TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 781

MEDICA - ODONTOLOGICA

$GABA_A$ RECEPTORS IN EXPERIMENTAL MODELS OF EPILEPSY IN THE DEVELOPING AND ADULT RAT BRAIN

by

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Turku 2007

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ISBN 978-951-29-3429-4 (PRINT)
ISBN 978-951-29-3430-0 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2007

To my family

ABSTRACT

Hanna Laurén: $GABA_A$ receptors in experimental models of epilepsy in the developing and adult rat brain. Department of Pharmacology, Drug Development and Therapeutics, University of Turku, and Drug Discovery Graduate School. Annales Universitatis Turkuensis, Medica-Odontologia, Painosalama Oy Turku, Finland, 2007.

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain and it binds to ionotropic GABA type A ($GABA_A$) receptors that are pentameric compelexes composed of a variety of different subunits. The receptor subtypes have different pharmacological properties, and the α subunit variant mainly determines the binding properties to $GABA_A$ receptors. Changes in the structure and function of $GABA_A$ receptors may contribute to epileptogenesis and reduced GABAergic inhibition is proposed to be a key element for seizure generation in epilepsy. Several studies have demonstrated changed expression of multiple $GABA_A$ receptor subunits after seizures in the adult rat brain that may result in receptors with altered functional and pharmacological properties. The postnatal $GABA_A$ receptor subunit messenger RNA (mRNA) expression in the rat brain, including the hippocampus, exhibits a unique temporal and regional developmental profile *in vivo*. The influence of epilepsy on the expression of $GABA_A$ receptor subunits in the immature, developing brain is not well known

Here, the expression and pharmacology of $GABA_A$ receptor subunits were studied in different experimental models of epilepsy. For studies of the developmental subunit expression, as well as the impact of epileptiform activity on subunit expression in the immature rat brain, two separate models were applied: organotypic hippocampal slice cultures that serve as an *in vitro* model where the structural organization is retained and can be compared to the *in vivo* development, and immature 9-day old rats, in which status epilepticus (SE) is induced by the excitatory glutamate receptor agonist kainate, and roughly function as an *in vivo* epilepsy model for newborn children. In addition to these acute models, long-term alterations in the expression of $GABA_A$ receptor subunits were examined in chronically epileptic adult rats, in which epilepsy was induced by electrical stimulation of the amygdala.

As a conclusion the results show that SE region- and subunit-specifically alterered the subunit mRNA and protein expression as well as pharmacology of the $GABA_A$ receptor in the immature rat brain during the sensitive postnatal period. Especially, SE disturbed the normal developmental α1 and α2 subunit expression patterns. This may have long-term consequences on the strictly developmentally-regulated maturation of $GABA_A$ receptors and lead to receptors with altered function and pharmacology. Chronic treatment of cultured hippocampal slices with drugs that affect the balance between excitation and inhibition altered the mRNA expression of several subunits in a region-specific manner, some of which are comparable to the epileptic hippocampus. In adult rats with chronic epilepsy, region- and subunit-selective changes of $GABA_A$ receptor subunit mRNAs were found in the hippocampus, however different from changes after acute seizures and those found in immature rats. The decreased expression of the α2 and α4 subunits and the increased expression of the β3 subunit in certain hippocampal subfields of adult epileptic rats, may represent compensatory responses to seizure activity.

Keywords: epilepsy, $GABA_A$ receptor, hippocampus, immature brain, status epilepticus

TIIVISTELMÄ

Hanna Laurén: $GABA_A$ reseptorimuutokset epilepsian kokeellisissa malleissa kehittyvien ja aikuisten rottien aivokudoksessa. Farmakologia, lääkekitys ja lääkehoito, Turun yliopisto, ja Drug Discovery Graduate School. Annales Universitatis Turkuensis, Medica-Odontologia, Painosalama Oy Turku, Finland, 2007.

Muutokset γ-aminovoihappo tyyppi A ($GABA_A$) reseptorin rakenteessa ja toiminnassa voivat johtaa epilepsian syntyyn. Vähentyneen GABAergisen inhibition oletetaankin olevan eräs avaintekijä epileptisten kohtausten syntyessä aikuisilla rotilla. Monien $GABA_A$ reseptorialayksikköjen ilmentyminen on muuttunut aikuisten rottien aivoissa epileptisten kohtausten jälkeen. Nämä muutokset voivat johtaa sellaisten reseptorien muodostumiseen, joiden toiminnalliset ja farmakologiset ominaisuudet poikkeavat normaalista, ja siten ne voivat edesauttaa epilepsian syntyä. Ne voivat myös vaikuttaa antiepileptisten lääkkeiden tehoon. Kehityksen kuluessa rottien aivoissa, mukaan lukien hippokampus, tapahtuu ainutlaatuisia ajallisia ja alueellisia muutoksia $GABA_A$ reseptorialayksikköjen lähetti-RNA:n ilmentymisissä, mutta epileptisten kohtausten vaikutukset alayksikköjen ilmentymiseen kehittyvissä aivoissa tunnetaan huonosti.

Tässä työssä tutkittiin $GABA_A$ reseptorialayksikköjen ilmentymistä ja reseptorin farmakologisia ominaisuuksia kolmen erilaisen kokeellisen epilepsian mallin avulla. Kahta eri mallia käytettiin tutkittaessa alayksikköjen kehityksellistä ilmentymistä ja epilepsian kaltaisen sähköisen aktiviteetin vaikutusta alayksikköjen ilmentymiseen kehittyvissä aivoissa: organotyyppiset hippokampuksen kudosviljelmät *in vitro* ja 9-vrk:n ikäiset rotat *in vivo*. Hippokampusten kudosviljelmissä kaikki päähermosolutyypit säilyvät hyvin ja erilaistuvat kuten *in vivo* olosuhteissa. Myös kudoksen perusrakenne ja sen sisäiset hermoyhteydet kehittyvät kuten *in vivo* ja siten viljellyissä kudoksissa tapahtuvia muutoksia voidaan verrata aivoissa tapahtuvaan *in vivo* kehitykseen. Käsittelemällä viljelmiä ja kehittyviä eläimiä sellaisilla farmakologisilla aineilla, jotka stimuloivat hermosolujen toimintaa saadaan sekä *in vitro* että *in vivo* epilepsiamalleja. 9-vrk:n ikäiset rotat, joille aiheutettiin status epileptikus (SE) eksitoivalla glutamaattireseptoriagonistilla kainaatilla, vastaavat karkeasti ottaen vastasyntynyttä lasta. Näiden akuuttimallien lisäksi tutkittiin $GABA_A$ reseptorialayksiköiden ilmentymisissä tapahtuvia pitkäaikaisia muutoksia aikuisilla, kroonista epilepsiaa sairastavilla rotilla.

Tulokset osoittavat, että SE muuttaa alue- ja alayksikkö-spesifisesti monien alayksikköjen $GABA_A$ reseptori lähetti-RNA:n ja proteiinin ilmentymistä. Myös reseptorin farmakologiset ominaisuudet muuttuvat 9-vrk:n ikäisen rotan aivoissa niiden herkässä kehitysvaiheessa. Kroonisen epilepsian mallissa aikuisilla rotilla havaittiin alue- ja alayksikkö-spesifisiä muutoksia $GABA_A$ reseptorin alayksikköjen lähetti-RNA:n ilmestymisessä hippokampuksessa. $GABA_A$ reseptoreiden monimuotoinen molekulaarinen rakenne antaa runsaasti kohteita ikä- ja kohdespesifisten lääkeaineiden kehittämiselle mm epilepsian hoitoon. Epileptisten kohtausten aiheuttamat ikäspesifiset muutokset $GABA_A$ reseptoreiden rakenteessa sekä akuuttien kohtauksien jälkeen että kroonisessa epilepsiassa tarjoavatkin haasteen uusien antiepileptisten lääkeainemolekyylien kehittämiseksi epilepsiaa sairastaville sekä aikuisille että lapsipotilaille.

Avainsanat: epilepsia, $GABA_A$ reseptori, hippokampus, kehittymättömät aivot, status epileptikus

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following publications, which are referred to in the text by the Roman numerals I – IV.

I. Laurén, H.B., Pitkänen, A., Nissinen, J., Soini, S.L., Korpi, E.R. and Holopainen, I.E. Selective changes in $GABA_A$ receptor subunits in the hippocampus in spontaneously seizing rats with chronic temporal lobe epilepsy. Neurosci Lett 349: 58-62, 2003.

II. Holopainen, I.E. and Laurén, H.B. Neuronal activity regulates $GABA_A$ receptor subunit expression in organotypic hippocampal slice cultures. Neuroscience 118: 967-974, 2003.

III. Laurén H.B., Lopez-Picon F.R., Korpi E.R., Holopainen I.E. Kainic acid-induced status epilepticus alters $GABA_A$ receptor subunit mRNA and protein expression in the developing rat hippocampus. J Neurochem 94: 1384-1394, 2005.

IV. Laurén, H.B., Lopez-Picon, F.R., Kukko-Lukjanov, T-K., Uusi-Oukari, M., Holopainen, I.E. Status epilepticus alters zolpidem sensitivity of [^{3}H]flunitrazepam binding in the developing rat brain. Neuroscience 146: 802-811, 2007.

ABBREVIATIONS

AED	antiepileptic drug
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BDNF	brain-derived neurotrophic factor
BZ	benzodiazepine
CA	Cornu Ammonis
CNS	central nervous system
DG	dentate gyrus
DIV	days *in vitro*
DNQX	6,7-dinitroquinoxaline-2,3-dione
EEG	electroencephalography
E	embryonic day
GABA	γ-aminobutyric acid
$GABA_A$ receptor	GABA type A receptor
GABARAP	$GABA_A$ receptor-associated protein
GABA-T	GABA transaminase
GAD	glutamic acid decarboxylase
GAT	GABA transporter
GDP	giant depolarizing potential
G-protein	heterotrimeric guanine nucleotide-binding protein
$5\text{-}HT_3$ receptor	5-hydroxytryptamine receptor
ILAE	International League Against Epilepsy
i.p.	intraperitoneal
IPSP	inhibitory postsynaptic potential
IPSC	inhibitory postsynaptic current
KA	kainic acid
K_i	equilibrium dissociation constant
KCC2	K-Cl co-transporter isoform 2
LTD	long-term depression
LTP	long-term potentiation
M	transmembrane spanning segment
mRNA	messenger RNA
MF	mossy fiber
mIPSC	miniature inhibitory postsynaptic current
NKCC1	Na-K-2Cl co-transporter isoform 1
NMDA	N-methyl-D-aspartate
P	postnatal day
PKC	protein kinase C
PFA	paraformaldehyde
Ro 15-4513	ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4] benzodiazepine -3-carboxylate
SE	status epilepticus
TBPS	*t*-butylbicyclophosphorothionate
THIP	4,5,6,7-tetrahydroisoazolo[5,4-c]pyridin-3-ol
TLE	temporal lobe epilepsy

1. INTRODUCTION

GABA is a neurotransmitter responsible for the major part of inhibition in the mammalian central nervous system (CNS). It acts mainly on $GABA_A$ receptors which are coupled to a ligand-gated Cl^- channel. $GABA_A$ receptors are the target for a variety of drugs that enhance GABAergic function for the treatment of diseases such as epilepsy, anxiety, sleep disorders and addiction (Sieghart, 1995; Korpi *et al.*, 2002). In the adult rat brain, activation of $GABA_A$ receptors increases Cl^- permeability of the neuronal membrane leading to hyperpolarization and decreased excitability of neurons. In the immature brain, the receptor activation leads to depolarization of neurons in all regions of the CNS studied (Cherubini *et al.*, 1991; Leinekugel *et al.*, 1997). During development GABA also acts as a trophic neurotransmitter by regulating nearly all the key developmental steps, from cell proliferation to circuit refinement (Demarque *et al.*, 2002).

The $GABA_A$ receptors are protein complexes consisting of five subunits, and there are 16 different receptor subunit types (Barnard *et al.*, 1998). This gives an almost unlimited amount of possible subunit combinations, forming receptors with very different binding properties, and the subunit composition (mainly the α subunit) determines the pharmacology of the receptor (Bonnert *et al.*, 1999; Korpi *et al.*, 2002). The receptors display a unique distribution in the mammalian CNS. The expression patterns of $GABA_A$ receptor subunits in the rat brain, including the hippocampus, also change region- and subunit-specifically during the embryonic and postnatal development (Laurie *et al.*, 1992; Poulter *et al.*, 1992; Fritschy *et al.*, 1994; Paysan & Fritschy, 1998). In particular, the expression of α1 and α2 subunits is strictly developmentally regulated, with a high α2 mRNA level of the embryonic phase that declines, whereas the α1 mRNA expression increases during the early postnatal development in the rat hippocampus (Laurie *et al.*, 1992; Poulter *et al.*, 1992; Fritschy *et al.*, 1994; Paysan & Fritschy, 1998). This switch has been suggested to indicate the appearance of a new, prevalent receptor subtype involved in synaptic inhibition (Fritschy *et al.*, 1994; Hevers & Lüddens, 2002).

Epilepsy is a fairly common chronic neurological disorder disturbing the normal function of the brain and the patient gets recurrent seizures. The cellular and molecular detrimental effects related to epilepsy are however not well known, especially during brain development. In adult rats, an important triggering factor for seizure generation is an impaired function of $GABA_A$ receptors (Tsunashima *et al.*, 1997; Bouilleret *et al.*, 2000; Andre *et al.*, 2001). Recent

investigations in adult patients with epilepsy and adult epileptic experimental animals show that the subunit composition, function, and pharmacology of the $GABA_A$ receptor changes after increased excitation, i.e. seizures (Brooks-Kayal *et al.*, 1998b; Nusser *et al.*, 1998a; Loup *et al.*, 2000; Andre *et al.*, 2001; Raol *et al.*, 2006b). However, it is still incompletely known whether and to what extent the subunit composition is modulated by seizures in the developing brain, with special interest to the hippocampus that is a region highly vulnerable to seizures.

The main objectives of this thesis were to study seizure-induced alterations in the subunit composition of $GABA_A$ receptor by using *in vitro* and *in vivo* experimental epilepsy models of the developing and adult rat brain. The goal was to find out the time course of seizure-induced changes in the expression of subunit mRNAs and to study how the changes may affect the receptor pharmacology. Understanding of these epilepsy-associated, regionally distinct changes in the $GABA_A$ receptor in different hippocampal neurons of the immature brain should permit development of new anti-epileptic drugs that specifically target receptor subtypes, which may predominate on the epileptic neuron in pediatric patients.

2. REVIEW OF LITERATURE

2.1 GABA

2.1.1 GABA as a neurotransmitter

The amino acid gamma-aminobutyric acid (GABA) has long been considered to be the main inhibitory neurotransmitter in the adult mammalian CNS. It regulates a neuron's ability to fire action potentials either through hyperpolarization of the membrane potential or through shunting of excitatory inputs. GABA was first identified in the mammalian brain during the 1950s (Roberts & Frankel, 1950). When GABA was applied to nerve and muscle cells of both vertebrates and invertebrates, it was generally found to have inhibitory effects and produce conductance changes with ion sensitivities similar to those observed after the activation of inhibitory nerves (Kuffler & Edwards, 1958; Krnjevic *et al.*, 1966). In the 1970s GABA was finally localized to mammalian nerve terminals (Bloom & Iversen, 1971), and antibodies raised against GABA-biosynthetic enzymes were shown to be localized preferentially to known inhibitory neurons (Ribak, 1978; Ribak *et al*., 1978). However, recent evidence suggests that GABA is not only an inhibitory neurotransmitter, but also act as an excitatory and trophic neurotransmitter in the immature brain and is involved in generating rhythmic activities in neuronal networks (Cherubini *et al.*, 1991; Ben-Ari *et al*., 1997; Demarque *et al.*, 2002).

2.1.2 Synthesis and metabolism

In the mammalian brain, the portion of GABA that functions as neurotransmitter is formed by a metabolic pathway commonly referred to as the GABA shunt. As with glutamate synthesis, the most common precursor for GABA formation is glucose. The first step in the GABA shunt is the conversion of α-ketoglutarate into glutamate by the action of GABA α-oxoglutarate transaminase (GABA transaminase or GABA-T) (Shank *et al.*, 1989). GABA is then synthesized primarily from glutamate in a reaction that is catalysed by two glutamic acid decarboxylase (GAD) enzymes, GAD65 and GAD67 (Martin & Rimvall, 1993) (Figure 1).

Like most neurotransmitters, GABA is packaged into vesicles in the presynaptic terminals by a vesicular GABA transporter. Upon stimulation, GABA is released from nerve terminals by calcium-dependent exocytosis (Gaspary *et al.*, 1998). Once released, GABA freely diffuses across the synaptic cleft to interact with its appropriate receptors on the postsynaptic membrane.

GABA signals are terminated by reuptake of the neurotransmitter from the cleft into nerve terminals by the actions of several types of plasma membrane GABA transporters (GATs). By this mechanism, GABA can be returned to GABAergic nerve terminals where it is repackaged for release (Conti *et al.*, 2004). The transport of GABA requires extracellular Na^+ and Cl^-; two Na^+ and one Cl^- ion are transported for each GABA molecule (Radian & Kanner, 1983). By molecular cloning techniques the genes for four highly homologous GABA transporters have been characterized, and they are expressed on nerve terminals and glial cell membranes throughout the nervous system (Conti *et al.*, 2004). GAT-1 is considered the predominant neuronal GABA transporter, whereas the others show a more ubiquitous distribution (Guastella *et al.*, 1990; Borden, 1996; Engel *et al.*, 1998).

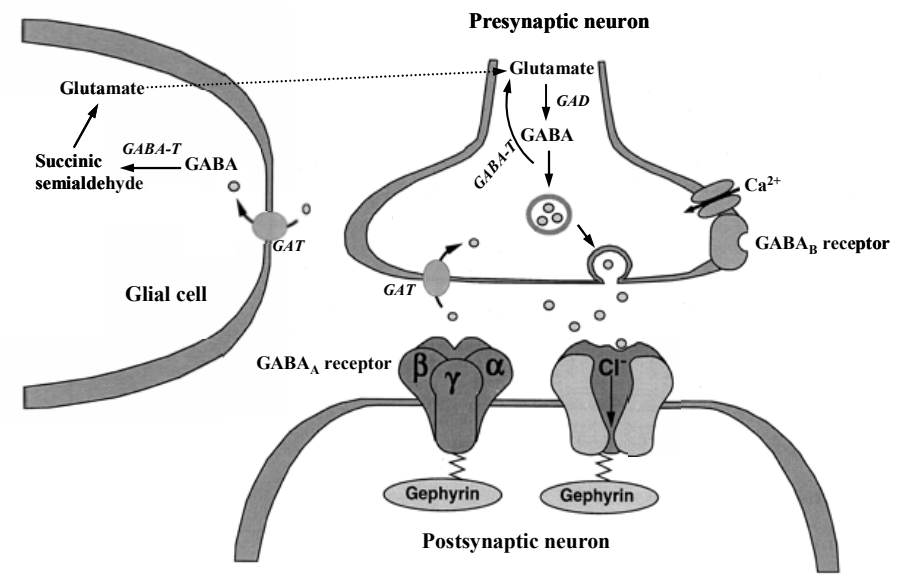

Figure 1. The GABAergic synapse. GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD) and stored in vesicles located in the presynaptic terminal. It is then released to the synaptic cleft and interacts with postsynaptic $GABA_A$ receptors apposed to GABAergic release sites. The $GABA_A$ receptor is linked to the postsynaptic membrane by anchoring proteins, such as gephyrin. GABA is removed from the synaptic cleft by specific transporters, GABA transporters (GATs), located both on presynaptic nerve terminals and on adjacent glial cell membranes. GABA is again converted into glutamate by GABA transaminase (GABA-T). In glial cells, glutamate can be further metabolized to glutamine, which is more easily taken up by neurons via amino acid transporters and reused for GABA synthesis. $GABA_B$ receptors, located on presynaptic GABAergic nerve terminals, suppress the release of GABA, by inhibiting Ca^{2+} influx. Modified from Möhler et al. (2002).

GABA can thus also be taken up by surrounding astrocytes where it is metabolized into succinic semialdehyde by GABA-T, which is again transformed into glutamate (GABA shunt) (Waagepetersen *et al.*, 2003). Because GAD is not present in glia, glutamate cannot be

converted into GABA and is instead transformed by glutamine synthetase into glutamine (Waagepetersen *et al.*, 2003; Schousboe & Waagepetersen, 2006). Glutamine is then transferred back to neurons by specific transporters (Varoqui *et al.*, 2000), where it can be converted by GAD to regenerate GABA (Schousboe & Waagepetersen, 2006).

2.1.3 $GABA_A$ receptor

The GABA receptors are divided into three groups: $GABA_A$, $GABA_B$ and $GABA_C$ (reviewed in Chebib & Johnston, 2000). The $GABA_B$ and $GABA_C$ receptors are shortly described later, whereas the $GABA_A$ receptor, the main interest of this work, will be further discussed here.

$GABA_A$ receptors are ligand-gated ion channels that belong to the same superfamily of receptors as the nicotinic acetylcholine receptor, glycine receptors, and the serotonin 5-HT_3 (5-hydroxytryptamine) receptor, although they exhibit a low sequence homology (approximately 10-25%) (reviewed in Leite & Cascio, 2001). The receptors are heteropentameric protein complexes that arrange around a central water-filled pore that constitutes the Cl^- channel (Chebib & Johnston, 2000). When GABA binds to $GABA_A$ receptors the channel responds by opening and mediating fast inhibitory neurotransmission (Figure 2A).

2.1.3.1 Subunit genes

The receptors are thus constructed of five subunits from several related genes or gene classes. The first of the $GABA_A$ receptor subunits was cloned in 1987 (Schofield *et al.*, 1987). Since then 16 different subunit genes have been identified and categorized into seven functionally distinct classes: α1-6, β1-3, γ1-3, δ, ε, θ, π (Sieghart, 1995; Barnard *et al.*, 1998; Bonnert *et al.*, 1999; Sinkkonen *et al.*, 2000; Korpi *et al.*, 2002; Whiting, 2003). All subunits are products of separate genes, but further variety is caused by several splice forms, e.g. for α6, β2 and γ2 subunits (Whiting *et al.*, 1990; Harvey *et al.*, 1994; Korpi *et al.*, 1994). The γ2 subunit exists in short (γ2S) and long (γ2L) forms distinguished by an additional 8 amino acids in the cytoplasmic loop of the longer form (Whiting *et al.*, 1990). There is a high conservativity among subunits at the amino acid level, with about 70% sequence similarity within classes and about 30% between classes (Korpi *et al.*, 2002). The subunits are polypeptides of approximately 50 kDa in size. They all possess a similar putative membrane topology, comprising a long extracellular N-terminal domain, four transmembrane spanning segments (M1-M4) (Schofield *et al.*, 1987), a long intracellular sequence between M3 and M4, and a short extracellular C-

terminal loop. The M2 region of each of the five subunits arranges to form the wall of the channel pore (Johnston, 2005) (Fig 2B).

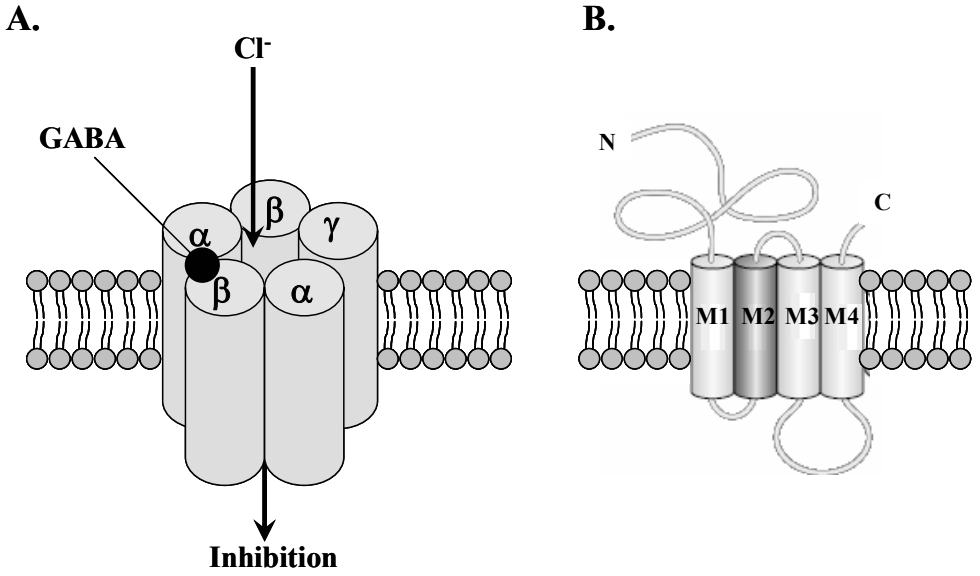

Figure 2. Schematic structure of the $GABA_A$ receptor. A. The receptors are heteropentameric proteins most commonly composed of 2α, 2β and 1γ subunit forming an integral Cl^- channel. Binding of GABA to its distinct binding site at the receptor allows influx of Cl^- ions leading to inhibitory neurotransmission. B. Each subunit polypeptide chain consists of a large extracellular N-terminal region, four transmembrane regions (M1-M4), and a short extracellular C-terminal portion.

2.1.3.2 Subunit expression

The large number of receptor subunits provides an enormous number of possible subunit combinations and calculations indicate that more than 2000 distinct $GABA_A$ receptors could exist (Moss & Smart, 2001; Johnston, 2005). However, subunit studies show that every combination is not possible and it seems that only certain subunit combinations are preferred in the CNS (Barnard *et al.*, 1998; Whiting, 2003). The number of combinations is reduced by the limited spatiotemporal overlap in the subunit expression patterns (Laurie *et al.*, 1992; Wisden *et al.*, 1992; Fritschy & Möhler, 1995) and by the rules determining subunit assembly into functional receptors (reviewed by Moss & Smart, 2001). Coexpression of α and β subunits in heterologous cells is enough to assemble GABA-gated ion channels on the cell surface (Günther *et al.*, 1995; Fritschy *et al.*, 1997), but these receptors lack the full range of electrophysiological and pharmacological properties. Evidence from both native and recombinant receptors indicate that a single receptor complex commonly contains two α, two β and one γ subunit, with the δ, ε,

π and θ subunits being able to substitute for the γ-subunit (Chang *et al.*, 1996; Tretter *et al.*, 1997; Baumann *et al.*, 2001). Also receptors containing two isoforms of a subunit class, e.g. certain co-localized α subunit pairs, on various neurons have been detected with immunofluorescent stainings (Fritschy *et al.*, 1992).

In the adult brain, the α1 is the most prevalent subunit and the α1β2γ2 combination is widely expressed and represents the major subtype of $GABA_A$ receptors (Laurie *et al.*, 1992; Wisden *et al.*, 1992; Whiting, 2003). In contrast, most other subunits display a unique regional and/ or cellular distribution in the mammalian CNS, and this will determine the functional and pharmacological properties of the receptor (Sieghart, 1995; Bonnert *et al.*, 1999; Korpi *et al.*, 2002). For example, the α4 subunit is concentrated in the thalamus and hippocampal dentate gyrus (DG) (Wisden *et al.*, 1992; Sperk *et al.*, 1997). Other subunits expressed in the hippocampus are the α2 and α5 subunits, that are predominantly expressed in pyramidal cells, most commonly combined with β3 and γ2 (Laurie *et al.*, 1992; Wisden *et al.*, 1992; Fritschy & Möhler, 1995; Sperk *et al.*, 1997; Pirker *et al.*, 2000; Brünig *et al.*, 2002a; Christie *et al.*, 2002). The α2 subunit is expressed only weakly in interneurons, whereas immunoreactivity for the α1 subunit is found over the entire hippocampus (Laurie *et al.*, 1992; Fritschy & Möhler, 1995; Sperk *et al.*, 1997; Pirker *et al.*, 2000). α1 is strongly expressed especially in parvalbumine-positive GABAergic interneurons, colocalized with the β2 and γ2 subunits (Fritschy & Möhler, 1995; Sperk *et al.*, 1997), but also found in principal cells (Nusser *et al.*, 1995, 1996). Some neuronal populations, such as the dentate granule cells, contain virtually all $GABA_A$ receptor subunit mRNAs (Laurie et al., 1992; Wisden et al., 1992). The α6 subunit, on the other hand, is only expressed in cerebellar granule cells (Lüddens *et al.*, 1990; Nusser *et al.*, 1998b). γ1 is a rather rare subunit, that still seems to be the major subunit e.g. in the hypothalamus, septum, and amygdala (Wisden *et al.*, 1992; Pirker *et al.*, 2000). All $GABA_A$ receptors are thought to contain β subunits, with the possible exception of the θ subunit-containing receptors which are unlikely to be present in the hippocampus (Sinkkonen *et al.*, 2000). The ε and θ subunits are fairly similarly expressed in the noradrenergic locus coeruleus of the brainstem (Bonnert *et al.*, 1999; Sinkkonen *et al.*, 2000). Additionally, the π subunit is abundant in reproductive tissues, but is virtually absent in the brain (Hedblom & Kirkness, 1997).

Furthermore, $GABA_A$ receptors with different subunit compositions seem to be distributed to different cellular compartments. This has been well documented in hippocampal pyramidal

neurons containing α1, α2, and α5 subunits together with β1-3 and γ2 subunits, suggesting that they can coexpress several distinct receptor subtypes in the same cell (Fritschy & Möhler, 1995; Pirker *et al.*, 2000). Parvalbumine-positive basket cells make synapses with receptors that contain α1 subunits equally distributed on neuronal soma, proximal and distal dendrites, spines, and axon initial segment of pyramidal cells (Nusser *et al.*, 1996; Nyíri *et al.*, 2001; Klausberger *et al.*, 2002). In contrast, receptors that contain α2 subunits are preferentially localized at axo-axonic synapses on the axon initial segment of Cornu Ammonis (CA) field 1 (CA1) pyramidal cells, (Nusser *et al.*, 1996; Fritschy *et al.*, 1998), known to be innervated by parvalbumine-immunoreactive cells (Nyíri *et al.*, 2001). To a lesser extent α2 subunit-containing receptors are found postsynaptically at axo-somatic synapses formed by cholecystokinin-positive (parvalbumine-negative) basket cells (Nusser *et al.*, 1996; Nyíri *et al.*, 2001).

Although most $GABA_A$ receptors are located postsynaptically, some structurally and functionally distinct subtypes are also differentially located to extrasynaptic membrane sites (Mody, 2001; Fritschy & Brünig, 2003). The targeting of $GABA_A$ receptors to these sites has been extensively analysed in cerebellar granule cells (Nusser *et al.*, 1998b) where receptors that contain δ subunits, mainly α4βδ and α6βδ, are specifically targeted to extrasynaptic domains (Jones *et al.*, 1997; Nusser *et al.*, 1998b; Sur *et al.*, 1999). α5 subunits are predominantly expressed at extrasynaptic regions of pyramidal neurons in the CA1 and CA3 regions of the hippocampus (Fritschy *et al.*, 1998; Brünig *et al.*, 2002a), but also at synapses (Christie & de Blas, 2002). Furthermore, there is also evidence for presynaptic $GABA_A$ receptors e.g. at hippocampal mossy fibers (MFs) (Ruiz *et al.*, 2003). The mechanism that underlies differential subcellular targeting of $GABA_A$ receptor isoforms remains largely speculative. Studies in hippocampal pyramidal cell cultures, however, have shown that in the absence of GABAergic innervation mismatched $GABA_A$ receptor clusters were formed opposite to presynaptic glutamate-containing terminals, suggesting the importance of presynaptic factors for proper synapse formation (Brünig *et al.*, 2002b; Christie *et al.*, 2002).

2.1.3.3 Receptor assembly and intracellular trafficking

Neurotransmitter receptors have to accumulate postsynaptically for efficient synaptic transmission, and studies have demonstrated that functional $GABA_A$ receptors are clustered opposite to GABAergic terminals at postsynaptic sites (Maccaferri *et al.*, 2000; Ruiz *et al.*, 2003). The γ2 subunit has a central role in the clustering of synaptic $GABA_A$ receptors, not only

in embryonic cells undergoing synapse formation (Essrich *et al.*, 1998) but also in more mature neurons with existing synaptic contacts (Schweizer *et al.*, 2003). In fact, the γ2 subunit is part of all naturally occurring postsynaptically located $GABA_A$ receptor subtypes identified to date, most commonly associated with the α1, α2, or α3 subunit (Essrich *et al.*, 1998; Fritschy *et al.*, 1998; Brünig *et al.*, 2002a). The mechanisms how $GABA_A$ receptors are maintained at synapses are still poorly understood. Several intracellular scaffolding proteins have shown to interact with the receptors for clustering at their synaptic locations (Moss & Smart, 2001; Fritschy & Brünig, 2003), and the functionally best characterized scaffolding proteins are discussed below.

Gephyrin is a 93-kDa, highly concentrated protein in the subsynaptic compartment of inhibitory synapses, and it was first identified to colocalize with glycine receptors (Kirsch *et al.*, 1991). It interacts with tubulin of the cytoskeleton (Kirsch *et al.*, 1991), and forms a hexagonal lattice that serves as a subsynaptic scaffold (Schwarz *et al.*, 2001). In the brain, gephyrin shows a dominant role for postsynaptic clustering of $GABA_A$ receptors, probably together with the γ2 subunit (Kneussel *et al.*, 1999; Brünig *et al.*, 2002a; Brünig *et al.*, 2002b; Christie & de Blas, 2002), seen in both immature and mature synapses (Essrich *et al.*, 1998; Schweizer *et al.*, 2003). It also seems essential for proper localization of receptors containing the α2 subunit (Essrich *et al.*, 1998; Kneussel *et al.*, 1999). No direct biochemical interaction between $GABA_A$ receptors and gephyrin has, however, been demonstrated, so there might be other possible intermediate proteins involved (Fritschy & Brünig, 2003).

Dystrophin is a protein restricted to postsynaptic sites that has been associated with a subset of $GABA_A$ receptors containing α1 or α2 subunit, e.g. in the hippocampus (Knuesel *et al.*, 1999). Studies in mutant mouse (*mdx*) that lacks dystrophin, show no change in gephyrin clustering, but a significant reduction in $GABA_A$ receptor clusters. Dystrophin might therefore regulate the stability of $GABA_A$ receptors at synapses (Knuesel *et al.*, 1999). It is suggested to cluster selectively opposite to GABAergic terminals, unlike gephyrin that together with $GABA_A$ receptors may be mistargeted to glutamatergic synapses (Brünig *et al.*, 2002b).

A protein called $GABA_A$ receptor-associated protein (GABARAP), that specifically interacts with the cytoplasmic domain of the γ2 subunit as well as with microtubules and gephyrin, has recently been identified (Wang *et al.*, 1999), and is implied to have a role for clustering of $GABA_A$ receptors. However, GABARAP is concentrated in the Golgi complex and other vesicular bodies including subsynaptic cisternae and is present only scarcely at inhibitory synapses (Kittler *et al.*, 2001). Thus it might therefore facilitate the intracellular transport of

$GABA_A$ receptors between the Golgi apparatus and synaptic sites (Moss & Smart, 2001). The mechanisms responsible for this targeting remain unknown but likely involve several anchoring proteins.

Additional mechanisms involving the intracellular trafficking of $GABA_A$ receptors have been detected. Palmitoylation of residues on the cytoplasmic loop of the γ2 subunit by a novel $GABA_A$ receptor-associated membrane protein named Golgi apparatus-specific protein with the DHHC Zn^{2+} finger domain, represents a new, reversible post-translational modification that is important for normal clustering of $GABA_A$ receptors at synapses (Keller *et al.*, 2004). $GABA_A$ receptor function can also be modulated through phosphorylation. Several protein kinases including cAMP-dependent protein kinase, protein kinase C (PKC) can phosphorylate key residues within the intracellular loop of β1-3 and γ2 subunits (McDonald *et al.*, 1998; Connolly *et al.*, 1999). The phosphorylation has been suggested to be important in functional regulation (ranging from enhancement to inhibition of the GABA-mediated anionic currents) and cell-surface stability of $GABA_A$ receptors (McDonald *et al.*, 1998; Connolly *et al.*, 1999).

It was recently shown that $GABA_A$ receptors are constitutively internalized by clathrin-dependent endocytosis (Kittler *et al.*, 2000) and are constantly cycling from synaptic sites on the membrane to internal endocytic structures, endosomes (Connolly *et al.*, 1999; van Rijnsoever *et al.*, 2005). During endocytosis, $GABA_A$ receptors interact with the adaptin complex AP2, constituting of adaptin proteins that are one of the main components of the clathrin-coated pits subsequently converting into endosomes (Kittler *et al.*, 2000). Individual subunits or incomplete $GABA_A$ receptors are retained within the endoplasmic reticulum and are quickly degraded (Connolly *et al.*, 1999). Only receptors that are fully assembled as pentamers (α and β or α, β, and γ) reach the cell surface (Connolly *et al.*, 1999). PKC may regulate αβγ receptor expression at the cell surface by recycling them from endosomes (Connolly *et al.*, 1999).

2.1.4 $GABA_A$ receptor function

For the $GABA_A$ receptor class of receptors, ligand binding is followed by a conformational change in the channel protein that allows a net inward or outward flow of ions through the membrane-spanning pore of the channel, depending on the electrochemical gradient of the particular permeant ion. $GABA_A$ receptors carry primarily Cl^- ions; however, other anions, such as bicarbonate (HCO_3^-), can also permeate the channel pore, although less efficiently (Kaila, 1994). Activation of $GABA_A$ receptors by GABA in the adult rat brain increases Cl^-

permeability of the neuronal membrane, allowing an inward Cl^- flow that leads to a hyperpolarizing postsynaptic response, the inhibitory postsynaptic potential (IPSP), and decreased excitability of the neuron. As mentioned earlier the receptor has been found both pre- and postsynaptically and Cl^--dependent $GABA_A$ receptor-mediated synaptic inhibition can occur at both synaptic sites.

2.1.4.1 Types of inhibitory action

Most neurons in the brain show two different types of inhibitory action for the $GABA_A$ receptor: phasic and tonic inhibitory activity. These two types of inhibition correlate with the structurally and functionally distinct $GABA_A$ receptor subtypes that are differentially located to postsynaptic and extrasynaptic membrane sites (Mody, 2001; Fritschy & Brünig, 2003). Neurotransmitter receptors located traditionally at the synapse mediates the phasic neurotransmission, where postsynaptic receptors are activated by a GABA transient that is rapidly diffused away from the release site (Brickley *et al.*, 1996). Thereby, miniature inhibitory postsynaptic currents (mIPSCs) with a rapid onset are generated, followed by deactivation of the receptor and decay of the inhibitory postsynaptic current (IPSC) when the ion channel closes and the ligand is removed (Brickley *et al.*, 1999). As mentioned earlier, some neurotransmitter receptors, on the other hand, are located in the membrane outside the synapse (extrasynaptic) where they respond to neurotransmitter spilled out of the synapse and hence mediate tonic activity (Mody, 2001). Consistent with such a paracrine activity, these receptors have a high affinity for GABA, and are therefore activated by the low concentrations of the neurotransmitter found outside the synapse, and they desensitize more slowly.

Tonic activity was first identified in cerebellar granule cells and these cells, where synapses made with Golgi cells constitutes the only inhibitory input, have provided a good model of the inhibitory actions (Brickley *et al.*, 1996; Nusser *et al.*, 1998b). Electrophysiological and anatomical evidence from cerebellar granule neurons suggests that phasic inhibitory currents are mediated by postsynaptic $GABA_A$ receptors that contain the $\gamma 2$ subunit in combination with diverse α and β subunits (Nusser *et al.*, 1998b; Brickley *et al.*, 1999). On the other hand, receptors containing the δ subunit together with the $\alpha 6$ subunit preferentially localize to extrasynaptic sites, where they mediate tonic inhibition (Jones *et al.*, 1997; Nusser *et al.*, 1998b; Brickley *et al.*, 1999; Mody, 2001; Wisden *et al.*, 2002). Tonic currents mediated by extrasynaptic $GABA_A$ receptors have subsequently been found also in several other brain

regions, such as α5 subunit-containing receptors in hippocampal CA1 pyramidal cells (Collinson *et al.*, 2002; Wisden *et al.*, 2002), and in receptors containing the δ subunit, likely together with the α4 subunit in granule cells of the DG (Nusser & Mody, 2002).

2.1.5 Molecular pharmacology

The era of $GABA_A$ receptor pharmacology started in the 1970s, when researchers discovered that that the convulsant alkaloid bicuculline antagonizes certain inhibitory actions of GABA (Curtis *et al.*, 1971). Since then it has been found that the multitude of $GABA_A$ receptor subunits forms receptors with different subtypes and many different drug binding sites, and they are indeed pharmacological targets for many groups of drugs (Figure 3). Drugs acting on $GABA_A$ receptors are in clinical use for treating anxiety, epilepsy, sleep disorders, cognitive and mood disorders, alcohol withdrawal, and for induction and maintenance of anesthesia (Korpi *et al.*, 2002; Johnston, 2005). These drugs mainly act to increase GABA-mediated synaptic inhibition either by directly activating $GABA_A$ receptors or, more commonly, by enhancing the action of GABA on $GABA_A$ receptors. This latter action is known as positive allosteric modulation, and the drugs act as agonists on allosteric sites that are remote from the GABA site on the receptor, thereby increasing the affinity of the receptor for GABA at its own binding site and consequently also the frequency of channel openings. The benzodiazepine (BZ) site is such an allosteric site (Sieghart, 1995; Korpi *et al.*, 2002). BZs act as agonists on $GABA_A$ receptors (Johnston, 2005). They are widely used therapeutic drugs in the treatment of anxiety disorders, sleep disturbances, and epilepsy including SE. Still, the use of BZs is limited because they may produce tolerance and dependence.

Drugs that reduce the action of GABA on $GABA_A$ receptors are known as negative allosteric modulator, or inverse agonists, that have the opposite actions to classical BZs. They decrease both the frequency of channel openings and the efficacy of GABA binding. Inverse agonists, such as ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine -3-carboxylate (Ro 15-4513) (Sieghart *et al.*, 1987), tend to be convulsant and anxiogenic. Agents that block the actions of both positive and negative allosteric modulators are known as neutralizing allosteric modulators or antagonists, e.g. the classical BZ antagonist flumazenil. They bind to and occlude the BZ-binding site, but do not affect channel function and can be used to reverse the actions of an agonist or inverse agonist, as in the treatment of an overdose with a BZ agonist.

Selective competitive agonists that directly activate $GABA_A$ receptors are, in addition to GABA, conformationally restricted analogs such as muscimol and 4, 5, 6, 7-tetrahydroisoxazolo[5, 4-c] pyridine-3-ol (THIP). Muscimol acts as a full agonist in most $GABA_A$ receptor subtypes, whereas THIP seems to act as a partial agonist (Ebert *et al.*, 1997). Several competitive antagonists of $GABA_A$ receptors are also known, including bicuculline and SR 95531 (Heaulme *et al.*, 1986). In addition, the $GABA_A$ receptor antagonists picrotoxin, pentylenetetrazol, and *t*-butylbicyclophosphorothionate (TBPS) bind at or near the Cl^- channel at an assumed "convulsant site" and non-competitively block GABA-gated Cl^- currents (Squires *et al.*, 1983). These compounds also cause convulsions in animals.

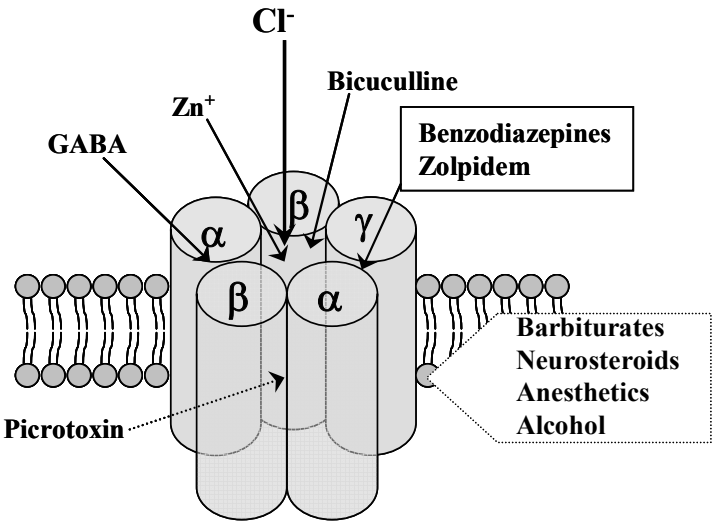

Figure 3. Major drug-binding sites of the $GABA_A$ receptor. The binding sites for GABA and benzodiazepines are located extracellularly at the interface of the α/β and α/γ2 subunits, respectively. Some sites, e.g. for barbiturates, neurosteroids, anesthetics and alcohol, may be located at the M regions, whereas other binding sites, e.g. for Zn^{2+} and picrotoxin, are most likely located near the Cl^- channel pore.

$GABA_A$ receptors are also the site of action for a large number of sedative-hypnotic and anesthetic agents, including barbiturates, ethanol, and volatile anesthetics. They facilitate GABA's ability to activate the receptor by prolonging the time that the Cl^- channel remains open. Barbiturates bind to a distinctly separate location, most likely near the Cl^- channel pore. The mechanism of ethanol-induced $GABA_A$ receptor enhancement is unclear, and different studies have produced controversial results.

Furthermore, there are many endogenous ligands that may influence $GABA_A$ receptor function *in vivo* including metal ions such as Zn^{2+}, steroids , and chemicals derived from the diet

such as flavonoids (reviewed by Korpi *et al.*, 2002; Johnston, 2005). Zn^{2+} has an opposite effect to barbiturates, thus slowing the onset and accelerating the deactivation kinetics of the receptor. It does not competitively interact with the GABA recognition site and probably has a different recognition site formed by the interaction of several subunits (Berger *et al.*, 1998). Neuroactive steroids and their synthetic derivatives may be synthesized endogenously in the brain, and modulate brain function by still unknown mechanisms. However, neurosteroids can allosterically regulate $GABA_A$ receptor function to enhance or attenuate Cl^- conductance. Neurosteroids recognize a distinct site on the $GABA_A$ receptor, probably dependent on certain subunit combinations (Siegwart *et al.*, 2002).

2.1.5.1 Subunit-selective drugs

Classical BZs, such as diazepam and flunitrazepam, are thought to act on $GABA_A$ receptors at a binding pocket at the interface between the γ2 subunit and α subunits (α1, α2, α3 and α5 subunits), that contain a conserved histidine residue at the amino acid position 101 in the BZ binding domain on the extracellular N-terminus. $GABA_A$ receptors containing α4 or α6 subunits have an arginine at the corresponding position and they are relatively insensitive to classical BZs (Wieland *et al.*, 1992). When the BZ-sensitive histidine residue was replaced to an arginine in the α1 subunit these receptors became diazepam-insensitive (Wieland *et al.*, 1992). By this kind of "knock-in" approach of the BZ-insensitive residue into several α subunits, the behavioural roles of these subtypes have been defined. Concluded from these studies it seems that the α1-containing receptors are important for the sedative, amnesic and partially the anticonvulsant actions of BZs (Rudolph *et al.*, 1999; Crestani *et al.*, 2002) whereas α2-containing receptors seem to mediate anxiolytic effects (Rudolph *et al.*, 1999; Löw *et al.*, 2000). Mice lacking the α5 subunit, which is mainly located in the hippocampus, showed decreased GABA-mediated synaptic inhibition and enhanced learning (Collinson *et al.*, 2002).

The α subunit variant thus mainly determines the binding properties of BZ site on the receptor (Pritchett *et al.*, 1989; Pritchett & Seeburg, 1990; Lüddens *et al.*, 1995), and these sites have been divided into types I (α1) and II (α2, α3 and α5) according to their α subunit affinity (Pritchett *et al.*, 1989; Pritchett & Seeburg, 1990; Lüddens *et al.*, 1995). Classical BZs are nonselective for type I and II BZ receptors. In addition, a third group of receptors were found that bound RO 15-4513 with high affinity, but were insensitive to diazepam (Sieghart *et al.*,

1987). RO 15-4513 is a BZ site antagonist somewhat selective for cerebellar α6-containing $GABA_A$ receptors (Lüddens *et al.*, 1990). This drug reduces ataxia produced by ethanol without altering other actions of ethanol. Ligands, such as zolpidem and CL 218,872 have high affinity for type I receptors, whereas they show low affinity for type II receptors (Pritchett *et al.*, 1989; Pritchett & Seeburg, 1990; Lüddens *et al.*, 1995). They can further be distinquished as zolpidem prefers receptors containing the α1 and γ2 subunits together with a β subunit whereas CL 218,872 has a higher affinity for receptors with the α1, γ3 and β subunits (Lüddens *et al.*, 1995). Zolpidem is a non-BZ, clinically used sedative-hypnotic drug that has provided a basis for further classification of the BZ-sensitive $GABA_A$ receptors (Korpi *et al.*, 1997) into three distinct $GABA_A$ receptor subtypes with high (α1), low (α2, α3), and very low (α5) affinity for zolpidem (Ruano *et al.*, 1992; Benavides *et al.*, 1993).

2.1.6 Other GABA receptors

Other members of the GABA receptor family are the metabotropic $GABA_B$ receptor and the ionotropic $GABA_C$ receptor. Similarities as well as differences can be found when comparing these receptors with the $GABA_A$ receptor. They have also been shown to interact with $GABA_A$ receptors.

2.1.6.1 $GABA_B$ receptor

At the beginning of the 1980s, a bicuculline-insensitive, Cl^--independent GABA response was discovered in the brain. This was shown to be mediated by a metabotropic receptor termed the $GABA_B$ receptor (Bowery *et al.*, 1980). $GABA_B$ receptors belong to the superfamily of heterotrimeric guanine nucleotide-binding protein (G-protein) coupled seven-transmembrane-domain receptors that inhibit adenylyl cyclase via the $G_{\alpha i/o}$ subunits of the G-protein (Holopainen & Wojcik, 1993). $GABA_B$ receptors are localized both pre- and postsynaptically, and they use different mechanisms at these locations to regulate cell excitability (Bettler *et al.*, 2004). In the hippocampus, presynaptic $GABA_B$ receptors located both on inhibitory (GABAergic) and excitatory (glutamatergic) terminals are proposed to be tonically activated by ambient levels of GABA (Kubota *et al.*, 2003).

$GABA_B$ receptors were not cloned until 1997 and thus remained the last of the major neurotransmitter receptors to be characterized at the molecular level (Kaupmann *et al.*, 1997). They show sequence homology with the metabotropic glutamate receptors (mGluRs)

(Kaupmann *et al.*, 1997). Two molecular subunits of the $GABA_B$ receptor, $GABA_{B1}$ and the $GABA_{B2}$, have been characterized and both subunits seem to be required to assemble functional heterodimeric receptors (Bettler *et al.*, 2004). In the rat brain, $GABA_{B1}$ mRNA is detectable in almost all neuronal cell populations, with highest levels of expression in hippocampus, thalamic nuclei, and cerebellum (Liang *et al.*, 2000). High levels of $GABA_{B2}$ mRNA are found in cortex, hippocampus, thalamus, and in cerebellar Purkinje cells (Liang *et al.*, 2000).

$GABA_B$ receptors are considered to be promising drug targets (Bettler *et al.*, 2004). They are activated by baclofen, a competitive agonist, and inhibited by phaclofen, a competitive antagonist. Baclofen is a lipophilic derivative of GABA and is used to treat spasticity and skeletal muscle rigidity in patients with spinal cord injury, multiple sclerosis, and cerebral palsy (Wojcik & Holopainen, 1992; Bettler *et al.*, 2004). A number of studies have also shown the involvement of $GABA_B$ receptors in the etiology of epilepsies. A dysfunctional $GABA_B$ system is proposed to be one of the causes of temporal lobe epilepsy (TLE) (Scanziani *et al.*, 1994; Gambardella *et al.*, 2003).

2.1.6.2 $GABA_C$ receptor

A third related ionotropic GABA receptor, termed the $GABA_C$ receptor, has also been identified (Johnston *et al.*, 1975; Polenzani *et al.*, 1991). This receptor is a Cl^--selective ion-channel but differs from the $GABA_A$ receptor by having a smaller single channel conductance, meaning a longer lasting inhibition (Feigenspan & Bormann, 1994), and a higher affinity for GABA than the $GABA_A$ receptors (Feigenspan & Bormann, 1994; Wang *et al.*, 1994). $GABA_C$ receptors are believed to be homo- or heteropentameric proteins that are composed of ρ-subunits, of which three subunits have been identified, the ρ1, ρ2 and ρ3 subunits (Cutting *et al.*, 1991; Alakuijala *et al.*, 2005). Little is known about their function, but as the ρ-subunits share sequence homology (30-38%) with the so far identified $GABA_A$ receptor subunits, $GABA_C$ receptors can be considered as pharmacological variants of $GABA_A$ receptors. The functional characteristics of these receptors are nearly identical to extrasynaptic $GABA_A$ receptors composed of α6 and δ subunits at the cerebellar Golgi cell-granule cell synapse (Alakuijala *et al.*, 2006). In addition, extrasynaptic $GABA_C$ receptors possessing tonic inhibitory activity have already been found in the retina (Cutting *et al.*, 1991; Polenzani *et al.*, 1991; Enz *et al.*, 1995). Recent evidence indicates that the receptor is also expressed widespread in the CNS, including the cortex,

thalamus, cerebellum, hippocampus and spinal cord (Johnston *et al.*, 1975; Enz *et al.*, 1995; Alakuijala *et al.*, 2006).

The pharmacology of the $GABA_C$ receptors also differs from that of the $GABA_A$ receptors. Agents like muscimol and cis-4-aminocrotonic acid act on $GABA_C$ receptors as agonists (in addition to GABA) (Johnston *et al.*, 1975; Alakuijala *et al.*, 2006), that suppress postsynaptic excitability and increase the membrane conductance, and they are antagonized by Cl^--channel blockers such as picrotoxin (Feigenspan & Bormann, 1994; Wang *et al.*, 1994). On the other hand, $GABA_C$ receptors are insensitive to potent $GABA_A$ receptor modulators such as BZs, barbiturates and neurosteroids (Feigenspan & Bormann, 1994; Wang *et al.*, 1994), and they neither respond to the $GABA_A$ receptor antagonist bicuculline nor to the $GABA_B$ receptor agonist baclofen (Cutting *et al.*, 1991; Polenzani *et al.*, 1991; Wang *et al.*, 1994). The major indications for drugs acting on $GABA_C$ receptors are in the treatment of epilepsy, as well as visual, sleep and cognitive disorders (Johnston *et al.*, 2003).

2.1.7 GABA in the developing brain

As GABA is able to depolarize postsynaptic cells in the early postnatal rat brain, it has been suggested that fast excitatory synaptic transmission is mediated by $GABA_A$ receptors (Cherubini *et al.*, 1991; Ben-Ari *et al.*, 1997). Later in development, around the end of the second postnatal week in the rat, GABA becomes mainly hyperpolarizing (Ben-Ari *et al.*, 1989; Khazipov *et al.*, 2004). The depolarizing effects of $GABA_A$ receptor-mediated responses in immature neurons are due to a high intracellular Cl^- concentration (Kaila, 1994; Payne *et al.*, 2003). During neuronal maturation the intracellular Cl^- concentration decreases, causing a shift in the equilibrium potential of Cl^- to more negative values (–60 to –70), which allows the effect of GABA to become progressively inhibitory (Rivera *et al.*, 2005). Consistent with this is the differential expression of cation-chloride co-transporters during different developmental stages (Rivera *et al.*, 1999). The main transporter mediating Cl^- uptake in immature neurons is the inwardly directed Na-K-2Cl co-transporter isoform 1, NKCC1 (Kaila, 1994; Payne *et al.*, 2003). The developmental shift of GABA becoming hyperpolarizing is primarily attributed to the developmental up-regulation of the outwardly directed K-Cl co-transporter isoform 2, KCC2, by the end of the second postnatal week in the rat (Rivera *et al.*, 1999; Payne *et al.*, 2003). These developmental gradients have also been detected in primate neurons in utero, and can thus be compared to development in human fetuses and preterm babies (Khazipov *et al.*, 2001).

In hippocampal interneurons and pyramidal cells functional GABAergic synapses are the first to be formed, even before glutamatergic ones are established (Khazipov *et al.*, 2001; Hennou *et al.*, 2002). The majority of synaptic activity and functional connections in the neonatal rat and monkey neocortex and hippocampus are hence $GABA_A$ receptor-mediated (Khazipov *et al.*, 2001; Hennou *et al.*, 2002). Before the functional maturation of synapses, GABA also exerts its effects in a paracrine fashion via tonic $GABA_A$ receptor activation, because of higher agonist affinity and longer-lasting activation kinetics of these receptors (Demarque *et al.*, 2002). These responses are mediated by the activation of $GABA_A$, but also to a lesser extent by N-methyl-D-aspartate (NMDA) receptors (Demarque *et al.*, 2002). The endogenous GABAergic depolarization may induce synaptic plasticity by removing the Mg^{2+} block of NMDA receptors, resulting in an increased Ca^{2+} influx to the cell (Ben-Ari *et al.*, 1997; Leinekugel *et al.*, 1997). This function is attributed to α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors in the adult brain, but as the first glutamatergic synapses [appearing after postnatal day (P) 2] only consist of NMDA receptors, $GABA_A$ receptors play the role of AMPA receptors in the immature brain, acting in synergy with NMDA receptors (Ben-Ari *et al.*, 1997; Leinekugel *et al.*, 1997).

These events where NMDA receptor-mediated signals are potentiated by the depolarizing effect of GABA, called GDPs (giant depolarizing potentials), occurs during the first two postnatal weeks in the rat hippocampus (Ben-Ari *et al.*, 1989; Khazipov *et al.*, 2004). GDPs are a synchronized neuronal activity associated with intracellular Ca^{2+} oscillations, thought to play a central role in the formation of functional neuronal circuits and provide conditions for plasticity in developing synapses (Ben-Ari *et al.*, 1997; Leinekugel *et al.*, 1997). These events also generate long-term potentiation (LTP) and long-term depression (LTD) of developing GABAergic synapses (McLean *et al.*, 1996).

The endogenous $GABA_A$ receptor activation early in development where GABA acts in a paracrinic fashion on distal neurons (Demarque *et al.*, 2002), together with the depolarization-induced increase in intracellular Ca^{2+}, have provided knowledge about GABA acting as a trophic factor in brain development (Brickley *et al.*, 1996; Demarque *et al.*, 2002). The trophic actions of GABA can influence processes such as DNA synthesis (LoTurco *et al.*, 1995), neuronal migration (Manent *et al.*, 2005), neuronal differentation (Marty *et al.*, 1996), and synaptogenesis (Marty *et al.*, 2000). Brain-derived neurotrophic factor (BDNF) has been shown to be an important mediator of the trophic effects of GABA in the postnatal hippocampus

(Marty *et al.*, 1996; Marty *et al.*, 2000). Moreover, during early development $GABA_A$ receptors may exert dual, that is inhibitory as well as excitatory actions, depending on the context of their activation (Khazipov *et al.*, 2004). Thus, GABA will provide a sufficient degree of excitatory drive to modulate developmental processes without danger, because GABAergic synapses may also inhibit overexcitation.

Apart from changes in the properties of GABA and in the $GABA_A$ receptor subunit expression there seem to be maturational changes in other components of the GABAergic system in the immature brain including metabotropic $GABA_B$ receptors, GABA transporters and GADs. The $GABA_{B1}$ and $GABA_{B2}$ receptor subunits have been detected during early embryonic and postnatal development of the CNS, even before the formation of synaptic circuits (Fritschy *et al*., 1999; Lopez-Bendito *et al*., 2002). It has been suggested that $GABA_B$ receptors function as the main inhibitory receptors in the neonatal rat hippocampus (Gaiarsa *et al*., 1995; Mc Lean *et al*., 1996). There is also evidence implicating for $GABA_B$ receptors as mediators of motility signals for migrating embryonic cortical cells (Behar *et al*., 2001). GABA itself is expressed early during rat brain development [embryonic day (E) 15] and the activity of its synthesizing enzyme, GAD, increases with age (Coyle & Enna, 1976). Both GAD and the main GABA transporter, GAT-1, are present before birth (Dupuy & Houser, 1996; Jursky & Nelson, 1996). In interneurons they show a largely similar distribution throughout the postnatal rat hippocampal development (Frahm & Draguhn, 2001). It has been shown that GAT-1 is up-regulated during the first two postnatal weeks, followed by a down-regulation to adult levels (Jursky & Nelson, 1996; Yan *et al*., 1997).

2.1.7.1 $GABA_A$ receptor expression and assembly

Studies applying *in situ* hybridization and immunohistochemistry have revealed that individual $GABA_A$ receptor subunits have a different temporal expression pattern, apart from the spatial, in the CNS. It has been suggested that the change in GABA function from a trophic, excitatory neurotransmitter in the immature brain to an inhibitory neurotransmitter in the mature brain could be, to some extent, connected to the developmental regulation of $GABA_A$ receptor subunit expression (Poulter *et al.*, 1992; Fritschy *et al.*, 1994; Paysan & Fritschy, 1998; Vicini *et al.*, 2001). In the developing brain, there is a specific subunit expression, with the $\alpha 2$, $\alpha 3$, $\alpha 5$ and $\beta 2/3$ subunits predominantly expressed during embryonic development, whereas $\alpha 1$ is most prominent during postnatal development and $\alpha 2$ decreases during the first postnatal week

(Laurie *et al.*, 1992; Fritschy *et al.*, 1994; Brooks-Kayal *et al.*, 1998, 2001). The expression levels of the γ1 and γ3 subunits drop markedly during development, whereas γ2 expression increases after birth and remains mostly constant throughout development (Fritschy *et al.*, 1994).

During synaptogenesis, the α2, α3, and α5 subunits (Hutcheon *et al.*, 2004) as well as the β2/3 and γ2 subunits (Fritschy *et al.*, 1994; Scotti & Reuter, 2001) are expressed and clustered early on in the membrane of neurons even before synapses have been formed, and maturation then proceed by recruitment of the α1 subunit receptors to these sites (Hutcheon *et al.*, 2004). The α1 subunit expression thus increases with age and becomes increasingly associated with synaptophysin (a synaptic vesicle at the presynaptic terminal), indicating an increased number of synapses formed (Hutcheon *et al.*, 2004). Also the developmental clustering of the β2/3 and γ2 subunits become increasingly associated with presynaptic terminals, but even after 30 days in culture, large fractions of these complexes are not associated, and were presumed to be extrasynaptic receptors (Scotti & Reuter, 2001). $GABA_A$ receptor β3 and γ2 subunit knockout mice are confronted with a high lethality and serious epileptic seizures (Günther *et al.*, 1995; Homanics *et al.*, 1997), indicating that these subunits are obligatory at least during development. Unexpectedly, deficiency of two of the most abundant subunits, α1 and β2, are not associated with lethal or strong behavioural effects, which could be due to a functional compensation by other $GABA_A$ receptor subunits (Sur *et al.*, 2001).

Moreover, differences have been found in the expression of BZ-sensitive sites between the adult and neonatal rat brain e.g. in the cortex and hippocampus (Ruano *et al.*, 1992; Benavides *et al.*, 1993; Roberts & Kellogg, 2000). It has been shown that a relatively high level of $GABA_A$/BZ receptors is present in the rat brain at birth, the levels then alters region-specifically during the development, and the adult levels are achieved by about three weeks of age (Chisholm *et al.*, 1983; Daval *et al.*, 1991). The expression of BZ binding sites stabilizes after P10 in the cortex and hippocampal CA1 and CA3 layers, increases in the DG up to adulthood, but decreases in certain thalamic regions (Daval *et al.*, 1991). The developmental increase in BZ binding has been demonstrated to be due to an additional number of specific sites. There is a postnatal increase in BZ type I receptors (Chisholm *et al.*, 1983; Bacon *et al.*, 1991), and the levels of high affinity zolpidem sites increase between P6 and P14 in the hippocampus, whereas the highest levels of the low and very low affinity zolpidem sites have been detected in P7 rats

(Roberts & Kellogg, 2000). These changes in BZ receptor expression coincide with the developmental increase of the α1 subunit and with the decrease of the α2 in many brain regions during the development (Poulter *et al.*, 1992; Fritschy *et al.*, 1994; Brooks-Kayal *et al.*, 2001). The BZ enhancement of $GABA_A$ receptor activity also increases in multiple brain areas over postnatal development (Kapur & Macdonald, 1999).

2.2 Hippocampus

The hippocampus is a part of the brain located under the temporal lobe (humans and other mammals have two hippocampi, one in each side of the brain), forming a part of the limbic system. Memory formation and spatial navigation are associated with the hippocampus (Ekström *et al.*, 2003), and hippocampal synaptic plasticity has been regarded as a cellular model of memory formation (Lynch *et al.*, 2000). Studies in rats have shown that certain neurons in the hippocampus, called place cells, may fire when the animal finds itself in a particular location (Ekström *et al.*, 2003). Recent experiments demonstrate that selective hippocampal damage results in profound amnesia in adult humans (Astur *et al.*, 2002), and prevents spatial relational learning in adult rodents and monkeys (Lavenex *et al.*, 2006). Removal of most of the medial temporal lobes in children to relieve frequent epileptic seizures, may also result in memory impairment (Cronel-Ohayon *et al.*, 2006). In contrast, in monkeys with neonatal hippocampal lesions some functional brain reorganization takes place that enables spatial information to be acquired through the use of brain regions that normally do not subserve this function. These monkeys showed normal spatial relational learning and memory (Lavenex *et al.*, 2007b).

2.2.1 Structural and functional organization

The hippocampal formation consists of the DG, the CA fields CA1-CA3, the hilus (considered part of the DG), and the subiculum. The main input to the hippocampus is received from cortical and subcortical structures. Cortical input is mainly derived from the entorhinal cortex that in turn receives input from many subcortical regions including the amygdala, medial septum, and thalamus (reviewed in Knowles, 1992). It is widely accepted that each of these hippocampal subregions has a unique functional role in the information processing of the hippocampus, but still to date the specific contribution of each region is poorly understood. In the most classical glutamatergic pathway, cells of the entorhinal cortex are connected by the perforant pathway to

the DG. The dentate granule cells are thereby excited and their axons, called MFs, project to the pyramidal cells of the CA3 field that are further excited. The CA3 region sends connections to the CA1 region through a set of fibers called the Schaffer collaterals. The CA1 pyramidal cell axons are the main output from the hippocampus and they project to the subiculum as well as to the enthorhinal cortex (Figure 4).

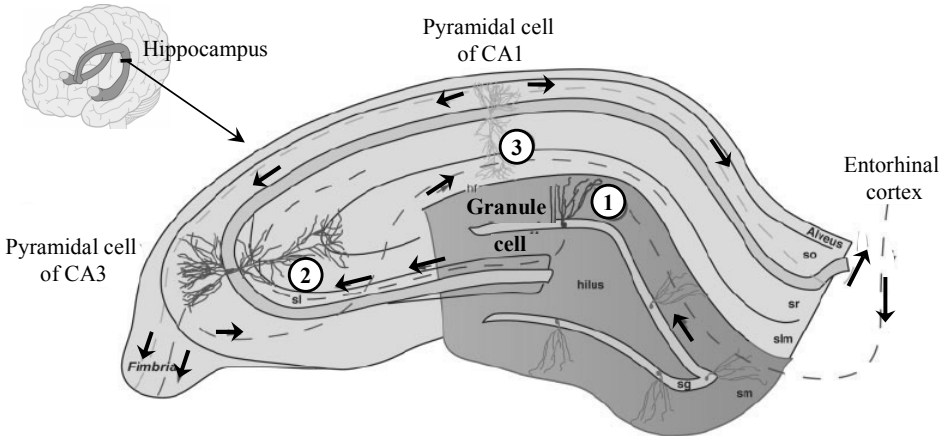

Figure 4. Structure and functional connections of the hippocampus. In the classical trisynaptic pathway granule cells of the dentate gyrus are excited by the perforant pathway coming from the entorhinal cortex (1). The granule cell axons, the mossy fibers excite the CA3 pyramidal cells (2), that in turn excite CA1 pyramidal cells via Schaffer collaterals (3). The axons of CA1 pyramidal cells are the main output from the hippocampus, that project back to the entorhinal cortex and the subiculum. Modified from Danglot *et al.* (2006).

2.2.1.1 Excitatory and inhibitory pathways

At all developmental stages of brain maturation, the strength of inhibitory and excitatory connections must be well equilibrated (Danglot *et al.*, 2006). In the adult brain, the vast majority of neurons are glutamatergic providing the main excitatory drive, whereas the inhibitory drive is provided by interneurons that constitute 10-15% of the neurons (Freund & Buzsaki, 1996). Upon excitation in the hippocampus, NMDA receptors are activated in principal cells and this is required for formation of LTP, a plasticity phenomenon important for the formation of memory (McLean *et al.*, 1996).

Interneurons are local circuit neurons responsible for inhibitory activity in the adult hippocampus, and despite their small amount, they control the activity of principal excitatory cells, i.e., pyramidal cells in the hippocampus proper and granule and mossy cells of the DG (Freund & Buzsaki, 1996), but some interneurons also inhibit each others (Freund & Buzsaki, 1996; Klausberger *et al.*, 2002). In the adult hippocampus, there are several types of GABAergic

interneurons whose morphological and physiological characteristics have been reviewed in detail (Freund & Buzsaki, 1996; Somogyi & Klausberger, 2005). The inhibitory interneurons are GABAergic. They inhibit principal cells through the activation of postsynaptic $GABA_A$ receptors, but they are also activated by excitatory afferents or by nearby glutamatergic neurons. Thereby, GABAergic interneurons establish local feedforward and feedback inhibitory circuits, respectively.

Moreover, subgroups of hippocampal interneurons establish connections with specific parts of the principal neurons (Freund & Buzsaki, 1996; Klausberger *et al.*, 2002) that may differentially affect the activity of excitatory neurons. A hippocampal pyramidal cell receives GABAergic innervation from several distinct interneurons (Freund & Buzsaki, 1996), e.g. the same postsynaptic domain of a pyramidal cell soma may be targeted by two distinct basket cells expressing specific calcium-binding proteins such as parvalbumin or cholecystokinin (Freund & Buzsaki, 1996; Nyíri *et al.*, 2001; Klausberger *et al.*, 2002). Indeed, the divergent axonal arborisation of interneurons allows them to contact hundreds of pyramidal cells (Sik et al., 1995). Furthermore, interneurons regulate not only the excitability of the hippocampus, but act also as stable oscillators, which is important for the generation of rhythmic activities in neuronal networks (Somogyi & Klausberger, 2005). By innervating perisomatic regions of pyramidal cells, hippocampal basket cells synchronize their activity thereby generating network oscillations (Somogyi & Klausberger, 2005).

2.2.2 Development of the hippocampus

Morphological studies have shown that comparable stages of hippocampal development occur much earlier in primates than in rodents with most of the developmental processes taking place in utero in human and in nonhuman primates (Bayer, 1980; Khazipov *et al.*, 2001; Lavenex *et al.*, 2007a). There is a hippocampal-dependent form of recognition memory in human and monkey babies soon after birth (Bachevalier *et al.*, 1993; Pascalis & de Schonen, 1994), and the hippocampal network is active before birth in monkey (Khazipov *et al.*, 2001). Early studies of the development of neuronal activity in the rat brain demonstrated that irregular electroencephalography (EEG) activity appears at the age of 5-6 days (Deza & Eidelberg, 1967, Gramsbergen, 1976). However, up to the 10th postnatal day EEG activity is interrupted by periods of electrical silence (Gramsbergen, 1976; Mares *et al*., 1979). A similar brain activity pattern was seen in preterm newborns but not in full-term human newborns (Dreyfus-Brisac &

Monod, 1966). Around P11 considerable developmental changes occur in the EEG of rats with a high amplitude pattern that adopts the adult basic rhythmic activity by the third postnatal week (Deza & Eidelberg, 1967; Gramsbergen, 1976; Mares *et al*., 1979). An important period of brain development is the so-called brain growth spurt, a transient period when the brain is growing most rapidly, with intense neuronal growth and synaptogenesis. This occurs in the first 2 postnatal weeks in the rat and between the third trimester of gestation and first 2 years of life in humans (Dobbing & Sands, 1979).

It is, however, very difficult to correlate developmental stages of immature rodents with humans, as different studies are based on age-related changes of various parameters. Older studies claimed that the 5-day-old rat is an appropriate model for the human newborn in terms of brain maturation based on the timing of peak velocity of accumulation of brain wet weight in both species (Dobbing & Sands, 1979). However, based on timing of ”growth spurt” as a vulnerable-period, Dobbing (1970) compared human babies from the last few weeks of gestation through the first few months of life to 10-12 days old rats. Roughly, the developmental stages between rodents and humans can be compared as a newborn rat pup to a third trimester fetus, a rat at 7-10 days of age similar to a full-term newborn baby, and a 3-week-old rat can be considered as a young child (Avishai-Eliner *et al*., 2002; Marsh *et al.*, 2006). The milestones in hippocampal development of human, rat and primates is reviewed by Avishai-Eliner *et al*. (2002).

All neuronal generation in the Ammon’s horn, however, occurs prenatally, whereas most granule cells of the DG are generated postnatally (85%) in rats (Bayer, 1980). The DG is also one of the few brain regions with postnatal neurogenesis throughout life (reviewed in Lavenex *et al.*, 2007a). Proliferation of neurons is first followed by axon and later by dendrite extension and spine formation. GABA-containing interneurons are generated prenatally in rats and monkeys (between E13 and E18), before the excitatory glutamatergic pyramidal neurons and astrocytes (Bayer, 1980; Khazipov *et al.*, 2001; Hennou *et al.*, 2002), but most of their morphological maturation extends to the postnatal period (Hennou *et al.*, 2002). In rodents, pyramidal cells are generated primarily during the last third of embryonic life, i.e. E 16-19 (Bayer, 1980), whereas in primates the second half of gestation is a principal period of pyramidal cell differentiation (Khazipov *et al.*, 2001).

The first GABAergic synapses are established in interneurons already during rat embryonic development (around E20), before they become expressed in pyramidal cells around birth

(Hennou *et al.*, 2002). The number of pyramidal neurons targeted by an interneuron increases during rat hippocampus development (Groc *et al.*, 2003). In the monkey hippocampus, immature pyramidal neurons start to receive sequentially established GABAergic synapses already at midgestation whereas the glutamatergic synaptic inputs appear later, and their expression coincides with the appearance of the first dendritic spines (Khazipov *et al.*, 2001).

2.3 Epilepsy

The prevalence of epilepsy is about 1% of humans worldwide, with the highest incidence in the first year of life and in old people (Pitkänen & Sutula, 2002). It is one of the most common neurological disorders (Pitkänen & Sutula, 2002). Epilepsy results from an electrical disturbance in the brain which is characterized by recurrent seizures (International League Against Epilepsy (ILAE), (Fisher *et al.*, 2005). Epileptic seizures are associated with abnormal high firing rates and synchronization of neurons in the defined regions of the brain (Fisher *et al.*, 2005). Epileptic activity may be conducted from all cortical areas to the hippocampus that is a region of high vulnerability to seizures (Ben-Ari, 1985; Bengzon *et al.*, 2002). The diseased hippocampus can also be a source of epileptic seizures. The electrical activity of the brain can be recorded by an EEG examination and it is used in the diagnosis of epilepsy. A seizure can last from a few seconds to SE, continuous seizures lasting more than 30 min, that is a life-threatening condition (Lowenstein, 1999).

Seizures are broadly classified into two large categories, partial and generalized seizures, according to their origin within the brain (Commission on Classification and Terminology of the ILAE, 1981). Partial seizures involve an electrical discharge only in a localized part or in one hemisphere of the brain, whereas generalized seizures involve both hemispheres of the brain. The two main categories include many individual seizure types, usually depending on the behavioural effects the seizure produces. One type of generalized seizures is tonic-clonic seizures that may prolong into SE, if there is no recovery between successive seizures. The incidence of SE is highest in the first year of life (including febrile SE) and in humans over age 60 years.

TLE is the most common form of human partial epilepsy (Engel, 1996), that has got its name from the seizures originating from the temporal lobe structures, particularly the hippocampus. It is typically refractory to anticonvulsant therapy but amenable to surgical interventions (Engel, 1996).

Epilepsy may be caused by certain postnatal insults such as brain trauma, CNS infections, SE, stroke, and brain tumors (reviewed in Pitkänen & Sutula, 2002). Some epilepsy syndromes may be related to heritable mutations. For example, mutations in $GABA_A$ receptor $\alpha 1$ (Cossette *et al.*, 2002) or $\gamma 2$ subunits have been detected in some forms of generalised epilepsies (Wallace *et al.*, 2001). Both from studies with experimental animals (Cavalheiro *et al.*, 1991; Mello *et al.*, 1993) and from patients with epilepsy (Pitkänen & Sutula, 2002) it has been shown that after the initial brain insult there is a latent period defined as to epileptogenesis. During the epileptogenic period, neurobiological events such as neuronal loss and abnormal synaptic reorganization may occur (Mello *et al.*, 1993). This reorganization of the neuronal integration leads to abnormally increased excitability and synchronization, and eventually to the occurrence of spontaneous seizures (Cavalheiro *et al.*, 1991), the chronic epileptic condition. The progression of seizure-induced neuropathology is illustrated in Figure 5.

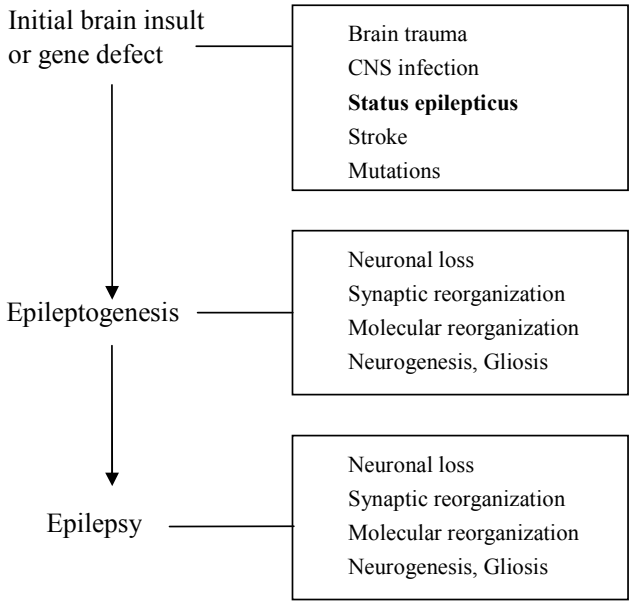

Figure 5. The process of epileptogenesis in the brain. Modified from Pitkänen and Sutula (2002).

2.3.1 Neuropathological alterations of epilepsy in the adult brain

There is a high risk of mortality (20-60%) as well as morbidity associated with SE, including epileptogenesis (Claassen *et al.*, 2002). In experimental models of adult rat, neuronal damage is apparent shotly after SE induction, and the extent of injury is correlated to SE duration (Ben-Ari, 1985; Covolan & Mello, 2000). It is, however, not well known for how long the neurodegenerative processes proceed after SE in different brain regions (the epileptogenic period) until full epilepsy has developed. Some recent histological data from rats indicate that SE-induced damage continues for several weeks (Pitkänen & Sutula, 2002), involving several brain structures including the hippocampal areas, amygdala, thalamus, and cortical regions (Tuunanen *et al.*, 1999; Covolan & Mello, 2000; Nissinen *et al.*, 2000). The hippocampus (Covolan & Mello, 2000; Smith & Dudek, 2001) as well as several nuclei of the amygdala (Tuunanen *et al.*, 1999; Nissinen *et al.*, 2000) may suffer from serious damage during epileptogenesis. In the hippocampal hilar, CA1 and CA3 subregions, the majority of neurons may undergo cell death shortly after SE (Buckmaster & Dudek, 1997; Bengzon *et al.*, 2002; Pitkänen & Sutula, 2002). During the epileptogenic process cell death has been proposed to occur through both apoptotic or necrotic pathways, or as a combination of both (Fujikawa *et al.*, 2000; Bengzon *et al.*, 2002). The outcome depends on the severity and duration of seizures that differently affect the various cell types in the brain (Fujikawa *et al.*, 2000; Bengzon *et al.*, 2002; Henshall & Simon, 2005).

The extensive neuronal loss is associated with synaptic reorganization in the hippocampus (Tauck & Nadler, 1985; Haas *et al.*, 2001). One of the most prominent reorganizations is MF sprouting, that refers to abnormal axonal growth, and the development of new excitatory circuits in the hippocampus (Buckmaster & Dudek, 1997; Smith & Dudek, 2001; Bengzon *et al.*, 2002). The relevance of the MF sprouting is however not clear, but it has been demonstrated that the fibers target granule cells and GABAergic interneurons, which might lead to abnormal excitability and the development of recurrent spontaneous seizures after SE (Brooks-Kayal *et al.*, 1998b; Nissinen *et al.*, 2000; Scharfman *et al.*, 2003)..

SE has also been shown to promote the neurogenesis of dentate granule cells, (that are neurons normally undergoing proliferation in adult brain) (Covolan *et al.*, 2000), which also complicates the outcome of cell death as it may reduce its impact (Sutula *et al.*, 2003). The neurogenesis may persist long after the induction of SE (days to weeks) as shown in the pilocarpine and kainic acid (KA) models of epilepsy (Covolan *et al.*, 2000; Sankar *et al.*, 2000).

Although these new granule cells develop normally, they show some abnormal features such as extensive growth of dendrites. However, they seem to be well integrated into local networks and could therefore be involved in seizure activity (Scharfman *et al.*, 2000).

The neuropathological changes associated with the kindling epilepsy animal model seem to be milder than with the experimental model of KA-induced SE (Mathern *et al.*, 1997; Nakagawa *et al.*, 2000; Morimoto *et al.*, 2004). However, there is neuronal loss in the hippocampal hilar, CA1 and CA3 subfields resembling that of hippocampal sclerosis in patients with TLE (Mathern *et al.*, 1997; Haas *et al.*, 2001; Kotloski *et al.*, 2002). The distribution of damage depends on the site of stimulation (Kotloski *et al.*, 2002) and changes have also been found in other temporal lobe structures including the amygdaloid complex (Nissinen *et al.*, 2000). MF sprouting has been detected in the DG (Lynch & Sutula, 2000; Haas *et al.*, 2001). Furthermore, kindling also induce neurogenesis of dentate granule cells (Nakagawa *et al.*, 2000), and proliferation and reorganization of glial cells, usually referred to as gliosis (Khurgel & Ivy, 1996).

Repeated SE results in progressively longer seizures and may produce further long-term cellular and network alterations which may lead to more severe damage to the hippocampus (Sarkisian *et al.*, 1997). Seizures evoked by kindling as well as SE evoked by convulsants also result in many molecular changes, such as in $GABA_A$ and glutamate receptor composition and gene expression, activation of glutamate receptors, cytokine activation, oxidative stress, and changes in plasticity (Pitkänen & Sutula, 2002; Sutula *et al.*, 2003). The neuronal loss in the hippocampus after repeated seizures is associated with progressive memory dysfunction (Sarkisian *et al.*, 1997; Kotloski *et al.*, 2002).

The consequences of SE in human are not that clear-cut. In some cases SE causes permanent injury but not in others (Sutula *et al.*, 2003). The prolonged seizure activity of SE may cause brain damage that can lead to the development of epilepsy and cognitive decline (Pitkänen & Sutula, 2002). Many patients with TLE have progression of hippocampal sclerosis, a unique neuropathological disturbance of atrophy with changes such as neuronal loss, MF sprouting and gliosis (Engel, 1996; Kälviäinen & Salmenperä, 2002). Changes such as neuronal cell death has been found in other brain regions as well, especially in the amygdala and enthorhinal cortex (Mikkonen *et al.*, 1998; Kälviäinen & Salmenperä, 2002).

2.3.1.1 Changes in the $GABA_A$ receptor

One main determinant for seizure generation in epilepsy is proposed to be reduced GABAergic inhibition (Rice *et al.*, 1996; Tsunashima *et al.*, 1997; Brooks-Kayal *et al.*, 1998b). Pharmacologically, this is proven by $GABA_A$ receptor agonists that suppress seizures in animal models, and antagonists that block the receptor activity leading to seizures. The neuronal loss caused by repeated seizures eventually involves hippocampal GABAergic interneurons and it could contribute to the reduced inhibition (Sayin *et al.*, 2003). These neurons comprise those components that provide axo-somatic and axo-axonic inhibition by regulating the propagation of activity into axons, and if they are lost, the presynaptic inhibitory drive to excitatory principal cells will be reduced (Tsunashima *et al.*, 1997; Bouilleret *et al.*, 2000; Andre *et al.*, 2001; Sayin *et al.*, 2003).

Also postsynaptic alterations in the GABAergic synapse have been found in the epileptic brain. These changes in the $GABA_A$ receptors presumably largely relate to the altered expression of genes encoding different $GABA_A$ receptor subunits as well as receptor proteins, as seen in adult experimental epileptic animals and in human with epilepsy (Rice *et al.*, 1996; Schwarzer *et al.*, 1997; Sperk *et al.*, 1997; Tsunashima *et al.*, 1997; Brooks-Kayal *et al.*, 1998b; Fritschy *et al.*, 1999; Loup *et al.*, 2000; Raol *et al.*, 2006b). Both up- and downregulations in the expression of different subunits have been detected after seizures (Rice *et al.*, 1996; Brooks-Kayal *et al.*, 1998b; Nusser *et al.*, 1998a; Loup *et al.*, 2000; Andre *et al.*, 2001; Zhang *et al.*, 2004; Raol *et al.*, 2006b). Different effects may be seen in different epilepsy models and whether the alterations are of acute or chronic origin. Seizure-induced alterations may be persistent, but not necessarily similar to those of an acute phase (Schwarzer *et al.*, 1997; Sperk *et al.*, 1997; Brooks-Kayal *et al.*, 1998b). At the chronic phase, i.e. 1-2 months after pilocarpine injection in adult rats, $\alpha 2$ expression has decreased in the CA1-CA3 regions (Rice *et al.*, 1996). On the other hand, the expression of $\alpha 4$ and $\beta 3$ subunits has increased, whereas $\alpha 1$ has either increased or decreased in granule cells 1-4 months after KA- and pilocarpine-induced seizures (Schwarzer *et al.*, 1997; Sperk *et al.*, 1997; Brooks-Kayal *et al.*, 1998b; Fritschy *et al.*, 1999). Neuronal loss detected in several epilepsy models, especially in the CA1 and CA3 pyramidal cell regions (Schwarzer *et al.*, 1997; Tsunashima *et al.*, 1997; Fritschy *et al.*, 1999; Nissinen *et al.*, 2000), could partly explain the decreased subunit expression but not the increased expression, indicating that real changes in $GABA_A$ receptor subunit expression occur in

surviving neurons. In agreement with the animal studies, loss of CA1 and CA3 interneurons as well as a decreased number of α1 immunopositive cells were reported in human tissue resected from patients who had undergone surgery for TLE, compared to autopsy controls (Loup *et al.*, 2000; Pirker *et al.*, 2003). There was also an increase in immunostainings of α1, α2, β2 and β3 subunits, particularly in the dentate granule cell layer.

The changes in subunits are also consistent with changes found in receptor pharmacology. Several studies have shown increased [^{3}H]flunitrazepam binding in the DG of adult rats subacutely after seizures (Shin *et al.*, 1985; Nobrega *et al.*, 1989; Titulaer *et al.*, 1995c). In the CA1 and CA3 regions, seizures caused decreased binding of [^{3}H]flunitrazepam (Titulaer *et al.*, 1995c; Rocha & Ondarza-Rovira, 1999), whereas the long-term effects of seizures on receptor binding are usually different. In dentate granule cells from animals with spontaneous seizures induced with pilocarpine, Zn^{2+} sensitivity was increased and zolpidem sensitivity decreased, findings that both could be due to the decrease in α1 subunit mRNA (Brooks-Kayal *et al.*, 1998b).

2.3.2 Epilepsy in the developing brain

The postnatal brain with its complex maturation can be expected to be more sensitive to disturbances and the majority of epilepsy syndromes in human start in infancy or childhood. There is thus a higher incidence of seizures and also seizure severity in the immature brain (Holmes & Ben-Ari, 2001), which according to different experimental seizure models is maximal between P10 and P12 in rats (Khazipov *et al.*, 2004). Despite the low seizure threshold, the immature hippocampus is quite resistant to SE-induced damage (Haas *et al.*, 2001; Holmes & Ben-Ari, 2001; Baram *et al.*, 2002; Zhang *et al.*, 2004) compared with the mature brain. In some studies, little or no neuronal damage was found in the hippocampus of experimental rats experiencing continous seizures early in life (Haas *et al.*, 2001; Wasterlain *et al.*, 2002; Bender *et al.*, 2003; Lopez-Picon *et al.*, 2004). Also, modest or no synaptic reorganisation, such as MF sprouting, was detected (and did not occur until after the third postnatal week) (Sankar *et al.*, 1998; Haas *et al.*, 2001; Wasterlain *et al.*, 2002; Bender *et al.*, 2003; Cilio *et al.*, 2003; Lopez-Picon *et al.*, 2004). Overall, the extent of hippocampal damage is small in the postnatal period and increases with maturation (Wasterlain *et al.*, 2002).

Other studies have shown that some degree of hippocampal injury may occur during early development but the pattern seems to be different from that in adult rats (Haas *et al.*, 2001;

Baram *et al.*, 2002; Wasterlain *et al.*, 2002). Neuronal damage has been detected in the hippocampus and other brain structures after SE in rats older than two weeks (Sankar *et al.*, 1998; Humphrey *et al.*, 2002; Dong *et al.*, 2003; Druga *et al.*, 2005). However, in some of these studies there was no cell loss in animals that survived to adulthood indicating that some effects may be reversible (Toth *et al*., 1998; Baram *et al.*, 2002), whereas other studies demonstrated the presence of irreversible damaged neurons in the hippocampus or other parts of the brain in animals younger than 3 weeks (Sankar *et al*., 1998; Kubova *et al*., 2001; Nairismägi *et al*., 2006). Available techniques may not be sensitive enough to detect limited neuronal loss, which can be present only in a subpopulation of surviving animals (Nairismägi *et al*., 2006). In fact, there are studies showing chronic hippocampal damage induced by SE in rats younger than three weeks (Wu *et al*., 2001; Nairismägi *et al*., 2006). During brain development there is a physiological programmed cell death, usually defined as apoptosis that may be accelerated by seizures (Kuan *et al.*, 2000; Wasterlain *et al.*, 2002). Neuronal damage in the CA3 region as well as MF sprouting has also been detected after KA-induced SE in cultured hippocampal slices, prepared from P6-7 rats and cultured for 1 week (Routbort *et al.*, 1999; Holopainen *et al.*, 2001a; Holopainen *et al.*, 2004), which corresponds to 14-days-old rats *in vivo*. In contrast to adult brain, seizures occurring in the immediate neonatal period (P0-7) reduce neurogenesis of dentate granule cells (McCabe *et al.*, 2001), but as early as P15, SE-induced dentate granule cell neurogenesis has been detected (Sankar *et al.*, 2000) and also in the CA3 region (Dong *et al.*, 2003).

SE early in life may increase the development of TLE in adulthood (Holmes & Ben-Ari, 1998; Dube *et al.*, 2000), and even increase the sensitivity to other insults later in life (Koh *et al.*, 1999; Bender *et al.*, 2003). In P20 rats experiencing seizures early in life by the kindling method, the susceptibility to seizures was higher in adulthood (Cilio *et al.*, 2003). It has also been observed that about 25% of rats with SE at P12 will develop spontaneous seizures within 3 months (Kubova *et al.*, 2004). However, the neural damage may be less severe prior to episodes of SE early in life which could be due to some plasticity-related neuroprotective effects (Sutula *et al.*, 2003).

There are also long-term effects such as impaired spatial learning and memory deficits at P1-25 in rodents after seizures, induced for example by KA (Lynch *et al.*, 2000) and pilocarpine (Kubova *et al.*, 2004). A cellular basis for these deficits is supported by findings that animals experiencing early-life seizures have impaired LTP (Lynch *et al.*, 2000). According to this,

some studies indicate that children with epilepsy are at risk for cognitive and learning deficits (Hermann *et al.*, 2006).

2.3.2.1 Changes in the $GABA_A$ receptor

The higher incidence of seizures in the immature brain is assumed to at least partly be due to the late maturation of a potent inhibitory GABAergic system and the early excitatory actions of GABA (Holmes & Ben-Ari, 1998; Khazipov *et al.*, 2004). There is also evidence that seizures early in life can cause alterations in inhibitory neurotransmission and in the $GABA_A$ receptor subunit expression. At the circuit level, increased hippocampal inhibition was seen after KA-induced seizures in P1-14 rats (Lynch *et al.*, 2000). At the molecular level, early-life KA- or pilocarpine-induced seizures produce subunit-specific alterations in the hippocampus of immature rats, such as an increased number of $GABA_A$ receptors and a selective increase in α1 subunit expression in several regions (Zhang *et al.*, 2004; Raol *et al.*, 2006b). These findings suggest that seizures disturb the normal developmental $GABA_A$ receptor subunit expression (in the hippocampus), and this could represent a compensatory response to epileptiform activity. Similarly to SE-induced regional changes in adult rats, the short-term developmental alterations can be different from those of chronic ones (Zhang *et al.*, 2004).

The seizure-induced disruption of normal developmental alterations in subunit expression may also disturb other developmental processes such as maturation of the inhibitory synaptic transmission and its strength (Marty *et al.*, 2000; Vicini *et al.*, 2001) or synaptogenesis, which is dependent on the normal developmental α2 subunit down-regulation (Fritschy *et al.*, 1994). There are also pharmacological changes after seizures in the immature hippocampus, including increased BZ binding (Werck & Daval, 1991; Rocha *et al.*, 2000), and more selectively, enhanced type I BZ augmentation consistent with the increased α1 subunit expression (Zhang *et al.*, 2004).

2.3.3 Experimental models of epilepsy

Ideally the research on human epilepsy should be carried out on humans with epilepsy, but this it not always possible for ethical or practical reasons and therefore experimental models of epilepsy and epileptic seizures in animals are essential. Experimental models of epilepsy can be distinguished between models of acute epileptic seizures that occur in a normal brain and do not necessarily indicate the presence of an epileptic condition, and chronic models of epilepsy that

are associated with a permanent epileptogenic condition. Several of these chronic models were created to reproduce certain types of human epilepsy, particularly the most common form, TLE. There are both *in vivo* and *in vitro* epilepsy models where acute versus chronic neuronal mechanisms in the epileptic brain can be studied, including the altered $GABA_A$ receptor expression and function, excitotoxicity, and synaptic reorganization. The results from animal studies are usually compared with investigations carried out in patients with epilepsy. Ultimately, the goal with these animal models is to develop more effective treatments for epilepsy, and to reveal the mechanisms for the development of recurrent seizures so this process could be prevented.

Although there are currently more than 100 seizure models available for epilepsy research, later spontaneous seizures, i.e. epilepsy, occur in only a few of them. Such models include systemic injection of a chemoconvulsant such as KA or pilocarpine (with or without lithium) (Turski *et al.*, 1983; Ben-Ari, 1985; Cavalheiro *et al.*, 1991), or electrical stimulation of the brain (Mathern *et al.*, 1997; Sayin *et al.*, 2003). Common for these models is that they can induce a state of chronic spontaneous recurrent seizures, reflecting the clinical and neuropathological features of TLE (Engel, 1996). Accordingly, these animal models are valuable imitations of human TLE (Turski *et al.*, 1983; Ben-Ari, 1985; Cavalheiro *et al.*, 1991), but with the chemoconvulsant models also more acute effects of SE can be studied.

In addition to SE, some of the most common factors causing epilepsy are traumatic brain injury and stroke (Hauser, 1997), and there are also animal models for these epileptogenic ethiologies. The weight drop and lateral-fluid percussion models represent two models for traumatic brain injury (Pitkänen & McIntosh, 2006). In models of stroke, thrombosis, thromboembolism, or vasoconstriction can be applied to cause stroke for epileptogenesis studies (Karhunen *et al*., 2005).

2.3.3.1 Chemically-induced status epilepticus

SE can be induced in animals by a number of different stimuli. KA is the most common neurotoxin used to cause SE by acting as an agonist for the KA subtype of ionotropic glutamate receptors and depolarizing neurons that express these receptors, such as CA3 pyramidal cells (Westbrook & Lothman, 1983) and interneurons of the hippocampus (Cossart *et al.*, 1998) of adult rat. The CA3 pyramidal neurons have a particularly low threshold for the generation of seizures by KA (Westbrook & Lothman, 1983; Ben-Ari, 1985). However, apart from

postsynaptic receptors, KA may affect presynaptic AMPA receptors on glutamatergic and GABAergic terminals and the underlying bases for these effects remains unclear (Huettner, 2003). However, at low concentrations KA activate mainly KA receptors, as shown in hippocampal slice cultures (Kristensen *et al.*, 2001). There is a high density of KA receptors in the developing rat hippocampus already at birth (Bahn *et al*., 1994; Ritter *et al*., 2002).

Systemic injection of KA gives rise to sustained limbic seizures (SE) lasting for several hours, which is then followed by a period of a few weeks with no obvious ictal activity (epileptogenesis). After this silent period, during which epileptogenesis occurs, adult animals start to display spontaneous recurrent limbic seizures with increasing frequency and no remission (Wasterlain *et al.*, 2002). In rats younger than 2 weeks, seizures of tonic-clonic type will appear, but no spontaneous seizures (Wasterlain *et al.*, 2002). KA can also be administrated through other routes, but the intraperitoneal (i.p.) as well as intracerebral injections, including intrahippocampal and intracerebroventricular, are the most common ones (Ben-Ari, 1985). Unfortunately, there is a high mortality with the approach of one single systemic KA injection in immature rats, as shown e.g. for P15 rats (Koh *et al*., 1999). This can be reduced by stopping the resulting SE with an antiepileptic compound such as paraldehyde or diazepam within a specified time window (Kubova *et al.*, 2004). Consequently, the seizure-induced alterations in such a model will represent the more acute phase of SE.

Systemic injection of pilocarpine, an agonist of muscarinic acetylcholine receptors, represents a model similar to that of KA, and produces persistent and long-lasting seizures in adult rats (Turski *et al.*, 1983). The dose of pilocarpine required to induce seizures is high but it can be decreased by pre-treatment with lithium chloride (Jope *et al.*, 1986). In contrast to the KA model, the lithium-pilocarpine model produces neuronal damage in the immature hippocampus of P12-14 rats (Sankar *et al*., 1998; Nairismägi *et al*., 2006), in addition to the adult rat hippocampus (Turski *et al.*, 1983). Also the pattern of hippocampal damage in adult rats seems to be different between the two models (Turski *et al.*, 1983; Sankar *et al*., 1998). After a latency period, spontaneous seizures occur in a subpopulation of P12-14 animals (Sankar *et al*., 1998; Kubova *et al*., 2004).

2.3.3.2 Electrical induction of status epilepticus

SE can also be induced by sustained electrical stimulation to specific sites of the brain, such as the perforant path, the ventral hippocampus, and the amygdala (Mathern *et al.*, 1997; Nissinen *et*

al., 2000; Sayin *et al.*, 2003). The most sensitive area for SE induction is the basolateral amygdala (Goddard *et al.*, 1969). In this stimulation procedure, seizures can be provoked by, most commonly, electrical stimuli through surgically implanted electrodes (Nissinen *et al.*, 2000). In adult rats, after repeated or a long-lasting (> 20 min) electrical stimulation, SE is induced, and after a latency period of approximately 1 month spontaneous generalized seizures will develop that continue for the rest of the animals' life (Nissinen *et al.*, 2000; Sayin *et al.*, 2003). The changes that take place during/ after the stimulation period can be characterized as epileptogenesis. Therefore, electrical stimulation can be used to study the cellular and molecular epileptogenic alterations induced by SE and the neuropathological changes resemble those of human TLE (Pitkänen *et al.*, 1998).

Kindling is another commonly used method, considered to be a chronic model of TLE (Goddard *et al.*, 1969). Seizures are induced by repeated administration of a subconvulsive chemical or repeated electrical stimulation of a defined brain structure, e.g. hippocampus (Mathern *et al.*, 1997) or amygdala (Goddard *et al.*, 1969), through implanted electrodes. At early stages of kindling, partial seizures appear that gradually after daily repetition of the stimulation become generalized. In adult rats, after many days of repeated electrical stimulation and approximately 90-100 kindled seizures, spontaneous generalized seizures will develop that continue for the rest of the animals' life (Sayin *et al.*, 2003). During the kindling procedure, the number of seizures is precisely controlled, and cellular alterations induced by a specific number of seizures can be studied. The long-term neuropathological changes in this model also resemble those of human TLE. (Lynch & Sutula, 2000; Pitkänen & Sutula, 2002; Sutula *et al.*, 2003).

2.3.3.3 In vitro model: Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures can be used as an *in vitro* model to study the cellular and molecular changes induced by enhanced excitatory activity. In these cultures, epileptiform activity can be induced e.g. by $GABA_A$ receptor antagonists such as picrotoxin and bicuculline, whereas the application of e.g. non-NMDA receptor antagonists blocks the increased activity (Scanziani *et al.*, 1994; Routbort *et al.*, 1999; Bausch & McNamara, 2000). Hippocampal slice cultures are prepared from postnatal rat (usually P6-7), and they can be cultured from one week up to 1-2 months (Stoppini *et al.*, 1991; Routbort *et al.*, 1999; Bausch & McNamara, 2000; Holopainen *et al.*, 2001a; Holopainen, 2005). Advantages of slice cultures are that they can survive for long time *in vitro*, the experimental conditions can be precisely controlled, and

confounding variables of *in vivo* models are eliminated. Furthermore, all major neuronal types survive and the native structural organization is retained (Caeser & Aertsen, 1991; Frotscher *et al.*, 1995; Gähwiler *et al.*, 1997). Consequently, the expression of receptors as well as synaptic contacts resembles those seen *in vivo* (Frotscher *et al.*, 1995; Gähwiler *et al*., 1997; Holopainen *et al*., 2005). However, the preparation of slices results in loss of normal afferent input to dentate granule cells, which upon culturing favors growth of aberrant, excitatory connections of MFs into the molecular layers of the DG (Caeser & Aertsen, 1991; Frotscher *et al.*, 1995; Bausch & McNamara, 2000). Despite this partial circuit reorganization, cultured hippocampal slices offer a relatively simple and pharmacologically accessible method to study changes in receptors, such as $GABA_A$ receptor subunit expression, cell death and morphological rearrangements (Gerfin-Moser *et al*., 1995; Holopainen *et al.*, 2001a; Holopainen, 2005).

2.3.4. Antiepileptic drugs acting through the GABAergic system

Many types of antiepileptic drugs (AEDs) are used to prevent epileptic seizures. They interact mainly with voltage-gated ion channels such as Na^+ and Ca^{2+} channels, metabolic enzymes and neurotransmitter transporters in the brain, thereby inhibiting epileptic bursting, synchronization and seizure spread (reviewed in Rogawski & Löscher, 2004). Several AEDs decrease synaptic excitation or enhance synaptic inhibition by blocking glutamate receptors or enhancing $GABA_A$ receptor activity, respectively.

Enhancement of GABA-mediated inhibition is indeed one of the major mechanisms of action of AEDs, affecting different steps of the metabolic cycle of GABA (GABA shunt). The BZs act as agonists on $GABA_A$ receptors enhancing their currents, and are used as first-line drugs in the treatment of SE. Vigabatrin is another anticonvulsant drug that act by irreversibly inhibiting the GABA-degrading enzyme GABA-T (De Biase *et al.*, 1991). This is assumed to lead to elevation of extracellular GABA levels in the brain (Löscher & Horstermann, 1994) and potentiation of the inhibitory effects of GABA on CNS function (Jackson *et al.*, 2000). Tiagabine is a selective competitive inhibitor of the high-affinity plasma membrane GABA transporter GAT-1 (Suzdak & Jansen, 1995) that prevents GABA uptake and prolongs IPSPs (Engel *et al.*, 1998). It seems to reduce partial seizures but shows complex responses to some other seizure types (Skardoutsou *et al.*, 2003). Loreclezole is an anticonvulsant with a broad spectrum that can act only on receptors with the β2 or β3 subunit but is inactive in β1-containing receptors (Siegwart *et al.*, 2002). One of the new AEDs is topiramate that shows

effects on $GABA_A$ receptor responses in vitro (Gordey *et al.*, 2000). However, it also increases the level of GABA in the brain and, therefore, it may use direct and indirect mechanisms to enhance GABAergic neurotransmission.

Despite advances in the treatment of epilepsy about 25% of patients, including children, have refractory epilepsy resistant to traditional drug therapy. Moreover, adverse effects of these drugs have been found in the immature brain, both in animals (Bittigau *et al.*, 2002; Raol *et al.*, 2005) and in children (Herranz *et al.*, 1988; Calandre *et al.*, 1990). There is still a growing concern about the current epilepsy treatment, and a need for more effective epilepsy therapy, especially in infants and children.

3. AIMS OF THE STUDY

Reduced GABAergic inhibition is proposed to be a key element for seizure generation in epilepsy in the adult brain, and seizures are known to change the structure and function of $GABA_A$ receptors that may contribute to epileptogenesis. However, it is not known whether or not the subunit expression is changed in response to seizures in the developing brain. In this thesis work, the objective was to characterize the influence of excessive neuronal activity, i.e. epileptic seizures, on the expression of $GABA_A$ receptor subunits in both the developing and adult rat brain. To address this question, different experimental models were used and the specific aims were:

1. to determine the long-term alterations in the expression of $GABA_A$ receptor subunits in the hippocampus of spontaneously seizing adult rats with chronic TLE

2. to analyze the expression patterns of $GABA_A$ receptor subunits during rat postnatal development in organotypic hippocampal slice cultures *in vitro* and in developing rat hippocampus *in vivo*

3. to study whether the $GABA_A$ receptor subunit expression is altered by neuronal activity in hippocampal slice cultures and by SE in developing rats

4. to reveal the distribution of BZ- and zolpidem-sensitive receptors in developing rats after SE

4. MATERIALS AND METHODS

4.1 Experimental epilepsy models

4.1.1 Animals (I-IV)

Adult male Harlan Sprague-Dawley rats (n=8, I) and 9-day old Sprague-Dawley rat pups (n=51, III; n=26, IV) were used in the experiments. Hippocampal slice cultures were prepared from 6-day-old Wistar rat pups (II). All experimental animal procedures were done with the permission of the Animal Use and Care Committees of the University of Kuopio or the University of Turku, and they were conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC. All attempts were made to minimize the pain, discomfort, and number of the experimental animals.

4.1.2 Status epilepticus induced by electrical stimulation of the amygdala (I)

The new model of SE induced by electrical stimulation of the amygdala leading to spontaneous seizures was used in the laboratory of Prof. Asla Pitkänen in University of Kuopio and developed by Nissinen et al. (2000). Briefly, stereotaxic instruments were used for implantation of electrodes in adult rats (n=4): one in the left lateral nucleus of the amygdala for stimulation, one in frontal cortex for recording the spread of electrographic seizures, and two electrodes in the cerebellum serving as ground and reference electrodes. In control rats (n=4), the electrodes were implanted in corresponding locations but animals were not stimulated. Two weeks after the surgery, SE lasting for 6-20 h was induced by stimulating the amygdala for 20-30 min. If the animals did not meet the criteria of clonic SE, the stimulation was repeated for another 5 to 10 min. Electrographic seizure activity was recorded 9 weeks after induction of SE to detect spontaneous seizures using video-EEG recording systems. Rats were used for *in situ* hybridization 3 months after SE.

4.1.3 Status epilepticus induced by kainic acid injection (III, IV)

P9 rats were chosen for the studies of $GABA_A$ receptor subunit expression in immature rat brain, because the expression of many subunits alters during the second postnatal week (Laurie *et al.*, 1992; Fritschy *et al.*, 1994). In rat pups, a single dose of 2 mg/kg of KA (Tocris Cookson Ltd, Avonmouth, UK or Ocean Produce International, Shelbourne, NS, Canada) administered i.p. leads to seizures. Rats were then carefully followed up to detect signs of seizures that

usually started to appear within 12-15 min. One hour after the KA injection, paraldehyde (0.3 ml/kg; i.p; Fluka Chemie AG, Buchs, Switzerland) was injected to stop SE, as described recently (Kubova *et al.*, 2001). To restore dehydration, 0.9% NaCl was injected (i.p.) to the pups soon after, at a dose of 2.5% of the body weight, and the well-fare of the pups was carefully controlled during the recovery period. After the follow-up for at least 2 h, pups were taken back to their lactating mothers until they were sacrificed for the studies at different time points. Age-matched control rats received the same volume of 0.9% NaCl as those of KA-treated, but in order to minimize the discomfort of the experimental animals, they did not receive any further injections and were otherwise treated like the KA-treated rats. Animals were used for the studies 6 h, 3 days or 1 week after KA injection.

4.1.4 Organotypic hippocampal slice cultures (II)

Hippocampal slice cultures were prepared from the hippocampi of P6 rat pups, using the modified method of Stoppini *et al*. (1991), and as described recently in detail by Holopainen *et al*. (2001). Briefly, hippocampi were isolated, slices (400 μm) were cut perpendicular to the septotemporal axis of the hippocampus, and placed on semipermeable membrane inserts (Millipore Corporation, Bedford, MA, USA). Inserts were kept on the membranes in a six-well plate, at the interface between culture medium and the air, which allows sufficient amounts of oxygen to diffuse into the slice. Slices were incubated at 37°C in 5% CO_2 for 7 days. The culture medium, containing no antibiotics or antimitotics, was changed twice a week. Cultures were chronically treated (for the entire culture period of 7 days) either with picrotoxin or with the non-NMDA receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) (20 μM) or the $GABA_A$ receptor antagonist picrotoxin (100 μM) (both from Sigma, St. Louis, MO, USA).

4.2 Receptor localization

4.2.1 In situ hybridization (I-III)

In situ hybridization was used for the detection of $GABA_A$ receptor subunit mRNA expression, with the aid of radiolabeled probes that are allowed to hybridize with their complementary mRNA molecules in brain tissue sections. The hybridized duplexes can then be detected on irradiation-sensitive films. The procedure was performed using the protocols of Wisden and Morris (1994) and Gerfin-Moser and Monyer (1994), and as modified by Sinkkonen *et al.* (2001). Shortly, brains were dissected, frozen in isopentane, and cut into coronal sections (14

μm) (I, III). Sections were mounted on poly-L-lysine-coated slides, fixed in 4% paraformaldehyde (PFA, Sigma, St Louis, MO), and stored in 95% ethanol at 4°C until used. Hippocampal slice cultures were fixed with PFA in the culturing plates, transferred to poly-L-lysine-coated slides, and stored in -20°C until used (II).

The 45-bases-long antisense oligonucleotide probes (Institute of Biotechnology, University of Helsinki, Finland), complementary to rat cDNA sequences for the $GABA_A$ receptor subunits, were 3' end labeled with 0.06 nM [α-^{33}P]dATP (NEN, Boston, MA, USA), and terminal transferase (Finnzymes Oy, Espoo, Finland or Boehringer Mannheim, Mannheim, Germany). Unincorporated nucleotides were separated by chromatography columns. Sections and slices were incubated (42°C, overnight) with hybridization buffer containing the labelled probe. After hybridization, sections were washed, dehydrated, and dried before exposure to autoradiographic film with a [^{14}C] labeled standard (II, III). Signal specificity of probes was determined with 100 x excess of unlabeled probe. For detection of cellular expression of $GABA_A$ receptor subunits, some sections were dipped in autoradiographic nuclear emulsion (I). The emulsion contains silver grains that precipitate over the cells in which radiolabeled molecules are hybridized to endogenous mRNA, and the density of silver grains can be quantified and correlated to the amount of mRNA.

4.2.2 Ligand binding autoradiography (IV)

Ligand binding autoradiography is a precise technique for the study of receptor populations, where a radioactively labelled ligand binds to its binding site in the receptor complex in brain sections. While freezing and sectioning of the brain destroys most neurons, receptor proteins can remain intact for several years if handled and stored properly. The specific binding of the radioligand can be determined in competition binding assays, by displacing the radioactively labelled ligand at a low, fixed concentration from its specific binding sites, with an unlabelled, competing ligand at a range of concentrations. This approach enables detection of receptor subpopulations, such as different $GABA_A$ receptor subunits. Here, displacement of [^{3}H]flunitrazepam binding by zolpidem was performed to reveal the distribution of the α1, α2/α3 or α5 subunit-containing receptors.

Brain samples were processed otherwise similarly as for *in situ* hybridization, but brain sections were mounted onto gelatin-coated glasses, and stored at -20°C without any fixation. For the binding, tissue sections were preincubated in ice-cold incubation buffer to remove

endogenous GABA. Then sections were incubated with 1 nM [^{3}H]flunitrazepam (PerkinElmer Life Sciences, Boston, MA) diluted in incubation buffer for 1 h. Zolpidem (10 nM – 100 μM) was used to estimate zolpidem-sensitivity of [^{3}H]flunitrazepam binding. Nonspecific binding was determined with 10 μM flumazenil (Hoffmann La-Roche, Basel, Switzerland). The incubation was terminated by washing the sections, followed by drying and exposure of sections together with a plastic ^{3}H standard to autoradiographic film for 6 weeks.

4.2.3 Immunocytochemistry (III, IV)

For the detection of $GABA_A$ receptor subunit proteins, the immunocytochemical staining procedure with perfusion-fixed brain sections was applied, as recently described in detail (Lopez-Picon *et al.*, 2004). Briefly, rats were anaesthetized with sodium pentobarbital (50 mg/kg; i.p.), transcardially rinsed with 0.9% NaCl, and thereafter perfused with 4% PFA. After that, brains were rapidly removed, postfixed, and then processed with an antigen retrieval protocol as published by Fritschy *et al*. (1998). Thereafter, brains were cryoprotected in 30% sucrose, and kept at -80°C until used.

For the immunostaining, brains were sectioned, collected in buffer (III, 40 μm thick sections, free-floating system), or put onto gelatine-coated glasses (IV, 20 μm thick sections). Slices were incubated in the blocking solution, and thereafter with the primary antibodies for 24 h. After washing with buffer containing Triton X-100, the free-floating slices were incubated with the biotin-SP-conjugated secondary antibodies (Jackson Immunoresearch Labs, Inc, USA) for 1 h (III). Thereafter slices were incubated with the avidin-peroxidase conjugate (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA), and the staining was detected with 3,3'-diaminobenzidinetetrahydrochloride (Sigma) under microscope. Finally sections were transferred to glasses that were coverslipped. Alternatively, after primary antibody incubation, glasses were incubated with the secondary fluorescent antibody Alexa 488 (Molecular Probes, Eugene, OR, USA), coverslipped and examined with confocal microscope.

4.3 Histological staining methods

4.3.1 Thionin staining (II, III)

Thionin staining was used to verify the severity of neuronal damage in various hippocampal regions after drug treatments in brain sections and cultured slices. The staining of cultured hippocampal slices (II), was carried out as earlier described in detail (Holopainen *et al.*, 2001a).

Briefly, slices on membrane inserts were first fixed with 4% PFA, detached, and transferred to glass slides. After rehydration, slices were treated in 0.1% Triton X-100 followed by 0.1% thionin (Sigma, St. Louis, MO). Finally, they were dehydrated, cleared in xylene, and coverslipped. The number of neurons were scored using defined criteria (scale from 0-4), according to the number of stained neurons.

For thionin staining of immature rat brain sections (III), the same procedure as used for immunocytochemistry was applied, i.e. perfusion-fixation, postfixation and cryoprotection. Brain sections (20 μm) were cut, mounted on glasses and rehydrated. Then sections were stained in 0.1% thionin, dehydrated, cleared in xylene, and coverslipped.

4.3.2 Fluoro-Jade B staining (III)

Fluoro-Jade B staining was used to study KA-induced neuronal degeneration (Schmued *et al.*, 1997), as previously described in detail (Holopainen *et al.*, 2004) with minor modifications. Brain sections (20 μm; prepared as for the thionin staining) were rehydrated, transferred to 0.06% potassium permanganate, and then to 0.001% Fluoro-Jade B solution. After the staining, sections were washed, dried, cleared in xylene, and coverslipped.

4.4 Data analysis

4.4.1 Quantification of autoradiographic films and dipped samples (I-IV)

The hybridization and ligand binding signals were quantified in film autoradiograms by using the MCID AIS image analysis devices and programs (Imaging Research, St. Catharines, Ontario, Canada). The binding densities for each brain area were measured, and the resulting binding values were converted to radioactivity levels estimated for gray matter areas (in nCi/g), with reference to the standard curve created from the calibrated [^{14}C] (II, III) or [^{3}H] (IV) standards. Dipped sections were imaged with dark-field microscopy (Leica DMR, Heerburg, Switzerland) connected to a grey-scale digital camera (Leica DC100), and the density of silver grains was quantified (Image-J Program, version 1.20s) (I).

4.4.2 Microscopy (II-IV)

Sections processed for thionin staining (II, III) and immunocytochemistry (III) were imaged under microscope using bright-field optics (Leica DMR, Heerbrugg, Switzerland or Olympus BX60, Olympus Optical, Tokyo, Japan). A computerized image analysis program (Cell A,

Olympus Soft Imaging System, Hamburg, Germany) was used to count immunopositive neurons with the Leica DMR microscope using a fluorescence filter for Alexa 488 (IV). A digital camera (Leica DC100, Leica, Olympus U-TV1 X or Olympus DP 70, Olympus Optical) was used to capture images.

The stainings with Fluoro-Jade B (III) and of the α1 subunit/ Alexa 488 complex (IV) were analysed with a confocal microscope (Leica, Heidelberg, Germany) equipped with an argon/krypton laser (Omnichrome; Melles Griot, Carlsbad, CA). The laser wavelength used for excitation of Fluoro-Jade B and Alexa 488 was 488 nm, and the emission detection window was 500–600 nm.

4.4.3 Statistical methods (I-IV)

All statistical analyses were carried out using Prism software (versions 3.0 and 4.0, GraphPad Software, San Diego, CA, USA). The level of significance was set at $p<0.05$. Student's independent two-tailed *t*-test (I, III, IV) was used to assess differences between two groups. When three or more groups were compared, one-way analysis of variance (ANOVA) with Tukey-Kramer Multiple Comparison as a post hoc test (I, II, III) or two-way ANOVA with the Bonferroni post hoc test (II, IV) were adapted. The nonlinear regression analysis (one site competition) was applied for calculating IC_{50} values (IV).

5. RESULTS

5.1 $GABA_A$ receptor subunit messenger RNA expression in rat hippocampus (I-III)

The $GABA_A$ receptor subunit mRNA expression was examined in four hippocampal regions with *in situ* hybridization in studies I, II and III. These regions were the pyramidal cell layers CA3c, CA3a/b and CA1, and the granule cell layers (both superior and inferior blade) of the DG.

5.1.1 Adult rat (I)

In adult control rats, mRNAs for $GABA_A$ receptor α2, α4, β3, and γ2 subunits was expressed in all the studied subfields of the hippocampus. Each subunit showed a varying subunit expression between the subfields, with a high expression in the DG and the lowest expression was found in the hilus for all subunits. The highest mRNA level was detected for γ2 subunit in the granule cell layer as well as in the CA3 and CA1 regions.

5.1.2 Developing rat (III)

In immature control rats, the expression levels of several $GABA_A$ receptor subunit mRNAs varied significantly during the postnatal development (from P9 to P16) in the hippocampal subregions. The expression of α1 showed an increased developmental expression profile between P9 and P16 in all subregions, whereas that of α2 showed a decreased one. Also the expression of α4 mRNA increased in CA1 and DG, whereas significantly decreased expression levels were detected of β3 mRNA between P9 and P12 and of γ2 mRNA between P12 and P16 in all subregions. No significant developmental changes were detected in the expression of α3, α5, β1, β2 and γ1 subunit mRNAs in any subregion.

5.1.3 Hippocampal slice cultures (II)

The subunit mRNA expression in control cultured hippocampal slices indicated significant regional differences. The most abundantly expressed subunits were α2 and α5 in all hippocampal subfields. These subunits were expressed in the CA3a/b and CA1 regions as well as the other studied subunits (α1, α4, α5, β1, β3, γ2 and γ3). The expression of all subunits, except for α4 and γ3, was detected in the DG and CA3c regions.

5.2 $GABA_A$ receptor subunit messenger RNA expression in the rat hippocampus in different experimental epilepsy models (I-III)

5.2.1 Adult rat (I)

In rats with chronic TLE, the expression of α2 and α4 mRNAs was decreased significantly in the CA3c and CA1 subfields, respectively, compared to that in controls. The expression of the β3 subunit, on the contrary, was elevated in all hippocampal subfields of epileptic rats with the highest increase in the granule cell layer and in the hilus, whereas the signal intensity of the γ2 mRNA subunit did not differ between the two groups in any hippocampal region. However, in stimulated animals the daily seizure frequency was varying from 0.2 to 26.8 seizures/ day.

5.2.2 Developing rat (III)

KA-induced SE significantly altered the normal developmental expression patterns of all studied α subunits (α1-5), as well as of β3 and γ2 subunits in all or some hippocampal subregions as compared to their age-matched control rats. The developmental down-regulations normally seen in the expression of mRNAs encoding α2, β3 and γ2 subunits, and the up-regulation of α1 subunit mRNA in the CA3 pyramidal cell regions were not detected in KA-treated rats. In KA-treated rats, the normal developmental $GABA_A$ receptor expression pattern was altered. Significant developmental changes were detected 1 week after SE in the expression of α3 subunit mRNA in all subregions, in α4 mRNA expression in the CA3 regions, and α5 expression in CA3a/b and CA1.

When comparing the $GABA_A$ receptor subunit mRNA expression between KA-treated and their age-matched controls at various time-points after SE further significant differences were found. The α1 mRNA expression was down-regulated in CA3c 1 week after SE, γ1 expression in the CA3c, CA1, and DG regions 6 h, and γ2 expression in all subregions 6 h and 3 days after SE. On the other hand, the β1 mRNA expression was up-regulated 6 h, and β3 subunit mRNA 1 week after SE, both in CA1.

5.2.3 Hippocampal slice cultures (II)

The chronic treatment of cultures either with DNQX or picrotoxin caused region- and subunit-specific changes in mRNA expression, when compared with untreated control cultures. The picrotoxin treatment significantly increased the mRNA expression of α1 in CA3a/b and CA1,

and of α5 and γ2 in CA1. The treatment with DNQX instead dramatically increased β1 subunit mRNA expression in DG, as well as also significantly the expression of α2 mRNA in CA3c and DG. The expression of the other studied subunits showed no significant changes after the treatments.

5.3 Immunostainings of $GABA_A$ receptor subunits in the developing rat brain and after kainic acid-induced status epilepticus (III, IV)

The immunostainings of α1, α2 and β3 subunits were studied in the hippocampus 6 h and 7 days after KA-induced SE together with their age-matched controls (III). Similarly to the mRNA expression, the α1 immunoreactivity increased, whereas the α2 and β3 immunoreactivities decreased during the maturation in control rats. The α1 subunit specific antibody immunostained mainly processes of the main cell types in CA1, CA3, and DG, whereas α2 showed pronounced immunostaining in cell bodies of CA3 pyramidal cells and dentate granule cells. The number and staining intensity of α1 interneurons increased in the CA3 and DG regions during the development. Also interneurons in the CA3 and DG were α2 immunopositive. β3 was moderately immunostainined at P9 and the staining was localized in the cell processes within the stratum oriens and radiatum of CA3 and CA1, and in the molecular layers of the DG.

The localization of the subunits were rather similar between the treated and control rats either 6 h or 7 days after SE. The developmental changes in the α1 and α2 immunoreactivities were less pronounced in KA-treated rats, than those in the control rats, in particular in the stratum oriens and radiatum of the CA3 and CA1 regions (III). In P16 KA-treated rats (1 week after SE), the α1 immunoreactivity was attenuated, and the amount of stained neurons was significantly decreased in the CA1 and CA3 pyramidal cell layers compared to control rats (IV). However, no clear differences were detected in the β3 immunostaining between either P9 or P16 treated and control rats (III).

5.4 Pharmacology of $GABA_A$ receptor subtypes in the developing rat brain

5.4.1 Developmental benzodiazepine receptor expression

The number of total [^{3}H]flunitrazepam binding sites was measured in P9 and P16 control rats in the following regions of the hippocampus: the CA3 and CA1 stratum pyramidale, oriens,

lacunosum moleculare, and the stratum granulare, and molecular layers of the DG, and additionally in the parietal cortex and thalamus. The total [^{3}H]flunitrazepam binding was more than two-fold higher in P16 than in P9 control rats in all these studied regions.

The displacement of [^{3}H]flunitrazepam binding by zolpidem was analyzed in P9 and P16 control rats. For the displacement 10, 30, 100, 300 nM, and 3 and 100 μM zolpidem was used, corresponding to the approximate equilibrium dissociation constants (K_i) for zolpidem displacement of [^{3}H]flunitrazepam binding; the high K_i = 2.7 nM, low K_i = 67 nM, and very low K_i = 4.1 μM affinity sites. The IC_{50} values were calculated from the displacement curve of [^{3}H]flunitrazepam binding by zolpidem. These values were also significantly higher in P16 than in P9 control rats in all other brain regions, except for in the CA3 stratum pyramidale and the parietal cortex. According to the proportion of zolpidem displacement of the total [^{3}H]flunitrazepam binding between the two age groups, the proportion of both the very low (3 μM), and low affinity (30 to 100 nM) zolpidem binding sites were higher in P16 than in P9 control rats in most hippocampal subregions and in the cortex. Within the high affinity site (10 nM), the displacement by zolpidem was more effective in P16 than in P9 control rats only in the CA1 stratum oriens, and CA3 stratum pyramidale.

5.4.2 Changes in benzodiazepine- and zolpidem-sensitive binding after kainic acid-induced status epilepticus

The total [^{3}H]flunitrazepam binding was significantly higher in P9 rats 6 h after KA-induced SE in the stratum oriens and stratum lacunosum of the CA3 region, but decreased 1 week after SE in the CA1 and CA3 stratum pyramidale, and in the stratum granulare of the DG.

The displacement of [^{3}H]flunitrazepam binding by zolpidem was analyzed in P9 rats 6 h, and 1 week after SE together with their age-matched control rats. In general, SE significantly attenuated the potency of zolpidem to displace [^{3}H]flunitrazepam binding in both age groups. In P9 rats 6 h after SE, zolpidem displaced [^{3}H]flunitrazepam binding significantly less within the low affinity binding range in the molecular and granule cell layers of the DG (100 nM), and in the parietal cortex (30 nM), and within the high affinity binding site in the thalamus (10 nM). The IC_{50} value was significantly increased 6 h after the KA treatment, in the granule and molecular cell layers of the DG.

In P16 rats, the displacement curves of [^{3}H]flunitrazepam binding by zolpidem were almost identical in control and KA-treated groups. However, in the CA1 and CA3 stratum pyramidale,

zolpidem displaced [^{3}H]flunitrazepam binding significantly less in KA-treated than in control rats within the high (10 nM) and low affinity binding sites (300 and 100 nM respectively).

5.5 Damage and loss of hippocampal cells in different experimental epilepsy models (I-III)

In rats with chronic TLE, neuronal cell loss was detected bilaterally in the hippocampus with thionin staining. The most severely affected subregions were the CA3 and CA1 regions (I).

The immature brain was evidently more resistant to the increased excitability. The neuronal cell layers were well preserved in treated hippocampal slice cultures as (II). Similarly in rats after KA-induced SE, no neuronal degeneration was detected with Fluoro-Jade B and no neuronal loss was observed with thionin staining at any time point. Thionin staining also showed that the hippocampal cell layers were well preserved in the studied age groups after KA-induced SE (III).

6. DISCUSSION

6.1 Methodological considerations

6.1.1 Epileptic in vivo animal models as models of human epilepsy

Experimental animal models of epilepsy try to mimic human epilepsy as much as possible. In the epilepsy models described here, a chemoconvulsant or electrical stimulation is administered to produce recurrent seizures in an unnatural way, whereas in human epilepsy there is a pathological process causing the seizures. There is also substantial variability between animals experiencing seizures. For example, the duration of seizures or the seizure frequency and type in animals after SE may be very different (Sankar *et al.*, 1998; Kubova *et al.*, 2004). Despite these differences, the animal seizure models provide information about neuropathological consequences of seizures, whereas they provide only rough measures of learning and memory. Furthermore, when handling immature rats it is important to consider also other factors than seizures that may influence the results. In neonatal rats exposed to recurrent seizures in addition to daily separation from their mothers, neuronal degeneration was more prominent, and long-term cognitive impairments were worse than in rats with neonatal seizures alone (Huang *et al.*, 2002). Likewise, P9 rats that also experienced repeated maternal separation maintained a more immature $GABA_A$ receptor phenotype, and exhibited more active responses to stress than did control rats (Hsu *et al.*, 2003). Therefore, in our study of SE in P9 rats, pups were taken back to their lactating mothers immediately after recovery of SE, and control and KA-treated rats were separated from their mother for an equal time.

In the KA model of epilepsy, an acute excitotoxic insult (SE) is later followed by spontaneous seizures in adult rats (Wasterlain *et al.*, 2002). In adult rats, SE will lead to more severe hippocampal injury, than with chronic models of TLE where repeated seizures are induced e.g. by electrical stimulation (Haas *et al.*, 2001). However, spontaneous seizures do not develop in immature rats after KA-induced seizures before P21, although they occur in adult rats (Wasterlain *et al.*, 2002). Therefore, in our model where seizures are induced in P9 rats by KA (III, IV), the effects of seizures thus represent those of more acute type, as no spontaneous seizures appear comparable to adult rats, during the time window we have studied. Also, a lesser extent of hippocampal damage has been detected in immature rats than in adult rats after KA-induced seizures (Haas *et al.*, 2001; Lopez-Picon *et al.*, 2004). This is however not due to an

absence of KA receptors (Berger *et al*., 1984), because there is a high density of KA receptors in the hippocampus of young rats (Bahn *et al*., 1994; Ritter *et al*., 2002).

Furthermore, seizures are more easily induced, and the dosage of KA to induce seizures is much lower (~2mg/kg) in immature rats than in adult animals (~10 mg/kg) (Stafström *et al.*, 1992; Lopez-Picon *et al.*, 2004). The seizures are also rather severe and frequently lethal for young animals, so in order to reduce the duration of SE a single dose of paraldehyde was administered shortly after KA injection, as described recently (Kubova *et al.*, 2001). The behavioral seizures are thereby suppressed, but ictal activity may still continue as has earlier been shown by EEG recordings (Kubova *et al.*, 2004; Druga *et al.*, 2005).

In the other animal model of epilepsy used in the present study (I), SE, lasting for several hours, was induced by a 20 to 30 min electrical stimulation of the lateral nucleus of the amygdala in adult rats (Nissinen *et al.*, 2000). The lateral nucleus of the amygdala can be considered a good stimulation site because of its low threshold for electrically induced seizures (Goddard *et al.*, 1969). Moreover, the most wide-spread intra-amygdaloid projections originate from the lateral nucleus of the amygdala, thus facilitating the spread of seizure activity (Pitkänen *et al.*, 1997). After a latency period of approximately 1 month, adult rats develop spontaneous seizures, the sequence of events mimicking the pattern of human TLE. The pattern and neuropathological changes of the seizures can then be followed up for long periods. The long follow-up period after SE enables testing of new AEDs, possibly for preventing epileptogenesis or the development of recurrent seizures. The effect of the AED vigabatrin to spontaneous seizures in this model appears similar to that found in the treatment of human epilepsy (Nissinen & Pitkänen, 2007).

Other advantages of the electrical stimulation-induced SE model include easy induction of seizures at an identified site, and with a known stimulus. With this model the toxic effects associated with chemical convulsants are avoided. Furthermore, the epileptogenic agent is acting only during application of current and it is not interfering with the epileptic brain activity. On the other hand, the electrical current stimulates all neurons in the stimulated tissue. For the local electrical stimulation procedure, good equipment and operator experience is required for reproducible experiments, and these experiments are also time-consuming and labor-extensive. Therefore, if the main interest is to study acute effects of seizures, the KA-model requiring less effort is preferred.

6.1.2 Organotypic hippocampal slice cultures as in vitro model of epilepsy

Organotypic hippocampal slice cultures are intact preparations of postnatal hippocampus that correspond to perinatal human hippocampal tissue (Caeser & Aertsen, 1991; Frotscher *et al.*, 1995). In these cultures, many of the intrinsic properties of the tissue are maintained, including important aspects of connectivity, and the morphological organization of the hippocampus is well preserved. During the first two weeks in culture, many developmental processes continue, e.g. proliferation of granule cells, and outgrowth and organizarion of axons and dendrites and the maturation of synapses and receptors resembles that seen *in vivo* (Caeser & Aertsen, 1991; Frotscher *et al.*, 1995; II). Therefore, the *in vitro* slice cultures are also valuable models to study developmental changes in cellular and molecular reorganizations, such as in $GABA_A$ receptor subunit expression. Furthermore, in contrast to acute slices, the organotypic slice cultures possess all of the neuron types that are present in the brain *in vivo* and they also can be further cultivated after pharmacological manipulations. However, the *in vitro* slice cultures are usually maintained in an artificial growth medium, supplied with serum, and extrinsic factors such as the immune signaling system is lost, so it is obvious that they may not be straightforward comparable to a brain *in vivo*.

Nevertheless, all slice preparations have undergone a period of ischemia and the normal afferent input to dentate granule cells has been severed (Caeser & Aertsen, 1991; Frotscher *et al.*, 1995). Upon culturing this leads to some reorganization of excitatory synaptic circuitry in slice cultures, i.e. the MFs sprout into the molecular layers of the DG, and the dentate granule cells show enhanced excitability (Bausch & McNamara, 2000). As result of the abnormal connections in slice cultures, seizure-like events can be induced in slice cultures, for example by application of $GABA_A$ receptor antagonists (Scanziani *et al.*, 1994; Bausch & McNamara, 2000). Furthermore, in mature hippocampal slice cultures (12-20 DIV) treated for 3 days with picrotoxin, there were morphological changes similar to those observed in the hippocampus of patients with epilepsy, such as loss of neurons and dendritic spines (Müller *et al.*, 1993). Thus, cultured hippocampal slices represent an ideal, carefully controlled system to study cellular and molecular rearrangements, such as region- and subunit-specific changes in $GABA_A$ receptor subunits, induced by seizure-like events. Some of the changes may even be comparable to those of the epileptic hippocampus.

6.2 Neuropathology of epilepsy in the developing and adult rat brain

In the amygdala stimulation model used in our study, the most severe damage has been shown to appear in the amygdala and surrounding cortex of adult rats (Nissinen *et al.*, 2000). Consistently, amygdaloid damage has been found in humans who died from SE (Pitkänen *et al.*, 1998) and damage of the entorhinal cortex has been found in humans with TLE (Mikkonen *et al.*, 1998). In addition, hippocampal damage was found in our model, including neuronal loss in the hilus as well as in the CA1 and CA3 areas, as also found in human TLE (Mikkonen *et al.*, 1998; Pitkänen *et al.*, 1998). In rats of this model, MF sprouting has been found (Nissinen *et al.*, 2000), the feature typical for patients with TLE (Engel, 1996; Kälviäinen & Salmenperä, 2002).

In adult rats, death of hippocampal neurons, particularly in the CA3 and CA1 regions (Fujikawa *et al.*, 2000; Zhang *et al.*, 2002), and also in the DG (Buckmaster & Dudek) has frequently been detected after KA-induced seizures. Even a brief, non-convulsive seizure evoked by kindling stimulation was found to produce apoptotic neurons in the rat DG (Bengzon *et al.*, 2002). In contrary, several studies have found the immature rat hippocampus to be resistant to seizure-induced neuronal death, where seizures were induced with KA at P1-16 (Lynch *et al.*, 2000; Haas *et al.*, 2001; Lopez-Picon *et al.*, 2004). However, in other studies neuronal death has been detected in the hippocampus after KA-induced seizures at P7 (Humphrey *et al.*, 2002; Dong *et al.*, 2003). Neuronal damage has also been found in hippocampal regions of P14 rats (Sankar et al., 1998) as well as in the thalamic region of P12 rats (Kubova *et al.*, 2001; Druga *et al.*, 2005) after seizures induced with lithium-pilocarpine. Neuronal death was also detected in cultured hippocampal slices started at P6-7 and treated with KA for 2 days (Holopainen *et al.*, 2001a; Holopainen *et al.*, 2004). Furthermore, in immature P10 rats hippocampal neurons were injured 24 h after febrile seizures, but 4 weeks after the seizures no significant neuronal death was detected (Toth *et al*., 1998; Baram *et al.*, 2002), indicating that some kind of recovery of damaged neurons may occur.

Sprouting of dentate granule cell MFs, has been detected besides in adult experimental animals after KA-induced seizures (Buckmaster & Dudek, 1997; Lynch & Sutula, 2000), also in epileptic humans (Engel, 1996; Kälviäinen & Salmenperä, 2002). Similarly to the rarely seen neuronal death, MF sprouting of varying degree has been detected in the immature brain. In our KA model of P9 rats, neither neuronal death nor MF sprouting was found 1-4 weeks after induction of seizures (Lopez-Picon *et al.*, 2004; III). On the contrary, prolonged febrile seizures induced at P10 resulted in MF sprouting 3 months later (Bender *et al.*, 2003). Also in cultured

hippocampal slices started at P10-12 and treated with KA for 2 days sprouting of MFs was detected (Routbort *et al*., 1999). On the other hand, the neuronal viability of cultured hippocampal slices was not affected by chronic treatment with a $GABA_A$ receptor antagonist picrotoxin or with a non-NMDA receptor antagonist DNQX (II). The discrepancy between different studies in the consequences of seizures may be due to the fact that available techniques are not sensitive enough to detect limited neuronal loss, which can be present only in a subpopulation of surviving animals (Nairismägi *et al*., 2006). However, factors such as the postnatal age of the animals, as well as the type of animal model used in the studies may also contribute to the differences in seizure outcome.

6.3 $GABA_A$ receptor expression in the rat brain

6.3.1 Receptor expression during development

In the developing rat hippocampus, GABA acts as an excitatory neurotransmitter (Ben-Ari, 2002). Around the end of the second postnatal week GABA switches from a depolarizing to a hyperpolarizing mode (Ben-Ari *et al.*, 1989; Khazipov *et al.*, 2004). During this period many other developmental changes also take place. An important feature is the expression patterns of $GABA_A$ receptor subunit mRNAs that change region- and subunit-specifically during the embryonic and postnatal development (Fritschy *et al.*, 1994; Brooks-Kayal *et al.*, 1998; II; III). This was verified in our *in situ* hybridization studies, both using organotypic hippocampal slice cultures *in vitro* (II) and in immature rat hippocampus *in vivo* (III). The developmental subunit expression in these two models can be compared, as the slice cultures were prepared from P6-7 rats and cultured for 7 days *in vitro* which corresponds roughly to P14 rats *in vivo*, whereas the studies in immature rats were performed during the postnatal brain development from P9 to P16. Compared to humans, these stages of hippocampal development in rodents can approximately be correlated to the development of a full-term newborn baby (Marsh *et al.*, 2006). This is an important period of brain development in both rodents and humans, when the brain is growing most rapidly, with intense neuronal growth and synaptogenesis (Dobbing & Sands, 1979).

Especially the strictly developmentally regulated expression patterns of α1 and α2 subunits (Laurie *et al.*, 1992; Poulter *et al.*, 1992; Fritschy *et al.*, 1994; Paysan & Fritschy, 1998) could be detected in our studies, and they were surprisingly similar in the slice cultures (II) to those observed in the rat hippocampus *in vivo* (III). The expression level of α2 mRNA decreased significantly between P9 and P16 in rat hippocampus, whereas α1 mRNA increased. Similarly,

in slice cultures, the expression level of α2 was also high, with the highest level in the CA3 region similar to the immature rats and the level of α1 mRNA was much lower. The highest mRNA level was detected for α5 subunit in slice cultures in all subregions and in accordance its expression was at its highest in the CA3 region of immature rats at P12, and then decreased.

Corresponding with the increased α1 mRNA expression, the amount of BZ-sensitive $GABA_A$ receptors increased in immature rat hippocampus between P9 and P16, as also shown in previous studies (Daval *et al.*, 1991; IV). The zolpidem displacement of [^{3}H]flunitrazepam binding within the high affinity site was also more pronounced in P16 than in P9 control rats within some regions of CA3 and CA1. This is in accordance with a postnatal increase of the α1β2γ2 $GABA_A$ receptor subtype, involved in synaptic inhibition and with high affinity for zolpidem (Brooks-Kayal *et al.*, 2001; Roberts & Kellogg, 2000; Hevers & Lüddens, 2002).

6.3.2 Effects of seizures in the immature brain

6.3.2.1 Changes in the expression of $GABA_A$ receptor subunits

By treating hippocampal slice cultures with the $GABA_A$ or glutamate receptor antagonists, picrotoxin respective DNQX, it was shown that a disruption of the balance between excitation and inhibition in neurons can alter the mRNA expression of $GABA_A$ receptor subunits in a region- and subunit-specific manner (II). There are only a few earlier studies on this specific question, and in those studies different treatment conditions have been applied (e.g. Gerfin-Moser *et al.*, 1995), so they are not straightforward comparable to the results of our study. The slice cultures in our study, were chronically treated with drugs during the entire culture period of 7 days. The time period when *in situ* hybridization was performed in these slice cultures, is a critical period in brain development when GABA is thought to change from an excitatory to inhibitory transmitter (Ben-Ari *et al.*, 1989; Khazipov *et al.*, 2004).

It is widely assumed that death of neurons is a direct consequence of excessive glutamatergic excitation that also causes the epileptiform discharge. This could be due to the activation of both NMDA and non-NMDA, i.e. AMPA and kainate, receptors that increases the intracellular Ca^{2+} (Holopainen *et al.*, 1989). Blockade of these receptors with a glutamate receptor antagonist such as DNQX is suggested to be neuroprotective (Drian *et al.*, 2001). KA-induced neuronal death was effectively decreased by low doses of DNQX at the early phase (within 12 h), but a longer exposure (24 h) significantly enhanced the damage in the CA3 regions (Holopainen, 2005).

How these mechanisms are related to the changes in subunit expression in our study remains unknown. The increased expression of α2 in the CA3 and DG regions after DNQX treatment, and the increased expression of α1 and γ2 in the CA3 and CA1 regions after picrotoxon treatment could represent some compensatory mechanisms associated with neuroprotection. In hippocampal slices cultured for 13 d in the presence of bicuculline, the density of GAD65-immunoreactive terminals was increased in the CA1 area when compared with control slices, whereas treatment with DNQX decreased their density in CA1 (Marty *et al.*, 2000). Tonic $GABA_A$ receptor activity is present in developing pyramidal cells (Demarque *et al.*, 2002). Under normal conditions, pharmacological blockade of tonic GABAergic inhibition with picrotoxin selectively enhances the excitability of interneurons leading to an increase in $GABA_A$ receptor-mediated tonic conductance in CA1 pyramidal cells (Semyanov *et al.*, 2003). The α5 subunit, up-regulated by picrotoxin in CA1 in our study (II), is often extra-synaptic, raising the possibility that blocking of $GABA_A$ receptors may cause increased inhibitory activity in CA1 pyramidal cells.

Similarly, KA-induced SE in immature 9-day-old rats altered in a time-dependent region-and subunit-specific manner the normal developmental expression pattern of $GABA_A$ receptor subunit mRNAs, especially those encoding α1, α2, β3 and γ2 subunits. The disruption of the normal expression pattern, may have long-term consequences on many developmental processes. Deficiency of the α1 subunits in knockout mice impairs normal development of dendritic spines (Heinen *et al.*, 2003), indicating that this subunit is needed for the development and maintenance of normal synaptic contacts. Furthermore, seizure activity can disturb the maturation of the inhibitory synaptic transmission and its strength (McLean *et al.*, 1996; Marty *et al.*, 2000). It has been shown that the α1 subunit is important at the inhibitory synapse by regulating the duration of IPSCs (Hevers & Lüddens, 2002). In studies using acutely dissociated cerebellar Purkinje cells from juvenile mice, gene deletion of the α1 subunit resulted in almost complete loss of spontaneous and evoked IPSCs (Sur *et al.*, 2001). The increased developmental expression of α1 subunit is proposed to be important for formation of the fast inhibitory synaptic currents (Vicini *et al.*, 2001). Conversely, the expression of α2 subunit is suggested to be down-regulated by the inhibitory synaptic activity (Hevers & Lüddens, 2002). Therefore, the enhanced excitatory activity induced by KA in our immature rats could disturb processes such

as synaptogenesis, which is suggested to temporally coincidence the developmental switch in α subunits (Fritschy *et al.*, 1994).

6.3.2.2 Changes in the $GABA_A$ receptor pharmacology

The alterations in subunit expression are associated with changes in both function and pharmacology of the $GABA_A$ receptor. There is evidence for enhanced inhibition after KA-induced seizures in the postnatal period (P1-14) (Lynch *et al.*, 2000). In our pharmacological study, the total [^{3}H]flunitrazepam binding was increased 6 h after KA-induced SE in P9 rats in the CA3 region (IV). This is in accordance with other studies showing augmented BZ-binding after seizures in the immature rat brain (Werck & Daval, 1991; Rocha *et al.*, 2000), as well as with our *in situ* hybridization study where the α1 mRNA level was increased acutely after SE in CA3 (III). One week after SE, the total [^{3}H]flunitrazepam binding was decreased in the CA1 and CA3 pyramidal cell layers. In accordance, at the same time point zolpidem displaced [^{3}H]flunitrazepam binding less effectively within the high zolpidem affinity binding site of the CA1 and CA3 pyramidal cell layers in KA-treated rats (IV). Altogether, these results could indicate an acute seizure-induced increase in α1 subunit expression already after 6 h, perhaps to enhance inhibition, but after 7 days there is a significant decline of α1 expression (III; IV). On the other hand, rats with pilocarpine-induced SE at P10 had increased α1 subunit expression 3 months later in dentate granule cells, and in accordance enhanced zolpidem augmentation (Zhang *et al.*, 2004). These rats did not become epileptic unlike adult rats that have a decreased α1 subunit expression (Gibbs *et al.*, 1997; Brooks-Kayal *et al.*, 1998b). Moreover, seizure-induced changes in the mRNA expression of most $GABA_A$ receptor subunits may closely be associated with the corresponding protein levels (Brooks-Kayal *et al.*, 2001), as we have shown for the α1, α2 and β3 subunits (III. IV).

6.3.3 Effects of seizures in the adult brain

In our study of adult rats with chronic TLE, the expression of several $GABA_A$ receptor subunits were found to undergo region-selective changes in the hippocampus after the development of spontaneous seizures (I). Changes in α subunits after seizures have been variable between different studies. We found that the mRNAs of α2 and α4 subunits were down-regulated in the CA3c and CA1 subfields of the hippocampus, respectively, in epileptic rats compared to controls. Similarly, α2 expression has decreased in the CA1-CA3 regions 1-2 months after KA

or pilocarpine injection in rats (Rice *et al.*, 1996; Tsunashima *et al.*, 1997). Other groups have found increases in the expression of α2 and α4. The α4 mRNA expression has increased in rat granule cells 1-4 months after pilocarpine- or electrically-induced SE (Brooks-Kayal *et al.*, 1998b; Nishimura *et al.*, 2005), whereas in our model it remained unchanged. Seven days after injection of KA, α4 expression has increased in dentate granule cells, but returned to the control level after 30 days (Tsunashima *et al.*, 1997). Likewise, α2 subunit mRNA expression has transiently increased in the CA1 and DG regions in the kindling model, and then returned to the control level at long-term (Kamphuis *et al.*, 1995). In our study, the amount of γ2 subunit did not change in any hippocampal subregion, which is consistent with the pilocarpine model where γ2 expression remained unchanged (Rice *et al.*, 1996). On the other hand, the expression of the β3 subunit, a subunit relatively highly expressed also in normal brain, was considerably increased in all hippocampal subregions in our rat model, suggesting that the β3 subunit may be of importance in controlling the neuronal excitability. The increased β3 subunit expression after seizures is in accordance with earlier *in situ* hybridization studies, using several different experimental animal models at both the acute and chronic phase (Kamphuis *et al.*, 1995; Schwarzer *et al.*, 1997; Brooks-Kayal *et al.*, 1998b; Sperk *et al.*, 1998; Nishimura *et al.*, 2005). Similar cell-specific as well as cell layer-specific changes in $GABA_A$ receptor subunit expression as in the hippocampus of epileptic animals has also been found in patients with TLE, with enhanced β3 expression and modest or no changes in γ2 expression (Loup *et al.*, 2000; Pirker *et al.*, 2000).

The discrepancy of the results obtained in various studies indicate that the seizure-induced acute alterations in the expression of $GABA_A$ receptor subunits are not necessarily similar to those of the chronic state. The changes detected here were studied in rats having chronic epilepsy (I), an exceptionally long period (3 months) after induction of seizures, whereas most other studies have focused on the effects of SE after a shorter time point (usually up to one month) (Schwarzer *et al.*, 1997; Sperk *et al.*, 1997; Brooks-Kayal *et al.*, 1998b). In addition, differences between the present and earlier findings in the subunit expression could partly be related to the animal model used as well as the method used to measure mRNA levels. If mRNA is measured by traditional *in situ* hybridization, changes in expression of individual neurons of a single cell type are not detectable, which is possible with the single-cell mRNA amplification approach.

Because of the neurodegeneration in the CA1 and CA3 regions in our model (I), the changes in subunit expression are more difficult to interpret than in granule cells that are relatively spared in experimental models. Lasting decreases of α2 and α4 subunits could at least partly be due to loss of pyramidal cells. However, the up-regulation of β3 expression in these regions supports the idea that the decreased expression of $GABA_A$ receptor subunits in epileptic tissue represents real changes occuring in the surviving neurons. The α2 subunit-containing receptors are usually located at axo-axonic synapses on CA1 pyramidal cells (Nusser *et al.*, 1996; Fritschy *et al.*, 1998), strategically located to effectively inhibit the generation of action potentials of these neurons (Maccaferri *et al.*, 2000; Fritschy & Brünig, 2003). These excitatory pyramidal cells are innervated by GABAergic interneurons that regulate their excitability through $GABA_A$ receptors (Freund & Buzsaki, 1996). If receptors containing α2 subunit are lost, the inhibitory activity is assumed to be affected. Accordingly, $GABA_A$ receptor-mediated inhibition seems to be impaired in the CA1 and CA3 regions in kindled experimental animals (Titulaer *et al.*, 1995a). The long-term alterations in $GABA_A$ receptor subunit α2, α4 and β3 expression may represent compensatory responses to seizure activity in the remaining pyramidal neurons, aiming at enhanced $GABA_A$ receptor-mediated neurotransmission to prevent the spontaneous seizures of the chronic epileptic state in our model.

The changes in subunit composition in adult rats may also contribute to changes in $GABA_A$ receptor pharmacology. As the subunits assemble into pentameric receptor complexes, usually having two α subunits, two β subunits, and one γ subunit, the seizure-induced changes in individual subunits are difficult to interpret (Korpi *et al.*, 2002). There seem to be a persistent seizure-induced decrease in BZ binding in the CA1 and CA3 regions (Titulaer *et al.*, 1995c; Rocha & Ondarza-Rovira, 1999). Binding studies in kindled rat brain, however, show increases in muscimol, BZ and TBPS binding in the DG, labeling the GABA, the BZ and the Cl^- channel sites of the $GABA_A$ receptor complex, respectively (Shin *et al.*, 1985; Nobrega *et al.*, 1989, 1990; Titulaer *et al.*, 1995b, c). These increases were significant shortly after kindling, but returned to control levels at 4 weeks (Shin *et al.*, 1985; Nobrega *et al.*, 1990). There is also initially increased GABAergic transmission in the DG of kindled rats (Buhl *et al.*, 1996; Gibbs *et al.*, 1997; Nusser *et al.*, 1998a).

Moreover, after repeated seizures, the inhibition and number of GABAergic interneurons seem to decrease (Bouilleret *et al.*, 2000; Sayin *et al.*, 2003). These interneurons provide axo-somatic and axo-axonic inhibition to granule cells of the DG, and due to their reduction the

excitatory output from granule cells to pyramidal neurons in CA3 could be enhanced and contribute to spontaneous seizures (Sayin *et al.*, 2003). The up-regulation of the β3 subunit could contribute to the increased $GABA_A$ receptor-dependent inhibition in the DG and may indicate that some compensatory subunit expression takes place. Since the α subunits (α1, α2, α3 and α5) confers sensitivity to BZs, it is possible that remaining receptors with reduced α2 and α4 subunit assembly might be compensated with other α subunits, or there might be a loss of functional receptors in some cells but not in others. Mouse lines in which the α1 subunit has been genetically deleted, exhibited a decreased number of type I BZ binding sites (Sur *et al.*, 2001; Kralic *et al.*, 2002b). These mice had instead a compensatory increase in type II BZ binding sites and in accordance increased $GABA_A$ receptor α2 and α3 subunit immunoprecipitation (Sur *et al.*, 2001; Kralic *et al.*, 2002b). The α5 subunit mRNA is decreased in the CA1-CA3 regions, but increased in the DG after seizures (Rice *et al.*, 1996), and could thereby give rise to increased BZ binding in the DG. As the α5 subunit most commonly forms receptors together with the β3 and γ2/3 subunits (Fritschy & Möhler, 1995), the increases in α5 and β3 subunit expression could contribute to the increased BZ binding in the DG.

Certainly, seizures may have an influence on many factors, such as those involved in the assembly and clustering of subunits, as well as presynaptic factors including release probability of GABA, and diffusion of GABA from the cleft, that may in turn affect the expression, function and pharmacology of $GABA_A$ receptors. Endocytosis of receptors is another important mechanism for regulation of their short and long-term expression on the cell surface (Connolly *et al.*, 1999; Kittler *et al.*, 2000). Indeed, there is an increase in $GABA_A$ receptor endocytosis during SE (Blair *et al.*, 2004). It was proposed that endocytosis provides synaptic plasticity by contributing to short-term regulation of GABAergic inhibitory transmission in direct response to acute changes in network activity (Kittler *et al.*, 2000; van Rijnsoever *et al.*, 2005).

6.4 Functional consequences of altered $GABA_A$ receptor expression in the immature and adult brain

The findings of this thesis together with other studies, suggest that the $GABA_A$ receptor subunit composition is different in the developing rat brain compared to the adult. Moreover, increased neuronal activity may disrupt the normal maturation of $GABA_A$ receptor subunits during early postnatal development. The effects of seizures on subunit expression are also region- and age-dependent and may lead to consequences on receptor function, the effects, however, being

different in the adult compared to the immature brain. Besides, GABA plays an important role during development. Thus in addition to their obvious role in controlling excitability in adult brain, a change in $GABA_A$ receptor function during development could be expected to elicit neurodevelopmental abnormalities perhaps leading to epilepsy.

The β3 subunit gene seems to be necessary for normal $GABA_A$ receptor function, as β3 knockout mice have spontaneous seizures with high mortality and a phenotype with marked similarities to patients with Angelman syndrome (Homanics *et al.*, 1997; DeLorey *et al.*, 1998; Holopainen *et al.*, 2001b). Angelman syndrome is a neurodevelopmental disorder with severe mental retardation, epilepsy and delayed motor development. The majority of patients have a mathernal deletion in the gene coding for β3 subunit (DeLorey *et al.*, 1998; Holopainen *et al.*, 2001b). Disruption of the γ2 subunit gene in mice likewise causes high lethality at birth, and a 94% reduction in binding to the BZ binding site on the $GABA_A$ receptor (Günther *et al.*, 1995). Genetic studies in humans revealed a point mutation of the γ2 subunit linked to childhood absence epilepsy and febrile seizures (Wallace *et al.*, 2001). In frog oocytes, this mutation showed diminished sensitivity to BZs (Wallace *et al.*, 2001). In addition, a mutation in the M3 region of the α1 subunit is related to a common epilepsy syndrome in humans, i.e. juvenile myoclonic epilepsy (Cossette *et al.*, 2002).

An interesting hypothesis is that the general pattern of $GABA_A$ receptor subunit expression, and its pharmacological profile is in many ways similar in immature brain to that of adult animals with TLE. The relative expression of α1 mRNA is significantly lower in immature than in adult dentate granule cells resulting in lower sensitivity to augmentation by zolpidem and higher sensitivity to Zn^{2+} inhibition, although zinc concentrations in dentate gyrus are low in immature rats (Brooks-Kayal *et al.*, 2001). In adult epileptic animals the expression of α1 mRNA and zolpidem augmentation are decreased in the DG, and the inhibition by Zn^{2+} is coincidently increased (Buhl *et al.*, 1996; Brooks-Kayal *et al.*, 1998b). The MFs sprout extensively into the inner molecular layer of the DG and could provide an increased source of Zn^{2+} (Tauck & Nadler, 1985), which could lead to the long-term failure of inhibition in epileptic tissue that facilitates seizure generation (Buhl *et al.*, 1996; Gibbs *et al.*, 1997; Brooks-Kayal *et al.*, 1998b; Cohen *et al.*, 2003).

Furthermore, the inhibitory GABAergic function can change to excitatory during seizure activity (Khazipov *et al.*, 2004). Depolarizing GABAergic responses have been recorded in pyramidal neurons from human epileptic hippocampal slices (Cohen *et al.*, 2002). This could be

due to the intracellular Cl^- concentration that seems to be altered in epileptic networks, such that the GABAergic function would become excitatory in the epileptic brain activity (Cohen *et al.*, 2002). The changed GABAergic function could be mediated by intracellular cascades that involve down-regulation of KCC2 which leads to impaired Cl^- extrusion from neurons, as shown in the hippocampus of kindled mice (Rivera *et al.*, 2002). The NKCC1 transporter that is highly expressed in the immature brain plays the opposite function, accumulating Cl^- in neurons (Kaila, 1994), and is, on the other hand, up-regulated in the rat cortex after kindling (Okabe *et al.*, 2002). These abnormal expression patterns of the Cl^--transporters causing altered Cl^- transport and decreased GABAergic inhibition, were also seen in hippocampal specimens from human TLE patients (Palma *et al.*, 2006). Thus, the adult epileptic brain seems to revert to a more immature state, that may be necessary for repair mechanisms that operate at the network level (Rivera *et al.*, 2005).

A fascinating approach is that altering $GABA_A$ receptor subunit expression could affect the development of epilepsy. This was successfully achieved by using a novel promoter, that was up-regulated after SE and successfully increased the expression of α1 subunit and reduced the incidence of seizures (Raol *et al.*, 2006a). Furthermore, the anticonvulsant efficacy of BZs is primarily mediated by receptors containing an α1 subunit (Rudolph *et al.*, 1999; Crestani *et al.*, 2002), which is also important for seizure sensitivity to $GABA_A$ antagonists (Kralic *et al.*, 2002a). It would be interesting to test if the anticonvulsant effects of BZs are conferred by the same α1 subunit-containing receptors in the immature brain for example by using transgenic immature animals with substitutions in α-subunits.

As GABA can have excitatory as well as trophic effects on immature neurons, an interfering compound on the immature GABA system, such as an AED, may have adverse effects on developmental processes (Ben-Ari & Holmes, 2005). Consequently, the use of classical BZs that potentiate the function of α1-, α2-, α3- and α5-containing receptors during the early development, may contribute to increased excitability (Ben-Ari *et al.*, 1997; Leinekugel *et al.*, 1997; Khazipov *et al.*, 2004). The enhancement by diazepam on $GABA_A$ receptor currents in dentate granule cells increases with postnatal development (Kapur & Macdonald, 1999). Certain types of neonatal and early childhood seizures are currently treated with BZs, as well as other AEDs. Adverse effects of some AEDs have been found in the immature brain, both in animals (Bittigau *et al.*, 2002; Raol *et al.*, 2005) and in children (Herranz *et al.*, 1988; Calandre *et al.*, 1990). They have even been shown to cause apoptotic neuronal death in the developing rat brain

(Bittigau *et al.*, 2002) and teratogenic effects as well as cognitive impairment in children born to mothers with epilepsy treated with AEDs during pregnancy (Calandre *et al.*, 1990; Meador *et al.*, 2006). Also in rat pups that were exposed to the AEDs vigabatrin, and valproate in utero, showed hippocampal and cortical dysplasias (Manent *et al.*, 2007).

At any rate, there are possible deleterious actions of drugs acting on $GABA_A$ receptors in the immature brain, and a need for more effective therapy in infants and children. Also the observation that the action of GABA reverses in the epileptic brain, sets our currently used AEDs in a new light and calls for new strategies in the development of new AEDs (Ben-Ari & Holmes, 2005). Better insight of the seizure-induced changes in $GABA_A$ receptor subunit expression is needed to be able to develop age- and subunit-specific drugs for patients with epilepsy.

7. SUMMARY AND CONCLUSIONS

In the current thesis project the main goal was to characterize seizure-induced alterations in $GABA_A$ receptor subunit expression in the developing and adult rat brain, specifically in the hippocampus. The developmental subunit expression was also verified. The main findings of the study are summarized.

1. Long-term epilepsy-induced changes in the $GABA_A$ receptor subunit expression were found in the hippocampus of adult rats with chronic TLE, i.e. having spontaneous seizures. These changes were both subunit- and subregion-selective and the most prominent ones were the decreased mRNA expression of the α2 and α4 subunits in the CA3c and CA1 regions, respectively, and the increased expression of the β3 subunit in all hippocampal subregions. These long-term alterations may lead to impaired GABAergic function, favoring the state of increased excitability that gives rise to spontaneous seizures. Another possibility is that the changes are compensatory responses to the chronic epileptic state in this model attempting to prevent seizure activity.

2. The strictly developmentally regulated expression patterns of $GABA_A$ receptor subunits during the second postnatal week were studied in organotypic hippocampal slice cultures *in vitro* and in developing rat hippocampus *in vivo*. This is a critical time period in development when the function of GABA is thought to change from excitatory to inhibitory. Comparison of the expression patterns of $GABA_A$ receptor subunit mRNAs in the two models indicated high expression of the α2 and α5 subunits, and low expression of the α1 subunit in cultured hippocampal slices, which is surprisingly similar to the subunit expression observed in rats of the corresponding age *in vivo*. In the immature rat hippocampus, α1 mRNA expression showed an increased profile between P9 and P16, whereas that of α2 and β3 showed a decreased one. Similarly, the α1 immunoreactivity increased, whereas the α2 and β3 immunoreactivities decreased during the development. Furthermore, the amount of BZ-sensitive $GABA_A$ receptors increased in the immature rat hippocampus between P9 and P16. Also the zolpidem displacement of [^{3}H]flunitrazepam binding within the high affinity site was more pronounced in P16 than in P9 control rats within certain subregions of CA3 and CA1. These findings indicate

that pharmacologically important changes occur in $GABA_A$ receptor subtypes during the development. They can be correlated to the increased developmental expression of the α1 subunit that is assumed to be involved in synaptic inhibition.

3. The $GABA_A$ receptor subunit expression was altered region- and subunit-specifically in cultured hippocampal slices, by disturbing neuronal activity with chronic treatment of a $GABA_A$ receptor antagonist picrotoxin, or with a non-NMDA receptor antagonist DNQX. Particularly, increased mRNA expression of the α1, α5 and γ2 subunits was found in the pyramidal cell layers of picrotoxin-treated cultures, and of the α2 subunit in the CA3 and DG and of the β1 subunit in the DG of DNQX-treated cultures. The picrotoxin-induced changes are comparable to changes in the epileptic hippocampus. The cultured hippocampal slices represent a potent, carefully controlled system to study seizure-induced cellular and molecular alterations, such as in the expression of $GABA_A$ receptors.

4. KA-induced SE in 9-day-old rats disturbed the normal developmental expression patterns of several $GABA_A$ receptor subunits in the rat hippocampus in a subunit- and subregion-specific manner during the critical second postnatal week. Especially, the normal developmental mRNA expression patterns of the α1, α2 and β3 subunits were abolished. The developmental changes in the α1 and α2 subunit immunoreactivities were also less pronounced in rats after SE, indicating that changes in the mRNA expression may be closely correlated to the corresponding protein levels. The changes in subunit expression were not due to neuronal loss in immature rats as they are resistant to neuronal damage. The pharmacological properties of BZ- and zolpidem-sensitive $GABA_A$ receptors were also region-specifically altered after SE in the immature rat brain during the postnatal period. The total [^{3}H]flunitrazepam binding decreased, and zolpidem displaced [^{3}H]flunitrazepam binding significantly less within the high affinity binding range in the CA1 and CA3 pyramidal cell layers of rats one week after SE. These results could imply a down-regulation of α1 subunit containing receptors in these specific regions one week after SE, which is consistent with the results both at the mRNA and protein level. Altogether, these findings suggest that the $GABA_A$ receptor subunit transcription is extremely sensitive to external stimuli during the early postnatal development. The SE-induced changes in the α1 and α2 subunit expression can profoundly affect the normal maturation of $GABA_A$ receptors that may

have long-term consequences on many developmental processes such as synaptogenesis, and lead to expression of receptors with abnormal function and pharmacology.

However, further research is needed to reveal possible alterations in $GABA_A$ receptor subtypes after acute seizures as well as after chronic epilepsy in the developing and adult brain. New information about the epilepsy-associated changes will enable to discover the molecular targets for age-specific antiepileptic drugs acting on specific receptor subtypes, which may predominate on epileptic neurons.

8. ACKNOWLEDGEMENTS

This thesis work was carried out in the Institute of Biomedicine, the Department of Pharmacology, Drug Development and Therapeutics, at the University of Turku. I am grateful to the heads of the departments, Professors Esa Korpi, Mika Scheinin and Risto Huupponen, for allowing me to use the departments' facilities. I want to thank Professor Sirpa Jalkanen for providing me the office place at Medicity Research Laboratory to complete my work. I also greatly appreciate the Drug Discovery Graduate School for education and financial support.

My supervisor Docent Irma Holopainen, I want to thank you for your guidance to complete this thesis work. I am sincerely grateful for your neverending support and encouragement. Your "sisu" and positive attitude, even during difficult times when nothing seemed to work, have made me believe in both my research work and in myself. You have inspired me that science can be fun!

I want to thank Professor Esa Korpi for opening me the doors to the peculiar world of $GABA_A$ receptors. Many thanks are also expressed to the other co-authors of my articles: Professor Asla Pitkänen, PhD Jari Nissinen, PhD Sanna Soini and Docent Mikko Uusi-Oukari. Mikko was the "head" of my pharmacological experiments and I admire his great knowledge about $GABA_A$ receptors. Ms. Eija Lehtovirta is highly appreciated for the very careful and skilful technical assistance.

I gratefully acknowledge the official reviewers of this thesis, Professor Pirjo Saransaari and Docent Hana Kubová, for their constructive criticism and encouraging comments.

I thank all the present and former members of the Department of Pharmacology, Drug Development and Therapeutics for friendship, help and a warm atmosphere at Farmis.

To the other members of the Hippo group, Niina Jalava, MSc, Juha Järvelä, MD, Tiina-Kaisa Kukko-Lukjanov, MSc and Francisco López-Picón, MSc, I want to express my greatest thanks for contributing to the pleasant atmosphere within our group and for being good friends. It has been very nice to share both scientific and personal interests with you and your support during this work has been invaluable.

Many warm thanks are owned to the Biotica girls, and also to all my Pargas friends, for sharing many nice moments and being there for me.

I want to give my sincerest thanks to my whole family. I am truly grateful to my mother and father for providing me with the stable foundation to stand on in life. You have always helped me to feel safe and given invaluable support in my everyday life. We are also extremely fortunate to have both Mommo and Fammo to call when we need some help with the children. Many thanks are also owned to my sister Pia and brother Peter and their families for always standing up for me and contributing to what I am today!

The biggest thanks are devoted to my girls Jill and Sara and my husband Max. Jill and Sara, you are the inspiration in my life. You have brought me joy even through the hardest times and always given me new inspiration to complete this work. Max, you have given me the greatest love and support in my life, in your own way, when I have needed it most. Your patience has been tested now and then during the thesis work time, and some of the honour to get here definitely goes to you!

The financial support of Academy of Finland, the Arvo and Lea Ylppö Foundation, the Finnish Cultural Foundation, the Oskar Öflund Foundation, the Research and Science Foundation of Farmos, the Sigrid Juselius Foundation, the Special State Grant for Clinical Research (EVO) of Hospital District on Southwest Finland, the Svenska Kulturfonden and the Turku University Foundation is gratefully acknowledged.

Turku, November 2007

Hanna Laurén

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