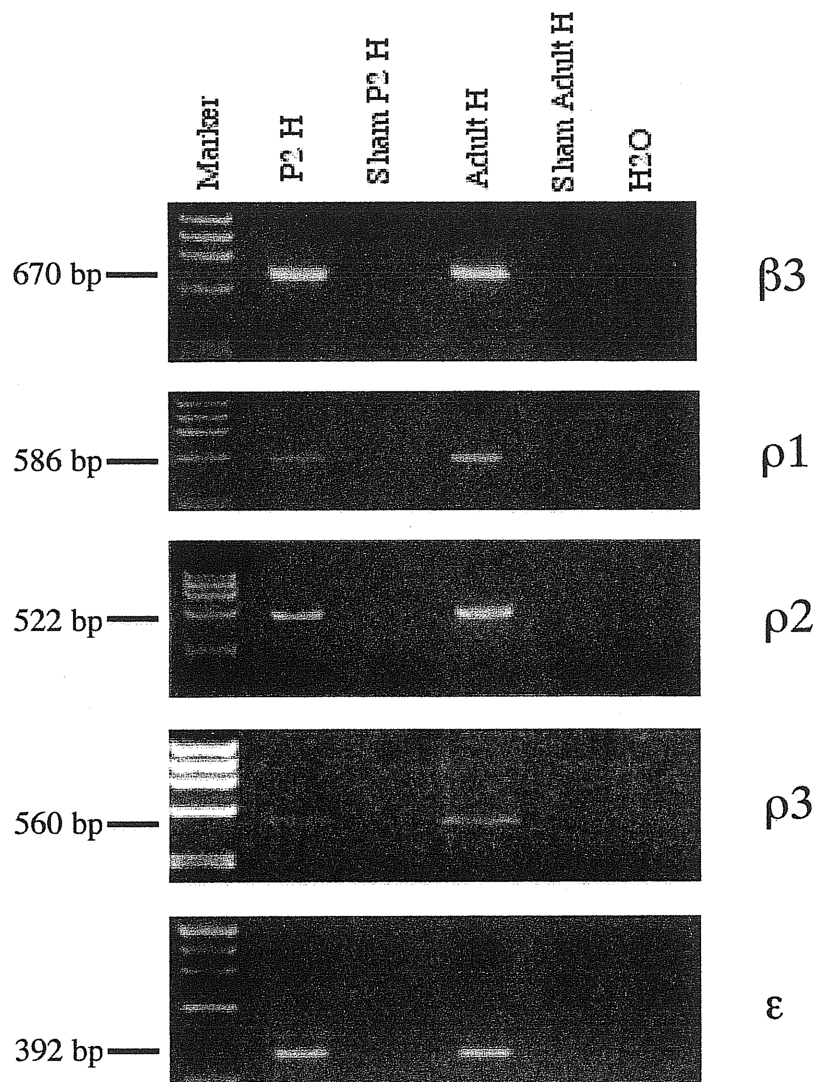



**Panels A and B**:. High expression is shown for the β3 subunit in both P2 and adult hippocampus.
**Panels C and D**: The δ subunit is also clearly present in P2- as well as in adult- hippocampus.
**Panels E and F**: The γ3 subunit shows a signal slightly above background at P2 and is clearly negative in adulthood.
Note that a weak labeling was consistently found for the sense probes on P2 hippocampal slices (**panel G**), while it is absent on adult slices (**panel H**).
P2 slices, scale bar = 125 μm; adult slices, scale bar = 200 μm.

## 4.6 RT-PCR ANALYSIS OF $GABA_A$ RECEPTOR GENE EXPRESSION

The use of the cDNA screening procedure described here allowed a semi-quantitative analysis of $GABA_A$ receptor subunit expression and showed that most of the $GABA_A$ receptor subunits are expressed from birth in the hippocampal formation. However, this strategy did not enable us to clone the ρ subunits or related genes that could be responsible for the unusual pharmacology of GABA receptors observed at early post-natal age. This could be partially due to the fact that the sensitivity of the cDNA library screening method is not as high as the RT-PCR, which has been previously used to show the presence of the ρ subunits in the CNS (Wegelius *et al.*, 1998; Boue-Grabot *et al.*, 1998). In particular, the existence of the ρ subunits in the neonatal or adult hippocampus has been documented (Boue-Grabot *et al.*, 1998; Wegelius *et al.*, 1998; Albrecht *et al.*, 1997), although contrasting results have been reported (see Discussion). On the contrary, no data are available concerning the presence of the ε subunit in the hippocampus during the early postnatal period (Whiting *et al.*, 1997).
Therefore, in order to determine the developmental expression pattern of ρ and ε subunits in the hippocampus, RT-PCR experiments were performed. For this purpose, a set of primers for each individual ρ and ε subunits was designed (see **Table 7**) and their gene expression level was analysed in both P2 and adult hippocampus.
**Figure 14** shows that all three ρ subunits were detected at both ages, although differences in amplification levels were consistently observed. Thus, the ρ2 specific PCR product was always found at higher levels with respect to ρ1 while a specific product for ρ3 transcript was obtained at extremely low levels. Moreover, the ρ1 and ρ2 PCR products appeared to be developmentally regulated since the intensity of the corresponding bands was higher in the adult samples, starting with equal amounts of mRNA in both cases. The ρ3 subunit however was barely detectable at any developmental stage. Although not truly quantitative, these experiments represent a good estimate of the ρ subunits' expression. Furthermore, using specific primers, the ε subunit was detected at similar amplification levels in both neonatal and adult hippocampus. The expression of this subunit was never reported in the neonatal hippocampus, while it was shown by Northern blot analysis and immunohistochemistry to be expressed in the adult hippocampus (Whiting *et al.*, 1997). As controls, β3 subunit transcripts were amplified. Two bands of equal intensity were observed in both neonatal and adult hippocampus. This is in line with the *in situ* hybridisation results, and suggests that the RT-PCR experiments could be considered as semi-quantitative. Sham reactions containing RNA but no cDNA were always negative (**Figure 14**). In order to confirm the identity of RT-PCR products, fingerprinting by appropriate restriction digestion was performed : for ρ1, *Ava* II; for ρ2, *Rsa* I, for ρ3, *Eco*R V. In all cases bands of the expected size were obtained (not shown).

**FIGURE 14. Expression of the ρ1, ρ2, ρ3 and ε subunits in P2 and adult rat hippocampus analysed by RT-PCR.**




Photographs show the products generated by PCR with P2 or adult hippocampus cDNA as template and specific primers. The sizes of the different PCR products are indicated on the left. **Marker**: ϕx174 HaeIII. **P2 H**: P2 hippocampus; **Adult H**: adult hippocampus.

## 4.7 SINGLE-CELL RT-PCR EXPERIMENTS

The different strategies we applied in order to understand the molecular basis of the GABA responses insensitive to bicuculline turned out to be unsuccessful. Although some useful information was obtained from the screening and RT-PCR experiments, these approaches only allow an examination of the expression at the mRNA level of different subunits in the tissue in its whole, but are neither restricted to a specific area nor to a defined cell type. Moreover, they do not give any information regarding the functional role of the subunits detected.

In order to overcome these limitations and try to directly correlate gene expression with receptor function, the single-cell RT-PCR approach was thought to be the appropriate experimental paradigm. This methodology should also help to answer the question: which subunits are responsible for $GABA_A$-ρ-like responses obtained in the hippocampus during an early stage of development (Strata & Cherubini, 1994)?

The bicuculline binding-site was already shown to overlap the binding-site of GABA and Sigel *et al.* (1992) have demonstrated that some mutations in the α subunits affect the binding of both bicuculline and GABA. In addition, the ρ subunits are known to confer bicuculline insensitivity to GABA-mediated responses. Thus, these data led us to concentrate on the expression of the α and ρ subunits in single CA3 hippocampal cells as these subunits may contribute to the bicuculline- and baclofen-insensitive GABA responses.

For that purpose, different sets of primers were used. First, for the α subunits, a set of primers designed by Berger *et al.* (1998) to analyse the expression of the α subunits in hippocampal basket cells was chosen. These primers are degenerate, and therefore are able to amplify in the same way α1, α2, α3, α4 and α5 subunits simultaneously. For the first round the protocol described by Berger *et al.* (1998) was utilised, i.e., the generic α-forward- and α-backward- primers allowing the generation of products for all the α subunits present in the cell analysed. For the second round however, an alternative protocol was chosen. Indeed, Berger *et al.* (1998) used a nested forward primer named α5'n and the same backward primers for the first amplification, namely α3'. After the second PCR amplification using this primer pair, they had to perform Southern-blots with internal oligonucleotide probes specific for each of α1, α2, α3, α4 and α5 to determine the identity of the α subunits present in the cells analysed. Although quite specific, this strategy appeared to be time-consuming and costly in terms of amount of work. It was therefore decided to alternatively use the oligonucleotides specific for the Southern-blot as PCR primers for the second round and therefore save an experimental step. The primers used, namely α1-SB, α2-SB, α3-SB, α4-SB and α5-SB were extensively tested for their ability to work as PCR primers on total cDNA (not shown). These primers were used here as backward

primers, and the forward primer was the α5' primer. The α2-SB, α4-SB, and α5-SB primers were efficient at the annealing temperature of 53°C (as for α3') while the α1-SB and α3-SB had to be used at 50°C as annealing temperature instead. Primers for the analysis of β and γ subunits were not used because these subunits were not thought to be involved in the receptor property we were interested in. In addition, the primers for the β subunits designed by Berger *et al.* were shown to compete for the amplification of other subunits, and therefore were not use to avoid unequal amplification efficiency during the PCR amplification steps.

For the ρ subunits, it was decided to use the same oligonucleotides as for the RT-PCR experiments in the first amplification since these primers were very good at amplifying the individual ρ1, ρ2 and ρ3 subunits. Another reason for this choice is that it was not possible to design nucleotide primers common for the three ρ subunits, in particular because the ρ3 subunit is relatively dissimilar to ρ1 and ρ2 at the nucleotide sequence level and so would have led to primers which were too degenerate. Concerning the second round, three new primers (see section 3.7.7.2) were designed as nested backward primers to give specific products for each of the ρ subunits. These primers were also tested and were used at 55°C with excellent results. With these sets of primers, co-amplification of the α and ρ subunits was done using a multiplex RT-PCR protocol where all primers are mixed in the first round reaction. During the second round, a single reaction was made for a single subunit analysed on an individual cell.

The first experiments carried out were done on harvested single hippocampal CA3 neurons from both P4-P5 and P15 old rats. These cells were not recorded in the presence of GABA, but simply harvested in order to set up the conditions for both harvesting procedure and especially for testing primers and the multiplex PCR protocol.

Although, of course, not particularly informative regarding the correlation between responses to GABA and gene expression, these preliminary experiments allowed us to assess the occurrence and coexistence of the different subunits in isolated cells of the same type. These parameters could be in some respects of functional importance as it will be discussed later. The two different ages were chosen because of the differential physiological properties regarding bicuculline insensitivity in order to try to find significant differences at the level of subunit composition.

The results of such experiments are shown in **Table 11**.

**TABLE 11. Expression of $GABA_A$ receptor subunits transcripts in individual hippocampal CA3 pyramidal cells.**

| | α1 | α2 | α4 | α5 | ρ2 | n° of cells |
|---|---|---|---|---|---|---|
| P4/P5 | 1 (20%) | 5 (100%) | 2 (40%) | 1 (20%) | 1 (20%) | **5** |
| P15 | 1 (10%) | 7 (70%) | 1 (10%) | 4 (40%) | 0 | **10** |

The first striking result was the presence of the ρ2 subunit in one of the five P4/P5 cells tested, and its absence in the more numerous (10) P15 cells. No ρ1 or ρ3 subunits transcripts could be detected, suggesting that they are probably absent or extremely rare in this type of cells. Furthermore, our data demonstrate that the α2 and α5 are the main subunits expressed in the postnatal period, the expression of α4 and α1 being lower. Interestingly, the α2 subunit was detected in all five P4/P5 cells and at a slightly lower frequency in the P15 cells, confirming the prominence of this subunit in the pyramidal cell type. It is also relevant to notice that the α3 subunit was not detected here. However, no striking difference concerning the α subunits distribution was found between the two postnatal ages examined. Another characteristics was that about half of the cells expressed two α subunits simultaneously; in one P4 cell, α2, α4, α5 and the ρ2 subunits were found to be coexpressed. This gives a hint of the complexity of the $GABA_A$ receptors combinations that can be formed in a single cell.

In a second set of experiments, bicuculline-insensitive responses to GABA were correlated with a particular pattern of gene expression. To this aim, CA3 pyramidal cells in P3-P4 hippocampal slices patch-clamped under visual control were identified according to the typical spiking pattern induced by membrane depolarization (under current-clamp conditions). Currents induced by pressure application of GABA (10 μM) were recorded (in voltage-clamp configuration) in the presence and in the absence of bicuculline (100 μM), from a holding potential of -70 mV. Tetrodotoxin (1μM) was routinely applied in the bath to block fast sodium spikes. With this procedure, in the presence of bicuculline, a small current (ranging from 30%, n=3, to 5%, n=3 of the initial response to GABA recorded in the absence of bicuculline) was observed in 6 (out of 19) neurons. This current was shown in a previous report (Martina et al., 1995) to be completely blocked by picrotoxin (100 μM). In several cells (n = 5), 1,2,5,6-tetrahydropyridine-4-yl methylphosphinic acid or TPMPA (10 μM), a selective antagonist of $GABA_A$-ρ receptors (Ragozzino *et al.*, 1996), was tested. Unlike the retinal receptors, this compound had no effect on the kinetics and amplitude of the residual bicuculline-resistant currents to GABA. It should be stressed that, in contrast to previous findings, bicuculline-insensitive responses were found only in a small percentage of neurons tested (30 %). This discrepancy may be

explained at least in part by the different preparation used (acutely dissociated neurons in previous experiments and thin hippocampal slices in the present ones) or by the different concentration of bicuculline applied. In previous work on the thick hippocampal slice preparation (Strata & Cherubini, 1994), conventional intracellular recordings in current clamp configuration, have revealed residual responses to GABA in the presence of 50μM bicuculline in almost every cell tested. It is therefore posssible that with this bicuculline concentration $GABA_A$-mediated responses are not completely blocked. The conditions we used here could not be identical to the ones of Strata & Cherubini (1994) because we had to fit to the requirements for the harvesting procedure, i.e., record the cells in the whole-cell configuration, being in a radically different configuration with respect to the intracellular recordings conditions. Following electrophysiological characterisation, the cytoplasm of the cells was harvested and RT-PCR was performed on the content of these cells.

A total of 19 cells were recorded and submitted to RT-PCR analysis. All the cells were harvested, reverse transcribed, and two rounds of PCR amplification as described above were made using all the primers for the α and ρ subunits. After agarose gel analysis, only 11 (out of 19) cells displayed a PCR product. The 8 negative cells were therefore not taken into account. Among these, two cells exhibited bicuculline-resistant responses whose current amplitude was 28 and 31% of the initial responses observed in the absence of bicuculline, respectively. The failure to detect any transcript in about half of the harvested cells can be explained by the numerous factors influencing this experiment. Almost every step contains a risk for the success of the experiment, from the recording step which must not be too long (to avoid mRNA degradation, to the harvesting procedure, the reverse-transcription (which must be very efficient in generating long cDNAs) as well as the two successive PCR amplification steps that have to be extremely efficient.
The results of these experiments are shown in **Table 12** and **Figure 15.**

**<u>TABLE 12.</u> $GABA_A$ receptor subunits transcripts detected in single CA3 hippocampal cells from P3-P4 rats recorded in the presence of GABA and bicuculline.**

| Cell n° | 1 | 2 | 3 | 6 | 7 | 10 | 11 | 14 | 17 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Subunits | α2<br>α5 | α2<br>ρ2 | ρ1 | α5 | α2<br>α5 | α2 | α2 | ρ2 | α2 | α2 | α2<br>α5 |
| Residual current in bicuculine (% of initial current) | 0 | 27 | 3.5 | 0.5 | 0 | 1.2 | 0 | 0 | 0 | 0 | 2 |

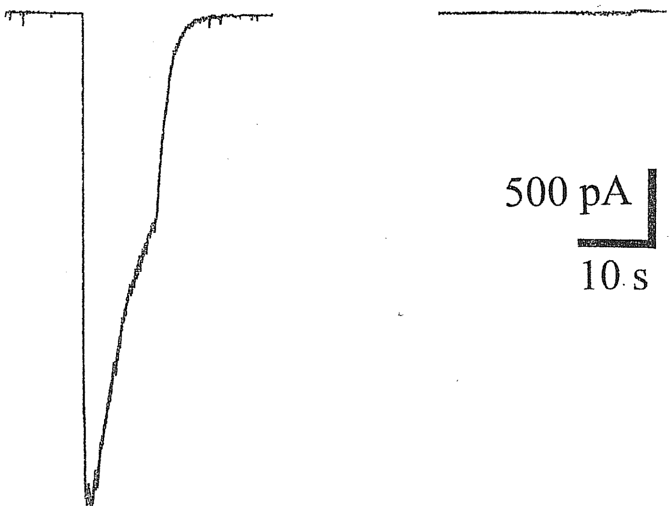
**FIGURE 15.** **$GABA_A$ receptor α and ρ subunits gene expression in single hippocampal CA3 neurons from P3-P4 rats showing bicuculline- sensitive and resistant responses to GABA.**

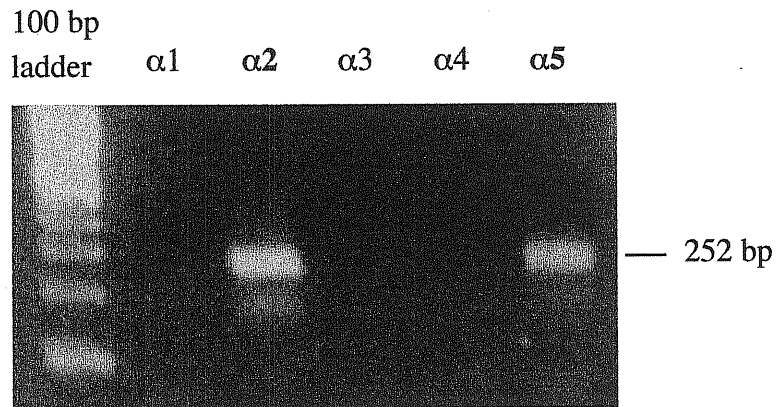
**A**. Cell 7: On the left part, current amplitude of the responses to GABA in the absence and presence of bicuculline. On the right part, agarose gel showing the presence of the α2 and α5 subunits in this cell.

**B**. Cell 2: On the left part, same as in A. On the right part, agarose gel showing the presence of α2 and ρ2 subunits in this cell.

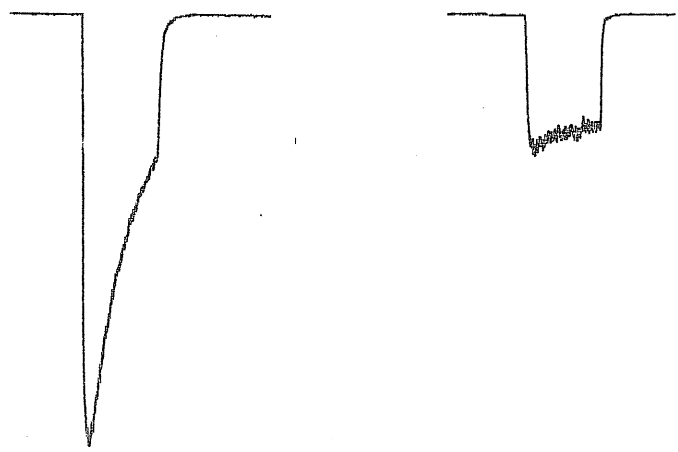
**FIGURE 15.**

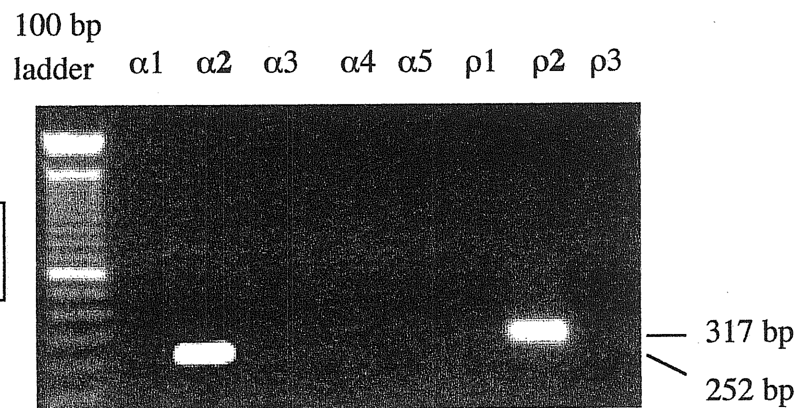
A




B



The single-cell RT-PCR perfomed on the 11 cells resulted primarily in the expression of the $\alpha2$ subunit in most of the cells (8/11), as suggested by preliminary experiments. Four cells exhibited the presence of the $\alpha5$ subunit.
More interestingly, cell n°2 which bears a very significant bicuculline-resistant response (27% of total GABA response) showed the expression the $\rho2$ subunit, together with the $\alpha2$ subunit. In addition, the $\rho1$ subunit was found in cell n° 3 which also had a bicuculline resistant component, although of very low amplitude. The $\rho2$ subunit was also detected in the cell n° 14, but this cell was completely blocked by bicuculline, no other subunits being detected in that case. The definite identity of the $\rho$ subunits in these cells was subsequently confirmed by Southern blot (see Materials & Methods) as shown in **Figure 16**.

**FIGURE 16. Expression of the $\rho1$ and $\rho2$ subunits in single hippocampal CA3 neurons from P3-P4.**

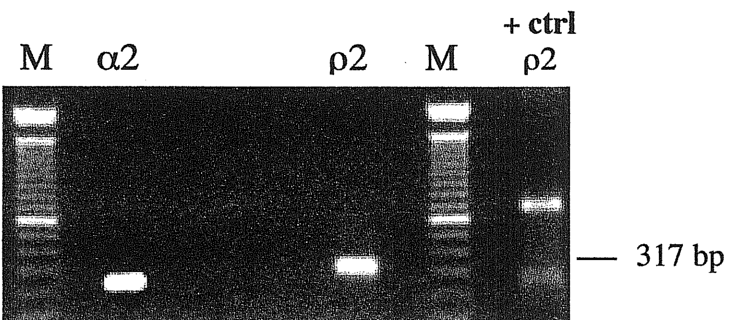
**A.** Agarose gel showing the expresion of the $\alpha2$ and $\rho2$ subunits in the cell number 2.
A southern blot analysis for the $\rho2$ subunits is also shown, together with a restriction fragment of $\rho2$ cDNA as a positive control (+ ctrl $\rho2$).

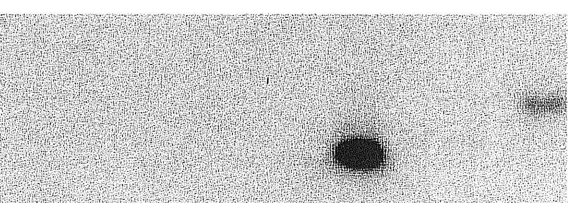
**B.** Agarose gel showing the expression of the $\rho1$ subunit in the cell number 3.
As in A., a southern blot analysis is presented for the $\rho1$ subunit, as well as a restriction fragment of $\rho1$ cDNA as a positive control (+ ctrl $\rho1$).

**FIGURE 16.**

**A.**

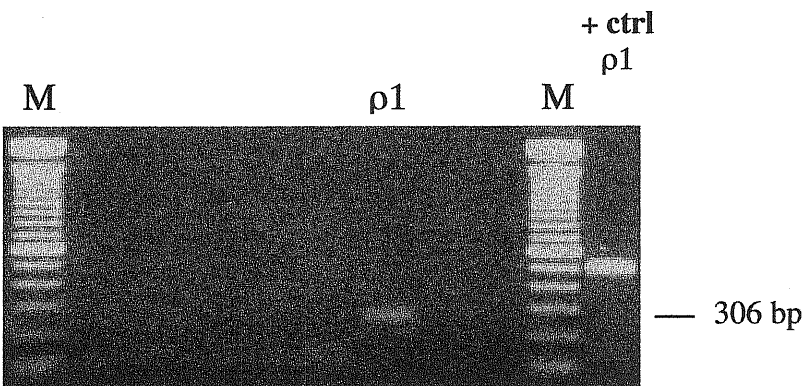
Cell n°2

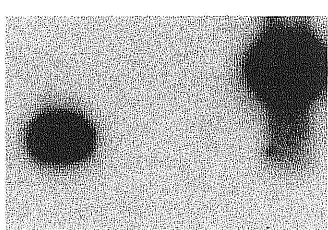


**B.**

Cell n°3



These data tentatively suggest or at least do not exclude a participation of the ρ subunits in the bicuculline-resistant responses identified in electrophysiological experiments. More precisely the association of ρ2 with an α subunit (here α2) could be at the origin of the phenomenon. The ρ2 subunit alone (as in the example of cell 14) may not be able to form functional receptor as suggested by Zhang *et al.* (1995), but may be able to assemble with α subunits. On the contrary, the ρ1 subunit alone was shown to form robust homomeric channels (Hackam *et al.*, 1997a & 1997b), and could well form bicuculline-resistant channels on its own in these cells. Other "classical" $GABA_A$ receptors formed by the α/β/γ, δ, ε subunits very lilkely represent the bicuculline-sensitive component of the response to GABA.
Other α subunits different from α2 and α5 were not identified in any of the cells tested. It is possible that these subunits, as predicted by preliminary results are less abundant in this particular cell type. A technical limitation however of the results shown here is that consistently the positive amplification of any of the ρ subunits was coupled to a less efficient detection of the α subunits. Indeed, of the three cells bearing a ρ subunit, only one showed the α2 subunit, but the corresponding band on the agarose gel was weak (not shown). This is likely due to the much higher $T_m$ of the oligonucleotides for the ρ subunits than for the α, therefore leading to much more stable primer-target association and subsequent more efficient PCR amplifications. Thus, it is likely that other α subunits coexist in these cells because classical GABA responses abolished by bicuculline are present and are therefore necessarily composed of at least one subtype of α subunit.
Unfortunately, the number of cells expressing the ρ subunits and the ones bearing a bicuculline resistant component are relatively rare and therefore do not facilitate the task of looking for such cells, the number of experiments to be done being very high. Indeed, more data would be needed to support the idea that indeed the ρ subunits are at the origin of the unusual GABA pharmacology in the hippocampus at an early developmental stage.

# 5. DISCUSSION

## 5.1 GABA$_A$ RECEPTOR SUBUNITS MEDIATING BICUCULLINE-INSENSITIVE RESPONSES IN THE HIPPOCAMPUS OF NEONATAL RATS

As already mentioned in the introduction, striking differences exist in GABA$_A$ receptor function between the neonatal and adult CNS. In adults, GABA acts as the main inhibitory neurotransmitter, while in neonates GABA acts as an excitatory neurotransmitter and neurotrophic factor. In the adult CNS, GABA inhibits neuronal firing by increasing a chloride conductance. This hyperpolarizes the membrane, by virtue of the higher extracellular chloride concentration, and effectively raises the threshold for action potential , thus making their generation more difficult. In the neonatal brain (until P8-P12) GABA depolarises and excites neuronal membranes by a reverse chloride gradient. Several hypotheses have been put forward to explain this change: i. a delayed maturation of a cation-chloride cotransporter which in adult neurones contributes to maintain intracellular chloride concentration below equilibrium (Misgeld *et al.*, 1986); ii. the presence of an inwardly directed bicarbonate current which would lead to membrane depolarisation even in the presence of an outwardly directed chloride current (Staley *et al.*, 1995). In this case, the reversal potential for GABA-induced current would shift from chloride equilibrium potential ($E_{Cl-}$) towards the more positive bicarbonate equilibrium potential ($E_{HCO3-}$) (Kaila *et al.*, 1993); iii. low expression or regulation of a particular type of chloride channel with properties similar to the ubiquitous distributed ClC-2 (Staley, 1994). This channel, originally cloned from the rat heart and brain (Thiemann *et al.*, 1992) is widely expressed in neuronal and non-neuronal tissue and is activated by cell swelling, acidic extracellular pH and hyperpolarisation (Jordt & Jentsch, 1997). At negative potentials this channel strongly rectifies in the inward direction leading to chloride efflux, thus preventing intracellular chloride accumulation. The recent finding that the ontogenic change in GABA-mediated responses from depolarising to hyperpolarising is coupled to the developmental induction of the neuronal chloride extruding $K^+$-$Cl^-$ cotransporter KCC2 (Rivera *et al.*, 1999) strongly supports the view that this cotransporter is involved in chloride homeostasis. In addition, our findings showing that the ClC-2 channel is poorly expressed during development (Mladinic *et al.*, 1999) suggest that also this channel plays a crucial role in maintaining a low intracellular chloride concentration in adulthood. While the chloride extrusion system would fulfill the "gradient-priming"

role necessary to maintain a low intracellular chloride concentration to allow chloride efflux through ligand-gated chloride channels (Payne, 1997), passive conductance systems such as the ClC-2 channels would assist in chloride homeostasis.
Furthermore, a novel chloride-mediated GABA response, bicuculline and baclofen insensitive, similar to that reported in the retina as $GABA_C$ or $GABA_A$-ρ, has been found in neonatal hippocampal neurons (Strata & Cherubini, 1994). This novel $GABA_A$-ρ-like response is detectable only during the first two weeks of postnatal development (P0-P12), a period critical for stabilization and formation of synapses. Although the functional significance of this response is not clear yet, it is possible that because of its slow desensitization and fast recovery from desensitization, GABA will prolong membrane depolarisation favouring calcium entry through voltage activated channels. This calcium signal may be essential for synaptogenesis during this early period of postnatal development.

This novel type of GABA receptor may be due to the existence of:

1) new types of $GABA_A$ receptor subunits (possibly of the ρ type)
2) existence of modified versions of already cloned $GABA_A$ receptor subunits (alternative splicing, RNA editing, post-translational modifications)
3) unexpected combination of already known $GABA_A$ receptor subunits, not co-existing in other situations.

Major changes in receptor distribution and subunit mRNA expression patterns occur during development (see **Figure 3**). It is known that numerous $GABA_A$ receptor subunits are expressed before birth in a region and age specific manner. During postnatal development, there is a switch in the subunit composition of $GABA_A$ receptors, suggesting the existence of molecularly distinct immature and adult forms (Fritschy *et al.*, 1994; Laurie *et al.*, 1992). As discussed later, the cloning of the $GABA_A$ receptor subunits from the neonatal hippocampus confirms this view.
However, the existence of a receptor responsible for the novel $GABA_A$-ρ-like response in the CA3 neonatal hippocampal neurons cannot be explained by the existence of a simple combination of α/β/γ, δ, ε subunits. No combination tested in expression studies has been found to mimic the new response observed. Therefore, on the basis of the similarities described above, initial efforts were focussed on the search of a ρ $GABA_A$ receptor subunit type in the neonatal rat brain, assuming that this ρ GABA receptor subunit could either be of a known type (ρ1, ρ2) or more likely, a new member of the ρ family.

## 5.1.1 ATTEMPTS TO CLONE A NEW $GABA_A$ RECEPTOR SUBUNIT GENE FROM NEONATAL HIPPOCAMPUS

The first step in this project was to confirm the presence or absence of the $GABA_A$ receptor ρ subunits in the rat brain, especially in neonates by *in situ* hybridization. All three probes used (ρ1, ρ2 and ρ1+ρ2) gave high hybridization signals in *in situ* hybridization on rat retinal neonatal sections. On brain sections however, only the ρ1+ρ2 probe gave strong signals in the neonates and in particular in the hippocampus. The negative result obtained with the ρ2 probe was understood later following an annalysis of the sequence of this probe derived from human and found to differ notably from the rat sequence. This however is in contrast with the positive signal obtained in *in situ* hybridization experiments on the retina. It could be that this probe recognized the ρ2 gene, even with many mismatches, or more likely that the ρ2 probe hybridized to an unrelated sequence from another gene, similarly to the ρ1+ρ2 probe (see section 4.3.1).
Attempts to clone the gene responsible for the specific pattern obtained in *in situ* hybridization with the ρ1+ρ2 probe by RACE cloning was unsuccessful, leading only to aspecific results. This was due to a methodological matter, since the short stretch of sequence used (ρ1+ρ2 oligo) was not necessarily identical to the gene of interest and was therefore probably inadequate using this PCR-based approach. In particular, the RACE cloning strategy may not have functioned because the homology between probe and gene sequence was not preserved at the sequence ends.
The construction of the cDNA library represented a way to overcome the problems of the RACE technique, and was also thought to be a very good tool for cloning the ρ subunits or related genes. In fact, this approach has been used in the past decade to identify and clone most of the ligand-gated channel subunit genes such as those of acetylcholine, glycine and the $GABA_A$ receptors. As shown in section 4.3.1, a novel $EFI_A$/dbpB/YB-1-like gene was cloned from the neonatal hippocampal cDNA library using the ρ1+ρ2 probe. This was the gene that gave positive signals in *in situ* hybridization experiments using the ρ1+ρ2 probe. Indeed, using this cDNA as a probe, the pattern of expression corresponded to that of the ρ1+ρ2 probe. This result was disappointing since we expected to clone a new ρ-like $GABA_A$ receptor subunit. 24 nucleotides out of 45 of the ρ1+ρ2 oligonucleotide sequence were homologous to the novel gene sequence showing the care that needs to be taken with *in situ* hybridization experiments.
The (partially sequenced) novel gene shares more than 96% sequence homology with the $EFI_A$/dbpB/YB-1 gene, and can therefore be considered to be a related gene. As stressed before, this is a group of DNA-binding proteins that probably act as transcription factors. It is very difficult to speculate about a possible function of the novel protein, but since its expression pattern overlaps with the appearance of the

$GABA_A$-ρ-like response from the neonatal hippocampus, there is a possibility that this gene is involved in the control of the expression of certain GABA receptor genes. It would be interesting to test this hypothesis by foot-printing analysis or analysis of the effects of (over)expression of this gene on $GABA_A$ receptor subunit expression in a suitable model system.
From this screening experiment, one can conclude that if the technique used allowed the cloning of a gene which shared such a low degree of homology (about 50%) with the probe used, then it would probably have allowed the cloning of the rat ρ $GABA_A$ receptor genes, which have sequence homology of >80% with the probe used (89% for ρ1 and 80% for ρ2) (see **Figure 6**). This represented a negative indication regarding the presence of the ρ subunits or homologues in the neonatal hippocampus, or at least in the cDNA library constructed.
The use of a single oligonucleotide was probably not the ideal approach to clone a putative new gene from which no stretch of sequence is known. Indeed, the homology between the target gene and the probe could be low in the region from which the oligonucleotide was designed, even if both sequences came from two genes of the same family. This potential problem was the motivation for changing probe and for using a full-length cDNA probe instead. This probe was also from human, but it was reasoned that inter-species homologies are extremely high, usually more than 90%, therefore do not represent a problem for cloning related genes from another species. In that particular case, between the rat and human ρ1 subunits genes, the homology is 88%. The screening with that probe led to the isolation of 19 clones. However, after partial sequencing, none of them were related to $GABA_A$ receptor subunit genes, but only constitutively expressed genes such as histones and tubulin.

Altogether the negative results from these attempts to clone a new $GABA_A$ receptor subunit gene suggest that such a new gene related to the ρ subunits do not exist in the hippocampus of neonatal rats. Likewise the ρ1, ρ2 and ρ3 subunits were therefore concluded to be probably absent from the developing hippocampus, or expressed at levels lower than the technique used is able to detect. However, these experiments do not strictly exclude the possibility that the ρ subunits or related gene(s) are present in the neonatal hippocampus. It could well be that this method was not suitable for cloning them. In fact, since the library was amplified once, less abundant clones could have been lost because the preferential amplification of some clones can not be controlled. This last point therefore prevented us from drawing any definitive conclusion about the presence of the ρ subunits in the hippocampus. This issue was therefore addressed with the RT-PCR experiments that will be discussed later.

## 5.1.2 $GABA_A$ RECEPTOR SUBUNITS GENES CLONED FROM THE DEVELOPING RAT HIPPOCAMPUS

The previous results were in contrast with the idea of the presence of $GABA_A$ receptor ρ subunits genes in the neonatal rat hippocampus. However, the idea of the presence of a completely new GABA receptor subunit (not exclusively of a ρ type), or partially transformed version of already cloned $GABA_A$ receptor subunits in the neonatal rat hippocampal formation during the critical period of the development, became more likely and represented a more general working frame. For this reason, all $GABA_A$ receptor subunits present in the neonatal hippocampus were cloned using oligonucleotide probes derived from the M2 domain of all rat $GABA_A$ receptor subunits. This part of these polypeptides is the most conserved region among the different $GABA_A$ receptor subunits and is generally accepted as lining the channel pore and therefore has a crucial importance for receptor function. This is confirmed by its high evolutionary conservation between subunits from very different species and its homology with the related region of the glycine receptors. It is very difficult to imagine a new $GABA_A$ receptor subunit which would be devoid of this region, or which would have a very different sequence in this region. The logic in the design of the oligonucleotide probes was explained earlier (see section 4.4)

The results of the screening are shown in **Table 10**. The number of clones encoding each single subunit gives some indications about the level of expression of the corresponding subunit. However, these numbers cannot be taken as an absolute indicator of subunit expression, as subunits were cloned using different oligonucleotide probes, with different percentages of degeneracy and GC content. So, hybridization conditions will also affect the cloning efficiency of each subunit. Furthermore, the cDNA library used for cloning the subunits was amplified once and there is a possibility that all genes were not amplified similarly. However, if the levels of expression are subdivided into four different groups (high, intermediate, low and no expression), the different subunits can be divided into the following categories:

1) high expression: α5 (the highest of all subunits), γ2, α2 and α4
2) intermediate expression: β2, α1, γ1, β1 and β3
3) low expression: α3, δ
4) no expression: α6, γ3, ρ1, ρ2, ρ3 and ε.

In general, these results are consistent with previous *in situ* hybridisation studies (see **Figure 12**; Gambarana *et al.*, 1991; Laurie *et al.*, 1992; Poulter *et al.*, 1992, 1993) with a couple of notable exceptions. The δ subunit which has been previously described as being virtually absent in the neonatal hippocampus (but present in the caudate putamen) was found, both in the library screening approach and using cRNA-based *in situ* hybridisation. In contrast, the γ3 subunit which was previously described

as being present (with oligonucleotide-mediated *in situ* hybridisation) was not found by the cDNA screening strategy and *in situ* hybridisation showed that this subunit is expressed at very low level in P2- but not in adult- hippocampus.

The experiments described here confirm the high diversity of $GABA_A$ receptor subunits expressed in the neonatal hippocampus. They also strongly suggest that native $GABA_A$ receptors would be mainly formed by combinations of α5, α2, α4, β2 and γ2. In keeping with this, single cell mRNA amplification experiments (Brooks-Kayal *et al.*, 1998) have shown that the majority of neurons express at least five subunits simultaneously. The predominance of α5 and α2 subunits, known to confer a high sensitivity to GABA (Levitan *et al.*, 1988, Malherbe *et al.*, 1990) would increase the efficacy of this neurotransmitter during a particular period of postnatal life, and this may contribute to the trophic action exerted by GABA during development (Cherubini *et al.*, 1991).

In addition, the hippocampal formation is remarkable in that subunits otherwise expressed in adult brain are also found in perinatal hippocampus. For example, the α1 and α4 subunits which are characteristic of the adult brain, can be found at intermediate (α1) and even high (α4) levels in the neonatal hippocampus.

The abundance of the δ and the scarcity of the γ3 subunit are likely to have functional significance. Recombinant studies have shown that the δ subunit contributes to the slow deactivation and desensitisation kinetics of $GABA_A$ currents (Haas & MacDonald, 1999), a feature characteristic of neonatal brain (Hollrigel & Soltesz, 1997). It is possible, for example, that the δ subunit assembles with the α4 subunit since their respective pattern of expression revealed by immunocytochemistry are overlapping in the adult hippocampus (Sperk *et al.*, 1997). Receptor combinations comprising these two subunits should therefore be extensively tested in recombinant system to assess their pharmacological and physiological properties.

The respective expression levels of the γ3 and δ subunits could partially explain the lack of responsiveness of neonatal hippocampal principal cells to benzodiazepines (Knoflach *et al.*, 1991; Rovira *et al.*, 1991) and their high sensitivity to zinc (Martina *et al.*, 1996; Krishek *et al.*, 1998). This also appears to be in contradiction with the strong neonatal expression of the γ2 subunit, known to be involved in both benzodiazepine potentiation and zinc insensitivity (Pritchett *et al.*, 1989; Krishek *et al.*, 1998). Immunocytochemical experiments showed, however, that the expression of the γ2 subunit in principal cells in the CA1-CA3 regions is relatively weak, while it is very abundant in interneurons and *stratum radiatum* and *stratum oriens*. Thus, this raises the possibility that the δ subunit is already present in the hippocampal principal cells from birth and competes with the γ subunits to participate in the formation of benzodiazepine-insensitive receptors. This idea however should be confirmed in immunocytochemical experiments using δ-subunit specific antibodies that are currently being developed in our laboratory.

Unusual subunit combinations could be the basis for the novel $GABA_A$ receptor type present during the critical period of postnatal development. However, as mentioned above, no combination of $GABA_A$ receptor subunits in *in vitro* expression studies has replicated the $GABA_A$-ρ-like physiological findings. Sequencing of the subunits which have been isolated from the neonatal hippocampus cDNA library could have revealed particular isoforms susceptible to generating functionally distinct receptors. Alternative splicing is very common among $GABA_A$ receptor subunit genes (see section 1.3.1.3) and it would not be surprising to find developmentally regulated novel splicing forms which account for the $GABA_A$-ρ-like response. The screening experiments allowed us to isolate two alternative spliced isoforms of the γ2 subunit, but these novel forms turned out to produce prematurely truncated proteins and were therefore not functional. In addition, further analysis of the clones isolated from the library by sequencing or PCR with primers encompassing start and stop codons did not show any other isoforms of $GABA_A$ receptor subunits. The coding region of all subunits clones was amplified with specific primers, but the length of the products obtained was always identical to the expected length of the published coding regions. This strongly suggests that the set of subunits and their isoforms is limited and that probably the bicuculline-insensitive responses do not originate from new hypothetical isoforms.

However, we can not exclude the possibility that RNA edited forms of the $GABA_A$ receptor subunits exist, like those for glutamate receptors (Scott, 1995). The pre-mRNA of the B subunit of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor (GluR-B) is edited from a glutamine (CAG) to an arginine (CGG) codon. This so-called Q/R site editing occurs in a channel-forming domain and markedly reduces the calcium permeability of the channel. A second RNA editing affects the kinetic properties of the AMPA receptors. In GluR-B, GluR-C, and GluR-D subunits, intron elements determine an arginine (AGA) to glycine (GGA) (R/G site editing) (Lomeli *et al.*, 1994). Edited channels recover much faster from desensitization and may integrate all incoming signals better. As mentioned above, the novel $GABA_A$-ρ-like response from the neonatal hippocampus is reminiscent of that obtained in *Xenopus* oocytes expressing an α1 subunit in which position 64 was occupied by Leu instead of Phe (Sigel *et al.*, 1992), a subunit cloned from a 7-day old rat brain cDNA library. A change of T to C at position 306 was the reason for the Phe64Leu mutation. As other α1 cDNAs coming from 2 days old, 7 days old and adult animals did not have this mutation, the authors suggested that the α1Phe64Leu subunit cDNA probably stems from mutated neonatal brains. Since U to C editing of RNA expressed in the nucleus has been described (apolipoprotein B mRNA in mammals, for example) it is possible that similar processes occur with the $GABA_A$ receptor α subunit mRNAs. In that respect, the sequences of some of the α subunits clones were checked by sequencing. The sequences obtained were never

found to carry such base changes, even if some of the clones were shown to bear conservative mutations, likely to be due to the reverse-transcription process known to be of low fidelity. However, as the number of clones to sequence was too high, the hypothesis of an edited form expressed in the postnatal hippocampus, although unlikely, cannot be definitively excluded.

On the basis of the results described above, hypotheses about the presence of partially transformed versions of GABA receptor subunits in the neonatal rat brain (alternatively spliced or RNA edited) and/or new $GABA_A$ receptor subunits appeared to be very unlikely. With the exception of the two non-functional spliced forms of the γ2 subunit, all subunit sequences were the same as those found in the adult brain. However, one further possibility to explain a distinct receptor function is the presence of new, unexpected combinations of $GABA_A$ receptor subunits in the neonatal hippocampus. If we accept the possibility of unusual subunit combinations, this must arise from the co-existence of subunits that would form very distinct $GABA_A$ receptors and otherwise never co-exist in the adult brain. For instance, the α5 subunit being very abundant in the neonatal hippocampus could be combined with other α subunits (e.g. α4) to form uncommon receptors, even if this remains to be verified. In addition, it was stressed earlier that different subunits are targeted to different regions of the cell, and that co-expression of subunits re-routes some subunits to different parts of the cell (it seems that β subunits have a role in controlling the subcellular distribution of other $GABA_A$ receptor subunits (Connolly *et al.*, 1996b). In that respect, it is possible that $GABA_A$ receptors appearing in the synaptic locations of CA3 pyramidal hippocampal cells have very unusual subunit combinations, which never occur in other neurons in the period after P8-P12. *In vitro* expression studies of different subunit combinations has never detected bicuculline-insensitive GABA response, except those including ρ subunits, but the further use of recombinant systems to test more subunits combinations could help in predicting the subunits that could assemble to form the native receptors.

## 5.2 EXPRESSION OF THE ρ SUBUNITS IN THE NEONATAL HIPPOCAMPUS AND IN THE BRAIN

The attempts to clone $GABA_A$ receptor subunits which could give rise to the unusual neonatal GABA pharmacology in the hippocampus from the rat neonatal hippocampal cDNA library using the ρ1+ρ2 oligonucleotide, the full-length ρ1 cDNA probe and oligonucleotide probes derived from transmembrane region two (TM2) of the known subunits, turned out to be unsuccessful. Indeed, no new gene or functionally distinct isoforms were found and the candidate ρ subunits were not isolated. These negative results were not in favour of the existence of such genes, and the presence of the $GABA_A$ receptor ρ subunits in the rat neonatal hippocampus remained an open question. Indeed, it could be that the expression levels of the ρ subunits are below the limits of detection of our screening procedure, a method known to be much less sensitive than RT-PCR in detecting relatively rare transcripts. This observation led us to change strategy, using the RT-PCR technique to address this question and try to assess the potential contribution of the ρ subunits to the diversity of $GABA_A$ receptor subunit expression in the hippocampus.

Our results from these RT-PCR experiments showed that all three ρ subunits are expressed in the hippocampus of neonatal as well as adult animals. Nevertheless, the ρ2 subunit PCR product was consistently found to be the most abundant. The ρ1 subunit showed a moderate expression as judged by the lesser intensity of the PCR products, whereas ρ3 was barely detectable at any of the neonatal and adult stages. In addition, ρ1 and ρ2 subunits appeared to be developmentally regulated, being more expressed in adulthood (see **Figure 14**). However, these data are not truly quantitative as PCR methodology can lead to unequal amplification of target mRNAs, even if the starting conditions are identical. In addition, the number of cycles applied (35) is quite high and therefore could tend to abolish or exacerbate small differences in mRNA levels. Nevertheless, because the data were reproduced several times on different batches of RNA, some confidence can be granted to these results. Moreover, the findings in RT-PCR experiments are in general agreement with the data from the literature. Most of the reports about expression of the $GABA_A$ receptor ρ subunits refer to their prominent retinal expression. However, the retinal expression is not absolute and several lines of evidence demonstrate the definitive presence of the ρ subunits in the CNS.

First of all, Cutting *et al.* (1991) cloned a ρ subunit (human ρ1 subunit) starting from the T84 cells (human colon tumor cell line). The Northern blot analysis (performed on RNA from different bovine tissues) revealed high expression of the cloned subunit in the bovine retina and much lower expression in the bovine cerebellum. Low intensity of hybridization was found also in the lung and thymus tissue as well as in

the RNA from T84 cells. These results, as well as the results of Enz *et al.* (1995) using the RT-PCR technique (this will be discussed later) were for a long time the only direct proof of the presence of ρ subunits in the CNS. However, these Northern blot results can be interpreted in line with the observations of Ogorusu *et al.* (1995) who cloned the rat ρ2 subunit and performed Northern blot analysis to reveal the tissue distribution of this subunit, using two different probes. With the first cDNA probe, corresponding to transmembrane regions two to three (M2-M3 domains), they obtained hybridization signals corresponding to the mRNA of 3.9kb in rat cerebellum, retina and spinal cord and two minor bands corresponding to mRNA of about 8 kb and 2.3 kb in retina. However, with another, more specific, cDNA probe corresponding to 36 amino acid residues at the amino terminus of the mature subunit where the lowest degree of amino acid similarity between ρ1 and ρ2 subunits occurs, only the hybridization band of an mRNA of 2.3 kb was detected in the retina, with no signals found in RNA prepared from cerebral cortex, cerebellum and spinal cord. These results indicate that the expression of the ρ2 subunit is prominent in the retina and that the signals obtained in other regions may be due to the non-specific hybridization which occurs when probes derived from well conserved regions (conserved even among other related genes from the same gene superfamily) are used.
The second direct proof for the existence of the ρ subunits, more precisely of the ρ2 subunit, in the rat CNS came from the experiments of Enz *et al.* (1995) that used the RT-PCR technique to analyse the expression of the ρ2 mRNAs in the rat CNS. These authors have been able to detect ρ2 mRNAs in the retina but also in almost all brain regions, with the highest level of expression in the hippocampus and cortex. The same authors, however, have never been able to detect $GABA_A$ receptor ρ subunit mRNAs in the CNS using *in situ* hybridization experiments. Enz *et al.* (1995) explained this discrepancy by the different levels of sensitivity of the techniques used, so that the rare mRNAs detected by the PCR technique can not be detected by *in situ* hybridization techniques. However, the finding that bicuculline-resistant responses were recorded in almost all neurons tested between P0 and P10 (Strata and Cherubini, 1994) indicates that transcripts for the ρ subunits, if responsible for these responses, should not be rare (but see also single-cell RT-PCR experiments). Furthermore, polyclonal antibodies against the N-terminus of the rat ρ1 subunit which recognised ρ1, ρ2 and ρ3 subunits were generated (Enz *et al.*, 1996) and used to study the distribution of ρ1, ρ2 and ρ3 $GABA_A$ receptor subunits. Even though strong in the retina, the immunocytochemical signals have never been detected in the CNS using this antiserum. To explain the discrepancy between the immunocytochemical and the RT-PCR signals, one can speculate that the existing mRNA transcripts are, for some reason, not translated into functional protein (but this leaves the question of the origin the $GABA_A$-ρ–like responses in the CNS

unanswered) or one can doubt the specificity of the PCR results. It is well known that the extreme sensitivity of PCR can lead to the introduction of false positives derived from inadvertently introduced templates.

More recently, a few reports shed more light on the expression of the ρ subunits in the CNS and allow a more detailed map of the regions in which they are expressed. However, some of these recent data are contradictory. Thus, in agreement with our data, ρ1 and ρ2 subunits have been detected in the neonatal and adult rat hippocampus using RT-PCR (with increased expression levels of ρ2 compared to ρ1) by Boue-Grabot *et al.* (1998), while a higher expression of ρ1 in neonates with respect to the adults has been observed by Wegelius *et al.* (1998) with the same methodology. It has also been recently reported that ρ2 is expressed in several regions of the human brain, while ρ1 was found at much lower levels (Enz *et al.*, 1999). In addition, high levels of ρ3 in the adult but not in the neonatal hippocampus were reported by Wegelius *et al.* (1998). These data are however in contrast with those of Boue-Grabot et al. (1998) and with our results that have shown that this subunit is minimally expressed in the hippocampus.

*In situ* hybridisation was also used to analyse the level of expression and the distribution of the transcripts for the ρ subunits. Several groups were not able to detect them in the hippocampus (Enz *et al.*, 1995; Albrecht *et al.*, 1997; Boue-Grabot *et al.*, 1998) while the ρ2 subunit has been found in the superior colliculus, and the ρ1 subunit was detected in the Purkinje cells of the cerebellum (Boue-Grabot *et al.*, 1998). Nevertheless, Wegelius *et al.* (1998) detected the ρ2 subunit in the adult hippocampus. On the whole these discrepancies indicate that the level of expression of the ρ2 subunit is probably relatively low and could be at the limit of detection by the *in situ* hybridisation technique.

Altogether, these data represent clear indications that indeed the ρ subunits are present in the CNS, and also outside regions involved in the visual pathway like the hippocampus. Our RT-PCR results are also in line with other published reports, and confirm that the ρ2 subunit is likely to be the most represented among the ρ subunits in the CNS and in the hippocampus.

These observations however do not allow a positive conclusion about a potential contribution for the ρ subunits to the baclofen- and bicuculline-insensitive responses to GABA, found in the hippocampus of neonatal rats (Strata & Cherubini, 1994; Martina *et al.*, 1995). Indeed, it should be stressed that the ρ subnits are present at a low level of expression, whereas the bicuculline-insensitive responses to GABA were found in almost all neurones tested. In addition, these peculiar responses disappeared in adulthood when the levels of expression of ρ1 and ρ2 subunits were found to be increased. Other experimental approaches should be employed to clarify this point; in that sense the single-cell RT-PCR represented a powerful alternative and gave a refined but not unequivocal idea about the role of the ρ subunits in the hippocampus.

Lastly, the recently cloned ε subunit was detected at similar levels in both neonatal and adult hippocampus. This subunit, whose role in native receptors is still unclear, when co-expressed in recombinant systems with α and β subunits forms functional channels which would exhibit rapid desensitisation kinetics and would be insensitive to benzodiazepines (Whiting *et al.*, 1997; Thompson *et al.*, 1998). Immunocytochemical experiments revealed that this subunit is expressed in the hilus as well as in the CA3 region of the adult hippocampus (Whiting *et al.*, 1997). These data could represent a first indication that this subunit is involved in the bicuculline-resistant resistant responses observed in the neonatal hippcampus. Thus, it would be of great interest to better characterize this subunit functionally and determine its subregional and temporal distribution in the developing hippocampus by *in situ* hybridisation and immunocytochemical techniques.

## 5.3 FUNCTIONAL ROLE OF THE ρ SUBUNITS IN THE NEONATAL HIPPOCAMPUS

Although the RT-PCR experiments have demonstrated the presence of ρ subunits in the rat hippocampus, this method however does not allow correlation between their expression and their functional role. The results obtained with single-cell RT-PCR represent the first step for understanding the function of these subunits in the hippocampus. In preliminary experiments not correlated with the electrophysiological ones, the observation that the ρ2 subunit is present in P4-P5 pyramidal cells of the CA3 region represents *per se* a novel finding. Indeed, only few studies have analysed $GABA_A$ receptor subunits expression in the CNS using single-cell RT-PCR technique (Berger *et al.* 1998; Ruano *et al*, 1997) and no one has addressed the question of the presence of ρ subunits.

Subsequently, ρ subunits (ρ1 and ρ2) were found also in pyramidal cells exhibiting bicuculline-insensitive responses to GABA. Since it is known that rat ρ2 subunits are not able to assemble into functional homomeric receptors (Zhang *et al.* 1995) it is possible that these subunits form functional receptors with other known $GABA_A$ receptor subunits. In line with this hypothesis is the observation that TPMPA, a specific antagonist of GABA ρ receptors (Ragozzino *et al.*, 1996) produced no effect on the residual current evoked by GABA in the presence of bicuculline. It should be stressed however that in one cell, in which GABA response was fully blocked by bicuculline, the ρ2 subunit was detected. This apparent discrepancy could be explained by the lack of a putative accessory $GABA_A$ receptor subunit necessary for the assembly of a functional receptor with mixed $GABA_A$ and $GABA_A$-ρ-like properties. In contrast, the ρ1 subunit alone was shown to form robust homomeric channels (Hackam *et al.* 1997b) and therefore could be by itself responsible for the

observed bicuculline-insensitive response. It should be emphasized that in all other cells, in which GABA responses were blocked by bicuculline, no ρ subunits were detected further stressing the possible contribution of the ρ subunits in the bicuculline-insensitive GABA responses.

In contrast with previous data (Strata & Cherubini, 1994; Martina *et al.* 1995), in the present electrophysiological experiments, only a small fraction of cells (15-20%) exhibited bicuculline insensitive responses to GABA. This contradiction is likely to be due to the different experimental conditions used. Firstly, 100 μM bicuculline instead of 50 μM were used. Secondly, GABA currents were recorded from thin (250 μm instead of 600 μm) hippocampal slices using the patch clamp technique in voltage clamp configuration (instead of conventional intracellular recordings in current clamp configuration). The conditions presently used, namely the reduced thickness of the slice and the higher concentration of bicuculline, could allow a better access of the drug to the cell membrane leading to a more efficient block of $GABA_A$ receptors by bicuculline.

Single cell RT-PCR experiments confirmed the data obtained by the cDNA library screening. Indeed, as in the screening experiments, we found that most hippocampal CA3 neurons express α2, α5 and α4 subunits. In some cells also the α1 subunit was detected while the α3 subunit was never observed. This is also consistent with *in situ* hybridization (Gambarana *et al.*, 1991; Laurie *et al.*, 1993; Poulter *et al.*, 1992) and immunocytochemical studies (Fritschy *et al.*, 1994). In particular, the α2 subunit has been demonstrated to be the main α subtype in the perinatal brain, gradually decreasing with age, while the expression of the α1 subunit increases notably until maturity. It is thought that the α2 (as well as α5) subunit confers a greater sensitivity to GABA and a lower desensitization (Levitan *et al.*, 1988, Malherbe *et al.*, 1990) to promote the neurotrophic effect of GABA released from the growth cones of immature neurons (Cherubini *et al.*, 1991).

Another interesting piece of information we can draw from the single cell RT-PCR data is that the concomitant expression of many subunits of the α type can occur in a single cell. This observation is an indication of the complexity of the $GABA_A$ receptors, not only at the level of the tissue, but more significantly at the single cell level where several different receptors could exist and have different roles in mediating the effects of GABA. The single-cell RT-PCR approach represents a first step in determination of the exact nature of native receptors, but does not give information at the protein level. In particular this method does not provide any data about the colocalization of the subunits as well as the exact subunit composition of the native receptors in the neonatal hippocampus. Experiments of co-immunofluorescence, immunoprecipitation and immunopurification (see section 1.3.3.2) could help to better define the assembly of $GABA_A$ receptor subunits in the neonatal hippocampus.

# 6. CONCLUSIONS AND PERSPECTIVES

The work described in this thesis is an attempt to understand the molecular origin of bicuculline-resistant responses to GABA found in the hippocampus during development. This research brought to light the ρ subunits as probable players in this phenomenon. Additional experiments however are now needed to definitively conclude on the exact identity of the $GABA_A$ receptor subunits involved. First, immunocytochemitry would be very helpful in determining whether the expression of the ρ subunits found by both RT-PCR and single-cell RT-PCR holds true at the protein level. This would represent a strong indication about their possible functional role. This could be achieved thanks to the development of highly specific antibodies against ρ1 and ρ2 in particular by the phage-display system or by more classical methods. In parallel to that, single-cell RT-PCR experiments could be pursued in order to increase the statistical significance of the results described in this thesis and to corroborate or negate the hypothesis according to which the ρ subunits are likely to be associated with the bicuculline-baclofen insensitive responses observed. In that respect, recombinant receptors could then help to reconstitute the native responses, and assess whether the ρ subunits are able to coassemble with other subunits.

A detailed pharmacological and kinetic characterization of the response to GABA in the neonatal rat CA3 pyramidal cells will also help to better understand the action of GABA during development. Finally, the search for functional $GABA_A$-ρ like receptors could be extended also to the adult hippocampus where the ρ subunits have been found.

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