IMAGING AND PROPERTIES OF EXTRASYNAPTIC $GABA_A$ RECEPTORS IN THE BRAIN

Saku Sinkkonen

Institute of Biomedicine, Pharmacology
University of Helsinki, Finland

and

Turku Graduate School of Biomedical Sciences and
Department of Pharmacology and Clinical Pharmacology
University of Turku, Finland

ACADEMIC DISSERTATION

To be presented, with the permission of the Medical Faculty of the University of Helsinki, for public examination in the Small Lecture Hall of Haartman Institute, Haartmaninkatu 3, on November 5th, 2004, at 12 o'clock.

Helsinki 2004

Supervised by

Professor Esa R. Korpi, M.D.
Institute of Biomedicine, Pharmacology
Faculty of Medicine
University of Helsinki, Finland

Reviewed by

Associate Professor Bryndis Birnir, Ph.D.
Department of Physiological Sciences
Faculty of Medicine
University of Lund, Sweden

and

Docent Petteri Piepponen, Ph.D. (Pharm.)
Department of Pharmacology and Toxicology
Faculty of Pharmacy
University of Helsinki, Finland

Dissertation opponent

Professor Emeritus Simo S. Oja, M.D., Ph.D., M.LL.
Brain Research Center
Medical School
University of Tampere, Finland

ISBN 952-91-7825-5 (paperback)
ISBN 952-10-2092-X (PDF http://ethesis.helsinki.fi)
University Printing House
Helsinki 2004

To Wera

TABLE OF CONTENTS

ABSTRACT 7
ORIGINAL COMMUNICATIONS 8
ABBREVIATIONS 9
1. INTRODUCTION 11
2. REVIEW OF THE LITERATURE 14
2.1. GABA as neurotransmitter 14
2.2. GABA receptors 14
2.3. $GABA_A$ receptor structure 15
2.3.1. Subunit genes 15
2.3.2. Subunit proteins 16
2.3.3. Receptor assembly 16
2.3.4. Heterologous receptor expression 17
2.3.5. Receptor subtypes in the native brain 19
2.4. $GABA_A$ receptor function 19
2.4.1. Phasic inhibition 21
2.4.2. Tonic inhibition 22
2.4.3. Inhibition in the cerebellar granule cell layer 24
2.4.4. Inhibition in the hippocampus 26
2.5. Molecular pharmacology of $GABA_A$ receptor binding sites 27
2.5.1. GABA 28
2.5.2. Picrotoxinin/TBPS 29
2.5.3. Benzodiazepines 31
2.5.4. DMCM 32
2.5.5. Furosemide 33
2.6. Tiagabine 34
2.7. $GABA_A$ receptors in disease 34
2.7.1. Anxiety 34
2.7.2. Epilepsy 35
2.8. $GABA_A$ergic drug therapy and its problems 38
3. AIMS OF THE STUDY 39
4. MATERIALS AND METHODS 40
4.1. Experimental animals 40
4.2. Ligand autoradiographic assays (I, II, III, IV, V) 40
4.2.1. Preparation of brain sections (I, II, III, IV, V) 40
4.2.2. [^{35}S]TBPS binding (I, II, III, IV, V) 41
4.2.3. [^{3}H]Ro 15-4513 binding (I, III, IV) 41
4.2.4. [^{3}H]Muscimol binding (I, III) 42
4.3. Localization of $GABA_A$ receptor subunits by *in situ* hybridization (II) 42
4.4. Quantitative autoradiographic film analysis (I, II, III, IV, V) 43
4.5. Recombinant $GABA_A$ receptors in HEK 293 cells (III) 43
4.5.1. Receptor expression 43
4.5.2. [^{35}S]TBPS binding assay 44
4.6. Electrophysiological recordings from acutely isolated mouse hippocampal CA1 pyramidal cells (IV) 44

4.7. Determination of receptor subunit composition (IV) 44
4.7.1. Preparation of receptor extracts 44
4.7.2. Immunoaffinity chromatography 44
4.7.3. Quantification of $GABA_A$ receptor subtypes by immunoprecipitation and [^{3}H]muscimol binding assays 45
4.8. Mouse behavioral studies (IV) 45
4.8.1. Observational functional analysis by the SHIRPA protocol 45
4.8.2. Light-dark choice test 46
4.8.3. T-maze test 46
4.8.4. Convulsion tests 47
4.9. Recombinant $GABA_A$ receptors in *Xenopus* oocytes (V) 47
4.9.1. Receptor expression 47
4.9.2. Electrophysiological recordings 47
4.10. Statistics (I, II, III, IV, V) 48
5. RESULTS AND DISCUSSION 49
5.1. Development of a receptor autoradiographic method to reveal GABA-insensitive [^{35}S]TBPS binding (I) 49
5.2. Localization and prevalence of GABA-insensitive [^{35}S]TBPS binding in the brain (I, II) 52
5.3. Roles of different $GABA_A$ receptor subunits in GABA-insensitive [^{35}S]TBPS binding 53
5.3.1. Correlation with receptor subunit mRNA localization by *in situ* hybridization 53
5.3.1.1. Adult rat (II) 53
5.3.1.2. Developing rat 54
5.3.2. GABA-insensitive [^{35}S]TBPS binding in genetically engineered mouse lines 54
5.3.2.1. The β3 subunit knockout mice (β3-/-) (II) 54
5.3.2.2. The δ subunit knockout mice (δ-/-) (II) 56
5.3.2.3. The α6 subunit knockout mice (α6-/-) (II) 56
5.3.2.4. Mice with ectopic α6 subunit expression (Thy1α6) (IV) 57
5.3.2.5. Mice heterozygous for the γ2 subunit deletion (γ2+/-) (III) 57
5.3.3. Demonstration of GABA-insensitive [^{35}S]TBPS binding in recombinant receptors expressed in HEK 293 cells (III) 58
5.4. A possible mechanism of atypical coupling between GABA and convulsant binding sites 60
5.5. Mice with ectopic α6 subunit expression (Thy1α6) as a model system of altered balance between synaptic and extrasynaptic inhibition (IV) 62
5.5.1. Functional α6 subunit expression and its effects on other subunit mRNA levels 62
5.5.2. $GABA_A$ receptor subtype alterations 63
5.5.3. Behavioral significance of altered balance between synaptic and extrasynaptic inhibition 64
5.5.3.1. Basic behavior 65
5.5.3.2. Convulsion tests 65

5.6. $GABA_A$ receptor subtype-selective actions of niflumate revealed by [^{35}S]TBPS autoradiography (V) 66
5.6.1. Effects in the brain 67
5.6.2. Actions on recombinant receptors 67
5.6.2.1. Negative modulation 67
5.6.2.2. Positive modulation 68
6. GENERAL DISCUSSION 69
6.1. Receptor subunit combinations leading to GABA-insensitive [^{35}S]TBPS binding in the brain 69
6.2. Properties and significance of extrasynaptic $GABA_A$ receptors 71
6.3. Use of GABA-insensitive [^{35}S]TBPS binding in monitoring alterations of $GABA_A$ergic system in animal models of neurological and psychiatric diseases 75
6.4. Use of [^{35}S]TBPS autoradiography in drug research 77
7. CONCLUSIONS 78
ACKNOWLEDGEMENTS 80
REFERENCES 82
ORIGINAL COMMUNICATIONS 97

ABSTRACT

The ionotropic γ-aminobutyric acid (GABA) type A receptor ($GABA_AR$) constitutes the major inhibitory neurotransmitter receptor type in the mammalian brain. The binding of GABA to the pentameric receptor complex results in opening of an integral anion channel, chloride influx, membrane hyperpolarization, and reduction in neuronal excitability. Synaptic $GABA_ARs$ mediate classic phasic "point-to-point" inhibition, whereas extrasynaptic $GABA_ARs$ give rise to persistent tonic inhibition. The exact roles of phasic and tonic inhibition in normal or pathological conditions, or in drug therapy, are unknown. $GABA_ARs$ contain allosteric binding sites for numerous clinically and experimentally important drugs, such as benzodiazepines (BZ), barbiturates, neuroactive steroids, anesthetics and convulsants. Many of these compounds are used to enhance $GABA_A$ergic activity for treating, e.g., epilepsy, anxiety and sleep disorders, and in general anesthesia. The pharmacological and functional diversity of $GABA_AR$ subtypes is a consequence of variable receptor subunit compositions originating from different brain regional/cellular subunit expression patterns. Different $GABA_AR$ subtypes mediate different behaviors, e.g., hypnotic effects of BZs by α1- and anxiolytic effects by α2-$GABA_ARs$. Targeting drug action to certain receptor subtypes might offer a possibility to avoid the problems of nonselective drugs, such as development of tolerance and dependence. Thus far, only the BZ site heterogeneity has been exploited in pharmacotherapy, as indicated by the good efficacy and low abuse potential of zolpidem-like α1-preferring hypnotics. However, a similar diversity is also expected to exist in the other allosteric drug binding sites of the $GABA_ARs$, which makes it important to discover novel pharmacological heterogeneities.

The aim of the thesis study was to develop a receptor autoradiographic method to better visualize the atypically weak displacement of the convulsant site ligand [^{35}S]TBPS by high concentrations of GABA [GABA-insensitive (GIS) [^{35}S]TBPS binding] in the cerebellar granule cell layer, and thereafter, to characterize the atypical receptors responsible for this heterogeneity. After development of the method, GIS-[^{35}S]TBPS was detected in human, rodent and avian brains, and also outside the cerebella. Using genetically engineered mice and recombinant receptors expressed in cell lines it was demonstrated that extrasynaptic α6β2/3, α6β2δ, α1β3 and α1β2δ receptors display GIS-[^{35}S]TBPS, as can also be suggested for α4β and α4βδ receptors, while inclusion of γ2 subunit abolishes GIS-[^{35}S]TBPS. The formation of GIS-[^{35}S]TBPS could be explained, at least in part, by the finding that GABA might act as a partial agonist in the native receptors responsible for GIS-[^{35}S]TBPS. In a mouse line with ectopic extrasynaptic α6 subunit expression in the hippocampus (Thy1α6) in conjunction with increased tonic but decreased phasic inhibition, expression of α6βγ2 and α6β receptors was detected. In Thy1α6 mice the altered balance between synaptic and extrasynaptic inhibition caused increased seizure sensitivity. Antiepileptic tiagabine at a low dose was more effective in the Thy1α6 than control mice, presumably due to increased tonic inhibition by heightened GABA levels, but at higher doses the tiagabine effect was smaller in the Thy1α6 mice. The results revealed the first behavioral correlate of tonic inhibition, but also emphasized the importance of phasic inhibition in circumstances with strong stimuli. In the last part of the project, GIS-[^{35}S]TBPS was used to reveal the dual actions of a non-steroidal anti-inflammatory drug, niflumate, on brain $GABA_ARs$. In α6-receptors it had negative, but in α1βγ2 receptors positive, effects on $GABA_AR$ function.

The results of the thesis project indicate that GIS-[^{35}S]TBPS can be used as a tool to visualize extrasynaptic $GABA_ARs$ in animal models of neurological and psychiatric diseases, and in drug development in a search for subtype-selective compounds.

ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by Roman numerals (**I-V**):

I Sinkkonen S.T., Uusi-Oukari M., Tupala E., Särkioja T., Tiihonen J., Panula P., Lüddens H., Korpi E.R. (2001a) Characterization of γ-aminobutyrate type A receptors with atypical coupling between agonist and convulsant binding sites in discrete brain regions. Molecular Brain Research 86:168-178.

II Sinkkonen S.T., Mihalek R.M., Homanics G.E., Lüddens H., Korpi E.R. (2001b) Altered atypical coupling of γ-aminobutyrate type A receptor agonist and convulsant binding sites in subunit-deficient mouse lines. Molecular Brain Research 86:179-183.

III Sinkkonen S.T., Lüscher B., Lüddens H., Korpi E.R. (2004) Autoradiographic imaging of altered synaptic αβγ2 and extrasynaptic αβ $GABA_A$ receptors in a genetic mouse model of anxiety. Neurochemistry International 44:539-547.

IV Sinkkonen S.T., Vekovischeva O.Y., Möykkynen T., Ogris W., Sieghart W., Wisden W., Korpi E.R. (2004) Behavioral correlates of an altered balance between synaptic and extrasynaptic $GABA_A$ergic inhibition in a mouse model. European Journal of Neuroscience 20:2168-2178.

V Sinkkonen S.T., Mansikkamäki S., Möykkynen T., Lüddens H., Uusi-Oukari M., Korpi E.R. (2003) Receptor subtype-dependent positive and negative modulation of $GABA_A$ receptor function by niflumic acid, a nonsteroidal anti-inflammatory drug. Molecular Pharmacology 64:753-763.

In addition, some unpublished data are presented. The original communications have been reproduced with permission of the copyright holders.

ABBREVIATIONS

α6-/-	mouse line devoid of $GABA_A$ receptor α6 subunit
ANOVA	analysis of variance
β3-/-	mouse line devoid of $GABA_A$ receptor β3 subunit
BZ	benzodiazepine
CA	cornu ammonis of the hippocampus
CRF	corticotropin releasing hormone
C-terminal	carboxy-terminal
δ-/-	mouse line devoid of $GABA_A$ receptor δ subunit
DIS	diazepam-insensitive
DMCM	methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate
DS	diazepam-sensitive
EBOB	4'-ethynyl-4-*n*[2,3-H_2]propyl-bicycloorthobenzoate
EC_{50}	concentration producing a half maximal effect
EPSC	excitatory postsynaptic current
ER	endoplastic reticulum
γ2+/+	γ2 subunit wild-type mouse line
γ2+/-	mice heterozygous for deletion of $GABA_A$ receptor γ2 subunit
γ2-/-	mouse line devoid of $GABA_A$ receptor γ2 subunit
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid type A receptor
$GABA_B$	γ-aminobutyric acid type B receptor
$GABA_C$	γ-aminobutyric acid type C receptor
GAD	glutamate decarboxylase
GAT	GABA transporter
GIS	GABA-insensitive
HEK 293	human embryonic kidney cells
HPA	hypothalamic-pituitary-adrenal axis
i.p.	intraperitoneal
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
ISH	*in situ* hybridization
K_d	equilibrium dissociation constant
LTP	long-term potentiation
mIPSC	miniature inhibitory postsynaptic current
NMDA	N-methyl-D-aspartate

NSAID	non-steroidal anti-inflammatory drug
N-terminal	amino-terminal
PET	positron emission tomography
Ro 15-4513	ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine -3-carboxylate
sIPSC	spontaneous inhibitory postsynaptic current
SPECT	single photon emission computed tomography
TBOB	*t*-butylbicycloorthobenzoate
TBPS	*t*-butylbicyclophosphorothionate
Thy1α6	transgenic mouse line expressing the $GABA_A$ receptor α6 subunit under Thy1.2 promoter
TM	transmembrane
Tris	tris(hydroxymethyl)aminomethane

1. INTRODUCTION

During the last hundred years or so, it has become evident that brain function can be ultimately explained by current flow across neuronal cell membranes. How this movement of charged particles, ions, results, e.g., in higher cognitive functions and emotions, such as memory and fear, is probably the outcome of a complex puzzle including electrochemical signal-transduction between neurons, spatial and temporal summation of currents and precise control of the whole neuronal network.

The mammalian central nervous system consists of brain and spinal cord. While the spinal cord is specialized to carry mainly sensory and motor information between the brain and rest of the body, in both directions, the brain is devoted to react to incoming information, to generate commands, and to execute them. To succeed in the challenge of creating meaningful orders to guarantee the survival of the individual, the brain has to compare, integrate and modulate the information it receives. For this, the mammalian brain is highly organized: it contains several functionally different units that are interconnected by certain pathways. The information reaching one of these units is usually modulated before it is conveyed to the next target. The brain can be divided into parts such as the brain stem, cerebellum, diencephalon and cerebrum. The brain stem is involved, e.g., in modulation of autonomic functions and regulation of sensory and motor information. The cerebellum modulates movements. In the diencephalon, the thalamus serves as processing station for information that is heading to the cerebral cortex, while the hypothalamus has neuroendocrine functions. The cerebrum itself consists of the cerebral cortex, the hippocampus, the amygdala and the basal ganglia. The cerebral cortex is primarily responsible for cognitive functions. The hippocampus has a role, e.g., in the memory and the organization of behavioral pathways, and the amygdala regulates responses according to different emotional values of inputs. The basal ganglia are involved in modulation of movements.

The brain, like the whole body, is a collection of different cells with extracellular matrix in between them. Neurons are cells that connect with each other to form tracts that enable information to move between different parts of the brain or from the brain to the periphery and back. A string called the axon originates from the neuronal body, i.e., the soma. At the distal end of the axon an expansion, the axon terminal, comes close to the neighboring neuron. Together, these two neurons form a synapse, which enables transfer of information between the presynaptic cell's axon and a postsynaptic cell's branch called a dendrite. As the dendrite is connected to the postsynaptic neuron's body, there is a functional link between the two neuronal bodies separated only by a tiny cleft in the synapse. In addition to neurons, other types of cells are also found in the brain. These glial cells do not deal directly with information transfer but are responsible for a variety of tasks that aim to maintain homeostasis in the brain, e.g., they, provide mechanical support, immunoprotection and vital nutrients for neurons.

In the resting state, a neuronal membrane is negatively charged as a result of differential distribution of anions and cations between the intracellular and extracellular spaces. Different permeability of ions across the neuronal membrane and active transport by Na^+-K^+-ATPase against the chemical gradients leads to low intracellular Na^+ and Cl^- concentrations, whereas the K^+ concentration becomes high. The starting point of any brain function is an action potential. Action potential is a summation of excitatory signals that cause Na^+ flux into the neuron. Na^+ influx and the resulting inward current shifts the membrane potential to a more positive value to the limit where "all-or-nothing" action potential is triggered. During the action potential the membrane potential is depolarized. Depolarization propagates along the axon to the axon terminal, where Ca^{2+} enters the cell and neurotransmitters are released to the synaptic cleft. Whether the neuron at the postsynaptic side of the synapse is excited or

inhibited depends on the nature of the neurotransmitters released by the presynaptic neuron. The postsynaptic cell in turn summates excitatory and inhibitory signals, and, if enough excitation is gained, action potential is triggered. This type of information flow, consisting of propagation of electrical impulses within neuron and conversion of electrical impulses to chemical signals in the synapses between neurons, offers several advantages that are essential for brain function. First, information is conveyed only in the anterograde direction, since synapses relay signals only from presynaptic to postsynaptic cells. Second, spatial and temporal summation of information flow is possible, because many presynaptic neurons have synapses with a single postsynaptic cell and action potential is generated only if enough excitation is achieved. Thirdly, while action potential carries only messages of one modality, depolarization, the chemical signal transduction in synapses enables this message to be converted to either an excitatory or an inhibitory one.

Excitation in the mammalian brain is mainly mediated by the neurotransmitter glutamate. Like all neurotransmitters, glutamate exerts its actions through specialized membrane proteins, receptors. Glutamate receptors fall into two main categories, the ionotropic ligand-gated ion channels and the metabotropic receptors that are coupled to intracellular G-proteins. Both categories can be further divided into several receptor types on the basis of their structural and functional properties. It is generally accepted that glutamate plays a pivotal role in many important brain functions, and its role in so-called “long-term potentiation” (LTP), which confers the capacity to learn and remember, has been particularly well studied. It is also know that overt glutamate-mediated excitation is toxic to cells, long-lasting excitation leading to cell death. When hyperexcitability is spread in the neuronal circuitry, it causes convulsions and ultimately death of the individual. Thus, while neurotransmitter-mediated excitation and the following action potentials are essential for any event to take place in the brain, an equally important factor in normal brain function is control of excitation.

The mechanism that controls excitation in the brain is called inhibition. It derives basically from outward currents that oppose depolarization and may lead to membrane hyperpolarization. Hyperpolarization shifts resting potential to a more negative value, at which more excitation is needed to trigger action potential. Inhibition has many ways to affect brain function. It directly balances neuronal excitability, coordinates various rhythmic activities, and modulates information flow in different circuits by complex feedback systems. Deficiencies in inhibition have been found in a number of brain diseases, e.g., generalized anxiety disorder and schizophrenia. Like the effects of excessive excitation, convulsions may result from lack of inhibition even in the presence of normal amounts of excitation. From the 1960’s, γ-amino butyric acid (GABA) has been suggested to be responsible for the major part of inhibition in the brain, and today we know that this is true. As in the case of glutamatergic transmission, the actions of GABA are mediated by specialized receptors. As brain function is a fine-tuned balance between receptor-mediated excitation and inhibition, it is understandable that the receptors for GABA are key-players in controlling brain activity, and ultimately the behavior of the individual.

More complexity to the brain function is brought about by the heterogeneity of any given neurotransmitter receptor system. The complexity of GABA receptors exceeds that of any other receptor type. While it offers an infinite field of research for many generations of neuroscientists, it, more importantly, enables a delicate control over excitation, since different receptor subtypes have different functional properties. The different properties are derived from different structures. In addition to functional heterogeneity, structural variability causes pharmacological heterogeneity. This means that a given drug doesn’t necessarily affect all receptor subtypes, or that its actions on different subtypes may vary. This forms the basis for modern drug design, where attempts are made to create selective ligands for certain receptor

subtypes with defined functional and/or behavioral roles. With selective drug therapy more effective and safer treatment may be achieved.

2. REVIEW OF THE LITERATURE

2.1. GABA as neurotransmitter

By definition, a neurotransmitter is a substance with specific presynaptic synthesis and storage, inactivation mechanisms after release, and comparable effects to a postsynaptic cell when applied exogenously as with electrical stimulation of a presynaptic cell. In addition, if the action of the neurotransmitter can be blocked with another compound after presynaptic release, the compound should also block the action of the exogenously applied transmitter. GABA fulfills all these criteria (reviewed in Oja and Kontro, 1987). It is synthesized from L-glutamate by the enzyme glutamate decarboxylase (GAD). From the cytoplasm, GABA is actively transported to the synaptic vesicles (Kish et al., 1989), where it is stored and released in a Ca^{2+}-dependent way by action potential. After release to the synaptic cleft, GABA is largely taken up by active GABA-transporters to neurons and glial cells. The uptake is not complete, since 0.1 – 0.8 μM GABA concentrations can be detected in the extracellular space depending on the brain region (Lerma et al., 1986; Tossman *et al.*, 1986). This is in accordance with theoretical calculations using transporter models, which suggest that GABA transporters are unable to lower extracellular GABA below 0.1 - 0.4 μM concentrations (Attwell et al., 1993; Richerson and Wu, 2003). The first demonstration of exogenous GABA mediating similar inhibitory effects on postsynaptic cells as normal neuronal activity came from Obata and colleagues (1967). Soon after this Obata and Highstein extended the studies by showing that picrotoxin blocks both the neuronal inhibition and the inhibitory effect of GABA on rabbit oculomotor neurons (1970). Since then, over 40,000 reports concerning GABA have been published, and its central role as an inhibitory neurotransmitter has been generally accepted.

It has been estimated that, depending on the brain region, up to 30 - 40 % of brain synapses use GABA as their transmitter (Bloom and Iversen, 1971; Iversen and Bloom, 1972). Immunohistochemical detection of GAD and GABA receptors made it possible to map GABAergic neurons and their pathways in the brain. From this extensive work carried out during recent decades it is obvious that GABAergic cells are found throughout the brain, and innervation is especially rich in, for example, the cerebral cortex, hippocampus, thalamus, substantia nigra, striatum and cerebellum. GABA seems to control brain excitation in two major patterns. First, inhibitory GABA transmission may be the main output of a brain circuit as seen in the cerebellar cortex, where Purkinje cells use GABA to inhibit the deep cerebellar neurons that further project to motor systems (Curtis et al., 1970). In the other type of inhibition, GABA has more restricted actions, as GABAergic inhibitory interneurons control the principal neurons' firing, and thus serve more as integrative tools in local circuits (Cobb et al., 1995).

In addition to the brain, GABAergic inhibition is important in the spinal cord (reviewed in Malcangio and Bowery, 1996). GABA also has peripheral actions, for it participates in the operation of the peripheral nervous system, it has endocrine functions, and it regulates various peripheral organs, such as female reproductive systems (reviewed in Ong and Kerr, 1990).

2.2. GABA receptors

GABA exerts its actions by interacting with specialized membrane proteins, receptors. From early on pharmacological data suggested the existence of at least two different types of receptors for GABA (Naik et al., 1976), and later it was shown that the fast component of inhibitory postsynaptic potentials was selectively blocked by bicuculline but the slow

component by phaclofen (Soltesz et al., 1988). Applying molecular cloning and following heterologous expression of recombinant receptors established the current view of two basic types of GABA receptors, which were named in order of their discovery as type A ($GABA_A$) and type B ($GABA_B$) receptors.

The $GABA_A$ receptors, together with nicotinic acetylcholine, strychnine-sensitive glycine and serotonin 5-HT_3 receptors, form the superfamily of ligand-gated ion channels (reviewed in Leite and Cascio, 2001). Characteristic to these receptors composed of five polypeptide subunits, agonist binding causes a conformational change in the receptor complex and opening of the integral ion channel. Depending on the ion selectivity of the channel, fast neuronal depolarization or hyperpolarization results. The $GABA_A$ receptors are the main targets for GABAergic drugs in various neurological and psychiatric diseases, and their properties and significance will be dealt with in forthcoming sections. Later on, another GABA receptor was found that was insensitive to selective $GABA_A$ and $GABA_B$ receptor ligands bicuculline and baclofen, respectively, and it was termed the $GABA_C$ receptor (Drew et al., 1984). To date, three different subunits (ρ1-3) for $GABA_C$ receptors have been cloned, and they form homo- or hetero-oligomeric GABA-gated chloride channels mainly in the retina (reviewed in Bormann, 2000). While it has been suggested that $GABA_C$ receptors should be considered as a subgroup of $GABA_A$ receptors, the classification to different categories is more applicable due to the unique pharmacological properties of these two receptor types.

$GABA_B$ receptors belong to the family of seven transmembrane domain G-protein coupled receptors. They are basically composed of a single polypeptide chain, but functionally they are coupled in dimeric units (reviewed in Couve et al., 2000). GABA binding leads to activation of inhibitory G-proteins that cause inhibition of adenylyl cyclase and agonist-induced inositol triphosphate synthesis, inhibition of Ca^{2+} channels and activation of inwardly rectifying K^+ channels. $GABA_B$ receptors may be located either pre- or postsynaptically, the former causing inhibition of neurotransmitter release and the latter hyperpolarization of postsynaptic membrane. Activation of $GABA_B$ receptors has long-lasting inhibitory effects, and it has been shown to contribute to several important phenomena, such as regulation of LTP (Davies et al., 1991) and rhythmic activity in the hippocampus (Scanziani, 2000). While widespread distribution of $GABA_B$ receptors in the brain offers possibilities to affect behavior, and alterations in $GABA_B$ergic function have been detected in animal models of depression, epilepsy and addiction (reviewed in Couve et al., 2000), currently the only pharmacotherapy targeted specifically to $GABA_B$ receptors consists of baclofen treatment of spasticity associated with multiple sclerosis (Beard et al., 2003).

2.3. $GABA_A$ receptor structure

2.3.1. Subunit genes

Pharmacological and biochemical studies (Squires et al., 1979; Sieghart and Karobath, 1980) suggested heterogeneity of $GABA_A$ receptors before cloning of different receptor subunits. Schofield et al. (1987) used partial amino acid sequences of benzodiazepine (BZ) column affinity chromatography purified proteins to design oligonucleotides that enabled them to clone the first $GABA_A$ receptor α and β subunits from bovine brain. Sequence homology made it possibly to screen brain cDNA libraries with oligonucleotide probes, which resulted in identification of a large repertoire of subunits in various species. Today, altogether 16 different mammalian subunits [α1-6, β1-3, γ1-3, δ, ε, θ, π (for references see Whiting et al., 1995; Davies et al., 1997; Hedblom and Kirkness, 1997; Whiting et al., 1997; Bonnert et al., 1999; Sinkkonen et al., 2000)] have been cloned. All the subunits are encoded by separate

genes, which are arranged in clusters in chromosomes (Russek, 1999). While it is likely that various receptor subunits are formed by duplication of a common ancestor, the need for such multiplicity is not totally clear. However, the different subunits confer individual properties to the receptor complex, and at least it allows differential regulation of subunit expression even if the gene products were largely similar.

The subunits display 70-80 % sequence homology within a subunit class and 30-40 % homology between classes (Macdonald and Olsen, 1994). In addition to numerous different subunits, the heterogeneity is increased by alternative exon splicing of the mRNA (two forms of α6: Korpi et al., 1994; two forms of β3: Kirkness and Fraser, 1993; three forms of γ2: Whiting et al., 1990; Kofuji et al., 1991; Jin et al., 2004a). The receptor subunit genes are expressed in the brain with well characterized spatial and temporal patterns (e.g., Laurie et al., 1992; Wisden et al., 1992; Sinkkonen et al., 2000). Other transcripts, such as α1 and β2 subunit mRNAs are found throughout the brain throughout life, whereas others, such as α6, ε and θ have extremely restricted spatiotemporal expression (Laurie et al., 1992; Wisden et al., 1992; Sinkkonen et al., 2000). The mechanisms controlling the expression patterns of the different subunits are largely unknown, and this study is in its early phase (reviewed in Steiger and Russek, 2004).

2.3.2. Subunit proteins

The $GABA_A$ receptor subunits display many features similar to the subunits of other members of the ligand-gated ion channel family (Schofield et al., 1987; McKernan and Whiting, 1996; Leite and Cascio, 2001). The subunits are 450-550 residue polypeptides with four hydrophobic transmembrane domains (TM1-4), and a large aminoterminal (N-terminal) tail containing a cysteine loop and agonist binding site. A large intracellular loop between TM3 and TM4 has the highest amino acid diversity between subunits and contains putative phosphorylation sites. The short carboxy-terminal (C-terminal) tail is also extracellular. Immature subunits contain an N-terminal signal sequence needed to address the subunits on cell membranes, and the sequence is presumably cleaved after membrane insertion.

2.3.3. Receptor assembly

The subunit translation, post-translational modification and receptor assembly of ligand-gated ion channels take place in the endoplastic reticulum (ER, reviewed in Green and Millar, 1995). Like other members of the ligand-gated ion channels, the $GABA_A$ receptors are composed of five subunits (Nayeem et al., 1994; Green and Millar, 1995; Tretter et al., 1997). To allow formation of receptor complexes composed of several subunits, sequences for specific interactions of subunits are required. Detailed analysis of the subunit residues responsible for the interaction has been performed, and the amino acids in the N-terminal extracellular domain of α, β and γ subunits are important (Taylor et al., 1999; Klausberger et al., 2000; Taylor et al., 2000; Klausberger et al., 2001; Sarto et al., 2002; Jin et al., 2004b). Bollan et al. (2003) provided a summary figure of the complex organization of assembly signals between the α, β and γ subunits. Currently no data is available for assembly signals of other receptor subunit classes.

After oligomerization in ER, $GABA_A$ receptor subunits are targeted to the cell surface (Connolly et al., 1996). While α1β2 and α1β2γ2 combinations are capable of leaving ER and be transported to the cell membrane, the single α1, β2 and γ2 subunits and binary combinations of α1γ2 and β2γ2 are retained in ER (Connolly et al., 1996; Kittler et al., 2000),

and rapidly degraded (Gorrie et al., 1997). This suggests that assembly of α1γ2 and β2γ2 receptors is stopped in the absence of β2 or α1 subunits, respectively. This indicates that strict, although partially unknown, rules for receptor assembly regulate the number of different $GABA_A$ receptor subtypes in the brain.

2.3.4. Heterologous receptor expression

$GABA_A$ receptors can be transiently or stably expressed in a number of different cells that are introduced to suitable RNA or DNA encoding for receptor subunits. *Xenopus laevis* oocytes can be individually injected with RNA purified from the brain (Smart et al., 1983), with synthetic subunit cRNAs (Schofield et al., 1987) or with subunit cDNAs (Ebert et al., 1994), which results in functional expression of $GABA_A$ receptors. The receptors can subsequently be studied with electrophysiological methods, but the oocytes do not provide enough membranes for simple binding studies with radioligands. Some binding studies have been performed with single oocytes. Another possibility for transient expression of $GABA_A$ receptors is to transfect cell lines (Lüddens and Korpi, 1997; e.g., human embryonic kidney cells, mouse fibroblasts, Chinese hamster ovary cells) with cDNA cocktails of different subunits. While both electrophysiological and binding studies can be used, only some of the cells express receptors, and it is possible that not all cells express all receptor subunits. A third possibility is to use cell lines that endogenously express $GABA_A$ receptors (Hales and Tyndale, 1994) or to produce cell lines with stable expression of desired subunits (e.g., α1β2γ2; Hamilton et al., 1993). The possible problems with these cell lines are low efficiency of receptor expression (Hamilton et al., 1993) or its long-term unstability (Valeyev et al., 1993). Common to all these approaches, recombinant technologies and stable cell lines yield subunits in artificial amounts, which might cause discrepancies in the data between different laboratories. The relative amounts of subunits may vary a lot because of different constructs, transfection methods or model systems. Thus, it is important to try to validate the expression levels of the different subunits using pharmacological or other methods (Boileau et al., 2002a). One possibility to control the coassembly of subunits in different transfection protocols is to use tandem-constructs, where different subunits are linked to each other by gene fusion (Baumann et al., 2001).

If there were no rules for receptor assembly, a theoretical maximum of how 16 different receptor subunits can be assembled in a pentamer results in over one million (16^5) receptor subtypes. This number would be still further increased by alternative splicing of subunits. Even though receptor assembly in many cases works properly in heterologous systems, it is possible that they might produce such receptors that would not exist in the native brain. This is the core problem of heterologous expression of $GABA_A$ receptors. It is easy to imagine that introduction of large quantities of cRNA to cells leads to mass production of proteins that are processed differentially from the native situation. This is the possible explanation for the results of electrophysiological studies which show that α1 (Blair et al., 1988; Pritchett et al., 1988; Verdoorn et al., 1990), α2 (Blair et al., 1988), α3 (Blair et al., 1988), β1 (Blair et al., 1988; Pritchett et al., 1988; Sigel et al., 1989), β2 (Verdoorn et al., 1990), β3 (Wooltorton et al., 1997), γ2 (Shivers et al., 1989; Verdoorn et al., 1990; Martínez-Torres and Miledi, 2004) and δ (Shivers et al., 1989) subunits are able to form functional receptors by themselves. However, no expression was observed in some other studies with single subunits (Schofield et al., 1987; Sigel et al., 1990), which might reflect different expression levels. Another possible explanation for the apparent functional expression of homomeric receptors in transfected cell lines are endogenously expressed subunits [e.g., small amounts of α1, β3 and γ2 subunit mRNAs in HEK (human embryonic kidney) 293 cells ; Fuchs et al., 1995].

When subunits from two different classes are co-expressed, receptors are formed more efficiently, they are more sensitive to GABA and have larger chloride currents than homomeric receptors (Sigel et al., 1990). This suggests a preferential subunit assembly of receptors composed of dual combinations over homomeric subunits. With subunits from two classes, a large number of combinations produces detectable levels of functional receptors in electrophysiological studies (reviewed in Sieghart, 1995). However, subunit composition seems to affect the expression efficiency, since αβ receptors are produced on a large scale, whereas data from αγ and βγ combinations are somewhat controversial, but they seem to form less functional receptors (Sigel et al., 1990; Verdoorn et al., 1990; Knoflach et al., 1996). This is supported by the findings that αγ and βγ combinations are retained in ER and only αβ reach the cell surface (Connolly et al., 1996; Kittler et al., 2000). Furthermore, it seems that αγ and βγ combinations mostly form dimmers, while αβ combination preferentially forms tetramers and pentamers (Tretter et al., 1997).

Transfection with subunits from three or more classes is efficient and makes it possible to produce receptors with many properties similar to native $GABA_A$ receptors (reviewed in Sieghart, 1995).

In addition to electrophysiological methods, recombinant receptors can be studied with binding studies. The results of binding studies (reviewed in Sieghart, 1995) using cell membrane homogenates are even more confusing, since, in addition to the receptors on cell membranes studied in electrophysiology, the radioligand binding also takes place in intracellular subunit combinations that would never reach the cell surface. Thus, having radioligand binding in a given recombinant receptor subtype on membrane homogenates does not necessarily mean that these receptors are found on the cell surfaces of native neurons. Using intact cells instead of cell membrane homogenates offer a possibility to study ligand binding to only those recombinant $GABA_A$ receptors that are properly transported to cell membranes. While intact cells have successfully been used with the water-soluble radioligand [^{3}H]muscimol (Chang et al., 2002), most other $GABA_A$ergic radioligands are lipid soluble (such as BZs and convulsant site ligands), and they easily pass through cell membranes, resulting in ligand binding to both membrane-targeted and intracellular receptors.

One possible problem common to all approaches concerning the heterologous expression of recombinant $GABA_A$ receptors is the lack of neuron-specific intracellular proteins that interact with the receptor complexes. Although many candidate proteins have been found that are suggested to affect the receptor clustering, trafficking and function of the native brain (reviewed in Lüscher and Keller, 2004), demonstration of their exact roles or direct interaction between these proteins and $GABA_A$ receptors in most cases is still lacking. However, recently Everitt et al. (2004) showed that in mouse L929 fibroblasts co-expression of $GABA_A$ receptor subunits with an intracellular $GABA_A$ receptor-associated protein (GABA-RAP), known to directly interact with the γ2 subunit (Wang et al., 1999), remarkably affected the functional properties of the receptors, which then closely resembled those detected in native neurons. This suggests that in the future, in addition to GABA-RAP, different neuron-specific intracellular proteins should be co-transfected with the $GABA_A$ receptor in order to obtain more physiological results.

The problems concerning heterologous receptor expression are obvious. However, recombinant receptors can be used in many applications, and owing to the largely homogenous pool of receptors, accurate information, e.g., about the pharmacological properties of the given receptor subtype, are obtained. However, one should preferentially use subunit combinations that are known to be formed in the native brain on the basis of immunohistochemical, biochemical or pharmacological data.

2.3.5. Receptor subtypes in the native brain

The exact number of $GABA_A$ receptor subtypes and their subunit stoichiometry in the native brain is still unknown. Several methods may be used to deduce the receptor subunit composition. *In situ* hybridization (ISH) can be used to localize different subunit mRNAs, and co-localization reveals the possibility for coassembly. However, mRNA expression does not necessarily mean protein translation, and a given cell type may express up to 12 different subunits, as is the case in hippocampal dentate granule cells (Wisden et al., 1992). A traditional but indirect method is to use receptor subtype specific pharmacological heterogeneities in receptor autoradiography or ligand binding studies with subsequent demonstration of the heterogeneity in recombinant receptors (e.g., Korpi et al., 1995a). Immunoprecipitation of solubilized receptors from brain homogenates with subunit-specific antibodies and subsequent Western blot analysis may be used to reveal protein level interactions (e.g., Benke et al., 1994). However, this method requires high-affinity and high-specificity antibodies, and its sensitivity is questionable. For example, solubilization and precipitation of the receptors may result in loss of about 50 % of the receptors from the original brain homogenate (Tretter et al., 2001). Similar to ligand binding studies of brain or cell homogenates, it is possible that immunoprecipitation detects intracellular receptors. Immunoaffinity columns with subsequent Western blot analysis also allow identification of two different subunits in the same receptor (e.g., Pöltl et al., 2003), but it has the disadvantage of possible detection of intracellular receptors. Still another possibility to identify different subtypes is to use direct localization of receptor subunits with immunofluoresence or electron microscopy. This has been successfully used to reveal many native receptor subtypes (e.g., Benke et al., 1994; Nusser et al., 1998a), but may have limited sensitivity. Despite the limitations of the detection methods, it has been possible, by combining the results of various studies, to determine the major $GABA_A$ receptor subtypes in different brain regions (Table 1). However, due to the limited sensitivity of the detection methods, many unknown low-abundant subtypes exist, and their roles in certain neuronal populations may be significant.

Colocalization of subunits in clusters on neuronal membranes, as in immunocytochemical studies, show that most native $GABA_A$ receptors are composed of α, β and γ subunits (Fritschy et al., 1992; Somogyi et al., 1996). The preferable stoichiometry of these receptors is 2α, 2β and 1γ subunits (Im et al., 1995a; Chang et al., 1996; Tretter et al., 1997; Farrar et al., 1999; Baumann et al., 2001). In some $\alpha4$ and $\alpha6$ subunit-containing receptors δ substitutes for the γ subunit (Quirk et al., 1995; Nusser et al., 1998a; Bencsits et al., 1999). Minimal structural requirements for membrane localization of the receptor are the α and β subunits (Connolly et al., 1996; Kittler et al., 2000), and the presence of $\alpha\beta$ receptors in the brain is confirmed (Bencsits et al., 1999). Suggested stoichiometry of $\alpha\beta$ receptors is 3α and 2β subunits or vice versa (Im et al., 1995a; Kellenberger et al., 1996; Baumann et al., 2001). The π and ε subunits are considered as possible substitutes for γ (Davies et al., 1997; Hedblom and Kirkness, 1997; Whiting et al., 1997). The θ seems to be able to replace β subunits in some receptors (Bonnert et al., 1999). Further multiplicity of receptor subtypes is provided by findings that two different subunits from the same subunit class can be assembled in the same receptor complex (e.g., different α subunits: Lüddens et al., 1991; Jechlinger et al., 1998, or different β subunits: Li and De Blas, 1997; Jechlinger et al., 1998).

2.4. $GABA_A$ receptor function

GABAergic cells have two distinct functions, both of which are mainly mediated by $GABA_A$ receptors. In the first case GABA is used by the principal neuron of the brain circuit, and thus the main output is inhibitory (Curtis et al., 1970). Secondly, in the more common type of

Table 1. Major $GABA_A$ receptor subtypes in the brain.

Subunit combination	Relative abundance in rat brain (%)	Examples of brain regional localization	References
α1β2γ2	43	Present in most brain areas; hippocampus, cortex and cerebellar Purkinje cells	Benke et al., 1991 Fritschy et al., 1992 Somogyi et al., 1996
α2β2/3γ2	18	Spinal cord motoneurones and hippocampal pyramidal cells	Benke et al., 1994 Fritschy et al., 1998
α3βγ2/3	17	Cholinergic and monoaminergic neurons	Fritschy et al., 1992 Quirk et al., 1994
α2β2/3γ1	8	Bergmann glia, nuclei of the limbic system	Quirk et al., 1994
α5β3γ2/3	4	Hippocampal pyramidal cells	McKernan et al., 1991 Fritschy and Möhler, 1995
α6βγ2	2	Cerebellar granule cells	Quirk et al., 1994 Nusser et al., 1998a
α6βδ	2	Cerebellar granule cells	Quirk et al., 1994 Nusser et al., 1998a
α4β	2	Thalamus and hippocampal dentate gyrus	Bencsits et al., 1999
α4βγ2	2	Thalamus and hippocampal dentate gyrus	Bencsits et al., 1999
α4βγδ	1	Thalamus and hippocampal dentate gyrus	Bencsits et al., 1999
Other minor subtypes	1		

Modified from McKernan and Whiting, 1996.

inhibition, GABAergic cells act as inhibitory interneurons that control principal neuron activity (Cobb et al., 1995). Interneurons can control principal cells in a variety of ways (reviewed in Paulsen and Moser, 1998; Freund, 2003). The inhibition may be of phasic or tonic nature (see next sections), and it may arise from feedback or feedforward circuits. In addition to directly controlling network oscillations, which are considered essential for higher brain functions, the interneurons regulate dendritic calcium influx and backpropagation of action potentials, which affects synaptic plasticity.

Knowledge about the actual $GABA_A$ receptor activation is derived from the predicted homology to nicotinic acetylcholine receptors. Binding of an agonist causes a small rotation of extracellular domains of subunits, which opens the channel formed by TM2 regions of adjacent subunits (Unwin, 1998). The homology to $GABA_A$ receptors is supported by the finding that in recombinant α1β1 receptors the α and β subunits rotate asymmetrically after GABA binding, which causes channel opening (Horenstein et al., 2001).

When the $GABA_A$ receptor-associated channel is opened, various anions may pass through it in both directions (Bormann et al., 1987). The fact that GABA usually causes neuronal hyperpolarization is a result of a favorable electro-chemical gradient for chloride influx, not that of channel selectivity (reviewed in Kaila, 1994). For this reason $GABA_A$ receptor activity may also cause depolarization, which is the case in the developing brain, where the intracellular chloride concentration is elevated (Cherubini et al., 1991). During hippocampal maturation, induction of the expression of the K^+/Cl^- co-transporter causes an

adult-like shift in chloride equilibrium, which turns GABA from excitatory to inhibitory (Rivera et al., 1999). By studying the permeability of different anions through $GABA_A$ receptors, it has been possible to predict a diameter of 5.6 Å for the anion pore (Bormann et al., 1987). So, in theory, all anions smaller than this may flow through $GABA_A$ receptors, but, in the native brain, the main anion passing through the channel is chloride, and the second most important is bicarbonate (Kaila and Voipio, 1987; Kaila, 1994).

2.4.1. Phasic inhibition

Phasic inhibition is crucial for normal brain function, since it shapes the amplitude and duration of excitatory post-synaptic currents (EPSC) of the principal cells in the feed-back and feed-forward loops. In this prototypic inhibition $GABA_A$ receptors enriched in the postsynaptic cell membranes are activated by GABA, which is released from presynaptic terminals by the action potential. Many synapses are activated simultaneously, which causes a rapidly activating inhibitory postsynaptic current (IPSC; Figure 1) and hyperpolarization of the postsynaptic cell. The IPSC is terminated within milliseconds when GABA is eliminated from the synapses by diffusion and uptake by transporters to neurons and glia (Clements, 1996). Another factor contributing to the magnitude of IPSC is the receptor desensitization caused by high agonist concentrations. During desensitization, the agonist is still bound to the receptor, but the channel enters a closed state. Since the GABA concentration in a synapse may rise to 300 μM or 3 mM (Mozrzymas et al., 1999; Perrais and Ropert, 1999), whereas the concentration producing a half maximal effect (EC_{50}) of the $GABA_A$ receptor subtypes is usually below 50 μM (reviewed in Hevers and Lüddens, 1998), some degree of receptor desensitization takes place, which additionally affects the size of the IPSCs (Jones and Westbrook, 1996).

Receptor enrichment to synaptic membranes exactly opposite to the presynaptic terminals is essential for efficient synaptic transmission. Like glycine receptors (Meier et al., 2001; Rosenberg et al., 2001), $GABA_A$ receptors are thought to diffuse freely around the plasma membrane after membrane insertion but before their aggregation to synaptic clusters. Gephyrin is a protein that causes anchoring of glycine receptors to synapses by interaction with the cytoskeleton (Kirsch et al., 1991). Gephyrin can be found colocalized with $GABA_A$ receptor α1, α2, α3 and γ2 subunits in many brain regions, but it does not colocalize with α6 or δ (Sassoè-Pognetto et al., 2000). Direct molecular interaction between gephyrin and $GABA_A$ receptor subunits has not been shown (Kannenberg et al., 1997), but gephyrin-deficient mice show decreased clustering of major $GABA_A$ receptor subtypes (Kneussel et al., 1999). However, it does not affect clustering of some other receptor subtypes, and therefore, other clustering proteins must also exist (Kneussel et al., 2001). Of the different $GABA_A$ receptor subunits, γ2 is essential for synaptic anchoring, since in the lethal phenotype of γ2 subunit knockout mice (γ2-/-), postsynaptic $GABA_A$ receptor clusters are dramatically decreased (Essrich et al., 1998). Gephyrin clusters are also abolished (Essrich et al., 1998). However, in the γ2-/- mice, overexpression of γ3 subunits is sufficient to restore synaptic receptor clustering (Baer et al., 1999), which suggests that other γ subunits are also capable of interaction with anchoring proteins, but γ2 is favored for some reason *in vivo*. GABA-RAP is another protein possibly involved in $GABA_A$ receptor clustering, and it has been shown to interact directly with the γ2 subunit (Wang et al., 1999). In addition to gephyrin and GABA-RAP, many other intracellular proteins have been proposed to affect $GABA_A$ receptor clustering and trafficking (reviewed in Luscher and Keller, 2004).

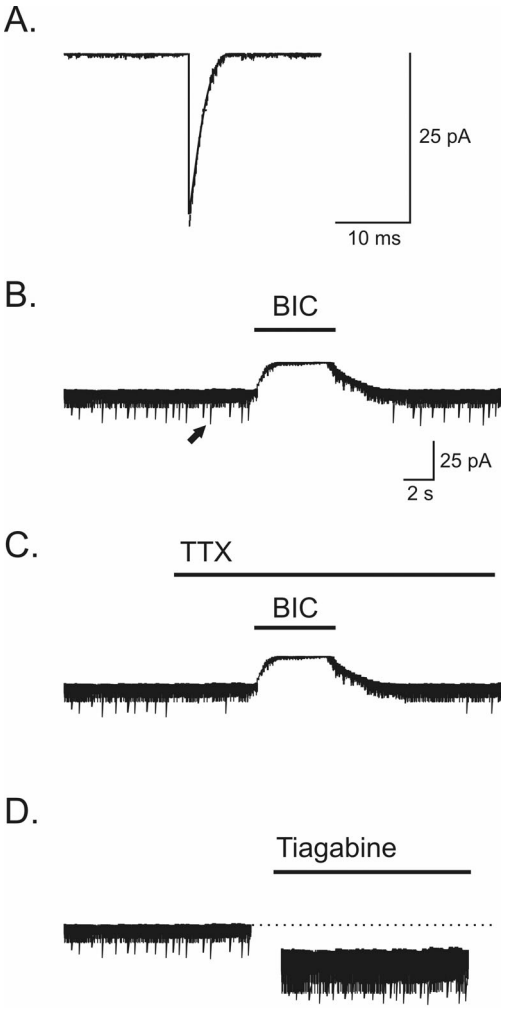

Figure 1. Schematic presentation of phasic and tonic $GABA_A$ergic currents recorded from a single neuron. **A.** Typical sIPSC arising from simultaneous activation of many synapses by action potential-triggered GABA release. **B.** $GABA_A$ergic currents composed of sIPSCs and mIPSCs (either of which indicated with an arrow), and tonic background conductance (revealed after application of the GABA antagonist bicuculline, *BIC*). mIPSCs represent the release of a single or a few neurotransmitter quanta from presynaptic terminal to postsynaptic receptors. It is action potential independent, and thus might be considered as a form of tonic conductance. Bicuculline (*BIC*) blocks all currents, which is illustrated by abolition of sIPSCs and mIPSCs, a decrease in background "noise", and a shift in mean membrane current. **C.** Tetrodotoxin (*TTX*) blocks action potential triggered GABA release and thus sIPSCs. mIPSCs are still detected. Bicuculline blocks mIPSCs and tonic conductance. **D.** The GABA uptake blocker tiagabine raises the extracellular GABA level, which increases background conductance (increased "noise" and shift in mean current) without affecting sIPSCs or mIPSCs. E.g., in the recordings by Bai et al. (2001) largely similar traces are shown.

2.4.2. Tonic inhibition

In addition to classical phasic synaptic transmission, $GABA_A$ receptors also exert their actions by tonic inhibition, which is common also to other neurotransmitters (reviewed in Zoli et al., 1999). Tonic $GABA_A$ergic conductance was first proposed by Birnir et al. (1994) in the hippocampal dentate gyrus. Thereafter it was found and characterized in the cerebellar granule layer (Kaneda et al., 1995; Brickley et al., 1996; Tia et al., 1996a; Wall and Usowicz, 1997; Rossi and Hamann, 1998; Hamann et al., 2002). Also the hippocampus (CA1 region: Birnir et al., 2000; Bai et al., 2001; Semyanov et al., 2003; Caraiscos et al., 2004; dentate gyrus: Nusser and Mody, 2002; Stell et al., 2003; Wei et al., 2003), the cerebral cortex (Salin and Prince, 1996), and the thalamus (Porcello et al., 2003) display tonic $GABA_A$ergic inhibition. The contribution of tonic conductance to the total inhibitory charge transfer varies depending on the brain region, but in the cerebellar granule cell layer the vast majority of the inhibition is of a tonic nature (Brickley et al., 1996; Wall and Usowicz, 1997; Hamann et al., 2002). The exact functional roles of the tonic inhibition are unknown, but it is suggested to set a background level for overall neuronal excitability (reviewed in Mody, 2001). It has been suggested to control the synaptic responses by regulating general membrane properties, such as membrane input resistance and time constant (Häusser and Clark, 1997; Mitchell and Silver, 2003). In the cerebellar granule cells, tonic inhibition is shown to decrease the number of excited cells, which is suggested to improve information storage capacity (Hamann et al., 2002). In the hippocampus, tonic inhibition may affect learning and memory processes (Caraiscos et al., 2004).

Tonic inhibition (Figure 1) results basically from the incapacity of GABA transporters to lower extracellular GABA concentration below 0.1 - 0.4 μM, and neurons seem to be in permanent agonist bath of ≥ 0.1 μM (Lerma et al., 1986; Tossman *et al.*, 1986; Attwell et al., 1993; Richerson and Wu, 2003). Extracellular GABA may originally have been spilled-over from neighboring synapses (Brickley et al., 1996; Rossi and Hamann, 1998) or released from the surface matrix reservoir that becomes exposed during vesicular exocytosis (Vautrin et al., 2000). Non-vesicular release of GABA from astrocytes or neurons is also possible (Liu et al., 2000; Rossi et al., 2003), which might be due to reverse operation of the GABA transporters (Gaspary et al., 1998). Thus, the functional state of the transporters seems to be very important in regulating tonic inhibition (Richerson and Wu, 2003; Wu et al., 2003). Anyway, the presence of permanent agonist stimulation results in continuous receptor activation causing tonic inhibition. Another possibility leading to tonic conductance is receptors that are constitutively active without agonist application. Such receptors are found at least in the hippocampus (Birnir et al., 2000).

A clear-cut distinction between the receptor subtypes responsible for phasic and tonic conductances is difficult, if not impossible to make, but some general points may be drawn. Tonic conductance may be derived from receptors located on both synaptic and extrasynaptic membranes, but owing to receptor properties, extrasynaptic receptors are primarily activated. Since ambient GABA concentrations are maximally in a low micromolar range, the receptor subtypes responsible for tonic inhibition need to have high affinity to GABA. In addition, despite the persistent presence of an agonist, the receptors must not desensitize, otherwise they would be nonconducting. Taking these considerations into account, and the results from genetically engineered animals, the roles of certain $GABA_A$ receptor subtypes in tonic inhibition may be addressed. Most convincing evidence indicates that α6 and δ subunit-containing receptors are responsible for tonic conductance in the granule cells of the cerebellum. The α6 subunit protein is found on both synaptic and extrasynaptic membranes (Nusser et al., 1998a). The α6 subunit-containing receptors have a high affinity to GABA [EC_{50} 0.2-2 μM depending on other subunits (Ducic et al., 1995; Knoflach et al., 1996; Saxena and Macdonald, 1996)], and they desensitize poorly (Tia et al., 1996b). Tonic current in cerebellar granule cells is detected after developmental induction of α6 subunit expression (Brickley et al., 1996), and is blocked by α6-specific antagonist furosemide (Hamann et al., 2002). In addition, tonic inhibition is abolished in the α6 knockout mice (Brickley et al., 2001), but it is enhanced after ectopic expression of α6 subunits in the hippocampal CA1 neurons of Thy1α6 mice (Wisden et al., 2002). The δ subunit-containing receptors are located exclusively extrasynaptically (Nusser et al., 1998a), have an extremely high affinity for GABA [EC_{50} 0.2-0.5 μM (Saxena and Macdonald, 1996; Hevers et al., 2000; Brown et al., 2002)] and desensitize poorly (Haas and Macdonald, 1999). In addition, tonic conductance is greatly reduced in the δ knockout mice (Stell et al., 2003). As δ is preferentially assembled with α6 (Jones et al., 1997), most of the tonic conductance in the cerebellar granule cells is probably mediated by α6βδ receptors.

Since α6 is exclusively expressed in the cerebellar granule cells (Wisden et al., 1992), it cannot explain the tonic conductance in forebrain areas. The δ subunit-containing receptors (presumably combined with α4; Sur et al., 1999) give rise to tonic conductance in hippocampal dentate granule cells (Stell et al., 2003) and thalamic relay neurons (Porcello et al., 2003). The α5 subunit in the hippocampal CA1 pyramidal cells is exclusively extrasynaptic (Brünig et al., 2002; Crestani et al., 2002a). The α5 subunit-containing receptors are more sensitive to GABA than α1-containing receptors, and they desensitize more slowly (Caraiscos et al., 2004). Tonic inhibition in the CA1 pyramidal cells is greatly reduced in α5-knockout mice (Caraiscos et al., 2004), but it is BZ-sensitive in the wild-type mice, which suggests the presence of γ2 subunit in the receptor complex (Bai et al., 2001; Lindquist et al., 2003). The α3 subunit is found both in synapses and on extrasynaptic

membranes (Brünig et al., 2002), and might thus cause tonic inhibition. In addition, pharmacological evidence suggests that the most prevalent and prototypic receptor subtype, $\alpha1\beta\gamma2$, also contributes to tonic inhibition in the hippocampal interneurons (Semyanov et al., 2003).

The interplay between phasic and tonic inhibition is still largely unknown, but recent studies with genetically engineered animals suggests a precise balance between these two components. In the GABA transporter (GAT) -1 knockout mice, which display increased tonic inhibition due to increased extracellular GABA levels, spontaneous IPSCs (sIPSC) are unchanged, but the frequency of miniature IPSCs (mIPSC) is reduced (Jensen et al., 2003). Somewhat similarly ectopic expression of the $GABA_A$ receptor $\alpha6$ subunit in the hippocampus of Thy1$\alpha6$ mice increases tonic inhibition but decreases sIPSC frequency and mIPSC amplitude (Wisden et al., 2002). In addition, in the $\alpha6$ knockout mice, where tonic $GABA_A$ergic inhibition in the cerebellar granule cells is abolished, induction of potassium leak conductance restores neuronal excitability (Brickley et al., 2001).

2.4.3. Inhibition in the cerebellar granule cell layer

The cerebellum is highly conserved across different species. It is devoted to maintaining balance and orientation of the body, adjusting and learning motor tasks, and it contributes to some aspects of cognition (Ito, 1972; Modianos and Pfaff, 1976; Fiez, 1996; Asanuma and Pavlides, 1997; Ito, 2002). Different cortical regions of the cerebellum share the same basic organization, which consists of circuits that are used to modulate the afferent information on its way through the cerebellar cortex to the descending systems (Figure 2; Ito, 2002). The cerebellum has two main inputs, both of which are excitatory, the climbing fibers arising from the inferior olivary nucleus, and the mossy fibers arising from the precerebellar nuclei. The only output of the cortex, from Purkinje cells to deep cerebellar neurons, is inhibitory and uses GABA as a transmitter. Each Purkinje cell receives input from just one climbing fiber, but each climbing fiber excites about 10 Purkinje cells (not drawn in the figure). Mossy fibers influence Purkinje cell firing indirectly through interneurons as they excite granule cells. Granule cells, in turn, excite several Purkinje cells by parallel fiber contacts. Parallel fibers also excite inhibitory Golgi interneurons, which inhibit granule cells and Purkinje cells. In addition, stellate cells and basket cells, which are also excited by parallel fibers, inhibit Purkinje cells. Purkinje cells have feed-back inhibition to basket cells. Thus, excitatory mossy fiber input is conferred to a complex excitation-inhibition of Purkinje cells, which is thought to enable spatial and temporal integration of information flow through the cerebellar cortex.

The diversity of $GABA_A$ receptors in the cerebellum has been reviewed extensively (Wisden et al., 1996). In brief, prototypical BZ-sensitive receptors composed of $\alpha1$, $\beta2/3$ and $\gamma2$ subunits are expressed predominantly in the molecular layer and in Purkinje cells (Wisden et al., 1992; Gutiérrez et al., 1994). In contrast, the cerebellar granule cells express a unique repertoire of subunits, namely $\alpha1$, $\alpha6$, $\beta2$, $\beta3$, $\gamma2$ and δ subunits (Wisden et al., 1992; Thompson et al., 1992; Benke et al., 1994; Gutiérrez et al., 1994). The cerebellar granule cells are the most abundant cells in the brain, amounting to about half of all neurons. They possess several distinct anatomical and physiological features. They receive GABAergic input to their distal dendrites from the Golgi interneurons. The excitatory mossy fibers meet the inhibitory connection and together they form a special structure called the glomerulus. In addition to synaptic $GABA_A$ receptors, the granule cells have extrasynaptic receptors on their cell soma and dendrites (Baude et al., 1992; Nusser et al., 1996). The organization of these receptors in synaptic and extrasynaptic locations gives rise to phasic and tonic inhibition, respectively

(Brickley et al., 1996; Wall and Usowicz, 1997; Brickley et al.,1999). Based on immunohistochemical data, Nusser et al. (1998a) have drawn a schematic representation of the differential distribution of $GABA_A$ receptor subtypes on granule cells: $\alpha6\beta\delta$ receptors (Caruncho and Costa, 1994; Quirk et al., 1994; Jones et al., 1997) are exclusively extrasynaptic (Nusser et al. 1998a), whereas $\alpha6\beta\gamma2$ receptors (Khan et al., 1994; Quirk et al., 1994; Pollard et al., 1995; Jones et al., 1997) are found in Golgi synapses, in some of the mossy fiber to granule cell synapses, and on extrasynaptic membranes (Nusser et al., 1998a). The $\alpha1\beta\gamma2$ receptors (Quirk et al., 1994; Jones et al., 1997; Nusser et al. 1998a) are mainly located in the Golgi synapses but at lower concentrations also on extrasynaptic membranes

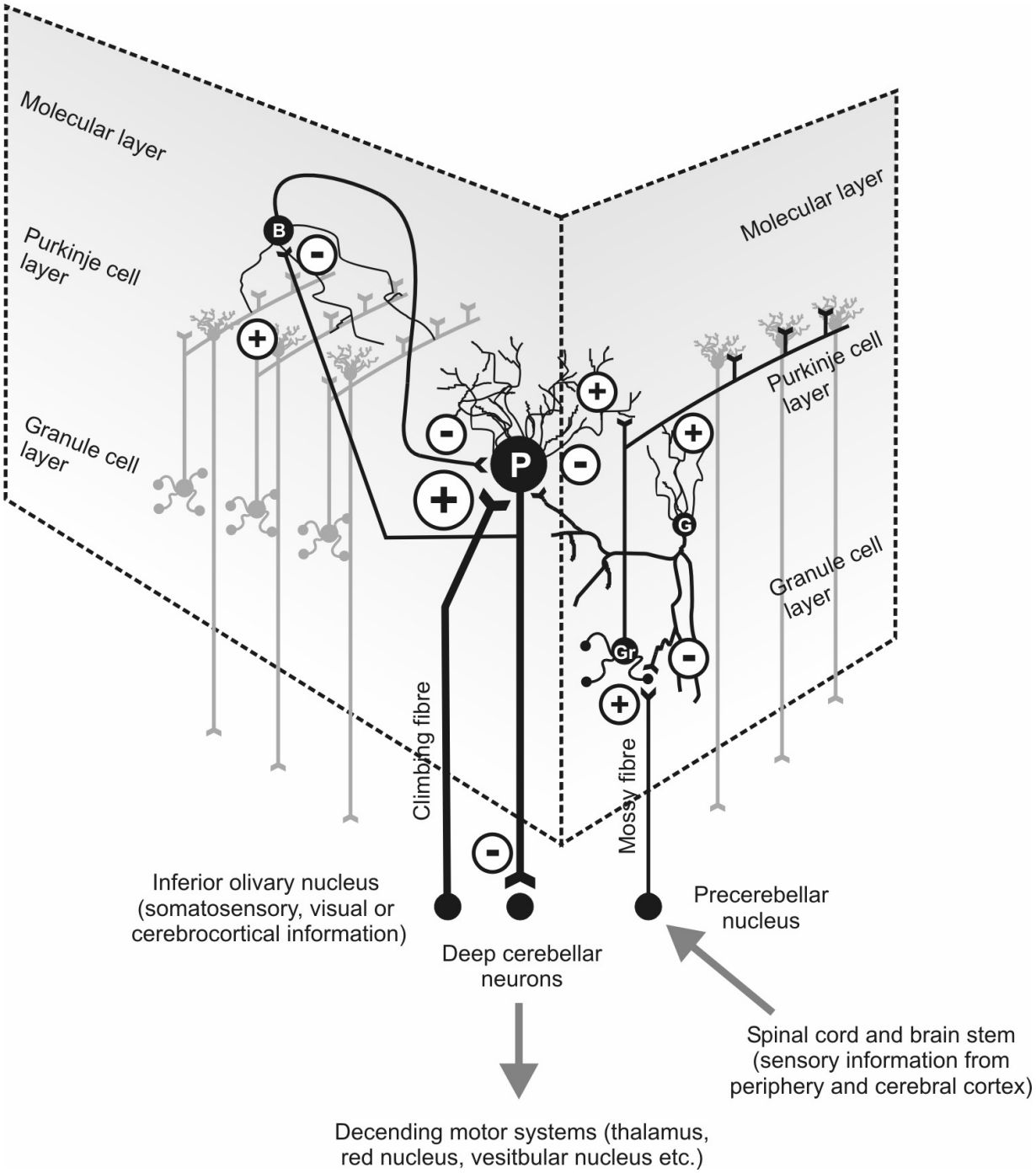

Figure 2. Information flow through the cerebellar cortex. *B*; basket cell; *G*, golgi interneuron; *Gr*, granule cell; *P*, Purkinje cell. (+), glutamatergic excitatory synapse; (-), GABAergic inhibitory synapse. Modified from Wisden et al. (1996).

(Nusser et al., 1995; Nusser et al. 1998a). The α1 and α6 subunits are colocalized in some Golgi synapses (Nusser et al., 1996), and immunoprecipitation data suggest that some receptors harbor both α1 and α6 subunits (Pollard et al., 1995; Khan et al., 1996). In the cerebellum granule cells, no coassembly of α1 and δ subunits is found (Quirk et al., 1994), or the abundance of α1βδ-receptors is very low (Pöltl et al., 2003).

2.4.4. Inhibition in the hippocampus

The basic anatomy and neurophysiology of the hippocampus has been reviewed extensively (e.g., Knowles, 1992). In brief, the hippocampus receives input from cortical and subcortical structures. Cortical input derives from, e.g., the controlateral hippocampus and entorhinal cortex, which in turn receives input from many regions, including the amygdala, the medial septum and the thalamus. The subcortical input derives from many brain areas, including the hypothalamus, the raphe nucleus and the locus coeruleus. Input from cortical areas uses glutamate to directly excite the hippocampus, whereas input from subcortical areas uses several kinds of transmitters and tends to have more indirect and modulatory actions.

In the classical glutamatergic trisynaptic pathway (Figure 3), granule cells of the dentate gyrus are excited by the perforant pathway coming from the entorhinal cortex. The granule cells excite the CA3 (cornu ammonis subregion 3) pyramidal cells by their axons called mossy fibers. CA3, in turn, excites CA1 pyramidal cells via Schaffer collaterals. The CA1 pyramidal cell axons are the main output from the hippocampus, and they project into, e.g., the subiculum, the entorhinal cortex, the nucleus accumbens, the amygdala and the hypothalamus. The projections to the subiculum and the entorhinal cortex provide indirect pathways to several cortical areas, and to the amygdala and thalamus. Excitation is mediated by α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) type glutamate receptors. The activation of NMDA receptors in principal cells is required for formation of LTP, which is considered as synaptic counterpart for formation of memory (Tsien et al., 1996).

In addition to synapses between the hippocampal principal cells, the granule cells and the pyramidal cells, glutamate is used in synapses between the principal cells and various types of interneurons. The interneurons are GABAergic and inhibit the principal cells by activating postsynaptic $GABA_A$ receptors in feedback and feedforward inhibitory loops (Figure 3; reviewed in Paulsen and Moser, 1998). Interneurons also inhibit each other by synaptic contacts and by direct electrical coupling through gap junctions (Skinner et al., 1999; Klausberger et al., 2002). In the feedback inhibition, axon collaterals from the principal cells excite inhibitory interneurons (e.g., basket cells), which in turn produce an IPSP in pyramidal cells. Feedback inhibition strongly controls the activity of the principal cells. In feedforward inhibition of CA1 pyramidal cells, Schaffer collaterals excite interneurons that directly inhibit CA1 pyramidal cells, which restrict the duration and strength of CA1 pyramidal cell excitation. Different types of interneurons have specific roles and they innervate different parts of the principal cells (Figure 3; Paulsen and Moser, 1998; Klausberger et al., 2003). Basically, perisomatic interneurons (basket and axo-axonic cells) control output and synchronize the firing of the principal cells, while dendritic interneurons control the efficacy and plasticity of the excitatory input. Basket cells can be divided into two populations with different properties and inputs (Freund, 2003). Parvalbumin-positive cells are interconnected and act as nonplastic synchronizers of pyramidal cells through α1 subunit-containing $GABA_A$ receptors (Klausberger et al., 2002; Freund, 2003). The other, cholecystokinin-positive basket cells, serve as sensors for mood and fine-tune firing of the pyramidal cells through α2 subunit-containing $GABA_A$ receptors (Nyíri et al., 2001; Freund, 2003), which are known to mediate the anxiolytic effects of BZs (Löw et al., 2000). Of the other receptor subunits, the α5

subunit is exclusively extrasynaptic in CA1 pyramidal cells (Brünig et al., 2002; Crestani et al., 2002a), and causes tonic inhibition (Lindquist et al., 2003; Caraiscos et al., 2004). The α3 is found both in synapses and on extrasynaptic membranes (Brünig et al., 2002). The α4 is present in pyramidal cells and dentate granule cells (Wisden et al., 1992; Sperk et al., 1997), but its subcellular localization is unknown. The δ subunit is expressed in dentate granule cells (Sperk et al., 1997) and gives rise to tonic conductance (Stell et al., 2003), which suggests an extrasynaptic localization similar to the cerebellar granule cells (Nusser et al., 1998a). Localization of δ in the forebrain is largely similar to the α4 subunit (Wisden et al., 1992; Sperk et al., 1997), and these subunits display a preferential coassembly (Sur et al., 1999). In addition to α and δ subunits, virtually all β and γ subunit variants are expressed in the hippocampal principal cells (Wisden et al., 1992; Sperk et al., 1997). In the interneurons, mainly α1, β2 and γ2 subunits are found (Schwarzer et al., 1997; Sperk et al., 1997).

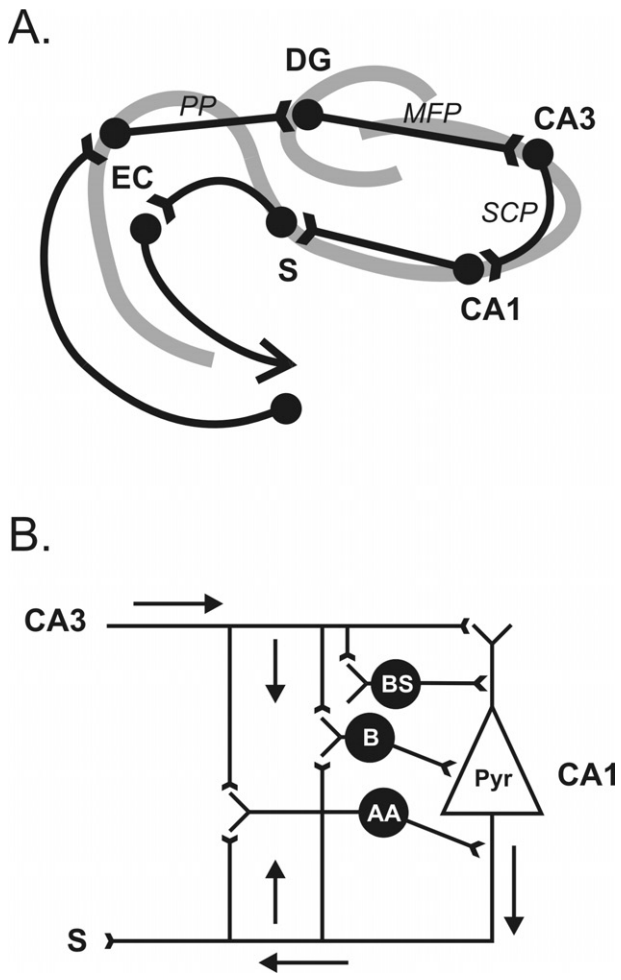

Figure 3. Information flow through the hippocampus (**A.**) and inhibitory loops in the hippocampal CA1 region (**B.**). **A.** The classical glutamatergic trisynaptic pathway and examples of its inputs and outputs are illustrated. *DG*, dentate granule cells; *EC*, entorhinal cortex; *MFP*, mossy fiber pathway; *PP*, perforant pathway; *S*, subiculum; *SCP*, Schaffer collateral pathway. The hippocampus receives input from cortical and subcortical structures. Output from CA1 regions goes to, e.g., the subiculum, the entorhinal cortex, the nucleus accumbens, the amygdala and the hypothalamus. The projections to the subiculum and the entorhinal cortex provide indirect pathways to several cortical areas, the amygdala and the thalamus. Based on Knowles (1992). **B.** The interneurons are excited by Schaffer collaterals from the CA3 region and inhibit CA1 pyramidal cells by activating postsynaptic $GABA_A$ receptors in feedback and feedforward inhibitory loops. Different types of interneurons have specific roles, and they innervate different parts of the principal cells. Interneuron-interneuron contacts, synapses and gap junctions (Skinner et al., 1999; Klausberger et al., 2002), are not shown. *AA*, axo-axonic cell; *B*, baskel cell; *BS*, bistriatal cell; Pyr, pyramidal cell. Modified from Paulsen and Moser (1998).

2.5. Molecular pharmacology of $GABA_A$ receptor binding sites

Extensive reviews of different $GABA_A$ receptor binding sites, their structural requirements, and the modes of action of different $GABA_A$ergic drugs have been published (e.g., Sieghart, 1995; Hevers and Lüddens, 1998; Korpi et al., 2002a), and thus only those compounds essential for the thesis are presented here. However, a simplified summary is provided in Figure 4.

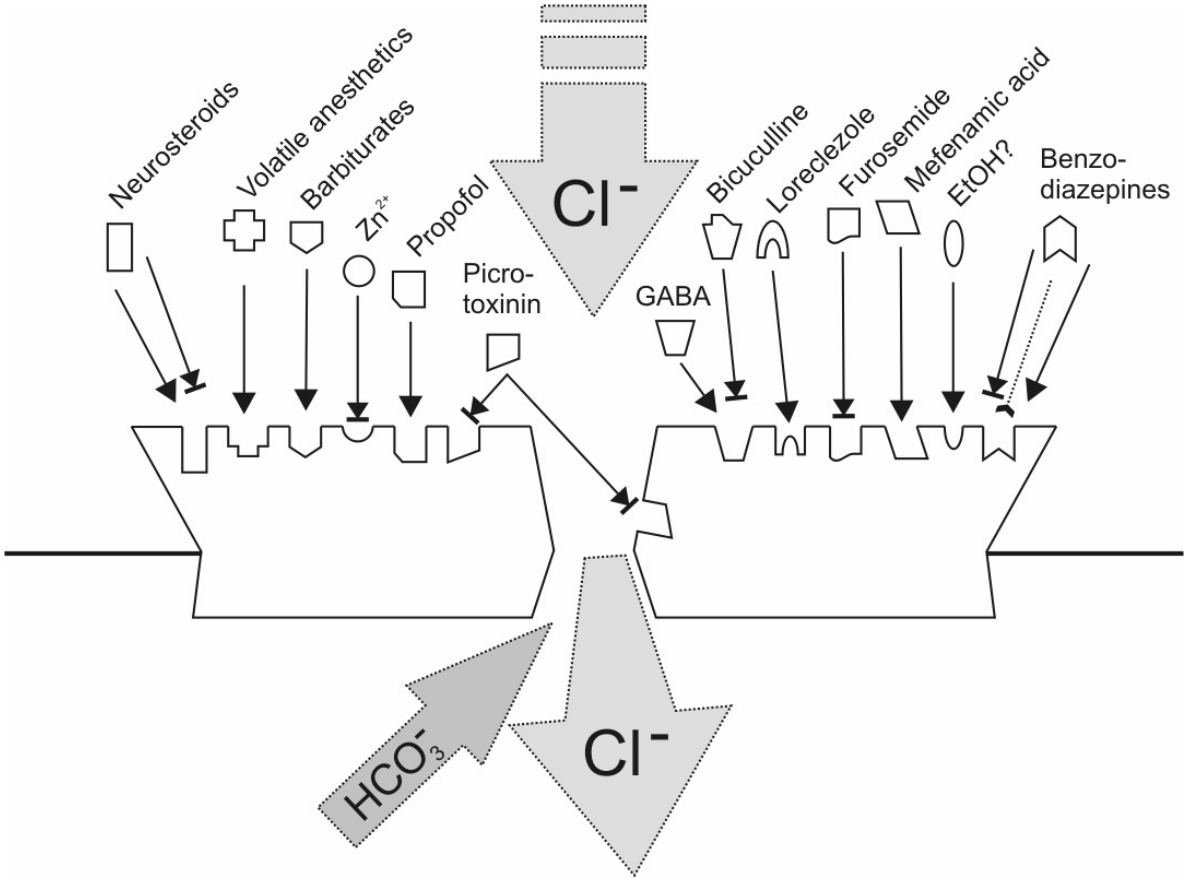

Figure 4. Most of the known $GABA_A$ergic drugs and their effects on receptor function in a hypothetical model. (→), positive modulation of the receptor function; (→|), negative modulation of the receptor function; (- - <), antagonistic action of the benzodiazepine binding site. Data are from Sieghart (1995) and Korpi et al. (2002a).

2.5.1. GABA

The $GABA_A$ receptor agonist binding sites can be labeled with [^{3}H]GABA or [^{3}H]muscimol. The receptor has both high- and low-affinity binding sites for GABA, the affinities being at low nanomolar and low micromolar range, respectively (Olsen et al., 1981; Agey and Dunn, 1989). The extracellular N-terminal amino acids Phe64 (phenylalanine at position 64; Sigel et al., 1992; Smith and Olsen, 1994), Arg66 (Boileau et al., 1999), Ser68 (Boileau et al., 1999), Arg120 (Westh-Hansen et al., 1999), Val178 (Newell and Czajkowski, 2003), Val180 (Newell and Czajkowski, 2003), and Asp183 (Newell and Czajkowski, 2003) of the α1 subunit form part of the low-affinity GABA binding pocket. These amino acids are otherwise conserved in all α subunits, but there is some variability of the amino acids in positions 68 (Boileau et al., 1999) and 183 (personal amino acid sequence comparison). In the β subunit, the extracellular amino acids (named after β2 amino acid numbering) Tyr97 (Boileau et al., 2002b), Leu99 (Boileau et al., 2002b), Tyr157 (Amin and Weiss, 1993), Thr160 (Amin and Weiss, 1993), Thr202 (Amin and Weiss, 1993), Ser204 (Wagner and Czajkowski, 2001), Tyr205 (Amin and Weiss, 1993), Arg207 (Wagner and Czajkowski, 2001) and Ser209 (Wagner and Czajkowski, 2001) are important for forming the low-affinity GABA binding site. The low-affinity GABA binding site is located in the extracellular interface between the α and β subunits (Smith and Olsen, 1995), and GABA binding to it is

important for channel opening, since mutation of the above mentioned residues affects channel activity, and there is a correlation between the GABA concentrations needed to activate the channel and the affinity of this site (Hevers and Lüddens, 1998).

The functional role of the high-affinity binding site is unclear, but the [^{3}H]muscimol autoradiographic data suggest that in the native brain it is associated with receptors containing the δ subunit together with α4 or α6 (Quirk et al., 1995; Mihalek et al., 1999; Korpi et al., 2002b). However, [^{3}H]muscimol binding in test tube conditions with brain homogenates or recombinant receptor preparations seems not to depend on the δ subunit (Tretter et al., 2001). It has been demonstrated that the Tyr62 amino acid of the β2 subunit is important for [^{3}H]muscimol binding, but not for channel gating (Newell et al., 2000).

The type of α subunit in the receptor complex largely defines the affinity for GABA (reviewed in Hevers and Lüddens, 1998). α6 subunit-containing receptors are the most sensitive (EC_{50} 0.2-2 μM: Ducic et al., 1995; Knoflach et al., 1996; Saxena and Macdonald, 1996), whereas EC_{50} for GABA is about ten or a hundred times higher in α3 subunit-containing receptors (Sigel et al., 1990; Ebert et al., 1994; Verdoorn, 1994). Receptors with α1, α2, α4 and α5 have intermediate affinity (e.g., Wafford et al., 1993; Ebert et al., 1994; Knoflach et al., 1996). The type of β and γ variant has a minor effect (Hevers and Lüddens, 1998), but the role of the δ subunit is clear: the receptors with δ instead of γ are ten times more sensitive to GABA (Saxena and Macdonald, 1996; Hevers et al., 2000; Brown et al., 2002). Inclusion of the γ subunit with αβ receptors reduces the affinity to GABA (Sigel et al., 1990; Horne et al., 1993).

In addition to GABA, the agonist site recognizes several conformationally restricted analogs, such as muscimol and thiomuscimol, which act as full agonists in most receptors (Ebert et al., 1997). THIP is a restricted GABA analog, which is a partial agonist in some receptor subtypes (e.g., α1βγ2: Ebert et al., 1994; Ebert et al., 1997), while it acts as a full agonist in α4βδ receptors (Brown et al., 2002). Interestingly, GABA is only a partial agonist in α4βδ receptors (Brown et al., 2002). In addition to agonists and partial agonists, several competitive antagonists, such as bicuculline and SR 95531, are known (Heaulme et al., 1986).

2.5.2. Picrotoxinin/TBPS

Blockage of ion flux through the $GABA_A$ receptor with various allosteric modulators, such as picrotoxin (an equimolar mixture of active picrotoxinin and inactive picrotin), *t*-butylbicyclophosphorothionate (TBPS; Squires et al., 1983), *t*-butylbicycloorthobenzoate (TBOB; Lawrence et al., 1985), 4'-ethynyl-4-*n*[2,3-H_2]propyl-bicycloorthobenzoate (EBOB; Kume and Albin, 1994), pentylentetrazol (Squires et al., 1983) and many insectides (e.g., lindane and dieldrin; Huang and Casida, 1996) cause convulsions. These compounds compete with each other, which has led to the assumption of a single "convulsant" binding site (Ticku and Maksay, 1983).

In electrophysiological studies, picrotoxinin block has been detected in homomeric receptors composed of α, β, γ or δ subunits (Shivers et al., 1989; Atkinson et al., 1992). However, [^{35}S]TBPS binding was detected only in homomeric β3 receptors (Slany et al., 1995), which might suggest somewhat different minimal structural requirements for the binding of these compounds. [^{35}S]TBPS binding and picrotoxinin block can be detected in various αβ, βγ and αβγ combinations (reviewed in Sieghart, 1995). Using site-directed mutagenesis and the cysteine accessibility method (Xu and Akabas, 1993) several amino acids in the pore-lining TM2 region of the α1 (Val257, Thr261, Thr267, Ser272), β2 (Thr246), β3 (Ala252, Leu253) or γ2 (Thr271, Thr277) subunits have been revealed to be important for picrotoxinin or TBPS sensitivity (Gurley et al., 1995; Xu et al., 1995; Perret et al., 1999;

Jursky et al., 2000; Buhr et al., 2001). These data strongly suggest that the convulsant binding site is located in the ionophore. Picrotoxinin and TBPS also block $GABA_C$ receptors in electrophysiological recordings (Vale et al., 1999), and may also bind [^{35}S]TBPS, but this has not been published.

[^{35}S]TBPS binding can be used to image the convulsant/ionophore sites (Squires et al., 1983; Ticku and Maksay, 1983; Supavilai and Karobath, 1984; Trifiletti et al., 1984). [^{35}S]TBPS binding is absolutely anion dependent (Squires et al., 1983; Trifiletti et al., 1984), and anion permeability though the $GABA_A$ receptor ionophore bears a direct relationship to [^{35}S]TBPS affinity (Havoundjian et al., 1986). Since changes in the chloride concentration affect mainly [^{35}S]TBPS binding association, but not dissociation, rate constants (Garrett et al., 1989), Maksay (1996) has speculated that chloride ions are needed for the formation of the binding site for [^{35}S]TBPS. In the presence of chloride, [^{35}S]TBPS associates with picrotoxinin-sensitive sites slowly, and equilibrium is reached in 3 h (Edgar and Schwartz, 1990). [^{35}S]TBPS dissociates in a monophasic way with a dissociation constant of 88 min (Edgar and Schwartz, 1990). [^{35}S]TBPS binding has a pH optimum at 7.5-8.5, and the optimal temperature is about 21 °C (Squires et al., 1983). Picrotoxinin block of GABA currents have been shown to be use-dependent (Yoon et al., 1993). In addition, Yoon et al. (1993) described a picrotoxinin block in dissociated rat hippocampal neurons that was not use-dependent and suggested two different binding sites in one receptor or in different receptors (Figure 4). Whether the same applies to receptors outside the hippocampus or to TBPS binding, is currently unknown. However, [^{35}S]TBPS binding is accelerated in the presence of nanomolar GABA concentrations (Korpi and Lüddens, 1993), and electrophysiological block by TBPS is achieved more rapidly with repeated or prolonged GABA applications (Van Renterghem et al., 1987; Dillon et al., 1995). [^{35}S]TBPS binding is inhibited by micromolar GABA concentrations (Squires et al., 1983), its dissociation correlating with the chloride flux (Im and Blakeman, 1991), and recovery from the block can be speeded up with repeated GABA applications in electrophysiological experiments (Van Renterghem et al., 1987). These data together show that TBPS (and picrotoxinin) dissociation is use-dependent, and association is accelerated by agonist application. Both TBPS and picrotoxinin are suggested to stabilize the inactivated state of the receptor, because they decrease the channel open probability without affecting the mean open times (Hamann et al., 1990; Newland and Cull-Candy, 1992). Thus, [^{35}S]TBPS binding represents resting or non-conducting receptors, whereas dissociation of the binding by GABA and other positive modulators serves as a biochemical measure of receptor activation (Im and Blakeman, 1991). While TBPS and picrotoxinin bind to both GABA-bound and unbound receptors, their affinity to the former is about 10 times higher (Dillon et al., 1995). This suggests that TBPS has better access to its binding site in the open configuration of the receptor.

While it is clear that $GABA_A$ receptor agonists and other positive modulators at high concentrations cause [^{35}S]TBPS dissociation (e.g., GABA: Squires et al., 1983; muscimol: Wong et al., 1984; benzodiazepine agonists: Gee et al., 1986; barbiturates: Squires et al., 1983), and antagonists or negative modulators inhibit the effect of GABA (e.g., bicuculline and SR 95531: Squires and Saederup, 1987; furosemide: Korpi et al., 1995a), it is not so clear how low agonist concentrations seem to enhance [^{35}S]TBPS binding (Supavilai and Karobath, 1984; Korpi and Lüddens, 1993). Studying the effects of GABA agonists and antagonists on [^{35}S]TBPS binding kinetics Maksay and Simonyi (1986) proposed a model for convulsant site binding, which explains these “low-dose hooks”. They assume the existence of two interconvertible states of the convulsant binding sites, one with slow and the other with rapid kinetics, representing the closed and open forms of the $GABA_A$ receptor ionophore, respectively. In the model, agonists accelerate both association and dissociation of [^{35}S]TBPS, low concentrations more inducing the association rate while and higher concentrations predominantly speeding up the dissociation rate. Bimodal actions seen in [^{35}S]TBPS binding

with different agonist concentrations are thus caused by a shift in receptor kinetics from slow ([^{35}S]TBPS bound) to rapid ([^{35}S]TBPS dissociated) when shifting from low to high agonist concentrations (Maksay and Simonyi, 1986). According to this model, agonists accelerate reaching of the binding equilibrium, whereas antagonists delay it. This stresses the importance of using equilibrium conditions (at least 180 min incubation in the presence of chloride) when probing for the effects of different compounds on [^{35}S]TBPS binding. Otherwise a "low-dose hook" (where there is more [^{35}S]TBPS binding in the presence of an agonist at nonequilibrium than at equilibrium) might cause misleading results (Maksay and Simonyi, 1986). However, if one uses [^{35}S]TBPS binding to reveal receptor subtype heterogeneities, nonequilibrium conditions may also be used, especially in autoradiography, when the tissue might deteriorate during prolonged incubation at room temperature.

2.5.3. Benzodiazepines

The $GABA_A$ receptor BZ binding site has been extensively studied due to it's clinical importance and due to it's heterogeneity, which has enabled development of subtype-preferring drugs and elucidation of the significance of certain receptor subtypes (reviewed in Rudolph et al., 2001). BZ site full agonists, such as diazepam and flunitrazepam, are anxiolytic, anticonvulsant, sedative and myorelaxant. They potentiate the actions of GABA (Macdonald and Barker, 1978) by increasing the frequency of channel openings (Study and Barker, 1981) and by increasing channel conductance (Eghbali et al., 1997) due to increased affinity to GABA (Skerritt et al., 1982; Hattori et al., 1986). In the absence of an agonist, however, BZs are without effect (Study and Barker, 1981; Hattori et al., 1986) and are thus unable to increase the maximal effect of GABA (Hattori et al., 1986), which makes them safe when used as a single therapeutic. In addition to full agonists, the entire spectrum of compounds from partial agonists (only partial efficacy; e.g., bretazenil; Martin et al., 1988) to antagonists (blocking the effect of agonists and inverse agonists without affecting the effect of GABA *per se*; e.g., flumazenil; Hunkeler et al., 1981) and inverse agonists (decreasing the effect of GABA, e.g., Ro 15-4513 (ethyl-8-azido-5,6- dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate); Bonetti et al., 1988) exist. The mode of action of the given compound varies depending on the receptor subtype (see below).

Prior to cloning of $GABA_A$ receptor subunits, the BZ binding sites were divided into types I and II, the former having a higher affinity to CL218,872 and zolpidem (Squires et al., 1979). In addition, a third group of receptors were found that bound Ro 15-4513 with high affinity, but were insensitive to classical BZs such as diazepam (Sieghart et al., 1987; Malminiemi and Korpi, 1989). Subsequent cloning of receptor subunits and heterologous expression of different subunit combinations provided a molecular explanation for the previous findings: the type of α subunit variant in the receptor complex largely determines the BZ pharmacology (Pritchett et al., 1989a; Lüddens et al., 1990). [^{3}H]Ro 15-4513 can be used to label all BZ binding sites (Sieghart et al., 1987). However, it can be displaced by full agonists, such as diazepam, only from receptors containing α1, α2, α3 or α5 subunits. These subunits differ from α4 and α6 in having a critical amino acid (His in the position of about 100 in α1, α2, α3 and α5; Arg in position 99 and 100 in α4 and α6, respectively), which makes α4/6βγ2 receptors diazepam-insensitive (Wieland et al., 1992). Vice versa, introduction of arginine to α1 makes receptors diazepam-insensitive (Wieland et al., 1992), and α6 receptors can be converted into diazepam-sensitive ones by exchanging histidine for glutamine (Korpi et al., 1993). While Ro 15-4513 is a partial inverse agonist (i.e., it decreases the effect of GABA; Bonetti et al., 1988) in most receptor subtypes, it has an agonist effect in α4/6βγ2 receptors (Knoflach et al., 1996). Mutating the α1 subunit His101 can be used to convert flumazenil to a partial agonist and Ro 15-4513 to a partial agonist or antagonist,

depending on mutation, in the α1β2γ2 receptors (Dunn et al., 1999; Crestani et al., 2002b), which further demonstrates the importance of this residue in both BZ binding and signal transduction. Recently, a demonstration of the direct interaction of the diazepam molecule and α1 His101 was provided (Berezhnoy et al., 2004).

The key role of the aforementioned histidine/arginine heterogeneity of α subunits in the BZ binding domain pharmacology has been exploited to elucidate the significance of receptor subtypes with different α subunits. Replacing the natural histidine with arginine in α1, α2, α3 and α5 subunits, and subsequent generation of transgenic mouse lines with these "knock-in" mutations, have enabled a very elegant dissection of the different behavioral effects of BZ agonists. The α1 subunit-containing receptors are mainly responsible for sedation, anterograde amnesia and anticonvulsive effects (Rudolph et al., 1999; McKernan et al., 2000). The α2 subunit-containing receptors mediate anxiolysis selectively and myorelaxation partially (Löw et al., 2000; Crestani et al., 2001). The α3 subunit-containing receptors partially mediate myorelaxation (Löw et al., 2000; Crestani et al., 2001). The α5 subunit-containing receptors affect trace fear conditioning and exert partially myorelaxation (Crestani et al., 2002a). These findings at present contribute the best rationale for future subtype-selective compounds (Rudolph et al., 2001).

In addition to diazepam sensitivity, the type of α subunit also determines the BZ I and BZ II type pharmacology (Pritchett et al., 1989a). The α1 Gly201 confers high affinity to CL218,872 and zolpidem (Pritchett and Seeburg, 1991). If the amino acid volume in position 201 is increased from that of glycine, a suggested steric inhibition confers BZ pharmacology to type II (Wingrove et al., 2002). The α1 subunit preference of zolpidem has also been confirmed *in vivo* (Crestani et al., 2000).

In addition to the α subunit, γ2 is needed for high-affinity BZ binding (Pritchett et al., 1989b), and the BZ binding site is located at the interface between the α and γ2 subunits (reviewed in Smith and Olsen, 1995). The type of β subunit variant has no major role (Pritchett et al., 1989a). The δ subunit-containing receptors have no BZ binding sites (Quirk et al., 1995). In the γ2 subunit at least the amino acid residues Phe77 (Buhr et al., 1997) and Met130 (Buhr and Sigel, 1997) are shown to be important. Thr142 seems to be the critical amino acid in determining the efficacy of the BZ ligand, since mutations in this amino acid may convert negative or neutral modulators to positive ones (Mihic et al., 1994). Receptors containing γ1 or γ3 subunits also possess a BZ binding site, and the affinity and efficacy of BZ ligands are modulated by the type of γ variant (Knoflach et al., 1991; Puia et al., 1991; Herb et al., 1992; Wafford et al., 1993; Lüddens et al., 1994). Since the γ2 subunit is the major γ subunit in the brain, the γ1- and γ3-containing receptors are of minor importance in BZ modulation, as supported by the finding that in the γ2 knockout mice, 94 % of the brain BZ binding sites are abolished (Günther et al., 1995).

2.5.4. DMCM

DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) has two distinct binding sites in $GABA_A$ receptors. It is a BZ site inverse agonist in the receptors containing α subunit other than α6 (Im et al., 1995b; Knoflach et al., 1996; Saxena and Macdonald, 1996). Through BZ inverse agonism, DMCM has *in vivo* stimulant, anxiogenic and proconvulsant actions. Whether partial agonism of α6 subunit-containing receptors' BZ site takes place *in vivo*, is currently unknown. However, the His101Arg mutation of α1 subunit is enough to confer DMCM from an inverse agonist to a partial agonist *in vitro*, and leads to complete abolition of proconvulsant actions of DMCM *in vivo* (Crestani et al., 2002b).

In addition to the BZ site, DMCM acts as a low-affinity positive modulator independent of BZ binding (Im et al., 1995b). This action is dependent on the presence of the β2/3 subunit, since DMCM is inactive in the β1 subunit-containing receptors (Stevenson et al., 1995). Similarly to antiepileptic loreclezole (Wingrove et al., 1994), anesthetic etomidate (Belelli et al., 1997) and loop diuretic furosemide (see below), Asn290 in β2/3 (Ser in β1) is the crucial amino acid for low-affinity positive actions of DMCM (Stevenson et al., 1995). It is currently not known whether this residue forms a binding site for the compounds, or is merely a site for allosteric coupling. The potentiation is basically independent of the type of α subunit, but it is more pronounced in α6 subunit-containing receptors, possibly due to the lack of DMCM inverse agonism via the BZ site (Stevenson et al., 1995).

2.5.5. Furosemide

Furosemide is a loop diuretic widely used in cardiovascular diseases. Its diuretic actions are mediated by reversible binding to the $Na^+/2Cl^-/K^+$ transporter in the renal loops of Henle, which results in decreased reabsorption of NaCl and water. In addition to the peripheral effects, furosemide has been found to block GABA currents in frog spinal cord (Nicoll, 1978). However, since furosemide does not cross the blood-brain barrier, it does not have central effects *in vivo*. Korpi et al. (1995a) showed that furosemide interacts specifically with recombinant α6β2/3γ2 $GABA_A$ receptors and antagonizes the effects of GABA in the cerebellar granule cell layer, although it was inactive in other brain regions and in recombinant α6β1γ2 and α1β1/2/3γ2 receptors. Since another loop diuretic, bumetadine, was without an antagonistic effect, the furosemide antagonism was concluded to be independent of the ion transporter actions (Korpi et al., 1995a). Since furosemide did not interact with GABA, BZ or diazepam-insensitive Ro 15-4513 binding, a novel allosteric binding site was suggested (Korpi et al., 1995a). Later on furosemide actions were demonstrated to be independent of the presence of γ or δ subunits (Korpi and Lüddens, 1997). Antagonism has also been detected in recombinant α4β2γ2 receptors (Knoflach et al., 1996; Wafford et al., 1996), but the effect was not observed in [^{35}S]TBPS autoradiography in brain regions where an α4 subunit was expressed (Korpi and Lüddens, 1997). However, furosemide-sensitive GABA currents have been detected in hippocampal slices (Pearce, 1993).

Furosemide antagonism of α6 subunit-containing receptors has been studied with α1/α6 subunit chimeras and point mutations. It was first shown with chimeric constructs that the main determinant for furosemide action is located in the N-terminal part of the TM1 of the α6 subunit (Fisher et al., 1997; Jackel et al., 1998), and isoleusine at position 228 was later shown to be the most important amino acid (Thompson et al., 1999). However, all other α subunit variants have threonine in this position (personal observation), including the furosemide-sensitive α4 subunit, and thus additional factors conferring furosemide sensitivity must also exist. Furosemide has only intermediate sensitivity in α4 subunit-containing receptors (> 50 fold more sensitive than α1, α2, α3 or α5) when compared to α6 (> 500-fold more sensitive; Wafford et al., 1996), which might be explained by Ile228 in α6. Another domain of lower potency for furosemide might be present in the C-terminal part of the α6 subunit starting from TM2 (Jackel et al., 1998). Similar β subunit selectivity as for DMCM (see above), the antiepileptic loreclezole (Wingrove et al., 1994) and anesthetic etomidate (Belelli et al., 1997) suggested that furosemide shared the requirement for Asn in TM2 of the β2/3 subunits (Ser in β1), which was later on confirmed (Thompson et al., 1999).

2.6. Tiagabine

After synaptic release, GABA is taken up to neurons and glial cells by GABA transporters (GAT-1-4) together with Na^+ and Cl^- ions (1:2:1 ratio, respectively; reviewed in Borden, 1996). GAT-1 is the major transporter subtype throughout the brain. Tiagabine is a selective GAT-1 blocker (Borden et al., 1994), that is used in the treatment of epilepsy. Tiagabine exerts its antiepileptic actions by raising the extracellular GABA levels (Fink-Jensen et al., 1992) to the submicromolar range (Dalby, 2000). Tiagabine and/or other GAT-1 blockers have been shown to increase tonic background inhibition (Frahm et al., 2001; Overstreet and Westbrook, 2001; Nusser and Mody, 2002; Wisden et al., 2002; Jensen et al., 2003), and GAT-1 knockout mice display increased tonic inhibition (Jensen et al., 2003). In addition, tiagabine and/or other GAT-1 blockers have been shown to increase synaptic inhibition by prolonging the decay of evoked IPSCs and IPSPs (Dingledine and Korn, 1985; Roepstorff and Lambert, 1992; Thompson and Gähwiler, 1992; Draguhn and Heinemann, 1996; Overstreet and Westbrook, 2003). Usually, no changes in mIPSC amplitude or decay are detected (Jensen et al., 2003; Overstreet and Westbrook, 2003). These data suggest that GAT blockers do not affect individual synapses, but somehow regulate cross talk between synapses (Overstreet and Westbrook, 2003). However, Nusser and Mody (2002) found no effects on synaptic $GABA_A$ergic inhibition by these drugs.

2.7. $GABA_A$ receptors in disease

It is difficult to demonstrate a causative role for disturbed $GABA_A$ receptor function in the pathophysiology of any disease, but a connection between these two may be surmised on the basis of the following findings: 1) a genetic linkage between disease incidence and, e.g., receptor mutation, 2) altered $GABA_A$ receptor function (due to changes in receptor subunit composition or subunit expression) in the disease, 3) mouse models with selective $GABA_A$ receptor alterations displaying similarities to human disease, 4) good efficacy of $GABA_A$ergic drugs in the treatment of the disease. Based on these criteria, a role for the $GABA_A$ergic system may be proposed in a variety of neurological and psychiatric diseases. A detailed description of $GABA_A$ receptor function in anxiety and in epilepsy is given in the following sections. Other diseases linked with an altered $GABA_A$ergic system, or which can be efficiently treated with $GABA_A$ergic drugs, include at least alcoholism (Davies, 2003), Angelman's syndrome (DeLorey and Olsen, 1999), autism (Buxbaum et al., 2002), depression (Brambilla et al., 2003), premenstrual syndrome (Smith et al., 1998), schizophrenia (Benes, 1999) and sleep disorders (Lancel, 1999).

2.7.1. Anxiety

Anxiety is a normal response to stressful stimuli, but purposeless or prolonged anxiety is pathological. In stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated, including hypothalamic corticotropin releasing hormone (CRF) secretion and adrenal cortisol secretion. HPA is inhibited by cortisol via a negative feedback mechanism. In anxiety (including panic disorder, post-traumatic stress disorder and generalized anxiety disorder) elevated HPA activation persists, which affects the CRF-containing neurons in the hypothalamus, but also in many other brain regions such as the hippocampus, amygdala, locus coeruleus, prefrontal cortex and cingulate gyrus. These areas are important in the expression of anxiety (reviewed in Coplan and Lydiard, 1998). GABA, with other neurotransmitter systems, is important in keeping homeostasis during stress, for it opposes the actions of CRF on the HPA axis (reviewed in Lydiard, 2003).

Patients with anxiety have decreased BZ binding in many brain regions (Tiihonen et al., 1997; Malizia et al., 1998; Bremner et al., 2000a; Bremner et al., 2000b). These findings are supported by the decreased BZ binding in the mouse line heterozygous for $GABA_A$ receptor γ2 subunit deletion (γ2+/-), which displays a behavioral phenotype of the human generalized anxiety disorder (Crestani et al., 1999). Brain GABA levels have been shown to be decreased in anxious patients (Goddard et al., 2001), but the results of measuring plasma or cerebrospinal fluid GABA levels are controversial, and no firm conclusion about GABA levels can be drawn.

Strong evidence for a role of $GABA_A$ receptors in anxiety derives from its treatment. Traditionally, anxiety has been relieved by self-administration of ethanol. Barbiturates have also been found to efficiently decrease anxiety, but due to side effects their use in anxiety disorders is nowadays minimal. Elevating brain GABA levels with tiagabine has been shown to be promising approach to treat anxiety (Zwanzger et al., 2003). THIP has also been shown to have anxiolytic activity in animal models (Gulinello et al., 2003; Liang et al., 2004).

Today the BZs are by far the most widely used anxiolytic agents in Finland, as in 1995-1996 2.6 % of the population used them daily (Klaukka, 1999). Even though antidepressants are now suggested as the first choice treatment for anxiety (Gorman, 2003), largely due to their safety profile, BZs are very effective in short-term use. While full positive BZ site ligands, such as diazepam, are anxiolytic, inverse agonists are anxiogenic (reviewed in Korpi et al., 1997), which suggests a key role for the BZ binding site in the pathophysiology of anxiety. The aforementioned α subunit-dependent BZ binding site heterogeneity has made it possible to dissect the different behavioral actions of BZs to different receptor subtypes. In studies with transgenic mouse lines, an animal model with a point mutation in $GABA_A$ receptor α2 subunit was created that selectively lacked the anxiolytic effects of BZs, while other pharmacological effects of BZs were restored (Low et al., 2000). The α2 subunit-containing receptors are found mainly in the pyramidal cells of the hippocampus, where they are selectively modulated by a subset of interneurons (reviewed in Freund, 2003). These interneurons are modulated by serotonin, acetylcholine and cannabinoid systems, and act like sensors for mood. This link may explain the efficacy of antidepressants in the treatment of anxiety. Demonstration of the key role of the $GABA_A$ receptor α2 subunit in anxiolytic effects of BZs considerably strengthens the $GABA_A$ergic hypothesis of anxiety, and leads the way for selective drug design (Rudolph et al., 2001). Unfortunately, no α2-selective ligands are currently available, but they are supposed to largely lack the sedative, amnesic and muscle relaxant side effects of classical BZs, and hopefully possess a lower liability for abuse (Rudolph et al., 2001).

2.7.2. Epilepsy

Epilepsy is a heterogeneous neurological disorder characterized by involuntary seizures. The etiology of epilepsy varies from brain injuries or tumors to genetic abnormalities, and in many cases the cause is unknown. The exact mechanisms underlying seizure induction and propagation are still unrevealed, but, basically, epilepsy is a consequence of imbalance between excitation and inhibition leading to intense spontaneous local activity of neurons.

Seizures may be categorized as partial or generalized, the former usually not including disturbed consciousness, while the latter always does. In the normal brain, cortical pyramidal cell groups undergo rhythmic depolarizations mediated by glutamate and voltage-dependent channels resulting in synchronous bursts of action potentials (reviewed in Dichter, 1997). This depolarization shift is followed by prolonged hyperpolarization mediated at least by voltage-dependent potassium currents, calcium-dependent potassium currents, and $GABA_A$ and

$GABA_B$ receptors. In partial epilepsy the hyperpolarization phase is gradually decreased, and neurons are tonically depolarized. Depolarization leads to simultaneous and repetitive neuronal firing, which generates a seizure. Generalized seizures result from hypersynchrony of thalamocortical oscillations (Dichter, 1997), and are categorized to absence and tonic-clonic seizures. Absence seizures are characterized by short loss of consciousness without convulsions, and they originate from a disturbed thalamocortical loop. Generalized tonic-clonic seizures correspond to the most well-known "classical epileptic attack" with generalized motor seizures and loss of consciousness. Cortex, limbic structures (such as the hippocampus and amygdala) and the thalamus are important in the generation of clonic convulsions, whereas tonic convulsions originate from the brainstem (Browning and Nelson, 1986; Gale, 1992). However, both clonic and tonic seizures, once commenced, strongly activate the forebrain regions (Samoriski et al., 1997). Absence and motor seizures might share a common pathophysiology, as suggested by the finding that a rat line with genetic absence epilepsy is also more sensitive to convulsions produced by $GABA_A$ receptor negative modulators than a control rat line (Vergnes et al., 2000).

The role of $GABA_A$ receptors in the generation of seizures is supported by animal studies where convulsions may be produced by systemic administration of $GABA_A$ receptor negative modulators, such as DMCM and picrotoxinin. Cortical areas are most susceptible to picrotoxinin-induced convulsions (Vergnes et al., 2000), and the frontal cortex seems to be the lead area (Medvedev et al., 1996). In addition to cortical areas, the hippocampus and amygdala are also highly sensitive to the convulsive properties of DMCM (Vergnes et al., 2000). Picrotoxinin usually causes progressive convulsions, where writhing clonus (a brief episode of symmetrical forelimb clonus and a jerk of the neck) is followed by a generalized tonic-clonic convulsion, and then by a tonic hindlimb extensor convulsion. DMCM usually induces writhing clonus, which is only sometimes followed by a generalized tonic-clonic convulsion (Kosobud and Crabbe, 1990). During a prolonged seizure, status epilepticus, the $GABA_A$ receptor function has been shown to be decreased in the hippocampal CA1 region due to the reduced potency and efficacy of GABA and the reduced driving force for GABA-gated chloride currents (Kapur and Coulter, 1995).

In addition to acute seizures, which may be triggered by various stimuli, epilepsy may be examined from a developmental point of view. The hippocampus has proved a suitable model region in research of the development of epilepsy (reviewed in Avoli et al., 2002). During epileptogenesis, complex changes in the neuronal circuits take place, since hippocampal formation is one of the most seizure-prone areas in the brain. Synchronous ictal activity in the entorhinal cortex may paradoxically be initiated by the $GABA_A$ receptor-mediated mechanisms. From the entorhinal cortex, ictal activity propagates successively to the dentate gyrus, the CA3 and CA1 regions, and the subiculum. Seizures cause destruction of the CA1 and CA3 pyramidal cells and the dentate hilus, with relative sparing of the dentate granule cells and sprouting of their axon terminals (Figure 5). Axonal sprouting leads to recurrent excitation of dentate granule cells and increased excitability (Cronin et al., 1992). In addition to the dentate gyrus, extensive reorganization of axon collaterals has also been detected in the CA1 region, including formation of intra-CA1 and retrograde CA1-CA3 connections as well as retrograde connection between the subiculum and the CA1 region (Lehmann et al., 2001). In addition to the altered excitation, GABAergic inhibition in the epileptic hippocampus is impaired. Possible mechanisms include disconnection of inhibitory interneurons from excitatory inputs (Sloviter, 1987), use-dependent reduction of excitatory drive to inhibitory interneurons (Doherty and Dingledine, 2001), alterations in the number of $GABA_A$ receptors (Titulaer et al., 1994; Titulaer et al., 1995a, b; Nusser et al., 1998b), alterations in $GABA_A$ receptor subunit composition (Rice et al., 1996; Brooks-Kayal et al., 1998; Sperk et al., 1998), increased sensitivity to Zn^{2+} inhibition of $GABA_A$ receptors in the dentate granule cells (Buhl et al., 1996; Gibbs et al., 1997; Brooks-Kayal et al., 1998), and a

decrease in GABA transporter function (Williamson et al., 1995). In addition, mutations in $GABA_A$ receptor subunits have been described in patients with epilepsy. A point mutation in the γ2 subunit that greatly decreased the efficacy of GABA was detected in a family with inheritable generalized epilepsy (Baulac et al., 2001). Another mutation in the γ2 subunit, which abolishes the BZ sensitivity of the receptor *in vitro*, has been linked to childhood absence epilepsy and febrile seizures (Wallace et al., 2001). In juvenile myoclonic epilepsy, a mutation in α1 subunit, which decreases receptor function (Cossette et al., 2002; Fisher, 2004), has been detected (Cossette et al., 2002). Recently, a genetic variation in δ subunit, which decreased GABA-induced currents, was associated with generalized epilepsy with febrile seizures (Dibbens et al., 2004).

The essential role of $GABA_A$ receptors both in acute seizures and in epilepsy is supported by the mechanisms of action of anticonvulsive and antiepileptic drugs. In acute seizures, and during the status epilepticus, BZs are the most effective treatment. In the long term treatment of epilepsy, drugs that inhibit voltage-dependent sodium currents (e.g., carbamazepine, phenytoin) are most widely used, but also enhancement of the actions of GABA is also applied by, e.g., tiagabine, vigabatrin and topiramate.

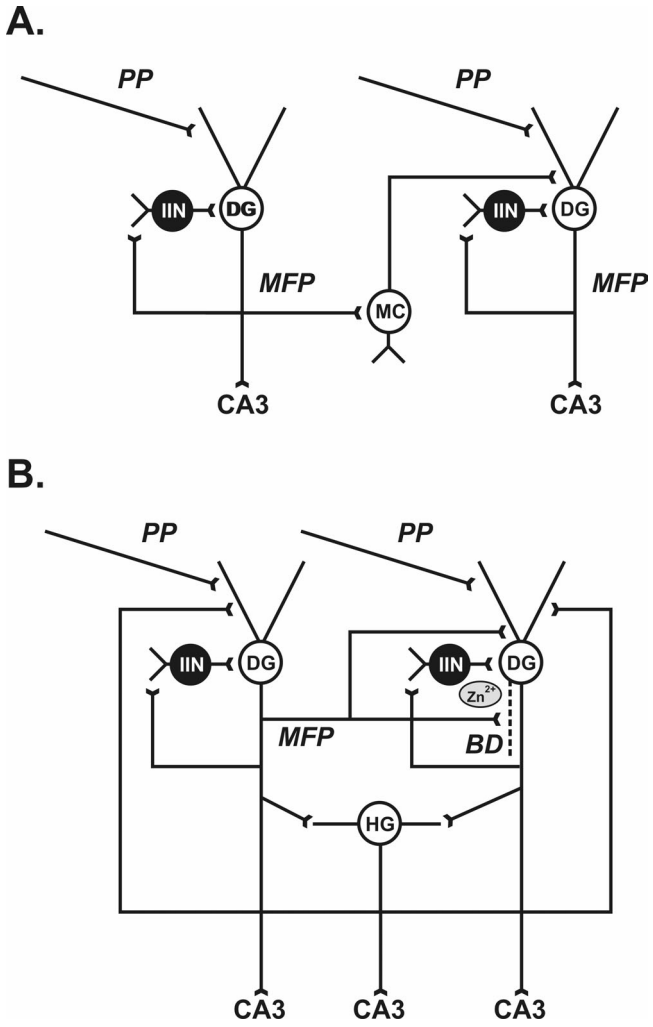

Figure 5. A simplified presentation of a dentate granule cell (*DG*) circuit in the normal (**A.**) and the epileptic (**B.**) hippocampus. **A.** Perforant pathway (*PP*) from the entorhinal cortex excites dentate granule cells, which relay excitation via the mossy fiber pathway (*MFP*) to the CA3 region. Mossy fibers also excite inhibitory interneurons (*IIN*) and mossy cells (*MC*), which project to other dentate granule cells. **B.** Seizures destroy mossy cells, which causes mossy fiber sprouting. Seizures also increase the rate of granule cell replication leading to generation of granule cells with both apical and basal dendrites (*BD*) and generation of ectopic hilar granule cells (*HG*). Also inhibitory interneurons are damaged (not shown). Mossy fiber collaterals form synapses with granule cell apical dendrites, with atypical basal dendrites and with ectopic hilar granule cells. Through these synapses granule cells excite other granule cells with glutamate. Zn^{2+} is co-released and it blocks inhibitory $GABA_A$ receptors of granule cells, leading to further excitation. Modified from Nadler (2003).

2.8. $GABA_A$ergic drug therapy and its problems

Drugs potentiating $GABA_A$ receptor function are widely used clinically to treat alcohol withdrawal, anxiety disorders, epilepsy, febrile convulsions, and sleep disorders. They are also used in general anesthesia. The problem of $GABA_A$ergic drug therapy is not insufficient efficacy but nonselective actions. The $GABA_A$ergic system is so widely distributed in the brain, that receptor subtype nonselective drug therapy by, e.g., classical BZs cause too powerful and nonspecific actions. Use of nonselective BZs, in addition to the desired anticonvulsive, muscle-relaxant, sedative and anxiolytic effects, leads to many side-effects (dizziness, ataxia, dysarthria, drowsiness, paradoxical anxiety/hostility, mild cognitive deficits and amnesia), development of dependence, tolerance and abuse, and to potentiation of the effects of other CNS-affecting drugs (reviewed in e.g., Lader, 1995). These are clinically important issues and have lead, e.g., to avoidance of BZ use in the long-term treatment of anxiety (Gorman, 2003), even though, in acute situations, BZs are the most potent anxiolytics.

As indicated in section 2.5.3., studying the heterogeneity of the BZ binding site has revealed that different behavioral effects of BZs are mediated by receptors containing different α subunits. Selective therapy against different subtypes might offer the possibility to escape the problems of nonselective drugs. This idea is strengthened by the finding that abuse of non-anxiolytic α1 subunit-preferring zolpidem-like sedatives is low (Jaffe et al., 2004). In addition to the BZ binding site, another heterogeneity, now in β subunits, has recently been introduced that mediates different behavioral effects. By mutating the residue in the β3 (Asn265 to Met) subunits that differentiates β1 from β2 and β3 in respect to loreclezole, DMCM, furosemide and etomidate sensitivity, resulted in decreased anesthetic actions of etomidate and propofol, but not clearly those of neurosteroids and inhalation anesthetics (Jurd et al., 2003). These data suggest that, in addition to BZ binding sites, significant heterogeneities exist in the other $GABA_A$ receptor effector sites as well. The therapeutic potential of the heterogeneity of these binding sites is still unrevealed.

In addition to the binding site heterogeneities, there is another possibility for receptor subtype specific drug treatment. It is offered by the differential subcellular distribution and special pharmacological properties of receptor subtypes in synaptic and extrasynaptic loci. Traditionally, $GABA_A$ergic drug therapy has been thought to affect synaptic receptors, but a recent study raised the possibility that the major site of actions of the anesthetic propofol might be the extrasynaptic receptors (Bieda and MacIver, 2004). With knowledge of the probable subunit composition of extrasynaptic receptors, it might be possible to selectively modulate tonic inhibition. This might lead to novel therapeutic strategies and safer treatment. This is supported by a recent finding that THIP, a GABA binding site partial agonist in most receptor subtypes but a full agonist in α4 and δ subunit-containing receptors (Brown et al., 2002), more efficiently relieved anxiety in a rat model of the premenstrual syndrome, where extrasynaptic α4βδ receptors are upregulated, than in control rats (Gulinello et al., 2003). In addition, there seems to be no development of tolerance to the anxiolytic effects of THIP (Liang et al., 2004).

Taken together, the future directions in $GABA_A$ergic drug research should include identification and characterization of novel receptor subtypes in the brain and elucidation of behavioral significance of already known receptor subtypes. Subsequent development of selective ligands for these receptor subtypes would offer the possibility to avoid the problems of nonselective therapy.

3. AIMS OF THE STUDY

The current project was targeted to characterize the atypically weak coupling between $GABA_A$ receptor agonist and convulsant binding sites (GIS-[^{35}S]TBPS binding) detected previously in the rat cerebellar granule cell layer (Korpi and Lüddens, 1993). The characterization was aimed to consist of a multilevel approach to ultimately elucidate, which receptor subtypes are responsible for GIS-[^{35}S]TBPS binding, and what are their properties and possible functions in the brain. For this, the following specific aims were placed:

1. to develop a receptor autoradiographic method to better visualize the GIS-[^{35}S]TBPS binding

2. to investigate the prevalence of GIS-[^{35}S]TBPS binding in different species

3. to reveal the molecular determinants and the mechanisms leading to GIS-[^{35}S]TBPS binding

4. to study the functional and behavioral significance of receptors showing GIS-[^{35}S]TBPS binding

5. to search for novel drug interactions in $GABA_A$ receptors with GIS-[^{35}S]TBPS binding autoradiography.

4. MATERIALS AND METHODS

All the experiments were done with the permission of the Institutional Animal Use and Care Committee of the University of Turku, Turku, Finland. When needed, permissions were obtained from the Western Finland Provincial Government.

4.1. Experimental animals

Male Wistar and Sprague-Dawley rats aged 4 months (**I**, *n* = 26; **II**, *n* = 8; **V**, *n* = 4) were used.

The human brain (**I**, *n* = 1) was obtained from clinical autopsy at the Department of Forensic Medicine, University of Oulu, Oulu, Finland. The brain autopsy was approved by the Ethics Committee of the University of Oulu and National Institute of Medicolegal Affairs, Helsinki, Finland. On the basis of medicolegal autopsy findings and medical records the case was determined as a normal control without medications affecting the central nervous system.

One-day-old White Leghorn chickens (**I**; *n* = 6) were used.

The following genetically-engineered mouse lines were used in the study and they were produced by collaborators as described below in the original references: three adult (one male and two females) β3-/- mice (Homanics et al., 1997a) with three wild-type controls (**II**); four male α6-/- (Homanics et al., 1997b), three male δ–/– (Mihalek et al., 1999) and five male wild-type control mice (**II**); ten adult male γ2+/- mice (Crestani et al., 1999) with nine littermate controls (**III**); male and female Thy1α6; Wisden et al., 2002; *n* = 85 and 61) and control (*n* = 86 and 47 for males and females, respectively) mice at the ages of 2 – 5 months or 16 – 20 days (**IV**).

Oocytes from female *Xenopus laevis* frogs were used (**V**).

4.2. Ligand autoradiographic assays (I, II, III, IV, V)

Receptor autoradiography of brain sections with selective ligands for $GABA_A$ receptor binding sites can be used to image the different $GABA_A$ receptor populations in the brain. Basically, thin sections are cut from frozen brain, and the sections are adhered to microscope slides. While most cells are destroyed during preparation, receptors remain intact. During incubation in optimized conditions, the radioactively labeled ligand binds to its binding site in the receptor complex on the brain section. After incubation, the unbound ligand is washed away in conditions that do not cause dissociation of the bound ligand. The washed sections are then dried, and microscope slides containing brain sections are exposed to irradiation-sensitive films. When the films are developed during the linear phase of exposure, the amount of irradiation detected by the film reflects the amount of ligand bound to the receptors in any particular brain area. All ligands have some nonspecific binding to brain tissue, but the amount of nonspecific binding can be determined by displacing the radioactively labeled ligand from its specific binding sites by competitive compounds.

4.2.1. Preparation of brain sections (I, II, III, IV, V)

To avoid degradation of receptor proteins, the brain samples were frozen on dry ice (solid CO_2) as soon as possible after dissection. Thereafter, they were wrapped in plastic and stored at - 80 °C until cut. Thin (14-µm for all other but 100-µm for human cerebral hemispheres)

frozen brain sections were cut in a cryostat at -20 °C. The sections were thaw-mounted onto gelatin-coated object glasses, air-dried, and stored frozen under desiccant at -20 °C. Freezing and sectioning of the brain destroy individual neurons, but when handled and stored properly, the receptor proteins on sections are preserved for several years. The macroscopic anatomy, i.e. the arrangement of the different brain regions on the sections, is also well preserved.

4.2.2. [^{35}S]TBPS binding (I, II, III, IV, V)

First autoradiographic studies with [^{35}S]TBPS showed that it might be used on brain sections (Gee et al., 1983; Wamsley et al., 1983). In slide-mounted cerebrocortical mash slice receptor autoradiography [^{35}S]TBPS had K_d of about 20 nM and only 2-4 % of the binding was nonspecific with 2 nM [^{35}S]TBPS (Edgar and Schwartz, 1990). In the same study with brain sections, nonspecific binding was not detectable.

In the present study, two procedures for [^{35}S]TBPS autoradiography were used. In general, the frozen object glasses with brain sections were thawed and encircled with a PAP-pen. Thereafter, sections were preincubated in ice-cold buffer containing 50 mM Tris [tris(hydroxymethyl)aminomethane]-HCl (pH 7.4) supplemented with 120 mM NaCl for 15 min to remove endogenous GABA. In the final incubation at room temperature for 90 min, an 800-µl bubble of incubation medium was applied on sections that were placed horizontally on an incubation platter. This reduced the required amount of radioactive ligand. In the standard assay (**III;** Edgar and Schwartz, 1990; Olsen et al., 1990; Korpi et al., 1995b) the final incubation in preincubation buffer was done with [^{35}S]TBPS (PerkinElmer Life Sciences Inc., Boston, MA, USA; diluted with cold TBPS to a final concentration of 6 nM, about 150 dpm/µl). After incubation, the sections were washed 3 x 15 s in ice-cold 10 mM Tris-HCl (pH 7.4). The sections were then dipped in distilled water, air-dried at room temperature, and exposed with a plastic ^{14}C-standard to autoradiographic films for 2 weeks. Nonspecific binding was determined with 100 µM picrotoxinin, which competitively bound to the same binding site, and displaced [^{35}S]TBPS.

A modified [^{35}S]TBPS binding assay (**I, II, III, IV, V**) was developed to reveal the GABA-insensitive [^{35}S]TBPS binding component. The procedure was otherwise similar to the standard assay, but final incubation was performed with 3-6 nM (as high as possible without the brain labeling exceeding the activity of the highest ^{14}C-standard) hot [^{35}S]TBPS (about 350 – 800 dpm/µl), and the washing time was lengthened to 3 x 30 min. Sections were exposed to autoradiographic films with ^{14}C-standard for 2 days to several weeks.

Modulation of [^{35}S]TBPS binding by various $GABA_A$ receptor agonists and antagonists in both procedures was studied by incubating those compounds together with [^{35}S]TBPS. Because we always used TBPS concentrations (3-6 nM) well below K_d of about 50 nM under our experimental conditions (Figure 7, Sinkkonen S.T. et al., unpublished results), our results only partially mirror the total amount of [^{35}S]TBPS binding sites in the brain. As well, we used pre-equilibrium binding of 90 min, which, in the absence of positive $GABA_A$ receptor modulators, yields only about 70 % of the total binding (Maksay and Simonyi, 1986). However, as discussed above, pre-equilibrium conditions may be used deliberately when screening for receptor subtype heterogeneities.

4.2.3. [^{3}H]Ro 15-4513 binding (I, III, IV)

The autoradiographic procedure for [^{3}H]Ro 15-4513 binding to $GABA_A$ receptor BZ binding site was performed as described (Olsen et al., 1990; Mäkelä et al., 1997). BZ binding site

hereterogeneity prevents labeling of all sites with full agonists such as flunitrazepam, but [^{3}H]Ro 15-4513 binds to all known BZ sites (Sieghart et al., 1987). In autoradiography, the brain sections were preincubated in ice-cold incubation buffer (50 mM Tris-HCl supplemented with 120 mM NaCl; pH 7.4) to remove endogenous GABA. Thereafter 5-10 nM [^{3}H]Ro 15-4513 (PerkinElmer Life Sciences Inc., Boston, MA, USA) binding in plastic 20-ml incubation containers was performed at 0-4 °C for 60 min. Since [^{3}H]Ro 15-4513 can be used to photoaffinity label BZ sites (Sieghart et al., 1987), incubation was performed in the dark. Nonspecific binding was determined with the BZ site competitive antagonist flumazenil, which displaced [^{3}H]Ro 15-4513 totally. After incubation, the sections were washed, air-dried, and exposed with a plastic ^{3}H-standard to autoradiographic films. Since only one concentration of [^{3}H]Ro 15-4513 near the K_d (Korpi et al., 1993; Löw et al., 2000) was used, our results with [^{3}H]Ro 15-4513 binding only partially reflect the total amount of BZ binding sites in the brain.

4.2.4. [^{3}H]Muscimol binding (I, III)

The autoradiographic procedure for [^{3}H]muscimol binding to $GABA_A$ receptor GABA binding site was carried out as described (Olsen et al., 1990; Mäkelä et al., 1997). While it is meaningful that all native $GABA_A$ receptors contain an agonist binding site, [^{3}H]muscimol under our experimental conditions seems to preferentially label receptors containing α6 and/or δ subunit (Korpi et al., 2002b). In the autoradiographic procedure, to remove endogenous GABA, slides were preincubated in ice-cold 0.31 M Tris-citrate (pH 7.1) for 15 min. Final incubation in the preincubation buffer was performed with 6-10 nM [^{3}H]muscimol (Amersham Biosciences Corp.) at 0-4 °C for 30 min in plastic 20-ml incubation containers. [^{3}H]Muscimol binding was performed at low temperature without sodium ions to inactivate the GABA transport system. Since [^{3}H]muscimol can be used to photoaffinity label GABA sites (Cavalla and Neff, 1985), incubation was performed in the dark. Nonspecific binding was determined with GABA, which bound competitively to the GABA sites, and displaced [^{3}H]muscimol totally. After incubation, sections were washed, air-dried, and exposed with a plastic ^{3}H-standard to autoradiographic films. Since only one concentration of [^{3}H]muscimol near the K_d (Uusi-Oukari and Korpi, 1989; Wisden et al., 1991) was used, our results only partially reflect the total amount of high-affinity [^{3}H]muscimol binding sites in the brain.

4.3. Localization of $GABA_A$ receptor subunits by *in situ* hybridization (II)

In the *in situ* hybridization (ISH) procedure a radioactively labeled nucleic acid probe is allowed to hybridize with its complementary mRNA molecule on brain sections. The probe-mRNA duplex formed can be visualized on irradiation-sensitive films similar to ligand binding receptor autoradiography. Since proteins are synthesized according to the directions encoded in mRNAs, radioactive signals from ISH can be used to map the cells and brain areas that express the proteins of interest. As with receptor proteins, mRNA remains intact and detectable on brain sections when processed properly. When working with mRNA, contamination by the RNA degrading enzyme RNAse must be avoided by using plastic gloves, sterile equipment, and diethyl-pyrocarbonate-treated and autoclaved solutions. Brain samples were processed identically to ligand autoradiography, but poly-L-lysine-coated microscope slides and storage at -80 °C were used. Paraformaldehyde fixation inactivates endogenous RNAse. After fixation, the sections were stored in 95 % ethanol at 4 °C until used.

ISH for detection of $GABA_A$ receptor subunit transcripts was done using the protocol described by Wisden and Morris (1994). Briefly, 36-45 base-long antisense oligonucleotide probes were synthesized complementary to rat (Institute of Biotechnology, University of Helsinki, Helsinki, Finland) $GABA_A$ receptor subunit cDNA sequences. Probes were [α-^{33}P] (NEN Life Science Products, Boston, MA, USA) 3'end-labeled with terminal transferase. Unincorporated nucleotides were separated by chromatography columns. Hybridization buffer containing labeled probe was applied to each slide, and hybridized under parafilm coverslips overnight at 42 °C. Sections were then washed, dehydrated, dried and exposed to autoradiographic films with ^{14}C standards. Specificity of probes was determined with 100 x excess of unlabeled probes.

4.4. Quantitative autoradiographic film analysis (I, II, III, IV, V)

To get quantitative measures of ligand binding and $GABA_A$ receptor subunit mRNA expression levels, autoradiography and ISH films were quantified using MCID AIS image analysis devices and programs (Imaging Research Inc., St. Catharines, Ontario, Canada) as described (Korpi et al., 1995b). Films were digitized and the plastic standards exposed simultaneously with the brain sections were used as references to create a standard curve. The binding densities for each brain area were quantified, and the resulting binding values were converted to radioactivity levels estimated for gray matter areas (in nCi/mg or nCi/g) using the standard curve. The specific binding or hybridization values were determined by subtracting the nonspecific binding or hybridization values from the corresponding total binding or hybridization values, respectively.

4.5. Recombinant $GABA_A$ receptors in HEK 293 cells (III)

Human embryonic kidney cells (HEK 293) are immortalized cancer cells that can be transfected with exogenous cDNA to produce various proteins. Protein targeting takes place according to the signal sequence of the transfected cDNA. Transfection with different $GABA_A$ receptor subunits results in receptor expression on cell surfaces. The receptor subtypes formed were then used in binding studies.

4.5.1. Receptor expression

Expression vectors (Shivers et al., 1989; Pritchett and Seeburg, 1990) for rat $GABA_A$ subunits were transfected into HEK 293 cells using a calcium phosphate precipitation method (Chen and Okayama, 1987) as described (Lüddens and Korpi, 1997). HEK 293 cells were plated on tissue culture dishes filled with medium. Cultures were incubated at 37 °C in a humidified 95% O_2/5% CO_2 atmosphere for 2-3 days. In the transfection process, vectors containing the desired subunit cDNAs were mixed with phosphate-buffered saline and $CaCl_2$. Calcium phosphate precipitates containing an arbitrary mixture of cDNA vectors were taken up by HEK 293 cells, which resulted in transient expression of recombinant $GABA_A$ receptors. Two days after transfection the cells were harvested, homogenized, washed by centrifugation and stored at –80 °C until binding experiments.

4.5.2. [^{35}S]TBPS binding assay

The membrane pellets were thawed and resuspended in 50 mM Tris/citrate buffer, pH 7.3. Resuspended cell membranes were incubated at room temperature with 6 nM [^{35}S]TBPS in 50 mM Tris-HCl (pH 7.3) supplemented with 200 mM NaCl in the absence or presence of GABA. Non-specific binding was determined by picrotoxinin. After incubation, the bound fraction of [^{35}S]TBPS was separated by rapid filtration of the membrane homogenates with ice-cold 10 mM Tris/HCl onto glass fiber filters under reduced pressure. Thereafter, the filters were dried, immersed in scintillation fluid, and the radioactivity determined in a liquid scintillation counter.

4.6. Electrophysiological recordings from acutely isolated mouse hippocampal CA1 pyramidal cells (IV)

In order to study the possible functional expression of α6 subunit in the Thy1α6 mouse hippocampus, mice at the ages of 16 – 20 days were decapitated, their brains were dissected, and 300 – 400 μm thick coronal brain slices were cut. The CA1 neurons were dissociated by gentle mechanical trituration, and the whole-cell patch-clamp method (Hamill et al., 1981) on CA1 pyramidal cells was applied as described (Möykkynen et al., 2003). Cells were patch-clamped at a holding potential of –60 mV. During the recording, the cells were constantly superfused with recording solution. Drugs were diluted in recording solution and applied to the cells with a multi-barrel fast solution application system. Currents evoked by drug application were recorded, filtered and digitized. Current amplitudes were measured from the baseline to the peak of the current trace using pClamp 8.0 software (Axon Instrument, Inc., Union City, CA, USA).

4.7. Determination of receptor subunit composition (IV)

To determine the abundance of αβ $GABA_A$ receptors in the hippocampi of control C57BL/6 and mutant Thy1α6 mice, all receptors were first extracted from hippocampal brain membranes. Receptors were then immunoaffinity chromatographed with a series of columns containing subunit-specific antibodies. The subunit composition of the remaining receptors was then determined by immunoprecipitation with subunit-specific antibodies, and their amount was quantified with subsequent [^{3}H]muscimol binding.

4.7.1. Preparation of receptor extracts

C57BL/6 and Thy1α6 adult mouse hippocampi in pools of 10 or 5, respectively, were used. The pools were suspended in deoxycholate buffer. The suspension was homogenized and incubated with intensive stirring for 1 hour at 4° C. After centrifugation at 150,000 *g* for 45 min, the clear supernatant was used in immunoaffinity chromatography.

4.7.2. Immunoaffinity chromatography

Immunoaffinity columns were prepared by coupling 3-5 mg of the purified antibodies to ImmunoPure immobilized protein A using the ImmunoPure Protein A IgG Orientation Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The hippocampal

extract was chromatographed slowly at 4° C up to two times on the same affinity column in order to completely remove the receptors containing the respective subunit from the extract, regenerating the respective column after each chromatography step by washing it.

4.7.3. Quantification of $GABA_A$ receptor subtypes by immunoprecipitation and [^{3}H]muscimol binding assays

For immunoprecipitation of the $GABA_A$ receptors present in the hippocampal extract, a sample of immunoaffinity column efflux was mixed with a solution containing subunit-specific antibodies in order to precipitate receptors containing the respective subunit (Pöltl et al., 2003). For determination of the total amount of receptors before and after immunoaffinity columns, an antibody mixture containing antibodies for β1, β2, β3 and α1 was mixed with the original extract or column efflux. This antibody composition was used because all functional $GABA_A$ receptors are supposed to contain at least one of the three β subunits, and most of them contain an α1 subunit (Tretter et al., 2001; Pöltl et al., 2003). The mixtures were then incubated overnight with gentle shaking at 4 °C. The receptors were then precipitated, and the precipitate was centrifuged for 5 min at 2 300 *g,* and washed three times.

In order to quantify the amount of receptors in the original hippocampal extract or column efflux, or the amount of receptors precipitated by subunit-specific antibodies (α4, α6, γ1-3, δ; Pöltl et al., 2003) from the column efflux, [^{3}H]muscimol binding was performed (Pöltl et al., 2003). The precipitated receptors were suspended in 1 ml of a solution containing 0.1 % Triton X-100, 50 mM Tris/citrate buffer (pH 7.1) and 40 nM [^{3}H]muscimol (29.5 Ci/mmol, PerkinElmer Life Sciences) in the absence or presence of 1 mM GABA. After incubation for 1h at 4 °C, the suspensions were rapidly filtered through Whatman GF/B filters, washed twice with 3.5 ml of 50 mM Tris/citrate buffer (pH 7.1) and subjected to liquid scintillation counting. Binding in the presence of 1 mM GABA (nonspecific binding) was then subtracted from binding in the absence of GABA (total binding), resulting in specific binding to the precipitated $GABA_A$ receptors.

4.8. Mouse behavioral studies (IV)

4.8.1. Observational functional analysis by the SHIRPA protocol

Even though transgenic animals offer the possibility to determine the role of a given gene in animal behavior, the effects of virtually selective genomic rearrangements might be widespread and nonselective. For this reason it is important to perform a basic observational functional analysis of new transgenic mouse lines before starting a detailed behavioral test.

A modification of the primary screen of the SHIRPA protocol (www.mgu.har.mrc.ac.uk/mutabase/shirpa_summary.html) was used to analyze the physiology and basic neurological and behavioral properties of the Thy1α6 mice and their C57BL/6 controls, as described (Vekovischeva et al., 2004). Mice were observed with the bare eye in a viewing glass jar, in an open arena and on a grid floor. Mouse body position, spontaneous activity, respiratory rate and presence of tremor were evaluated in a viewing glass jar. The first behavioral reaction of a mouse placed at the centre of an open arena (transfer arousal), the number of crossed squares, the gait, the pelvic and the tail elevations were assessed. The grid floor was used as a support for the viewing jar and to measure tail suspension, grip strength and the negative geotaxis reflex. The negative geotaxis reflex was assessed as the possibility of a mouse to turn around and climb up on the grid floor quickly raised to the vertical plane. The sensorymotor response consisted of pinna, corneal and toe

pinch reflexes. Pinna and corneal reflexes were provoked by a stainless steel wire with a gentle touch on the ear lobe or the cornea of the eye. The toe pinch reaction was measured by pinching the central toe of the right hind paw with forceps. A click box generating a sudden tone at 90 dB was held 30 cm above the mouse to measure the startle reflex. Touch-escape behavior was determined as a response to a finger stroke of the back. Struggle-escape behavior combined the body position, touch-escape response and aggression toward an experimenter and the mouse vocalization provoked by handling.

4.8.2. Light-dark choice test

Mice and other rodents have a congenital aversion of brightly illuminated areas but simultaneously display spontaneous exploratory behavior. In the light-dark choice test (Crawley and Goodwin, 1980), mice have to make a decision between a "familiar - safe" dark area and a "new - fearful" illuminated area. Normally, mice have a high tendency to explore, and they often visit the illuminated area even though they prefer the dark compartment. Anxiety heightens the aversion to the novel environment (Crestani et al., 1999), and the light-dark choice test can be used to test for this disturbance (Crawley and Goodwin, 1980). The light-dark test was started by placing a mouse in the light compartment of two-compartment box divided into one dark and one lit area with an open door between them (Crawley and Goodwin, 1980). The time before the first crossing to the dark compartment (latency), the number of crossings between compartments, and the time spent in the light compartment (minus the latency) were recorded (Vekovischeva et al., 2004).

4.8.3. T-maze test

Similar to the light-dark choice test, the T-maze test is based on the mouse's natural desire to explore its territory. Exploration is important for learning and memory, which, in turn, are crucial for survival, because they enable the animal to know, e.g., where food is to be found and how to avoid predators. The T-maze alternation procedure allows measuring of exploratory behavior in an unbiased way (Gerlai, 1998). The T-maze consisted of three arms with three removable guillotine doors separating a short compartment at the beginning of the start arm and the other arms. The procedure consisted of one forced trial followed by choice trials during 10 min. Mice were individually placed in the short compartment. After 5 s of confinement, the door was lifted and the mouse was allowed to explore the start arm and one of the goal arms. Entry to the other goal arm was blocked. After this first, "forced" trial, the mice explored the areas available to them and eventually re-entered the start arm and moved down to the start compartment. When they entered the start compartment they were confined there for 5 s by closing the start compartment door. Before reopening the door, the door blocking one of the goal arms was also lifted. The start compartment door was then lifted and the first free choice trial began: the mice could choose between the two goal arms. After the mouse had chosen and entered half way down one goal arm, the other arm was blocked by the door. The mice left the explored goal arm and moved down to the start box again. They were confined there for 5 s and the testing cycle continued with another free choice trial as described above. The number of free choice trials was calculated as the level of mouse activity. An important aspect of the procedure was that the mice were not allowed to go from the chosen goal arm directly to the opposite goal arm. Once they had entered one of the goal arms at a particular choice trial, the opposite arm was blocked by closing the entrance door. This door was opened for the subsequent choice trial, which presented a novel change for the mouse at each choice trial. Since the presence of a novel stimulus in the goal arm has been

shown to elicit exploration of that goal arm, the mice were expected to explore the arm that was blocked at the previous trial. Consecutive choices made by the mice were measured, and the overall alternation rate was calculated (0 % = no alternation, 100 % = alternation at each trial).

4.8.4. Convulsion tests

$GABA_A$ receptor antagonists block GABA-induced chloride currents and cause neuronal hyperexcitability leading to brain seizures and involuntary muscle jerks called convulsions. Latency to convulsions after $GABA_A$ receptor antagonist administration can be used to determine the sensitivity of the mice to convulsions (Kosobud and Crabbe, 1990). In the present experiments, mice were injected intraperitoneally (i.p.) with different doses of either DMCM or picrotoxinin. After injection, the mice were placed in a transparent Plexiglass cylinder, their tails were tape-fixed, and latency to different convulsions was measured (Kosobud and Crabbe, 1990). The anticonvulsive effect of tiagabine was tested with i.p. injections 30 min prior to picrotoxinin administration. Mice were killed by cervical dislocation immediately after observing a generalized tonic-clonic convulsion or at the end of the 15 min observation period, whichever came first.

4.9. Recombinant $GABA_A$ receptors in *Xenopus* oocytes (V)

Xenopus oocytes can be used as protein expression machines, since they express and target proteins according to the RNA injected into their cytoplasm. Injection with a mixture of different $GABA_A$ receptor subunit cRNAs results in the formation of different receptor populations on the cell surface. The properties of these receptor subtypes can then be studied using electrophysiological techniques.

4.9.1. Receptor expression

Capped cRNAs coding for rat $GABA_A$ receptor subunits (Shivers et al., 1989; Ymer et al., 1989; Seeburg et al., 1990; Jackel et al., 1998) were transcribed *in vitro* from pRK5 plasmids using mMessage mMachine kit (Ambion Inc., Austin, TX, USA). Oocytes from adult *Xenopus laevis* females were injected with mixtures of subunit cRNAs. After injection the oocytes were collagenase-treated to remove the follicular cell layer and incubated at 19 °C until recordings.

4.9.2. Electrophysiological recordings

To allow production of receptors, the electrophysiological recordings were made 1-3 days after cRNA injection. The function of recombinant $GABA_A$ receptors was studied using the two-electrode voltage-clamp method as described (Kuner et al., 1993). Oocytes were impaled with two microelectrodes and voltage-clamped at – 50 mV. Agonist application to the oocytes caused receptor activation and an inward current. The inward current needed to maintain the potential at -50 mV was measured. Currents were modulated by simultaneous drug applications. The amplitudes of the peak currents induced by GABA + drug applications were determined from the recorded traces, normalized to the corresponding GABA-induced peak currents. The peak currents induced by various GABA concentrations for each oocyte were

normalized by setting the saturating GABA-current at 100 %, and the GABA concentration-response curves were generated using nonlinear regression fit.

4.10. Statistics (I, II, III, IV, V)

Statistics were performed with GraphPad Prism (GraphPad Software, San Diego, CA, USA) and SAS-STAT (SAS Institute, Cary, NC, USA) software programs. When the statistical significance of the difference between two groups was assessed, Student's *t*-test was used. One-way analysis of variance (ANOVA) and Dunnett or Newman-Keuls post tests were used when the means of three or more groups were compared. Two-way ANOVA (factors: mouse line, brain region) with a Bonferroni post test was used to assess the statistical significance of the difference in ligand autoradiography of different brain regions between the γ2+/+ and γ2+/- mice. Behavioral tests were analyzed by two-way ANOVA (factors: gender and mouse line). Three-way ANOVA (factors: gender, mouse line, drug dose) was used to analyze the data from the convulsion experiments. When ANOVA revealed significant differences ($p < 0.05$), it was followed by decreased-factor ANOVA or Dunnett post tests.

5. RESULTS AND DISCUSSION

5.1. Development of a receptor autoradiographic method to reveal GABA-insensitive [^{35}S]TBPS binding (I)

$GABA_A$ receptors in the brain can be imaged using radioactively labeled ligands for specific binding sites. Ligand binding can be studied with brain homogenates, brain sections or with PET or SPECT. Experimental studies are usually carried out with brain homogenates or sections, while PET and SPECT can be applied in clinical studies with living patients. Working with brain section receptor autoradiography instead of ligand binding to brain homogenates offers the advantage of spatial resolution. Since $GABA_A$ receptors have tremendous heterogeneity according to the brain region where they are expressed, brain sections should be used whenever possible to avoid summation of the effects by the various receptor subtypes in the brain homogenates. Anyhow, the biochemical and pharmacological characterization of ligand binding is more convenient with brain homogenates because of the easier preparation of samples, the simpler binding assay procedures and the faster analysis of data. For these reasons, binding of most of the radioactive ligands to the picrotoxinin binding site of the $GABA_A$ receptor has been characterized with brain homogenates. Nevertheless, binding kinetics in brain sections and in homogenates are largely similar.

The picrotoxin derivative [^{3}H]α-dihydropicrotoxin has low affinity (K_d about 1 μM) and yields high (~ 80 %) nonspecific binding (Ticku et al., 1978) in brain homogenates. [^{3}H]EBOB has a high affinity (K_d about 5 nM) and 3-6 % of the binding is nonspecific (Kume and Albin, 1994). [^{3}H]TBOB has a relatively high affinity (K_d 60 nM), and 10-20 % of the total binding is nonspecific (Lawrence et al., 1985). In brain homogenates, ^{35}S-labeled *t*-butylbicyclophosphorothionate ([^{35}S]TBPS) has a high affinity (K_d about 15 nM) and about 30 % nonspecific binding (Squires et al., 1983). However, with brain sections, nonspecific binding is very low (Edgar and Schwartz, 1990). The ^{35}S label emits high energy β radiation as compared to ^{3}H, which enables shorter exposure periods. For these reasons [^{35}S]TBPS has proved superior to the other compounds in the picrotoxinin site receptor autoradiography, and so was chosen for the present studies.

[^{35}S]TBPS binding is considered to represent resting receptors, whereas its dissociation by GABA and other positive modulators is thought to be a biochemical measure of receptor activation, i.e., chloride flux (Im and Blakeman, 1991). Usually, low micromolar GABA leads to displacement of [^{35}S]TBPS binding from brain homogenates or sections (Squires et al., 1983; Edgar and Schwartz, 1990; Olsen et al., 1990; Korpi et al., 1995b), whereas GABA antagonists reverse the inhibitory effect of GABA (Squires et al., 1983; Squires and Saederup, 1987). However, in the cerebellar granule cell layer, a minor receptor population with [^{35}S]TBPS binding insensitive to the inhibitory effect of GABA has been reported (Edgar and Schwartz, 1990; Korpi and Lüddens, 1993). Edgar et al. (1990) found that in the presence of 50 mM K_2HPO_4/ NaH_2PO_4 and 200 mM NaCl, 1 μM GABA displaced only 11 % of the [^{35}S]TBPS binding in the internal rim of the cerebellar granule cell layer, while it was more effective in the molecular layer and the external rim of the granule cell layer. Korpi and Lüddens (1993) demonstrated that [^{35}S]TBPS binding in the cerebellar granule cell layer was actually insensitive to 50 μM GABA in 50 mM Tris-HCl buffer supplemented with 120 mM NaCl, while, in all other brain regions, the binding was displaced.

The first purpose of the thesis study was to develop an autoradiographic method to better visualize the GABA-insensitive component of [^{35}S]TBPS binding (Korpi and Lüddens, 1993). For this purpose, we modified the standard incubation conditions (Edgar and Schwartz, 1990; Olsen et al., 1990; Korpi and Lüddens, 1993). To improve the sensitivity of the autoradiographic assay we increased the specific activity of the radioligand and always used

as high [^{35}S]TBPS concentrations (3-6 nM) as possible without exceeding the activity of the ^{14}C standard. 90 min incubation with [^{35}S]TBPS in the standard buffer (50 mM Tris-HCl supplemented with 120 mM NaCl, pH 7.4) at room temperature was used, since it allows association of high basal [^{35}S]TBPS binding but still maintains the integrity of brain sections so that they tolerate the prolonged washing. A washing protocol of 3 x 30 min in ice-cold hypotonic washing buffer (10 mM Tris-HCl, pH 7.4), instead of 3 x 30 s in the standard incubation buffer, decreased nonspecific binding without affecting specific binding (**I: Fig. 2**). These modifications improved the signal-to-noise ratio of the assay, and we were able to use long film exposure times without an excessive background (**I: Fig. 1**).

GABA decreased [^{35}S]TBPS binding already at 10 μM concentration in most brain regions, but longer film exposure revealed that in certain areas the [^{35}S]TBPS binding persisted in the presence of 1 mM GABA (**I: Fig. 1**). Differential responses were detected, e.g., in the inferior colliculus and the thalamus. Both regions displayed high basal [^{35}S]TBPS binding; some binding was left in the presence of 30 μM GABA in both areas but, in the presence of 1 mM GABA, [^{35}S]TBPS binding was detected only in the thalamus. However, the GABA-insensitive [^{35}S]TBPS binding component represented only a small fraction of the total binding, since most of the binding was GABA-sensitive in the thalamus also. Increasing the GABA concentration to 10 mM did not cause further reduction in the [^{35}S]TBPS binding (data not shown). Thus, this binding component was named the GABA-insensitive (GIS) [^{35}S]TBPS binding.

Regardless of our modifications of the standard autoradiographic procedure, the fundamental characteristics of the [^{35}S]TBPS binding still remained. These included competitive displacement by picrotoxinin (**I: Fig. 1**), reversibility of the binding (Figure 6; Sinkkonen S.T. et al., unpublished results) and the absolute anion dependence of the binding, since no specific binding was detected when incubation was performed in 1 mM HEPES [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) ; pH 7.4] in the absence of halide anions (data not shown). These results show that the modified autoradiographic procedure represents normal [^{35}S]TBPS binding. This was confirmed by a direct comparison between the standard procedure and our modified autoradiographic procedure with mouse brain sections (**III**). Basal [^{35}S]TBPS binding in wild-type (γ2+/+) mice was largely similar in these two protocols (**III: Figs. 2 and 3**).

Even though some glycine receptor isoforms are picrotoxinin-sensitive (Elster et al., 1998), [^{35}S]TBPS binding to glycine receptors has not been detected (Rienitz et al., 1987). In electrophysiological recordings, picrotoxinin also blocks $GABA_C$ receptors (Enz and Cutting, 1999), but [^{35}S]TBPS binding to these receptors has not been studied. However, since $GABA_C$ receptors are predominantly expressed in the retina (reviewed in Bormann, 2000), they cannot be related to GIS-[^{35}S]TBPS binding. These results indicate that the modified autoradiographic procedure with high sensitivity makes it possible to study minor $GABA_A$ receptor populations, and that receptors with poor coupling between the agonist and convulsant sites are also present outside the cerebellum (Korpi and Lüddens, 1993).

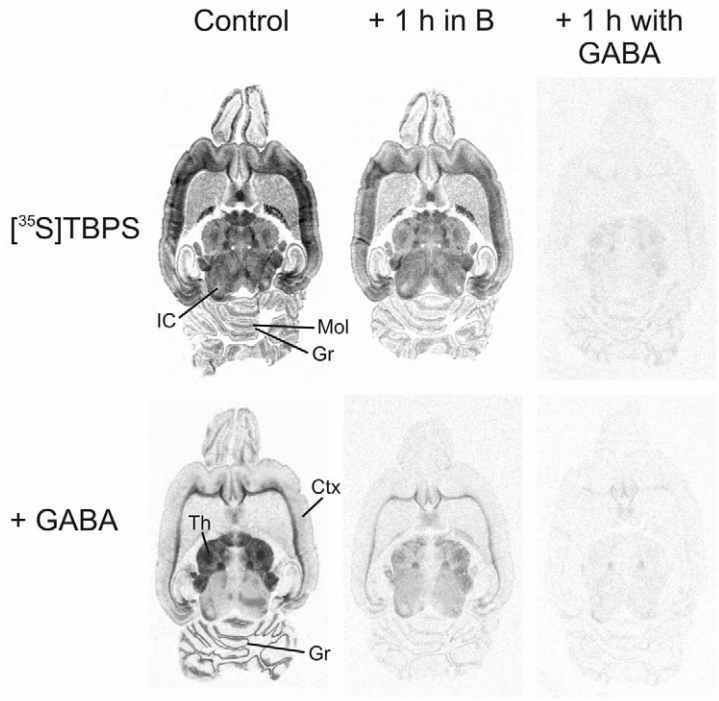

Figure 6. Reversibility of GIS-[^{35}S]TBPS binding on rat brain sections. *Control*, the modified autoradiographic procedure (4.2.2.); *+ 1 h in B*, standard 90-min incubation (in the absence or presence of 1 mM GABA, the upper and lower rows, respectively) followed by an additional 1-hour incubation in the fresh incubation buffer; *+ 1 h with GABA*, standard 90-min incubation (in the absence or presence of 1 mM GABA, the upper and lower rows, respectively) followed by an additional 1-hour incubation in the fresh incubation buffer supplemented with 1 mM GABA. *Ctx*, cerebral cortex; *Gr*, cerebellar granule cell layer; *IC*, inferior colliculus; *Mol*, cerebellar molecular layer; *Th*, thalamus. Two upper left images are from a film exposed for 2 days, while the other images are from a film exposed for 12 weeks, and, therefore, these two sets of images were processed using different scalings. Similar results were obtained from 3 rats.

To exclude the possibility that the GIS-[^{35}S]TBPS binding in selected brain regions is due to different [^{35}S]TBPS binding affinities in different brain regions, we studied the displacement of [^{35}S]TBPS by cold TBPS in the modified autoradiographic conditions (Figure 7; Sinkkonen S.T. et al., unpublished results). The nonlinear regression fit of [^{35}S]TBPS binding saturation curves revealed largely similar [^{35}S]TBPS binding affinities (K_d about 40 nM) throughout the rat brain. Thus, the affinity of basal [^{35}S]TBPS binding cannot explain the brain regional heterogeneity in GABA-sensitivity.

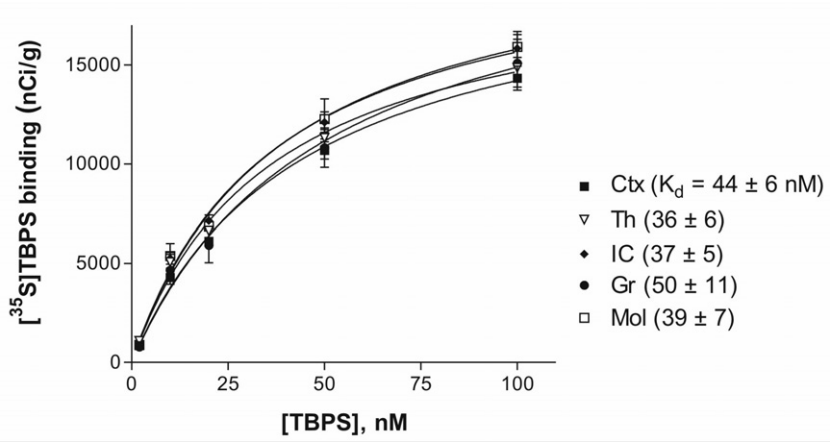

Figure 7. Regional [^{35}S]TBPS binding affinity on rat brain sections. Two nM [^{35}S]TBPS was incubated for 90 min with various cold TBPS concentrations to generate binding saturation curves. Data are mean ± standard error, n = 4. *Ctx*, cerebral cortex; *Gr*, cerebellar granule cell layer; *IC*, inferior colliculus; *Mol*, cerebellar molecular layer; *Th*, thalamus. 100 µM picrotoxinin was used to determine nonspecific binding. Nonlinear regression fit (GraphPad Prism) of [^{35}S]TBPS binding saturation curves revealed similar K_d values in different brain regions.

5.2. Localization and prevalence of GABA-insensitive [^{35}S]TBPS binding in the brain (I, II)

When the inhibitory effect of high (1 mM) GABA concentration on [^{35}S]TBPS binding was studied in rat, mouse, human and chicken brain sections, two main findings were made: 1) a minor population of $GABA_A$ receptors with poor coupling between agonist and convulsant binding sites is present in all the species studied in some but not in all brain regions, and 2) these receptors are highly conserved in the cerebellar granule cell layer, the deep layers of the neocortex and the thalamus.

The GIS-[^{35}S]TBPS binding was detected in the rat (**I: Fig. 3**), human (**I: Fig. 4**), chicken (**I: Fig. 4**) and mouse cerebellar granule cell layer (**II: Fig. 2**), and amounted to 6-16 % of the basal [^{35}S]TBPS binding. As mentioned in the section Material and methods (4.2.), the basal [^{35}S]TBPS binding has not reached equilibrium during the 90-min incubation (presumably about 70 % of the equilibrium binding level), but binding in the presence of 1 mM GABA should represent the binding level in equilibrium (Maksay and Simonyi, 1986). This makes it difficult to estimate the precise proportion of the GIS-[^{35}S]TBPS binding component in the total basal binding. Another confounding factor is that using one concentration of [^{35}S]TBPS in the binding assay does not allow direct estimation of the total receptor number, even though the amount of [^{35}S]TBPS binding is suggestive of it. Taking these two factors into account, it seems safe to estimate that a minor but fairly constant proportion, probably more than a few but less than ten percent, of all the [^{35}S]TBPS binding in the cerebellar granule cell layer, is GABA-insensitive. In addition to the cerebellar granule cell layer, GIS-[^{35}S]TBPS binding was detected in the thalamus and the deep layers of the neocortex in rodent and human brains but not in chicken brain. In these regions, it constituted a few percentages of the basal binding in these incubation conditions.

Comparison of the brain regional localization of GIS-[^{35}S]TBPS binding with two other well-known $GABA_A$ receptor pharmacological heterogeneities, the high-affinity [^{3}H]muscimol and the DIS-[^{3}H]Ro 15-4513 binding, revealed similarities (**I, Fig. 5**). All three pharmacological fingerprints were detected in the cerebellar granule cell layer. In addition, GIS-[^{35}S]TBPS and [^{3}H]muscimol colocalized in the thalamus and in the geniculate area, but they differed in the cerebrocortical distribution, as GIS-[^{35}S]TBPS was enriched in the deep layers whereas [^{3}H]muscimol was found mainly in the external layers. In the cortex, DIS-[^{3}H]Ro 15-4513 binding was distributed uniformly. DIS-[^{3}H]Ro 15-4513 failed to colocalize with GIS-[^{35}S]TBPS binding in the geniculate area, subiculum, or thalamus, but was present in the hippocampus, which was almost devoid of GIS-[^{35}S]TBPS binding.

These findings together show that the GIS-[^{35}S]TBPS binding formed a novel pharmacological heterogeneity of $GABA_A$ receptors with unique brain regional localization. Although GIS-[^{35}S]TBPS binding constituted only a minor receptor population in selected brain regions, several things suggest that these receptors may be of biological significance. Firstly, this kind of prevalence has been detected for various minor $GABA_A$ receptor subtypes (Table 1). Secondly, from the evolutionary point of view, conservation of a biological process (GIS-[^{35}S]TBPS binding) across different species usually argues for functional relevance. Thirdly, the $GABA_A$ receptors in these brain regions might contribute to the regulation of such processes as attention, vigilance and memory, sensory information processing, and/or motor coordination.

5.3. Roles of different $GABA_A$ receptor subunits in GABA-insensitive [^{35}S]TBPS binding

5.3.1. Correlation with receptor subunit mRNA localization by *in situ* hybridization

5.3.1.1. Adult rat (II)

To find out which $GABA_A$ subunits could be responsible for the pharmacological fingerprint of the GIS-[^{35}S]TBPS binding, ligand autoradiography and *in situ* hybridization (ISH) techniques were combined. Although ISH gives information on the subunit mRNA level and ligand autoradiography on the protein level (usually subunit complexes), these measures have correlations, e. g., between α4 and α6 subunits and DIS-[^{3}H]Ro 15-4513 binding (Lüddens et al., 1990; Wisden et al., 1991) and between δ and α6 subunits and high-affinity [^{3}H]muscimol binding (Quirk et al., 1995; Mäkelä et al., 1997). Thus, comparison of ISH and receptor autoradiography signals is relevant. But one has to bear in mind the detection limit of ISH, and that small amounts of mRNA might be sufficient to produce enough protein for a minor receptor population. Localization of $GABA_A$ receptor subunit transcripts in the rat brain has been published previously (Wisden et al., 1992; Sinkkonen et al., 2000).

The α1 subunit mRNA expression was detected throughout the brain, including all the areas of GIS-[^{35}S]TBPS binding (**II: Fig. 1**). However, high α1 mRNA expression was also detected in brain regions with normal GABA sensitivity of [^{35}S]TBPS binding. The α2 mRNA colocalized with the GIS-[^{35}S]TBPS binding in the cortex, but only α3 and α5 were enriched in the deep layers of the cortex similarly to GIS-[^{35}S]TBPS binding. The α4 mRNA expression had the best regional correlation with the GIS-[^{35}S]TBPS binding in the forebrain, with expression in the thalamus and in the geniculate area. However, α4 mRNA was expressed in the striatum and the external parts of cortex, where GIS-[^{35}S]TBPS binding was not detected. The globus pallidus was devoid of an α4 mRNA signal. The α6 mRNA colocalized with the GIS-[^{35}S]TBPS binding in the granule cell layer of the cerebellum. Superimposing figures of α4 and α6 mRNA expression patterns yields a map almost identical to the GIS-[^{35}S]TBPS binding localization, with the exceptions of the striatum, globus pallidus and different cerebrocortical distribution.

The β1 subunit mRNA expression was low outside the hippocampus, but some expression was seen in the cortex. The β2 subunit mRNA colocalized in many brain areas with GIS-[^{35}S]TBPS binding, but was not enriched in the deep layers of the cortex. The β3 mRNA was detected in the cerebellar granule cell layer and cerebral cortex but not in other regions of the GIS-[^{35}S]TBPS binding.

Of the γ subunit family, only γ2 was detected in the areas of the GIS-[^{35}S]TBPS binding, but it was also expressed in most other brain regions.

The δ subunit mRNA expression in the cerebellar granule cell layer, thalamus and geniculate area was largely similar to the localization of GIS-[^{35}S]TBPS binding. However, δ mRNA in the cerebral cortex was distributed in the outer layers rather than in the deep layers (where the GIS-[^{35}S]TBPS binding was), a difference that was similar to that between GIS-[^{35}S]TBPS and [^{3}H]muscimol binding. The δ mRNA was present in the striatum and the hippocampus, which were devoid of GIS-[^{35}S]TBPS binding. In contrast, δ mRNA was not expressed in the globus pallidus, which displayed GIS-[^{35}S]TBPS binding. Altogether, the expression pattern of δ mRNA was in good, but not in perfect, correlation with the GIS-[^{35}S]TBPS binding.

The ε and θ subunits were not expressed in those regions that had GIS-[^{35}S]TBPS binding, which was in accordance with their restricted expression patterns and their enrichment in the brain stem locus coeruleus (Sinkkonen et al., 2000).

5.3.1.2. Developing rat

To find out whether GIS-[^{35}S]TBPS binding is present during brain development, rats of three different ages (P0, P6 and P12; P, postnatal day) were tested. Horizontal sections at the thalamus level and an identical autoradiographic procedure to that used in adult rats were used. Basal picrotoxinin-sensitive [^{35}S]TBPS binding was seen throughout the brain in all age groups (data not shown). One mM GABA displaced all the binding from the P0 and P6 brain sections, and only a little binding was left on the P12 sections (Figure 8; Sinkkonen S.T. et al., unpublished results). In a striking difference from the adult brain, this GIS-[^{35}S]TBPS binding had a totally different localization. In contrast to the binding component seen in the cerebellar granule cell layer and thalamus of the adult brain, these areas were not labeled in the P12 brain in the presence of 1 mM GABA. The strongest binding was seen in the external layer of the infralimbic cortex, which is not particularly labeled in the adult brain. It was impossible to find any $GABA_A$ receptor subunit mRNA enriched in the external layer of the infralimbic cortex (Figure 8). There was some GIS- [^{35}S]TBPS binding also in the deep layers of the cortex and the entorhinal cortex. The entorhinal cortex expressed α1, α2, α4, α5, β2, β3, γ2 and γ3 mRNAs, the α1 mRNA signal was especially strong at P12. The deep layers of the cortex contained particularly α1, α3, α5, β2, β3, γ2 and θ subunit mRNAs. In contrast to the "development" of the pharmacological fingerprint after post-natal day 12, the $GABA_A$ receptor subunit mRNAs have adult-like expression already at this age (**II: Fig. 1**; Figure 8). So, the "development" of GIS-[^{35}S]TBPS binding cannot be explained simply by induction of the expression of any known subunit. Instead, it has to be seen as a change at a later step of the receptor formation (e.g., post-translational modification, receptor complex assembly, interaction with intracellular proteins), or due to some as yet unknown receptor subunit (with late developmental expression).

5.3.2. GABA-insensitive [^{35}S]TBPS binding in genetically engineered mouse lines

On the basis of the brain regional distribution of the GIS-[^{35}S]TBPS, [^{3}H]muscimol and DIS-[^{3}H]Ro 15-4513 binding, and the mRNA expression pattern of the different subunits, candidate receptor subunits were revealed and their roles were probed using brain sections from genetically engineered mouse lines. The mouse lines were provided by collaborators. The α4 subunit-deficient mouse line is unfortunately not available at present, and thus the role of the α4 subunit could thus not be studied.

5.3.2.1. The β3 subunit knockout mice (β3-/-) (II)

Recombinant homomeric β3 receptors display [^{35}S]TBPS binding that is largely insensitive to the inhibitory effect of GABA (Slany et al., 1995; **III: Table 1**). In addition, the β3 subunit mRNA is strongly expressed in the cerebellar granule cell layer, and is detectable in the cerebral cortex. However, expression in the thalamus is negligible (**II: Fig. 1**). For these reasons, we tested whether homomeric β3 receptors could be responsible for GIS-[^{35}S]TBPS binding in the brain, and applied brain sections from β3 subunit knockout (β3-/-) mice

together with their wild-type controls for autoradiographic assay (**II: Fig. 2**). The proportion of the GIS binding component in the basal [^{35}S]TBPS binding was similar in the two genotypes. This suggests that, in the native brain, homomeric β3 receptors are rare, if they exist at all, and cannot explain the GIS-[^{35}S]TBPS binding.

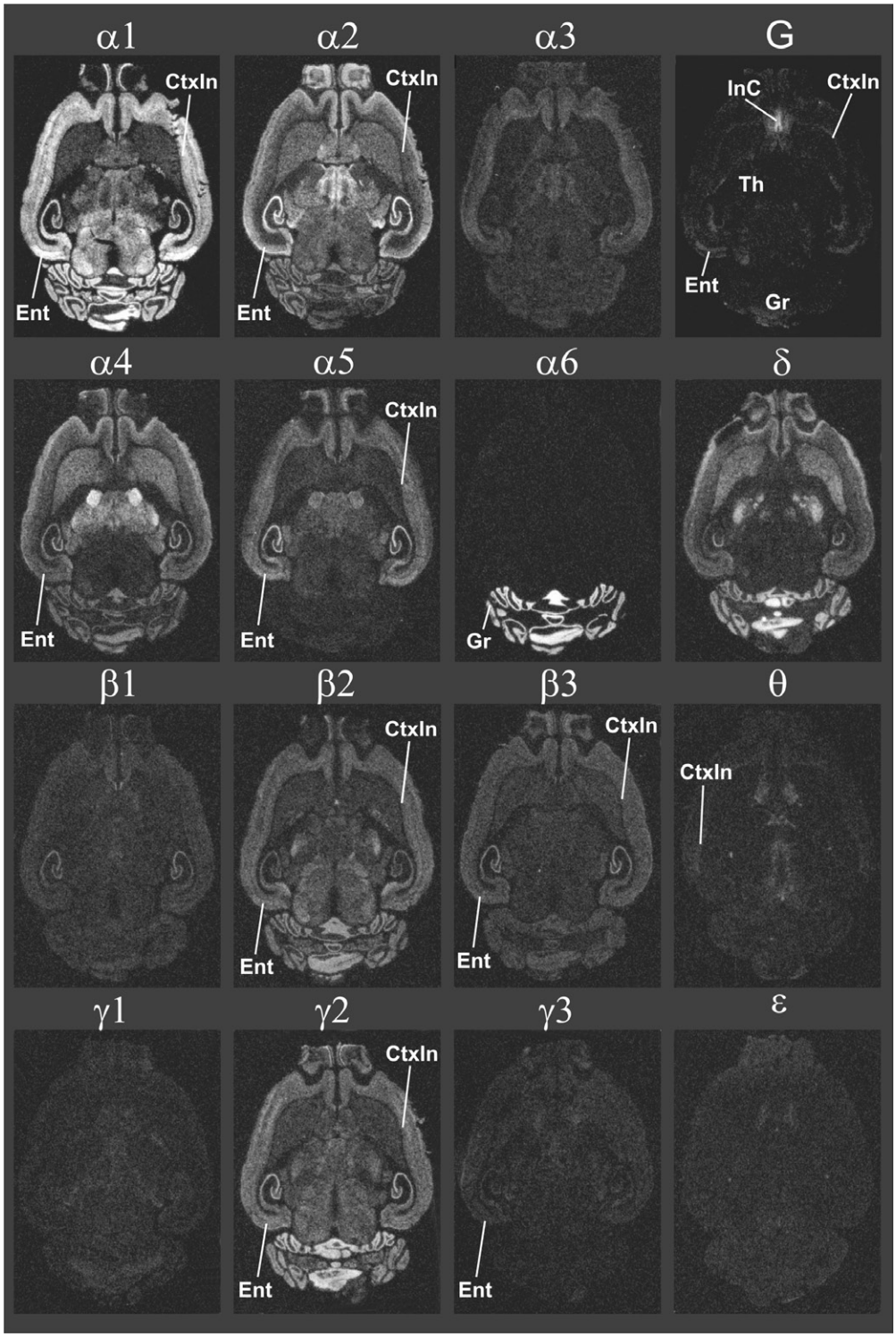

Figure 8. Comparison of the regional localization of the $GABA_A$ receptor subunit mRNAs with the GIS-[^{35}S]TBPS binding (*G*, upper right corner) in serial P12 rat brain sections. Images are processed using identical scaling for brightness and contrast to (**I: Fig. 1**) to allow comparison of transcription levels of different receptor subunits and amount of GIS-[35S]TBPS binding between 12-day-old and adult brains. *CtxIn*, internal rim of cortex; *Ent*, entorhinal cortex; *Gr*, cerebellar granule cell layer; *InC*, external part of the infralimbic cortex; *Th*, thalamus.

5.3.2.2. The δ subunit knockout mice (δ-/-) (II)

A good correlation between GIS-[^{35}S]TBPS binding and δ subunit-associated high-affinity [^{3}H]muscimol binding, together with δ mRNA localization, suggested a role for the δ subunit in GIS-[^{35}S]TBPS binding after exclusion of the role of homomeric β3 receptors. In addition to complete deletion of δ subunit expression, δ-/- mice have complex alterations in their $GABA_A$ergic system. In the thalamus, the δ subunit knockout mice have decreased high-affinity [^{3}H]muscimol binding but increased total [^{3}H]Ro 15-4513 binding. The forebrain γ2 subunit expression is upregulated but the α4 subunit expression is decreased (Korpi et al., 2002b; Peng et al., 2002). Nevertheless, the formation of α4βγ2 receptors is increased, as revealed by immunoprecipitation studies and the increased proportion of DIS-[^{3}H]Ro 15-4513 binding (Korpi et al., 2002b). In the cerebellum, δ-/- mice display increased [^{3}H]Ro 15-4513 binding and an increased DIS-[^{3}H]Ro 15-4513 binding component, but a decreased amount of high-affinity [^{3}H]muscimol binding (Tretter et al., 2001; Korpi et al., 2002b). Simultaneously, γ2 subunit expression is upregulated and coassembly of α6 and γ2 subunits is increased. In contrast to cerebellar extracts of control mice, where 97 % of the receptors were of αβγ2 or αβδ subtypes, and the numbers of γ1 and γ3 subunit-containing receptors are negligible, in the δ-/- mice 24 % of all cerebellar $GABA_A$ receptors are devoid of γ or δ subunits (Tretter et al., 2001).

When [^{35}S]TBPS binding autoradiography was applied to δ-/- and their wild-type control mouse brain sections, high basal binding was detected throughout the brains of both genotypes (**II: Fig. 2**). However, when incubation was performed in the presence of 1 mM GABA, the cerebellar granule cell layer of the δ-/- mice had 24.4 ± 1.5 % (mean ± standard error) of basal [^{35}S]TBPS binding left, which was double that of the wild-type controls (12.2 ± 1.5 %, $p < 0.01$, one-way ANOVA and Newman-Keuls Multiple post-hoc test). The δ-/- mice also had a higher proportion of GIS-[^{35}S]TBPS binding in the thalamus (4.3 ± 0.2 vs. 3.0 ± 0.3 %, $p < 0.01$), whereas the amount of GIS-[^{35}S]TBPS binding was similar in brain regions devoid of δ expression, e.g., in the inferior colliculus the GIS-[^{35}S]TBPS binding amounted to 0.6 % of the basal binding in both mouse lines. These results indicate that the GIS-[^{35}S]TBPS binding can be formed without δ subunits. However, owing to the compensatory alterations observed in the δ-/- mouse brains (Tretter et al., 2001; Korpi et al., 2002b; Peng et al., 2002), one cannot rule out the possibility that δ subunit-containing receptors might participate in the formation of GIS-[^{35}S]TBPS binding in the wild-type animals.

5.3.2.3. The α6 subunit knockout mice (α6-/-) (II)

GIS-[^{35}S]TBPS binding is detected in the cerebellar granule cell layer together with high-affinity [^{3}H]muscimol and DIS-[^{3}H]Ro 15-4513 binding, and together with α6 subunit expression. There are currently two α6 subunit knockout lines available, both of which have a neomycin gene insertion in exon 8 of the α6 subunit gene (Homanics et al., 1997b; Jones et al., 1997). Deletion of the α6 subunit in the mouse genome results in complex but similar adaptations in the $GABA_A$ergic systems of both knockout mouse lines. In the cerebellar granule cell layer, the α6-/- mice have about 80 % reduced translation of δ subunit protein despite normal amounts of δ mRNA (Jones et al., 1997), and the remaining δ subunits are not detected on the plasma membrane (Nusser et al., 1999). Protein levels for the β2, β3 and γ2 subunits are decreased by about 50, 20 and 40 %, respectively, and the total number of receptors is decreased by about 50 % (Nusser et al., 1999). DIS-[^{3}H]Ro 15-4513 binding is totally abolished and high-affinity [^{3}H]muscimol binding is greatly reduced (Mäkelä et al., 1997).

In line with the previous report of [^{35}S]TBPS binding to α6-/- brain sections (Mäkelä et al., 1997), the basal binding in the cerebellar granule cell layer did not significantly differ from the wild-type control values (332 ± 86 vs. 416 ± 45 nCi/g for α6-/- and wild-type controls, respectively), whereas the binding was decreased in virtually all the other brain regions when compared to wild-type control mice (**II: Fig. 2**). One mM GABA decreased [^{35}S]TBPS binding to 5.1 ± 0.7 %, which was significantly reduced from that of the wild-type controls (12.2 ± 1.5 %, $p < 0.01$). The strongly reduced GIS-[^{35}S]TBPS binding component of α6-/- mice in the cerebellar granule cell layer suggests a role for α6 in the formation of this pharmacological fingerprint. In addition to changes in the cerebellar granule cell layer, both α6-/- mouse lines display decreased expression of α1 and β2 subunits in the forebrain due to the suppression of the neighboring β2-α6-α1-γ2 gene cluster expression by the neomycin gene (Uusi-Oukari et al., 2000). These nonspecific changes were also demonstrated in our experiments by reduced amount of GIS-[^{35}S]TBPS binding in the forebrains of α6-/- mice.

5.3.2.4. Mice with ectopic α6 subunit expression (Thy1α6) (IV)

If the α6 subunit participates in the formation of GIS-[^{35}S]TBPS binding, as suggested by the effects of its deletion, then ectopic expression of the α6 subunit outside its natural environment, the cerebellar granule cell layer, should produce GIS-[^{35}S]TBPS binding. For this reason, brain sections from the transgenic Thy1α6 mouse line (Wisden et al., 2002), where the $GABA_A$ receptor α6 subunit is expressed under the control of the panneuronal Thy-1.2 promoter (Caroni, 1997), were studied. Depending on the accidental incorporation position of the transgene in the mouse genome, inheritable transgene expression patterns in random brain regions are produced. In the case of Thy1α6 mice, regionally limited expression of the α6 subunit protein was achieved (Wisden et al., 2002). The α6 protein is detected in some of the cells in selected brain regions, such as the deep cerebellar nuclei, the deep layers of the neocortex, subiculum, hippocampal CA1 and CA3 pyramidal cells and the hippocampal dentate granule cells. In the forebrain, α6 coassembled with the α1, α3, β1, β2, β3 and γ2 subunits, whereas no coassembly with the δ subunit was detected (Wisden et al., 2002). In accordance with ectopic α6 subunit expression and incorporation to receptor complexes, DIS-[^{3}H]Ro 15-4513 binding was increased in the hippocampal CA1 region and the deep layers of the cerebral cortex (Wisden et al., 2002).

[^{35}S]TBPS autoradiography revealed high basal binding throughout the Thy1α6 and their C57BL/6 control mouse brain sections (**IV: Fig. 1**). Brain regional binding density was otherwise similar, but Thy1α6 had 28 % less binding in the hippocampal CA1 region than their control animals ($p < 0.001$, unpaired *t*-test). One mM GABA produced a typical forebrain GIS-[^{35}S]TBPS binding pattern in control mouse brain sections, since it resulted in almost complete abolition of [^{35}S]TBPS binding (e.g., 0.7 ± 0.1 % of the basal binding left in the hippocampal CA1 region) of all other brain regions but the thalamus. In contrast, the hippocampal CA1 region of the Thy1α6 mice still had 8.9 ± 0.4 % of basal binding left. This result argues strongly for a role of the α6 subunit in the formation of GIS-[^{35}S]TBPS binding in the native cerebellum.

5.3.2.5. Mice heterozygous for the γ2 subunit deletion (γ2+/-) (III)

The α6 subunit-containing receptors cannot explain the GIS-[^{35}S]TBPS binding outside the cerebellum. Nevertheless, the γ2 subunit confers GABA-sensitivity of [^{35}S]TBPS binding on the α6β2 receptors (Korpi and Lüddens, 1993; Im et al., 1994). Functionally, the γ2 subunit enhances the efficacy of GABA by promoting higher conductance and a longer lifetime of

activated channels (Lorez et al., 2000). For these reasons, we wanted to probe the effects of γ2 subunit deletion on GIS-[^{35}S]TBPS binding. The mice homozygous for the deletion of the γ2 subunit have normal embryonic development, including normal levels of other receptor subunits, but die soon after birth (Günther et al., 1995). The mice heterozygous for the γ2 knockout (γ2+/-) have a decreased number of BZ binding sites, but a normal total number of $GABA_A$ receptors (Crestani et al., 1999). However, γ2 subunit-dependent receptor clustering to the postsynaptic membranes is decreased. Electrophysiologically, the γ2+/- mice display an increased proportion of low-conductance channel activity, suggestive of functional expression of receptors composed only of α and β subunits (Crestani et al., 1999).

[^{35}S]TBPS autoradiography with adult γ2+/- mouse brain sections revealed high convulsant site binding throughout the brain. Basal binding was increased in the cortex, thalamus and hippocampus, as compared with the wild-type controls (**III: Fig. 3**). Adding 1 mM GABA revealed a wide-spread increase in the GIS-[^{35}S]TBPS binding component of the mutant mice, a change that was virtually opposite to the decreased [^{3}H]Ro 15-4513 binding (**III: Fig. 1**). Since the δ subunit-related high-affinity [^{3}H]muscimol binding remained unchanged (**III: Fig. 4**), these results suggest that αβ $GABA_A$ receptors might participate in the formation of GIS-[^{35}S]TBPS binding in the brain. E.g., in the inferior colliculus, where only the α1 subunits among the α subunit family are highly expressed (Korpi et al., 2002a), these receptors might be of α1β2/3 composition. In addition, these results were supported by the finding that GABA modulation of [^{35}S]TBPS binding to the γ2+/- mouse forebrain was further decreased by Zn^{2+}, while it had fewer effects in the wild-type controls. Zn^{2+} is known to inhibit αβ receptors at higher potency than αβδ or αβγ receptors (Krishek et al., 1998).

5.3.3. Demonstration of GABA-insensitive [^{35}S]TBPS binding in recombinant receptors expressed in HEK 293 cells (III)

Based on the results with genetically engineered mouse lines, various subunit combinations were expressed in HEK 293 cells in order to determine the GABA-sensitivity of [^{35}S]TBPS binding in these receptor subtypes. As previously shown, the α6β2 receptors devoid of γ2 subunits display [^{35}S]TBPS binding that is insensitive to the inhibitory effect of at least 10 μM GABA (Korpi and Lüddens, 1993; Im et al., 1994). Also, α6β3 receptors displayed [^{35}S]TBPS binding that was partially (55 %) insensitive to 10 μM GABA (Korpi and Lüddens, 1997). In the present experiments, a third of the [^{35}S]TBPS binding was not abolished in the presence of 1 mM GABA in the α6β3 receptors, whereas [^{35}S]TBPS binding to the α6β3γ2 receptors was fully displaced by GABA (**III: Table 1**; Figure 9). These and previous data (Korpi and Lüddens, 1993; Im et al., 1994, Korpi and Lüddens, 1997) clearly indicate that the α6β2/3 receptors form [^{35}S]TBPS binding sites with poor coupling to agonist binding sites, while γ2 confers the full GABA sensitivity.

The γ2+/- mice showed widespread enhancement of their GIS-[^{35}S]TBPS binding. Since α1 is the major α subunit in the brain, it was hypothesized that the γ2 subunit affects the GABA-sensitivity of [^{35}S]TBPS binding to the α1-receptors similarly to the α6 subunit-containing receptors. In agreement, about 20-30 % of the [^{35}S]TBPS binding to the α1β3 receptors was insensitive to 1 mM GABA, when the same amount of subunit plasmids for both subunits was used in the transfection. When the amount of the α1 plasmid was raised or the γ2 subunit was cotransfected, the GABA-sensitivity of [^{35}S]TBPS binding was restored (**III: Table 1**; Figure 9). When β2 subunit was cotransfected with the α1 subunit (**III: Table 1)**, [^{35}S]TBPS binding was totally GABA-sensitive, which was in accordance with previous results (Korpi and Lüddens, 1993; Im et al., 1994). [^{35}S]TBPS binding to homomeric β2 receptors was weak and fully displaceable by GABA (**III: Table 1**).

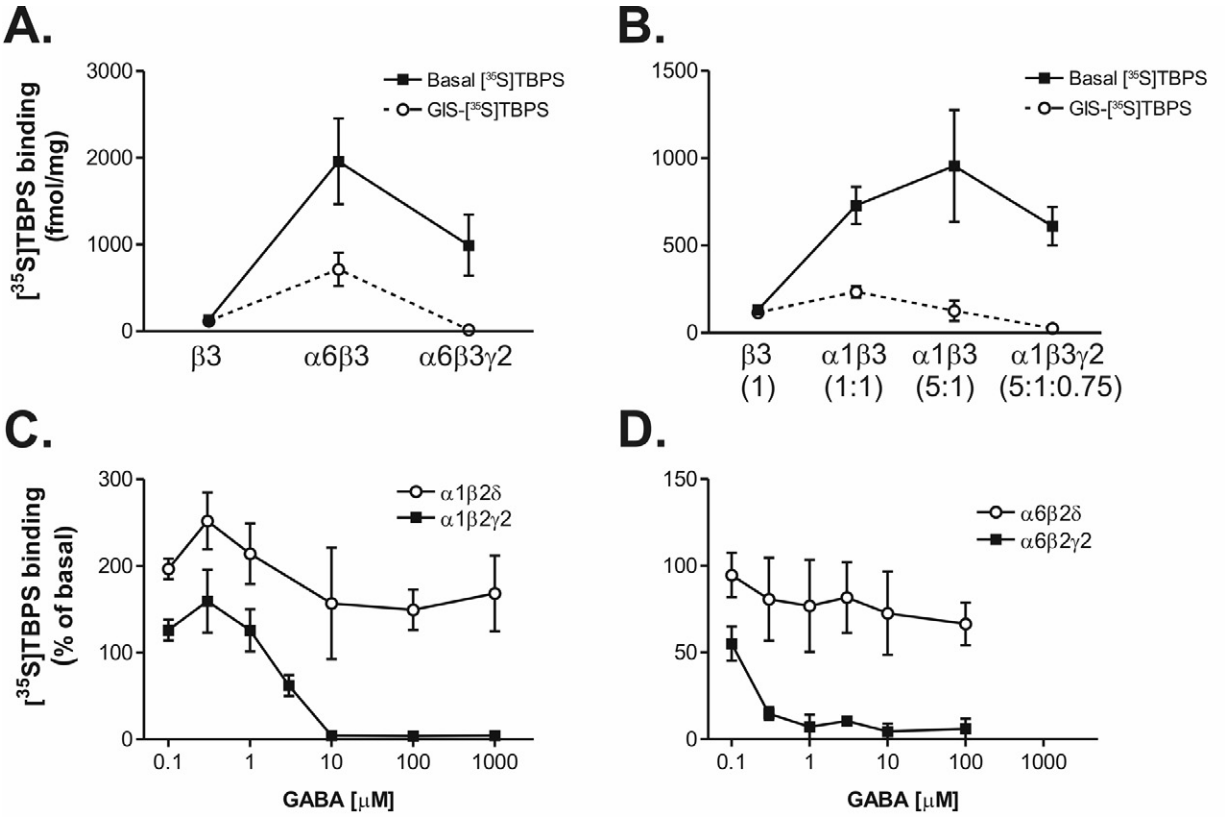

Figure 9. [^{35}S]TBPS binding and its modulation by GABA in various recombinant receptor subtypes expressed in HEK 293 cells. **A.** Inclusion of γ2 subunit into α6β3 receptor complexes changes [^{35}S]TBPS binding sensitive to 1 mM GABA. Data are mean ± standard error, n = 3 transfections for each subunit combination (**III: Table 1**). **B**. Also α1β3 receptors display partially GIS-[^{35}S]TBPS binding, but increasing the amount of α1 subunit plasmid in transfection procedure or inclusion of γ2 subunit into α1β3 receptor complexes convert [^{35}S]TBPS binding sensitive to the inhibitory effect of 1 mM GABA. Data are mean ± standard error, n = 3 - 7 transfections for each subunit combination (**III: Table 1**). In parenthesis the amount of respective subunit (in µg plasmid/plate) used in transfections. **C.** Substituting γ2 subunit with δ in α1 subunit-containing receptors confers [^{35}S]TBPS binding from GABA-sensitive to GABA-insensitive. Note that α1β2 receptors are fully GABA sensitive (Korpi and Lüddens, 1993; Im et al., 1994; **III: Table 1**), which confirms incorporation of δ subunits in the receptor complexes. Data are mean ± standard error, n = 2 - 5 transfections for both subunit combinations. The basal [^{35}S]TBPS binding levels for α1β2γ2 and α1β2δ combinations were 88 ± 21 and 54 ± 13 fmol/mg protein. The amounts of subunit plasmids (in µg plasmid/plate) used in transfections were α1: 2, β2: 10, γ2: 0.2, δ: 0.3. These experiments were carried out by Korpi E.R. in the University of Heidelberg, Germany. **D.** Similar to α1 subunit-containing receptors, substitution of γ2 by δ seems to confer [^{35}S]TBPS binding to α6-receptors GABA-insensitive. However, also α6β2 receptors display GIS-[^{35}S]TBPS binding (Korpi and Lüddens, 1993; Im et al., 1994), which makes judgement of the incorporation of δ subunits difficult. Data are mean ± standard error, n = 2 - 4 transfections for both subunit combinations. The basal [^{35}S]TBPS binding levels for α6β2γ2 and α6β2δ combinations were 54 ± 13 and 63 ± 5 fmol/mg protein. The amounts of subunit plasmids (in µg plasmid/plate) used in transfections were α6: 2, β2: 10, γ2: 0.2, δ: 0.3. These experiments were carried out by Korpi E.R. in the University of Heidelberg, Germany.

It has been shown that 65 % of [^{35}S]TBPS binding to α6β3δ receptors is insensitive to the inhibitory effect of 10 µM GABA (Korpi and Lüddens, 1997), but there are currently no published data on [^{35}S]TBPS binding to δ subunit-containing receptors in the presence of higher GABA concentrations. One possible explanation for this is the decreasing effect of $GABA_A$ receptor ligand binding by δ subunit transfection, which makes binding studies with recombinant δ-$GABA_A$ receptors difficult. Hevers et al. (2000) were able to

electrophysiologically demonstrate the functional expression of the δ subunit in αβδ and αβγ2δ combinations, but, in ligand binding assays with cell homogenates, cotransfection of δ with the αβγ2 combination greatly reduced the binding levels to the GABA agonist, BZ and convulsant binding sites. The effect was detected irrespective of the type of α subunit present in tranfection. E.g., [^{35}S]TBPS binding to α4 and α6 subunit-containing receptors was decreased to almost background levels, and it was also clearly decreased in α1 subunit-containing receptors (Hevers et al., 2000). Nevertheless, when it was managed to express δ subunit in HEK 293 cells together with α1 and β2, the α1β2δ receptors formed displayed relatively low basal [^{35}S]TBPS binding, as expected, but as a striking difference from control α1β2γ2 receptors, [^{35}S]TBPS binding was not decreased by GABA (Figure 9; Korpi E. R., unpublished results). One has to bear in mind that recombinant β2 and α1β2 receptors display totally GABA-sensitive [^{35}S]TBPS binding (Korpi and Lüddens, 1993; Im et al., 1994; **III: Table 1**), which suggests that incorporation of δ subunit to receptor complexes is responsible for the GIS-[^{35}S]TBPS binding detected in these experiments. In the native brain, δ is preferentially assembled with the α6 and α4 subunits (Jones et al., 1997; Sur et al., 1999). Due to technical difficulties in obtaining reliable expression levels of the α4 subunit in HEK 293 cells, we have not been able to study recombinant receptors comprising this subunit. In contrast, δ subunit expression together with α6 and β2 subunits was successful and revealed largely similar results to the α1 subunit-containing receptors: low basal [^{35}S]TBPS binding together with low sensitivity to the inhibitory effect of GABA. [^{35}S]TBPS binding to the α6β2γ2 receptors was fully GABA-sensitive, as expected (Korpi and Lüddens, 1993). Anyhow, the results obtained with coexpression of α6, β2 and δ subunits do not necessarily imply formation of α6β2δ receptors, since α6β2 receptors are able to form the GIS-[^{35}S]TBPS, as has previously been shown (Korpi and Lüddens, 1993; Im et al., 1994). However, these results do not rule out the possibility that α6βδ receptors display GIS-[^{35}S]TBPS binding, which is supported by the finding that [^{35}S]TBPS binding to α6β3δ was only partially displaced by 10 μM GABA (Korpi and Lüddens, 1997). Although the results presented here with recombinant δ subunit expression are preliminary, they suggest that $GABA_A$ receptors containing the δ subunit might participate in forming the GIS-[^{35}S]TBPS pharmacology in the native brain.

5.4. A possible mechanism of atypical coupling between GABA and convulsant binding sites

During the initial characterization of the GIS-[^{35}S]TBPS binding in the native brain, we probed the effects of other GABA site agonists on [^{35}S]TBPS binding. Muscimol, as a prototypical and potent agonist, produced an identical pharmacological fingerprint at 1 mM concentration (data not shown). The effect of the endogenous amino acids β-alanine and taurine, which are known to potentiate both $GABA_A$ and glycine receptors (Horikoshi et al., 1988), were tested up to 100 mM concentrations. At this highest concentration, β-alanine revealed a similar [^{35}S]TBPS binding component to 1 mM GABA or muscimol, whereas the taurine effect at 100 mM concentration was comparable to the effects of 10-30 μM GABA (data not shown).

Even though other GABA site agonists failed to displace more [^{35}S]TBPS binding from the brain sections than GABA, the residual binding could still be modulated by various $GABA_A$ receptor allosteric modulators. The thalamic component of GIS-[^{35}S]TBPS binding was further decreased by drugs acting on BZ, barbiturate, loreclezole and neurosteroid binding sites, and by Mg^{2+} (**I: Table 1**). The GIS-[^{35}S]TBPS binding in the cerebellar granule cell layer was largely similarly affected, but it was insensitive to Mg^{2+} modulation. The selective $GABA_C$ receptor agonist *cis*-4-aminocrotonic acid did not affect GIS-[^{35}S]TBPS

binding. Even though some predictions of receptor subunit composition might be made on the basis of this pharmacology, it is safer, owing to the high drug concentrations and long incubation periods, to make only two basic assumptions: 1) the receptor population responsible for GIS-[^{35}S]TBPS binding is regionally heterogeneous, and 2) GABA seem not to have full efficacy on these receptors, since the effect of a saturating (1 mM) concentration can still be potentiated. While the heterogeneity of GIS-[^{35}S]TBPS binding can be explained by the differential subunit composition of the receptors, it is harder to imagine the circumstances in which the natural agonist does not possess full efficacy. However, Brown et al. (2002) provided an interesting finding when they showed that THIP, a restricted GABA analog and a partial agonist in $\alpha1\beta2/3\gamma2$ and $\alpha4\beta3\gamma2$ receptors (Ebert et al., 1994; Ebert et al., 1997; Brown et al., 2002), was more effective in the $\alpha4\beta3\delta$ receptors than GABA. Unfortunately, Brown et al. (2002) did not demonstrate competition between GABA and THIP, which would have established the idea of GABA being only a partial agonist in recombinant $\alpha4\beta3\delta$ receptors.

Nevertheless, on the basis of the idea that THIP might be more efficacious than GABA by acting as a full agonist of the GABA site, we applied THIP in an [^{35}S]TBPS autoradiographic assay with mouse brain sections. As shown in Figure 10 (Sinkkonen S.T. et al., unpublished results), THIP was more effective than GABA in displacing [^{35}S]TBPS binding from the cerebellar granule cell layer and the thalamus. At 10 mM concentration, it displaced virtually all [^{35}S]TBPS binding. The effect of THIP could be inhibited by simultaneous application of GABA, which demonstrates competitive displacement of THIP from the agonist binding site. These results suggest that GABA is not a full agonist, at least in respect of [^{35}S]TBPS dissociation, in the receptors responsible for GIS-[^{35}S]TBPS binding in the native brain. Thus, this might provide the first demonstration of native receptors, where GABA acts as a partial agonist.

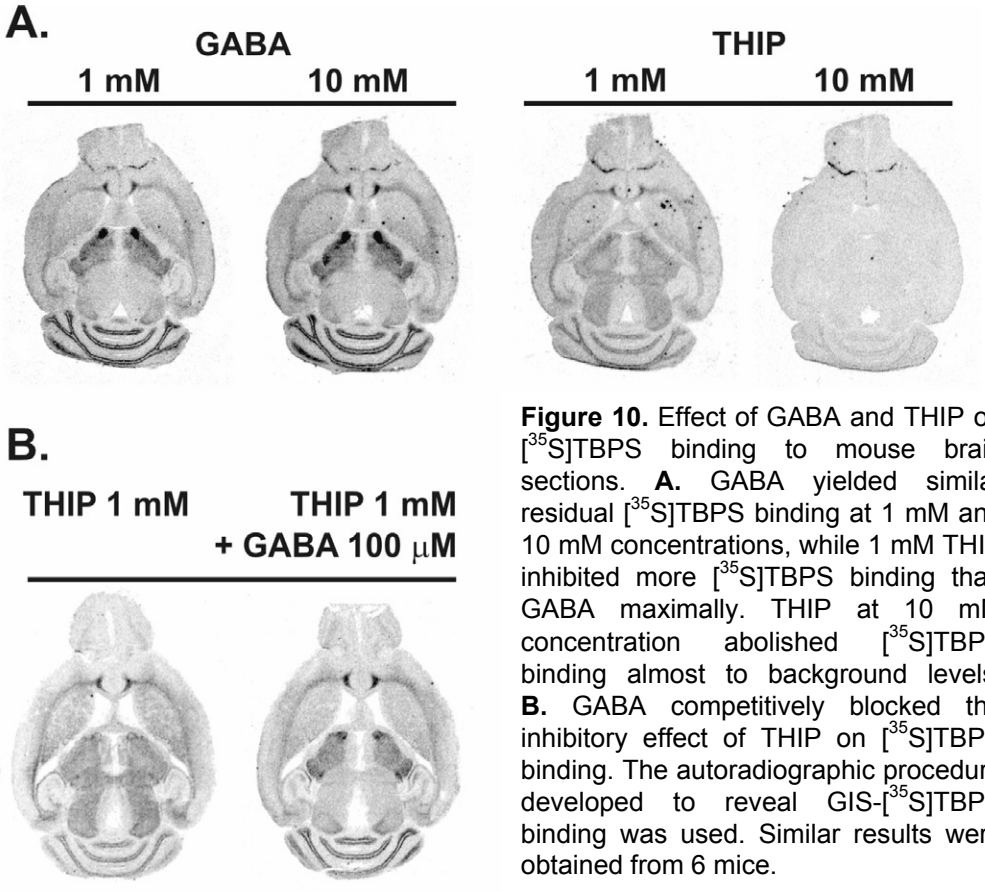

Figure 10. Effect of GABA and THIP on [^{35}S]TBPS binding to mouse brain sections. **A.** GABA yielded similar residual [^{35}S]TBPS binding at 1 mM and 10 mM concentrations, while 1 mM THIP inhibited more [^{35}S]TBPS binding than GABA maximally. THIP at 10 mM concentration abolished [^{35}S]TBPS binding almost to background levels. **B.** GABA competitively blocked the inhibitory effect of THIP on [^{35}S]TBPS binding. The autoradiographic procedure developed to reveal GIS-[^{35}S]TBPS binding was used. Similar results were obtained from 6 mice.

5.5. Mice with ectopic α6 subunit expression (Thy1α6) as a model system of altered balance between synaptic and extrasynaptic inhibition (IV)

As explained in section 5.3.2.4., the transgenic Thy1α6 mouse line has ectopic α6 subunit expression in some brain regions. But, as a striking difference from its normal synaptic enrichment in the natural environment (cerebellar granule cell layer), the transgene product is almost solely located on the extrasynaptic membranes of the soma and the dendrites (Wisden et al., 2002). Together with ectopic α6 expression, Thy1α6 mice have increased $GABA_A$ergic extrasynaptic inhibition. However, they display a simultanous decrease in synaptic inhibition, since the sIPSC frequency and mIPSC amplitude are decreased by 37 and 30 %, respectively. The α6-immunoaffinity column showed coassembly of α1, α3, β1, β2, β3 and γ2 subunits together with α6, whereas no δ subunit was detected, presumably reflecting the non-overlapping expression patterns of the δ and Thy1α6 genes. As indicated by the immunoaffinity column results and the increased DIS-[^{3}H]Ro 15-4513 binding, many of the α6 subunits were assembled in α6βγ2 receptors (Wisden et al., 2002). However, our preliminary screening revealed the GIS-[^{35}S]TBPS binding in the brain regions of ectopic α6 expression, which suggested the additional presence of α6β receptors. For this reason, we extended the characterization of these ectopic receptor subtypes, and performed behavioral analysis with the Thy1α6 mice.

5.5.1. Functional α6 subunit expression and its effects on other subunit mRNA levels

ISH with an oligonucleotide probe against mouse α6 subunit mRNA revealed a consistent α6 expression pattern in Thy1α6 mouse frontal brain sections (Figure 11; Sinkkonen S.T. et al., unpublished results). The strongest expression was detected in the hippocampal CA1 region. Other areas of clear expression were the hippocampal dentate gyrus and the CA3 area, the deep layers of the neocortex and the amygdala. No α6 subunit mRNA was detected in the forebrain of wild-type mice, as expected. To confirm the functional expression of α6 subunit-containing $GABA_A$ receptors in the forebrain of Thy1α6 mice, whole-cell patch-clamp was applied to acutely isolated CA1 pyramidal cells from control and Thy1α6 mice. The α4/6-selective non-competitive antagonist furosemide blocked the GABA-induced currents only in cells from Thy1α6 mice (**IV: Fig. 3**), which confirmed the functional expression of the Thy1α6 transgene.

To test whether ectopic α6 expression in the hippocampus changes other receptor subunit mRNA levels expressed in the hippocampus (Wisden et al., 1992; Sperk et al., 1997), oligonucleotide probes against α1-5, γ2 and δ mRNAs were used on both male and female Thy1α6 (n = 8 and 13 for males and females, respectively) and control (n = 7 and 11 for males and females, respectively) mouse brain sections. The expression levels of β1-3 subunit mRNAs were not examined; although they are hippocampally expressed, the type of β subunit variant does not crucially influence the receptor's physiological properties (Korpi et al., 2002a), and thus their mutual relations were not of particular interest in the present study. ISH revealed clear expression patterns for subunit mRNAs in the hippocampal CA1 area and the dentate gyrus (Figure 11). Since different oligonucleotide probes have different specific activities and hybridization properties, the expression levels of the different subunit mRNAs are not comparable. In contrast, comparison between different mouse lines is justified. Expression levels in CA1 for control and Thy1α6 mice were (in nCi/g, n = 14 - 21) for α1 229 ± 21 vs. 217 ± 22 (mean ± standard error, expression level for Thy1α6 was 95 % of that of the control), α2 120 ± 11 vs. 117 ± 6 (97 %), α3 5.6 ± 0.4 vs. 5.7 ± 0.4 (100 %), α4 8.1 ± 0.9 vs. 7.6 ± 0.4 (94 %), α5 79 ± 4 vs. 74 ± 3 (93 %), γ2 169 ± 8 vs. 160 ± 5 (95 %) and for δ

3.4 ± 0.1 vs. 3.1 ± 0.2 (89 %), respectively. In the dentate gyrus, the differences in expression levels between the mouse lines were even smaller (data not shown). Three-way ANOVA (factors: gender, mouse line, subunit) revealed no differences in receptor subunit mRNA levels between genders or mouse lines [$F(1,254) = 0.16$, ns, and $F(1,254) = 0.34$, ns, for mouse line factors in CA1 and the dentate gyrus, respectively]. Thus, functional expression of α6 subunits in the forebrain of Thy1α6 mice does not interfere with the expression of other $GABA_A$ receptor subunit mRNAs. However, the possible changes in subunit translation and/or subunit assembly cannot be ruled out on the basis of the ISH findings.

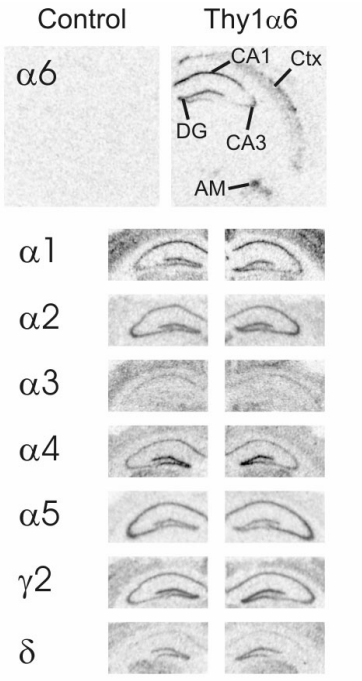

Figure 11. $GABA_A$ receptor subunit expression in the hippocampus of control and Thy1α6 mice. ISH with mouse brain sections was performed otherwise similarly to rat brain sections (see 4.3.), excepct that oligonucleotide probes were synthesized (CyberGene Ab, Huddinge, Sweden) complementary to mouse subunit cDNA sequences as follows: α1 (cDNA nucleotides 1331-1375; GenBank accession number M86566), α2 (1144-1188; M86567), α3 (1404- 1448; M86568), α4 (542-586; NM_010251), α5 (1477-1521; BC062112), α6 (1291-1335; NM_008068), γ2 (1352-1396; BC031762), δ (1160-1204; NM_008072). Due to different specific activities and hybridization properties of oligonucleotide probes expression levels of different subunit mRNAs can not be comparared. However, comparison between different mouse lines is justified, and thus representative ISH autoradiographs for both mouse lines are processed from the same films with identical scaling for brightness and contrast. Quantification of subunit expression levels revealed no statistically significant differences between mouse lines either in the CA1 area or dentate gyrus *AM*, amygdala; *CA1* and *CA3*, respective hippocampal areas; *Ctx*, cerebral cortex; *DG*, dentate gyrus.

5.5.2. $GABA_A$ receptor subtype alterations

Since the α6 subunit was incorporated into the functional receptors of the Thy1α6 mouse hippocampal pyramidal cells, it should also be possible to detect those receptors in ligand autoradiographic assays. In fact, increased forebrain DIS-[^{3}H]Ro 15-4513 binding suggesting upregulation of α6βγ2 receptors was detected on Thy1α6 mouse brain sections (Wisden et al., 2002; **IV: Fig. 1).** In the previous study (Wisden et al., 2002) the coassembly of α6 and γ2 subunits was confirmed by applying extracted forebrain $GABA_A$ receptors from Thy1α6 mice on an α6-immunoaffinity column, and by Western blotting of receptors retained by the column with the γ2 antibody. Further analysis with [^{3}H]muscimol binding indicated that most α6 subunit-containing $GABA_A$ receptors in Thy1α6 forebrain contained γ2 subunits. No δ subunit with α6 could be detected. However, in addition to upregulated DIS-[^{3}H]Ro 15-4513 binding, GIS-[^{35}S]TBPS binding was also detected in the forebrain regions of Thy1α6 mice, suggesting the presence of α6β receptors (**IV: Fig. 1**). The highest level of GIS-[^{35}S]TBPS binding was detected in the hippocampal CA1 region, which was also the region with the greatest increase in DIS-[^{3}H]Ro 15-4513 binding. In this region, about 10 % of the basal [^{35}S]TBPS binding was not displaced by GABA.

Previous analysis of the subunit content of Thy1α6 mouse $GABA_A$ receptors was performed with whole forebrain extracts. Due to the large number of $GABA_A$ receptors in many other brain regions besides hippocampus, and due to the detection limit of the assay, it is possible that these experiments failed to detect minor receptor subtypes. For example,

solubilization and precipitation of the receptors from brain homogenates may cause a loss of about 50 % of the receptors (Tretter et al., 2001). In order to get more precise estimates of the hippocampal $GABA_A$ receptors of the Thy1α6 and control mice, bare hippocampi from both mouse lines were dissected and analyzed (**IV: Fig. 2**). The total amount of receptors in the hippocampal extracts was determined by immunoprecipitation with a mixture of α1, β1, β2 and β3 antibodies and subsequent [^{3}H]muscimol binding. In parallel, hippocampal extracts (hippocampi from 10 or 5 control or Thy1α6 animals, respectively, were pooled) were chromatographed on immunoaffinity columns containing antibodies against the γ1, γ2, γ3, and δ subunits. The column efflux was subjected to immunoprecipitation with a mixture of α1, β1, β2 and β3 antibodies and subsequent [^{3}H]muscimol binding. After chromatography, roughly 30 % of all the receptors present in control or in the Thy1α6 hippocampus extracts, respectively, could still be found in the column efflux. Due to the unexpectedly high proportion of receptors devoid of γ or δ subunits in both mouse lines, the subunit composition of the receptors remaining in the efflux was studied. For this, immunoprecipitation with subunit-specific antibodies and subsequent [^{3}H]muscimol binding assays were applied. Immunoprecipitation of control mouse hippocampal extracts indicated that most of the receptors still present in the efflux contained γ2 subunits, indicating that the anti-γ2 column could not completely retain all the γ2-containing receptors. No α6, γ1, γ3, or δ subunit-containing receptors could be identified in this efflux. So, no significant amounts of αβ receptors could be detected in the hippocampi of the control mice. In the experiments with Thy1α6 mouse hippocampal extracts, about 60 % of the receptors in the column efflux still contained the γ2 subunits. This indicates that, like the situation with the control mice, the anti-γ2 column could not completely retain all the γ2-containing receptors. However, no γ1, γ3 or δ subunits were detected. These data indicate that about 10 % of the $GABA_A$ receptors in the Thy1α6 hippocampi were of αβ stoichiometry. Furthermore, most of the receptors found in the column efflux of the Thy1α6 mice contained α4 and α6 subunits, thus suggesting that most, if not all, αβ receptors contained α4 or α6 subunits.

In the previous study (Wisden et al., 2002) Scatchard analysis of [^{3}H]Ro 15-4513 binding to mouse forebrain homogenates indicated a 28 % increase in BZ binding in the Thy1α6 mice, 80 % of which was due to the increased DIS binding component. This suggests that the number of non-α6βγ2 receptors was not decreased. Our autoradiographic assay showed a 25 % increase in [^{3}H]Ro 15-4513 binding (64 ± 1 vs. 80 ± 4 nCi/mg for control and Thy1α6 mice, respectively; mean ± standard error, $n = 5$, $p = 0.0045$, unpaired *t*-test) but a 28 % decrease in [^{35}S]TBPS binding (335 ± 13 vs. 240 ± 9 nCi/mg, for control and Thy1α6 mice, respectively, $n = 7$ and 8, $p < 0.001$, unpaired *t*-test) in the CA1 area of the Thy1α6 mice. The autoradiographic assay does not allow direct estimation of total $GABA_A$ receptor numbers in the hippocampus of Thy1α6 and control mice, and in the Scatchard analysis of [^{3}H]Ro 15-4513 binding whole forebrain membranes were used (Wisden et al., 2002). In these circumstances, and without knowledge of the subcellular localization of [^{35}S]TBPS or [^{3}H]Ro 15-4513 binding, it is not safe to speculate about changes in the total $GABA_A$ receptor levels of the Thy1α6 mice. However, these data together clearly demonstrate the presence of α6βγ2 and α6β receptors, which, according to the immunohistochemical data (Wisden et al., 2002), are exclusively extrasynaptic.

5.5.3. Behavioral significance of altered balance between synaptic and extrasynaptic inhibition

To test the functional significance of the changed balance between synaptic and extrasynaptic inhibition, Thy1α6 and control mice were exposed to behavioral assays. Since most of our knowledge of the altered $GABA_A$ergic system in the Thy1α6 mice derives from the

hippocampal CA1 region (Wisden et al., 2002), the area of highest expression of ectopic α6-containing receptors according to our receptor pharmacological studies, hippocampus-related behavioral tests were applied. The Thy1α6 transgene has regionally restricted expression, and in the brain regions where it is expressed, only part of the cells are immunopositive (Wisden et al., 2002), in keeping with 35 % of the CA1 pyramidal cells showing furosemide sensitivity (**IV: Fig. 3**). For these reasons, no drastic phenotype was expected.

5.5.3.1. Basic behavior

Thy1α6 mice look normal, and grow and breed normally. All behavioral tests were run on both male and female control and Thy1α6 mice, and no significant gender differences were detected. Thus data from the two genders were pooled. The Thy1α6 mice displayed grossly normal behavior in the basic behavioral assessment made according to the SHIRPA protocol (Rogers et al., 1997). Differences from controls were detected only in the startle reflex and the struggle-escape behavior. In the startle reflex, the Thy1α6 mice responded to abrupt tones more actively (**IV: Fig. 4**), acoustic stimuli usually producing a jump. The startle reflex is a protective reaction that is mediated by a brain-stem circuit, but modulated by forebrain areas such as the amygdala (Koch, 1999). The hippocampus, in turn, may modulate the startle reflex via projections from the ventral hippocampus to the amygdala (Pitkänen et al., 2000). As the α6 transgene is expressed both in the amygdala and in the hippocampus of Thy1α6 mice (Figure 11), it might be involved in the changed startle reflex. Thy1α6 mice also had enhanced struggle-escape behavior (**IV: Fig. 4**), meaning heightened touch reflex, aggression and vocalization towards the observer. Struggle-escape behavior involves complex behavioral responses and cannot be linked to any particular brain region.

In patients with panic disorder disturbed hippocampal $GABA_A$ergic system has been reported (Kaschka et al., 1995). In the $GABA_A$ergic animal model of human anxiety (γ2+/- mouse line; Crestani et al., 1999) the phenotype is caused by reduced synaptic clustering of $GABA_A$ receptors and the resulting diminished synaptic inhibition (Crestani et al., 1999). Moreover, an enhanced startle reflex, similar to that observed in Thy1α6 mice, has been detected in another mouse model of pathological anxiety, the BALB mice (Plappert and Pilz, 2002). Thus, Thy1α6 mice were studied in the light-dark test, a common paradigm for testing baseline anxiety (Crawley and Goodwin, 1980) to see whether decreased synaptic inhibition caused an altered phenotype. However, Thy1α6 had a performance similar to their control animals, and thus revealed no differences in background anxiety levels. The hippocampus is also of crucial importance in other cognitive functions such as exploratory behavior, learning and memory. To probe for these hippocampal functions in Thy1α6 mice, the T-maze test with continuous alternation task protocol (Gerlai, 1998) was applied. As with the light-dark test, Thy1α6 mice had a similar performance to their control animals in all the parameters measured. In conclusion, the basic behavioral experiments without external stimuli revealed no differences between control and Thy1α6 mouse phenotypes. This indicates that in the Thy1α6 mice, there are no widespread deficits in $GABA_A$ receptor function, and that the compensatory decrease in the inhibitory synaptic transmission after increased tonic inhibition sustains adequate hippocampal function under normal circumstances.

5.5.3.2. Convulsion tests

In the basic behavioral tests, the Thy1α6 displayed disturbances only after strong external stimuli, a sudden loud noise (startle reflex) or the hand of the observer (struggle escape behavior). Next the inhibitory capacity of the altered $GABA_A$ergic system in Thy1α6 mice

was studied with strong chemical stimuli, the $GABA_A$ receptor antagonists DMCM and picrotoxinin injected intraperitoneally (i.p.). Both compounds cause convulsions by blocking $GABA_A$ergic inhibition in the cortex, amygdala and hippocampal formation (Vergnes et al., 2000). Convulsant actions of DMCM are mediated solely by α1 subunit-containing $GABA_A$ receptors (Crestani et al., 2002b). In α6-containing receptors, DMCM has agonist effects *in vitro* (Im et al., 1995b; Stevenson et al., 1995; Knoflach et al., 1996; Saxena and Macdonald, 1996). Whether this happens *in vivo*, is currently not known. When giving DMCM we thus inhibited mainly synaptic α1βγ2 receptors in the hippocampus of both mouse lines, and possibly also positively modulated α6βγ2 receptors in the Thy1α6 mice. However, the Thy1α6 mice had a shorter latency to the writhing clonus induced by DMCM (**IV: Fig. 5**). This demonstrates that reducing the already decreased synaptic inhibition in Thy1α6 mice was critical for the onset of convulsions. Similar to DMCM, the $GABA_A$ receptor subtype non-selective antagonist picrotoxinin caused (generalized tonic-clonic) convulsions in the Thy1α6 with shorter latency as compared with their control animals (**IV: Fig. 5**). These data demonstrate that the Thy1α6 mice are more sensitive to the convulsions produced by $GABA_A$ receptor antagonists.

In electrophysiological recordings of CA1 pyramidal cells the increased background inhibition of the Thy1α6 mice was revealed after an increase of extracellular GABA concentration by GABA uptake blocker NO-711 (Wisden et al., 2002). By administering another GABA uptake blocker, the antiepileptic drug tiagabine (Fink-Jensen et al., 1992; Dalby, 2000), we hoped to increase the function of high-affinity α6-receptors in the extrasynaptic loci similar to the *in vitro* situation. Interestingly, tiagabine (i.p., 30 min prior to i.p. picrotoxinin) delayed the picrotoxinin-induced generalized tonic-clonic convulsions already at a 3.2 mg/kg dose in the Thy1α6 mice, but not in their control animals (**IV, Fig. 6**). This suggests that tonic $GABA_A$ergic conductance was increased by the elevated GABA level *in vivo*, similar to that detected *in vitro* (Wisden et al., 2002), and demonstrates the behavioral function of increased tonic conductance for the first time. However, higher tiagabine doses had an antiepileptic effect in the control mice also, and the overall effect of tiagabine was greater in the control mice (**IV: Fig. 6**). In addition to delaying convulsions, the highest tiagabine dose prevented convulsions in 6 out of 7 control mice for the 900-s observation period, whereas it prevented convulsions in only 2 out of 11 Thy1α6 mice.

In conclusion, functional extrasynaptic α6 subunit expression in α6βγ2 and α6β combinations in the hippocampal CA1 region of Thy1α6 mice increases tonic but decreases phasic inhibition without compensatory alterations in other $GABA_A$ receptor subunit mRNA levels. The compensation of increased tonic conductance by a decrease in synaptic transmission sustains normal behavior, but in controlling strong excitation the synaptic component of inhibition dominates.

5.6. $GABA_A$ receptor subtype-selective actions of niflumate revealed by [^{35}S]TBPS autoradiography (V)

In a search for compounds affecting the GIS-[^{35}S]TBPS binding, the effects of niflumate (niflumic acid; 2-[3-(trifluoromethyl)-phenyl]aminonicotinic acid), a non-steroidal anti-inflammatory drug (NSAID), on the pharmacological fingerprint was tested on the rat brain autoradiography. Previously, direct actions by niflumate on $GABA_A$ receptors have not been reported, but both negative and positive modulation of GABA-induced currents in *Xenopus* oocytes injected with rat brain total RNA (White and Aylwin, 1990) or with rat cortical poly(A)$^+$RNA (Woodward et al., 1994) have been found. In addition, niflumate had been shown to decrease both [^{35}S]TBPS and [^{3}H]EBOB bindings to convulsant binding sites in rat brain homogenates (Evoniuk and Skolnick, 1988; Maksay et al., 1998).

5.6.1. Effects in the brain

The original experiment revealed that the GIS-[^{35}S]TBPS binding was increased in the cerebellar granule cell layer by 100 μM niflumate (not shown). No effect on the forebrain in the presence of 1 mM GABA was detected. This was somewhat surprising, since a decreasing effect of [^{3}H]EBOB binding to cerebellar membranes was previously detected (Maksay et al., 1998). In the following experiments, the effects of various niflumate concentrations on [^{35}S]TBPS binding were tested without GABA or with 3 μM or 1 mM GABA (**V: Fig. 2**). Brain regional heterogeneity of the effects was revealed, as niflumate potentiated the inhibitory effect of 3 μM GABA on [^{35}S]TBPS binding in most brain regions, but in the cerebellar granule cell layer it inhibited the effect of GABA (**V: Table 2**). These effects were detected up to a niflumate concentration of 1 000 μM, which decreased [^{35}S]TBPS also in the cerebellar granule cell layer. In the presence of 1 mM GABA, only the GIS-[^{35}S]TBPS component was visible, and in the cerebellar granule cell layer it was increased by nifumate concentration-dependently up to 300 μM, but at 1000 μM concentration it was decreased (**V: Table 3**). The thalamic component of GIS-[^{35}S]TBPS binding was increased by 10 and 30 μM niflumate, whereas 100 and 300 μM concentrations were inactive and 1000 μM niflumate decreased the binding.

5.6.2. Actions on recombinant receptors

The brain regional variation of niflumate effects on [^{35}S]TBPS binding led to a search for structural correlates for it. In the case of $GABA_A$ receptors, the pharmacological heterogeneities can usually be explained by different receptor subunit combinations. For this reason, we selected candidate subunits and expressed them in *Xenopus* oocytes. The direct actions of niflumate on the different recombinant $GABA_A$ receptor subtypes were then studied using two-electrode voltage-clamp recordings.

5.6.2.1. Negative modulation

Furosemide selectively and non-competitively inhibited the effects of GABA on recombinant receptors containing α4/6 and and β2/3 subunits (see 2.5.8.) and on [^{35}S]TBPS binding in the cerebellar granule cell layer and the thalamus (**V: Fig. 2**), similarly to the effects of niflumate. Niflumate and furosemide also share some structural similarity (**V: Fig. 1**). For these reasons the negative modulation by niflumate in the cerebellar granule cell layer was hypothesized to share the same structural requirements as the furosemide actions, and α6 and β2 subunit-containing receptors were selected. Niflumate alone was without effect or had only minor effects with high concentrations on α6 subunit-containing receptors. Niflumate inhibited GABA-induced currents in a concentration-dependent manner in α6β2 and α6β2γ2 $GABA_A$ receptors (**V: Figs. 3 and 7**). In α6β2γ2 receptors, niflumate reduced the maximal GABA currents but did not affect the EC_{50} for GABA (**V: Fig. 4**) suggesting a non-competitive mechanism of action similar to furosemide (Korpi et al., 1995a). A chimeric α1 subunit, where a 258 bp fragment including the TM1 and TM2 domains of the α1 subunit is substituted with that of the α6 subunit gene to confer furosemide sensitivity (Jackel et al., 1998), was enough to introduce the negative modulation by niflumate to α1β2γ2 receptors, which strengthened the idea of furosemide and niflumate sharing the same binding/effector site on the α6 subunit. These results indicate that niflumate acts as a negative non-competitive modulator in native and recombinant $GABA_A$ receptors composed of α6 and β2 subunits with or without γ2 subunits.

5.6.2.2. Positive modulation

The GABA-potentiating effect of niflumate was seen throughout the brain in the [^{35}S]TBPS autoradiography. For this reason, the prototypical and most abundant $\alpha1\beta2\gamma2$ $GABA_A$ receptor subtype (McKernan and Whiting, 1996) was selected for studies on positive modulation. Niflumate dose-dependently potentiated the 3 μM GABA-induced currents by about 60 % in $\alpha1\beta2\gamma2$ receptors with EC_{50} of 31 ± 3 μM (**V: Fig. 3**). Niflumate did not increase the efficacy of GABA, but increased its potency, indicating an allosteric mechanism of action (**V: Fig. 4**). In $\alpha1\beta2$ receptors, niflumate did not have any effect up to 100 μM concentrations, but 1000 μM niflumate inhibited the GABA response by 30 % (**V: Fig. 7**). This shows that positive modulation by niflumate is dependent on the presence of the $\gamma2$ subunit together with a suitable α subunit. Since niflumate potentiation in $\alpha1\beta2\gamma2$ receptors was not blocked by the BZ binding site antagonist flumazenil (**V: Fig. 8**), these data suggest that niflumate potentiation is mediated via a novel site of the $GABA_A$ receptors dependent on α, β and γ subunits.

6. GENERAL DISCUSSION

6.1. Receptor subunit combinations leading to GABA-insensitive [^{35}S]TBPS binding in the brain

[^{35}S]TBPS binding insensitive up to 50 μM GABA was first reported in the cerebellar granule cell layer (Edgar and Schwartz, 1990; Korpi and Lüddens, 1993). In the present study this minor proportion of the total [^{35}S]TBPS binding was found to be totally GABA-insensitive, since GABA up to 10 mM could not displace it. Thus, it was named the GABA-insensitive (GIS) [^{35}S]TBPS binding, and it was also found in some forebrain areas, most notably the thalamus (**I**). In a search for the subunit combinations causing this pharmacological fingerprint in the brain, strong evidence for a positive correlation with the α6 subunit and a negative correlation with the γ2 subunit was revealed in mutant mouse lines (**II**, **III**). In the present experiments recombinant β3, α6β3, α1β3 and α1β2δ $GABA_A$ receptors bound [^{35}S]TBPS in a way that was totally or partially insensitive to the inhibitory effect of GABA (**III: Table 1;** Figure 9). Previously, α6β2 (Korpi and Lüddens, 1993; Im et al., 1994), and α6β3 and α6β3δ (Korpi and Lüddens, 1997) receptors had displayed [^{35}S]TBPS binding that was totally or partially insensitive to the inhibitory effect of 10 μM GABA. However, [^{35}S]TBPS binding to α1β2, α1βγ2 and α6βγ2 receptors is GABA-sensitive (**III**; Korpi and Lüddens, 1993). These results indicate that there are four main factors which determine the modulatory effect of GABA on [^{35}S]TBPS binding. Firstly, the α6 subunit induces GABA-insensitivity when compared to the α1 subunit, as indicated by GIS-[^{35}S]TBPS binding to α6β2 but not to α1β2 receptors. Secondly, inclusion of the γ2 subunit in the receptor complex converts [^{35}S]TBPS binding to α1β3 and α6β2/3 receptors GABA-sensitive. Thirdly, δ makes [^{35}S]TBPS binding even to α1β2 receptors insensitive to GABA. Fourthly, β3 subunits are more susceptible than β2 subunits to causing poor coupling between GABA and convulsant sites, at least in the presence of α1 subunit, as indicated by GIS-[^{35}S]TBPS binding to α1β3 receptors but not to α1β2 receptors. Also, receptors composed only of β3 subunits displayed GIS-[^{35}S]TBPS binding, but homomeric β2 receptors did not. What is the exact mechanism leading to GABA-insensitivity of [^{35}S]TBPS binding to these receptor subtypes is currently unknown, but it may include atypical conformation of agonist or convulsant binding sites, or atypical coupling between these.

From the above-summarized results it can be deduced that, in the native brain, at least the greatest part of the GIS-[^{35}S]TBPS binding in the cerebellar granule cell layer is caused by α6β2/3 and/or α6β2/3δ receptors. While data for α6βδ receptors is preliminary (Figure 9), and it is not totally certain that δ subunits are incorporated into recombinant α6β2δ receptors in the present set of experiments, or in the previous experiments with α6β3δ receptors (Korpi and Lüddens, 1997), since α6β receptors would already display GIS-[^{35}S]TBPS binding, the incorporation of δ to α1β2δ receptors is certain (Figure 9). Since δ is able to confer GIS-[^{35}S]TBPS binding to α1β2 receptors, it is presumable that α6βδ displays also GIS-[^{35}S]TBPS binding. So, in the native brain, both α6β2/3 and α6β2/3δ combinations resulting in GIS-[^{35}S]TBPS binding are possible. Using subunit-specific antibodies and combining immunoaffinity chromatography with immunoprecipitation and Western blot analysis with subsequent binding studies, it is possible to determine and quantify the $GABA_A$ receptor subtypes in the brain. In the cerebellum, the major receptor subtypes are α1βγ2, α6βγ2, α1α6βγ2, α6βδ and α1α6βδ (Quirk et al., 1994; Pöltl et al., 2003). The α6βδ receptors constitute about 23 % of all receptors in the cerebellum (Quirk et al., 1994). Coassembly of α1 and δ subunits is very limited (Quirk et al., 1994), but a minor population of α1βδ receptors may also be present (Pöltl et al., 2003), and altogether 97 % of receptors contain γ2 or δ subunit (Tretter et al., 2001). However, the quantitation techniques have limited sensitivity. The solubilization and precipitation of the receptors from brain membranes cause

loss of about 50 % of the receptors (Tretter et al., 2001), and each precipitation step with subunit-specific antibodies has its own sensitivity. Thus, detection of minor receptor subtypes is difficult (Quirk et al., 1994). Also, detection of a minor subtype of cerebellar granule cell layer is further hampered by the receptors of the molecular layer. Nevertheless, if α6β receptors exist *in vivo*, they seem to constitute a minor population, a few percentages, of the total number of cerebellar receptors. Since GIS-[^{35}S]TBPS binding consists of 6-15 % of the basal [^{35}S]TBPS binding in the cerebellar granule cell layer, depending on the species studied (**I**, **II**), it seems likely that both α6β and α6βδ receptors participate in its formation. Since we do not know how GABA actually modulates [^{35}S]TBPS binding in these receptors (total or partial insensitivity of [^{35}S]TBPS binding), it is possible that the about 10 % of the GIS-[^{35}S]TBPS binding component represents the α6βδ receptors with a prevalence of 23 % of the total cerebellar receptor population (Quirk et al., 1994). However, in the δ-/- mice there is a remarkable correlation between the number of receptors devoid of γ or δ subunits (24 %; (Tretter et al., 2001) and the proportion of GIS-[^{35}S]TBPS binding of the total binding (24 %; **II**), which suggests that, in these mice, GIS-[^{35}S]TBPS binding represents α6β and possibly other αβ3 receptors.

In addition to the cerebellar granule cell layer, the GIS-[^{35}S]TBPS binding is also found in selected forebrain regions, mainly the thalamus and deep layers of the cortex, where it represent a few percentages of the total [^{35}S]TBPS binding (**I**). Its localization is in good correlation with the expression of the δ and α4 subunits (**II**). In the thalamus, α4βδ receptors compose 13 % and α4βγ2 8 % of all $GABA_A$ receptors (Sur et al., 1999), but about 50 % of the α4 subunit-containing receptors in the brain are devoid of γ or δ subunits (Bencsits et al., 1999). Due to the difficulties with α4 subunit expression in HEK 293 cells, we have not been able to study the GABA sensitivity of [^{35}S]TBPS binding in different α4 subunit-containing $GABA_A$ receptors. Furthermore, mouse lines devoid of α4 do not exist. However, since the α4 subunit is the closest subunit to α6 in respect of pharmacological properties (insensitive to BZ full agonists; Benke et al., 1997, but sensitive to furosemide; Knoflach et al., 1996) and preferential assembly with the δ subunit (Sur et al., 1999), and even α1β2δ receptors display GIS-[^{35}S]TBPS binding, it is highly likely that native α4β and α4βδ receptors would bind [^{35}S]TBPS in a GABA-insensitive manner. The role of α4βδ receptors is supported by the finding that THIP, a partial agonist in α1/4βγ2 receptors (Ebert et al., 1994; Ebert et al., 1997; Brown et al., 2002) but a full agonist in α4βδ receptors (Brown et al., 2002), displaces all [^{35}S]TBPS binding from the thalamus at high concentrations, while GABA competitively decreases the effect of THIP (Figure 10).

In addition to the previously discussed receptor subtypes, recombinant α3β3γ2/3 and α5β3γ2/3 receptors also bind [^{35}S]TBPS in the presence of 1 mM GABA (Lüddens and Korpi, 1995). The α3 and α5 are the only subunits whose expression is enriched in the deep layers of the cerebral cortex similarly to the distribution of GIS-[^{35}S]TBPS binding (**II: Fig. 1**). The α5 is additionally expressed in the hippocampus, where it might participate in forming the tiny proportion of GIS-[^{35}S]TBPS binding detected (**II: Fig. 1**). α5 seems to be located exclusively extrasynaptically (Brünig et al., 2002; Crestani et al., 2002a). This is in line with tonic $GABA_A$ergic inhibition being reduced in the hippocampus of a mouse line devoid of α5 subunits (Caraiscos et al., 2004). The α3 is located on both synaptic and extrasynaptic membranes (Brünig et al., 2002). In addition, THIP is as effective as GABA in α5β3γ2 receptors, but in α3β3γ2 receptors it has only about 50 % of the efficacy of GABA (Ebert et al., 1994). As the receptor subtypes previously proposed to produce GIS-[^{35}S]TBPS binding (α6β2/3, α6β2/3δ, α4β, α4βδ) are also extrasynaptic (δ subunit-containing receptors exclusively extrasynaptic; Nusser et al., 1998a), and the γ2 subunit is required for postsynaptic clustering (Crestani et al., 1999), these data suggest that GIS-[^{35}S]TBPS binding preferentially represents receptors on extrasynaptic loci. Why α3β3γ2 and α5β3γ2 receptors

display GIS-[^{35}S]TBPS binding despite the presence of γ2 subunits is currently unknown, but it might be at least partially related to the β3 subunit variant.

All the evidence presented above about the roles of the different subunits in GIS-[^{35}S]TBPS binding is indirect. Also the subcellular localization of GIS-[^{35}S]TBPS binding on extrasynaptic membranes is deduced from the presumed subunit combinations. [^{35}S]TBPS binding cannot be studied at the cellular level owing to its dissociation with photographic emulsion. Only photoaffinity labeling of receptors with [^{35}S]TBPS or similar derivatives and subsequent electron microscopic detection of the signal would allow direct demonstration of the subcellular localization directly. Unfortunately, no suitable photoaffinity ligand for the convulsant binding site is available, even though irreversible binding of some TBOB derivatives has been suggested (Lewin et al., 1989). On the other hand, it is unlikely that the allosteric interaction, or lack of it, between the agonist and the convulsant sites (GIS-[^{35}S]TBPS binding) could be demonstrated with photoaffinity labeling. Thus, in this respect we have at present to rely on indirect and deductive data.

6.2. Properties and significance of extrasynaptic $GABA_A$ receptors

Extrasynaptic receptors give rise to tonic inhibition, which is common to all neurotransmitter systems (reviewed in Zoli et al., 1999). Only a small fraction of the postsynaptic neuronal membrane area participates in forming synapses. After subunit assembly in the cytoplasm, the receptors are inserted to neuronal membranes, where they diffuse somewhat arbitrarily. In order to achieve the receptor enrichment on postsynaptic membranes there must be ways to retain receptors juxtaposed to the presynaptic terminal. Gephyrin participates in this process with $GABA_A$ receptors (Kneussel et al., 1999) and is colocalized with α1, α2, α3 and γ2 subunits in many brain regions, but not with α6 or δ subunits (Sassoè-Pognetto et al., 2000). Lack of colocalization with gephyrin does not necessarily mean extrasynaptic localization, since other clustering proteins are also proposed (Kneussel et al., 2001). The γ2 subunit is essential for synaptic anchoring of the major $GABA_A$ receptor subtypes as deduced from dramatically reduced postsynaptic receptor clusters in γ2 knockout mice (Essrich et al., 1998). On the basis of immunohistochemical studies it has been suggested that α6βδ receptors are exclusively extrasynaptic. The α6βγ2 receptors are found in both synaptic and extrasynaptic membranes, while the α1βγ2 receptors are mainly located in synapses, but at lower concentrations also on extrasynaptic membranes (Nusser et al., 1998a). The previous considerations about the structural requirements of synaptic receptors (γ2 subunits) fit with the immunohistochemical data, and also indicate that the α6β receptors proposed in the current study would be extrasynaptic. Their presence is supported by detection of α6β receptors, but not α6βδ, in the hippocampus of Thy1α6 mice together with GIS-[^{35}S]TBPS binding (**IV;** Wisden et al., 2002). Also, in the δ-/- mice GIS-[^{35}S]TBPS is found in the cerebellar granule cell layer, indicating that α6βδ receptors are not the only possible receptors responsible for the pharmacological fingerprint. Receptor subtypes with both α1 and α6 subunits also exist, mainly in synaptic compartments, but they will not be dealt with here. However, one should not be too pragmatic in biology, and it is likely that all the receptor subtypes exist in both synaptic and extrasynaptic loci, but their proportions in the two compartments varies, depending on subunit composition.

Tonic $GABA_A$ergic conductance was first proposed by Birnir et al. (1994) in the hippocampal dentate gyrus. Thereafter, it was found and characterized in the cerebellar granule cell layer (Kaneda et al., 1995; Brickley et al., 1996; Tia et al., 1996a; Wall and Usowicz, 1997). Some properties of synaptic and extrasynaptic receptors in the cerebellar granule cell layer are listed in Table 3. When interpreting the functional correlates of GIS-[^{35}S]TBPS binding, it has to be remembered that the receptors responsible for GIS-[^{35}S]TBPS

binding are actually highly sensitive to low concentrations of GABA, but that [^{35}S]TBPS binding to these receptors is not efficiently displaced by GABA. This suggests that the "problem" is not in the agonist binding *per se*, but rather in the coupling between agonist and convulsant sites and/or channel activation. One interpretation of the situation is that GABA is not a full agonist in the receptors responsible for GIS-[^{35}S]TBPS binding. What might be the rationale behind the scheme where a natural agonist has only partial efficacy? The explanation may be related to the localization of these receptors outside synapses. For synaptic receptors, which have access to the agonist for only a very limited period of time, it is important to be able to activate as large and fast postsynaptic response as possible. In those receptors the response is proportional to the efficacy of the agonist. However, for the extrasynaptic receptors, which are permanently bathed in submicromolar GABA (Lerma et al., 1986; Tossman *et al.*, 1986; Attwell et al., 1993; Richerson and Wu, 2003), and which will usually not encounter much higher agonist concentrations, the important property might be not the full but the permanent activation, which is ensured by the high affinity for GABA and the low receptor desensitization (Table 2). Here, it is important to note that "typical" receptors with lower GABA sensitivity may also participate in forming tonic conductance; those receptors just have a lower open probability in the presence of low agonist concentrations. On the other hand, low ambient agonist concentrations do not cause desensitization, which would take place at higher agonist concentrations, and receptors would remain functional despite permanent stimulation.

What might be the functional correlate of the findings that GIS-[^{35}S]TBPS binding can be totally abolished by either application of THIP instead of GABA, or by inclusion of the γ2 subunit in the receptor complex? It is possible that partial and full agonists affect receptor channel conductance or mean open time differentially (Lindquist et al., 2003; Mortensen et al., 2004). Similarly, inclusion of the γ2 subunit into αβ receptors may affect channel conductance and mean open time (Lorez et al., 2000). However, the totally different time scales of [^{35}S]TBPS binding (min - hour) and channel kinetics (ms) makes it impossible to deduce any solid conclusion about the receptor channel functional correlate of GIS-[^{35}S]TBPS binding.

The presence of GIS-[^{35}S]TBPS binding in the cerebellar granule cells with well-known tonic inhibition (**I**), its decrease in the α6-/- mice together with decreased tonic inhibition (**II**), and its induction in the hippocampal CA1 pyramidal cells together with an increase in tonic inhibition in the Thy1α6 mice (**IV;** Wisden et al., 2002), show that GIS-[^{35}S]TBPS binding can be used to image the extrasynaptic receptors causing tonic conductance. However, there is an important exception, the δ-/- mice. In these mice GIS-[^{35}S]TBPS binding in the cerebellar granule cell layer is increased (**II**), but the tonic $GABA_A$ergic inhibition is practically abolished (Stell et al., 2003). Also, GIS-[^{35}S]TBPS binding is very low in some areas with marked tonic inhibition, e.g., the hippocampus (CA1 region: Birnir et al., 2000; Bai et al., 2001; Semyanov et al., 2003; Caraiscos et al., 2004; dentate gyrus: Nusser and Mody, 2002; Stell et al., 2003; Wei et al., 2003). Thus, even though, in many cases GIS-[^{35}S]TBPS binding is suggestive of the presence of pronounced tonic $GABA_A$ergic inhibition, even without the δ subunit, as indicated by the Thy1α6 mice (**IV;** Wisden et al., 2002), it should not be considered obligatory for it.

In general, tonic inhibition deriving from extrasynaptic receptors is suggested to set a background level for overall neuronal excitability by controlling membrane properties, such as membrane input resistance and time constant (Häusser and Clark, 1997; Mody, 2001; Mitchell and Silver, 2003). More specific functions in most brain areas are still unrevealed, but in the cerebellar cortex the role of tonic inhibition is beginning to be solved. Tonic inhibition of the granule cells, which contributes more than 90 % of the total inhibitory charge transfer (Brickley et al., 1996; Hamann et al., 2002), decreases the number of excited granule

Table 2. Properties of selected $GABA_A$ receptor subtypes in the cerebellar granule cells.

Receptor subtype	α1β2/3γ2	α6β2/3γ2	α6β2/3δ	α6β2/3	References
Subcellular localization	synaptic	synaptic & extrasynaptic	extrasynaptic	extrasynaptic	Nusser et al., 1998a; **IV**
Form of inhibition	phasic	phasic & tonic	tonic	tonic (?)	Nusser et al., 1998a; **IV**
Autoradiographic imaging	DS-[^{3}H]Ro 15-4513	DIS-[^{3}H]Ro 15-4513	[^{3}H]musc & GIS-[^{35}S]TBPS	GIS-[^{35}S]TBPS	Korpi and Lüddens, 1997; Mäkelä et al., 1997; Korpi et al., 2002b; **II, III, IV,** Figure 9
GABA EC_{50} (μM)	8.0	1.8	0.19	0.46	**V;** Saxena and Macdonald, 1996
Desensitization	fast	poor	poor	poor	Tia et al., 1996b; Haas and Macdonald, 1999
Modulation by drugs					
GABA	++	++	+ (?)	+ (?)	**I, II, III, IV,** Figure 9
THIP	+	++	++ (?)	++ (?)	Ebert et al., 1997, Figure 10
Diazepam	++	0	0	0	Saxena and Macdonald, 1996
DMCM	-	+	+	+	Im et al., 1995b; Knoflach et al., 1996; Saxena and Macdonald, 1996
picrotoxinin	-	-	-	-	Saxena and Macdonald, 1996
furosemide	0	-	-	-	Korpi et al., 1995a; Korpi and Lüddens, 1997; **V**
niflumate	++	-	- (?)	-	**V**

DS, diazepam-sensitive; *DIS*, diazepam-insensitive; *[^{3}H]musc*, high-affinity [^{3}H]muscimol autoradiographic procedure. (++), full agonist or full positive modulator; (+), partial agonist or weak positive modulator; (0), inactive; (-), negative modulator. (?), hypothesized.

cells *in vitro* (Hamann et al., 2002). Recently it was demonstrated that tonic inhibition also reduces spontaneous firing rates of granule cells *in vivo* (Chadderton et al., 2004). In addition, it allows temporal summation of excitatory postsynaptic currents, which is important in regulating granule cell bursting (Chadderton et al., 2004). Thus, tonic inhibition improves the signal-to-noise ratio of sensory information transfer through the cerebellar cortex (Chadderton et al., 2004). Reduction of the number of granule cells activated decreases the rate of Purkinje cell firing, which is suggested to improve the information storage capacity of the cerebellum (Hamann et al., 2002). Vice versa, reduction of tonic conductance enables single mossy fiber

action potential to be transferred through the granule cell layer, which makes the network sensitive to minor stimuli when required (Chadderton et al., 2004). Thus, tonic inhibition in the cerebellar granule cells seems to be crucial for normal performance of the cerebellar cortex. This is supported by the finding that in the α6-/- mice, where $GABA_A$ergic tonic inhibition is abolished (Brickley et al., 2001) together with decreased GIS-[^{35}S]TBPS binding (**II**), normal motor performance is maintained by induction of potassium "leak" conductance (Brickley et al., 2001). In this context, it seems likely that in the δ-/- mice, which display normal motor performance but decreased $GABA_A$ergic tonic conductance (Stell et al., 2003), some as yet unrevealed compensation may take place.

Inhibition in the hippocampus is reviewed in Section 2.4.7. Normally, CA1 pyramidal cells possess tonic inhibition (Birnir et al., 2000; Bai et al., 2001; Semyanov et al., 2003; Caraiscos et al., 2004), but its contribution to the total inhibitory charge transfer has not been quantified. Since CA1 pyramidal cells constitute the main output of the hippocampus, the tone setting tonic inhibition presumably has a large influence on hippocampal function as suggested by homology with the cerebellar function. For this reason the Thy1α6 mice offer, for the first time, an intriguing possibility to study the effects of different forms of inhibition on animal behavior. A hypothetical model of the $GABA_A$ergic system in the Thy1α6 mouse CA1 pyramidal cells leading to behavioral consequences may be provided (**IV:Fig. 7**). The Thy1α6 model suggests that increasing extracellular GABA levels upregulates tonic inhibition *in vivo,* similar to the effects detected *in vitro* (Wisden et al., 2002), which has the lengthened latency to convulsions as its behavioral correlate (**IV: Fig. 6**). If tonic inhibition were more important in absolute terms than phasic inhibition in CA1 pyramidal cell function, it would be likely that the Thy1α6 would benefit from tiagabine more than their control animals in the whole dose range tested. Since this is not the case (**IV: Fig. 6**), it has to be concluded that tonic inhibition cannot substitute for phasic inhibition in circumstances with strong excitation. Nevertheless, the Thy1α6 mice might serve as a useful tool in future studies of the pathophysiology of epilepsy, and in research on antiepileptic drugs. In fact, recently a structural variation in the δ subunit gene was found that caused a decrease in GABA-gated currents, and was genetically linked to generalized epilepsy (Dibbens et al., 2004). This may indicate that tonic inhibition, or its disturbance, is important in the expression of human epilepsy. However, it is important to keep in mind that, in the native hippocampal environment, the α6 subunit does not exist, and thus the results presented here have to be considered as suggestive rather than conclusive.

While many basic physiological characteristics of $GABA_A$ergic tonic inhibition are still unclear, the chief question at the moment concerns drug therapy: where are the primary targets of $GABA_A$ergic drugs, in synapses or on extrasynaptic membranes? $GABA_A$ergic drugs usually exert their actions by enhancing the agonistic effect of GABA. It has been estimated that peak GABA concentrations in the synaptic cleft reach 0.3 - 3 mM (Mozrzymas et al., 1999; Perrais and Ropert, 1999), but most $GABA_A$ receptor subtypes have an EC_{50} for GABA below 50 μM (Hevers and Lüddens, 1998). The discrepancy between the receptors' affinity for GABA and the apparently overwhelming agonist concentrations in the synaptic cleft makes it tempting to speculate that the primary targets for $GABA_A$ergic drugs are the extrasynaptic receptors. In these receptors, positive modulators would have powerful effects on inhibitory tone in the presence of low ambient GABA. In fact, their effects would be independent of synaptic activity. Also, another interesting point arises when thinking about the penetration of drugs to their effectory sites. The drugs initially diffuse throughout the extracellular space, and thus their concentrations on extracellular membranes are presumably similar to those in synapses. Later on, drugs are probably concentrated in synapses due to enrichment of the receptors with high affinity to the drugs. Anyhow, it has been demonstrated that the tonic conductance in CA1 pyramidal neurons of rat hippocampal slices is potentiated by midazolam and propofol more than the phasic conductance (Bai et al., 2001). Vigabatrin,

an antiepileptic drug that increases brain GABA levels, reduces phasic but increases tonic inhibition (Overstreet and Westbrook, 2001; Wu et al., 2003). In addition, neurosteroid modulation was reduced in the δ-/- mouse hippocampal dentate gyrus in conjunction with deletion of extrasynaptic δ-receptors (Stell et al., 2003). THIP, which is currently developed as a hypnotic, has a markedly higher affinity, and also a higher efficacy, on extrasynaptic receptors than on prototypical synaptic α1βγ2 receptors (Ebert et al., 1994; Ebert et al., 1997; Brown et al., 2002), which might make it relatively selective for increasing tonic inhibition *in vivo*. At least its clinically effective concentration (~1 μM; Madsen et al., 1983; Faulhaber et al., 1997) is far closer to the EC_{50} of the extrasynaptic receptors, and 2 μM THIP has been shown to activate extrasynaptic $GABA_A$ receptors in CA1 pyramidal neurons of hippocampal slices (Lindquist et al., 2003). Taking all these things together, and accepting the fact that the subunit composition of the receptor determines its subcellular localization, there should be possibilities to introduce novel compounds acting selectively on phasic or tonic conductance. With these compounds, selective and possibly safer treatment could be feasible. This is supported by the recent finding that no tolerance was developed to the anxiolytic actions of THIP (Liang et al., 2004), which were markedly enhanced in an anxious rat model of the premenstrual syndrome with upregulated hippocampal α4βδ receptors (Gulinello et al., 2003). Considering only some of the brain regions where tonic conductance could be selectively modulated, novel treatments affecting e.g. memory performance and seizure threshold (hippocampus), sleep and anesthesia (thalamus) and motor coordination (cerebellum) could be envisaged. In addition, selective compounds could be used to elucidate the significance of the complex balance between the two forms of $GABA_A$ergic inhibition. However, if the unidentified compensatory mechanisms seen in the Thy1α6 (Wisden et al., 2002) and GAT-1 deficient mice (Jensen et al., 2003), which balance the increased tonic inhibition with reduction in phasic inhibition, apply also for drug therapy, the issue is obviously more complex. This seems to hold true for vigabatrin (Overstreet and Westbrook, 2001; Wu et al., 2003).

6.3. Use of GABA-insensitive [^{35}S]TBPS binding in monitoring alterations of $GABA_A$ergic system in animal models of neurological and psychiatric diseases

In order to better understand the pathophysiology of neurological and psychiatric diseases, and to obtain better cure for them, we constantly need basic research and laboratory animals. Hopefully in the future many things can be solved with cell or organ cultures, but in the case of such a complex organism as the brain, we shall still be, for a long period of time, dependent on experimental animals. In addition, with the advances in gene manipulation technology and knowledge of genetic liability to the diseases, more and more accurate animal models for human neurological and psychiatric diseases can be produced. In many cases the animal models are so similar to human diseases that they offer a fairly straight-forward link to the crucial changes leading to human pathological conditions. In a recent report, Zambrowicz and Sands (2003) retrospectively analyzed the knockout mouse phenotypes of the drug targets of the 100 best-selling drugs, and found that the drug efficacy could have been predicted from the respective mouse phenotype. Even though the matter may not be this simple, it clearly shows the potential of animal modeling in biomedical research and drug discovery.

Thus, presumably the number of animal models, mostly mouse lines, of different neurological and psychiatric diseases will increase dramatically in the future. Additionally, crossbreeding of different mouse lines will create a new source of research models. Considering the complexity and plasticity of the brain, there will be a great need for techniques that can be used for rapid characterization of basic neurotransmitter systems in different disease models. Thus far, most researchers have concentrated their work on some

particular neurotransmitter and a subclass of its receptors, but I think that when getting deeper into the pathophysiology of diseases, we should be more aware of the other systems as well. Manipulating, e.g., NMDA type glutamate receptors might cause alterations in the GABAergic system also. Considering the wide range of approaches, from single cell RT-PCR revealing the subunit mRNA levels of individual neurons to electrophysiological studies with certain neuronal populations or detailed behavioral characterization of living animals, it will be almost impossible to perform even all the relevant studies to characterize the animal models. In this scenario, an approach that describes receptor level alterations with brain regional resolution would be a good starting point. Ligand autoradiography with protocols revealing different receptor subtypes should be ideal for this purpose, since with a limited number of brain samples, enough serial sections for many parallel assays are obtained. In general, ligand autoradiographic procedures do not require processing of the tissue, and they are technically relatively easy to perform. Still, they reveal relevant information about receptor populations in different brain regions, and could be used to direct future studies. As an example we may consider the autoradiographic "screening" of the γ2+/- mice (**III**), an animal model of the human generalized anxiety disorder (Crestani et al., 1999). Solely based on the receptor autoradiographic results, it could be predicted that the amount of synaptic γ2-receptors in many brain regions is decreased (reduced [^{3}H]Ro 15-4513 binding) together with regionally similar upregulation of extrasynaptic αβ receptors (increased GIS-[^{35}S]TBPS binding). In addition, [^{3}H]muscimol binding was unchanged, which suggests that the δ subunits do not replace the reduced γ2 subunits. With this panel of three receptor autoradiographic protocols, we have a fairly accurate estimate of the $GABA_A$ receptor system in γ2+/- mice, and would be able to direct the following studies according to these findings. Taking all these considerations into account, here I propose that, similar to the SHIRPA protocol in behavioral studies, there should be a basic screening protocol for receptor level changes that should be performed for all new animal models with $GABA_A$ergic system alterations. Before proceeding to more detailed studies with the model, a basic characterization of the major $GABA_A$ receptor subtype levels in different brain regions should be performed. This would build a solid ground for the following studies, and allow judgment of the significance of the findings from, e.g., behavioral studies.

Could poor coupling with agonist and convulsant sites of the $GABA_A$ receptor be used to image the possible changes in the $GABA_A$ergic system in human patients? Currently PET and SPECT can be used to image BZ binding sites, and, for example, a decrease in BZ binding in patients with generalized anxiety disorder (SPECT; Tiihonen et al., 1997) and in patients with Angelman' syndrome (PET; Holopainen et al., 2001) has been reported. However, no proper PET or SPECT ligands for other $GABA_A$ receptor binding sites have been introduced. Convulsant site ligand TBOB, or its derivatives, have been tested, but they have too rapid clearance from the brain, and they are too lipophilic to be useful for PET or SPECT (Culbert et al., 1993; Snyder et al., 1995). Moreover, while [^{11}C]flumazenil, as a BZ antagonist, can safely be used in PET or SPECT studies, it is not so clear that channel blockers, such as TBPS, could ever be suitable ligands *in vivo,* despite the low tracer amounts needed for human binding. In addition, reaching similar conditions *in vivo* that we apply *in vitro* to reveal the GIS-[^{35}S]TBPS binding (90 min in the presence of 1 mM GABA) is not realistic. Thus, in order to be able to image extrasynaptic $GABA_A$ receptors with PET or SPECT *in vivo*, we should probably think about the possibilities offered by such compounds as THIP, which seem to have a much higher affinity for the extrasynaptic than the synaptic receptors (Ebert et al., 1994; Brown et al., 2002). In fact, in receptor autoradiography, [^{3}H]THIP seems to label only a subset of GABA binding sites (Hösli et al., 1985), but the subcellular localization of these sites has not been determined.

6.4. Use of [^{35}S]TBPS autoradiography in drug research

[^{35}S]TBPS has a high affinity and specificity for native $GABA_A$ receptors, which make it a good ligand in receptor autoradiography with brain sections. [^{35}S]TBPS binding dissociates in a good correlation with $GABA_A$ receptor activation, as measured by chloride flux (Im and Blakeman, 1991). Thus, [^{35}S]TBPS binding and its dissociation can be used as a biochemical measure of receptor activation. Basically [^{35}S]TBPS dissociation and receptor activation are caused by GABA, but compounds that modulate the effect of GABA, in a positive or a negative way, also affect [^{35}S]TBPS. Thus [^{35}S]TBPS binding and its modulation can be used to reveal allosteric interactions in the receptor complex. These properties together make [^{35}S]TBPS autoradiography a powerful tool in drug research.

When a heterogeneity in [^{35}S]TBPS binding on brain section autoradiography is revealed (e.g., by a novel drug compound), two important general assumptions are justified: 1) there are at least two structurally different receptor subtypes in respect to the binding site of the novel compound, or in respect to coupling between the convulsant and the drug binding site, or in respect to coupling between GABA and the convulsant binding sites, and 2) the ligand autoradiographic heterogeneity probably represents receptor functional heterogeneity. From the first assumption it follows that heterogeneity can usually be replicated in [^{35}S]TBPS binding to recombinant receptors, once correct structural needs (i.e., correct subunit combination) are met, which may be used to confirm the findings with native receptors. From the second assumption it follows that the heterogeneity represents a property that potentially bears functional significance. The above described theoretical considerations of [^{35}S]TBPS autoradiography may be exemplified by the work that aimed to clarify the possible actions of niflumate on brain $GABA_A$ receptors (**V**). Both positive and negative modulation of receptor function (as deduced by modulation of the GABA effect on [^{35}S]TBPS binding with brain sections) had correlates on recombinant receptor function. The proper subunit combinations were originally chosen on the basis of brain regional heterogeneity of niflumate effects offered by the spatial resolution of receptor autoradiography with brain sections. Subsequent studies with recombinant receptors revealed that negative modulation was mediated by the furosemide binding site, whereas the binding site responsible for positive modulation has not been described before. Thus, the pharmacotherapeutically attractive idea of positive-negative modulation of different receptor subtypes by one compound was initially revealed by a simple autoradiographic procedure. Similarly, the original finding that GABA is not a full agonist in all native $GABA_A$ receptors (Figure 10) was provided by [^{35}S]TBPS autoradiography.

[^{35}S]TBPS autoradiography is a suitable method for high-throughput screening of novel $GABA_A$ergic compounds. Given the multiplicity of the available genetically engineered mouse lines, the pharmacology of which may also be conveniently studied, [^{35}S]TBPS autoradiography offers a simple and inexpensive way to rapidly characterize the receptor subtype specific actions of various compounds in the natural neuronal environment. Most importantly, the pharmacological heterogeneities revealed by the autoradiographic procedure are likely to bear functional correlates.

7. CONCLUSIONS

The aim of the current project was to characterize the atypical property of poor coupling between the $GABA_A$ receptor agonist and convulsant binding sites, named the GABA-insensitive (GIS) [^{35}S]TBPS binding. The main results and conclusions of the study were:

1. A modification of the standard receptor autoradiographic procedure was achieved, which allowed reproducible imaging of GIS-[^{35}S]TBPS binding on brain sections.

2. The GIS-[^{35}S]TBPS binding is conserved during evolution, since it was detected in human, rodent and avian cerebellar granule cell layer. The GIS-[^{35}S]TBPS binding was also detected in the thalamus and in the deep layers of the neocortex in human and rodent brain. It can be estimated that 6-16 % (quantified proportion of GIS-[^{35}S]TBPS binding) of the basal [^{35}S]TBPS binding in the cerebellar granule cell layer represents a minor population of native $GABA_A$ receptors. The proportion of receptors displaying GIS-[^{35}S]TBPS binding among all $GABA_A$ receptors in the forebrain regions is smaller. The prevalence of a receptor population displaying GIS-[^{35}S]TBPS binding is typical for many $GABA_A$ receptor subtypes.

3. The present experiments revealed that several receptor subunits affect the agonist modulation of [^{35}S]TBPS binding. 1) The α6 subunit induces GIS-[^{35}S]TBPS binding when compared to the α1 subunit. 2) Inclusion of the γ2 subunit in the receptor complex abolishes the GIS-[^{35}S]TBPS binding. 3) Inclusion of the δ subunit induces GIS-[^{35}S]TBPS binding. 4) The β3 subunits are more likely than the β2 subunits to induce GIS-[^{35}S]TBPS binding. Thus, in the native brain, at least the majority of the GIS-[^{35}S]TBPS binding in the cerebellar granule cell layer is caused by α6β2/3 and/or α6β2/3δ receptors. The identity of the receptors forming the forebrain GIS-[^{35}S]TBPS binding component is uncertain, but it is likely that α4β, α4βδ, α3β3γ2/3 and α5β3γ2/3 $GABA_A$ receptors participate in forebrain GIS-[^{35}S]TBPS binding. Since THIP, a partial GABA site agonist in α1/4βγ2 receptors but full a agonist in α4βδ receptors, was able to competitively displace GIS-[^{35}S]TBPS binding, it is possible that GIS-[^{35}S]TBPS binding in the brain represents native receptors, where GABA is only a partial agonist.

4. Since the γ2 subunit is generally required for synaptic enrichment of $GABA_A$ receptors, and δ subunit-containing receptors are exclusively extrasynaptic, the receptor subtypes displaying GIS-[^{35}S]TBPS in the native brain are deduced to be extrasynaptic. These receptors are high-affinity sensors of extracellular GABA, and, at least in the cerebellar granule cell layer, are responsible for the major part of the $GABA_A$ergic inhibition. Induction of GIS-[^{35}S]TBPS binding together with formation of α6β (and α6βγ2) receptors in the mouse hippocampus after ectopic α6 subunit expression (Thy1α6 mice), and following increased tonic but decreased phasic inhibition, resulted in increased sensitivity to convulsions. While increasing extracellular GABA level delayed convulsions in the Thy1α6 mice, presumably owing to increased tonic inhibition, it was not able to fully counteract the decreased phasic inhibition. These results reveal the first behavioral correlate of tonic inhibition, but highlight the significance of phasic inhibition during strong excitation.

5. Niflumate, a NSAID, was shown to decrease the inhibitory effect of GABA on [^{35}S]TBPS binding in the cerebellar granule cell layer, while on other brain regions it potentiated the effect of GABA. Using recombinant receptors expressed in *Xenopus laevis* oocytes, it was demonstrated that negative modulation had the same structural requirement as furosemide action, i.e., the α6 subunit, while positive modulation was dependent on the presence of α1 and γ2 subunits. The binding site for positive modulation has not been described before. These results indicate that GIS-[^{35}S]TBPS binding, like [^{35}S]TBPS autoradiography in general, can be used to screen receptor subtype-selective drug actions, and to reveal novel allosteric binding sites of the $GABA_A$ receptors.

In conclusion, the autoradiographic procedure revealing the GIS-[^{35}S]TBPS binding can be used to image extrasynaptic $GABA_A$ receptors. In these receptors, GABA possibly acts only as a partial agonist. The method can generally be used to image and quantify brain regional alterations in the extrasynaptic $GABA_A$ergic system in various animal models of neurological and psychiatric diseases. In addition, it can be used in reveal novel allosteric interactions in the receptor complex, and thus in the search for novel $GABA_A$ receptor subtype-selective drugs.

ACKNOWLEDGEMENTS

This thesis work was carried out in the Institute of Biomedicine, Department of Pharmacology and Clinical Pharmacology, at the University of Turku, and in the Institute of Biomedicine, Pharmacology, at the University of Helsinki during the years 1998-2004. I am grateful to the heads of the departments, Professors Esa Korpi and Mika Scheinin, for allowing me to use the departments' facilities. I also wish to thank Professor Olli Lassila, director of the Turku Graduate School of Biomedical Sciences, for education and support.

I have had the tremendous privilege to have Professor Esa Korpi as my supervisor. Esa's true devotion to brain research and neuropharmacology has impressed me deeply. Devotion combined with exceptional intelligence and vast experience in conducting sound experiments makes Esa an ideal supervisor, and neuroscientist, too. Esa has encouraged me to learn various techniques and try new things without hesitation. I think this has been essential for the advancement of the project. Most valuably, Esa has always had time for me when it comes to new results. One cannot fake the enthusiastic grin he has on his face when one has something novel to report. I sincerely thank Esa for his guidance and inspiration during all these years.

I am grateful to Professor Pertti Panula for being the other member of my Supervisory Committee. Pertti has always offered his help, his enormous knowledge, and his laboratory facilities when needed. It has been very instructive to observe how a firm neuroscientist works.

During the thesis work, I twice had the great chance to visit the laboratory of Professor Hartmut Lüddens, University of Mainz, Germany. Those two months taught me a great deal in scientific terms. Hartmut's overwhelming knowledge of molecular biology of the $GABA_A$ receptors and his contribution to the planning and executing of the experiments, were indispensable for the thesis project. I wish to express my deepest gratitude to Raymonde and Toni Picard for their friendship, and for taking care of that lone blond Finn during my visits.

The major part of the project was based on the genetically engineered mouse lines. For those I am very grateful to Professors Gregg Homanics and Bernhard Lüscher, and Dr William Wisden. Also other scientific collaborators Professors Werner Sieghart and Jari Tiihonen, and Drs Robert Mihalek, Waltraud Ogris, Terttu Särkioja and Erkki Tupala, and Salla Mansikkamäki are gratefully acknowledged for their contributions.

Professor Bryndis Birnir and Docent Petteri Piepponen are gratefully acknowledged for their review and constructive criticism of the thesis manuscript.

I thank all the personnel of the Department of Pharmacology and Clinical Pharmacology at the University of Turku for help, and for creating an inspiring atmosphere. Especially, I wish to thank Professor Markku Koulu, Renate Hakkarainen, Paula Heinonen, Ulla Hurme, Matti Karvonen, Sirkka Kiiskilä, Janne Lähdesmäki, Tuire Olli-Lähdesmäki, Sanna Palovaara, Eriika Savontaus, Anja Similä, Jukka Tuohimaa and Hanna Tuominen. Special thanks to Jori Ruuskanen for sharing the room during the Neuroscience meetings, and for playing the air-guitar on so many occasions.

I thank all the personnel of the Institute of Biomedicine, Pharmacology, at the University of Helsinki for help and encouragement. Especially, I wish to thank Eeva Harju, Esko Kankuri, Juha Ketonen, Kim Lemberg, Saara Merasto, Eero Mervaala, Anna-Kaisa Pere, Pekka Rauhala, Ilkka Reenilä and Antti Väänänen.

Being a member of Professor Korpi's research group has offered good surroundings for obtaining a scientific education. This is due to the diverse educational backgrounds of the group members. It is easy to predict a successful future to this bunch of talented people. Sincere thanks to Docent Mikko Uusi-Oukari, who taught me my hands-on molecular

biology, and, at least equally importantly, good laboratory practice. I wish to thank Drs Tommi Möykkynen, Sanna Soini and Holger Rabe (University of Mainz) for teaching me electrophysiology. Dr Olga Vekovischeva is gratefully acknowledged for introducing me to the fascinating world of behavioral studies. To Eija Lehtovirta and Aira Säisä I owe my deepest gratitude for providing me with their excellent technical assistance. Dr Anni-Maija Lindén is acknowledged for valuable advice during preparation of the thesis manuscript. I wish to express my sincere thanks to all the other present and former group members, and especially Teemu Aitta-Aho, Anu Heikkilä, Jari Heikkilä, Anne Heikkinen, Irma Holopainen, Aapo Honkanen, Kimmo Ingman, Maarit Juusti, Hanna Laurén, Sirpa Lehti-Koivunen, Elli Leppä, Francisco López-Picón and Martin Ranna.

Thanks are due to the friends from the Turku Medical School. Together we have had good times, and carried out many rather interesting clinical experiments in facilitating $GABA_A$ergic inhibition by self-administration of neuroactive substances. I guess the results of these experiments will never be published. Still, it has been important to have you there. Sincere thanks to the guys from Viherlaakso and Tapiola high school; I greatly appreciate the friendship we have had during all these years.

Thanks to my mother Liisa for your unconditional love. You have always helped me in my efforts and choices, and taken care of so many things during these years. Thanks to my father Juha; I guess the inspiration for science comes from you. You have always supported me on this road. Thanks also to Monika and Enna. I wish to thank my big brother Jonne for your trust and stand-by. Sincere thanks to Wera's family, Tanja, Asko, Kallu, Irina, Matti and Olga, for your continuous aid and belief in me.

The biggest thanks are devoted to my beloved wife Wera. After all, it is you who have paid the price for the long office hours. I am deeply grateful for your patience and support, which were, unfortunately, every now and then tested. I look eagerly to the future, since life with you is so exciting: dreaming, learning and doing. Let's see what we can reach together.

Financial support for the project was provided by the Finnish Medical Association Duodecim, the Academy of Finland, the Turku University Foundation, the Emil Aaltonen Foundation, the Lundbeck Foundation, the Finnish-Norwegian Medical Foundation, the German Academic Exchange Service, and the Drug Research Foundation.

Helsinki, October 2004

Saku Sinkkonen

REFERENCES

Agey MW, Dunn SM (1989) Kinetics of [^{3}H]muscimol binding to the $GABA_A$ receptor in bovine brain membranes. Biochemistry 28:4200-4208.

Amin J, Weiss DS (1993) $GABA_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. Nature 366:565-569.

Atkinson AE, Bermudez I, Darlison MG, Barnard EA, Earley FG, Possee RD, Beadle DJ, King LA (1992) Assembly of functional $GABA_A$ receptors in insect cells using baculovirus expression vectors. Neuroreport 3:597-600.

Attwell D, Barbour B, Szatkowski M (1993) Nonvesicular release of neurotransmitter. Neuron 11:401-407.

Avoli M, D'Antuono M, Louvel J, Kohling R, Biagini G, Pumain R, D'Arcangelo G, Tancredi V (2002) Network and pharmacological mechanisms leading to epileptiform synchronization in the limbic system in vitro. Prog Neurobiol 68:167-207.

Baer K, Essrich C, Benson JA, Benke D, Bluethmann H, Fritschy JM, Luscher B (1999) Postsynaptic clustering of γ-aminobutyric acid type A receptors by the γ3 subunit *in vivo*. Proc Natl Acad Sci U S A 96:12860-12865.

Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF, Orser BA (2001) Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by γ-aminobutyric $acid_A$ receptors in hippocampal neurons. Mol Pharmacol 59:814-824.

Baude A, Sequier J-M, McKernan RM, Olivier KR, Somogyi P (1992) Differential subcellular distribution of the α6 subunit versus the α1 and β2/3 subunits of the $GABA_A$/benzodiazepine receptor complex in granule cells of the cerebellar cortex. Neuroscience 51:739-748.

Baulac S, Huberfeld G, Gourfinkel-An I, Mitropoulou G, Beranger A, Prud'homme JF, Baulac M, Brice A, Bruzzone R, LeGuern E (2001) First genetic evidence of $GABA_A$ receptor dysfunction in epilepsy: a mutation in the γ2-subunit gene. Nat Genet 28:46-48.

Baumann SW, Baur R, Sigel E (2001) Subunit arrangement of γ-aminobutyric acid type A receptors. J Biol Chem 276:36275-36280.

Beard S, Hunn A, Wight J (2003) Treatments for spasticity and pain in multiple sclerosis: a systematic review. Health Technol Assess 7:40.

Belelli D, Lambert JJ, Peters JA, Wafford K, Whiting PJ (1997) The interaction of the general anesthetic etomidate with the γ-aminobutyric acid type A receptor is influenced by a single amino acid. Proc Natl Acad Sci U S A 94:11031-11036.

Bencsits E, Ebert V, Tretter V, Sieghart W (1999) A significant part of native γ-aminobutyric $acid_A$ receptors containing α4 subunits do not contain γ or δ subunits. J Biol Chem 274:19613-19616.

Benes FM (1999) Evidence for altered trisynaptic circuitry in schizophrenic hippocampus. Biol Psychiatry 46:589-599.

Benke D, Mertens S, Trzeciak A, Gillessen D, Möhler H (1991) $GABA_A$ receptors display association of γ2-subunit with α1- and β2/3-subunits. J Biol Chem 266:4478-4483.

Benke D, Fritschy J-M, Trzeciak A, Bannwarth W, Mohler H (1994) Distribution, prevalence, and drug binding profile of γ-aminobutyric acid type A receptor subtypes differing in the β-subunit variant. J Biol Chem 269:27100-27107.

Benke D, Michel C, Möhler H (1997) $GABA_A$ receptors containing the α4-subunit: prevalence, distribution, pharmacology, and subunit architecture in situ. J Neurochem 69:806-814.

Berezhnoy D, Nyfeler Y, Gonthier A, Schwob H, Goeldner M, Sigel E (2004) On the benzodiazepine binding pocket in $GABA_A$ receptors. J Biol Chem 279:3160-3168.

Bieda MC, MacIver MB (2004) A Major Role For Tonic $GABA_A$ Conductances In Anesthetic Suppression Of Intrinsic Neuronal Excitability. J Neurophysiol 12:12.

Birnir B, Everitt AB, Gage PW (1994) Characteristics of $GABA_A$ channels in rat dentate gyrus. J Membr Biol 142:93-102.

Birnir B, Everitt AB, Lim MS, Gage PW (2000) Spontaneously opening $GABA_A$ channels in CA1 pyramidal neurones of rat hippocampus. J Membr Biol 174:21-29.

Blair LA, Levitan ES, Marshall J, Dionne VE, Barnard EA (1988) Single subunits of the $GABA_A$ receptor form ion channels with properties of the native receptor. Science 242:577-579.

Bloom FE, Iversen LL (1971) Localizing ^{3}H-GABA in nerve terminals of rat cerebral cortex by electron microscopic autoradiography. Nature 229:628-630.

Boileau AJ, Evers AR, Davis AF, Czajkowski C (1999) Mapping the agonist binding site of the $GABA_A$ receptor: evidence for a β-strand. J Neurosci 19:4847-4854.

Boileau AJ, Baur R, Sharkey LM, Sigel E, Czajkowski C (2002a) The relative amount of cRNA coding for γ2 subunits affects stimulation by benzodiazepines in $GABA_A$ receptors expressed in *Xenopus* oocytes. Neuropharmacology 43:695-700.

Boileau AJ, Newell JG, Czajkowski C (2002b) $GABA_A$ receptor β2 Tyr97 and Leu99 line the GABA-binding site. Insights into mechanisms of agonist and antagonist actions. J Biol Chem 277:2931-2937.

Bollan K, King D, Robertson LA, Brown K, Taylor PM, Moss SJ, Connolly CN (2003) $GABA_A$ receptor composition is determined by distinct assembly signals within α and β subunits. J Biol Chem 278:4747-4755.

Bonetti EP, Burkard WP, Gabl M, Hunkeler W, Lorez H-P, Martin JR, Möhler H, Osterrieder W, Pieri L, Polc P, Richards JG, Schaffner R, Scherschlicht R, Schoch P, Haefely WE (1988) Ro 15-4513: Partial inverse agonism at the BZR and interaction with ethanol. Pharmacol Biochem Behav 31:733-749.

Bonnert TP, McKernan RM, Farrar S, le Bourdellès B, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJ, Brown N, Wafford KA, Whiting PJ (1999) θ, a novel γ-aminobutyric acid type A receptor subunit. Proc Natl Acad Sci U S A 96:9891-9896.

Borden LA, Murali Dhar TG, Smith KE, Weinshank RL, Branchek TA, Gluchowski C (1994) Tiagabine, SK&F 89976-A, CI-966, and NNC-711 are selective for the cloned GABA transporter GAT-1. Eur J Pharmacol 269:219-224.

Borden LA (1996) GABA transporter heterogeneity: pharmacology and cellular localization. Neurochem Int 29:335-356.

Bormann J, Hamill OP, Sakmann B (1987) Mechanism of anion permeation through channels gated by glycine and γ-aminobutyric acid in mouse cultured spinal neurones. J Physiol 385:243-286.

Bormann J (2000) The 'ABC' of GABA receptors. Trends Pharmacol Sci 21:16-19.

Brambilla P, Perez J, Barale F, Schettini G, Soares JC (2003) GABAergic dysfunction in mood disorders. Mol Psychiatry 8:721-737.

Bremner JD, Innis RB, Southwick SM, Staib L, Zoghbi S, Charney DS (2000a) Decreased benzodiazepine receptor binding in prefrontal cortex in combat-related posttraumatic stress disorder. Am J Psychiatry 157:1120-1126.

Bremner JD, Innis RB, White T, Fujita M, Silbersweig D, Goddard AW, Staib L, Stern E, Cappiello A, Woods S, Baldwin R, Charney DS (2000b) SPECT [I-123]iomazenil measurement of the benzodiazepine receptor in panic disorder. Biol Psychiatry 47:96-106.

Brickley SG, Cull-Candy SG, Farrant M (1996) Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of $GABA_A$ receptors. J Physiol 497:753-759.

Brickley SG, Cull-Candy SG, Farrant M (1999) Single-channel properties of synaptic and extrasynaptic $GABA_A$ receptors suggest differential targeting of receptor subtypes. J Neurosci 19:2960-2973.

Brickley SG, Revilla V, Cull-Candy SG, Wisden W, Farrant M (2001) Adaptive regulation of neuronal excitability by a voltage-independent potassium conductance. Nature 409:88-92.

Brooks-Kayal AR, Shumate MD, Jin H, Rikhter TY, Coulter DA (1998) Selective changes in single cell $GABA_A$ receptor subunit expression and function in temporal lobe epilepsy. Nat Med 4:1166-1172.

Brown N, Kerby J, Bonnert TP, Whiting PJ, Wafford KA (2002) Pharmacological characterization of a novel cell line expressing human $\alpha4\beta3\delta$ $GABA_A$ receptors. Br J Pharmacol 136:965-974.

Browning RA, Nelson DK (1986) Modification of electroshock and pentylenetetrazol seizure patterns in rats after precollicular transections. Exp Neurol 93:546-556.

Brünig I, Scotti E, Sidler C, Fritschy JM (2002) Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. J Comp Neurol 443:43-55.

Buhl EH, Otis TS, Mody I (1996) Zinc-induced collapse of augmented inhibition by GABA in a temporal lobe epilepsy model. Science 271:369-373.

Buhr A, Baur R, Sigel E (1997) Subttle changes in residue 77 of the γ subunit of $\alpha1\beta2\gamma2$ $GABA_A$ receptors drastically alter the affinity for ligands of the benzodiazepine binding site. J Biol Chem 272:11799-11804.

Buhr A, Sigel E (1997) A point mutation in the γ2 subunit of γ-aminobutyric acid type A receptors results in altered benzodiazepine binding site specificity. Proc Natl Acad Sci U S A 94:8824-8829.

Buhr A, Wagner C, Fuchs K, Sieghart W, Sigel E (2001) Two novel residues in M2 of the γ-aminobutyric acid type A receptor affecting gating by GABA and picrotoxin affinity. J Biol Chem 276:7775-7781.

Buxbaum JD, Silverman JM, Smith CJ, Greenberg DA, Kilifarski M, Reichert J, Cook EH, Jr., Fang Y, Song CY, Vitale R (2002) Association between a *GABRB3* polymorphism and autism. Mol Psychiatry 7:311-316.

Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing γ-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 101:3662-3667.

Caroni P (1997) Overexpression of growth-associated proteins in the neurons of adult transgenic mice. J Neurosci Methods 71:3-9.

Caruncho HJ, Costa E (1994) Double-immunolabelling analysis of $GABA_A$ receptor subunits in label-fracture replicas of cultured rat cerebellar granule cells. Receptors Channels 2:143-153.

Cavalla D, Neff NH (1985) Photoaffinity labeling of the $GABA_A$ receptor with [^{3}H]muscimol. J Neurochem 44:916-921.

Chadderton P, Margrie TW, Häusser M (2004) Integration of quanta in cerebellar granule cells during sensory processing. Nature 428:856-860.

Chang Y, Wang R, Barot S, Weiss DS (1996) Stoichiometry of a recombinant $GABA_A$ receptor. J Neurosci 16:5415-5424.

Chang Y, Ghansah E, Chen Y, Ye J, Weiss DS (2002) Desensitization mechanism of GABA receptors revealed by single oocyte binding and receptor function. J Neurosci 22:7982-7990.

Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7:2745-2752.

Cherubini E, Gaiarsa JL, Ben-Ari Y (1991) GABA: an excitatory transmitter in early postnatal life. Trends Neurosci 14:515-519.

Clements JD (1996) Transmitter timecourse in the synaptic cleft: its role in central synaptic function. Trends Neurosci 19:163-171.

Cobb SR, Buhl EH, Halasy K, Paulsen O, Somogyi P (1995) Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. Nature 378:75-78.

Connolly CN, Krishek BJ, McDonald BJ, Smart TG, Moss SJ (1996) Assembly and cell surface expression of heteromeric and homomeric γ-aminobutyric acid type A receptors. J Biol Chem 271:89-96.

Coplan JD, Lydiard RB (1998) Brain circuits in panic disorder. Biol Psychiatry 44:1264-1276.

Cossette P, Liu L, Brisebois K, Dong H, Lortie A, Vanasse M, Saint-Hilaire JM, Carmant L, Verner A, Lu WY, Wang YT, Rouleau GA (2002) Mutation of *GABRA1* in an autosomal dominant form of juvenile myoclonic epilepsy. Nat Genet 31:184-189.

Couve A, Moss SJ, Pangalos MN (2000) $GABA_B$ receptors: a new paradigm in G protein signaling. Mol Cell Neurosci 16:296-312.

Crawley J, Goodwin FK (1980) Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav 13:167-170.

Crestani F, Lorez M, Baer K, Essrich C, Benke D, Laurent JP, Belzung C, Fritschy JM, Lüscher B, Möhler H (1999) Decreased $GABA_A$-receptor clustering results in enhanced anxiety and a bias for threat cues. Nat Neurosci 2:833-839.

Crestani F, Martin JR, Möhler H, Rudolph U (2000) Mechanism of action of the hypnotic zolpidem *in vivo*. Br J Pharmacol 131:1251-1254.

Crestani F, Löw K, Keist R, Mandelli M, Möhler H, Rudolph U (2001) Molecular targets for the myorelaxant action of diazepam. Mol Pharmacol 59:442-445.

Crestani F, Keist R, Fritschy JM, Benke D, Vogt K, Prut L, Blüthmann H, Möhler H, Rudolph U (2002a) Trace fear conditioning involves hippocampal $\alpha5$ $GABA_A$ receptors. Proc Natl Acad Sci U S A 99:8980-8985.

Crestani F, Assandri R, Täuber M, Martin JR, Rudolph U (2002b) Contribution of the $\alpha1$-$GABA_A$ receptor subtype to the pharmacological actions of benzodiazepine site inverse agonists. Neuropharmacology 43:679-684.

Cronin J, Obenaus A, Houser CR, Dudek FE (1992) Electrophysiology of dentate granule cells after kainate-induced synaptic reorganization of the mossy fibers. Brain Res 573:305-310.

Culbert PA, Chan S, Wearring AV, Chamberlain MJ, Hunter DH (1993) A potential imaging agent for the $GABA_A$ receptor: 4-t-butyl-1-(4-[123I]iodophenyl)-2,6,7-trioxabicyclo-[2.2.2]octane. Nucl Med Biol 20:469-475.

Curtis DR, Duggan AW, Felix D (1970) GABA and inhibition of Deiters' neurones. Brain Res 23:117-120.

Dalby NO (2000) GABA-level increasing and anticonvulsant effects of three different GABA uptake inhibitors. Neuropharmacology 39:2399-2407.

Davies CH, Starkey SJ, Pozza MF, Collingridge GL (1991) $GABA_B$ autoreceptors regulate the induction of LTP. Nature 349:609-611.

Davies M (2003) The role of $GABA_A$ receptors in mediating the effects of alcohol in the central nervous system. J Psychiatry Neurosci 28:263-274.

Davies PA, Hanna MC, Hales TG, Kirkness EF (1997) Insensitivity to anaesthetic agents conferred by a class of $GABA_A$ receptor subunit. Nature 385:820-823.

DeLorey TM, Olsen RW (1999) GABA and epileptogenesis: comparing gabrb3 gene-deficient mice with Angelman syndrome in man. Epilepsy Res 36:123-132.

Dibbens LM, Feng HJ, Richards MC, Harkin LA, Hodgson BL, Scott D, Jenkins M, Petrou S, Sutherland GR, Scheffer IE, Berkovic SF, Macdonald RL, Mulley JC (2004) *GABRD* encoding a protein for extra or peri-synaptic $GABA_A$ receptors is a susceptibility locus for generalised epilepsies. Hum Mol Genet 28:28.

Dichter MA (1997) Basic mechanisms of epilepsy: targets for therapeutic intervention. Epilepsia 38:S2-6.

Dillon GH, Im WB, Carter DB, McKinley DD (1995) Enhancement by GABA of the association rate of picrotoxin and *tert*-butylbicyclophosphorothionate to the rat cloned $\alpha1\beta2\gamma2$ $GABA_A$ receptor subtype. Br J Pharmacol 115:539-545.

Dingledine R, Korn SJ (1985) γ-aminobutyric acid uptake and the termination of inhibitory synaptic potentials in the rat hippocampal slice. J Physiol 366:387-409.

Doherty J, Dingledine R (2001) Reduced excitatory drive onto interneurons in the dentate gyrus after status epilepticus. J Neurosci 21:2048-2057.

Draguhn A, Heinemann U (1996) Different mechanisms regulate IPSC kinetics in early postnatal and juvenile hippocampal granule cells. J Neurophysiol 76:3983-3993.

Drew CA, Johnston GA, Weatherby RP (1984) Bicuculline-insensitive GABA receptors: studies on the binding of (-)-baclofen to rat cerebellar membranes. Neurosci Lett 52:317-321.

Ducic I, Caruncho HJ, Zhu WJ, Vicini S, Costa E (1995) γ-Aminobutyric acid gating of Cl^- channels in recombinant $GABA_A$ receptors. J Pharmacol Exp Ther 272:438-445.

Dunn SMJ, Davies M, Muntoni AL, Lambert JJ (1999) Mutagenesis of the rat $\alpha1$ subunit of the γ-aminobutyric $acid_A$ receptor reveals the importance of residue 101 in determining the allosteric effects of benzodiazepine site ligands. Mol Pharmacol 56:768-774.

Ebert B, Wafford KA, Whiting PJ, Krogsgaard-Larsen P, Kemp JA (1994) Molecular pharmacology of γ-aminobutyric acid type A receptor agonists and partial agonists in oocytes injected with different α, β, and γ receptor subunit combinations. Mol Pharmacol 46:957-963.

Ebert B, Thompson SA, Saounatsou K, McKernan R, Krogsgaard-Larsen P, Wafford KA (1997) Differences in agonist/antagonist binding affinity and receptor transduction using recombinant human γ-aminobutyric acid type A receptors. Mol Pharmacol 52:1150-1156.

Edgar PP, Schwartz RD (1990) Localization and characterization of ^{35}S-t- butylbicyclophosphorothionate binding in rat brain: an autoradiographic study. J Neurosci 10:603-612.

Eghbali M, Curmi JP, Birnir B, Gage PW (1997) Hippocampal $GABA_A$ channel conductance increased by diazepam. Nature 388:71-75.

Elster L, Banke T, Kristiansen U, Schousboe A, Wahl P (1998) Functional properties of glycine receptors expressed in primary cultures of mouse cerebellar granule cells. Neuroscience 84:519-528.

Enz R, Cutting GR (1999) $GABA_C$ receptor ρ subunits are heterogeneously expressed in the human CNS and form homo- and heterooligomers with distinct physical properties. Eur J Neurosci 11:41-50.

Essrich C, Lorez M, Benson JA, Fritschy JM, Lüscher B (1998) Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the $\gamma2$ subunit and gephyrin. Nat Neurosci 1:563-571.

Everitt AB, Luu T, Cromer B, Tierney ML, Birnir B, Olsen RW, Gage PW (2004) Conductance of recombinant $GABA_A$ channels is increased in cells co-expressing $GABA_A$ receptor-associated protein. J Biol Chem 279:21701-21706.

Evoniuk G, Skolnick P (1988) Picrate and niflumate block anion modulation of radioligand binding to the γ-aminobutyric acid/benzodiazepine receptor complex. Mol Pharmacol 34:837-842.

Farrar SJ, Whiting PJ, Bonnert TP, McKernan RM (1999) Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. J Biol Chem 274:10100-10104.

Faulhaber J, Steiger A, Lancel M (1997) The $GABA_A$ agonist THIP produces slow wave sleep and reduces spindling activity in NREM sleep in humans. Psychopharmacology (Berl) 130:285-291.

Fiez JA (1996) Cerebellar contributions to cognition. Neuron 16:13-15.

Fink-Jensen A, Suzdak PD, Swedberg MD, Judge ME, Hansen L, Nielsen PG (1992) The γ-aminobutyric acid (GABA) uptake inhibitor, tiagabine, increases extracellular brain levels of GABA in awake rats. Eur J Pharmacol 220:197-201.

Fisher JL, Zhang J, Macdonald RL (1997) The role of α1 and α6 subtype amino-terminal domains in allosteric regulation of γ-aminobutyric $acid_A$ receptors. Mol Pharmacol 52:714-724.

Fisher JL (2004) A mutation in the $GABA_A$ receptor α1 subunit linked to human epilepsy affects channel gating properties. Neuropharmacology 46:629-637.

Frahm C, Engel D, Draguhn A (2001) Efficacy of background GABA uptake in rat hippocampal slices. Neuroreport 12:1593-1596.

Freund TF (2003) Interneuron Diversity series: Rhythm and mood in perisomatic inhibition. Trends Neurosci 26:489-495.

Fritschy JM, Benke D, Mertens S, Oertel WH, Bachi T, Möhler H (1992) Five subtypes of type A γ-aminobutyric acid receptors identified in neurons by double and triple immunofluorescence staining with subunit-specific antibodies. Proc Natl Acad Sci USA 89:6726-6730.

Fritschy JM, Möhler H (1995) $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. J Comp Neurol 359:154-194.

Fritschy JM, Weinmann O, Wenzel A, Benke D (1998) Synapse-specific localization of NMDA and $GABA_A$ receptor subunits revealed by antigen-retrieval immunohistochemistry. J Comp Neurol 390:194-210.

Fuchs K, Zezula J, Slany A, Sieghart W (1995) Endogenous [^{3}H]flunitrazepam binding in human embryonic kidney cell line 293. Eur J Pharmacol 289:87-95.

Gale K (1992) Subcortical structures and pathways involved in convulsive seizure generation. J Clin Neurophysiol 9:264-277.

Garrett KM, Blume AJ, Abel MS (1989) Effect of halide ions on *t*-[^{35}S]butylbicyclophosphorothionate binding. J Neurochem 53:935-939.

Gaspary HL, Wang W, Richerson GB (1998) Carrier-mediated GABA release activates GABA receptors on hippocampal neurons. J Neurophysiol 80:270-281.

Gee KW, Wamsley JK, Yamamura HI (1983) Light microscopic autoradiographic identification of picrotoxin/barbiturate binding sites in rat brain with [^{35}S]t-butyl-bicyclophosphothionate. Eur J Pharmacol 89:323-324.

Gee KW, Lawrence LJ, Yamamura HI (1986) Modulation of the chloride ionophore by benzodiazepine receptor ligands: influence of γ-aminobutyric acid and ligand efficacy. Mol Pharmacol 30:218-225.

Gerlai R (1998) A new continuous alternation task in T-maze detects hippocampal dysfunction in mice. A strain comparison and lesion study. Behav Brain Res 95:91-101.

Gibbs JW 3rd, Shumate MD, Coulter DA (1997) Differential epilepsy-associated alterations in postsynaptic $GABA_A$ receptor function in dentate granule and CA1 neurons. J Neurophysiol 77:1924-1938.

Goddard AW, Mason GF, Almai A, Rothman DL, Behar KL, Petroff OA, Charney DS, Krystal JH (2001) Reductions in occipital cortex GABA levels in panic disorder detected with ^{1}H-magnetic resonance spectroscopy. Arch Gen Psychiatry 58:556-561.

Gorman JM (2003) Treating generalized anxiety disorder. J Clin Psychiatry 64:24-29.

Gorrie GH, Vallis Y, Stephenson A, Whitfield J, Browning B, Smart TG, Moss SJ (1997) Assembly of $GABA_A$ receptors composed of α1 and β2 subunits in both cultured neurons and fibroblasts. J Neurosci 17:6587-6596.

Green WN, Millar NS (1995) Ion-channel assembly. Trends Neurosci 18:280-287.

Gulinello M, Gong QH, Smith SS (2003) Progesterone withdrawal increases the anxiolytic actions of gaboxadol: role of α4βδ $GABA_A$ receptors. Neuroreport 14:43-46.

Gurley D, Amin J, Ross PC, Weiss DS, White G (1995) Point mutations in the M2 region of the α, β, or γ subunit of the $GABA_A$ channel that abolish block by picrotoxin. Receptors Channels 3:13-20.

Gutiérrez A, Khan ZU, De Blas AL (1994) Immunocytochemical localization of γ2 short and γ2 long subunits of the $GABA_A$ receptor in the rat brain. J Neurosci 14:7168-7179.

Günther U, Benson J, Benke D, Fritschy JM, Reyes G, Knoflach F, Crestani F, Aguzzi A, Arigoni M, Lang Y, Bluethmann H, Möhler H, Lüscher B (1995) Benzodiazepine-insensitive mice generated by targeted disruption of the γ2 subunit gene of γ-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 92:7749-7753.

Haas KF, Macdonald RL (1999) $GABA_A$ receptor subunit γ2 and δ subtypes confer unique kinetic properties on recombinant $GABA_A$ receptor currents in mouse fibroblasts. J Physiol (Lond) 514:27-45.

Hales TG, Tyndale RF (1994) Few cell lines with $GABA_A$ mRNAs have functional receptors. J Neurosci 14:5429-5436.

Hamann M, Desarmenien M, Vanderheyden P, Piguet P, Feltz P (1990) Electrophysiological study of *tert*-butylbicyclophosphorothionate-induced block of spontaneous chloride channels. Mol Pharmacol 37:578-582.

Hamann M, Rossi DJ, Attwell D (2002) Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. Neuron 33:625-633.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp technique for high resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391:85-100.

Hamilton BJ, Lennon DJ, Im HK, Im WB, Seeburg PH, Carter DB (1993) Stable expression of cloned rat $GABA_A$ receptor subunits in a human kidney cell line. Neurosci Lett 153:206-209.

Hattori K, Oomura Y, Akaike N (1986) Diazepam action on gamma-aminobutyric acid-activated chloride currents in internally perfused frog sensory neurons. Cell Mol Neurobiol 6:307-323.

Havoundjian H, Paul SM, Skolnick P (1986) The permeability of γ-aminobutyric acid-gated chloride channels is described by the binding of a "cage" convulsant, *t*-butylbicyclophosphoro[^{35}S]thionate. Proc Natl Acad Sci U S A 83:9241-9244.

Heaulme M, Chambon JP, Leyris R, Molimard JC, Wermuth CG, Biziere K (1986) Biochemical characterization of the interaction of three pyridazinyl-GABA derivatives with the $GABA_A$ receptor site. Brain Res 384:224-231.

Hedblom E, Kirkness EF (1997) A novel class of $GABA_A$ receptor subunit in tissues of the reproductive system. J Biol Chem 272:15346-15350.

Herb A, Wisden W, Lüddens H, Puia G, Vicini S, Seeburg PH (1992) The third γ subunit of the γ-aminobutyric acid type A receptor family. Proc Natl Acad Sci U S A 89:1433-1437.

Hevers W, Lüddens H (1998) The diversity of $GABA_A$ receptors. Pharmacological and electrophysiological properties of $GABA_A$ channel subtypes. Mol Neurobiol 18:35-86.

Hevers W, Korpi ER, Lüddens H (2000) Assembly of functional α6β3γ2δ $GABA_A$ receptors in vitro. Neuroreport 11:4103-4106.

Holopainen IE, Metsähonkala EL, Kokkonen H, Parkkola RK, Manner TE, Någren K, Korpi ER (2001) Decreased binding of [^{11}C]flumazenil in Angelman syndrome patients with $GABA_A$ receptor β3 subunit deletions. Ann Neurol 49:110-113.

Homanics GE, DeLorey TM, Firestone LL, Quinlan JJ, Handforth A, Harrison NL, Krasowski MD, Rick CE, Korpi ER, Mäkelä R, Brilliant MH, Hagiwara N, Ferguson C, Snyder K, Olsen RW (1997a) Mice devoid of γ-aminobutyrate type A receptor β3 subunit have epilepsy, cleft palate, and hypersensitive behavior. Proc Natl Acad Sci U S A 94:4143-4148.

Homanics GE, Ferguson C, Quinlan JJ, Daggett J, Snyder K, Lagenaur C, Mi Z-P, Wang X-H, Grayson DR, Firestone LL (1997b) Gene knockout of the α6 subunit of the γ-aminobutyric acid type A receptor: lack of effect on responses to ethanol, pentobarbital, and general anesthetics. Mol Pharmacol 51:588-596.

Horenstein J, Wagner DA, Czajkowski C, Akabas MH (2001) Protein mobility and GABA-induced conformational changes in $GABA_A$ receptor pore-lining M2 segment. Nat Neurosci 4:477-485.

Horikoshi T, Asanuma A, Yanagisawa K, Anzai K, Goto S (1988) Taurine and β-alanine act on both GABA and glycine receptors in *Xenopus* oocyte injected with mouse brain messenger RNA. Brain Res 464:97-105.

Horne AL, Harkness PC, Hadingham KL, Whiting P, Kemp JA (1993) The influence of the γ2L subunit on the modulation of responses to $GABA_A$ receptor activation. Br J Pharmacol 108:711-716.

Huang J, Casida JE (1996) Characterization of [^{3}H]ethynylbicycloorthobenzoate ([^{3}H]EBOB) binding and the action of insecticides on the γ-aminobutyric acid-gated chloride channel in cultured cerebellar granule neurons. J Pharmacol Exp Ther 279:1191-1196.

Hunkeler W, Möhler H, Pieri L, Polc P, Bonetti EP, Cumin R, Schaffner R, Haefely W (1981) Selective antagonists of benzodiazepines. Nature 290:514-516.

Häusser M, Clark BA (1997) Tonic synaptic inhibition modulates neuronal output pattern and spatiotemporal synaptic integration. Neuron 19:665-678.

Hösli E, Krogsgaard-Larsen P, Hösli L (1985) Autoradiographic localization of binding sites for the γ-aminobutyric acid analogues 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP)isoguvacine and baclofen on cultured neurons of rat cerebellum and spinal cord. Neurosci Lett 61:153-157.

Im WB, Blakeman DP (1991) Correlation between γ-aminobutyric $acid_A$ receptor ligand-induced changes in *t*-butylbicyclophosphoro[^{35}S]thionate binding and $^{36}Cl^-$ uptake in rat cerebrocortical membranes. Mol Pharmacol 39:394-398.

Im WB, Pregenzer JF, Thomsen DR (1994) Effects of GABA and various allosteric ligands on TBPS binding to cloned rat $GABA_A$ receptor subtypes. Br J Pharmacol 112:1025-1030.

Im WB, Pregenzer JF, Binder JA, Dillon GH, Alberts GL (1995a) Chloride channel expression with the tandem construct of α6-β2 $GABA_A$ receptor subunit requires a monomeric subunit of α6 or γ2. J Biol Chem 270:26063-26066.

Im HK, Im WB, Carter DB, McKinley DD (1995b) Interaction of β-carboline inverse agonists for the benzodiazepine site with another site on $GABA_A$ receptors. Br J Pharmacol 114:1040-1044.

Ito M (1972) Neural design of the cerebellar motor control system. Brain Res 40:81-84.

Ito M (2002) Historical review of the significance of the cerebellum and the role of Purkinje cells in motor learning. Ann N Y Acad Sci 978:273-288.

Iversen LL, Bloom FE (1972) Studies of the uptake of ^{3}H-gaba and [^{3}H]glycine in slices and homogenates of rat brain and spinal cord by electron microscopic autoradiography. Brain Res 41:131-143.

Jackel C, Kleinz R, Mäkelä R, Hevers W, Jezequel S, Korpi ER, Lüddens H (1998) The main determinant of furosemide inhibition on $GABA_A$ receptors is located close to the first transmembrane domain. Eur J Pharmacol 357:251-256.

Jaffe JH, Bloor R, Crome I, Carr M, Alam F, Simmons A, Meyer RE (2004) A postmarketing study of relative abuse liability of hypnotic sedative drugs. Addiction 99:165-173.

Jechlinger M, Pelz R, Tretter V, Klausberger T, Sieghart W (1998) Subunit composition and quantitative importance of hetero-oligomeric receptors: $GABA_A$ receptors containing α6 subunits. J Neurosci 18:2449-2457.

Jensen K, Chiu CS, Sokolova I, Lester HA, Mody I (2003) GABA transporter-1 (GAT1)-deficient mice: differential tonic activation of $GABA_A$ versus $GABA_B$ receptors in the hippocampus. J Neurophysiol 90:2690-2701.

Jin P, Zhang J, Rowe-Teeter C, Yang J, Stuve LL, Fu GK (2004a) Cloning and characterization of a $GABA_A$ receptor γ2 subunit variant. J Biol Chem 279:1408-1414.

Jin P, Walther D, Zhang J, Rowe-Teeter C, Fu GK (2004b) Serine 171, a conserved residue in the γ-aminobutyric acid type A ($GABA_A$) receptor γ2 subunit, mediates subunit interaction and cell surface localization. J Biol Chem 279:14179-14183.

Jones MV, Westbrook GL (1996) The impact of receptor desensitization on fast synaptic transmission. Trends Neurosci 19:96-101.

Jones A, Korpi ER, McKernan RM, Pelz R, Nusser Z, Mäkelä R, Mellor JR, Pollard S, Bahn S, Stephenson FA, Randall AD, Sieghart W, Somogyi P, Smith AJ, Wisden W (1997) Ligand-gated ion channel subunit partnerships: $GABA_A$ receptor α6 subunit gene inactivation inhibits δ subunit expression. J Neurosci 17:1350-1362.

Jurd R, Arras M, Lambert S, Drexler B, Siegwart R, Crestani F, Zaugg M, Vogt KE, Ledermann B, Antkowiak B, Rudolph U (2003) General anesthetic actions in vivo strongly attenuated by a point mutation in the $GABA_A$ receptor β3 subunit. Faseb J 17:250-252.

Jursky F, Fuchs K, Buhr A, Tretter V, Sigel E, Sieghart W (2000) Identification of amino acid residues of $GABA_A$ receptor subunits contributing to the formation and affinity of the *tert*-butylbicyclophosphorothionate binding site. J Neurochem 74:1310-1316.

Kaila K, Voipio J (1987) Postsynaptic fall in intracellular pH induced by GABA-activated bicarbonate conductance. Nature 330:163-165.

Kaila K (1994) Ionic basis of $GABA_A$ receptor channel function in the nervous system. Prog Neurobiol 42:489-537.

Kaneda M, Farrant M, Cull-Candy SG (1995) Whole-cell and single-channel currents activated by GABA and glycine in granule cells of the rat cerebellum. J Physiol 485:419-435.

Kannenberg K, Baur R, Sigel E (1997) Proteins associated with α1-subunit-containing $GABA_A$ receptors from bovine brain. J Neurochem 68:1352-1360.

Kapur J, Coulter DA (1995) Experimental status epilepticus alters γ-aminobutyric acid type A receptor function in CA1 pyramidal neurons. Ann Neurol 38:893-900.

Kaschka W, Feistel H, Ebert D (1995) Reduced benzodiazepine receptor binding in panic disorders measured by iomazenil SPECT. J Psychiatr Res 29:427-434.

Kellenberger S, Eckenstein S, Baur R, Malherbe P, Buhr A, Sigel E (1996) Subunit stoichiometry of oligomeric membrane proteins: $GABA_A$ receptors isolated by selective immunoprecipitation from the cell surface. Neuropharmacology 35:1403-1411.

Khan ZU, Gutiérrez A, DeBlas AL (1994) The subunit composition of a $GABA_A$/benzodiazepine receptor from rat cerebellum. J Neurochem 63:371-374.

Khan ZU, Gutiérrez A, De Blas AL (1996) The α1 and a6 subunits can coexist in the same cerebellar $GABA_A$ receptor maintaining their individual benzodiazepine-binding specificities. J Neurochem 66:685-691.

Kirkness EF, Fraser CM (1993) A strong promoter element is located between alternative exons of a gene encoding the human γ-aminobutyric acid-type A receptor β3 subunit (GABRB3). J Biol Chem 268:4420-4428.

Kirsch J, Langosch D, Prior P, Littauer UZ, Schmitt B, Betz H (1991) The 93-kDa glycine receptor-associated protein binds to tubulin. J Biol Chem 266:22242-22245.

Kish PE, Fischer-Bovenkerk C, Ueda T (1989) Active transport of γ-aminobutyric acid and glycine into synaptic vesicles. Proc Natl Acad Sci U S A 86:3877-3881.

Kittler JT, Wang J, Connolly CN, Vicini S, Smart TG, Moss SJ (2000) Analysis of $GABA_A$ receptor assembly in mammalian cell lines and hippocampal neurons using γ2 subunit green fluorescent protein chimeras. Mol Cell Neurosci 16:440-452.

Klaukka T (1999) Psyykenlääkkeet sairausvakuutuksen näkökulmasta. In: Psykofarmakologian historia Suomessa (Kähkönen S, Partonen T, eds), pp 79-86: Kustannus Oy Duodecim.

Klausberger T, Fuchs K, Mayer B, Ehya N, Sieghart W (2000) $GABA_A$ receptor assembly. Identification and structure of γ2 sequences forming the intersubunit contacts with α1 and β3 subunits. J Biol Chem 275:8921-8928.

Klausberger T, Sarto I, Ehya N, Fuchs K, Fürtmuller R, Mayer B, Huck S, Sieghart W (2001) Alternate use of distinct intersubunit contacts controls $GABA_A$ receptor assembly and stoichiometry. J Neurosci 21:9124-9133.

Klausberger T, Roberts JD, Somogyi P (2002) Cell type- and input-specific differences in the number and subtypes of synaptic $GABA_A$ receptors in the hippocampus. J Neurosci 22:2513-2521.

Klausberger T, Magill PJ, Márton LF, Roberts JD, Cobden PM, Buzsáki G, Somogyi P (2003) Brain-state- and cell-type-specific firing of hippocampal interneurons in vivo. Nature 421:844-848.

Kneussel M, Brandstätter JH, Laube B, Stahl S, Müller U, Betz H (1999) Loss of postsynaptic $GABA_A$ receptor clustering in gephyrin-deficient mice. J Neurosci 19:9289-9297.

Kneussel M, Brandstätter JH, Gasnier B, Feng G, Sanes JR, Betz H (2001) Gephyrin-independent clustering of postsynaptic $GABA_A$ receptor subtypes. Mol Cell Neurosci 17:973-982.

Knoflach F, Rhyner T, Villa M, Kellenberger S, Drescher U, Malherbe P, Sigel E, Möhler H (1991) The γ3-subunit of the $GABA_A$-receptor confers sensitivity to benzodiazepine receptor ligands. FEBS Lett 293:191-194.

Knoflach F, Benke D, Wang Y, Scheurer L, Lüddens H, Hamilton BJ, Carter DB, Möhler H, Benson JA (1996) Pharmacological modulation of the diazepam-insensitive recombinant γ-aminobutyric $acid_A$ receptors α4β2γ2 and α6β2γ2. Mol Pharmacol 50:1253-1261.

Knowles WD (1992) Normal anatomy and neurophysiology of the hippocampal formation. J Clin Neurophysiol 9:252-263.

Koch M (1999) The neurobiology of startle. Prog Neurobiol 59:107-128.

Kofuji P, Wang JB, Moss SJ, Huganir RL, Burt DR (1991) Generation of two forms of the γ-aminobutyric acid$_A$ receptor γ2-subunit in mice by alternative splicing. J Neurochem 56:713-715.

Korpi ER, Kleingoor C, Kettenmann H, Seeburg PH (1993) Benzodiazepine-induced motor impairment linked to point mutation in cerebellar GABA$_A$ receptor. Nature (Lond) 361:356-359.

Korpi ER, Lüddens H (1993) Regional γ-aminobutyric acid sensitivity of *t*-butylbicyclophosphoro[^{35}S]thionate binding depends on γ-aminobutyric acid$_A$ receptor α subunit. Mol Pharmacol 44:87-92.

Korpi ER, Kuner T, Kristo P, Köhler M, Herb A, Lüddens H, Seeburg PH (1994) Small N-terminal deletion by splicing in cerebellar α6 subunit abolishes GABA$_A$ receptor function. J Neurochem 63:1167-1170.

Korpi ER, Kuner T, Seeburg PH, Lüddens H (1995a) Selective antagonist for the cerebellar granule cell-specific γ-aminobutyric acid type A receptor. Mol Pharmacol 47:283-289.

Korpi ER, Wong G, Lüddens H (1995b) Subtype specificity of γ-aminobutyric acid type A receptor antagonism by clozapine. Naynyn Schmiedeberg's Arch Pharmacol 352:365-373.

Korpi ER, Lüddens H (1997) Furosemide interactions with brain GABA$_A$ receptors. Br J Pharmacol 120:741-748.

Korpi ER, Mattila MJ, Wisden W, Lüddens H (1997) GABA$_A$ receptor subtypes: clinical efficacy and selectivity of benzodiazepine site ligands. Ann Med 29:275-282.

Korpi ER, Gründer G, Lüddens H (2002a) Drug interactions at GABA$_A$ receptors. Prog Neurobiol 67:113-159.

Korpi ER, Mihalek RM, Sinkkonen ST, Hauer B, Hevers W, Homanics GE, Sieghart W, Lüddens H (2002b) Altered receptor subtypes in the forebrain of GABA$_A$ receptor δ subunit-deficient mice: recruitment of γ2 subunits. Neuroscience 109:733-743.

Kosobud AE, Crabbe JC (1990) Genetic correlations among inbred strain sensitivities to convulsions induced by 9 convulsant drugs. Brain Res 526:8-16.

Krishek BJ, Moss SJ, Smart TG (1998) Interaction of H^+ and Zn^{2+} on recombinant and native rat neuronal GABA$_A$ receptors. J Physiol (Lond) 507:639-652.

Kume A, Albin RL (1994) Quantitative autoradiography of 4'-ethynyl-4-*n*-[2,3-$^3H_2$]propylbicycloorthobenzoate binding to the GABA$_A$ receptor complex. Eur J Pharmacol 263:163-173.

Kuner T, Schoepfer R, Korpi ER (1993) Ethanol inhibits glutamate-induced currents in heteromeric NMDA receptor subtypes. Neuroreport 5:297-300.

Lader M (1995) Clinical pharmacology of anxiolytic drugs: past, present and future. Adv Biochem Psychopharmacol 48:135-152.

Lancel M (1999) Role of GABA$_A$ receptors in the regulation of sleep: initial sleep responses to peripherally administered modulators and agonists. Sleep 22:33-42.

Laurie DJ, Wisden W, Seeburg PH (1992) The distribution of thirteen GABA$_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci 12:4151-4172.

Lawrence LJ, Palmer CJ, Gee KW, Wang X, Yamamura HI, Casida JE (1985) *t*-[^{3}H]Butylbicycloorthobenzoate: new radioligand probe for the γ-aminobutyric acid-regulated chloride ionophore. J Neurochem 45:798-804.

Lehmann TN, Gabriel S, Eilers A, Njunting M, Kovacs R, Schulze K, Lanksch WR, Heinemann U (2001) Fluorescent tracer in pilocarpine-treated rats shows widespread aberrant hippocampal neuronal connectivity. Eur J Neurosci 14:83-95.

Leite JF, Cascio M (2001) Structure of ligand-gated ion channels: critical assessment of biochemical data supports novel topology. Mol Cell Neurosci 17:777-792.

Lerma J, Herranz AS, Herreras O, Abraira V, Martin del Rio R (1986) In vivo determination of extracellular concentration of amino acids in the rat hippocampus. A method based on brain dialysis and computerized analysis. Brain Res 384:145-155.

Lewin AH, de Costa BR, Rice KC, Skolnick P (1989) *meta*- and *para*-isothiocyanato-*t*-butylbicycloorthobenzoate: irreversible ligands of the γ-aminobutyric acid-regulated chloride ionophore. Mol Pharmacol 35:189-194.

Li M, De Blas AL (1997) Coexistence of two β subunit isoforms in the same γ-aminobutyric acid type A receptor. J Biol Chem 272:16564-16569.

Liang J, Cagetti E, Olsen RW, Spigelman I (2004) Altered pharmacology of synaptic and extrasynaptic GABA$_A$ receptors on CA1 hippocampal neurons is consistent with subunit changes in a model of alcohol withdrawal and dependence. J Pharmacol Exp Ther 310: 1234-1245.

Lindquist CE, Ebert B, Birnir B (2003) Extrasynaptic GABA$_A$ channels activated by THIP are modulated by diazepam in CA1 pyramidal neurons in the rat brain hippocampal slice. Mol Cell Neurosci 24:250-257.

Liu QY, Schaffner AE, Chang YH, Maric D, Barker JL (2000) Persistent activation of GABA$_A$ receptor/Cl^- channels by astrocyte-derived GABA in cultured embryonic rat hippocampal neurons. J Neurophysiol 84:1392-1403.

Lorez M, Benke D, Lüscher B, Möhler H, Benson JA (2000) Single-channel properties of neuronal GABA$_A$ receptors from mice lacking the γ2 subunit. J Physiol 527:11-31.

Lüddens H, Pritchett DB, Köhler M, Killisch I, Keinänen K, Monyer H, Sprengel R, Seeburg PH (1990) Cerebellar GABA$_A$ receptor selective for a behavioural alcohol antagonist. Nature 346:648-651.

Lüddens H, Killisch I, Seeburg PH (1991) More than one ALPHA variant may exist in a GABA$_A$/benzodiazepine receptor complex. J Receptor Res 11:535-551.

Lüddens H, Seeburg PH, Korpi ER (1994) Impact of β and γ variants on ligand-binding properties of γ-aminobutyric acid type A receptors. Mol Pharmacol 45:810-814.

Lüddens H, Korpi ER (1995) GABA antagonists differentiate between recombinant $GABA_A$/benzodiazepine receptor subtypes. J Neurosci 15:6957-6962.

Lüddens H, Korpi ER (1997) Methods for transient expression of hetero-oligomeric ligand-gated ion channels. Methods in Molecular Biology, ed Challis RAJ, Humana Press, Totowa, New Jersey 83:55-63.

Lüscher B, Keller CA (2004) Regulation of $GABA_A$ receptor trafficking, channel activity, and functional plasticity of inhibitory synapses. Pharmacol Ther 102:195-221.

Lydiard RB (2003) The role of GABA in anxiety disorders. J Clin Psychiatry 64:21-27.

Löw K, Crestani F, Keist R, Benke D, Brünig I, Benson JA, Fritschy JM, Rülicke T, Bluethmann H, Möhler H, Rudolph U (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. Science 290:131-134.

Macdonald R, Barker JL (1978) Benzodiazepines specifically modulate GABA-mediated postsynaptic inhibition in cultured mammalian neurones. Nature 271:563-564.

Macdonald RL, Olsen RW (1994) $GABA_A$ receptor channels. Annu Rev Neurosci 17:569-602.

Madsen SM, Lindeburg T, Følsgård S, Jacobsen E, Sillesen H (1983) Pharmacokinetics of the γ-aminobutyric acid agonist THIP (Gaboxadol) following intramuscular administration to man, with observations in dog. Acta Pharmacol Toxicol (Copenh) 53:353-357.

Maksay G, Simonyi M (1986) Kinetic regulation of convulsant (TBPS) binding by GABAergic agents. Mol Pharmacol 30:321-328.

Maksay G (1996) From kinetics and thermodynamics of $GABA_A$ receptor binding to ionophore function. Neurochem Int 29:361-370.

Maksay G, Korpi ER, Uusi-Oukari M (1998) Bimodal action of furosemide on convulsant [^{3}H]EBOB binding to cerebellar and cortical $GABA_A$ receptors. Neurochem Int 33:353-358.

Malcangio M, Bowery NG (1996) GABA and its receptors in the spinal cord. Trends Pharmacol Sci 17:457-462.

Malizia AL, Cunningham VJ, Bell CJ, Liddle PF, Jones T, Nutt DJ (1998) Decreased brain $GABA_A$-benzodiazepine receptor binding in panic disorder: preliminary results from a quantitative PET study. Arch Gen Psychiatry 55:715-720.

Malminiemi O, Korpi ER (1989) Diazepam-insensitive [^{3}H]Ro 15-4513 binding in intact cultured cerebellar granule cells. Eur J Pharmacol 169:53-60.

Martin JR, Pieri L, Bonetti EP, Schaffner R, Burkard WP, Cumin R, Haefely WE (1988) Ro 16-6028: a novel anxiolytic acting as a partial agonist at the benzodiazepine receptor. Pharmacopsychiatry 21:360-362.

Martínez-Torres A, Miledi R (2004) Expression of functional receptors by the human γ-aminobutyric acid A γ2 subunit. Proc Natl Acad Sci U S A 101:3220-3223.

McKernan RM, Quirk K, Prince R, Cox PA, Gillard NP, Ragan CI, Whiting P (1991) $GABA_A$ receptor subtypes immunopurified from rat brain with α subunit-specific antibodies have unique pharmacological properties. Neuron 7:667-676.

McKernan RM, Whiting PJ (1996) Which $GABA_A$ receptor subtypes really occur in the brain? Trends Neurosci 19:139-143.

McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P, Garrett L, Bristow L, Marshall G, Macaulay A, Brown N, Howell O, Moore KW, Carling RW, Street LJ, Castro JL, Ragan CI, Dawson GR, Whiting PJ (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the $GABA_A$ receptor α1 subtype. Nat Neurosci 3:587-592.

Medvedev A, Mackenzie L, Hiscock JJ, Willoughby JO (1996) Frontal cortex leads other brain structures in generalised spike-and-wave spindles and seizure spikes induced by picrotoxin. Electroencephalogr Clin Neurophysiol 98:157-166.

Meier J, Vannier C, Sergé A, Triller A, Choquet D (2001) Fast and reversible trapping of surface glycine receptors by gephyrin. Nat Neurosci 4:253-260.

Mihalek RM, Banerjee PK, Korpi ER, Quinlan JJ, Firestone LL, Mi ZP, Lagenaur C, Tretter V, Sieghart W, Anagnostaras SG, Sage JR, Fanselow MS, Guidotti A, Spigelman I, Li Z, DeLorey TM, Olsen RW, Homanics GE (1999) Attenuated sensitivity to neuroactive steroids in γ-aminobutyrate type A receptor delta subunit knockout mice. Proc Natl Acad Sci U S A 96:12905-12910.

Mihic SJ, Whiting PJ, Klein RL, Wafford KA, Harris RA (1994) A single amino acid of the human γ-aminobutyric acid type A receptor γ2 subunit determines benzodiazepine efficacy. J Biol Chem 269:32768-32773.

Mitchell SJ, Silver RA (2003) Shunting inhibition modulates neuronal gain during synaptic excitation. Neuron 38:433-445.

Modianos DT, Pfaff DW (1976) Brain stem and cerebellar lesions in female rats. I. Tests of posture and movement. Brain Res 106:31-46.

Mody I (2001) Distinguishing between $GABA_A$ receptors responsible for tonic and phasic conductances. Neurochem Res 26:907-913.

Mortensen M, Kristiansen U, Ebert B, Frølund B, Krogsgaard-Larsen P, Smart TG (2004) Activation of single heteromeric $GABA_A$ receptor ion channels by full and partial agonists. J Physiol 557: 389-413.

Mozrzymas JW, Barberis A, Michalak K, Cherubini E (1999) Chlorpromazine inhibits miniature GABAergic currents by reducing the binding and by increasing the unbinding rate of $GABA_A$ receptors. J Neurosci 19:2474-2488.

Mäkelä R, Uusi-Oukari M, Homanics GE, Quinlan JJ, Firestone LL, Wisden W, Korpi ER (1997) Cerebellar γ-aminobutyric acid type A receptors: pharmacological subtypes revealed by mutant mouse lines. Mol Pharmacol 52:380-388.

Möykkynen T, Korpi ER, Lovinger DM (2003) Ethanol inhibits α-amino-3-hydyroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function in central nervous system neurons by stabilizing desensitization. J Pharmacol Exp Ther 306:546-555.

Nadler JV (2003) The recurrent mossy fiber pathway of the epileptic brain. Neurochem Res 28:1649-1658.

Naik SR, Guidotti A, Costa E (1976) Central GABA receptor agonists: comparison of muscimol and baclofen. Neuropharmacology 15:479-484.

Nayeem N, Green TP, Martin IL, Barnard EA (1994) Quaternary structure of the native $GABA_A$ receptor determined by electron microscopic image analysis. J Neurochem 62:815-818.

Newell JG, Davies M, Bateson AN, Dunn SM (2000) Tyrosine 62 of the γ-aminobutyric acid type A receptor β2 subunit is an important determinant of high affinity agonist binding. J Biol Chem 275:14198-14204.

Newell JG, Czajkowski C (2003) The $GABA_A$ receptor α1 subunit Pro^{174}-Asp^{191} segment is involved in GABA binding and channel gating. J Biol Chem 278:13166-13172.

Newland CF, Cull-Candy SG (1992) On the mechanism of action of picrotoxin on GABA receptor channels in dissociated sympathetic neurones of the rat. J Physiol 447:191-213.

Nicoll RA (1978) The blockade of GABA mediated responses in the frog spinal cord by ammonium ions and furosemide. J Physiol 283:121-132.

Nusser Z, Roberts JD, Baude A, Richards JG, Somogyi P (1995) Relative densities of synaptic and extrasynaptic $GABA_A$ receptors on cerebellar granule cells as determined by a quantitative immunogold method. J Neurosci 15:2948-2960.

Nusser Z, Sieghart W, Stephenson FA, Somogyi P (1996) The α6 subunit of the $GABA_A$ receptor is concentrated in both inhibitory and excitatory synapses on cerebellar granule cells. J Neurosci 16:103-114.

Nusser Z, Sieghart W, Somogyi P (1998a) Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J Neurosci 18:1693-1703.

Nusser Z, Hájos N, Somogyi P, Mody I (1998b) Increased number of synaptic $GABA_A$ receptors underlies potentiation at hippocampal inhibitory synapses. Nature 395:172-177.

Nusser Z, Ahmad Z, Tretter V, Fuchs K, Wisden W, Sieghart W, Somogyi P (1999) Alterations in the expression of $GABA_A$ receptor subunits in cerebellar granule cells after the disruption of the α6 subunit gene. Eur J Neurosci 11:1685-1697.

Nusser Z, Mody I (2002) Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. J Neurophysiol 87:2624-2628.

Nyíri G, Freund TF, Somogyi P (2001) Input-dependent synaptic targeting of α2-subunit-containing $GABA_A$ receptors in synapses of hippocampal pyramidal cells of the rat. Eur J Neurosci 13:428-442.

Obata K, Ito M, Ochi R, Sato N (1967) Pharmacological properties of the postsynaptic inhibition by Purkinje cell axons and the action of γ-aminobutyric acid on deiters neurones. Exp Brain Res 4:43-57.

Obata K, Highstein SM (1970) Blocking by picrotoxin of both vestibular inhibition and GABA action on rabbit oculomotor neurones. Brain Res 18:538-541.

Oja SS, Kontro P (1987) Neurochemical aspects of amino acid transmitters and modulators. Med Biol 65:143-152.

Olsen RW, Bergman MO, Van Ness PC, Lummis SC, Watkins AE, Napias C, Greenlee DV (1981) γ-Aminobutyric acid receptor binding in mammalian brain. Heterogeneity of binding sites. Mol Pharmacol 19:217-227.

Olsen RW, McCabe RT, Wamsley JK (1990) $GABA_A$ receptor subtypes: Autoradiographic comparison of GABA, benzodiazepine, and convulsant binding sites in the rat central nervous system. J Chem Neuroanat 3:59-76.

Ong J, Kerr DI (1990) GABA-receptors in peripheral tissues. Life Sci 46:1489-1501.

Overstreet LS, Westbrook GL (2001) Paradoxical reduction of synaptic inhibition by vigabatrin. J Neurophysiol 86:596-603.

Overstreet LS, Westbrook GL (2003) Synapse density regulates independence at unitary inhibitory synapses. J Neurosci 23:2618-2626.

Paulsen O, Moser EI (1998) A model of hippocampal memory encoding and retrieval: GABAergic control of synaptic plasticity. Trends Neurosci 21:273-278.

Pearce RA (1993) Physiological evidence for two distinct $GABA_A$ responses in rat hippocampus. Neuron 10:189-200.

Peng Z, Hauer B, Mihalek RM, Homanics GE, Sieghart W, Olsen RW, Houser CR (2002) $GABA_A$ receptor changes in δ subunit-deficient mice: altered expression of α4 and γ2 subunits in the forebrain. J Comp Neurol 446:179-197.

Perrais D, Ropert N (1999) Effect of zolpidem on miniature IPSCs and occupancy of postsynaptic $GABA_A$ receptors in central synapses. J Neurosci 19:578-588.

Perret P, Sarda X, Wolff M, Wu TT, Bushey D, Goeldner M (1999) Interaction of non-competitive blockers within the γ-aminobutyric acid type A chloride channel using chemically reactive probes as chemical sensors for cysteine mutants. J Biol Chem 274:25350-25354.

Pitkänen A, Pikkarainen M, Nurminen N, Ylinen A (2000) Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. Ann N Y Acad Sci 911:369-391.

Plappert CF, Pilz PK (2002) Difference in anxiety and sensitization of the acoustic startle response between the two inbred mouse strains BALB/cAN and DBA/2N. Genes Brain Behav 1:178-186.

Pollard S, Thompson CL, Stephenson FA (1995) Quantitative characterization of α6 and α1α6 subunit-containing native γ-aminobutyric $acid_A$ receptors of adult rat cerebellum demonstrates two α subunit per receptor oligomer. J Biol Chem 270:21285-21290.

Porcello DM, Huntsman MM, Mihalek RM, Homanics GE, Huguenard JR (2003) Intact synaptic GABAergic inhibition and altered neurosteroid modulation of thalamic relay neurons in mice lacking δ subunit. J Neurophysiol 89:1378-1386.

Pritchett DB, Sontheimer H, Gorman CM, Kettenmann H, Seeburg PH, Schofield PR (1988) Transient expression shows ligand gating and allosteric potentiation of $GABA_A$ receptor subunits. Science 242:1306-1308.

Pritchett DB, Lüddens H, Seeburg PH (1989a) Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. Science 245:1389-1392.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, Seeburg PH (1989b) Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. Nature 338:582-585.

Pritchett DB, Seeburg PH (1990) γ-Aminobutyric $acid_A$ receptor α5-subunit creates novel type II benzodiazepine receptor pharmacology. J Neurochem 54:1802-1804.

Pritchett DB, Seeburg PH (1991) γ-Aminobutyric acid type A receptor point mutation increases the affinity of compounds for the benzodiazepine site. Proc Natl Acad Sci U S A 88:1421-1425.

Puia G, Vicini S, Seeburg PH, Costa E (1991) Influence of recombinant γ-aminobutyric acid-$_A$ receptor subunit composition on the action of allosteric modulators of γ-aminobutyric acid-gated Cl^- currents. Mol Pharmacol 39:691-696.

Pöltl A, Hauer B, Fuchs K, Tretter V, Sieghart W (2003) Subunit composition and quantitative importance of $GABA_A$ receptor subtypes in the cerebellum of mouse and rat. J Neurochem 87:1444-1455.

Quirk K, Gillard NP, Ragan CI, Whiting PJ, McKernan RM (1994) Model of subunit composition of γ-aminobutyric acid A receptor subtypes expressed in rat cerebellum with respect to their α and γ/δ subunits. J Biol Chem 269:16020-16028.

Quirk K, Whiting PJ, Ragan CI, McKernan RM (1995) Characterisation of δ-subunit containing $GABA_A$ receptors from rat brain. Eur J Pharmacol 290:175-181.

Rice A, Rafiq A, Shapiro SM, Jakoi ER, Coulter DA, DeLorenzo RJ (1996) Long-lasting reduction of inhibitory function and γ-aminobutyric acid type A receptor subunit mRNA expression in a model of temporal lobe epilepsy. Proc Natl Acad Sci U S A 93:9665-9669.

Richerson GB, Wu Y (2003) Dynamic equilibrium of neurotransmitter transporters: not just for reuptake anymore. J Neurophysiol 90:1363-1374.

Rienitz A, Becker CM, Betz H, Schmitt B (1987) The chloride channel blocking agent, *t*-butyl bicyclophosphorothionate, binds to the γ-aminobutyric acid-benzodiazepine, but not to the glycine receptor in rodents. Neurosci Lett 76:91-95.

Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lämsä K, Pirvola U, Saarma M, Kaila K (1999) The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397:251-255.

Roepstorff A, Lambert JD (1992) Comparison of the effect of the GABA uptake blockers, tiagabine and nipecotic acid, on inhibitory synaptic efficacy in hippocampal CA1 neurones. Neurosci Lett 146:131-134.

Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ, Martin JE (1997) Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. Mamm Genome 8:711-713.

Rosenberg M, Meier J, Triller A, Vannier C (2001) Dynamics of glycine receptor insertion in the neuronal plasma membrane. J Neurosci 21:5036-5044.

Rossi DJ, Hamann M (1998) Spillover-mediated transmission at inhibitory synapses promoted by high affinity α6 subunit $GABA_A$ receptors and glomerular geometry. Neuron 20:783-795.

Rossi DJ, Hamann M, Attwell D (2003) Multiple modes of GABAergic inhibition of rat cerebellar granule cells. J Physiol 548:97-110.

Rudolph U, Crestani F, Benke D, Brünig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Möhler H (1999) Benzodiazepine actions mediated by specific γ-aminobutyric $acid_A$ receptor subtypes. Nature 401:796-800.

Rudolph U, Crestani F, Möhler H (2001) $GABA_A$ receptor subtypes: dissecting their pharmacological functions. Trends Pharmacol Sci 22:188-194.

Russek SJ (1999) Evolution of $GABA_A$ receptor diversity in the human genome. Gene 227:213-222.

Salin PA, Prince DA (1996) Spontaneous $GABA_A$ receptor-mediated inhibitory currents in adult rat somatosensory cortex. J Neurophysiol 75:1573-1588.

Samoriski GM, Piekut DT, Applegate CD (1997) Differential spatial patterns of Fos induction following generalized clonic and generalized tonic seizures. Exp Neurol 143:255-268.

Sarto I, Wabnegger L, Dögl E, Sieghart W (2002) Homologous sites of $GABA_A$ receptor α1, β3 and γ2 subunits are important for assembly. Neuropharmacology 43:482-491.

Sassoè-Pognetto M, Panzanelli P, Sieghart W, Fritschy JM (2000) Colocalization of multiple $GABA_A$ receptor subtypes with gephyrin at postsynaptic sites. J Comp Neurol 420:481-498.

Saxena NC, Macdonald RL (1996) Properties of putative cerebellar γ-aminobutyric $acid_A$ receptor isoforms. Mol Pharmacol 49:567-579.

Scanziani M (2000) GABA spillover activates postsynaptic $GABA_B$ receptors to control rhythmic hippocampal activity. Neuron 25:673-681.

Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA, Seeburg P.H., Barnard E.A. (1987) Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. Nature 328:221-227.

Schwarzer C, Tsunashima K, Wanzenböck C, Fuchs K, Sieghart W, Sperk G (1997) $GABA_A$ receptor subunits in the rat hippocampus II: altered distribution in kainic acid-induced temporal lobe epilepsy. Neuroscience 80:1001-1017.

Seeburg PH, Wisden W, Verdoorn TA, Pritchett DB, Werner P, Herb A, Lüddens H, Sprengel R, Sakmann B (1990) The $GABA_A$ receptor family: molecular and functional diversity. Cold Spring Harbor Symp Quant Biol 55:29-40.

Semyanov A, Walker MC, Kullmann DM (2003) GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. Nat Neurosci 6:484-490.

Shivers BD, Killisch I, Sprengel R, Sontheimer H, Köhler M, Schofield PR, Seeburg PH (1989) Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. Neuron 3:327-337.

Sieghart W, Karobath M (1980) Molecular heterogeneity of benzodiazepine receptors. Nature 286:285-287.

Sieghart W, Eichinger A, Richards JG, Möhler H (1987) Photoaffinity labeling of benzodiazepine receptor proteins with the partial inverse agonist [^{3}H]Ro 15-4513: a biochemical and autoradiographic study. J Neurochem 48:46-52.

Sieghart W (1995) Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes. Pharmacol Rev 47:181-234.

Sigel E, Baur R, Malherbe P, Möhler H (1989) The rat β1-subunit of the $GABA_A$ receptor forms a picrotoxin-sensitive anion channel open in the absence of GABA. FEBS Lett 257:377-379.

Sigel E, Baur R, Trube G, Möhler H, Malherbe P (1990) The effect of subunit composition of rat brain $GABA_A$ receptors on channel function. Neuron 5:703-711.

Sigel E, Baur R, Kellenberger S, Malherbe P (1992) Point mutations affecting antagonist affinity and agonist dependent gating of $GABA_A$ receptor channels. EMBO J 11:2017-2023.

Sinkkonen ST, Hanna MC, Kirkness EF, Korpi ER (2000) $GABA_A$ receptor ε and θ subunits display unusual structural variation between species and are enriched in the rat locus ceruleus. J Neurosci 20:3588-3595.

Skerritt JH, Willow M, Johnston GA (1982) Diazepam enhancement of low affinity GABA binding to rat brain membranes. Neurosci Lett 29:63-66.

Skinner FK, Zhang L, Velazquez JL, Carlen PL (1999) Bursting in inhibitory interneuronal networks: A role for gap-junctional coupling. J Neurophysiol 81:1274-1283.

Slany A, Zezula J, Tretter V, Sieghart W (1995) Rat β3 subunits expressed in human embryonic kidney 293 cells form high affinity [^{35}S]*t*-butylbicyclophosphorothionate binding sites modulated by several allosteric ligands of γ-aminobutyric acid type A receptors. Mol Pharmacol 48:385-391.

Sloviter RS (1987) Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. Science 235:73-76.

Smart TG, Constanti A, Bilbe G, Brown DA, Barnard EA (1983) Synthesis of functional chick brain GABA-benzodiazepine-barbiturate/receptor complexes in mRNA-injected *Xenopus* oocytes. Neurosci Lett 40:55-59.

Smith GB, Olsen RW (1994) Identification of a [^{3}H]muscimol photoaffinity substrate in the bovine γ-aminobutyric $acid_A$ receptor α subunit. J Biol Chem 269:20380-20387.

Smith GB, Olsen RW (1995) Functional domains of $GABA_A$ receptors. Trends Pharmacol Sci 16:162-168.

Smith SS, Gong QH, Li X, Moran MH, Bitran D, Frye CA, Hsu FC (1998) Withdrawal from 3α-OH-5α-pregnan-20-one using a pseudopregnancy model alters the kinetics of hippocampal $GABA_A$-gated current and increases the $GABA_A$ receptor α4 subunit in association with increased anxiety. J Neurosci 18:5275-5284.

Snyder SE, Kume A, Jung YW, Connor SE, Sherman PS, Albin RL, Wieland DM, Kilbourn MR (1995) Synthesis of carbon-11-, fluorine-18-, and iodine-125-labeled $GABA_A$-gated chloride ion channel blockers: substituted 5-*tert*-butyl-2-phenyl-1,3-dithianes and -dithiane oxides. J Med Chem 38:2663-2671.

Soltesz I, Haby M, Leresche N, Crunelli V (1988) The $GABA_B$ antagonist phaclofen inhibits the late K^+-dependent IPSP in cat and rat thalamic and hippocampal neurones. Brain Res 448:351-354.

Somogyi P, Fritschy JM, Benke D, Roberts JDB, Sieghart W (1996) The γ2 subunit of the $GABA_A$ receptor is concentrated in synaptic junctions containing the α1 and β2/3 subunits in hippocampus, cerebellum and globus pallidus. Neuropharmacology 35:1425-1444.

Sperk G, Schwarzer C, Tsunashima K, Fuchs K, Sieghart W (1997) $GABA_A$ receptor subunits in the rat hippocampus I: immunocytochemical distribution of 13 subunits. Neuroscience 80:987-1000.

Sperk G, Schwarzer C, Tsunashima K, Kandlhofer S (1998) Expression of $GABA_A$ receptor subunits in the hippocampus of the rat after kainic acid-induced seizures. Epilepsy Res 32:129-139.

Squires RF, Benson DI, Braestrup C, Coupet J, Klepner CA, Myers V, Beer B (1979) Some properties of brain specific benzodiazepine receptors: new evidence for multiple receptors. Pharmacol Biochem Behav 10:825-830.

Squires RF, Casida JE, Richardson M, Saederup E (1983) [^{35}S]*t*-butylbicyclophosphorothionate binds with high affinity to brain-specific sites coupled to γ-aminobutyric $acid_A$ and ion recognition sites. Mol Pharmacol 23:326-336.

Squires RF, Saederup E (1987) $GABA_A$ receptor blockers reverse the inhibitory effect of GABA on brain-specific [^{35}S]TBPS binding. Brain Res 414:357-364.

Steiger JL, Russek SJ (2004) $GABA_A$ receptors: building the bridge between subunit mRNAs, their promoters, and cognate transcription factors. Pharmacol Ther 101:259-281.

Stell BM, Brickley SG, Tang CY, Farrant M, Mody I (2003) Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing $GABA_A$ receptors. Proc Natl Acad Sci U S A 100:14439-14444.

Stevenson A, Wingrove PB, Whiting PJ, Wafford KA (1995) β-Carboline γ-aminobutyric $acid_A$ receptor inverse agonists modulate γ-aminobutyric acid via the loreclezole binding site as well as the benzodiazepine site. Mol Pharmacol 48:965-969.

Study RE, Barker JL (1981) Diazepam and (-)-pentobarbital: fluctuation analysis reveals different mechanisms for potentiation of γ-aminobutyric acid responses in cultured central neurons. Proc Natl Acad Sci U S A 78:7180-7184.

Supavilai P, Karobath M (1984) [^{35}S]-t-butylbicyclophosphorothionate binding sites are constituents of the γ-aminobutyric acid benzodiazepine receptor complex. J Neurosci 4:1193-1200.

Sur C, Farrar SJ, Kerby J, Whiting PJ, Atack JR, McKernan RM (1999) Preferential coassembly of α4 and δ subunits of the γ-aminobutyric $acid_A$ receptor in rat thalamus. Mol Pharmacol 56:110-115.

Taylor PM, Thomas P, Gorrie GH, Connolly CN, Smart TG, Moss SJ (1999) Identification of amino acid residues within $GABA_A$ receptor β subunits that mediate both homomeric and heteromeric receptor expression. J Neurosci 19:6360-6371.

Taylor PM, Connolly CN, Kittler JT, Gorrie GH, Hosie A, Smart TG, Moss SJ (2000) Identification of residues within $GABA_A$ receptor α subunits that mediate specific assembly with receptor β subunits. J Neurosci 20:1297-1306.

Thompson CL, Bodewitz G, Stephenson FA, Turner JD (1992) Mapping of $GABA_A$ receptor α5 and α6 subunit-like immunoreactivity in rat brain. Neurosci Lett 144:53-56.

Thompson SM, Gähwiler BH (1992) Effects of the GABA uptake inhibitor tiagabine on inhibitory synaptic potentials in rat hippocampal slice cultures. J Neurophysiol 67:1698-1701.

Thompson SA, Arden SA, Marshall G, Wingrove PB, Whiting PJ, Wafford KA (1999) Residues in transmembrane domains I and II determine γ-aminobutyric acid type A receptor subtype-selective antagonism by furosemide. Mol Pharmacol 55:993-999.

Tia S, Wang JF, Kotchabhakdi N, Vicini S (1996a) Developmental changes of inhibitory synaptic currents in cerebellar granule neurons: role of $GABA_A$ receptor α6 subunit. J Neurosci 16:3630-3640.

Tia S, Wang JF, Kotchabhakdi N, Vicini S (1996b) Distinct deactivation and desensitization kinetics of recombinant $GABA_A$ receptors. Neuropharmacology 35:1375-1382.

Ticku MK, Ban M, Olsen RW (1978) Binding of [^{3}H]α-dihydropicrotoxinin, a γ-aminobutyric acid synaptic antagonist, to rat brain membranes. Mol Pharmacol 14:391-402.

Ticku MK, Maksay G (1983) Convulsant/depressant site of action at the allosteric benzodiazepine-GABA receptor-ionophore complex. Life Sci 33:2363-2375.

Tiihonen J, Kuikka J, Räsänen P, Lepola U, Koponen H, Liuska A, Lehmusvaara A, Vainio P, Könönen M, Bergstrom K, Yu M, Kinnunen I, Åkerman K, Karhu J (1997) Cerebral benzodiazepine receptor binding and distribution in generalized anxiety disorder: a fractal analysis. Mol Psychiatry 2:463-471.

Titulaer MN, Kamphuis W, Pool CW, van Heerikhuize JJ, Lopes da Silva FH (1994) Kindling induces time-dependent and regional specific changes in the [^{3}H]muscimol binding in the rat hippocampus: a quantitative autoradiographic study. Neuroscience 59:817-826.

Titulaer MN, Kamphuis W, Lopes da Silva FH (1995a) Autoradiographic analysis of [^{35}S]t-butylbicyclophosphorothionate binding in kindled rat hippocampus shows different changes in CA1 area and fascia dentata. Neuroscience 66:547-554.

Titulaer MN, Kamphuis W, Lopes da Silva FH (1995b) Long-term and regional specific changes in [^{3}H]flunitrazepam binding in kindled rat hippocampus. Neuroscience 68:399-406.

Tossman U, Jonsson G, Ungerstedt U (1986) Regional distribution and extracellular levels of amino acids in rat central nervous system. Acta Physiol Scand 127:533-545.

Tretter V, Ehya N, Fuchs K, Sieghart W (1997) Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. J Neurosci 17:2728-2737.

Tretter V, Hauer B, Nusser Z, Mihalek RM, Höger H, Homanics GE, Somogyi P, Sieghart W (2001) Targeted disruption of the $GABA_A$ receptor δ subunit gene leads to an up-regulation of γ2 subunit-containing receptors in cerebellar granule cells. J Biol Chem 276:10532-10538.

Trifiletti RR, Snowman AM, Snyder SH (1984) Solubilization and anionic regulation of cerebral sedative/convulsant receptors labeled with [^{35}S] *tert*-butylbicyclophosphorothionate (TBPS). Biochem Biophys Res Commun 120:692-699.

Tsien JZ, Huerta PT, Tonegawa S (1996) The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell 87:1327-1338.

Unwin N (1998) The nicotinic acetylcholine receptor of the *Torpedo* electric ray. J Struct Biol 121:181-190.

Uusi-Oukari M, Korpi ER (1989) Cerebellar $GABA_A$ receptor binding and function in vitro in two rat lines developed for high and low alcohol sensitivity. Neurochem Res 14:733-739.

Uusi-Oukari M, Heikkilä J, Sinkkonen ST, Mäkelä R, Hauer B, Homanics GE, Sieghart W, Wisden W, Korpi ER (2000) Long-range interactions in neuronal gene expression: evidence from gene targeting in the $GABA_A$ receptor β2-α6-α1-γ2 subunit gene cluster. Mol Cell Neurosci 16:34-41.

Valeyev AY, Barker JL, Cruciani RA, Lange GD, Smallwood VV, Mahan LC (1993) Characterization of the γ-aminobutyric $acid_A$ receptor-channel complex composed of α1β2 and α1β3 subunits from rat brain. J Pharmacol Exp Ther 265:985-991.

Van Renterghem C, Bilbe G, Moss S, Smart TG, Constanti A, Brown DA, Barnard EA (1987) GABA receptors induced in *Xenopus* oocytes by chick brain mRNA: evaluation of TBPS as a use-dependent channel-blocker. Brain Res 388:21-31.

Vautrin J, Maric D, Sukhareva M, Schaffner AE, Barker JL (2000) Surface-accessible GABA supports tonic and quantal synaptic transmission. Synapse 37:38-55.

Vekovischeva OY, Aitta-Aho T, Echenko O, Kankaanpää A, Seppälä T, Honkanen A, Sprengel R, Korpi ER (2004) Reduced aggression in AMPA-type glutamate receptor GluR-A subunit-deficient mice. Genes Brain Behav 3:253-265.

Verdoorn TA, Draguhn A, Ymer S, Seeburg PH, Sakmann B (1990) Functional properties of recombinant rat $GABA_A$ receptors depend upon subunit composition. Neuron 4:919-928.

Verdoorn TA (1994) Formation of heteromeric γ-aminobutyric acid type A receptors containing two different α subunits. Mol Pharmacol 45:475-480.

Vergnes M, Boehrer A, Reibel S, Simler S, Marescaux C (2000) Selective susceptibility to inhibitors of GABA synthesis and antagonists of $GABA_A$ receptor in rats with genetic absence epilepsy. Exp Neurol 161:714-723.

Wafford KA, Bain CJ, Whiting PJ, Kemp JA (1993) Functional comparison of the role of γ subunits in recombinant human γ-aminobutyric acid$_A$/benzodiazepine receptors. Mol Pharmacol 44:437-442.

Wafford KA, Thompson SA, Thomas D, Sikela J, Wilcox AS, Whiting PJ (1996) Functional characterization of human γ-aminobutyric acid$_A$ receptors containing the α4 subunit. Mol Pharmacol 50:670-678.

Wagner DA, Czajkowski C (2001) Structure and dynamics of the GABA binding pocket: A narrowing cleft that constricts during activation. J Neurosci 21:67-74.

Wall MJ, Usowicz MM (1997) Development of action potential-dependent and independent spontaneous GABA$_A$ receptor-mediated currents in granule cells of postnatal rat cerebellum. Eur J Neurosci 9:533-548.

Wallace RH, Marini C, Petrou S, Harkin LA, Bowser DN, Panchal RG, Williams DA, Sutherland GR, Mulley JC, Scheffer IE, Berkovic SF (2001) Mutant GABA$_A$ receptor γ2-subunit in childhood absence epilepsy and febrile seizures. Nat Genet 28:49-52.

Wamsley JK, Gee KW, Yamamura HI (1983) Comparison of the distribution of convulsant/barbiturate and benzodiazepine receptors using light microscopic autoradiography. Life Sci 33:2321-2329.

Wang H, Bedford FK, Brandon NJ, Moss SJ, Olsen RW (1999) GABA$_A$-receptor-associated protein links GABA$_A$ receptors and the cytoskeleton. Nature 397:69-72.

Wei W, Zhang N, Peng Z, Houser CR, Mody I (2003) Perisynaptic localization of δ subunit-containing GABA$_A$ receptors and their activation by GABA spillover in the mouse dentate gyrus. J Neurosci 23:10650-10661.

Westh-Hansen SE, Witt MR, Dekermendjian K, Liljefors T, Rasmussen PB, Nielsen M (1999) Arginine residue 120 of the human GABA$_A$ receptor α1 subunit is essential for GABA binding and chloride ion current gating. Neuroreport 10:2417-2421.

White MM, Aylwin M (1990) Niflumic and flufenamic acids are potent reversible blockers of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes. Mol Pharmacol 37:720-724.

Whiting P, McKernan RM, Iversen LL (1990) Another mechanism for creating diversity in γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of γ2 subunit, one of which contains a protein kinase C phosphorylation site. Proc Natl Acad Sci U S A 87:9966-9970.

Whiting PJ, McKernan RM, Waffor0d KA (1995) Structure and pharmacology of vertebrate GABA$_A$ receptor subtypes. Int Rev Neurobiol 38:95-138.

Whiting PJ, McAllister G, Vassilatis D, Bonnert TP, Heavens RP, Smith DW, Hewson L, O'Donell R, Rigby MR, Sirinathsinghji DJ, Marshall G, Thompson SA, Wafford KA, Vasilatis D (1997) Neuronally restricted RNA splicing regulates the expression of a novel GABA$_A$ receptor subunit conferring atypical functional properties. J Neurosci 17:5027-5037.

Wieland HA, Lüddens H, Seeburg PH (1992) A single histidine in GABA$_A$ receptors is essential for benzodiazepine agonist binding. J Biol Chem 267:1426-1429.

Williamson A, Telfeian AE, Spencer DD (1995) Prolonged GABA responses in dentate granule cells in slices isolated from patients with temporal lobe sclerosis. J Neurophysiol 74:378-387.

Wingrove PB, Wafford KA, Bain C, Whiting PJ (1994) The modulatory action of loreclezole at the γ-aminobutyric acid type A receptor is determined by a single amino acid in the β2 and β3 subunit. Proc Natl Acad Sci U S A 91:4569-4573.

Wingrove PB, Safo P, Wheat L, Thompson SA, Wafford KA, Whiting PJ (2002) Mechanism of α-subunit selectivity of benzodiazepine pharmacology at γ-aminobutyric acid type A receptors. Eur J Pharmacol 437:31-39.

Wisden W, Herb A, Wieland H, Keinänen K, Lüddens H, Seeburg PH (1991) Cloning, pharmacological characteristics and expression pattern of the rat GABA$_A$ receptor α4 subunit. FEBS Lett 289:227-230.

Wisden W, Laurie DJ, Monyer H, Seeburg PH (1992) The distribution of 13 GABA$_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci 12:1040-1062.

Wisden W, Morris BJ (1994) *In situ* hybridization with synthetic oligonucleotide probes. In '*In situ* hybridization protocols for the brain', Wisden and Morris (eds), Academic Press, London: 9-34.

Wisden W, Korpi ER, Bahn S (1996) The cerebellum: a model system for studying GABA$_A$ receptor diversity. Neuropharmacology 35:1139-1160.

Wisden W, Cope D, Klausberger T, Hauer B, Sinkkonen ST, Tretter V, Lujan R, Jones A, Korpi ER, Mody I, Sieghart W, Somogyi P (2002) Ectopic expression of the GABA(A) receptor α6 subunit in hippocampal pyramidal neurons produces extrasynaptic receptors and an increased tonic inhibition. Neuropharmacology 43:530-549.

Wong DT, Threlkeld PG, Bymaster FP, Squires RF (1984) Saturable binding of ^{35}S-t-butylbicyclophosphorothionate to the sites linked to the GABA receptor and the interaction with GABAergic agents. Life Sci 34:853-860.

Woodward RM, Polenzani L, Miledi R (1994) Effects of fenamates and other nonsteroidal anti-inflammatory drugs on rat brain GABA$_A$ receptors expressed in *Xenopus* oocytes. J Pharmacol Exp Ther 268:806-817.

Wooltorton JR, Moss SJ, Smart TG (1997) Pharmacological and physiological characterization of murine homomeric β3 GABA$_A$ receptors. Eur J Neurosci 9:2225-2235.

Wu Y, Wang W, Richerson GB (2003) Vigabatrin induces tonic inhibition via GABA transporter reversal without increasing vesicular GABA release. J Neurophysiol 89:2021-2034 Epub 2002 Dec 2027.

Xu M, Akabas MH (1993) Amino acids lining the channel of the γ-aminobutyric acid type A receptor identified by cysteine substitution. J Biol Chem 268:21505-21508.

Xu M, Covey DF, Akabas MH (1995) Interaction of picrotoxin with GABA$_A$ receptor channel-lining residues probed in cysteine mutants. Biophys J 69:1858-1867.

Ymer S, Schofield PR, Draguhn A, Werner P, Köhler M, Seeburg PH (1989) $GABA_A$ receptor β subunit heterogeneity: functional expression of cloned cDNAs. EMBO J 8:1665-1670.

Yoon K, Covey DF, Rothman SM (1993) Multiple mechanisms of picrotoxin block of GABA-induced currents in rat hippocampal neurons. J Physiol 464:423-439.

Zambrowicz BP, Sands AT (2003) Knockouts model the 100 best-selling drugs - will they model the next 100? Nat Rev Drug Discov 2:38-51.

Zoli M, Jansson A, Sykova E, Agnati LF, Fuxe K (1999) Volume transmission in the CNS and its relevance for neuropsychopharmacology. Trends Pharmacol Sci 20:142-150.

Zwanzger P, Eser D, Padberg F, Baghai TC, Schule C, Rötzer F, Ella R, Möller HJ, Rupprecht R (2003) Effects of tiagabine on cholecystokinin-tetrapeptide (CCK-4)-induced anxiety in healthy volunteers. Depress Anxiety 18:140-143.