EXPRESSION OF FUNCTIONAL $GABA_C$ RECEPTORS IN THE BRAIN

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Abstract

γ-aminobutyric acid (GABA) is the main inhibitory transmitter in the nervous system and acts via three distinct receptor classes: A, B, and C. $GABA_C$ receptors are ionotropic receptors comprising ρ subunits. In this work, we aimed to elucidate the expression of ρ subunits in the postnatal brain, the characteristics of ρ_2 homo-oligomeric receptors, and the function of $GABA_C$ receptors in the hippocampus.

In situ hybridization on rat brain slices showed ρ_2 mRNA expression from the newborn in the superficial grey layer of the superior colliculus, from the first postnatal week in the hippocampal CA1 region and the pretectal nucleus of the optic tract, and in the adult dorsal lateral geniculate nucleus. Quantitative RT-PCR revealed expression of all three ρ subunits in the hippocampus and superior colliculus from the first postnatal day. In the hippocampus, ρ_2 mRNA expression clearly dominated over ρ_1 and ρ_3. $GABA_C$ receptor protein expression was confirmed in the adult hippocampus, superior colliculus, and dorsal lateral geniculate nucleus by immunohistochemistry. From the selective distribution of ρ subunits, $GABA_C$ receptors may be hypothesized to be specifically involved in aspects of visual image motion processing in the rat brain.

Although previous data had indicated a much higher expression level for ρ_2 subunit transcripts than for ρ_1 or ρ_3 in the brain, previous work done on *Xenopus* oocytes had suggested that rat ρ_2 subunits do not form functional homo-oligomeric $GABA_C$ receptors but need ρ_1 or ρ_3 subunits to form hetero-oligomers. Our results demonstrated, for the first time, that HEK 293 cells transfected with ρ_2 cDNA displayed currents in whole-cell patch-clamp recordings. Homomeric rat ρ_2 receptors had a decreased sensitivity to, but a high affinity for picrotoxin and a marked sensitivity to the $GABA_C$ receptor agonist CACA. Our results suggest that ρ_2 subunits may contribute to brain function, also in areas not expressing other ρ subunits.

Using extracellular electrophysiological recordings, we aimed to study the effects of the $GABA_C$ receptor agonists and antagonists on responses of the hippocampal neurons to electrical stimulation. Activation of $GABA_C$ receptors with CACA suppressed postsynaptic excitability and the $GABA_C$ receptor antagonist TPMPA inhibited the effects of CACA. Next, we aimed to display the activation of the $GABA_C$ receptors by synaptically released GABA using intracellular recordings. GABA-mediated long-lasting depolarizing responses evoked by high-frequency stimulation were prolonged by TPMPA. For weaker stimulation, the effect of TPMPA was enhanced after GABA uptake was inhibited. Our data demonstrate that $GABA_C$ receptors can be activated by endogenous synaptic transmitter release following strong stimulation or under conditions of reduced GABA uptake. The lack of $GABA_C$ receptor activation by less intensive stimulation under control conditions suggests that these receptors are extrasynaptic and activated via spillover of synaptically released GABA.

Taken together with the restricted expression pattern of $GABA_C$ receptors in the brain and their distinctive pharmacological and biophysical properties, our findings supporting extrasynaptic localization of these receptors raise interesting possibilities for novel pharmacological therapies in the treatment of, for example, epilepsy and sleep disorders.

Original Publications

This thesis is based on the following publications, referred to in the text by their Roman numerals.

I Alakuijala, A., Palgi, M., Wegelius, K., Schmidt, M., Enz, R., Paulin, L., Saarma, M., Pasternack, M. GABA receptor ρ subunit expression in the developing rat brain. *Brain Res. Dev. Brain Res.*, 154:15–23, 2005.

II Alakuijala, A., TalviOja, K., Pasternack, A. and Pasternack, M. Functional characterization of rat ρ_2 subunits expressed in HEK 293 cells. *Eur. J. Neurosci.*, 21:692–700, 2005.

III Alakuijala, A., Alakuijala, J. and Pasternack, M. Evidence for a functional role of $GABA_C$ receptors in the rat mature hippocampus. *Eur. J. Neurosci.*, 23:514–520, 2006.

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Abbreviations

The long pharmacological names of the compounds mentioned in the text are given only in this list, while all other abbreviations are explained the first time they appear.

2-MeTACA	2-*trans*-4-amino-2-methylbut-2-enoic acid
3-APA	3-aminopropylphosphinic acid
3-APMPA	3-aminopropyl(methyl)-phosphinic acid
3-APS	3-aminopropanesulphonic acid
5α-THDOC	allotetrahydrodedoxycorticosterone
5β-THDOC	tetrahydrodedoxycorticosterone
5-HT_3	5-hydroxytryptamine (or serotonin) receptor type 3
ACSF	artificial cerebrospinal fluid
AP5	D-2-amino-5-phosphonopentoate
ATP	adenosine trisphosphate
BIM	bicuculline methiodide
CA1, CA3, CA4	*cornu ammonis* 1, 3, and 4
CACA	*cis*-4-amino crotonic acid
(±)-CAMP	(±)-*cis*-2-(aminomethyl)cyclopropanecarboxylic acid
cDNA	complementary deoxyribonucleic acid
CGP 36742	(3-aminopropyl-*n*-butyl)phosphinic acid
CGP 46381	(3-aminopropyl)(cyclohexylmethyl)phosphinic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
dLGN	dorsal lateral geniculate nucleus
DRG	dorsal root ganglia
DVN	dorsal vagal nucleus
EC_{50}	half-maximal effective agonist concentration
E_{Cl}	reversal potential for chloride
EEG	electroencephalography
EMG	electromyography
ERG	electroretinogram
GABA	γ-aminobutyric acid
GAT-1, GAT-3	GABA transporter-1 and -3
GDNSP	GABA-mediated depolarizing non-synaptic potential
GDPSP	GABA-mediated depolarizing postsynaptic potential

GLYT-1E/F	glycine transporter (C-terminal) splice variant
HEK 293	human embryonic kidney cell line
HFS	high-frequency stimulation
hIPSP	hyperpolarizing inhibitory postsynaptic potential
I4AA	imidazole-4-acetic acid
IC_{50}	half-maximal effective antagonist concentration
INL	inner nuclear layer
K_b	estimated binding constant of the antagonist
KCC2	potassium-chloride co-transporter
LTP	long-term potentiation
MAP1B	microtubule-associated protein 1B
mRNA	messenger ribonucleic acid
MTN	median terminal nucleus of the accessory optic tract
NKCC1	sodium-potassium-chloride co-transporter
NOT	pretectal nucleus of the optic tract
P	postnatal day
P4MPA	(piperidine-4-yl)methylphosphinic acid
P-4-S	piperidine-4-sulphonic acid
pEPSP	population excitatory postsynaptic potential
PiTX	picrotoxin
PKC	protein kinase C
pSpike	population spike
qRT-PCR	quantitative reverse-transcriptase polymerase chain reaction
REM	rapid eye movement
SGL	superficial grey layer
SKF 89976A	N-(4,4,-diphenyl-3-butenyl)-3-piperidinecarboxylic acid
SR95531	2-(3-carboxypropyl)-3-amino-6-(*p*-methoxyphenyl)pyridazinium bromide or gabazine
STC-1	gut neuroendocrine tumour cell line
SuC	superior colliculus
TACA	*trans*-4-amino crotonic acid
TAMP	*trans*-2-(aminomethyl)cyclopropanecarboxylic acid
TBPS	*t*-butylbicyclophosphorothionate
THIP	4,5,6,7,-tetrahydroisoxazolo{5,4-*c*}pyridin-3-ol or gaboxadol
TPMPA	(1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid

1. Introduction

γ-aminobutyric acid (GABA) is the main inhibitory transmitter in the nervous system. GABA acts via three distinct receptor classes: A, B, and C. $GABA_A$ and $GABA_C$ receptors are ionotropic receptors, i.e. they are ligand-gated ion channels (Fig. 1). The vast group of ligand-gated ion channels is divided into three superfamilies: the nicotinicoid superfamily, the excitatory glutamate receptors, and ATP receptors. The nicotinicoid superfamily encompasses several families of receptors, including nicotinic acetylcholine receptors, 5-HT_3 receptors, $GABA_A$ receptors, $GABA_C$ receptors, strychnine-sensitive glycine receptors, and some invertebrate anionic glutamate receptors. All members of the superfamily are considered to be pentamers.

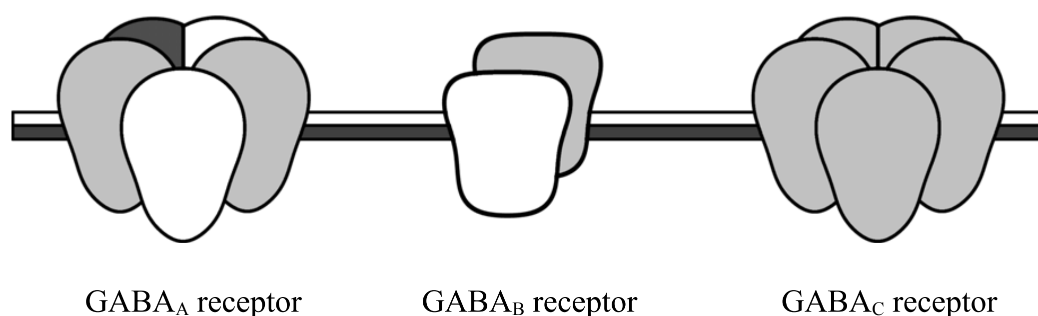


Fig. 1. GABA receptor classes.
$GABA_A$ receptors are heteropentameric ionotropic receptors, $GABA_B$ receptors are heterodimeric metabotropic receptors coupled to second messenger systems and $GABA_C$ receptors are homo- or heteropentameric ionotropic receptors.

Each $GABA_A$ or $GABA_C$ receptor subunit consists of four transmembrane domains, a long N-terminal extracellular domain with a cysteine loop, and an intracellular loop between the third and fourth transmembrane domains. The second transmembrane part lines the integral anion channel and has the highest proportion of conserved amino acids of all ionotropic GABA receptors. Binding sites for GABA and other ligands as well as for antagonists, picrotoxin block, and oligomerization signals have all been connected to particular areas of interest on the structure of the receptor, either in the transmembrane region or in the N-terminal domain (reviewed by Chebib and Johnston, 2000).

$GABA_B$ receptors are metabotropic, i.e. they act via a G-protein-coupled second messenger system, and their activation regulates inwardly rectifying potassium channels on the cell membrane. $GABA_B$ receptors can also function as presynaptic autoreceptors, reducing calcium current, and subsequently, transmitter release at the nerve terminal. They are slower than ionotropic receptors and usually modulate the response of the neuron rather than participating in the fast messaging between pre- and postsynaptic neurons, which is classically taken as the task for fast-acting ionotropic receptors. $GABA_B$ receptors are considered to be heterodimers consisting of $GABA_{B1}$ and $GABA_{B2}$ subunits (reviewed by Cryan and Kaupmann, 2005).

$GABA_C$ receptors are pharmacologically defined by their insensitivity to $GABA_A$ receptor antagonist bicuculline and to $GABA_B$ receptor agonist baclofen. The first evidence for the existence of $GABA_C$ receptors came in 1975 from cat spinal cord

interneurons, where bicuculline-insensitive, CACA-sensitive responses were discovered (Johnston *et al.*, 1975). In 1984, CACA-sensitive responses were shown to be baclofen-insensitive in the rat cerebellum (Drew *et al.*, 1984). Gradually, these and other findings led to the idea of a third subclass of GABA receptors, termed $GABA_C$ receptors (Drew *et al.*, 1984). As the first subunit of these new receptors was cloned from a retinal cDNA library, they were named ρ after retina (Cutting *et al.*, 1991). The nomenclature is still under debate, however, and will be further discussed at the end of this thesis.

More than a decade of intensive studies concerning ρ subunits and $GABA_C$ receptors had revealed a great deal of information, especially on their molecular structure, but less on their function and very little on their role outside the retina and superior colliculus. The aim of this study was to elucidate the expression and characteristics of functional $GABA_C$ receptors in the brain both during development and with particular emphasis on the adult hippocampus, the centre for learning and memory, with established neuronal networks.

2. Review of the Literature

2.1. $GABA_A$ receptors

The classical picture of $GABA_A$ receptors is of postsynaptic receptors rapidly activating, deactivating, and desensitizing. Prototypic $GABA_A$ receptors are known to be inhibited competitively by bicuculline and non-competitively by picrotoxin and potentiated by benzodiazepines, barbiturates, ethanol, and neuroactive steroids (reviewed by MacDonald and Olsen, 1994). Still, in different brain cells and regions, there is a vast variety of different $GABA_A$ receptor subtypes with distinct characteristics depending on the subunits involved. Johnston (1996a) has described $GABA_A$ receptors as the most complicated of the superfamily of ligand-gated ion channels in terms of the large number of receptor subtypes and the variety of ligands that interact with specific sites on the receptors.

2.1.1. $GABA_A$ receptor subunits

To date, 16 $GABA_A$ receptor subunit genes have been characterized: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, and π (Barnard *et al.*, 1998; Bonnert *et al.*, 1999; Sinkkonen *et al.*, 2000). The variety is further extended by several splice variants, among which the short and long isoforms of γ_2 are perhaps the most studied (Whiting *et al.*, 1990). Yet, the two most abundant $GABA_A$ receptor subtypes in the adult brain are supposed to consist of either two α_1, two β_2, and one γ_2 subunit, or one α_2, one α_3, two β_3, and one γ_2 subunit (McKernan and Whiting, 1996). Although many $GABA_A$ receptor subunits can inefficiently assemble to form homo-oligomeric receptors, when transfected alone in heterologous expression systems, these receptors have small currents and distorted pharmacology, and native $GABA_A$ receptors seem to always need α and β subunits for functionality. Extensive studies with recombinant receptors and genetically engineered mice have shed some light on the role of various subunits (reviewed by Korpi *et al.*, 2002 and Vicini and Ortinski, 2004).

Of the six distinct α subunits, α_1, α_2, and α_3 are thought to be localized at synapses, while α_4, α_5, and α_6 dominate the sites outside the synapse and have a higher sensitivity to GABA than their synaptic counterparts. α_1 and α_4 subunits produce fast current decay, α_2 and α_3 prolong current deactivation (i.e. returning to the baseline current level after an agonist has left the receptor), α_5 subunit currents desensitize less, while α_6 subunit currents deactivate very slowly and lack desensitization (i.e. they stay open even with a very long agonist application). Naturally, all of these properties are also strongly affected by the presence of γ or δ subunits. Extrasynaptic receptors will be discussed in more detail in Section 2.1.2. α_1 subunit-containing receptors characteristically possess high affinity to benzodiazepines, but these drugs seem to have subtype-specific effects on other α subunits as well. Receptors with various α subunits have been differentially connected to memory, sedation, anxiety, and cortical plasticity (reviewed by Rudolph and Möhler, 2006).

Of the three β subunits, β_3 (preferentially assembled with $\alpha_{2/3}$) seems to be crucial in the developing brain, and β_3 knock-out mice have a short life span and severe loss of GABA receptors together with functional deficits, resembling some features of

Angelman's syndrome, a human neurodevelopmental disorder. β subunits are involved in the action of anaesthetics, β_2 subunit-containing receptors mediating sedation and β_3 receptors anaesthetic immobility in mouse models (Siegwart *et al.*, 2003; Zeller *et al.*, 2007).

γ subunits, on the other hand, carry the signals for targeting and clustering $GABA_A$ receptors at synapses as well as for forming the binding site for benzodiazepines, mediating their allosteric action on the adjacent GABA binding sites, and thus, generating benzodiazepine sensitivity. While mice lacking the γ_2 subunit die young, heterozygous γ_2 mice have reduced $GABA_A$ receptor clustering, benzodiazepine binding, and an anxious phenotype. The δ subunit is abundantly expressed outside synaptic clefts and will be discussed further below. To date, not much is known about the role of ε, θ, and π subunits, with the last subunit perhaps only expressed in peripheral tissues (reviewed by Korpi *et al.*, 2002 and Vicini and Ortinski, 2004).

2.1.2. Extrasynaptic $GABA_A$ receptors

The classical type of GABAergic transmission appears in a way that, following depolarization of the presynaptic terminal, GABA is briefly released from transmitter vesicles and binds to postsynaptic $GABA_A$ receptors. Intriguingly, quantitative studies have revealed that, even in the case of a highly synapse-enriched $GABA_A$ receptor subtype, more receptors are found outside than inside synaptic junctions (Nusser *et al.*, 1995). No $GABA_A$ receptor subtype has yet been found to have an exclusive synaptic location, but δ subunit-containing receptors were shown to be present only at extra- and perisynaptic locations (Nusser *et al.*, 1998; Wei *et al.*, 2003). The δ subunit seems to form these purely extrasynaptic receptors specifically with the α_6 subunit in cerebellar granule cells and with the α_4 subunit in the dentate gyrus, thalamus, and neostriatum (Barnard *et al.*, 1998). Recently, this specific subunit partnership has been challenged by results showing the δ subunit together with the α_1 subunit at least in hippocampal interneurons, in which these subunits seem to produce extrasynaptic receptors (Glykys *et al.*, 2007). Another known, predominantly extrasynaptic $GABA_A$ receptor subtype, present in hippocampal pyramidal cells and interneurons, is formed by α_5 and γ_2 subunits, despite the latter commonly promoting synaptic localization (Crestani *et al.*, 2002; Semyanov *et al.*, 2003).

$GABA_A$ receptors containing α_4 or α_6 subunits have an EC_{50} for GABA of around 0.1 – 0.7 μM, which is an order of magnitude lower than the EC_{50} of the most abundant, "classical" $GABA_A$ receptor subtypes and even a bit lower than that of $GABA_C$ receptors (Farrant and Nusser, 2005). However, the apparent affinities of native $GABA_A$ receptors remain unknown, since in certain experimental situations, GABA sensitivities are much greater than those found in recombinant experiments (Lindquist and Birnir, 2006). Yet, GABA has a low efficacy for δ subunit-containing receptors, i.e. it gates these ion channels less efficiently, leading to slow and minimal desensitization (Bianchi and Macdonald, 2003). Extrasynaptic receptors are insensitive to zolpidem, and all but $\alpha_5\gamma_2$ receptors also to diazepam, but sensitive to some inhalation anaesthetics (Barnard *et al.*, 1998). In addition, the δ subunit is speculated to carry high affinity for ethanol and neurosteroids (Sundstrom-Poromaa *et al.*, 2002; Bianchi and Macdonald, 2003; Stell *et al.*, 2003). Moreover, α_6 subunit-containing receptors are specifically antagonized by

furosemide (Korpi *et al.*, 1995), while THIP is a superagonist on α_4 subunit-containing receptors (Krogsgaard-Larsen *et al.*, 2004).

The properties of extrasynaptic receptors are perfectly suited to their function in tonic inhibition. As opposed to fast-acting, phasic inhibition mediated by synaptic receptors, extrasynaptic receptors are activated on slower time scales by the spillover of the transmitter from synapses on the neighbouring cells or by the ambient extracellular GABA concentration maintained by GABA transporters and non-synaptic release from glia (Rossi *et al.*, 2003). In addition to constant currents, transmitter spillage to extrasynaptic areas can also underlie slow-rising GABAergic inhibitory postsynaptic currents. The single-channel conductance of $GABA_A$ receptors mediating a tonic current in hippocampal neurons was estimated to be 6 pS, which is significantly lower than that of hippocampal $GABA_A$ receptors mediating phasic inhibition (Bai *et al.*, 2001). To date, tonic currents mediated by extrasynaptic $GABA_A$ receptors have been found in cerebellar granule cells (Hamann *et al.*, 2002), hippocampal interneurons and pyramidal cells (Semyanov *et al.*, 2003; Yeung *et al.*, 2003; Caraiscos *et al.*, 2004), dentate gyrus granule cells (Nusser and Mody, 2002; Wei *et al.*, 2003), and thalamic relay neurons (Porcello *et al.*, 2003).

The role of the tonic inhibition is not yet thoroughly established, but several theories have been tendered. In the cerebellum, tonic inhibition alters the gain of transmission of the network, potentially improving the information storage capacity (Hamann *et al.*, 2002). In hippocampal interneurons, tonically active GABA receptors regulate the excitability, and thus, the extent of network oscillations, which are crucial for distributed signal processing (Semyanov *et al.*, 2003; 2004). In the hippocampus, adult pyramidal cells, in contrast to interneurons, possess apparent tonic currents only if GABA uptake is blocked. This is in line with the contribution of GABA uptake to the extrasynaptic transmitter concentrations being more important than diffusion, as in the *stratum pyramidale* there are abundant synapses releasing transmitters and a limited volume of extracellular space, whereas in the *strata radiatum* and *oriens* the extracellular volume is relatively large and more prone to diffusion (Semyanov *et al.*, 2004). It has been demonstrated that, where available, tonic inhibition carries larger overall inhibitory charge transfer than phasic inhibition, even with frequent synaptic events (reviewed by Farrant and Nusser, 2005).

2.2. $GABA_C$ receptors

2.2.1. Biophysical properties

$GABA_C$ receptors are about 10-fold more sensitive to GABA than classical $GABA_A$ receptors and their Hill slopes are steeper, reflecting the presence of more ligand binding sites on $GABA_C$ receptors and more cooperative binding. $GABA_C$ receptor currents are smaller and they activate and deactivate more slowly. $GABA_C$ receptors do not desensitize even with long agonist applications. They are permeable to chloride and bicarbonate, similar to $GABA_A$ receptors, and their pore size is approximately the same (Feigenspan *et*

al., 1993; Kusama *et al.*, 1993a; Qian and Dowling, 1993; Feigenspan and Bormann, 1994; Wang *et al.*, 1994; Johnston, 1996b).

At the single-channel level, $GABA_C$ receptors are characterized by 6-fold longer mean open times than $GABA_A$ receptors (Feigenspan *et al.*, 1993). The single-channel conductance of native $GABA_C$ receptors in rat retinal bipolar cells was 7 pS, whereas that of $GABA_A$ receptors in the same area was 27 pS (Feigenspan *et al.*, 1993; Feigenspan and Bormann, 1994). In goldfish retinal bipolar cells, the smaller conductance of $GABA_C$ receptors compared with $GABA_A$ receptors appeared to be compensated by a greater number of activated receptors per synapse (Palmer, 2006). The single-channel conductance of native $GABA_C$ receptors in white perch retina was only 1.36 pS, which lies between the values derived for homomeric ρ_1 and ρ_2 receptors (Zhu *et al.*, 2007).

2.2.2. Pharmacology

$GABA_C$ receptors are defined by their insensitivity to $GABA_A$ receptor antagonist bicuculline and to $GABA_B$ receptor agonist baclofen. The main pharmacological characteristics of GABA receptor classes are summarized in Table 1 (reviewed by Johnston, 1996b and Bormann, 2000).

Table 1. Essential pharmacology of GABA receptor classes.

Agonist/ antagonist	**$GABA_A$ receptors**	**$GABA_B$ receptors**	**$GABA_C$ receptors**
GABA	EC_{50} = 0.1 – 30 μM	EC_{50} = 1.6 – 2.4 μM[1]	EC_{50} = 0.8 – 7 μM
Muscimol	Potent agonist	Inactive	Partial agonist
TACA	Potent agonist	Inactive	Potent agonist
CACA	Inactive	Inactive	Partial agonist
Baclofen	Inactive	Agonist	Inactive
Phaclofen	Inactive	Competitive antagonist	Inactive
Saclofen	Inactive	Competitive antagonist	Inactive
Bicuculline	Competitive antagonist	Inactive	Inactive
Picrotoxin	Non-competitive antagonist	Inactive	Competitive antagonist
TPMPA	Weak antagonist	Weak agonist	Potent antagonist

[1] Affinity of $GABA_B$ receptors for GABA is high only in the presence of calcium (Sodickson and Bean, 1996; Galvez *et al.*, 2000)

2.2.2.1. Agonists

As a simple rule, GABA in a partially folded conformation and also partially folded GABA analogues can activate $GABA_C$ receptors, while the fully extended configuration of GABA or its analogues is needed for $GABA_A$ receptor activation (Fig. 2). Besides GABA, the most potent $GABA_C$ receptor agonists are TACA and muscimol. These two compounds can exist in both conformations, thus being effective $GABA_A$ receptor agonists as well.

The partially folded *cis*-isomer, CACA, is more selective and widely used as a $GABA_C$ receptor agonist, but it is only a partial agonist, showing 70–80% of the efficacy of GABA (Kusama *et al.*, 1993a; Woodward *et al.*, 1993). Unfortunately, higher concentrations of CACA have been demonstrated to activate also $GABA_A$ receptors at rat retinal bipolar cell terminals (≥ 500 µM; Pan and Lipton, 1995) or cerebellar granule cells (≥ 50 µM; Wall, 2001). Moreover, CACA is a weak substrate for the GAT-3 transporter, and it also stimulates the passive release of GABA and β-alanine (Chebib and Johnston, 1997).

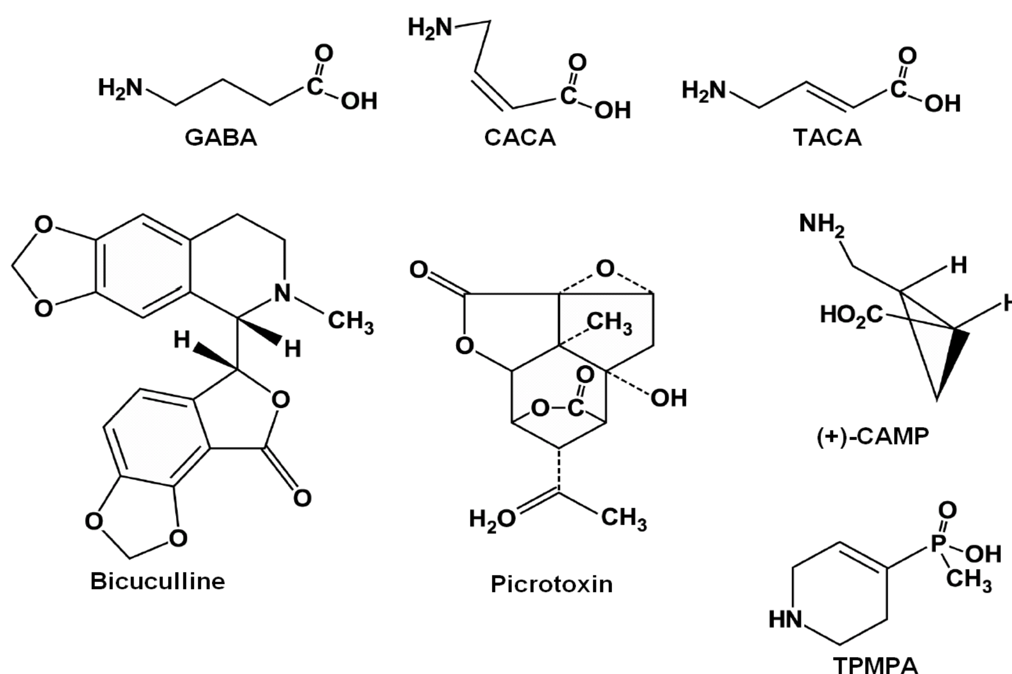


Fig. 2. Main GABAergic molecules referred to in the text.
GABA, CACA, TACA and (+)-CAMP all act as agonists on $GABA_C$ receptors, GABA and TACA also on $GABA_A$ receptors. Bicuculline is a $GABA_A$ receptor antagonist and TPMPA a $GABA_C$ receptor antagonist, while picrotoxin acts as a channel blocker on both receptor types. Modified with permission from Chebib and Johnston, 2000. © American Chemical Society.

To date, the most selective agonist at $GABA_C$ receptors is (±)-CAMP, more specifically (+)-CAMP (Duke *et al.*, 2000). The order of agonist/partial agonist potency at $GABA_C$ receptors can be summarized as follows: TACA > GABA > muscimol > I4AA > TAMP >> (±)-CAMP ≈ CACA > isoguvacine (Chebib and Johnston, 2000). Although

muscimol is weaker than GABA at $GABA_C$ receptors, it is nevertheless more potent at $GABA_C$ than $GABA_A$ receptors expressed in *Xenopus* oocytes (Kusama *et al.*, 1993a; b; Woodward *et al.*, 1993). TAMP, a *trans*-enantiomer of (±)-CAMP, 2-MeTACA, and I4AA are interesting compounds, as they seem to distinguish $GABA_C$ receptors composed of different ρ subunits (Kusama *et al.*, 1993a; b; Chebib *et al.*, 1998; Vien *et al.*, 2002).

In addition to GABA and its analogues, recombinant ρ_1 homomeric receptors have been shown to respond to glycine and β-alanine, albeit the EC_{50} being in the millimolar range. As for glycine, even low concentrations seemed to potentiate GABA-induced responses, but the physiological significance of the finding remains elusive (Calvo and Miledi, 1995).

2.2.2.2. Antagonists

The most specific $GABA_C$ receptor antagonist is TPMPA (Fig. 2), although it is also a weak antagonist of $GABA_A$ receptors and a weak agonist of $GABA_B$ receptors. TPMPA was a competitive antagonist of homo-oligomeric rat ρ_1 receptors expressed in *Xenopus* oocytes, with a K_b of around 2 μM, but slightly less potent on homo-oligomeric human ρ_2 receptors, with a K_b of around 15 μM, while rat cortical $GABA_A$ receptors expressed in oocytes had a K_b of around 320 μM and rat hippocampal $GABA_B$ receptors an EC_{50} of around 500 μM (Ragozzino *et al.*, 1996). Recently, a new antagonist, (±)-*cis*-3-ACPMPA, was reported to have stronger affinity than TPMPA for human ρ_2 homo-oligomers (Chebib *et al.*, 2007).

The chloride channel blocker picrotoxin (PiTX), which is a racemic mixture of picrotin and the active agent picrotoxinin, is effective on $GABA_A$, $GABA_C$, and glycine receptors, but $GABA_C$ receptors are less sensitive to it than $GABA_A$ receptors. Furthermore, native $GABA_C$ receptors in the retina are less sensitive to it than homo-oligomeric ρ_1 receptors expressed in heterologous systems (Feigenspan *et al.*, 1993; Zhang *et al.*, 1995; Enz and Cutting, 1999). The inhibitory mechanism of PiTX in the ligand-gated anion channels is a complex phenomenon and, putatively, a mixed antagonism of non-competitive and competitive inhibition (Woodward *et al.*, 1993; Qian and Dowling, 1994; Wang *et al.*, 1995b; Dong and Werblin, 1996; Qian *et al.*, 2005). PiTX seems to interact with two binding sites, the emphasis of which varies between different receptor types (Newland and Cull-Candy, 1992; Yoon *et al.*, 1993; Dong and Werblin, 1996; Dibas *et al.*, 2002). In $GABA_A$ receptors, PiTX acts mainly as a non-competitive antagonist, whereas PiTX inhibition of $GABA_C$ ρ_1 receptors is mainly competitive and use-facilitated (MacDonald and Olsen, 1994; Wang *et al.*, 1995b; Dong and Werblin, 1996; Dibas *et al.*, 2002; but see Goutman and Calvo, 2004). The relationships between PiTX block and certain ion channel-lining amino acids in the GABA receptors will be discussed further in Section 6.1. A related compound, TBPS, blocks ρ_1 homo-oligomeric receptors expressed in *Xenopus* oocytes, but much more weakly than $GABA_A$ receptors (Feigenspan and Bormann, 1994).

In addition to ineffective bicuculline, other competitive $GABA_A$ receptor antagonists, such as strychnine and SR95531 (gabazine), are much weaker at inhibiting $GABA_C$ receptors (Woodward *et al.*, 1993; Feigenspan and Bormann, 1994). Some more or less broadly used $GABA_A$ receptor agonists, like THIP (also known as gaboxadol), P4S, isonipecotic acid, and 3-APS, have inhibitory effects at $GABA_C$ receptors (Woodward *et al.*, 1993). Several $GABA_B$ receptor agonists, such as 3-APA and 3-APMPA, are potent

antagonists at $GABA_C$ receptors, while phaclofen and saclofen have no effect (Woodward *et al.*, 1993; Chebib *et al.*, 1997).

2.2.2.3. Modulators

$GABA_C$ receptors are insensitive to benzodiazepines and barbiturates, known to modulate the responses of classical $GABA_A$ receptors (Feigenspan and Bormann, 1994). Compared with classical $GABA_A$ receptors, $GABA_C$ receptors are relatively insensitive to neuroactive steroids, but at high (µM range) concentrations steroids can modulate $GABA_C$ receptors. The 5α-steroids (e.g. allopregnanolone and 5α-THDOC) modulate positively both $GABA_A$ and $GABA_C$ receptors, while 5β-steroids (e.g. pregnanolone and 5β-THDOC) are negative modulators of $GABA_C$ receptors, but positive modulators of $GABA_A$ receptors (Morris *et al.*, 1999). Although loreclezole is a very efficient positive modulator of $GABA_A$ receptors, it is a potent negative modulator of ρ_1 $GABA_C$ receptors, and has been described as a simple functional marker for them (Thomet *et al.*, 2000). In sharp contrast to $GABA_A$ receptors, ethanol seems to be a weak competitive inhibitor at ρ_1 homomeric $GABA_C$ receptors (Mihic and Harris, 1996). Flavonoids were shown to inhibit ρ_1 homo-oligomeric $GABA_C$ receptors similarly to $GABA_A$ receptors, even though these substances of plant origin have been correlated with benzodiazepines because of their effects on sleep, motility and pain (Goutman *et al.*, 2003).

Zinc and protons are physiologically interesting modulators, as they are endogenously present in the brain, and zinc is putatively co-released with transmitters on synaptic terminals (Dong and Werblin, 1995; 1996). Quite opposite to the $GABA_A$ receptor currents, extracellular acidification inhibits $GABA_C$ receptor currents; a change from pH 7.4 to 6.4 decreased the GABA-activated current by 52% in HEK 293 cells expressing rat ρ_1 homo-oligomeric receptors (Wegelius *et al.*, 1996). Rat ρ_1 homomeric receptors are sensitive to protons throughout the pH range, whereas the human ρ_1 counterparts are insensitive to alkaline pH levels, implying that these receptors lack one of the two binding sites for protons present in rat receptors (Wegelius *et al.*, 1996; Rivera *et al.*, 2000). Zinc potently inhibits and slows down $GABA_C$ receptor currents. Modulation of $GABA_C$ receptors by zinc is pH sensitive, decreasing with a decrease in pH. This is probably due to protons and zinc ions sharing a binding site, possibly a histidine residue in the extracellular N-terminal domain (Calvo *et al.*, 1994; Chang *et al.*, 1995; Dong and Werblin, 1995; 1996; Wang *et al.*, 1995a; Rivera *et al.*, 2000).

Phosphorylation by PKC has also been found to down-modulate $GABA_C$-mediated responses. The mechanism seems not to act through the direct phosphorylation of the consensus phosphorylation sites of the $GABA_C$ receptor, acting instead through the phosphorylation-dependent internalization of the receptor complex, with the internalized receptors later able to return to the cell surface (Kusama *et al.*, 1998; Filippova *et al.*, 1999; 2000).

2.2.3. ρ subunits

$GABA_C$ receptors are believed to consist only of ρ subunits, either of a single type of ρ subunit as a homo-oligomeric receptor or of a mix of different ρ subunits, i.e. a hetero-oligomeric receptor, sometimes also called a pseudohomo-oligomeric receptor to

distinguish it from $GABA_A$ receptors, which need subunits from at least two different groups of subunits (α and β). To date, three different ρ subunits have been cloned from rat retina and two from human retina, together with some known parts of the human ρ_3 subunit sequence (reviewed by Enz, 2001 and Zhang *et al.*, 2001). In white perch (*Morone Americana*), a total of five ρ subunits have been sequenced and their distinctive pharmacology demonstrated (Qian *et al.*, 1998; Pan *et al.*, 2005).

The sequence identity across species is highest among ρ_1 subunits (e.g. 94.1% between human and rat ρ_1) and lowest among ρ_3 subunits. In the human genome, the genes encoding ρ_1 and ρ_2 are located in the same chromosomal region (6q13-q16.3), whereas the gene encoding ρ_3 is located in chromosome 3 (3q11-q13.3). These findings suggest that the ρ_3 subunit has diverged earlier from a common ancestral gene (Cutting *et al.*, 1992; Bailey *et al.*, 1999a; b). Based on amino acid sequences, ρ receptors are considered phylogenetically old GABA receptors together with β, δ, θ, and π subunits, the sequence identity between these subunits being around 40% (Whiting *et al.*, 1999). The phylogenetic tree of GABA receptor subunits is seen in Figure 3. The ancestor of all ligand-gated ion channels was probably homo-oligomeric (Ortells and Lunt, 1995), which is still apparent in $GABA_C$ receptors. In line with archaic properties, most ρ subunits are found in older parts of the brain. Interestingly, the Rdl subunit of the GABA receptor of the fruit fly displays rather high homology at the amino acid level to the rat ρ_1 subunit, together with many similar characteristics (Hosie *et al.*, 1997), further supporting the phylogenetic archaicness of ρ subunits.

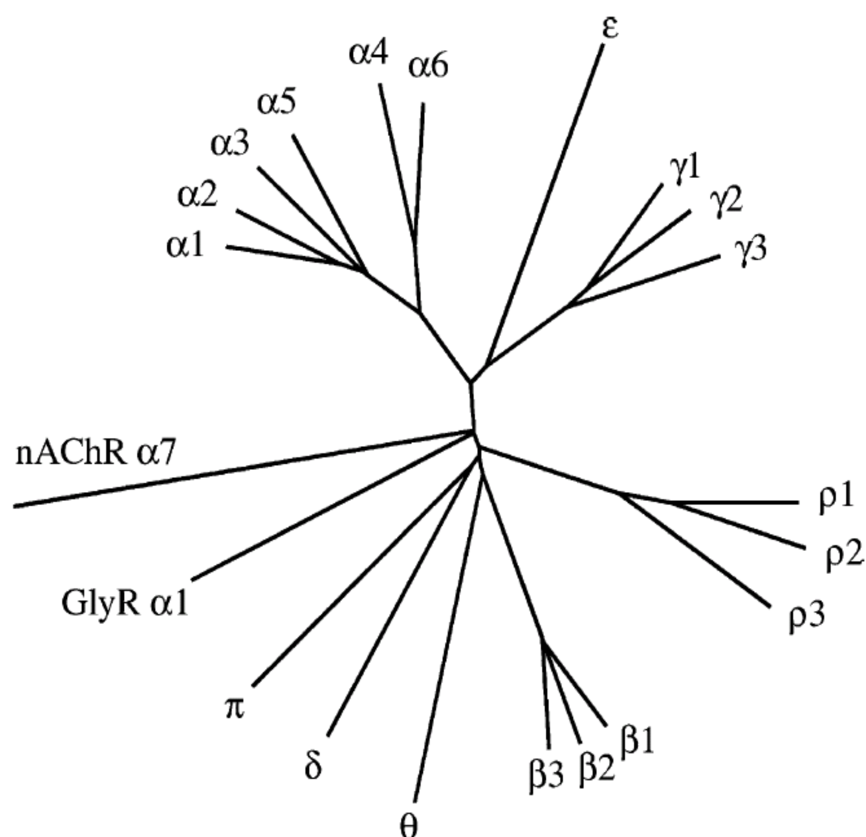
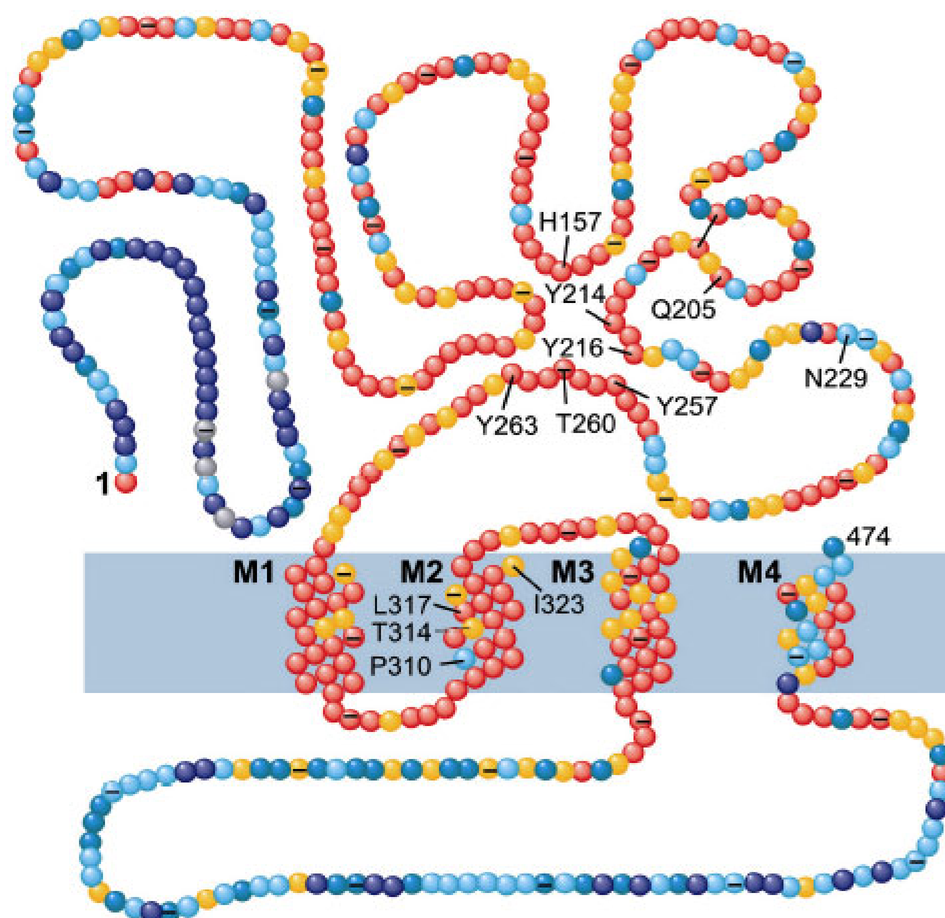


Fig. 3. The phylogenetic tree of GABA receptor subunit proteins as a cladogram.
The subunit sequences being compared are those of the rat, except for mouse θ. The distance between the subunits represents the variations of the amino acid sequences of the GABA receptor subunit proteins plus the glycine receptor α_1 (GlyR α_1) and the nicotinic acetylcholine receptor α_7 (nAChR α_7) subunits. Based on amino acid sequences, ρ subunits are relatives to β, θ, δ, and π subunits, as well as to glycine α_1 receptors. Reproduced with permission from Korpi *et al.*, 2002. © Elsevier Science Ltd.

A core structure is conserved among ρ subunits of all species, including the proximal two-thirds of the extracellular N-terminal region and the four membrane-spanning regions M1–M4. The least conserved regions are the distal one-third of the extracellular N-terminal domain and the intracellular loop between M3 and M4 (Fig. 4; Zhang *et al.*, 2001). The small differences in amino acid sequences in the channel-lining second transmembrane domain M2 between ρ subunits will be discussed further in Section 6.1.



Amino acid residues	Functional effects
Q205, Y214, Y216, Y257, T260, Y263	EC_{50}, agonist binding
H157	Zn^{2+} inhibition, pH
N299	Glycosylation
≈ 100 residues flanking cysteine loop	Receptor assembly efficiency
P310, P314	PiTX sensitivity
P310, P314, L317	Channel gating, agonist binding
I323	Barbiturate sensitivity

Fig. 4. Schematic of the molecular structure of ρ subunits.
Highly conserved amino acid residues, shared by a large fraction of known ρ subunits, are shown in orange and red, while residues that are only weakly conserved among ρ subunits are shown in light blue, dark blue and grey. Residues known to underlie specific functions of $GABA_C$ receptors are indicated by a single letter amino acid abbreviation, followed by the position of the amino acid within the sequence (on rat $ρ_1$ subunit). Every tenth amino acid is marked by a horizontal bar to facilitate counting. The functional effects of the labelled amino acids are indicated in the lower part of the figure. Modified with permission from Zhang *et al.*, 2001. © Elsevier Science Ltd.

2.2.3.1. ρ_1 subunit

The ρ_1 subunit is easily expressed in heterologous systems, resulting in large whole-cell currents. While TACA and muscimol are potent partial agonists, CACA is markedly less potent at ρ_1 homo-oligomeric receptors. The EC_{50} for CACA was around 70 µM on mammalian ρ_1 subunits expressed in *Xenopus* oocytes, and 131 µM when expressed in HEK 293 cells (Kusama *et al.*, 1993a; Woodward *et al.*, 1993; Enz and Cutting, 1999). The only essential difference between human and rat ρ_1 seems to be pH sensitivity (see Section 2.2.3). Two shorter splice variants of ρ_1 subunit have been found to date, $\rho_1\Delta51$ being more sensitive to GABA and zinc, whereas $\rho_1\Delta450$ was not functional (Martínez-Torres *et al.*, 1998; Demuro *et al.*, 2000). ρ_1 mRNA has been discovered in retinal bipolar cells, superior colliculus (SuC), hippocampus, cerebellum, and spinal cord (Enz *et al.*, 1995; Boué-Grabot *et al.*, 1998; Enz and Cutting, 1999; Didelon *et al.*, 2002; Rozzo *et al.*, 2002). Rat ρ_1 homo-oligomeric receptors are more sensitive to PiTX than native $GABA_C$ receptors in the rat retina. Because of this and other pharmacological differences, mammalian retinal receptors are considered to be $\rho_1\rho_2$ hetero-oligomers (Feigenspan *et al.*, 1993; Zhang *et al.*, 1995; Enz and Cutting, 1999). The single-channel conductance was shown to be 0.65 pS in human ρ_1 and 0.2 pS in white perch ρ_{1A} homo-oligomeric receptors (Wotring *et al.*, 1999; Zhu *et al.*, 2007). A summary of pharmacological properties of ρ subunits is given in Table 3 of section 6.1.

2.2.3.2. ρ_2 subunit

The ρ_2 subunit has been enigmatic in $GABA_C$ receptor studies because rat ρ_2 subunits did not form homomeric receptors when expressed alone in *Xenopus* oocytes, and yet, outside the retina, it is expressed more than ρ_1. There are even areas in the central nervous system where it is expressed alone without any other ρ subunit (see Section 2.2.5). This has raised questions of the role of $GABA_C$ receptors outside the retina (Boué-Grabot *et al.*, 1998; Zhang *et al.*, 2001). In contrast, mouse and human ρ_2 subunits did form homomeric receptors in *Xenopus* oocytes, although abundant ρ_2 cDNA was needed for the transfection to get even a small current (Kusama *et al.*, 1993b; Greka *et al.*, 1998; Enz and Cutting, 1999). Human ρ_2 receptors are slightly more sensitive to most agonists than ρ_1 receptors. EC_{50} for CACA at human ρ_2 homo-oligomers expressed in HEK 293 cells was 62 µM, while TACA was the most potent agonist, followed by GABA and muscimol (Kusama *et al.*, 1993b; Enz and Cutting, 1999). Intriguing differences in ρ_2 subunits between species have been reported. The presence of rat ρ_2 subunits in hetero-oligomeric $\rho_1\rho_2$ or $\rho_2\rho_3$ receptors has been suggested to result in insensitivity to PiTX (Zhang *et al.*, 1995; Ogurusu *et al.*, 1999), while human ρ_2 homo-oligomers were more sensitive to PiTX than ρ_1 homo-oligomers (Wang *et al.*, 1995b). The pharmacology of the ρ_2 subunit will be discussed in more detail in Sections 5.2 and 6.1. The single-channel conductance of mammalian ρ_2 subunits has not been published, but it was 3.2 pS in white perch ρ_{2A}, and 3.5 pS in perch ρ_{2B} homo-oligomeric receptors (Zhu *et al.*, 2007).

2.2.3.3. ρ_3 subunit

The rat ρ_3 subunit, but not the white perch ρ_3 subunit, formed homomeric receptors when expressed in *Xenopus* oocytes (Shingai *et al.*, 1996; Qian *et al.*, 1998; Ogurusu *et al.*, 1999, Vien *et al.*, 2002). The ρ_3 subunit has some distinctive pharmacological characteristics, such as muscimol and TACA being more potent than GABA and full agonists. The potency of CACA is lower, as the EC_{50} for CACA at homomeric ρ_3 receptors expressed in oocytes was shown to be either 139 µM (Vien *et al.*, 2002) or 65 µM (Ogurusu *et al.*, 1999). Homo-oligomeric ρ_3 receptors are relatively sensitive to PiTX (Shingai *et al.*, 1996; Ogurusu *et al.*, 1999). Most interestingly, MeTACA seems to be a moderately potent antagonist at ρ_1, a partial agonist at ρ_2, and inactive at ρ_3 (Chebib *et al.*, 1998; Vien *et al.*, 2002), whereas TAMP and I4AA act as partial agonists at ρ_1 (here I4AA acts somewhat more potently as an antagonist, though) and ρ_2, but potent, non-competitive antagonists at ρ_3 (Vien *et al.*, 2002). In the adult rat brain, ρ_3 mRNA was present in the mesencephalon (midbrain), hippocampus, cerebellum, thalamus and basal ganglia, and in the rat retina it has been detected in the ganglion cell layer (Ogurusu *et al.*, 1997; 1999). Intriguingly, the expression of ρ_3 mRNA was eight times higher in the rat brain at embryonic day 16 than in the adult brain (Ogurusu *et al.*, 1999).

2.2.4. Assembly properties

In vivo, the $\rho_1\rho_2$ hetero-oligomeric $GABA_C$ receptor in retinal bipolar cells is the only combination of ρ subunits detected thus far (Feigenspan *et al.*, 1993; Zhang *et al.*, 1995; Enz and Cutting, 1999). Extensive studies by many researchers had led to the belief that mammalian wild-type ρ subunits do not assemble *in vivo* with $GABA_A$ or glycine receptor subunits into functional receptors, most likely due to different assembly signals in their N-termini (reviewed by Enz, 2001). Recent accumulating evidence, however, supports the opposite conclusion. Mutated ρ_1 subunits were found to assemble with $GABA_A$ receptor subunit γ_{2S} and the glycine receptor subunits α_1 and α_2, but these receptors had constantly open ion channels (Pan *et al.*, 2000). In fish, the ρ_{1B} subunit cloned from white perch retina could be co-assembled with γ_2 subunit (either white perch or human), and the heterologously expressed hetero-oligomeric receptors had similar pharmacological characteristics to native $GABA_C$ receptors in white perch bipolar cells (Qian and Ripps, 1999; Qian and Pan, 2002).

Immunoprecipitation experiments, together with yeast two-hybrid and *Xenopus* oocyte expression system methods, demonstrated that protein-protein and functional interactions were possible also between rat ρ_1 and γ_2 subunits (Ekema *et al.*, 2002). In the rat brainstem, the co-localization of α_1 and ρ_1 subunits was shown to be possible at light and electron microscopy levels, and an association of ρ_1 with $GABA_A$ receptor α_1 and γ_2 subunits was found in immunoprecipitation experiments (Milligan *et al.*, 2004). Co-immunoprecipitation of ρ_1 subunits with α_1 subunits was also detected in cerebellum lysates (Harvey *et al.*, 2006). Of course, these findings of co-localization and co-immunoprecipitation do not necessarily imply that the receptors act together; no direct evidence exists that co-assembly of functional channels is true for any mammalian ρ subunit. Still, there are several demonstrations of receptors with unusual pharmacology in the hippocampus, spinal cord, cerebellum, amygdala, and brainstem (discussed in more detail in Section 2.2.5.), some of which could be explained by co-assembly of ρ_1 and

$GABA_A$ receptor subunits. It is noteworthy that no evidence for co-assembly applies to the ρ_2 subunit, at least not yet.

To prevent receptors from laterally diffusing on the cell membrane, anchoring mechanisms are proposed to connect the receptors to cytoskeleton proteins. Since, especially in the retina, different receptors are located on different synapses within the same neuron (Fletcher *et al.*, 1998; Koulen *et al.*, 1998), a variety of selective anchor proteins is needed. To date, two anchoring proteins for ρ_1 subunit have been identified: microtubule-associated protein MAP1B and glycine transporter splice variant GLYT-1E/F. They bind to distinct sites in the long intracellular loop of ρ_1, and MAP1B interacts to some extent with ρ_2, too, but not with $GABA_A$ receptor subunits (Hanley *et al.*, 1999; 2000; Billups *et al.*, 2000). However, as no difference was apparent in retinal $GABA_C$ receptor staining between MAP1B-deficient and wild-type mice, MAP1B does not seem to be crucial for $GABA_C$ receptor clustering (Meixner *et al.*, 2000).

2.2.5. Expression and function of native $GABA_C$ receptors

Unlike widely distributed $GABA_A$ receptors, $GABA_C$ receptors are selectively expressed. Of the three ρ subunits, ρ_2 is the most abundant. Quantitatively, $GABA_C$ receptor subunits are expressed at the highest level in the retina and superior colliculus (SuC). The function of $GABA_C$ receptors is also best known in those two areas. All functional studies where CACA has been used at a concentration of 200 μM or more should be interpreted with caution, as there are data showing an influence on $GABA_A$ receptors and on passive release of GABA (Pan and Lipton, 1995; Chebib and Johnston, 1997; Wall, 2001).

2.2.5.1. Retina

As visual signals pass through the retina from the photoreceptors through bipolar cells to the ganglion cells, they are modified by synaptic inputs from horizontal cells and amacrine cells, which are GABAergic interneurons. Horizontal cells form synaptic contacts with photoreceptor axon terminals and bipolar cell dendrites at the outer plexiform layer, whereas amacrine cells form synaptic contacts with bipolar cell axon terminals and ganglion cell dendrites at the inner plexiform layer. $GABA_A$ receptors are found in almost every type of neuron in the retina, where they exist in a variety of subtypes with different compositions and locations. $GABA_C$ receptors, in contrast, are present mainly – and ρ_1 transcripts exclusively– in bipolar cells, primarily at their axon terminals. ρ subunit mRNA has been found in the retina of all vertebrate species investigated (reviewed by Enz, 2001 and Lukasiewicz *et al.*, 2004).

In the rat retina, all three ρ subunit mRNAs have been shown by *in situ* hybridization to be present in the inner nuclear layer (INL), which contains the somata of the bipolar cells (Enz *et al.*, 1995). Strong, punctate immunofluorescence of the ρ subunit protein was found in the inner plexiform layer, indicating a synaptic clustering at bipolar axon terminals (Enz *et al.*, 1996). In addition, rat and chick amacrine and ganglion cells have ρ_2 mRNA, and rat ganglion cells also ρ_3 mRNA (Albrecht and Darlison, 1995; Ogurusu *et al.*, 1997), but, to date, neither ρ protein nor $GABA_C$ receptor-mediated currents have been detected in them. Some horizontal cells in fish, but not in mammals, have been found to contain ρ subunit mRNA, ρ protein and functional $GABA_C$ receptors,

and rod-driven horizontal cells of white perch are the only known neurons in which GABA responses are mediated exclusively by $GABA_C$ receptors (Qian and Dowling, 1993; 1994; Dong *et al.*, 1994). In rodent and porcine cone photoreceptors, ρ subunit protein expression was also revealed together with MAP1B by immunohistochemistry (Pattnaik *et al.*, 2000). $GABA_A$ receptors have been shown to be often located within the same bipolar neurons, but not on the same synapses as $GABA_C$ receptors (Fletcher *et al.*, 1998; Koulen *et al.*, 1998). In addition to synaptic receptors, extrasynaptic $GABA_C$ receptors have been observed on tiger salamander and goldfish retinal slices by using GABA transporter blockers (Ichinose and Lukasiewicz, 2002; Hull *et al.*, 2006). During development, ρ_2 mRNA has been reported to appear around postnatal day 9 (P9), peak at P15 and remain at that level through adulthood in the mouse retina, while ρ_1 mRNA was found already at P6 (Greka *et al.*, 2000; Wu and Cutting, 2001). Immunostaining with pan-ρ antibody revealed distinct labelling of rat bipolar cells at P7, and strong, punctate, adult-like labelling at P19 (Koulen *et al.*, 1998).

In the retina, $GABA_C$ receptors are involved in temporal inhibition of bipolar cells (reviewed by Lukasiewicz *et al.*, 2004). The retinal ganglion cells are excited at the onset and offset of light stimuli, and this transient response is caused by a delayed feedback inhibition from amacrine cells to bipolar cell terminals. Different types of bipolar cells have been demonstrated to express different proportions of $GABA_A$ and $GABA_C$ receptors, the rod bipolar cells having the highest and OFF cone bipolar cells the lowest ratio of $GABA_C$ to $GABA_A$ receptors (Euler and Wässle, 1998; Shields *et al.*, 2000). While the excitatory pathway from rod photoreceptors to rod bipolar cells has slower kinetics than the cone pathway, the characteristics of $GABA_C$ receptors are nicely matched. When taking into consideration that bipolar cells use graded potentials instead of action potentials as their means of signal transmission, the high-affinity, non-desensitizing $GABA_C$ergic responses are well-suited to fine-tune these signals. In addition to temporal inhibition, $GABA_C$ receptors are also thought to modulate the centre-surround antagonism of the ganglion cells, thus participating in spatial inhibition (reviewed by Enz, 2001 and Lukasiewicz *et al.*, 2004). Moreover, selective $GABA_C$ receptor inhibition has been shown to increase oscillatory discharges in dimming-detector ganglion cells, leading to potentiated escape behaviour in frogs (Ishikane *et al.*, 2005).

Based on the level of picrotoxin inhibition and other pharmacological properties of native vs. recombinant $GABA_C$ receptors, the mammalian retinal $GABA_C$ receptors are considered to be hetero-oligomers consisting of ρ_1 and ρ_2 subunits (Feigenspan and Bormann, 1994; Zhang *et al.*, 2001). In contrast, the bicuculline-insensitive currents evoked in the mouse cone photoreceptors were quite sensitive to picrotoxin, suggesting the absence of ρ_2 subunits (Pattnaik *et al.*, 2000). When the expression of the ρ_1 subunit was eliminated using a gene knock-out method, the ρ_2 subunit was also shown to be absent from the inner and outer plexiform layers and no $GABA_C$ receptor immunoreactivity was seen, but the morphology of the retina was normal at light microscopic level (McCall *et al.*, 2002). The rod bipolar cells in ρ_1-null mice were demonstrated to lack a sustained, $GABA_C$-mediated response to focally applied GABA and to brief light flashes, and no compensatory up-regulation of $GABA_A$ or glycine receptors, present normally in these cells, was detected. Furthermore, the overall visual processing, measured by a dark-adapted electroretinogram (ERG), was altered, namely, the oscillatory potentials were larger, implying enhanced transmission from bipolar to ganglion cells (McCall *et al.*, 2002).

2.2.5.2. Visual system

Outside the retina, ρ_2 is the most abundant subunit, while ρ_1 and ρ_3 subunits seem to exist at much lower expression levels (Enz *et al.*, 1995; Boué-Grabot *et al.*, 1998; Wegelius *et al.*, 1998; Enz and Cutting, 1999; Ogurusu *et al.*, 1999; Didelon *et al.*, 2002; Rozzo *et al.*, 2002).

The superior colliculus (SuC) has the highest concentration of both GABA as a transmitter and ρ subunit mRNA expression in the whole brain (Mize, 1992; Boué-Grabot *et al.*, 1998; Wegelius *et al.*, 1998). SuC is a multi-layered midbrain nucleus involved in the control of saccadic eye movements. In the superficial grey layer (SGL) of SuC, excitatory retinal and cortical afferents activate not only efferent projection neurons to the thalamus and brainstem but also a large number of local inhibitory interneurons that induce feed forward inhibition to the projection neurons. In the SGL, inhibitory interneurons comprise about half of the neuron population (Mize, 1992), and strong, punctate ρ-immunolabelling is largely restricted to this layer (Pasternack *et al.*, 1999). In contrast to the retina, the physiological role of $GABA_C$ receptors in the SuC is excitation, or rather disinhibition, of the SGL projection neurons (Arakawa and Okada, 1988; Pasternack *et al.*, 1999; Boller and Schmidt, 2001; Schmidt *et al.*, 2001). This is consistent with the preferential, or even exclusive, location of these receptors in the local GABAergic interneurons (Pasternack *et al.*, 1999; Schmidt *et al.*, 2001). $GABA_C$ receptors may also contribute to GABA-induced long-term potentiation (LTP) in the SuC (Platt and Withington, 1998).

In the SGL of SuC, ρ_1 subunits co-localize only partly with the synaptic protein synaptophysin (Clark *et al.*, 2001). In agreement with this, no $GABA_C$-driven inhibitory postsynaptic currents were found in SGL piriform or stellate cells with optic fibre stimulation, even though $GABA_C$ receptors on these cells were activated with exogenous agonists (Schmidt *et al.*, 2001). Furthermore, only a minor portion of spontaneous inhibitory postsynaptic currents were bicuculline-resistant or TPMPA-sensitive in collicular slices (Boller and Schmidt, 2003; Kirischuk *et al.*, 2003). These findings suggest that, in addition to synaptic receptors, a subpopulation of $GABA_C$ receptors could be extrasynaptic and activated by the spillover of the synaptically released GABA (Boller and Schmidt, 2003; Kirischuk *et al.*, 2003).

At P0, ρ_1 and ρ_2 mRNAs were shown to be expressed in chick optic tectum, the avian counterpart of the SuC (Albrecht *et al.*, 1997). In the SuC of the rat, ρ_1 protein expression was seen as early as from birth (Clark *et al.*, 2001). In neonatal rat SuC cultures, on the other hand, no functional $GABA_C$ receptors appeared to contribute to excitatory GABA responses (White and Platt, 2002). With single-cell patch-clamp recordings, functional $GABA_C$ receptors could be detected already at P4, but they did not significantly influence the response at the cell population level until the third postnatal week, putatively due to immature local GABAergic connections (Boller and Schmidt, 2001).

Strikingly, $GABA_C$ receptor expression has been found in many brain regions related to vision: SuC, dorsal lateral geniculate nucleus (dLGN), pretectal nucleus of the optic tract (NOT), median terminal nucleus of the accessory optic tract (MTN), and the sixth layer of the visual cortex (Boué-Grabot *et al.*, 1998; Wegelius *et al.*, 1998; Enz and Cutting, 1999; Ogurusu *et al.*, 1999; Pasternack *et al.*, 1999). These subcortical nuclei expressing ρ subunits are all retinorecipient (Van der Want *et al.*, 1992). The dLGN relays visual information to the cortex, while NOT and MTN are involved in the generation of optokinetic nystagmus. Punctate ρ-immunoreactivity surrounding putative unstained cell

bodies has been illustrated in the dLGN, NOT and MTN (Wegelius, 2000). Similarly to SuC, in the dLGN, $GABA_C$ receptors have been demonstrated to be localized in GABAergic interneurons and, in line with this, to mediate disinhibition of geniculocortical relay cells (Zhu and Lo, 1999; Schlicker *et al.*, 2004). Electrical stimulation of MTN was shown to cause bicuculline-insensitive GABAergic responses in the NOT (Van der Togt and Schmidt, 1994). Similarly to SuC, at least part of the $GABA_C$ receptors seem to be located away from synaptic sites in the NOT (Boller and Schmidt, 2003).

2.2.5.3. Hippocampus

Outside the visual system, the clearest $GABA_C$ receptor mRNA expression and ρ immunolabelling have been found in the hippocampus, where all three ρ subunit mRNAs have been detected by RT-PCR, ρ_2 being predominant (Boué-Grabot *et al.*, 1998; Wegelius *et al.*, 1998; Didelon *et al.*, 2002). With single-cell RT-PCR of hippocampal cultures and slices, most pyramidal cells were shown to co-express all three ρ mRNAs, while granule cells seemed to express mainly ρ_3 mRNA (Liu *et al.*, 2004). On the other hand, when the single-cell RT-PCR approach was used for individual CA3 pyramidal neurons, only very few exhibited ρ_2 mRNA (Didelon *et al.*, 2002). *In situ* hybridization displayed ρ_2 mRNA in the adult rat CA1 pyramidal layer (Wegelius *et al.*, 1998; Ogurusu *et al.*, 1999), but also in the interneurons throughout the different hippocampal subfields (Rozzo *et al.*, 2002), the latter group reporting also overlapping, but weaker expression of ρ_1 mRNA. Immunohistochemistry on adult hippocampal sections showed a few positive scattered interneurons and weakly positive dentate gyrus granular cells and CA1 pyramidal neurons (Rozzo *et al.*, 2002).

Postnatally, ρ_1 and ρ_2 transcripts have been detected at P5 and P8 with RT-PCR in the CA1 area (Boué-Grabot *et al.*, 1998; Wegelius *et al.*, 1998; Ogurusu *et al.*, 1999). In contrast, Rozzo and co-workers (2002) could detect ρ_1 and ρ_2 mRNAs in the *stratum pyramidale* of all CA regions and in the granule cell layer of the dentate gyrus at a very low level at P1. At P7, both subunit transcripts were strongly expressed in the *stratum pyramidale* of the CA1 and CA4 areas and the hilus, but also in cells, most likely interneurons, located within the *strata oriens* and *radiatum* of the CA1 and CA3 subfields, whereas after the first postnatal week the expression was downregulated (Rozzo *et al.*, 2002). Didelon *et al.* (2002) demonstrated all three ρ subunits at P2 with RT-PCR, but they reported upmodulation of ρ_1 and ρ_2 in the postnatal rat hippocampus.

Information on $GABA_C$ receptor function in the developing hippocampus has been discrepant. In the early postnatal hippocampus, some bicuculline-insensitive responses were seen in the CA3 area, disappearing after the second postnatal week (Strata and Cherubini, 1994; Martina *et al.*, 1995). As these receptors *1)* had lower agonist affinity and *2)* similar single-channel conductance as conventional $GABA_A$ receptors, and *3)* they were potentiated by zinc at low concentrations, but *4)* neither distinguished by CACA (although 300 μM or more was used) *5)* nor antagonized by TPMPA (Strata and Cherubini, 1994; Martina *et al.*, 1995; 1996; Didelon *et al.*, 2002), they do in fact not resemble $GABA_C$ receptors.

Three groups have studied cultured hippocampal neurons taken from rat brains at different ages. In hippocampal neurons taken from the brains of 17-day-old rat embryos and cultured for 10 – 14 days, a clear effect on ammonia-induced accumulation of chloride by CACA, TPMPA and PiTX, but not bicuculline was detected, indirectly indicating the

presence of functional native $GABA_C$ receptors (Irie *et al.*, 2001). In neurons taken from the brains of 1- to 2-day-old rat pups, only a negligible fraction of currents were bicuculline-insensitive, whereas CACA-sensitive currents were not seen at all (Cheng *et al.*, 2001a; b). In neurons from P3 to P5, a bicuculline-insensitive current was observed, but this current deactivated rapidly and was not blocked by $GABA_C$ receptor competitive antagonist 3-APA (Filippova *et al.*, 2001). The same group did not detect bicuculline-insensitive currents in cultured hippocampal slices from P7. Surprisingly, an unusual type of GABA receptor was found in pyramidal cells of acute hippocampal slices from P8 to P18, as it was activated by CACA (100 – 1000 μM), but inhibited by both TPMPA (60 μM) and bicuculline (30 μM), thus possessing pharmacological properties of $GABA_A$ and $GABA_C$ receptors (Hartmann *et al.*, 2004).

Few studies are available to date on functional $GABA_C$ receptors in the adult hippocampus. In acutely isolated rat hippocampal pyramidal neurons, a population of GABA receptors was found to have a high affinity for GABA and to be inhibited by protons at physiological pH levels (Pasternack *et al.*, 1996), resembling the properties of $GABA_C$ receptors (Rivera *et al.*, 2000). In the guinea-pig hippocampus, a large increase in the holding current of pyramidal neurons in the CA1 area was detected with a perfusion of 50 μM CACA, and this increase was not affected by pentobarbital, consistent with the presence of $GABA_C$ receptors in pyramidal neurons (Semyanov and Kullmann, 2002). In hippocampal interneurons, on the other hand, the same authors demonstrated atypical GABA responses; these responses had a relatively small single-channel conductance and were somewhat insensitive to 100 μM PiTX and sensitive to 50 μM CACA, but also sensitive to 10 μM bicuculline and 100 μM pentobarbital (Semyanov and Kullmann, 2002).

The ionotropic GABA receptors mediating fast inhibitory synaptic currents in the hippocampal CA1 area have been shown to be bicuculline-sensitive in a number of studies (Collingridge *et al.*, 1984; Lambert *et al.*, 1991), indicating that only $GABA_A$ receptors are present in these synapses. However, slowly activating GABA responses evoked by multiple synaptic stimulations have been reported in the hippocampal CA1 area. These responses were only partially blocked by a $GABA_B$ receptor antagonist, and hence, could be mediated by $GABA_C$ receptors (Thomson and Destexhe, 1999).

2.2.5.4. Cerebellum

The question of $GABA_C$ receptors in the cerebellum is controversial. In the adult rat cerebellum, ρ_1 and ρ_2 mRNAs were found in Purkinje and basket cells by *in situ* hybridization (Boué-Grabot *et al.*, 1998; Rozzo *et al.*, 2002), and RT-PCR revealed the same ratio between ρ_1 and ρ_2 mRNA (1:2) in the cerebellum and retina (Boué-Grabot *et al.*, 1998; Enz and Cutting, 1999). In addition, the ρ_2 subunit was cloned from the bovine cerebellum, the encoded receptors being functional in *Xenopus* oocytes (López-Chavez *et al.*, 2005). Immunocytochemical studies revealed ρ protein expression predominantly in Purkinje cell somata and proximal dendritic compartments (Boué-Grabot *et al.*, 1998; Harvey *et al.*, 2006).

CACA was found to bind to adult rat cerebellar membranes, and this binding could not be displaced by bicuculline or baclofen (Drew *et al.*, 1984; Drew and Johnston, 1992). Later on, α_6 subunit-containing $GABA_A$ receptors were demonstrated to have a low sensitivity to bicuculline (Thompson *et al.*, 1996); thus, these receptors may have

contributed to earlier findings. In cultured cerebellar granule cells, both bicuculline-sensitive and -insensitive receptors were found, but the latter group had lower affinity for GABA, unlike $GABA_C$ receptors (Martina *et al.*, 1997). More recently, 50 – 1000 µM CACA was shown to activate α_6 subunit-containing $GABA_A$ receptors in the granule cells of cerebellar slices. As CACA and GABA currents were blocked by bicuculline, PiTX and furosemide, but not TPMPA, no signs of $GABA_C$ receptors were visible (Wall, 2001). In cerebellar Purkinje cells, however, no α_6 subunit-containing $GABA_A$ receptors have been detected (Wisden *et al.*, 1996), but, still, CACA (50 – 500 µM) was able to evoke non-desensitizing currents inhibited by both TPMPA (100 µM) and bicuculline (50 µM; Harvey *et al.*, 2006). While co-immunoprecipitation studies suggested that ρ subunits could form complexes with $GABA_A$ receptor α_1 subunits in the cerebellar cortex, the authors speculated about a receptor population with a mixed pharmacology. Evidence for extrasynaptic $GABA_C$ receptors was sought, but not found, as TPMPA inhibited phasic, but not tonic transmission in Purkinje cells (Harvey *et al.*, 2006).

2.2.5.5. $GABA_C$ receptors in other brain areas and functions

In the rat central amygdala, some GABA-activated currents that were relatively insensitive to bicuculline and PiTX were found. As these responses were antagonized by TPMPA, but also by diazepam and flurazepam, the possibility of the co-assembly of ρ subunits with $GABA_A$ receptor subunits has been tendered (Delaney and Sah, 1999). These atypical currents were detected on the dendrites of neurons, where also conventional $GABA_A$ receptor currents were present (Delaney and Sah, 2001). Thus far, no molecular biology data exist on $GABA_C$ receptor subunit distribution in the amygdala.

In the rat brainstem, RT-PCR has revealed all three ρ mRNAs, whereas *in situ* hybridization showed only ρ_1 mRNA, suggesting its predominance (Milligan *et al.*, 2004). ρ_1 mRNA was seen on all neurons in the dorsal vagal nucleus (DVN) and on about two-thirds of neurons in *nucleus tractus solitarii*, and this neuronal localization was confirmed by immunohistochemistry. Furthermore, immunoelectron microscopy showed ρ_1 immunoreactivity adjacent to the postsynaptic side of the synaptic junction. CACA (100 – 800 µM) was shown to depolarize DVN neurons, but the responses were inhibited by both TPMPA (40 – 160 µM) and bicuculline (10 µM) and potentiated by sodium pentobarbitone and zolpidem, thus displaying unusual pharmacology with characteristics of $GABA_C$ and $GABA_A$ receptors. Moreover, the authors demonstrated the possibility of co-localization of α_1 and ρ_1 at an identical site on the postsynaptic membrane in DVN neurons by immunoelectron microscopy and co-immunoprecipitation of α_1, γ_2 and ρ_1 in brainstem lysates (Milligan *et al.*, 2004).

In the early postnatal rat brainstem, more precisely in the rostral nucleus of the solitary tract, bicuculline-insensitive, PiTX-sensitive responses were detected in the first three postnatal weeks, implying the transient expression of functional $GABA_C$ receptors (Grabauskas and Bradley, 2001). In addition, in brainstem auditory neurons (*nucleus magnocellularis*) of the young (P5 – P14) chick, some bicuculline-insensitive GABA responses were shown (Hyson *et al.*, 1995).

In the bovine caudate nucleus of the basal ganglia, ρ_1 and ρ_2 mRNAs, and ρ protein were demonstrated together with cloned ρ_1 receptors expressed in oocytes showing typical $GABA_C$ receptor properties (López-Chavez *et al.*, 2005; Rosas-Arellano *et al.*, 2007). The sensitive RT-PCR has also indicated ρ_2 mRNA in the rat epithalamus, thalamus and

mesencephalon (Enz *et al.*, 1995; Ogurusu *et al.*, 1999), and *in situ* hybridization in the *pars compacta* of the *substantia nigra* (Ogurusu *et al.*, 1999), but no signs of functional $GABA_C$ receptors have thus far been detected.

Interestingly, TPMPA has been demonstrated to have an influence on sleep in *in vivo* studies. Rats were given infusions of different TPMPA concentrations into the fourth ventricle and their sleep-waking behaviour was scored according to electroencephalography (EEG) and electromyography (EMG). TPMPA noticeably increased non-active waking and decreased sleep, both slow-wave sleep and REM sleep (Arnaud *et al.*, 2001). In addition, bilateral infusion of TPMPA in the *locus coeruleus* inhibited REM sleep (Gottesmann, 2004). These effects can be hypothesized to correspond to $GABA_C$ receptors in the brainstem dorsal raphe and *locus coeruleus* nuclei. This hypothesis is supported by the finding that a microperfusion of PiTX, but not of bicuculline, onto the dorsal raphe blocked REM sleep (Levine and Jacobs, 1992; Nitz and Siegel, 1997; Gottesmann, 2002; but see Gervasoni *et al.*, 2000). The sleep-waking generating processes have been shown to be modulated by various receptor systems, including GABA and all three of its receptor classes (reviewed by Gottesmann, 2002; 2004).

Contrary to $GABA_A$ receptors, $GABA_C$ receptors seem to have an inhibitory function in short-term memory formation in young chicks, as selective $GABA_C$ receptor antagonists TPMPA and P4MPA injected into the multimodal association area of the forebrain had an enhancing effect (Gibbs and Johnston, 2005). In agreement, CGP 36742, an antagonist of both $GABA_B$ and $GABA_C$ receptors, had a positive influence on learning and memory in mice, rats, and monkeys, unlike selective $GABA_B$ receptor antagonists (Froestl *et al.*, 1995; Genkova-Papakova *et al.*, 2000).

2.2.5.6. Spinal cord

Although the story of $GABA_C$ receptors originated in the spinal cord, their function in this structure has only recently been studied in more detail. RT-PCR displayed expression of all three ρ mRNAs, ρ_2 being the strongest (Enz *et al.*, 1995; Wegelius, 2000). In *in situ* hybridization studies, motoneurons were labelled on the newborn sections, but both interneurons and motoneurons were labelled at P7 and in adults, the ρ_2 staining being stronger than ρ_1. Adult spinal neurons displayed strong ρ-immunostained puncta in their dendrites and cell somata, where ρ_1 and ρ_2 mRNAs were also found (Wässle *et al.*, 1998; Rozzo *et al.*, 2002). In addition, immunoreactivity displayed strong membrane labelling on lamina I and II neurons of the superficial dorsal horn (Zheng *et al.*, 2003). Central nociceptive neurons are located in lamina I and II, where afferent fast and slow pain fibres also predominantly terminate. Many of the neurons in lamina I respond exclusively to noxious stimulation and project to higher brain centres, whereas many lamina II neurons are interneurons modulating the pain sensation.

Neonatal spinal motoneurons were shown to have functional $GABA_C$ receptors, which, although responding only to exogenous GABA and not to synaptic activity evoked by dorsal root stimulation (Rozzo *et al.*, 2002), contributed to spontaneous bursting activity following blocking of $GABA_A$ and glycine receptors (Rozzo *et al.*, 1999). In lamina II neurons, a sustained, bicuculline-insensitive component of GABA current was detected, but as it was potentiated by thiopental and flunitrazepam, and unaffected by I4AA (Park *et al.*, 1999), conventional $GABA_C$ receptors are not involved.

The cell bodies of all of the primary nociceptive neurons are located in the dorsal root ganglia (DRG). In the adult DRG, all three ρ mRNAs were found at relatively high levels by RT-PCR (Wegelius *et al.*, 1998; Wegelius, 2000), and ρ-immunoreactivity was shown to be quite uniform, labelling the DRG neurons of different sizes (Zheng *et al.*, 2003). In DRG, some bicuculline-resistant GABA-responses have been detected, but not further identified (Valeyev *et al.*, 1996).

The expression pattern suggested a role for the ρ_1 subunit in modulating spinal cord pain input and transmission, and this was nicely demonstrated *in vivo* with the generation of $\rho_1^{-/-}$ knock-out mice. In mice completely lacking the ρ_1 (and, at least in spinal cord, also ρ_2) subunit, the gross neuroanatomical structure of spinal cord and DRG was unchanged and the animals seemed healthy, but the spinal cord response to electric stimulation was markedly less sensitive to GABA. Moreover, the mechanical pain threshold was significantly decreased, putatively causing increased sensitivity and overexcitation of the primary nociceptive neurons, thus resembling allodynia in humans (Zheng *et al.*, 2003).

2.2.5.7. $GABA_C$ receptors outside the central nervous system

In the rat pituitary gland, ρ_1 and ρ_2 mRNAs were shown, the level of the former being considerably higher. Furthermore, ρ-immunoreactivity was found on thyrotropin-secreting cells and growth-hormone-secreting cells, and bicuculline-insensitive but desensitizing currents were detected using patch-clamp recordings (Boué-Grabot *et al.*, 2000; Gamel-Didelon *et al.*, 2003).

Intriguingly, a peripheral, subcutaneous administration of CACA to a hind paw elicited a local antinociceptive effect in the paw pressure test, and this effect was antagonized by TPMPA and picrotoxin, but not by potassium channel blockers (Reis and Duarte, 2007).

In the myenteric plexus of the rat gastrointestinal tract, the expression of all three ρ subunit mRNAs and ρ-immunolabelling mainly on intrinsic sensory and inhibitory motor neurons were evident (Fletcher *et al.*, 2001). Functional native $GABA_C$ receptors have been demonstrated on gut neuroendocrine tumour cell line STC-1 cells, where CACA induced calcium influx and increased cholecystokinin secretion, effects antagonized by TPMPA (Jansen *et al.*, 2000). In the neurons of the rat major pelvic ganglia, both $GABA_A$ and $GABA_C$ receptors were shown to have distinctive roles in mediating biphasic GABA responses (Akasu *et al.*, 1999). The sensitive RT-PCR has also shown ρ_2 mRNA expression in the heart, liver, gonadal endocrine tissues, adrenal gland and placenta, but no signs of functional $GABA_C$ receptors have been revealed thus far (Boué-Grabot *et al.*, 1998; Akinci and Schofield, 1999; Rozzo *et al.*, 2002).

2.3. Depolarizing GABA responses

GABA is known to have an excitatory action in the developing nervous system because the reversal potential for chloride (E_{Cl}) is more depolarized than the resting membrane potential and the action potential threshold in immature neurons (reviewed by Ben-Ari, 2002). The main underlying factors leading to a depolarization, instead of a hyperpolarization, when $GABA_A$ receptors are activated, are the distinctive contributions

of cation-chloride co-transporters in immature neurons compared with mature neurons. First to appear in cells is the sodium-potassium-chloride co-transporter NKCC1, which raises the intracellular chloride concentration and is downregulated during development (Plotkin *et al.*, 1997), while the potassium-chloride co-transporter KCC2, which actively lowers the intracellular chloride concentration, is upregulated after the first postnatal week (Rivera *et al.*, 1999). Probably, the role of this depolarization is to promote the growth of synapses and neuronal networks, as GABA can act as a trophic factor and GABAergic synapses appear first in the development (Owens and Kriegstein, 2002). During most of the embryonic phase, GABA-mediated giant depolarizing potentials provide the majority of the activity, as the network is not sufficiently developed to generate more elaborate patterns of activity (Khazipov *et al.*, 2001; Crépel *et al.*, 2007). Gradually and in an activity-dependent manner, the expression of KCC2 begins, chloride levels decrease and GABA responses become predominantly hyperpolarizing (reviewed by Ben-Ari, 2002).

There are a few situations where GABA mediates depolarizing responses also in adult neurons. Already decades ago, GABA was shown to have an excitatory action on DRG cells (Eccles *et al.*, 1963), and, more recently, DRG cells were demonstrated to maintain high expression of NKCC1 and low expression of KCC2 throughout development (reviewed by Stein and Nicoll, 2003). Furthermore, under certain circumstances, GABAergic transmission on any cortical neuron can have an excitatory action, and the net effect depends not only on the resting membrane potential, but also on the location and timing of the GABAergic input relative to the excitatory (glutamatergic) input (Gulledge and Stuart, 2003; Lamsa and Taira, 2003). In temporal lobe epilepsy, depolarizing GABA responses have been implicated in pathological, interictal epileptic activity (Cohen *et al.*, 2002).

A special case of depolarizing GABA responses is the biphasic, prolonged response following vigorous stimulation of hippocampal interneurons at *stratum radiatum* (Kaila *et al.*, 1997; Smirnov *et al.*, 1999; Voipio and Kaila, 2000; Ruusuvuori *et al.*, 2004; Rivera *et al.*, 2005). A high-frequency stimulation (HFS) train in the presence of ionotropic glutamate receptor antagonists results in a massive, synchronous excitation of hippocampal pyramidal neurons, which is either named GABA-mediated depolarizing postsynaptic potential (GDPSP; Fig. 7 in Materials and methods) or non-synaptic potential (GDNSP). The mechanisms behind this phenomenon have been debated (Staley *et al.*, 1995; Perkins, 1999; Staley and Proctor, 1999; Voipio and Kaila, 2000), but the most accepted theory suggests an essential role for bicarbonate-ion and the enzyme carbonic anhydrase (Ruusuvuori *et al.*, 2004; Rivera *et al.*, 2005). This qualitative change of action of GABAergic transmission takes place not only in response to HFS, but, putatively, also during epileptiform activity and the induction of LTP, showing once more the unique plasticity of neurotransmission under different conditions (Rivera *et al.*, 2005).

3. Aims of the Study

A decade of intensive studies on ρ subunits and $GABA_C$ receptors had revealed a great deal of information, especially on their molecular structure, but less on their function and very little on their role outside the retina and superior colliculus (SuC). In this work, we aimed to elucidate the expression of ρ subunits in the postnatal brain, the characteristics of ρ_2 homo-oligomeric receptors, and the function of $GABA_C$ receptors in the hippocampus. The aims of the individual studies were as follows.

I. Although the distribution of ρ subunit mRNAs in the adult brain had been examined using RT-PCR and *in situ* hybridization, information on the developmental regulation was scattered and incomplete. We wanted to investigate the expression of all three known ρ subunits in the brain throughout the postnatal period, with particular emphasis on developmental changes in subunit expression in the hippocampus and SuC. In these areas, our aim was also to get a quantitative assessment of putative $GABA_C$ receptor subunit combinations.

II. Previous work done on *Xenopus* oocytes had suggested that rat ρ_2 subunits do not form functional homo-oligomeric $GABA_C$ receptors but need ρ_1 or ρ_3 subunits to form hetero-oligomers with relatively low sensitivity to PiTX. Because previous data had, however, indicated a much higher expression level for the ρ_2 subunit transcripts than for ρ_1 or ρ_3 in the brain, we wanted to test whether ρ_2 subunits can be functionally expressed as homo-oligomers in mammalian cells. In addition, we aimed to characterize the properties of these homo-oligomeric receptors, especially their picrotoxin sensitivity.

III. As both $GABA_C$ receptor subunit mRNA and protein were shown to be expressed in the *stratum pyramidale* in the CA1 area of the adult rat hippocampus, but no conclusive evidence for functional receptors there had been demonstrated, we wanted to study the effects of $GABA_C$ receptor agonists and antagonists on responses of the hippocampal neuron population to electrical stimulation. Next, we aimed to display the activation of the $GABA_C$ receptors by synaptically released GABA. Fast inhibitory synaptic currents in the hippocampal CA1 area had previously been shown to be exclusively mediated by $GABA_A$ receptors, but we hypothesized that $GABA_C$ receptors in hippocampal CA1 neurons might be extrasynaptic and possibly activated by the spillage of synaptic GABA.

4. Materials and Methods

Detailed descriptions of the materials and methods applied in this work are given in the original publications (see Table 2 for references). A variety of methods focusing on different aspects were chosen to improve the reliability of the results. More specifically, with *in situ* hybridization one can detect a specific mRNA in a section, whereas immunocytochemistry localizes certain proteins in a given tissue section. Quantitative RT-PCR quantifies mRNA expression, but as RT-PCR is a very sensitive method, it should always be combined with other approaches to avoid physiologically less relevant results. Patch-clamp recordings in transfected cells enable detailed characterization of receptor subunits, while recordings in brain slices yield information on the function of the whole neuronal network in a given structure. Because the hippocampal CA1 area possesses a wide variety of transmitter receptors, it is crucial to combine the results from slice recordings with the data from other experiments in order to see the contribution of $GABA_C$ receptors in the hippocampus.

Table 2. The methods used in the original studies.

Method	Study
In situ hybridization	**I**
Quantitative RT-PCR	**I**
Immunocytochemistry	**I**
Expression of ρ subunits in HEK 293 cells	**II**
Immunofluorescence staining	**II**
Patch-clamp recordings in transfected cells	**II**
Hippocampal slices	**III**
Extracellular recordings in brain slices	**III**
Intracellular recordings in brain slices	**III**

As an exception, the method of preparing hippocampal slices is not fully described in **III** and is given here. P21 – P47 Wistar rats were anaesthetized with a mixture of ketamine and medetomidine given intraperitoneally. They were transcardially perfused with ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $MgSO_4$, 2 $CaCl_2$, and 10 glucose, continuously gassed with 5% CO_2 – 95% O_2. Within 4 min of perfusion, the brains of the animals were cooled down to diminish the metabolic rate and to prevent the degradation of brain tissue. The rats were then decapitated with a guillotine and the brains were exposed within 60 s. The tissues were placed in ice-cold, previously gassed, low NaCl solution containing (in mM) 3 KCl, 8 $MgCl_2$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose, and 230 sucrose, both during the exposition of the brains and while 400-µm-thick transverse slices of the hippocampi were made using a vibratome. The slices were allowed to recover in continuously gassed ACSF at 32°C for 30 min and then at room temperature for at least 30 min before use.

During the recordings, a stimulation electrode was placed in the CA1 area to stimulate the Schaffer collaterals in the *stratum radiatum* (Fig. 5). Extracellular responses (Fig. 6) were recorded from the *stratum pyramidale*. Membrane input resistance was measured in intracellular recordings using injections of hyperpolarizing current. GDPSPs (Fig. 7) were elicited by HFS and recorded inside pyramidal neurons. All of the chemicals used in patch-clamp and slice recordings are listed in Table 3 together with the concentrations used.

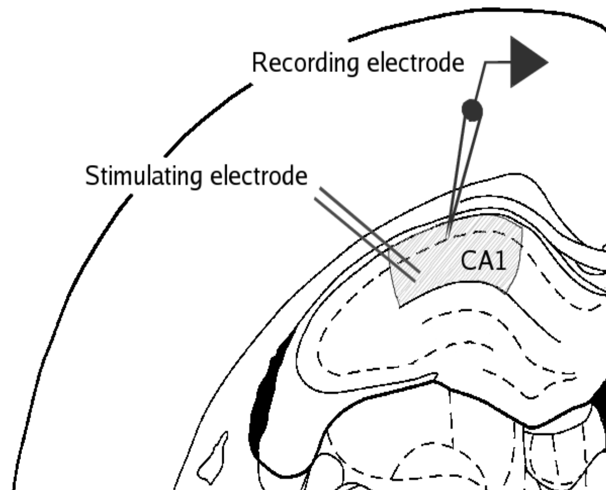


Fig. 5. Schematic of the recording site in the hippocampal slice.
The stimulus electrode was placed in the *stratum radiatum* and the recording electrode in the *stratum pyramidale*. HFS trains were given closer to the pyramidal neuron, from which the voltage was recorded.

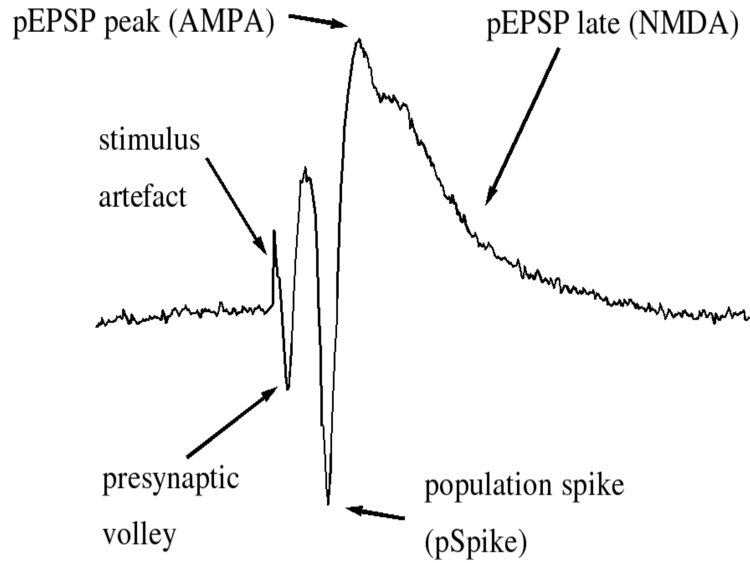


Fig. 6. An example of the extracellular field potential response showing the pEPSP and pSpike referred to in Section 5.3.1.

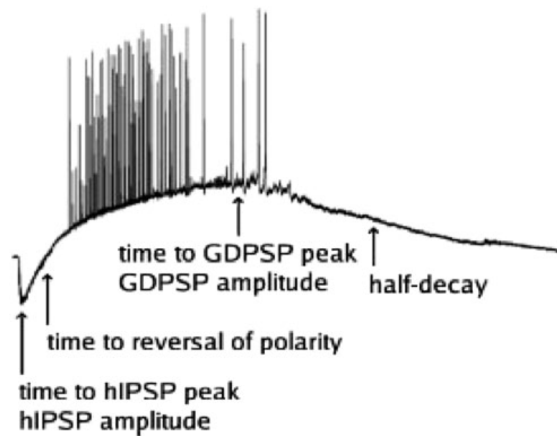


Fig. 7. High-frequency stimulation of local inhibitory interneurons in the presence of ionotropic glutamate receptor and $GABA_B$ receptor blockers evokes GABA-mediated long-lasting depolarizing responses. An example of the GDPSP response showing the different parameters referred to in Section 5.3.3. Reproduced with permission from Alakuijala *et al.*, 2006. © Federation of European Neuroscience Societies and Blackwell Publishing Ltd.

Table 3. Chemicals used in different kinds of electrophysiological recordings.

Drug	Effect	Concentration(s) used
AP5	Ionotropic glutamate receptor antagonist	40 µM (intracellular recordings)
BIM	$GABA_A$ receptor antagonist	1–5 µM (extracellular recordings), 100 µM (intracellular recordings)
CACA	$GABA_C$ receptor agonist	1–50 µM (patch-clamp recordings), 0.75–5 µM (extracellular recordings), 2–20 µM (intracellular recordings)
CGP 46381	$GABA_B$ receptor antagonist	50–100 µM (intracellular recordings)
CNQX	Ionotropic glutamate receptor antagonist	20 µM (intracellular recordings)
GABA	GABA receptor agonist	0.3–100 µM (patch-clamp recordings)
PiTX	$GABA_A$ and $GABA_C$ receptor blocker	0.01–100 µM (patch-clamp recordings), 100 µM (intracellular recordings)
SKF 89976A	GABA transporter blocker	10 µM (intracellular recordings)
TPMPA	$GABA_C$ receptor antagonist	1–10 µM (extracellular recordings), 10–50 µM (intracellular recordings), 50–500 µM (intracellular recordings with BIM)

5. Results

5.1. Expression of ρ subunits in the developing rat brain (I)

5.1.1. ρ_2 subunit mRNA expression in the developing rat retina and brain

Of the three $GABA_C$ receptor ρ subunits tested in the adult rat brain, our *in situ* hybridization revealed only ρ_2 subunit mRNA. While the ρ_1 *in situ* probe gave positive hybridization in the adult retina, the ρ_3 probe was negative in both the adult retina and brain.

In the developing retina, no expression of ρ_2 mRNA was seen at P0, but high expression could be detected in the inner nuclear layer (INL) of the retina at P8, P14, and P60 (Fig. 1A in **I**). These findings are in line with previous reports, demonstrating the specificity of our hybridization probe.

In the developing superior colliculus (SuC), *in situ* hybridization displayed strong expression of ρ_2 mRNA in the superior grey layer (SGL) from P8 to P60, the expression level being maximal in the adult SuC (Fig. 1B in **I**). Already at the time of birth, weak expression was seen, limited to the superficial border of SuC.

Between P8 and P60, ρ_2 transcripts were also visible in the CA1 region of the hippocampus (Fig. 1B in **I**). The hippocampal expression level increased during early postnatal development, was maximal after the second postnatal week and decreased somewhat towards adulthood, when it was clearly less prominent than in the SuC.

In postnatal developmental stages, ρ_2 subunit mRNA was also apparent in various other parts of the visual system: NOT from the second postnatal week to adulthood (Fig. 1B in **I**), dLGN at P60, and MTN from P14 to P60. In addition, ρ_2 transcripts were seen in three layers of the adult frontal cortex.

5.1.2. Quantitative ρ subunit mRNA detection in the hippocampus and SuC

qRT-PCR results indicated expression of all three ρ subunit mRNAs at varying levels from the newborn to adult hippocampus and SuC (Fig. 4 in **I**). In the hippocampus, the highest expression levels were evident for ρ_2 transcripts, confirming the observation made with *in situ* hybridization. In the hippocampus, ρ_3 was expressed at a higher level than ρ_1, with the exception of P14, while in the SuC the opposite was observed, except at P7, when ρ_3 was more abundant. Interestingly, the total amount of ρ subunit mRNA peaked at P7 and P21 in both the hippocampus and SuC. These findings are consistent with our *in situ* hybridization results during the postnatal development, with the only exception occurring at P14, when qRT-PCR showed decreased, but *in situ* increased, expression levels in both the hippocampus and SuC.

5.1.3. ρ subunit protein expression in the adult brain

An antibody recognizing all three ρ subunits was used to investigate whether $GABA_C$ receptor protein is expressed in those areas of the brain showing ρ subunit mRNA expression. The strongest immunoreactivity of all structures inspected was in the SuC, where a prominent density gradient was apparent across its different layers, the highest density of ρ subunits being found in the SGL (Fig. 5A,B in **I**). Immunolabelling with lower density was also visible within the intermediate and deep layers of the SuC, but the distribution of ρ subunits seemed not to follow the map of the visual field representation within the SuC.

In addition to SuC, $GABA_C$ receptor protein was illustrated in the adult dLGN (Fig. 5C in **I**), hippocampus (Fig. 5D-G in **I**), and MTN, all areas where ρ_2 mRNA was detected by *in situ* hybridization. In the dLGN, immunostaining appeared to form layers with mediolateral orientation. In the hippocampus, staining was restricted to the CA1 area. Immunopositive puncta were to a large extent restricted to the *stratum pyramidale*, although some puncta were also visible in the *stratum radiatum* (Fig. 5E-G in **I**).

5.2. Characterization of ρ_2 homo-oligomeric receptors (II)

5.2.1. ρ_2 subunits formed homo-oligomeric $GABA_C$ receptors

HEK 293 cells transfected with either ρ_1, ρ_2, or both ρ_1 and ρ_2 subunit cDNAs all displayed GABA-induced currents in whole-cell patch-clamp recordings (Fig. 1A in **II**). The maximum currents observed in cells expressing homomeric ρ_2 receptors were about two orders of magnitude smaller than in cells expressing homomeric ρ_1 receptors. Homo-oligomeric ρ_2 receptors also needed at least 3 days to mature after transfection before any GABA-induced currents could be detected, while currents with ρ_1 receptors were readily seen after 24 – 48 h.

This late maturation was confirmed with surface expression studies. FLAG-tagged homomeric ρ_2 receptors appeared in the endoplasmic reticulum within 42 h after transfection and the expression peaked at 66 h, decreasing slightly at 90 h (Fig. 2 in **II**). In contrast, the surface expression of ρ_2 was seen only at 66 h and increased until 90 h. In addition, the relatively weak surface expression of ρ_2 was strongly improved by heteromeric expression with either ρ_1 or ρ_3, while all constructs displayed similar intracellular staining by anti-FLAG antibody under permeabilized conditions (Fig. 3 in **II**).

5.2.2. Agonist sensitivity of homo-oligomeric ρ_2 and hetero-oligomeric $\rho_1\rho_2$ receptors

Homomeric ρ_2 receptors were more sensitive to GABA than ρ_1 receptors, and heteromeric receptors ranked between the ρ_1 and ρ_2 receptors in their sensitivity to GABA (Fig. 1B in **II**). After GABA application, the homomeric ρ_2 receptors deactivated more slowly than

the others, while the hetero-oligomeric $\rho_1\rho_2$ receptors were the fastest in deactivation kinetics.

Furthermore, compared with ρ_1 subunit-containing receptors, rat ρ_2 homo-oligomeric receptors expressed in HEK 293 cells were far more sensitive to CACA, which acted as a partial agonist with an EC_{50} of ≈ 6 μM, the maximum current being 65% of that observed with saturating GABA concentration (Fig. 4 in **II**). The deactivation after CACA application was faster than that seen with GABA in all subunits.

5.2.3. Picrotoxin sensitivity of homo-oligomeric ρ_2 and hetero-oligomeric $\rho_1\rho_2$ receptors

The homomeric ρ_2 receptors were noticeably less sensitive than ρ_1 receptors to PiTX (Fig. 5A in **II**). 100 μM PiTX inhibited only half of the maximum GABA current of the ρ_2 receptors and two-thirds of that of the hetero-oligomeric $\rho_1\rho_2$ receptors. Surprisingly, the affinity of ρ_2 receptors for PiTX was still very high, the IC_{50} of the picrotoxin-sensitive component being only about one-tenth of the IC_{50} of the ρ_1 receptors (Fig. 5B in **II**).

5.3. $GABA_C$ receptors in the rat mature hippocampus (III)

5.3.1. Effects of CACA and TPMPA on excitability

In extracellular recordings from the *stratum pyramidale* in the hippocampal CA1 area, the $GABA_C$ receptor agonist CACA potently inhibited stimulus-evoked pEPSP and pSpike amplitudes at very low concentrations (Fig. 8). The inhibition of the pSpikes and pEPSPs produced by CACA was reversed by the $GABA_C$ receptor antagonist TPMPA in a concentration-dependent manner (Fig. 1B in **III**), demonstrating that functional $GABA_C$ receptors are expressed in the CA1 area. Moreover, CACA was able to reduce multiple, epileptiform discharges produced by the $GABA_A$ receptor antagonist BIM by one-third, showing that the inhibitory effect of CACA was not mediated by $GABA_A$ receptors.

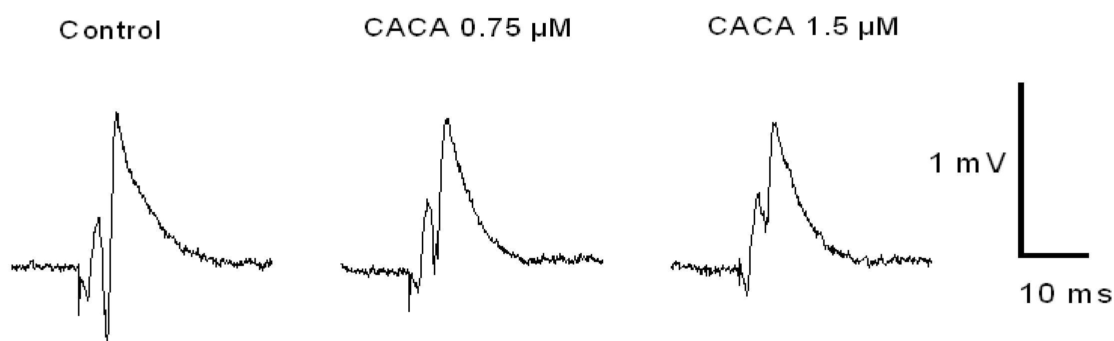


Fig. 8. $GABA_C$ receptor activation reduces excitability in the hippocampus. CACA inhibits pEPSPs and pSpikes at low concentrations.

5.3.2. Effects of CACA on membrane input resistance

In intracellular current-clamp recordings, CACA induced a small but significant decrease in the input resistances of neurons calculated from the voltage deflections produced by injections of hyperpolarizing current into the cell (Fig. 3A in **III**). The CACA-induced change in input resistance corresponds to approximately 100 channels/neuron, assuming a single-channel conductance of about 10 pS for ρ subunit-containing receptors (Feigenspan and Bormann, 1994). Small voltage fluctuations were observed during the experiments, which precluded the observation of possible minor potential changes elicited by activated $GABA_C$ receptors with a reversal potential rather close to the resting membrane potential.

5.3.3. GDPSP experiments

A HFS train given in the *stratum radiatum* in the presence of ionotropic glutamate receptor blockers results in a biphasic GABA response termed GDPSP or GDNSP (see Section 2.3 and Figure 7 in Materials and methods). We hypothesized that $GABA_C$ receptors in the hippocampal CA1 area would be extrasynaptic, possibly activated by the spillover of synaptic GABA following this strong stimulation (Isaacson *et al.*, 1993). In a total of 17 neurons from different slices, the GDPSP was clearly prolonged by TPMPA, indicating that $GABA_C$ receptors are activated by synaptically released GABA and that they contribute to the attenuation of the depolarizing phase of the GDPSP (Fig. 9A-C). During the HFS train, TPMPA affected the hyperpolarizing response within the first 100 ms (Fig. 9D), but the difference in the voltage between TPMPA and the control grew gradually larger over the course of the following 500 ms, suggesting accumulating activity of the $GABA_C$ receptors.

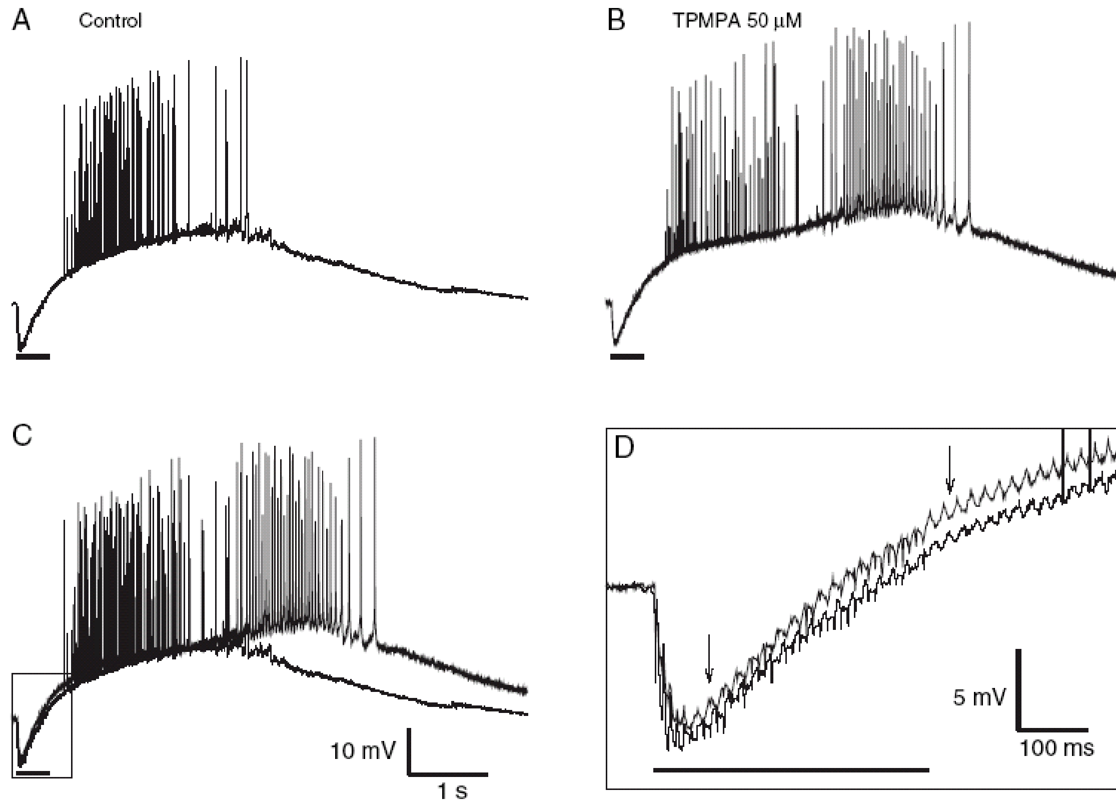


Fig. 9. $GABA_C$ receptors contribute to depolarizing GABA responses.
(A,B) GDPSPs evoked in the presence of ionotropic glutamate receptor and $GABA_B$ receptor antagonists are prolonged by 50 μM TPMPA. (C) The original traces are superimposed. Thin line, control; thicker line, TPMPA. The average effect of TPMPA was +16 ± 7.1% (P = 0.04) on time to hIPSP peak, +24 ± 8.0% (P = 0.009) on time to GDPSP peak, and + 30 ± 8.0% (P = 0.002) on half-decay of GDPSP. The box indicates where the magnification (D) has been taken. (D) A magnification of the first 650 ms of the response to emphasize the effects of TPMPA on the hyperpolarizing part of the response. The difference in the average voltage between TPMPA and control in this cell was 1.05 mV within 100 ms starting from the left arrow and 1.69 mV within 100 ms starting from the right arrow. Horizontal bars represent the time course of the HFS train. Reproduced with permission from Alakuijala *et al.*, 2006. © Federation of European Neuroscience Societies and Blackwell Publishing Ltd.

With smaller stimulus intensity, insufficient to elicit a GDPSP, no TPMPA effect was seen (Fig. 10A,B). As the spillover of the neurotransmitter outside the synaptic cleft can be increased either by vigorous stimulation or by preventing the transmitter uptake after synaptic release, we further tested the effect of SKF 89976A, a selective blocker of GAT-1, which is the major GABA transporter in the hippocampus (Ali *et al.*, 1985). In the presence of 10 μM SKF 89976A, we saw a small depolarizing GABA response with a stimulus train of five stimuli and – in clear contrast to the observation made in the presence of GABA uptake – TPMPA had a clear enhancing effect on the GDPSP amplitude and the GDPSP half-decay (Fig. 10C,D). When GAT-1 was blocked by SKF 89976A, the effect of TPMPA was most prominent with small stimulus intensity and

decreased with increasing stimulus intensity to levels similar to those observed in the absence of GAT-1 block (Table 1 in **III**). This implies that at higher stimulus intensities $GABA_C$ receptors are already maximally activated by synaptically released GABA, even without GABA uptake inhibition. Taken together, our data indicate that $GABA_C$ receptors in the hippocampal CA1 area are extrasynaptic and activated intrinsically by synaptically released GABA via spillover.

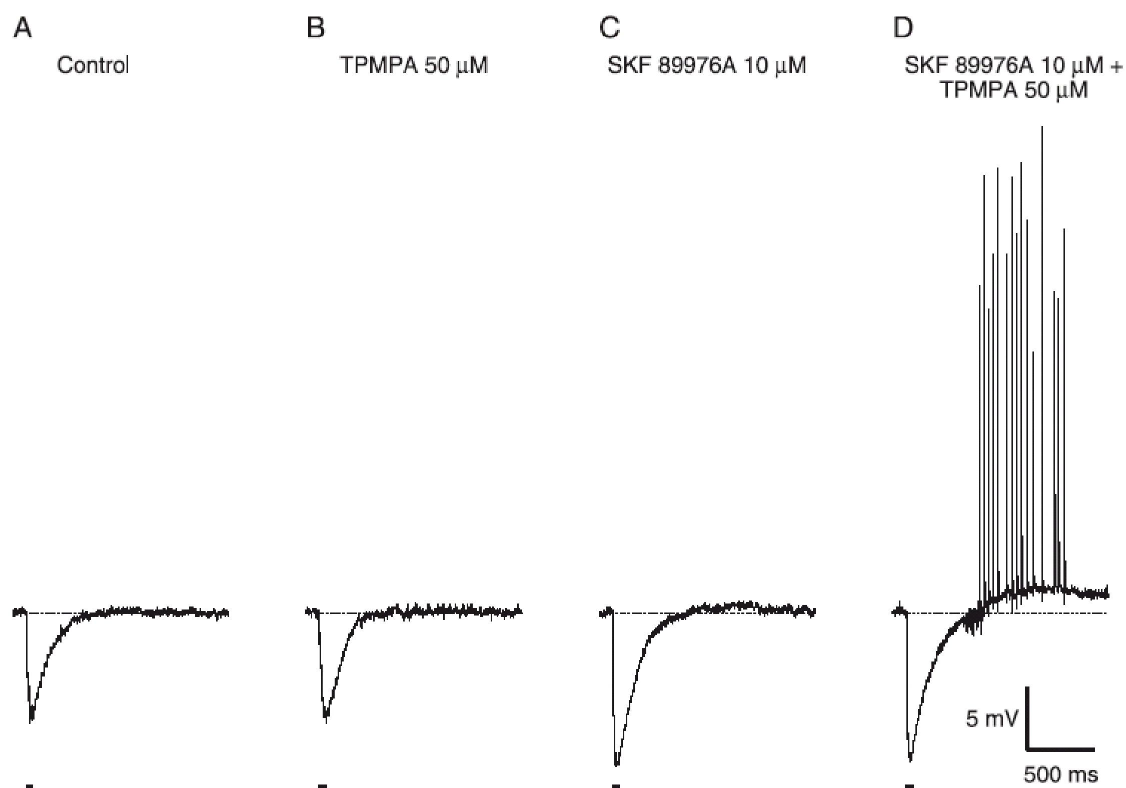


Fig. 10. Blocking GABA uptake results in $GABA_C$ receptor activation with a few stimuli.
(A,B) When five consecutive stimuli are given at 100 Hz, only a hyperpolarizing response is seen in control (A) or in 50 μM TPMPA (B). (C) GAT-1 transporter blocker SKF 89976A, 10 μM, increases the amount of the transmitter in the vicinity of the synapse density and a minor depolarizing response is detected. (D) In the presence of GAT-1 blocker, TPMPA increases the amplitude and duration of the GDPSP sufficiently to introduce spiking. Horizontal bars represent the time course of the HFS train. Note that traces (A,B) and (C,D) are from separate experiments, thus, traces (B) and (C) cannot be compared. Reproduced with permission from Alakuijala *et al.*, 2006. © Federation of European Neuroscience Societies and Blackwell Publishing Ltd.

6. Discussion

6.1. ρ_2 subunits dominate in the postnatal brain

Based on the literature and our results with *in situ* hybridization and qRT-PCR, in the brain ρ_2 is the most abundant $GABA_C$ receptor subunit, present more widely than previously suspected, but still selectively. Moreover, $GABA_C$ receptor protein immunostaining coincides fully with the expression of ρ_2 subunit transcripts.

Similar expression levels of the ρ_2 subunit in the hippocampus and SuC were revealed by qRT-PCR, as expected from the *in situ* hybridization. The expression of ρ_3 was in general higher in the hippocampus than in the SuC, while ρ_1 was clearly more abundant in the SuC. These data imply that the $GABA_C$ receptor subunit composition may differ between these two regions. The low levels of both ρ_1 and ρ_3 mRNAs in the hippocampus suggest that adult hippocampal $GABA_C$ receptors might predominantly be ρ_2 homo-oligomers. Of course, we cannot exclude the possibility of co-assembly of ρ_2 subunits with $GABA_A$ receptor subunits, but, hitherto, no evidence supporting ρ_2 hetero-oligomerization with other than ρ subunits anywhere in the nervous system has been presented. In SuC, ρ_1 expression levels were similar to ρ_2, suggesting a receptor subunit composition similar to that in the retina, i.e. $\rho_1\rho_2$ hetero-oligomers (Boué-Grabot *et al.*, 1998; Greka *et al.*, 2000).

As for the localization of the $GABA_C$ receptors in the hippocampus, we demonstrated that ρ_2 expression is restricted to the CA1 area throughout postnatal development. Our *in situ* data show that the majority of the somata expresses ρ_2 mRNA in the CA1 *stratum pyramidale* (Fig. 8B), where the proportion of inhibitory interneurons is estimated to be only about 7% of all neurons (Aika *et al.*, 1994); thus, $GABA_C$ receptors are putatively localized at least to a major subpopulation of the pyramidal neurons in the CA1 area. Our results are in line with some previous reports on the developing and adult hippocampus (Boué-Grabot *et al.*, 1998; Wegelius *et al.*, 1998; Ogurusu *et al.*, 1999), but in contrast to one report showing the most prominent staining in the interneurons throughout the different subfields, although the ρ_2 staining was the strongest in the CA1 area (Rozzo *et al.*, 2002). These discrepancies may be due to the different sensitivity levels of hybridization probes used in the studies.

The significance of $GABA_C$ receptors outside the retina had been questioned because even though ρ_2 subunits dominate in the rat brain, they did not form functional receptors when expressed alone in *Xenopus* oocytes (Zhang *et al.*, 1995; 2001; Boué-Grabot *et al.*, 1998). The lower temperature used in oocyte incubation or the different intracellular environment in oocytes compared with mammalian cells may somehow disturb the maturation of the ρ_2 protein or its transport to the cell membrane, as was further illustrated with our surface expression studies. Human and mouse ρ_2 homo-oligomeric receptors, on the other hand, were functional in oocytes, albeit the GABA-induced currents in them were small (Kusama *et al.*, 1993b; Greka *et al.*, 1998).

While their expression is challenging, ρ_2 subunits carry several unique biophysical and pharmacological properties. Table 4 summarizes the essential pharmacology of the three ρ subunits based on the literature and our studies. ρ_2 homomeric receptors seem to be one of the most sensitive among all GABA receptors to their natural agonist, together with

some α_4 or α_6 subunit-containing extrasynaptic $GABA_A$ receptors. As for CACA, the relatively high affinity of rat ρ_2 receptors implies that at concentrations below 10 µM, CACA activates these receptors highly selectively. This only applies to rat ρ_2 receptors, though, as human homo-oligomeric ρ_2 receptors have a tenfold higher EC_{50} for CACA (Kusama *et al.*, 1993a; Enz and Cutting, 1999).

Table 4. Pharmacological characteristics of individual ρ subunits.

Agonist/antagonist	**ρ_1 subunit**	**ρ_2 subunit**	**ρ_3 subunit**
GABA EC_{50} (µM)	Rat: 1.1–2.3 [1,**II**], human: 0.81–1.7 [2,3]	Rat: 0.96 (**II**), human: 1.8 [3]	Rat: 4.0–7.5 [4,5]
CACA EC_{50} (µM)	Human: 41–131 [6,7]	Rat: 6.3 (**II**), human 62–70 [8,7]	Rat: 65–139 [9,5]
TPMPA K_b (µM)	Human: 2.3 [10]	Human: 14.9 [10]	Rat: 4.5 [10]
PiTX IC_{50} (µM) + GABA 10 µM	Rat: 6.7 (**II**)	Rat: 0.60 (**II**), but only partial antagonism	Rat: 3.2 [9]
PiTX IC_{50} (µM) + GABA 20 µM	Rat: 40 [1], human: 48 [11]	Human: 4.7 [11]	Rat: 5.9 [10]

References: [1] Zhang *et al.*, 1995, [2] Chang and Weiss, 1999, [3] Wang *et al.*, 1994, [4] Shingai *et al.*, 1996, [5] Vien *et al.*, 2002, [6] Carland *et al.*, 2004, [7] Enz and Cutting, 1999, [8] Kusama *et al.*, 1993a, [9] Ogurusu *et al.*, 1999, [10] Chebib *et al.*, 2007, [11] Wang *et al.*, 1995b

In addition to agonist sensitivity, human and rat ρ_2 receptors differ also in their sensitivity to PiTX, and in their kinetic properties; human ρ_2 receptors are more picrotoxin-sensitive and deactivate faster than human ρ_1 receptors (Enz and Cutting, 1999), while the opposite was seen in rats in our study. Interestingly, the only differences in the channel-lining second transmembrane domain between rat and human ρ_2 are the 6' methionine and 12' serine residues in rat, as opposed to threonine in man. The only differences between rat ρ_2 and ρ_1 are the 2' serine instead of a proline in ρ_1 and the same 6' methionine residue, which is, in fact, unique among all GABA receptors (Fig. 11; Cutting *et al.*, 1992; Zhang *et al.*, 1995; Korpi *et al.*, 2002). These are the very same amino acid residue positions that have been demonstrated to affect picrotoxin sensitivity in many substitution studies (Gurley *et al.*, 1995; Wang *et al.*, 1995b; Xu *et al.*, 1995; Zhang *et al.*, 1995; Zhorov and Bregestovski, 2000). Furthermore, the single-channel conductance of ρ receptors has recently been shown to depend on the 2' amino acid residue in the second transmembrane domain, as well (Zhu *et al.*, 2007). Identically to mammalian subunits, the sequence in the white perch ρ_1 subunit contains proline and in the ρ_2 subunit serine at the 2' position. The homo-oligomeric receptors formed by ρ_2 subunits gated a channel with a 16-fold conductance compared with receptors formed by ρ_1 subunits, and mutating the 2' serine to proline reduced the conductance to a level similar to that of the wild-type ρ_1 receptor (Zhu *et al.*, 2007).

	Position	2' 6' 12'
ρ_1	Rat	P A R V P L G I T T V L T M S T I I T G V
	Human	P A R V P L G I T T V L T M S T I I T G V
ρ_2	Rat	P A R V S L G I M T V L T M S T I I T G V
	Human	P A R V S L G I T T V L T M T T I I T G V
ρ_3	Rat	P A R V S L G I T T V L T M S T I V T G V

Fig. 11. Comparison of amino acid sequences in the channel-lining second transmembrane domain between human and rat ρ subunits.
The different amino acid residues between rat and human ρ_2 subunit and between rat ρ_2 and ρ_1 are underlined. The second transmembrane domain in rat and human ρ_1 subunits is identical.

In our experiments, no concentration of PiTX could fully block the GABA current of ρ_2 subunit-containing homo- or hetero-oligomeric receptors. Still, the picrotoxin-sensitive component displayed a very high affinity. This high-affinity partial picrotoxin antagonism seems to be unique to rat ρ_2 receptors. PiTX has been suggested to interact with two binding sites (see Section 2.2.2.2.). The simplest explanation for our findings would be that rat ρ_2 receptors – with the missing threonine ring at the 6' position in the second transmembrane domain – lack the non-competitive binding site to GABA, and only the allosteric binding mechanism is left, but acting with a very high affinity. The allosteric mechanism deteriorates the efficacy of channel opening, but can never block it completely, thus resulting in partial antagonism. In the future, it would be highly interesting to determine the characteristics of a mutated ρ_2 subunit where the 6' methionine has been changed to threonine.

Rat hetero-oligomeric $\rho_1\rho_2$ receptors seemed to be relatively close to ρ_2 receptors in their low and partial sensitivity to PiTX, implying high picrotoxin affinity as a dominant trait. Previously, in their study on heteromeric ρ receptors, Zhang and co-workers (1995) reported that rat $\rho_1\rho_2$ receptors are picrotoxin-resistant, but, actually, their results are equally consistent with a single picrotoxin binding site, as suggested by our data. Because homomeric ρ_2 receptors were not functional in *Xenopus* oocytes, they used ρ_1 25% : ρ_2 75% hetero-oligomeric receptors and observed a highly decreased sensitivity to PiTX. A simple explanation, in light of our data, is that their results also contain a single, high-affinity, low-efficacy PiTX effect, seen at very low concentrations of PiTX to already affect the GABA response. Mouse ρ_2 homo-oligomeric receptors were only tested with 100 µM PiTX, which blocked 5–22% of the 1 µM GABA response, again in accord with our results (Greka *et al.*, 1998).

6.2. Hippocampal $GABA_C$ receptors

Our findings from hippocampal slices with conventional stimulation indicate that functional $GABA_C$ receptors are present in the CA1 area and can be activated by a specific

agonist even in bicuculline-mediated hyperexcited conditions. Consistent with small chloride conductance of $GABA_C$ receptor channels, the effect of $GABA_C$ receptor activation was not large; these receptors seem to modulate, rather than block, the overall excitability of the pyramidal neurons.

More impressively, our results from the GDPSP experiments demonstrate that $GABA_C$ receptors can be activated intrinsically, either with strong inhibitory synaptic activity or by a weaker stimulus when GABA uptake is blocked. In the absence of GAT-1 transporter blocker, only strong stimulation could markedly activate $GABA_C$ receptors. However, when GABA uptake was inhibited, a less vigorous stimulus train was sufficient, implying that uptake inhibition increased transmitter spillage to the extrasynaptic areas, resulting in the $GABA_C$ receptor activation. Our hypothesis about hippocampal $GABA_C$ receptors being extrasynaptic is also supported by the lack of desensitization and the high affinity for GABA of both $GABA_C$ receptors and extrasynaptic $GABA_A$ receptors (see Section 2.1.2.). Furthermore, putatively extrasynaptic $GABA_C$ receptors have been found in the retina, SuC, and NOT (Schmidt *et al.*, 2001; Ichinose and Lukasiewicz, 2002; Boller and Schmidt, 2003; Kirischuk *et al.*, 2003). Figure 12 is a schematic illustration of synaptic GABA spillover.

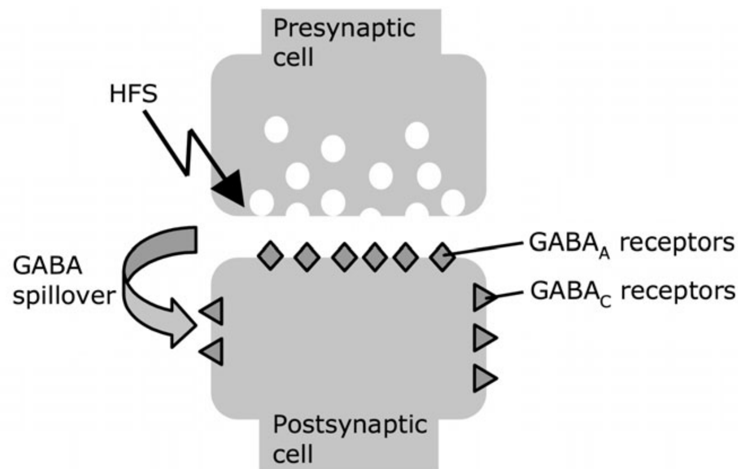


Fig. 12. Schematic of the concept of GABA spillover.
According to our hypothesis, $GABA_C$ receptors in the hippocampal pyramidal cells are extrasynaptic and activated by the spillage of GABA outside the synaptic clefts following HFS or when GABA uptake is blocked. In addition, $GABA_C$ receptor activation can be heterosynaptic, i.e. transmitter spilled over from synapses in adjacent neurons can sometimes activate sensitive $GABA_C$ receptors.

Previously, extrasynaptic $GABA_A$ receptors have been demonstrated to mediate tonic currents in interneurons and pyramidal cells in the hippocampus (Semyanov *et al.*, 2003; Yeung *et al.*, 2003; Caraiscos *et al.*, 2004). Also in our experiments, rapid initial hyperpolarization was enhanced by GAT-1 blocker, and this enhancement was TPMPA-insensitive, supporting the idea of $GABA_A$ receptor activation by spillover. Thus, in experiments like ours, the vigorous stimulation leading to spillover of GABA to extrasynaptic membranes may also activate putative extrasynaptic $GABA_A$ and $GABA_B$ receptors. However, in our study, the contribution of $GABA_C$ receptors was

pharmacologically isolated with the presence of the $GABA_B$ antagonist CGP 46381 and confirmed by using small concentrations of TPMPA (see Section 2.2.2.2.).

Our results from GDPSP experiments support the theory of the mechanism of biphasic GABA-mediated responses being a bicarbonate- and carbonic anhydrase-dependent transmission, non-synaptic in nature (see Section 2.3.). Inhibitory $GABA_C$ receptors in the pyramidal neuron, from which the measurement is made, do not sensitize, and thus, the effect of TPMPA is more prominent towards the end of the late depolarizing phase of the GDPSP, when $GABA_A$ receptors are desensitized. Hence, the smaller $GABA_C$ receptor conductance is more apparent in the voltage recording, inhibiting slightly but clearly the long-lasting depolarization of the pyramidal neuron. An alternative explanation might be that TPMPA inhibits putative $GABA_C$ receptors in interneurons and has an effect on their GABA release, but we find this explanation unlikely, as our immunostainings did not reveal a location of $GABA_C$ receptors in interneurons (but see Rozzo *et al.*, 2002).

Since our results are consistent with the known pharmacological properties of $GABA_C$ receptors, receptors comprising exclusively ρ subunits apparently exist in the adult hippocampal pyramidal cells, as well as in retinal bipolar cells and collicular interneurons in the SGL. By contrast, a mixed $GABA_A$/$GABA_C$ receptor population possibly is present in hippocampal interneurons, juvenile hippocampal pyramidal cells, cerebellar Purkinje cells, lamina II neurons in the spinal cord, brainstem DVN neurons and the amygdala (see Section 2.2.5.).

6.3. Visual system specificity

The highly specific expression of ρ_2 subunits in brain regions related to vision is noteworthy. In addition to the retina, expression of ρ_2 mRNA and ρ protein are seen in the SGL of the SuC, the NOT, the MTN of the accessory optic tract, the dLGN, and the visual cortex. These nuclei are retinorecipient, i.e. receive direct input from directionally selective retinal ganglion cells and are heavily interconnected by GABAergic connections (Van der Want *et al.*, 1992). Moreover, they all analyse information about the motion of single visual objects or the entire visual image. Based on our findings and previous results demonstrating $GABA_C$ receptors at transcript, protein and functional levels in the nuclei of visual pathways, $GABA_C$ receptors may be assumed to be specifically involved in aspects of visual image motion processing in the rat brain. The hippocampus as a base for visuospatial memory fits well with this assumption.

In the SGL of the SuC, $GABA_C$ receptors are preferentially, or exclusively, expressed by local GABAergic interneurons. Similarly, in dLGN, $GABA_C$ receptors have been detected in GABAergic interneurons. In SGL, inhibitory interneurons comprise about half of the neuron population, and a similar proportion of cells expresses ρ_2 mRNA. Consistently, the function of $GABA_C$ receptors is disinhibitory in the SuC, as well as in dLGN, as reflected by the preferential expression of $GABA_C$ receptors by local GABAergic interneurons.

6.4. Nomenclature of GABA receptors

Whether there actually are "$GABA_C$ receptors" or "$GABA_A$ receptors consisting of ρ subunits" in the nervous system has been a subject of discussion ever since their discovery. Arguments corroborating the separate groups of A and C comprise strong pharmacological differences: $GABA_C$ receptors are insensitive to bicuculline, barbiturates and benzodiazepines, have specific agonists and antagonists, and are more sensitive to GABA than classical $GABA_A$ receptors. In addition, $GABA_C$ receptors display a low single-channel conductance, activate and deactivate more slowly and do not desensitize. In most of these respects, $GABA_C$ receptors do, however, more strongly resemble the recently discovered extrasynaptic $GABA_A$ receptors. As a matter of fact, both $GABA_A$ and $GABA_C$ receptors are heterogeneous groups of different subtypes with distinctive pharmacology, subcellular localization and function. As concluded in this work, one cannot briefly describe a typical $GABA_C$ receptor any better than a typical $GABA_A$ receptor.

Based on amino acid sequences, ρ receptors are considered phylogenetically old GABA receptors, together with β, δ, θ, and π subunits (Whiting *et al.*, 1999). As for the phylogenetically oldest, invertebrate GABA receptors, they do not fit into these vertebrate receptor categories, because, for example, crustacean ionotropic GABA receptors share pharmacological properties of both $GABA_A$ and $GABA_C$ receptors (Takeuchi and Onodera, 1972; Jackel et al., 1994; Wegelius, 2000). It is noteworthy that the genes for $GABA_A$ and $GABA_C$ receptors are differentially localized within the genome, thus promoting the distinction. Four chromosomes (4, 5, 15 and X) in the human genome each contain a cluster of genes for α, β and γ subunits in a stoichiometry that accounts for most of the native $GABA_A$ receptors. In contrast, the genes for ρ subunits lie on chromosomes 3 and 6 (Bormann, 2000).

While findings of distinct intracellular anchoring proteins for $GABA_A$ and $GABA_C$ receptors support the separation, recent evidence for the possibility of co-assembly of ρ and γ subunits strongly disagree with it. Not only fish but also rat ρ_1 subunit was shown to be associated with $GABA_A$ receptor subunits in brainstem and cerebellar lysates. Moreover, several confusing results about native receptors with mixed pharmacological properties of both $GABA_A$ and $GABA_C$ receptors cannot be explained by such a close localization of both receptor types that one could find a $GABA_A$ receptor and a $GABA_C$ receptor on the same patch, as suggested by Hartmann and co-workers (2004).

Still, if one considered the concept of $GABA_C$ receptors equivocal in light of the recent data, what could be proposed in its place? An IUPHAR committee has recommended the use of the term $GABA_{A0r}$ to describe bicuculline-insensitive retinal receptors and, for example, $GABA_{A0r12}$ for $\rho_1\rho_2$ hetero-oligomeric $GABA_C$ receptors (Barnard *et al.*, 1998), but no authors have used this clumsy nomenclature in any publication. In fact, despite these recommendations, no change in the nomenclature of any GABA receptor subtypes has been seen. As long as the subunit composition of the different types of native GABA receptors is unknown, it would perhaps be simpler to continue with the already established nomenclature instead of introducing a new, possibly inadequate system.

7. Conclusions

Evidence of functional putatively homo-oligomeric hippocampal $GABA_C$ receptors is the key finding in this thesis. As $GABA_C$ receptors are most abundantly expressed in the retina and SuC, these areas have received major attention in $GABA_C$ receptor studies, and the prevalence of $GABA_C$ receptors outside these structures has been more or less questioned. Our results demonstrate that, among many other receptor types, the *stratum pyramidale* in the CA1 area of the hippocampus possess functional $GABA_C$ receptors that contribute to endogenous GABA responses.

During the past decade or so, the concept of tonic inhibition has become an essential part of network functions, side by side with phasic, conventional inhibition. Tonic and spillover inhibition mediated by receptors outside synaptic sites modulate the overall excitability of nerve cells in a way that can increase the information storage capability, while sheltering from excessive excitation. $GABA_C$ receptors are characterized by their lack of desensitization and their high agonist affinity; as such, they represent ideal candidates for the mediation of tonic or spillover inhibition. The first evidence supporting their role in spillover inhibition has been revealed in the hippocampus by our group, and in the retina, SuC and NOT by others, while their contribution to tonic inhibition has not yet been shown. $GABA_C$ receptors are, by no means, exclusively extrasynaptic, however, their localization in synapses and their contribution to synaptic transmission have been thoroughly established in retinal bipolar cells. All in all, $GABA_C$ receptors seem to compose a surprisingly heterogeneous group of receptors when taking into consideration pharmacological characteristics and subcellular localization, as well as their functional role in the nervous system, as shown partly in this work and in the review of the literature.

The contribution of the ρ_2 subunit to $GABA_C$ergic transmission in the central nervous system is, undoubtedly, the second main finding in this study. ρ_2 subunits were demonstrated to dominate in both the hippocampus and SuC during postnatal development, and the expression of ρ_2 also coincided fully with the $GABA_C$ receptor protein, as illustrated by immunocytochemistry. Our results display, for the first time, the functionality of the rat ρ_2 homo-oligomeric $GABA_C$ receptors. Taken together with the unique pharmacological and biophysical characteristics of the ρ_2 homo-oligomeric receptors detected, many previous questions concerning native $GABA_C$ receptors appear to have found their answers, one example being the picrotoxin resistance of retinal $GABA_C$ receptors.

The low levels of both ρ_1 and ρ_3 mRNAs in the hippocampus, combined with our results of the distinctive pharmacology of ρ_2 receptors expressed in HEK 293 cells and the pharmacology demonstrated in hippocampal slices, suggest that adult hippocampal $GABA_C$ receptors might predominantly be ρ_2 homo-oligomers. Because the currents of the homo-oligomeric ρ_2 receptors were small and their maturation slow but strongly improved by heteromeric expression, possibly the role of the ρ_2 subunits is to combine with some other subunits and modulate their properties. In some parts of the nervous system, ρ_1 subunits seem to co-assemble with ρ_2 subunits, and in other parts putatively with $GABA_A$ receptor subunits. Similarly, ρ_2 subunits may profit from an as yet unknown subunit for efficient functionality as a part of an inhibitory network. Whichever is the case, ρ_2 subunits can no longer be overlooked when either the function of the hippocampus or the $GABA_C$ receptors anywhere in the nervous system is studied.

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