Disease-related posttranscriptional modifications of glycine receptor mRNA under physiological and pathophysiological conditions

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Für meine Mutter und meinen Bruder

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Aim of this study

Impaired inhibition in the hippocampus is known to underlie temporal lobe epilepsy (TLE) because deregulation of the excitation/inhibition balance critically affects hippocampal network activity. Ictogenesis frequently is associated with hippocampal neurodegeneration and involves apoptosis and excitotoxicity as executive mechanisms. The majority of studies addresses the role of $GABA_AR$ in epileptogenesis and some mutations in *GABR* genes have already been identified as molecular markers of TLE. Just as $GABA_A$ receptors ($GABA_AR$) glycine receptors (GlyR) are chloride permeable ligand-gated ion channels that participate in hippocampal inhibition, and they were recently uncovered to exert anticonvulsive effects. However, hippocampal molecular mechanisms in TLE, particularly those concerning GlyR, were not investigated so far. In addition, the impact of posttranscriptional mechanisms on the pathophysiology of TLE is largely neglected. Therefore, the aim of this study was to characterize the role of posttranscriptional mechanisms at *GLRA* messengers in TLE.

1. Introduction

1.1 Neuronal systems

1.1.1 Excitation and inhibition, the Glu/GABA ratio

Excitation and inhibition are crucial events for information transfer in the brain. They influence each other to maintain a physiological neuronal circuit. A functional balance between excitatory and inhibitory synapses (Glu/GABA ratio) is established during development and maintained throughout life (Turrigiano and Nelson, 2004). The ability of a neuronal network to control its activity by itself is crucial in order to avoid runaway excitation or quiescence (Turrigiano and Nelson, 2004). As illustrated in figure 1, a neuron possesses a variety of homeostatic mechanisms for keeping its output firing rate within a narrow window (Turrigiano and Nelson, 2004).

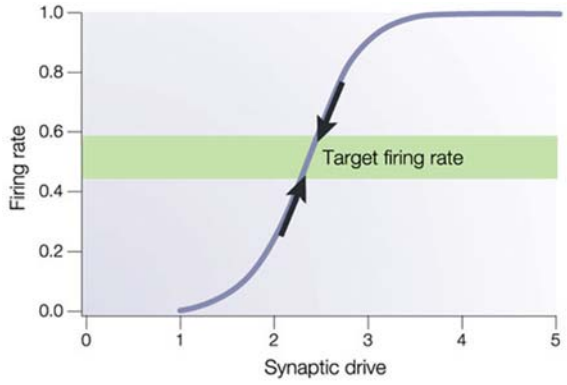

Figure 1: A single neuron keeps its firing rate within a narrow range. Homeostatic mechanisms (marked with arrows) up- and downscale the synaptic drive to keep the neuron in the target firing rate to ensure optimal neuronal information transfer (Turrigiano and Nelson, 2004).

Homeostasis involves changes in the number of synapses, activity-dependent regulation of neurotransmitter receptor and scaffolding protein expressions (Prange et al., 2004), mobility of neurotransmitter receptors in the plasma membrane and control of the amount of neurotransmitter released into the synaptic cleft. For example, the Glu/GABA ratio can be enhanced in response to chronic neuron silencing in order to avoid neuronal quiescence (Ramakers et al., 1990; Ramakers et al., 1994) or will be decreased in response to enhanced neuronal output (El Hassar et al., 2007).

Many neurological diseases are afflicted with a deregulated inhibition. For instance, impairment of glycine uptake results in deficits in respiration (Gomeza et al., 2003) and can provoke hyperexcitability or glycine encephalopathy (Harvey et al., 2008), and non-functional glycine receptors were also shown to be involved in startle disease (Shiang et al., 1993;

Shiang et al., 1995; Rees et al., 2001; Rees et al., 2002). Hyperexcitability disorders such as epilepsy are often related to an increased Glu/GABA ratio. Decreased Glu/GABA ratios are also associated with nervous system disorders, such as autism or mental retardation (for an overview see (Eichler and Meier, 2008)).

1.1.2 Excitation: Glutamate receptors

Glutamate is the main excitatory neurotransmitter in the CNS mediating fast as well as slow excitatory currents. Most excitatory neurons (~90%) use glutamate as transmitter. Synaptic vesicles are loaded with glutamate by vesicular glutamate transporters (VGluT) (Herzog et al., 2001). Glutamate removal from the synaptic cleft is mediated by excitatory amino acid transporters (EAAT), present in glia cells and in presynaptic terminals. Glutamate is synthesized from its precursor glutamine in the glutamine cycle and acts also as precursor for GABA synthesis.

There are two major groups of glutamate receptors in the brain, ionotropic and metabotropic receptors. The metabotropic glutamate receptors are G-protein coupled and can modulate the postsynaptic signaling through secondary messenger pathways. Ionotropic glutamate receptors are non-selective cation channels allowing the passage of sodium, potassium and in some cases of calcium ions through the plasma membrane. Three types of ionotropic glutamate receptors can be distinguished: kainate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid receptors (AMPAR) and *N*-methyl-*D*-aspartic acid receptors (NMDAR). Most neurons express both AMPAR and NMDAR, and their current ratio remains nearly constant later in development (Turrigiano and Nelson, 2004). However, a decreased ratio between AMPAR and NMDAR can reduce the threshold for LTP induction (see 1.1.3.1) and synaptic plasticity, but can also produce silent synapses. An increased ratio ensures basal synaptic transmission at the expense of plasticity, and pathological ratios are found for example in Alzheimer's disease (Snyder et al., 2005) and schizophrenia (Mohn et al., 1999).

1.1.2.1 AMPAR

The AMPAR is widely distributed throughout the CNS and expressed in many different neuron types. There are four subunits available to form a receptor channel, GluR1-4. GluR5-6 subunits are called kainate receptors, but their functional role is less well understood. This receptor type has a fast desensitizing kinetic and is suggested to play a role in the

modification of synaptic strength (Gillessen et al., 2003) and possibly serves as a feedback system for glutamate regulation (Purves et al., 2007). Generally, binding of glutamate results in channel opening and leads to passage of sodium and potassium ions, resulting in excitatory postsynaptic current (EPSC) with fast kinetics.

Alternative splicing (Steinhauser and Seifert, 2002), RNA editing (Steinhauser and Seifert, 2002; Seeburg and Hartner, 2003; Hassel and Dingledine, 2006) and assembly of different subunits create a large functional variety of AMPAR. The two splice variants flip and flop differ in their desensitization kinetic as well as in their distribution in the CNS. Most importantly, calcium permeability is controlled by RNA editing of the GluR2 subunit (Seeburg and Hartner, 2003; Hassel and Dingledine, 2006), and calcium-permeable AMPAR are typically found in GABAergic interneurons (Seeburg and Hartner, 2003).

1.1.2.2 NMDAR

In contrast to AMPAR, NMDAR are highly permeable to calcium ions and have slower kinetic compared with AMPAR. Their decay can last up to hundreds of milliseconds and therefore provides a good opportunity for temporal and spatial summation, a basis of learning and memory (see 1.1.3.1). Conventional heteromeric NMDAR composed of NR1 and NR2A-D subunits require dual agonists, glutamate and glycine, for activation, and current flow across the membrane requires removal of the magnesium block through depolarization. Synaptic NMDAR are localized to post synaptic densities (PSD), which also provide a platform for AMPAR, cell adhesion molecules and other constituents of intracellular signaling pathways, including kinases, phosphatases and others (Hassel and Dingledine, 2006; Lau and Zukin, 2007). PSD-95 interconnects NMDAR and neuronal nitric oxide synthase (nNOS) (Kim and Sheng, 2004), the two key players in glutamate-mediated neurotoxicity (see 1.1.3.2).

1.1.3 The two faces of NMDAR

1.1.3.1 Learning and memory

Memory formation requires NMDAR because they are coincidence detectors of synaptic activity due to magnesium block. Removal of the magnesium block by depolarization through AMPAR for example is a critical step towards the induction of long-term potentiation (LTP). LTP is a strong increase of synaptic strength produced by high-frequency stimulation (100 Hz or higher) over long periods and/or coincident synaptic depolarization or back-propagating

action potentials (APs). Meanwhile, more than 100 molecules are known to be involved in LTP (Sanes and Lichtman, 1999). The high-frequency stimulation leads to prolonged depolarization and in consequence to an elevated calcium influx through NMDAR. Calcium is a second messenger required to activate calcium/calmodulin-dependent protein kinase (CaMKII) during early phases of LTP (Sanes and Lichtman, 1999; Tsien, 2006). On the long run protein kinase A (PKA) is activated and different processes modulating synaptic plasticity, including posttranscriptional modifications, alterations in metabolisms, transcription of genes, recruitment of AMPAR and growth of new synapses (or even elimination) are engaged.

In contrast to LTP, long-term depression (LTD) decreases synaptic strength as a result of low-frequency stimulation (around 1 Hz). LTD involves activation of extrasynaptic NMDAR (Massey et al., 2004), and it is possible that these receptors are coupled to other signaling pathways than the synaptic counterpart; both LTP and LTD were found to be coupled to different calcium concentrations. LTD was found with a small increase and LTP with a large increase of intracellular calcium concentrations in cerebellar granule cells (Gall et al., 2005), therefore possibly mediating different effects on memory consolidation.

In addition to learning and memory, NMDAR receptors promote neuronal survival and maturation as well as neuronal migration, but they also mediate excitotoxicity (see also 1.1.3.2; (Chen and Lipton, 2006; Hassel and Dingledine, 2006; Tsien, 2006; Zhang et al., 2007; Purves et al., 2007)).

Neurodegeneration is mostly associated with overstimulation of NMDAR (Zhang et al., 2007), while low to intermediate levels of NMDAR activation promote survival and are crucial for physiological neuron function. For example, in consequence of stroke (disruption of blood supply in one part of the brain) or head injury glutamate is released from damaged cells in large amounts, initiating a cascade of events driving neurons into death even hours or days after the initial insult (Chen and Lipton, 2006). Overstimulation of NMDAR and in part also of calcium-permeable AMPAR (Rivera-Cervantes et al., 2004; Chen and Lipton, 2006) results in excitotoxicity; depending on the glutamate concentration neuronal death is referred to as apoptosis or necrosis (Bonfoco et al., 1995).

1.1.3.2 Excitotoxicity

Excitotoxicity "is defined as excessive exposure to the neurotransmitter glutamate or overstimulation of its membrane receptors, leading to neuronal injury or death" (Chen and Lipton, 2006).

Excitotoxicity can occur after brain injury or a heart attack, which can cause ischemia and deprives the brain from oxygen and glucose. Ischemia is followed by an accumulation of glutamate and aspartate in the extracellular fluid. Overstimulation of NMDAR leads to an enhanced activity of these receptors resulting in high calcium levels and additional recruitment of other calcium channels (e.g. voltage-gated ones). This cascade activates a number of enzymes and associated pathways (phospholipases, endonucleases and proteases), thereby damaging cell structures (cytoskeleton, membrane, DNA). Energy depletion and calcium overload of mitochondria disrupt the respiratory chain through enhanced production of reactive oxygen species (ROS) (Gillessen et al., 2003; Chen and Lipton, 2006).

Today, there are two models for excitotoxicity. The first one schedules synaptic versus non-synaptic glutamate receptor activation, while the second one deals with interaction of the NMDAR with PSD-95 and associated nNOS.

Model 1: Synaptic versus non-synaptic NMDAR

Extrasynaptic NMDAR are activated by neuronal ambient glutamate due to spillover or from astrocytic origins, whereas synaptic NMDAR are activated by synaptic glutamate release. Both receptors are coupled to different calcium signaling pathways and therefore mediate different effects (Zhang et al., 2007). It is conceivable that synaptic NMDAR induce a concomitant upregulation of pro-survival genes and downregulation of pro-death genes. According to this, extrasynaptic NMDAR would therefore fail to activate neuroprotective programs and instead trigger the expression of a putative calcium-activated chloride channel that is able to induce neuronal cell death (Zhang et al., 2007).

Model 2: Interaction between NMDAR and PSD-95

At postsynaptic sites, NMDAR are anchored to the postsynaptic protein scaffold via interaction with PSD-95. PSD-95 also binds nNOS, which converts L-arginine into citrulline and thereby produces nitric oxide (NO) (Gillessen et al., 2003). NO itself is nontoxic. Its

cellular effects are regulated by chemical reactions with target molecules and spontaneous formation of nitrite (Gillessen et al., 2003).

In this model, NO is considered to play a critical role in NMDAR-dependent excitotoxicity (Aarts et al., 2002; Gillessen et al., 2003; Cao et al., 2005). Due to excessive calcium influx through NDMAR, nNOS will concomitantly produce NO. Enhanced NO production is known to lead to neuronal cell damage and death, i.a. due to spontaneous and rapid reaction with superoxide anion (ROS) to form peroxynitrite, which is neurotoxic and induces e.g. DNA damage. A recent study demonstrated that uncoupling of NMDAR and PSD-95 rescues cells from glutamate-mediated excitotoxicity without blocking synaptic activity or calcium influx (Aarts et al., 2002).

1.1.3.2.1 Apoptosis

Programmed cell death, apoptosis, is a natural process, which occurs during development and metamorphose. A range of processes controls apoptosis, involving many different pathways and molecules. The trigger of apoptosis can originate from the cell itself, from surrounding tissue or the immune system. Apoptosis is a quiet process; apoptotic cells do usually not contribute to, or stimulate immune responses (Janeway et al., 2001). After initiation, apoptosis is processed by caspases (cysteine-aspartyl-proteases). Caspases are separated into two different functional groups: Initiation caspases (caspase-2, -8, -9 and -10) receive the initial signal and initiate apoptosis through cleavage and activation of downstream effector caspases (caspase-3, -6 and -7). The final step is activation of caspase-3.

Apoptotic cells undergo morphological changes that can be visualized under a microscope: the cell shrinks, the nuclear plasma membrane blebs, the chromatin condensates and fragmentizes through activation of endonucleases. In addition, karyorrhexis (Segerer et al., 2002) occurs (fragmentation of the nucleus seen as pyknotic nucleus (Janeway et al., 2001)). During apoptosis the cell dies without damaging the organism, whereas during necrosis pathogens are often released from the degenerated cell (for necrosis see below). As a last step, the apoptotic cell expresses phosphatidylserine on its membrane surface, which is the signal for ingestion by macrophages and microglia (Mattson and Bazan, 2006).

1.1.3.2.2 Necrosis

Induction of necrosis is due to physical or chemical injury (e.g. oxygen deprivation during heart attack and ischemia, or bacteria). Necrosis is characterized by cell and organelle swelling as a result of water influx following sodium and calcium ions that have entered the cell. Further characteristics of necrosis are loss of ion homeostasis, ATP depletion and karyolysis (Segerer et al., 2002) (breakup of nucleus, coagulation of proteins). At the end, the membrane is disrupted due to swelling so that pathogenic and toxic cellular contents are released into the extracellular fluid, which in turn affects surrounding cells and spreads out, leading to a vicious circle. The dead or necrotic cell/tissue is taken up and degraded by phagocytic cells. This form of cell death is very rapid and often is associated NMDAR-mediated excitotoxicity.

1.1.4 Inhibition: Glycine and GABA

There are two major inhibitory neurotransmitters in the CNS, γ-aminobutyric acid (GABA) and glycine. GABA is the main inhibitory neurotransmitter in the brain. It is synthesized by the enzyme glutamic acid decarboxylase (GAD), which converts glutamate into GABA. Glycine is the main inhibitory neurotransmitter in the spinal cord and brain stem (Olsen and Betz, 2006), but it is also found in other regions of the central nervous system (Malosio et al., 1991b). The enzyme serine hydroxymethyltransferase uses serine as a substrate to synthesize glycine. Synaptic vesicles are loaded with GABA and/or glycine by the vesicular inhibitory amino acid transporter (VIAAT). Therefore, both GABA and glycine can be coreleased from the same as well as from different presynaptic terminals (Meier, 2003). In fact, GABA and glycine can even be coreleased from the same synaptic vesicle (Jonas et al., 1998). GABA and glycine removal from the synaptic cleft is mediated by GABA transporters (GAT) and glycine transporters (GlyT), present in glia cells and in presynaptic terminals.

1.1.4.1 Glycine receptors

The glycine receptor (GlyR) shows structural homology to the nicotinic acetylcholine receptor (nAChR), the $GABA_{A/C}$ receptor and the serotonin receptor 5-HT_3 (Breitinger and Becker, 2002). Each subunit consists of a large extracellular N-terminus, which is involved in agonist binding and features two disulfide bridges, which are characteristic for the ligand-gated ion channel superfamily (LGIC). Further, the receptors contain four transmembrane domains with a large intracellular loop between transmembrane domain 3 and 4 as well as an extracellular C-terminus (figure 2). The transmembrane domain 2 is predicted to line the

channel pore (Breitinger and Becker, 2002). Moreover, the receptor contains several sites for phosphorylation, glycosylation and ubiquitination (Legendre, 2001; Breitinger and Becker, 2002).

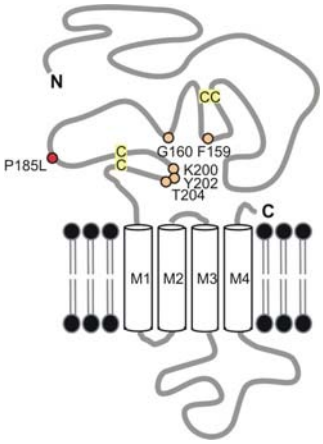

Figure 2: Structure of glycine receptor α subunit. Glycine receptor consists of a large N-terminus, four transmembrane domains and a small C-terminus. Orange dots mark amino acids involved in glycine and taurine binding, disulfide bridges are shown in yellow and the red dot marks position of α3 editing site, which results in an amino acid sustitution from proline to leucine (Meier et al., 2005).

Today, five genes (*GLRA1-4* and *GLRAB*) coding for different subunits are known. The α subunits can assemble into homopentameric channels. Heteromeric pentamers are formed together with the β subunit with a stoichiometry of two α subunits and three β subunits (Grudzinska et al., 2005). The β subunit modulates ligand binding properties of the receptor and anchors them to the postsynaptic cytoskeleton via gephyrin binding (Meyer et al., 1995; Moss and Smart, 2001; Legendre, 2001; Breitinger and Becker, 2002; Meier, 2003; Levi et al., 2004; Meier and Grantyn, 2004a). Therefore, homomeric receptors without β are mainly excluded from postsynaptic sites and expressed at extrasynaptic locations (Meier et al., 2001; Meier, 2003).

Upon binding of its agonists GlyR increase plasma membrane permeability for chloride ions (Breitinger and Becker, 2002). Besides glycine other less potent ligands are known: β-alanine > taurine > L-serine (Legendre, 2001), whereas GABA is largely ineffective (Olsen and Betz, 2006). In spinal cord and brain stem GlyR contribute to motor rhythm generation and locomotor behavior as well as regulation of spinal reflexes (Legendre, 2001; Olsen and Betz, 2006). Furthermore, the receptor is known to be involved in vision (glycinergic interneurons in the retina) (Haverkamp et al., 2003; Wassle, 2004; Olsen and Betz, 2006), processing of acoustic stimuli (Olsen and Betz, 2006; Dlugaiczyk et al., 2008) as well as of spinal nociceptive signals (Harvey et al., 2004).

GlyR undergo posttranscriptional modifications at individual subunits. GlyRα1-3 are known to be alternative spliced. Splicing of *GLRA1* and *GLRA3* messengers concern the large intracellular cytoplasmic loop between the transmembrane domain 3 and 4, while α2 is

alternatively spliced in the N-terminal ligand binding domain. In detail, compared with α1Δ, α1INS contains eight additional amino acids (Malosio et al., 1991a) (figure 3A), and addition of 15 amino acids into GlyRα3K generates α3L (Nikolic et al., 1998) (figure 3B). While the role of GlyRα1 splicing remains obscure, it was found that the L-sequence within α3L decelerates receptor desensitization kinetics (Nikolic et al., 1998).

```
α1INS 290 CLLFVFSALLEYAAVNFVSRQHKELLRFRRKRRHHKSPMLNLFQDDEGGEGRFNFSAYGMGPACL
α1Δ   290 CLLFVFSALLEYAAVNFVSRQHKELLRFRRKRRHHK--------DDEGGEGRFNFSAYGMGPACL

α1INS 355 QAKDGISVKGANNNNTTNPPPAPSKSPEEMRKLFIQRAKKIDKISRIGFP
α1Δ   347 QAKDGISVKGANNNNTTNPAPAPSKSPEEMRKLFIQRAKKIDKISRIGFP
```

Figure 3A: Protein sequence alignment of rat GlyRα1, according to amplified region by PCR (compare table 5). The α1INS subunit differs in eight amino acids compared with α1Δ. Amino acid numbering is according to the mature protein sequence.

```
α3L 171 GYTMNDLIFEWQDEAPVQVAEGLTLPQFLLKEEKDLRYCTKHYNTGKFTCIEVRFHLERQMGYYLIQ
α3K 171 GYTMNDLIFEWQDEAPVQVAEGLTLPQFLLKEEKDLRYCTKHYNTGKFTCIEVRFHLERQMGYYLIQ

α3L 238 MYIPSLLIVILSWVSFWINMDAAPARVALGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMAVCLLF
α3K 238 MYIPSLLIVILSWVSFWINMDAAPARVALGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMAVCLLF

α3L 305 VFSALLEYAAVNFVSRQHKELLRFRRKRKNKTEAFALEKFYRFSDTDDEVRESRFSFTAYGMGPCLQ
α3K 305 VFSALLEYAAVNFVSRQHKELLRFRRKRKNK--------------DDEVRESRFSFTAYGMGPCLQ

α3L 372 AKDGVVPKGPNHAVQVMPKSPDEMRKVFIDRA
α3K 357 AKDGVVPKGPNHAVQVMPKSPDEMRKVFIDRA
```

Figure 3B: Protein sequence alignment of rat GlyRα3, according to amplified region by PCR (compare table 5). The α3L subunit differs in 15 amino acids compared with α3K. Amino acid numbering is according to the mature protein sequence.

Alternative splicing of *GLRA2* messengers generates α2A and α2B, resulting in a substitution of two amino acids; valine-threonine (VT, α2A) for isoleucine-alanine (IA, α2B) (figure 3C) (Kuhse et al., 1991). Compared with α2A, agonists have increased potencies at GlyRα2B (Miller et al., 2004).

```
α2A 52   INSFGSVTETTMDYRVNIFLRQQWNDSRLAYSEYPDDSLDLDPSMLDSIWKPDLFFANEKGANFHDV
α2B 52   INSFGSIAETTMDYRVNIFLRQQWNDSRLAYSEYPDDSLDLDPSMLDSIWKPDLFFANEKGANFHDV

α2A 120 TTDNKLLRISKNGKVLYSIRLTLTLSCPMDLKNFPMDVQTCTMQLESFGYTMNDLIFEWLSDGPVQV
α2B 120 TTDNKLLRISKNGKVLYSIRLTLTLSCPMDLKNFPMDVQTCTMQLESFGYTMNDLIFEWLSDGPVQV

α2A 187 AE
α2B 187 AE
```

Figure 3C: Protein sequence alignment of rat GlyRα2, according to amplified region by PCR (compare table 5). The α2A and α2B subunit differs in a substitution of two amino acids: valine-threonine (VT, α2A) for isoleucine-alanine (IA, α2B). Amino acid numbering is according to the mature protein sequence.

Besides alternative splicing, *GLRA3* transcripts were found to be RNA-edited at a position corresponding to amino acid proline 185. As a result of enzymatic cytidine deamination a

leucine codon is generated. GlyR$\alpha 3^{185L}$ is a gain-of-function receptor, its apparent affinities for glycine and taurine are increased by 15- and 60-fold, respectively (Meier et al., 2005). As this amino acid substitution does not influence desensitization kinetics (Meier et al., 2005), and because GlyR are mainly extrasynaptic in supramedullar brain regions (Meier and Grantyn, 2004a), these gain-of-function receptors are ideally suited for generating and mediating tonic chloride currents in response to cellular ambient glycine. Because of high degree of sequence homology around the edited position, it is speculated that $\alpha 2^{192L}$ would be also a high affinity receptor.

1.1.4.2 GABA receptors

As GlyR, ionotropic GABA receptors ($GABA_{A/C}R$) are ligand-gated chloride channels. Besides chloride, they are permeable for bicarbonate (Cossart et al., 2005). Metabotropic GABA receptors ($GABA_BR$) are G-protein coupled and contribute to postsynaptic signaling through secondary messenger pathways.

There are different subunits known (α1-6, β1-3, γ1-3 and δ) that can assemble to form a heteropentameric chloride channel (Moss and Smart, 2001). For synaptic transmission high amounts of GABA (0.3 - 1mM) are required, and accordingly the apparent affinities of synaptic receptors for GABA (α1-3, β1-3, γ1-3) are quite low (Mody and Pearce, 2004), while receptors involved in tonic inhibition are high affinity receptors (α4-6 and δ). Both synaptic and non-synaptic receptors share β1-3 subunits, which are not considered to modulate agonist sensitivity.

Messengers of only one $GABA_AR$ gene is known to undergo posttranscriptional processing; due to alternative splicing of the $GABA_AR$ γ2 subunit, a short (γ2S) and a long variant (γ2L) are generated. The long variant contains eight additional amino acids (LLRMFSFK) in the large cytoplasmic loop between transmembrane domains 3 and 4. A deregulated γ2S/γ2L ratio was shown to be involved in schizophrenia (Huntsman et al., 1998) and a recent report suggested preferential synaptic targeting of $GABA_AR$ by γ2L (Meier and Grantyn, 2004b). However, a direct interaction between the synaptic scaffolding molecule gephyrin and the γ2 subunit could not be demonstrated so far (for a review see (Fritschy et al., 2008)), although the γ2 subunit is required for synaptic location. In γ2 knockout mice it was shown that loss of $GABA_AR$ clusters is paralleled by loss of gephyrin and synaptic GABAergic function

(Essrich et al., 1998). Interestingly, in gephyrin knockout mice, postsynaptic $GABA_AR$ clustering appears normal in the hippocampus, albeit at somewhat reduced levels (Levi et al., 2004). Although a recent report could demonstrate a direct interaction between the α2 and gephyrin (Tretter et al., 2008), a hierarchy of the functional relevance of the α2, γ2 and gephyrin with respect to synaptic receptor clustering cannot be determined today.

Generally, δ subunit containing $GABA_AR$ are excluded from synaptic sites (Stell et al., 2003; Mody and Pearce, 2004). This subunit is part of non-synaptic receptors with very high affinity for GABA (Brown et al., 2002), which is in the range of micromolar concentrations. Due to these properties and because these receptors have, in addition, slower desensitization kinetics (Brown et al., 2002) δ subunit containing $GABA_AR$ are well adapted to generate and maintain tonic chloride currents in response to ambient GABA. As mentioned above, $GABA_AR$ involved in tonic transmission contain α4, α5 or α6, in addition to β and δ subunits (Mody, 2001; Wisden et al., 2002; Mody and Pearce, 2004; Caraiscos et al., 2004).

1.1.4.3 The two faces of tonic inhibition

The impact of tonic inhibition on neuronal excitability can be more than three times larger than that mediated through phasic transmission (Mody and Pearce, 2004). Most importantly, the effects of tonic receptor activation on neuronal excitability are mainly mediated through a mechanism called shunt (Mitchell and Silver, 2003). Shunt inhibition acts as a cellular mechanism for gain control (input-output relationship) in cells with highly variable synaptic inputs (Mitchell and Silver, 2003). For example, image acquisition devises (e.g. video cameras) are modeled on this neuronal mechanism, whereby an automatic gain control is used to adapt the camera to different conditions of brightness.

Therefore, tonic inhibition plays an important role in the control of neuronal excitability (Mody and Pearce, 2004). According to the developmental state of neurons however, homeostatic regulation of neuronal output may have a completely different impact on the neuronal network. In the developing brain GABAergic transmission is still excitatory due to reduced expression of the potassium/chloride-cotransporter 2 (KCC2) (Rivera et al., 1999). In that condition chronic neuron silencing is detrimental because the synaptic network is going to be established, and this requires activity-dependent functional adjustment of developing glutamatergic and GABAergic synapses. If prohibited through exaggerated tonic inhibition, GABAergic synapses are downscaled according to the proposed default elimination pathway

for “un-synchronisable” GABAergic synapses (Tao and Poo, 2005), and neurons that appear not to fit into the developing network could therefore be ultimately eliminated. This rule may also apply to the epileptic brain (see below), where neuronal hypoactivity occurs (Sloviter et al., 2006) and which was found to adopt an earlier developmental status in terms of KCC2 downregulation and associated impairment of chloride extrusion (Rivera et al., 2002; Wake et al., 2007). Therefore, it is not surprising that under certain circumstances GABAergic tonic inhibition fails to prevent excitotoxicity (Yeh et al., 2005). By contrast, in the adult neuronal tissue, neuron silencing provokes concomitant upregulation of both glutamatergic and GABAergic synapses, which maintains the Glu/GABA ratio of synaptic input (Burrone et al., 2002).

Another way of silencing neurons comes from potassium channels. In humans suffering from Parkinson’s disease enhanced activity of potassium channels was found to trigger loss of dopaminergic neurons (Michel et al., 2007). Most intriguingly, deficits in tonic $GABA_AR$-mediated inhibition due to $GABA_AR$ $\alpha6$ knockout are compensated for by a continuously active potassium conductance of equal magnitude (Brickley et al., 2001).

1.1.4.4 Regulation of intracellular chloride during development and in disease

Early in development there is a high chloride concentration in the cell because the sodium/potassium/chloride-cotransporter 1 (NKCC1) pumps chloride into the cell (Rivera et al., 2005; Purves et al., 2007) and the expression of KCC2 is rather low (Rivera et al., 1999). $GABA_AR$ mediate depolarizing effects in the immature brain, and this produces large-scale network activity, termed giant depolarizing potentials (GDPs). These GDPs are crucial for establishment of neuronal circuits during development (Ben Ari, 2001). Depolarizing $GABA_AR$ activity itself was shown to promote KCC2 expression (Ganguly et al., 2001), but other groups published opposite results (Blum and Mann, 2002; Titz et al., 2003). Therefore, the mechanisms that govern developmental upregulation of KCC2 expression still have to be elucidated. By contrast, it is well known that activation of tyrosine kinase B (TrkB) receptor by brain-derived neurotrophic factor (BDNF) downregulates KCC2 expression for example in the epileptic hippocampus and in various kinds of neuronal trauma, respectively (Rivera et al., 2004). In the course of downregulation of KCC2 expression in epileptic tissue there is also an upregulation of NKCC1 (Palma et al., 2006). Together, these mechanisms shift the chloride equilibrium potential to more positive values (high intracellular chloride) and thus GABA again becomes depolarizing. In conclusion these events mirror an early stage of development

and produce a re-establishment of an immature neuronal network activity. It is speculated that this is a basis for renewal of neuronal circuits in damaged tissue under energy saving conditions (Rivera et al., 2005).

1.1.5 Hippocampus and temporal lobe epilepsy

The hippocampus (Greek word for seahorse) belongs to the forebrain, which is located in the medial temporal lobe. It plays major roles in short term memory and spatial navigation. In Alzheimer's disease the hippocampus is one of the first brain regions concerned, and memory deficits as amnesia (inability to form or retain new memories) and disorientation are among the first symptoms. Damage to the hippocampus can also result from oxygen deprivation (anoxia), encephalitis, or medial temporal lobe epilepsy.

1.1.5.1 Anatomy and regions

The hippocampus is an evolutionary old structure located in the temporal lobe, which is the medial part of telencephalon and belongs to the limbic system. The hippocampus can be divided into three regions: dentate gyrus, cornu ammonis and subiculum. The circuit in the hippocampus derives its input from the entorhinal cortex into the dentate gyrus, which forwards the input to subregions of the cornu ammonis that in turn transfer it into the subiculum. Finally, the information is sent back into the neocortex. The hippocampus is a highly organized structure with interconnected pyramidal cells and at least 12 different types of interneurons using six different transmitters (Sanes and Lichtman, 1999).

The granule cells located within the stratum granulare of the dentate gyrus receive input from the entorhinal cortex. Granule cells project via the so called mossy fibers to the region CA3 (see below). The *Cornu Ammonis* (CA) is subdivided into four regions, CA1-4. Neurons located there are called pyramidal cells and have a glutamatergic phenotype, as have the granule cells. From the CA3 region Schaffer collaterals (axons of pyramidal cells in CA3) project to the region CA1. From CA1 the input is transferred to the *subiculum*, which is the output structure of the hippocampus (figure 4).

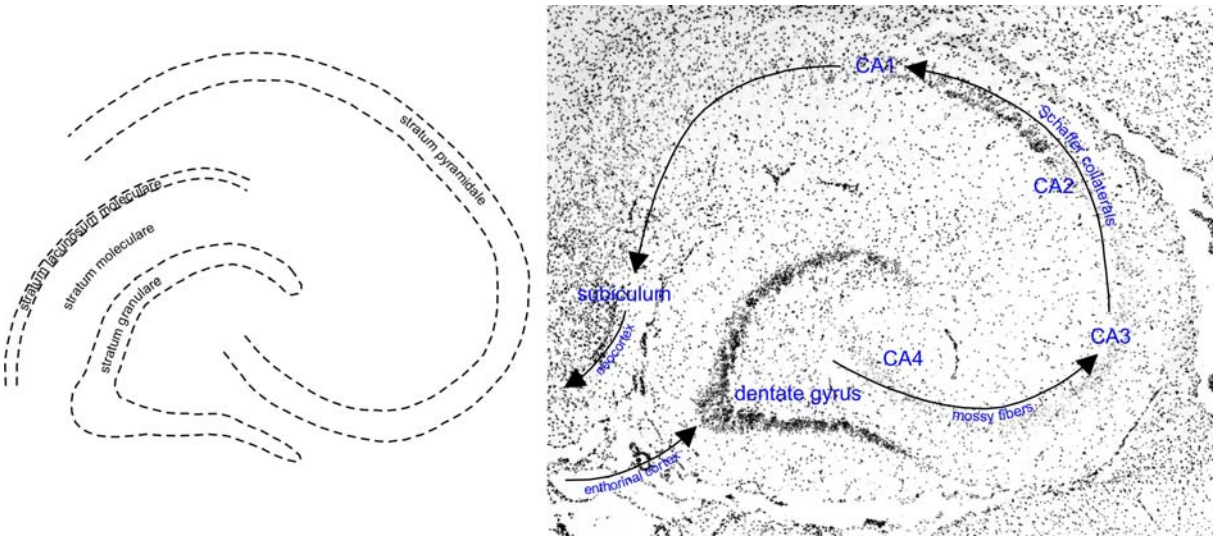

Figure 4: Overview of hippocampal regions. Cryosection of hippocampus of an adult mouse. Nuclei are marked with DAPI and shown in black. Left: Hippocampal regions are drawn schematically, matching those represented in the hippocampus cryosection (right). Right: Arrows mark pathway of information transfer through the hippocampus.

1.1.5.2 Epilepsy

Epilepsy is a common neurological disorder characterized by the occurrence of seizures, which are periodic and unpredictable. Today about 50 million people are affected worldwide. A seizure is a synchronous and rhythmic firing of many neurons at one time, thereby eventually stimulating surrounding neurons in other brain regions (secondary generalized). There are many different forms of epilepsy with different time scales; in case of absence epilepsy seizures can vanish after age of teenager. Over 50% of all human epilepsies have a genetic background (Gibb and McNamara, 2006). Mutations occur in genes that code for ion channels, voltage- as well as ligand-gated channels.

Epilepsy is classified in three groups according to seizure types: partial seizures, generalized epileptic seizures and unclassified seizures (table 1). Partial epileptic seizures start focally. Generalized epileptic seizures have discharges from both hemispheres and comprise besides others tonic clonic seizures. This is characterized by sustained muscular contraction (tonic) and followed by relaxation (clonic). If this occurs in consequence of a partial seizure, the seizure is secondarily generalized.

1. **Partial seizures**
    - A. Simple (consciousness not impaired)
        - A1. With motor manifestations
        - A2. With sensory manifestations
        - A3. With autonomic manifestations
        - A4. With psychic manifestations
    - B. Complex
        - B1. With simple partial features (as above A1 – A4) at onset followed by impairment of consciousness
        - B2. With impairment of consciousness at onset (both B1 and B2 may be followed by automatism)
    - C. Secondarily generalized
        - C1. Simple partial seizure evolving to generalized seizure
        - C2. Complex partial seizure evolving to generalized seizures
        - C3. Simple partial seizure evolving to complex partial seizure evolving to generalized seizure
2. **Generalized seizures**
    - A. Absence seizures (may be typical or atypical)
    - B. Myoclonic seizures
    - C. Clonic seizures
    - D. Tonic seizures
    - E. Tonic-clonic seizures
    - F. Atonic seizures
3. **Unclassified seizures**

Table 1: Epilepsy classification. Epilepsy is classified in three groups to seizure types: Partial, generalized and unclassified seizures. Partial seizures are further subdivided into simple, complex and secondarily generalized seizures with further subgroup classification.

1.1.5.3 Temporal lobe epilepsy

One of the most common forms is the temporal lobe epilepsy (TLE). It represents 1/3 of all epilepsies. The seizures have their origin in the mesial temporal lobe (hippocampus or amygdala, rarely from other regions). Possible reasons for TLE can be: benign tumors, stroke, vascular deformation, craniocerebral injury, inflammation, atrophy or sclerosis. Rarely a heritable component can be determined for this type of epilepsy, and in about half of the patients seizures arise spontaneously or from an obscure or unknown cause (idiopathic). One of the histopathological signs of temporal lobe epilepsy (TLE) is dispersion of granule cell layer, which occurs only within a narrow time window (first four years in life) (Lurton et al., 1998).

Seizures (see table 1) often start focally, can spread and affect other brain regions (secondary generalized tonic clonic seizures). The origin of the focus can be detected by electroencephalography (EEG) recording. The seizure itself starts and ends gradually and lasts 1-2 minutes. Sometimes seizures are accompanied by 10-20 seconds lasting akinesia, often

followed by automatisms (smack, lick), rarely lalopathy and seizure attacks often are accompanied by impaired consciousness (simple *versus* complex, table 1).

A large number of patients develop therapy resistance during the course of medication. In these cases the last therapeutic option is partial resection of the brain region where the focus is located (hippocampus). Using histopathological analysis the degree of damage within the resected hippocampal specimen is determined according to the Wyler classification (Wyler et al., 1992; Blumcke et al., 2007). As shown in table 2 this includes five grades (W0-4) according to the percentage of neuronal loss in hippocampal subfields and gliosis (proliferation of astrocytes in damaged brain areas).

Class	Description	Pathology
Wyler 0 (W0)	No mesial temporal lesion	• No cell loss or sclerosis
Wyler 1 (W1)	Slight mesial temporal lesion	• Gliosis with <10% cell loss • Affects regions CA1, CA3 and/ or CA4
Wyler 2 (W2)	Moderate mesial temporal lesion	• Gliosis with 10-50% cell loss • Affects regions CA1, CA3 and/or CA4 • Affecting only CA3 and CA4 = end folium sclerosis
Wyler 3 (W3)	Classical hippocampal sclerosis	• Gliosis with >50% cell loss • Affects regions CA1, CA3 and CA4 • Sparing CA2
Wyler 4 (W4)	Severe hippocampal sclerosis	• Gliosis with >50% cell loss • Affects all regions • Total Ammons horn sclerosis

Table 2: Wyler classification. Histopathological analysis of the degree of damage within the resected hippocampal specimen is determined according to the Wyler classification. Five grades of damage according to percentage of neuronal loss and gliosis can be distinguished.

1.1.5.4 Potential TLE mechanisms at a hippocampal network level

The frequency of network oscillations is controlled by interneurons (Gloveli et al., 2005a). GABAergic interneurons are involved in feedback inhibition and thus control network synchronization of principle cell populations, because the firing pattern of each cell type is phase-locked (for an overview see (Monyer and Markram, 2004; Gloveli et al., 2005a; Gloveli et al., 2005b)). Interneurons are classified according to their discharge frequencies. Fast spiking interneurons (e.g. basket cells) drive and control γ oscillations (> 30 Hz) and typically express the calcium binding protein parvalbumin, while interneurons discharging at low frequencies (θ oscillations, 4-7 Hz, e.g. oriens-lacunosum moleculare (O-LM) cells) express among other histological markers the peptide hormone cholecystokinin (CCK).

Therefore, any mechanism involved in control of interneuron discharge can have a strong impact on the transition from physiological to pathophysiological network activity. In fact, spatially localized increases in the power of γ frequency oscillations have been observed as precursors of seizures in TLE patients (Fisher et al., 1992; Bragin et al., 2007; Dugladze et al., 2007).

1.1.5.5 Potential TLE mechanisms at the neuron level

Deficits in GABAergic neuronal mechanisms are frequently observed in TLE and thus increased Glu/GABA ratios accompany the phenomenology of TLE (Baulac et al., 2001; Palma et al., 2005; Stief et al., 2007; Eugene et al., 2007; Eichler and Meier, 2008). Also, posttranscriptional processes were found to be involved in TLE. For example, RNA editing at the R/G site of AMPAR (Vollmar et al., 2004) was shown to produce channels with faster desensitization and resensitization kinetics (Krampfl et al., 2002), thus well suited to contribute to hyper-synchronous high frequency neuronal activity as it occurs in TLE (Fisher et al., 1992; Bragin et al., 2007; Dugladze et al., 2007).

An increase in the Glu/GABA ratio may not generally account for seizures because it depends on the type of neuron that is concerned. For example, the studies by Dugladze et al. (2007) showed that O-LM interneurons, which usually fire phase-locked during θ oscillations, can increase their firing rates up to γ frequencies in a mouse model of TLE. Although Glu/GABA ratio of synaptic input to O-LM cells was estimated to be decreased, based on frequency analysis of miniature excitatory and inhibitory postsynaptic currents, in epileptic animals (mEPSC and mIPSC, respectively), these afferent cells were found to discharge at increased frequencies. Thus synaptic drive of the O-LM cells shift output from θ to γ frequency, thereby contributes to hyper-synchronous γ oscillations in the epileptic hippocampus and therefore potentially also to seizure activity. However, using frequency analysis of miniature currents as a measure for the number of glutamatergic synapses may not be conclusive in shunted neurons, because glutamatergic mEPSCs with fast kinetics may not show up due to filtering, favored by the decreased membrane resistance. Experimental support for this concern derives from the considerations that $GABA_AR\alpha5$ subunit involved in tonic inhibition was found to be strongly upregulated in epileptic mice (Bouilleret et al., 2000), but after all this mechanism is unlikely to apply to O-LM cells because their membrane resistance was increased in epileptic animals (Dugladze et al., 2007).

This exemplifies that several levels of complexity underlie the TLE phenomenology because alterations of output discharge frequencies of hippocampal (inter)neurons can have completely different meanings with regard to epileptogenesis, according to the cell type that is concerned.

"Despite previous misconceptions" (Song et al., 2006), functional GlyR are present in the hippocampus (Chattipakorn and McMahon, 2002; Chattipakorn and McMahon, 2003; Kirchner et al., 2003; Song et al., 2006), but their functional role still has to be determined.

1.2 Non-neuronal systems

1.2.1 Molybdenum cofactor (Moco) and gephyrin

Molybdenum cofactor (Moco) is essential for molybdenum-dependent enzymes that are involved in redox reactions such as reduction of nitrate to nitrite and oxidation of sulfite to sulfate (Fritschy et al., 2008); the respective enzymes are sulfite oxidase, aldehyde oxidase and xanthine oxidoreductase (Schwarz, 2005). Moco-synthesizing enzymes are predominantly found in liver tissue, where they contribute to metabolic detoxification. Loss of Moco results in early childhood death (Reiss and Johnson, 2003; Schwarz, 2005), but was also associated with neurodegeneration and untreatable seizures (Schwarz, 2005). Generally, a genetic background characterizes the patients with reduced Moco levels, and most patients harbor mutations in the *MOCS1* gene (Lee et al., 2002).

There are two bacterial proteins which are well known to catalyze Moco synthesis (Xiang et al., 2001), these are homologues to domains of gephyrin protein. Gephyrin consists of three domains (G, E and C), which were named after the bacterial homologues Mo**g**A (G) and Mo**e**A (E) – the **c**entral domain (C) is vertebrate-specific and connects N-terminal G and C-terminal E domains (Lardi-Studler et al., 2007). And in fact, the experiments by Stallmeyer et al. (1999) demonstrated that gephyrin has retained the capacity of the bacterial homologous sequences to Moco synthesis.

Besides gephyrin expression in liver tissue (Paarmann et al., 2006), where it can contribute to Moco synthesis, gephyrin is highly expressed throughout the brain. As described above (see

1.1.4.1 and 1.1.4.2) the function of brain gephyrin so far was restricted to postsynaptic anchoring of $GABA_AR$ and GlyR. Considering the symptomatology of Moco-deficient patients the question of additional, enzymatic gephyrin functions in the brain arises. There is indeed evidence that Moco synthesis also occurs in the brain (Feng et al., 1998). There are several gephyrin splice variants known and some of them contain spliced exons in the enzymatically active G domain (Paarmann et al., 2006; Fritschy et al., 2008). Therefore regulation of brain Moco synthesis by splicing has to be considered, not only because different brain areas have different gephyrin splice variants (Meier and Grantyn, 2004a), but also because glial cells were suggested to express a specific set of gephyrin splice variants (Paarmann et al., 2006). Therefore, it is necessary to clarify the molecular basis for a catalytic function of gephyrin in the brain, in addition to the well known gephyrin-mediated Moco synthesis in liver tissue.

1.2.2 Glia cells and tumor

1.2.2.1 Glia cells

Glia cells contribute to a variety of functions in the brain including maintenance of the chemical milieu, ensheathing axons, synaptogenesis and synaptic transmission as well as metabolic support to neurons (Raine, 2006; Purves et al., 2007).

There are three types of glia cells: astrocytes, oligodendrocytes and microglia. Microglial cells are sometimes referred to as a specialized macrophage cell type because they share some common features with them (e.g. can modulate inflammatory processes) (Purves et al., 2007). Microglial cells derive from the immune system and are responsible for immune defense in the brain because antibodies are unable to cross the blood-brain barrier. Usually, microglia are inactive, but are activated following injury and migrate to the injured area where they remove the cellular debris (Raine, 2006; Purves et al., 2007). Oligodendrocytes contribute to information transfer in the brain because they myelinate the axons of neurons and therefore match with the Schwann cells of the peripheral nervous system. Astrocytes have a fundamental role in the brain because they act primarily as connective tissue or skeleton. Due to trauma or injury they proliferate, swell and accumulate glycogen and undergo fibrosis (gliosis, see above 1.1.5.3, Wyler classification, table 2). Besides this, they mediate clearance of neurotransmitters from the synaptic cleft and extracellular milieu and therefore contribute to maintenance of the chemical milieu. In addition, they are involved in metabolic processes in reference to neuronal function (e.g. glutamine cycle).

1.2.2.2 Tumor

A tumor *per se* is a development, a swelling or lesion by an abnormal growth of cells (neoplasm) (Alberts et al., 2008). Tumors can be differentiated into two groups: benign and malignant tumors. Benign tumors push away surrounding tissue by their growth, but do not infiltrate it or produce metastases. Malignant tumors infiltrate surrounding tissue thereby destroying it and can generate metastases. A tumor is not the same as cancer. Per definition only malignant tumors are cancer related neoplasms. A specialized form of malignant brain tumor is called glioblastoma.

1.2.2.3 Glioblastoma

The glioblastoma multiform is the most aggressive brain tumor known because it falls into category IV (World Health Organization, WHO). Following diagnosis, patients have a predicted mean life span of 12 months (Holland, 2000), even after complete resection and anti-tumor treatment. It is suggested that glioma cells derive from oligodendrocytic and astrocytic progenitor cells (Holland, 2001; Sontheimer, 2003), but also from grade III (WHO) astrocytomas (Perez-Ortiz et al., 2000). The tumor cells maintain a high proliferation rate and have to reduce the brain mass due to space constraints. The tissue surrounding the tumor displays gliosis and associated inflammatory mechanisms, also necrotic areas are observed (Synowitz et al., 2001). The necrotic area possibly results from excitotoxicity as glioma cells feature the release of high amounts of glutamate (Takano et al., 2001; Sontheimer, 2003), which is supposed to elicit excitotoxicity and in turn induces seizures often seen in patients afflicted with glioblastoma (Senner et al., 2004; Kohling et al., 2006).

2. Material and methods

2.1 Preface

2.1.1 Chemicals

Acetic acid glacial	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Agar-Agar	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Agarose	Invitrogen GmbH, Karlsruhe, Germany
Antibiotic-Antimycotic	Invitrogen GmbH (Gibco), Karlsruhe, Germany
Ammonium chloride	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ampicillin	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
AraC	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
B27-Supplement	Invitrogen GmbH (Gibco), Karlsruhe, Germany
Calcium chloride 2-hydrate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Chloroform	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DEPC	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
Diethyl ether	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
N,N-Dimethyl formamide	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dimethyl sulfoxide	Serva Electrophoresis GmbH, Heidelberg, Germany
DMEM	Invitrogen GmbH (Gibco), Karlsruhe, Germany
DNQX	Tocris, Bristol, UK
dNTP-Mix	Bioline GmbH, Luckenwalde, Germany
dTTP	Fermentas Life Science, St. Leon-Rot, Germany
EDTA	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethidium bromide	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
FCS	Invitrogen GmbH (Gibco), Karlsruhe, Germany
Gelatine	VWR International GmbH, Germany
D-(+)-Glucose	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
L-Glutamine	Invitrogen GmbH, Karlsruhe, Germany
Glycine	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
HEPES	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Horse serum	Invitrogen GmbH (Gibco), Karlsruhe, Germany

Hydrochloric acid	VWR International GmbH, Germany
IPTG	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Isopropanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
K252a	Sigma-Aldrich, Deisenhofen, Germany
Kanamycin	Invitrogen GmbH (Gibco), Karlsruhe, Germany
Magnesium chloride 6-hydrate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Magnesium sulphate 7-hydrate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
MEM (+) Earle's	Invitrogen GmbH (Gibco), Karlsruhe, Germany
MEM + Hanks'	Invitrogen GmbH (Gibco), Karlsruhe, Germany
β-Mercaptoethanol	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
Methanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
MK801	Tocris, Bristol, UK
Neurobasal Medium	Invitrogen GmbH (Gibco), Karlsruhe, Germany
Nifedipine	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
O.C.T TissueTec	Sakura Finetec, USA
$Oligo(dT)_{13,\ 15,\ 18}$	BioTeZ Berlin Buch GmbH
Paraformaldehyde	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
PBS-CMF	Invitrogen GmbH (Gibco), Karlsruhe, Germany
Penicillin-Streptomycin	Invitrogen GmbH (Gibco), Karlsruhe, Germany
Peptone	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Poly-DL-ornithine hydrobromide	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
Potassium chloride	VWR International GmbH, Germany
Potassium di-dydrogen phosphate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
RNA Hippocampus (human, post mortem)	Clontech/Takara Bio Europe, St. Germain en Laye, France
Sodium chloride	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium hydrogen carbonate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
di-Sodium hydrogen phosphate 2-hydrate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium hydroxide	VWR International GmbH, Germany
Sodium pyruvate	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
Sucrose	VWR International GmbH, Germany
TEA	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
TRIS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Triton X-100	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
TRIzol Reagent	Invitrogen GmbH, Karlsruhe, Germany
Vectashield Mounting Medium	Vector Laboratories, Inc., Burlingame, CA, USA
Vitamin B_{12}	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
X-Gal	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Yeast extract	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

2.1.2 Enzymes

Enzyme for cell culture:	
DNase (Type I)	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
Trypsin Type XII-S (from bovine pancreas)	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
Trypsin inhibitor Type I-S (from soybean)	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
Polymerases:	
RedTaq DNA Polymerase	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
TTH-Pyrophosphatase	Invitek GmbH Berlin-Buch, Germany
Restriction enzymes:	
BamHI	New England Biolabs GmbH, Frankfurt, Germany
BspEI	New England Biolabs GmbH, Frankfurt, Germany
EcoRV	New England Biolabs GmbH, Frankfurt, Germany
HindIII	New England Biolabs GmbH, Frankfurt, Germany

2.1.3 Kits

Effectene Transfection Reagent	Qiagen GmbH, Hilden, Germany
Superscript II	Invitrogen GmbH, Karlsruhe, Germany
Matrix Gel Extraction System	Marligen Biosciences, Ijamsville, MD, USA
Rapid DNA Ligation Kit	Fermentas Life Science, St. Leon-Rot, Germany

2.1.4 Bacteria

JM109 Competent Cells, high efficiency	Promega GmbH, Mannheim, Germany

2.1.5 Animals

E19 Wistar Han rats	Charles River Laboratories Berlin-Buch, Germany
Adult NMRI-mice	Charles River Laboratories Berlin-Buch, Germany

2.1.6 Software

ImageQuant	Molecular Dynamics, Sunnyvale, CA, USA
LCS	Leica Microsystems Heidelberg GmbH, Germany
Origin	Microcal, Northampton, MA, USA
MetaMorph	Visitron Systems GmbH, Puchheim, Germany
PAST	(Hammer et al., 2001)
ScionImage	Scion Corporation, Frederick, MD, USA

2.1.7 Equipment

Fluorescence microscopy: Olympus Life Science Europa GmbH, Hamburg, Germany

Systemmikroskop BX 51

Filter:

U-MSP100v2 MFISH DAPI Filter

U-MSP101v1 MFISH FITC Filter

U-MSP102v1 MFISH Cy3 Filter

U-MSP104v1 MFISH Cy5 Filter

Objectives:

UPLFLN40xPH/0.75 UPlan Semi Apo Phase, 40x phase contrast

UPlanApo 40x/1.00 oil immersion objective

Camera:

14bit-CCD camera system Spot PURSIUT

Lamp:

X-Cite-120W metal halide lamp

Confocal microscopy:

Laser scanning microscope, Leica DM TCS SP2	Leica, Solms, Germany
HCX PL APO 40x oil immersion objective, optical aperture 1.25	Leica Mikrosysteme, Wetzlar, Germany

Microtome:

Microtome CM 1850 UV	Leica, Solms, Germany
Superfrost Plus slides	Menzel GmbH & Co. KG, Braunschweig, Germany
DAKO Pen	Dako Deutschland GmbH, Hamburg, Germany
Glascuevettes	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Molecular- and microbiology:

Advanced Primus 96	PeqLab Biotechnologie GmbH, Erlangen, Germany
Biofuge Pico	Heraeus (Thermo Fisher Scientific, Inc.), Germany
Biofuge 28RS	Heraeus (Thermo Fisher Scientific, Inc.), Germany
BioPhotometer	Eppendorf Germany
GeneAmp PCR, System 2400	Perkin Elmer, Inc., Waltham, Massachusetts, USA
PerfectBlue Gelsystem	PeqLab Biotechnologie GmbH, Erlangen, Germany
Zentrifuge 5415R	Eppendorf Germany

Cell culture:

Incubator CB 150	Binder GmbH Labortechnik, Tuttlingen, Germany
Fuchs-Rosenthal counting chamber	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Microscope slides	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Cover slips, 13mm	Glaswarenfabrik Karl Hecht KG, Sandheim, Germany
Culture dishes, 35mm	TPP AG, Trasadingen, Switzerland
Culture flasks, T25	TPP AG, Trasadingen, Switzerland
24-well plates	TPP AG, Trasadingen, Switzerland

2.1.8 Media and solutions

2.1.8.1 Media and solutions for cell culture

2.1.8.1.1 Primary hippocampal cell culture from rat

NB-B27:

Neurobasal Medium
25µM β-Mercaptoethanol
2% B27-Supplement
1% FCS
0.25mM L-Glutamine
0.05% Penicillin-Streptomycin

DMEM-FCS:

DMEM
10% FCS

DNase-Ovomucoid:

MEM + Hanks'
1380U DNase/ml
1.8g/l Glucose
25mM HEPES
0.44mM $MgCl_2$ x $6H_2O$
50mM $MgSO_4$ x $7H_2O$
Trypsin-Inhibitor

PBS-CMF:

1x Antibiotic-Antimycotic
3.6g/l Glucose
15mM HEPES

PBS-CMF-EDTA:

1x Antibiotic-Antimycotic
1mM EDTA
3.6g/l Glucose
15mM HEPES

Poly-DL-ornithine hydrobromide (PO):

0.005% Poly-DL-ornithine hydrobromide in ddH_2O

Standard salt solution (SSS):

1x Antibiotic-Antimycotic

1.25mM $CaCl_2$ x $2H_2O$

4.5g/l Glucose

10mM HEPES

5mM KCl

135mM NaCl

1mM $NaHCO_3$

0.5mM $MgCl_2$ x $6H_2O$

0.5mM $MgSO_4$ x $7H_2O$

Trypsin:

1mg Trypsin

2.1.8.1.2 Substances for pharmacoligical manipulations (stock solutions)

DNQX:	10mM in ddH_2O and NaOH
K252a:	200µM in DMSO
KCl:	2M in ddH_2O
MK801:	2mM in ddH_2O
Nifedipine:	15mM in DMSO
TEA:	1.5M in ddH_2O

2.1.8.1.3 Media and solutions for HEK293 cells

Culture medium for HEK293 cells (pH 7.3):

MEM (+) Earle's
10% FCS
5g/l Glucose
0.25mM L-Glutamine
0-300µM Glycine (optionally)
10mM HEPES
0.23mM Sodium pyruvate
0.05% Penicillin-Streptomycin

Trypsin:

0.1% Trypsin in PBS-CMF (w/v)

2.1.8.1.4 Media and solutions for glia cells

MEM-HoS:

MEM (+) Earle's
5g/l Glucose
0.25mM L-Glutamine
10mM HEPES
0.23mM Sodium pyruvate
0.05% Penicillin-Streptomycin
10% Horse serum

KCl:

3M KCl in ddH_2O

2.1.8.1.5 Media and solutions for transfections

NB-Medium (transfection medium for neurons):

Neurobasal Medium
0.25mM L-Glutamine

Expression medium for neurons (pH 7.3):

MEM + Hanks'
25µM β-Mercaptoethanol
2% B27-Supplement
4.5g/l Glucose
5µM Glycine (optionally)
250µM L-Glutamine
10mM HEPES
230µM Sodium pyruvate
0.05% Penicillin-Streptomycin
2.5µM Vitamine B_{12}

Transfection solutions for HEK293 cells:

250mM $CaCl_2$ x $2H_2O$ in ddH_2O

2x HBS (pH 7.05):

2.16g/l Glucose
40mM HEPES
10mM KCl
274mM NaCl
1.4mM Na_2HPO_4 x $2H_2O$

2.1.8.2 Solutions for immunofluorescence

0.12% Gelatine in 1x PBS (w/v)

95% Methanol / 5% Acetic acid glacial (v/v)

50mM NH_4Cl in 1x PBS (w/v)

4% PFA / 4% Sucrose in 1x PBS (w/w)

8% Sucrose in 1x PBS (w/v)

0.12% Triton X-100 in 0.1% PBS-Gelatine (v/v)

PBS-solution:

1x PBS (Gibco)

2.1.8.3 Media and solutions for bacterial cultures

Agar plates:

1.5% Agar-Agar in LB-Medium (w/v), supplemented with 0.1mg/ml ampicillin or 40µg/ml kanamycin.

Ampicillin:

100mg/ml ampicillin in ddH_20

IPTG:

100mM IPTG in ddH20

Kanamycin:

40mg/ml kanamycin in ddH_20

LB-Medium:

0.5% Yeast extract (w/v)

1% Peptone (w/v)

1% NaCl (w/v)

X-Gal:

3% X-Gal in DMF (w/v)

73.5mM in DMF (3%, w/v)

For blue-white screening 50µl of a 100mM IPTG solution as well as 50µl of a 3% X-Gal solution were plated on agar plates. The average volume of one plate is about 20-25ml.

2.1.8.4 Solutions for molecular biology

DEPC-ethanol:

70% undenaturated ethanol in DEPC-treated water

DEPC-water:

ddH_2O was incubated with DEPC (1:2000) over night, constantly stirring. Afterwards the solution was autoclaved three times for 20min each time.

2.1.9 Antibodies

Primary antibodies:

Antibody	Host	Dilution	Supplier
α-c-myc	Mouse	1:200	Roche Diagnostics GmbH, Mannheim, Germany
α-GAD65	Mouse	1:100	Chemicon International (Millipore), MA, USA
α-Gephyrin (mAb7a)	Mouse	1:200	Synaptic Systems, Göttingen, Germany
α-GFAP (GA5)	Mouse	1:500	Cell Signaling Technologies, Inc., Danvers, MA, USA
α-GFP	Rabbit	1:200	Molecular Probes (Invitrogen), Invitrogen GmbH, Karlsruhe, Germany
α-GFP	Chicken	1:300	Chemicon International (Millipore), MA, USA
α-GlyR (mAb4a)	Mouse	1:200	Synaptic Systems, Göttingen, Germany
α-GlyR (pAbα3L)	Rabbit	1:200	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
α-GlyR α3	Rabbit	1:200	Chemicon International (Millipore), MA, USA
α-HA	Chicken	1:200	Bethyl Laboratories, Inc., Montgomery, TX, USA
α-KCC2	Rabbit	1:200	Upstate, Lake Placid, NY, USA
α-MAP2 (2a+2b)	Mouse	1:300	Sigma-Aldrich Logistik GmbH, Schnelldorf, Germany
α-Synapsin	Rabbit	1:200	Chemicon International (Millipore), MA, USA
α-VGluT1	Guinea pig	1:500	Chemicon International (Millipore), MA, USA
α-VGluT2	Guinea pig	1:500	Chemicon International (Millipore), MA, USA
α-VIAAT	Rabbit	1:200	Synaptic Systems, Göttingen, Germany

Table 3: Overview of used primary antibodies in alphabetical order

Secondary antibodies:

Antibody	Host	Dilution	Supplier
α-Chicken - Cy3	Donkey	1:200	
α-Chicken - FITC	Donkey	1:200	
α-Mouse - AMCA	Donkey	1:200	
α-Mouse - FITC	Donkey	1:200	
α-Mouse - Cy3	Goat	1:200	
α-Mouse - TRITC	Donkey	1:200	All secondary antibodies
α-Mouse - Cy5	Goat	1:200	were purchased from
α-Rabbit - AMCA	Donkey	1:200	Jackson ImmunoResearch Europe Ltd., Suffolk, UK
α-Rabbit - FITC	Goat	1:200	
α-Rabbit - Cy3	Donkey	1:200	
α-Rabbit - TRITC	Donkey	1:200	
α-Rabbit - Cy5	Donkey	1:200	
α-Guinea pig - Cy5	Donkey	1:200	

Table 4: Overview of used secondary antibodies in alphabetical order of host species

2.1.10 Oligonucleotides

Gene	Species	Sequence 5'-3'
GlyRα2		
GlyRα2-editing	Human	GGAAGGATCCACGGCTGGCG GTTCCTTCTCTTCTTTCAAAATAATCCGG
GlyRα2 Colony-PCR	Human	ACCCATCCATGCTAGACTCC
GlyRα2-editing	Rat	GTTGCTGAAGGACTCACCAAGC CTGCCTCTTCTGTAGGATCCGG
GlyRα2 Colony-PCR	Rat	GGACGGTTGTGATGCCAAGG
GlyRα2A Splicing forward GlyRα2B Splicing forward GlyRα2 Splicing reverse	Human	ATCAACAGTTTTGGATCAGTCA TCAACAGCTTTGGGTCAATAG CCTTCAGCAACTTGCACTGG
GlyRα3		
GlyRα3-editing	Human	GAGACGACCATGGATCCCAGAGTG CGTAAATCTTTTTCTTCTTTCAACAGTATCCGG
GlyRα3 Colony PCR	Human	GGAAACCTGATTTGTTCTTTGCC CATGGTCCTGCTGGAGTTCGTG
GlyRα3-editing	Rat	GGCTGAAGGACTCACTAAGC TTGACATAGGACACCTTTGG
GlyRα3 Colony-PCR	Rat	GTGCTGCATCCATGTTGATC
GlyRα3 Splicing	Human/Mouse	GGGTACACAATGAATGATCTC AGAGACTTAATCTTGCTGCTGATG

Gene	Species	Sequence 5'-3'
GlyR β		
GlyRβ	Human	TGAGCAAGCAGATGGGAAAGG TAACGTTGAAGAACAAGAAGCAG
KCC2		
KCC2	Human	CTGCTGGCTTACCTCTTCC CGTCTCATTTCCTTCCCAAG
Housekeeping genes		
β-Actin	Human	CGCTCGTTGCCAATAGTGATG TTGTAACAAACTGGGACGATATGG
GAPDH	Human	ATGGCACCGTCAAGGCTGAG CGACGCCTGCTTCACCACC
GAPDH	Mouse	CCACTCACGGCAAATTCAACG AGCCCAAGATGCCCTTCAGTG
GAPDH	Rat	CAGTATGACTCTACCCACGG CTCAGTGTAGCCCAGGATG
pBluescript vector		
T7		GTAATACGACTCACTATAGGGC
T3		CGCAATTAACCCTCACTAAAG

Table 5: Overview of used oligonucleotides

2.1.11 Plasmids

Plasmid	Gene	Origin
pBluescript II SK (+/-)	GlyRα3^{185P} GlyRα3^{185L}	Stratagene, USA
pCaspase3-sensor	NES-EYFP-NLS	BD Biosciences Clontech, Franklin Lakes, NJ, USA; provided by Heiko Luhmann (Univeristy of Mainz, Germany)
pEGFP-N1/pCMV	GlyRα3^{185P} GlyRα3^{185L} HA-GlyRα3L HA-GlyRα3K myc-GlyRβ	BD Biosciences Clontech, Franklin Lakes, NJ, USA
pMES	KCC2	Originally introduced by Katrin Krull (University of Missouri-Columbia, MO, USA); provided by Claudio Rivera (University of Helsinki, Finland) and Karl Kandler (University of Pittsburgh, PA, USA)
pTRACER-CMV2	GlyRα3^{185P} GlyRα3^{185L}	Invitrogen GmbH, Karlsruhe, Germany

Table 6: Overview of gene expression constructs and accordant plasmid background

2.2 Cell culture

2.2.1 Primary hippocampal neuronal cell culture from rat

2.2.1.1 Preparation

Preparation of the E19 embryos was previously described (Meier and Grantyn, 2004b).

The 13mm cover slips were coated with 0.005% Poly-DL-ornithine in a 24-well plate over night at 37°C. On preparation day, the Poly-DL-ornithine was removed and cover slips were dried for 20min at room temperature. Afterwards they were coated with DMEM-FCS for 30-90min at 37°C and 5% CO_2. Both serum proteins and positively charged ornithine provide a basis for neurons to bind to the glass surface.

The embryos were removed from the uterus and stored in ice cold standard salt solution (SSS). Scalp and skullcap were detached and brains were collected in fresh SSS on ice. From every brain the cerebellum was removed and the cortex was separated into two parts along the corpus callosum. The thalamus was removed so that the hippocampus was exposed. The pia mater surrounding the hippocampus was removed before isolation. All isolated hippocampi were collected in SSS on ice, sliced into pieces and transferred into PBS-CMF. After removing supernatant (except a small volume above the tissue), 1mg trypsin was dissolved in PBS-CMF-EDTA and applied to the hippocampi. Trypsination was carried out for 5min at 37°C and stopped through addition of trypsin inhibitor (DNase-Ovomucoid solution). The supernatant was discarded and hippocampi were triturated in NB-B27 and resuspended (trituration was carried out in three steps). After waiting for non-dissolved tissue to sedimentate, the solution was transferred into a fresh falcon tube.

Determination of cell count was done with a Fuchs-Rosenthal counting chamber. Eight squares were counted and the average of cells was determined. The calculation was carried out according to the following formula:

$$\boxed{\text{average of cell number} \cdot \text{dilution} \cdot 5000 = \text{cell number in 1ml suspension}}$$

The number 5000 is the predetermined factor of the chamber, which is necessary to adjust the volume to 1ml cell suspension.

At last, the DMEM-FCS was removed from the wells and cells were homogenously plated with an initial density of 68000/cm^2 in NB-B27. The neurons were cultured at 37°C and 5% CO_2. The growth medium was not changed.

For a glia cell free culture, neurons were plated with an initial density of 70000/cm^2 in FCS-free NB-B27 and cultured in T25 culture flasks. To inhibit glia cell proliferation 5µM AraC

(cytosine β-D-arabinofuranoside hydrochloride) was added two days after plating. The growth medium was not changed.

2.2.1.2 Effectene-based transfection of neurons

Transfection of primary hippocampal neurons was carried out on div 6 (div = *days in vitro*) using Effectene Transfection Reagent from Qiagen as previously described (Meier et al., 2005). Per cover slip 300ng DNA were mixed with 60µl of EC-buffer and 1.6µl Enhancer. The solution was shortly vortexed and incubated for 5min at room temperature. During incubation DNA is condensed by interaction with the Enhancer under stable buffer conditions provided by the EC buffer. After addition of 5µl Effectene the solution was mixed by vortexing. DNA and Effectene form a complex during the following 15min incubation step.

In the meantime, cover slips with cultured neurons were transferred into new wells containing 500µl prewarmed and equilibrated (2h, 37°C and 5% CO_2) NB-medium. Afterwards 200µl of warm and equilibrated NB-medium was added to the DNA-Effectene complex and 270µl were dropped carefully onto the neurons in NB-medium. Transfection time was 1h at 37°C and 5% CO_2. This procedure ensured moderate protein expression levels in 1% of hippocampal neurons. For protein expression the cover slips were transferred into wells containing equilibrated expression medium. Expression time was three days under defined glycine concentrations (without or with 5µM glycine, respectively).

To favor the natural stoichiometry between GlyRβ and GlyRα3 in heteropentamers (3:2) (Grudzinska et al., 2005), GlyRβ DNA was used in excess to GlyRα3 (5:1).

Due to different promotor activities of KCC2 and GlyRα3 coding plasmids a ratio of 1:5 was used. The KCC2 expression construct provided by Karl Kandler and Claudio Rivera uses a pMES vector backbone, which was originally produced by Catherine Krull (Swartz et al., 2001). This allowed the use of enhanced green fluorescent protein (EGFP) as an indicator for KCC2 expression (Lee et al., 2005).

Cotransfection of glycine receptors and pCaspase3-sensor was carried out with a ratio of 1:1.

Pharmacological manipulations were done by addition of following substances to the expression medium: 20µM DNQX, 1µM MK801, 15.4mM KCl, 15µM nifedipine, 5mM TEA as well as 200nM K252a. There was no change of medium.

2.2.2 Glia cell culture

For cultivation of glia cells, cortical cells (preparation adapted to 2.2.1.1) were plated with an initial density of 40000/cm^2 in MEM-HoS. Cultivation was carried out in T25 culture flasks at 37°C and 5% CO_2. Naturally, neurons do not survive under these culture conditions. Residual neurons were eliminated using 60mM KCl, applied to the culture for at least 6h. Culture medium was completely exchanged once a week.

2.2.3 Cocultivation of neurons and tumor cells

Neurons were initially plated with an initial density of 45000/cm^2 in NB-B27 on cover slips. After two weeks *in vitro*, tumor cells were added with an initial density of 2800/cm^2 (U373 cells, kindly provided and plated by Dr. Rainer Glass) and after 24h cover slips were processed for immunofluorescence. There was no change of medium.

2.2.4 HEK293 cells

2.2.4.1 Cultivation

Human embryonic kidney cells (HEK293) were cultured in HEK-medium at 37°C and 5% CO_2 in T25 culture flasks. As soon as the culture was confluent, the cells were trypsinated with 0.1% trypsin solution and plated with a dilution of 1:10 in T25 culture flasks or on cover slips, respectively.

2.2.4.2 Calcium phosphate-based transfection

HEK293 cells were transfected using a standard calcium phosphate protocol. The cells were cultured on 13mm cover slips in a 35mm culture dish. 2µg DNA were used per dish.
DNA was mixed with calcium chloride and supplemented with an equal volume of 2x HBS buffer. After 15min incubation at room temperature the mixture was applied onto the cover slips. Expression time was 16h at 37°C and 5% CO_2.

2.3 Tissue preparation

All animals were sacrified according the permit given by the Office for Health Protection and Technical Safety of the regional government of Berlin and in compliance with regulations laid down in the European Community Council Directive (LaGeSo T0122/07).
Analysis of resected human hippocampi was performed according to the rules laid down by the Ethics Commission (Charité, EA1/142/05), and informed consent was received from all subjects. Post-mortem hippocampus RNA was purchased from Clontech.

2.3.1 Preparation of human hippocampus

Resections of human hippocampi from therapy-resistant temporal lobe epilepsy patients were performed by Dr. Thomas-Nicolas Lehmann (Neurosurgery, Charité Berlin, Campus Virchow). After resection the tissue was immediately shock frozen in liquid nitrogen, and subsequently processed for RNA isolation (see 2.5.3).

2.3.2 Preparation of the mouse brain

After deep anesthesia with diethyl ether mice were sacrified by cervical dislocation (luxation of cervical spine by fast stretching) followed by decapitation. The skullcaps were removed and brains were transferred into ice-cold PBS solution. All following steps (cryosections of mouse brain see 2.3.4, isolation of brain regions for RNA-isolation see below) were carried out on ice.

2.3.3 Isolation of mouse brain regions for RNA isolation

After careful removal of the brain, the cerebellum as well as the brain stem was cut of the cortex. The cortex was divided into both hemispheres along the corpus callosum. Then, thalamus, hippocampus and frontal as well as residual cortices were recovered. For isolation of the spinal cord the spinal column was carefully opened prior to removal. All isolated areas were immediately shock frozen in liquid nitrogen.

2.3.4 Cryosections of mouse brain

For preparation of cryosections of the mouse brain, a 3-5mm thick coronal slice was cut out the brain at the level of the hippocampus and immediately fixed with a mixture of ice cold PFA/sucrose (4% both) for 1h at 4°C. After three times wash (5min each) with PBS solution the fixed slices were incubated in 8% sucrose over night at 4°C for cryoprotection.
Slices were then embedded in O.C.T Tissue Tec on dry ice, and 12µm thick cryosections were obtained using a cryomicrotome. The sections were finally mounted on Superfrost microscope slides and stored at -20°C until immuohistochemistry was performed.

2.4 Immunofluorescence

2.4.1 Fluorescence microscopy

The fluorochromes used in fluorescence microscopy absorb light in a specific range of the light spectrum and emit part of the absorbed energy as fluorescence at longer wavelengths. For fluorescence detection fluorochrome-specific emission filters have to be used in order to perform multiple labeling experiments.
To visualize proteins of interest, specific antibodies are used. These primary antibodies can then be visualized using fluorochrome-coupled secondary antibodies (indirect immunfluorescence). Use of a secondary antibody enhances the protein-associated signal because more than one secondary antibody binds to the primary one.
In the fluorescence microscope light of an external source (here a metal halide lamp was used) passes an excitation filter permeable for a specific wavelength range and scores a dichroic (semipermeable) mirror, which channels the light through the objective onto the object. As the emitted light has a longer wavelength it passes the dichroic mirror directly and can be detected using an ocular and/or a camera (here a 14-bit CCD-camera).
As the filter sets used here are characterized by a narrow permissive wavelength range the simultaneous detection of four target proteins/epitopes in one preparation is possible (multiple labeling).
For image acquisition and processing the software MetaMorph was used. For multiple labeling experiments monoclonal and polyclonal antibodies were combined. Secondary antibodies were AMCA- (7-amino-4-methylcumarin-3-acetic acid), FITC-

(fluorescein isothiocyanate), Cy3-/Cy5- (Carboxymethyl-indocyanine) or TRITC- (tetramethyl rhodamine isothiocyanate) coupled.

2.4.2 Confocal microscopy

Immunohistochemically processed cryosections of mouse brain (see 2.3.4) were analyzed with confocal microscopy. Here, the light sources are lasers with defined wavelengths. The emitted light is channeled through a pinhole and registered by a photomultiplier. Pinhole and focal layer are confocal (i.e. they are located in conjugated levels). Therefore only light from the focal point is able to pass the pinhole, and because excitation also takes place in a specific frame, disturbing signals (strayed light) above and beneath the target protein (Z-level) are nearly not existent.

For image acquisition the confocal laser-scanning microscope Leica DM TCS SP2 was used. All recordings were acquired sequentially with the following lasers: argon laser (488nm) and two helium-neon lasers (543 and 633nm). Each picture was recorded by scanning every frame six times each during simultaneous averaging of the signal using software LCS.

2.4.3 Immunocytochemistry

2.4.3.1 Surface staining

For surface staining antibodies were diluted into the culture medium and directly applied onto the living cells (30µl per cover slip). After a 5min incubation step at 37°C and 5% CO_2 in a humid chamber, cells were washed three times with culture medium prior to fixation with PFA (see below) or methanol (see below).

2.4.3.2 Methanol fixation

Cells were fixed in a mixture of methanol/glacial acetic acid (95:5) at -20°C for 10min. Methanol acts as hardener of tissue, whereas glacial acid acts as diluent and protects of rupture of membranes. After transferring cover slips onto parafilm in a humid chamber, cells were washed three times with PBS solution and blocked with 0.1% PBS-gelatine (see also section 2.4.3.4)

2.4.3.3 PFA fixation

For fixation with paraformaldehyde (PFA) cover slips were transferred in an ice cold mixture of PFA/sucrose (4% both) and incubated for 15min at room temperature. PFA fixes tissue by covalently introducing new bonds between free amino groups of the proteins and thus cross-links amino acids, and affects the accessibility of antibodies. Therefore, as longer the PFA fixation step as weaker the signal obtained with immunocyto-/histochemistry dependent on cell type (compare also section 2.4.3.4 and 2.4.4).

The following steps were carried out at room temperature. Cover slips were transferred in PBS solution on parafilm in a humid chamber and washed three times with PBS. After 15min incubation with 50mM ammonium chloride cells were washed twice with PBS, followed by block with 0.1% PBS-gelatine (5-10min). The incubation with ammonium chloride inactivates residual PFA, which reduces the fluorescence background especially in the range of green light.

A 4min incubation step with 0.12% Triton X-100 permeabilizes the cell membrane and renders intracellular epitopes accessible to the antibodies. Prior to reaction of cells with the primary antibodies, cells were washed at least two times with 0.1% PBS-gelatine.

2.4.3.4 Immunostaining

Gelatine unspecifically accumulates at free accessible protein epitopes and thus reduces unspecific binding of the primary antibodies to the proteins of interest (PBS-gelatine block).

Primary antibodies were diluted in 0.1% PBS-gelatine and cover slips were incubated for 1h at room temperature (see list of primary antibodies table 3).

After three wash steps in 0.1% PBS-gelatine incubation of secondary antibodies (again diluted in 0.1% PBS-gelatine, table 4) was carried out for 45min at room temperature.

Finally, cover slips were washed three times with 0.1% PBS-gelatine and two times with PBS. Washing with PBS removes residual gelatine, which in turn can enhance background signals, as does PFA.

After a brief dip into ddH_2O, cover slips were mounted on microscope slides with 3µl Vectashield Mounting medium and finally sealed with clear nail polish.

DAPI (4',6-diamidino-2-phenylindole) containing mounting medium was used by default to visualize cell nuclei, except if AMCA-conjugated secondary antibodies were present. Stainings can be stored at -20°C for years.

2.4.4 Immunohistochemistry

Cryosections were post-fixed for 5min in ice cold PFA/sucrose at room temperature. Then they were washed three times in PBS, incubated for 15min at room temperature in ammonium chloride, followed by an additional wash step in PBS. After 2h incubation in 0.1% PBS-gelatine at 4°C, the antibody solution was applied to cryosections. To reduce the antibody reaction volume, cryosections were contoured with a ring of silicone using the DakoPen. Primary antibodies were diluted in 0.1% PBS-gelatine and supplemented with 0.12% Triton X-100. Incubation of cryosections with primary antibodies was carried out over night at 4°C in a humid chamber.

After three washes with 0.1% PBS-gelatine, secondary antibodies (in PBS-gelatine) were applied to the cryosections. Incubation time was 1h at room temperature. Cryosections were washed three times with 0.1% PBS-gelatine, once with PBS and once with ddH_2O. Vectashield Mounting Medium (with or without DAPI, see above) was dropped on cryosections, which were finally overcasted with a cover slip and sealed with clear nail polish. Stainings can be stored at -20°C for years.

2.5 Molecular biology

All obtained cDNA-clones used in this work were sequenced. All used oligonucleotides and their sequences are listed in table 5. If not stated otherwise oligonucleotides were used with a final concentration of 0.2µM.

2.5.1 Gel extraction

Gel extraction was carried out using Matrix Gel Extraction System. The protocol was followed except that 5-20µl of silica gel were used (5µl for samples and 10-20µl for plasmids). Pellet was resuspended in 10mM Tris pH 8.0.

2.5.2 Generation of TA vector and TA-cloning

The principle of TA-cloning is based on the fact that polymerases without 3'-5' exonuclease activity autonomously stick a singular adenine to the 3'-end of a PCR-product. The resulting A-overhang can be ligated with the T-overhang of a TA vector.

The self-made TA-cloning vector used in this study was derived from pBluescript II SK (pBSK). It was linearized through restriction digest with EcoRV (2h at 37°C) generating blunt DNA ends. After gel extraction of the linearized pBSK single strand T-overhangs were obtained by incubation of the purified linearized vector with RedTaq DNA-polymerase and dTTPs at 70°C for 2h (a singular thymidine is added to the 3'-end). Final purification with gel extraction yielded the ready-to-use TA vector.

2.5.3 RNA isolation

For RNA isolation TRIzol Reagent was used. This phenol-based reagent decomposes cells and denaturates proteins, thereby preserving RNA integrity. Due to addition of chloroform and subsequent centrifugation two phases are generated, separated by the interphase. Upper and lower phases contain RNA and lipophilic cell components, respectively, while the interphase accumulates genomic DNA and proteins.

The aqueous, RNA-containing phase was transferred into a new RNase-free tube and mixed with isopropanol. After centrifugation, the precipitated RNA was recovered as a pellet, which was washed with 70% DEPC-ethanol (again centrifugation), air dried and resuspended in DEPC-water. DEPC inactivates RNases due to covalent binding to histidine residues within the catalytic domain of the enzyme.

The RNA concentration was determined by absorption measurement at 260 and 280nm. The resulting ratio between both values gives information about the purity of the RNA.

2.5.4 cDNA synthesis

To amplify RNA by PCR a complementary DNA copy has to be produced first. The reverse transcriptase used here was originally isolated from the RNA virus M-MLV (Moloney Murine Leukemia Virus).

For cDNA synthesis, 2µg total RNA was used; total RNA contains about 2% messenger RNA. To optimize the yield and length of open reading frame (ORF)-containing cDNAs reverse transcription of RNA was performed with 3' poly-A tail-anchored oligo(dT)$_{13, 15, 18}$. Because these oligo(dT)s end with any nucleotide except thymidine they bind directly at the beginning of the poly-A tail. Therefore the reverse transcriptase skips transcription of the sometimes very long poly-A tails, which increases the yield of full length ORF-containing cDNAs.

DEPC-water and RNA were mixed and incubated for 10min at 70°C (denaturation of RNA). After decreasing the temperature to 4°C (hybridization of RNA with oligo(dT)s), the reaction

mixture was added, containing 2mM dNTPs, 1x RT buffer and 10mM DTT. At beginning of the 42°C step, 100U reverse transcriptase (Superscript II) were added, and cDNA synthesis was allowed to occur for 1h. During the final 70°C (10min) step the enzyme is destroyed.

2.5.5 Detection of GlyRα2A und GlyRα2B splice variants in the human hippocampus

The relative amount of alternative spliced GlyRα2A and GlyRα2B subunits was determined by PCR. Annealing temperature was set to 58°C, elongation time was 1min, 30 cycles were run. The amplification was made in two different reaction tubes, because PCR products were of the same size.

PCR-fragments were separated using agarose gel electrophoresis, band intensity was determined using software ImageQuant and the ratio between α2A and α2B PCR products was determined. If the obtained ratio was above 1.5 mainly GlyRα2A subunit was considered to be expressed (designated as 'A'). If the value was between 0.5 and 1.5 GlyRα2A and α2B were considered to be equally expressed (designated as 'AB'), and if the value was below 0.5 mainly GlyRα2B subunit was expressed (designated as 'B'). The PCR was repeated three times per sample.

2.5.6 Detection of high affinity GlyRα2 und α3 mRNAs in human and rat hippocampi

Due to enzymatic deamination of cytosine at position 575 (α2) and 556 (α3) of GlyR mRNAs a uracil residue results (C-to-U RNA editing). This leads to amino acid substitution (proline to leucine) at positions 192 (GlyRα2) and 185 (GlyRα3), respectively. Nucleotide positions and amino acid numbering were carried out with respect to the mature protein, i.e. after cleavage of the signal peptide.

Because the 3'-end of the detection oligonucleotide binds adjacent to the edited position, a full HindIII (rat) or BspEI (human) restriction site emerges (figure 5). The second oligonucleotide binds approximately 300bp downstream (rat) or upstream (human) of the edited position. For cloning of the resulting PCR products, an endogenous cleavage site for BamHI (rat) was used or introduced by the oligonucleotide (human). PCR products can be cloned only if HindIII and BspEI restriction sites exist due to RNA editing. Parallel cloning of positive and negative fragments (derived from cDNA clones) were used to quantify the relative amount of edited *GLRA* messengers. Besides, only Taq polymerases without proofreading activity can be used; otherwise the mismatches introduced by the detection oligonucleotides will be corrected and the quantification may be falsified due to destruction of

HindIII and BspEI sites. To finally provide a proof of principle, calibration with known relative amounts of edited transcripts (defined mixtures of positive and negative control cDNA clones) was performed (data of P.K. Schäfermeier).

cDNA samples as well as controls were amplified using touch-up PCR. Annealing temperature was set to 52°C (α2) and 54°C (α3), respectively, during the first five cycles, followed by 30 cycles with 56°C (α2) and 58°C (α3), respectively. Elongation time was set to 1min.

PCR products were separated using agarose gel electrophoresis, extracted and purified (see 2.5.1). Determination of band intensities in the gel was performed using the software ImageQuant.

PCR products were digested with BamHI and HindIII (rat, 1h) or BamHI and BspEI (human, 2h), both in the presence of BSA at 37°C. Controls were digested with 10U each, and the amount of restriction enzymes was adjusted for samples according to the PCR products obtained from the control clones. Digested products were again purified with gel extraction.

Finally, a quantitative gel was run and the intensities of purified bands were determined using ImageQuant. Samples were ligated using a 3-fold molar excess of samples to the recipient vector (pBSK). For transformation of bacteria, highly competent (>10^8 cfu/µg) JM109 were heat shocked at 42°C for 1.5min.

The number of true-positive GlyR-containing cfu was determined by colony PCR (usually 10 colonies per sample) and blue-white screen. The relative amount of RNA-edited GlyRα2 and GlyRα3 mRNAs was then calculated as a percentage of true-positive cfu obtained with the respective positive control clone.

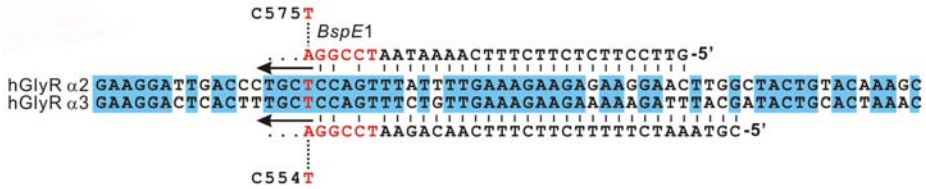

Figure 5: 3'-end of the detection oligonucleotide binds adjacent to the edited position and a full BspE1 restriction site emerges in case of edited transcripts. hGlyR: human GlyR.

2.5.7 Quantification of alternatively spliced GlyRα3 mRNAs

To quantify the expression of the long (L) and short (K) splice variants of the GlyRα3 subunit by PCR oligonucleotides were used flanking the alternative spliced exon 8A. Annealing temperature was set to 54°C, elongation time was 1min and 35 cycles were run for amplifying mouse cDNA. Analysis of human tissue was performed likewise, except that the annealing temperature was set to 52°C and 40 cycles were run using RedTaq DNA-Polymerase.

PCR fragments were separated using agarose gel electrophoresis and band intensities were quantified using ImageQuant. The amount of alternative spliced transcripts was expressed as the ratio between GlyRα3K and GlyRα3L PCR band intensities.

2.5.8 Semiquantitative PCR

To perform semiquantitative PCR, house-keeping genes (β-actin or GAPDH) are amplified together with the messengers of interest in the same PCR reaction tube. Due to nominal variations in expression levels of the house-keeping genes they can be used as a benchmark for comparison with the expression levels of the target mRNAs. Therefore the expression level of a target protein is determined in a semiquantitative way, i.e. relative to the amount of the house-keeping mRNA. In this study the house-keeping gene and the target gene were always amplified in the same PCR reaction tube using RedTag DNA-polymerase.

For semiquantitative determination of KCC2 expression (relative to β-actin) the annealing temperature was set to 58°C, elongation time was 1min and 30 cycles were run. To produce comparable band intensities with this competitive PCR, the concentrations of the β-actin oligonucleotides were adjusted to 0.1µM.

PCR products were separated with gel electrophoresis and band intensities were determined using software ImageQuant. The intensities are expressed as mean pixel intensities and the ratios were calculated according to the following formula:

$$\frac{\text{Intensity of KCC2 band}}{\text{Intensity of } \beta \text{ - actin band}}$$

The semiquantitative analysis of GlyRβ expression was performed likewise, except that GAPDH was used as house-keeping gene, that the annealing temperature was set to 54°C and that 35 cycles were run. Again, the concentrations of the GAPDH oligonucleotides were 0.025µM.

2.6 Analysis and statistics

2.6.1 Data analysis

In hippocampal cell culture experiments, neurons were sampled from at least three different cultures, each including two different coverslips. Molecular biology experiments conducted on TLE patients were performed in triplicate.

If not stated otherwise unpaired Student's *t*-test, ANOVA (analysis of variance) and Mann-Whitney were used for statistical analysis, performed with Origin and PAST. Table 7 gives an overview about application of statistical tests. Significance levels are indicated in the figures as * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Numerical data is reported as standard error of the mean (s.e.m.).

Type of Data	Measurement (from Gaussian Population)	Rank, score or measurement (from Non- Gaussian Population)
Describe one group	Mean, SD	Median, interquartile range
Compare one group to a hypothetical value	One-sample *t* test	Wilcoxon test
Compare two unpaired groups	Unpaired *t* test	Mann-Whitney test
Compare two paired groups	Paired *t* test	Wilcoxon test
Compare three or more unmatched groups	One-way ANOVA	Kruskal-Wallis test

Table 7: Overview on applications of statistical tests

2.6.2 Wyler classification

Human TLE hippocampi resected for therapeutic reasons were screened for elevated expression of high affinity GlyR. TLE patients were divided in two groups according to the severity of their course of disease (Wyler classification, (Wyler et al., 1992; Blumcke et al., 2007)). Patients without remarkable hippocampal cell loss were designated as Wyler 0-1, (cell loss <10% and a low frequency (<1/month) of secondarily generalized bilateral tonic-clonic seizures (SGTCS)). Patients with remarkable hippocampal cell loss were termed as Wyler 2-4 (cell loss > 10% or a high SGTCS frequency (≥1/month)). An overview about an anamnesis of all screened patients and according Wyler classification is listed in (Eichler et al., 2008).

2.6.3 Evaluation of neuronal cell death

The fraction of degenerated neurons which expressed $\alpha3^{185P}$ or $\alpha3^{185L}$ was evaluated using morphological criteria according to the presence of dendritic swellings and varicosities (Salama-Cohen et al., 2005; Singh et al., 2006) as well as shrunken somata and/or an intense lightened soma compared to belonging dendrites.

All transfected neurons on each cover slip were counted and classified as degenerated or alive. Classification was done blinded, without knowledge of the present condition.

2.6.4 Detection of apoptosis (pCaspase3-sensor)

pCaspase3-sensor was kindly provided by Heiko Luhmann (University of Mainz).

Apoptosis is marked through activation of proteolytic caspases, i.a. caspase-3. Due to transfection of cells with pCaspase3-sensor chimeric EYFP proteins were expressed. This chimera is N-terminal tagged with a nuclear export sequence (NES sequence) as well as C-terminal with a nuclear localization sequence (NLS sequence). The NES sequence is linked to the EYFP through a recognition site for activated caspase-3 (DEVD). Note that NES is dominant over NLS.

Due to cleavage of NES the chimeric construct is imported into the nucleus, therefore apoptotic cells can be identified. The present NLS favors the import. If the cell is apoptotic, EYFP is detectable in the nucleus. To enhance the signal EYFP was stained with an antibody directed against GFP, also recognizing YFP (see table 3).

2.6.5 Evaluation of apoptosis in $\alpha3^{185P}$- or $\alpha3^{185L}$-expressing neurons with pCaspase3-sensor

Neurons were cotransfected with pCaspase3-sensor and with either non-edited ($\alpha3^{185P}$) or edited ($\alpha3^{185L}$) form of GlyRα3. Per transfection, 50 cotransfected neurons were analyzed randomly and fraction of cells with negative and positive detectable EYFP signal in the nucleus was determined. Evaluation was carried out one, two and three days after transfection.

2.6.6 Colocalization

For colocalization experiments a circular region of interest (r.o.i.) with a diameter of 100µm was adducted. The soma was located in the center of this r.o.i. Evaluation of pre- and postsynaptic terminals was carried out as previously described (Salama-Cohen et al., 2005;

Singh et al., 2006). If two different terminals were congruent or overlap, this was termed as colocalization.

Transfected neurons from triple or fourfold stained preparations were photographed and evaluation of colocalization was carried out inside the r.o.i.

The analysis of terminals of GlyRß positive clusters was carried out likewise, except that only those clusters were analyzed which showed a clear overlap to the according GlyRα3 signal, and determined by arithmetic image processing using ScionImage.

2.6.7 Cluster analysis (slope and size)

Cluster analysis was performed using the software ScionImage and Origin. Using line scans pixel gray levels were acquired along GlyRα3-positive dendrite segments; the resulting values were plotted against the measured distance. If pixel gray levels were at least three times below the standard deviation of the mean pixel gray level along the entire axis of measurement (3 σ-criterion, (Meier and Grantyn, 2004a)) these pixel were defined as part of a receptor cluster. Cluster diameter was calculated as the distance between threshold-determined cluster edges. For analysis of GlyRα3L cluster shape, the declining phase of fluorescence gray levels of a cluster was considered for calculation of the relative change of consecutive pixel gray levels (cluster slope). The cluster slope was calculated as a function of the actual pixel gray level divided by the gray level of the previous pixel (cluster slope). To rule out bias of quantitative morphometric analysis of heteromeric α3/β-GlyR by non-overlapping HA-α3 and myc-β GlyR signals, mathematical image processing was applied to the corresponding pairs of images. In the resulting images only congruent HA-α3 and myc-β GlyR signals were then used for cluster and colocalization analyses.

3.1 Publication 1

S. A. Eichler and J. C. Meier

E-I balance and human diseases - from molecules to networking

Front.Mol.Neurosci. **1:2, 2008**

Publication is available online:
http://www.frontiersin.org/molecularneuroscience/paper/10.3389/neuro.02/002.2008/
DOI: 10.3389/neuro.02.002.2008

3.2 Publication 2

S.A. Eichler, S. Kirischuk, R. Juttner, P. K. Schafermeier, P. Legendre, T. N. Lehmann, T. Gloveli, R. Grantyn and J. C. Meier

Glycinergic tonic inhibition of hippocampal neurons with depolarizing GABAergic transmission elicits histopathological signs of temporal lobe epilepsy

J.Cell.Mol.Med. **12 (6B):2848-2866, 2008**

Publication is available online:
http://www3.interscience.wiley.com/journal/120119390/abstract
DOI: 10.1111/j.1582-4934.2008.00357.x

3.3 Manuskript 3

Sabrina A. Eichler, Benjamin Förstera, Birthe Smolinsky, René Jüttner, Michael Tsokos, Thomas-Nicolas Lehmann, Pascal Legendre, Günter Schwarz and Jochen C. Meier

Splice-specific roles of glycine receptor α3 in the hippocampus

submitted

Publication is available online:
Manuscript will be published in the European Journal of Neuroscience in 2009
DOI: 10.1111/j.1460-9568.2009.06903.x

3.4 Publication 4

B. Smolinsky, S. A. Eichler, S. Buchmeier, J. C. Meier, and G. Schwarz

Splice-specific functions of gephyrin in molybdenum cofactor biosynthesis

***J.Biol.Chem.* 283 (25):17370-17379, 2008**

Publication is available online:
http://www.jbc.org/cgi/content/abstract/283/25/17370
DOI: 10.1074/jbc.M800985200

3.5 Publication 5

S. R. Chirasani, D. S. Markovic, M. Synowitz, S. A. Eichler, P. Wisniewski, B. Kaminska, A. Otto, E. Wanker, M. Schafer, P. Chiarugi, J. C. Meier, H. Kettenmann and R. Glass

Transferrin-receptor-mediated iron accumulation controls proliferation and glutamate release in glioma cells

J.Mol.Med. **87 (2):153-167, 2009**

Publication is available online:
http://www.springerlink.com/content/a52r207g31115175/
DOI: 10.1007/s00109-008-0414-3

4. Discussion

In this study the role of posttranscriptional processing of GlyR mRNA under physiological and pathophysiological conditions was investigated. The results obtained can be summarized as follows:

- Proline residue at position 192 (α2) and 185 (α3) is highly conserved and responsible for low affinity of GlyR to glycine and taurine
- C-to-U RNA editing occurs in a cross-α-subunit-dependent way and increases GlyR affinity by an order of magnitude, probably through structural rearrangement of the ligand binding domain
- Activation of RNA-edited high affinity GlyR by hippocampal ambient glycine silences neuronal output through shunt inhibition
- Silencing of neurons with reduced KCC2 expression and a high chloride equilibrium potential elicits excitotoxicity
- KCC2 expression decreases chloride equilibrium potential and rescues neurons from excitotoxicity
- In addition to excitotoxicity, abnormal dendrite morphology of, and increased Glu/GABA ratio of synaptic input to, silenced hippocampal neurons is reminiscent of TLE histopathology
- GlyR C-to-U RNA editing is not species-specific and occurs in humans, and both high affinity GlyRα2 and α3 is upregulated in the hippocampus of TLE patients with a severe course of disease
- Alternative splicing of both GlyRα2 and α3 is altered in severely damaged TLE hippocampi, but concerns a non-overlapping group of patients

Further results were obtained to define a role for GlyR in non-neuronal systems:

- Cortical glia cells and neurons express different sets of gephyrin splice variants, and glia cells, but not neurons, synthesize molybdenum cofactor
- Glioblastoma elicits excitotoxicity through glutamate release and neuronal NMDAR activation

4.1 Neuronal systems

4.1.1 Proline residue at positions 192 (α2) and 185 (α3) is highly conserved and responsible for low affinity of GlyR to glycine and taurine

C-to-U editing of *GLRA3* transcripts results in the amino acid substitution proline to leucine at position 185 (Meier et al., 2005). Proline 185 is highly conserved across all GlyR subunits, and it is also present in some $GABA_AR$ subunits (β1-3, figure 6). Aliphatic hydrophobic neutral amino acids, such as leucine, are present in $GABA_AR$ known to be involved in tonic inhibition. As a rule, these receptors require a high affinity to their agonists, and indeed $GABA_AR$ α4, α6 and δ subunits are well known to be implicated in tonic inhibition (Mody, 2001; Wisden et al., 2002; Mody and Pearce, 2004; Caraiscos et al., 2004).

	179						185					
r GlyR α1	A	D	G	L	T	L	**P**	Q	F	I	L	K
r GlyR α2	A	E	G	L	T	L	**P**	Q	F	I	L	K
r GlyR α3	A	E	G	L	T	L	**P**	Q	F	L	L	K
r GlyR α3 185L	A	E	G	L	T	L	**L**	Q	F	L	L	K
r GlyR α4	A	E	G	L	T	L	**P**	Q	F	I	L	R
r GlyR β	-	E	K	I	A	L	**P**	Q	F	D	I	K
h nAchR α1	S	N	F	M	E	S	G	E	W	V	I	K
h nAchR α4	L	D	F	W	E	S	G	E	W	V	I	V
h nAchR α7	S	G	Y	I	P	N	G	E	W	D	L	V
h 5HT α	S	V	F	M	N	Q	G	E	W	E	L	L
r $GABA_AR$ α1	E	D	G	S	R	L	N	Q	Y	D	L	L
r $GABA_AR$ α2	P	D	G	S	R	L	N	Q	Y	D	L	L
r $GABA_AR$ α3	Q	D	G	S	R	L	N	Q	Y	D	L	L
r $GABA_AR$ α4	K	E	S	S	S	L	***V***	Q	Y	D	L	I
r $GABA_AR$ α5	E	D	G	S	R	L	N	Q	Y	H	L	M
r $GABA_AR$ α6	E	E	S	S	S	L	**L**	Q	Y	D	L	I
r $GABA_AR$ β1	V	N	K	I	E	L	**P**	Q	F	S	I	V
r $GABA_AR$ β2	V	T	K	I	E	L	**P**	Q	F	S	I	V
r $GABA_AR$ β3	V	E	R	I	E	L	**P**	Q	F	S	I	V
r $GABA_AR$ γ1	P	K	Y	W	R	L	Y	Q	F	A	F	V
r $GABA_AR$ γ2	T	R	S	W	R	L	Y	Q	F	S	F	V
r $GABA_AR$ γ3	Q	K	S	W	R	L	Y	Q	F	D	F	M
m $GABA_AR$ δ	L	D	R	L	Q	L	**A**	Q	F	T	I	T

Figure 6: Protein sequence alignment of LGIC superfamily members around conserved proline residue 185 (α3). Note that leucine is present in high affinity GlyRα3 (185L). Further, $GABA_AR$ subunits known to be involved in tonic inhibition also have hydrophobic residues at this site.

As GlyRα2 also contains a proline at this site, the question arises whether RNA editing would produce receptors with high affinity. Indeed, editing of *GLRA2* transcripts was found, and edited receptors have a high affinity (Eichler et al., 2008).

4.1.2 C-to-U RNA editing occurs in a cross-α-subunit-dependent way and increases GlyR affinity by an order of magnitude, probably through structural rearrangement of the ligand binding domain

GlyR are members of the LGIC superfamily, which comprises among others $GABA_{A/C}R$ and nicotinic acetylcholine receptor. All members share some common features, particularly in their ligand binding domains (figure 7).

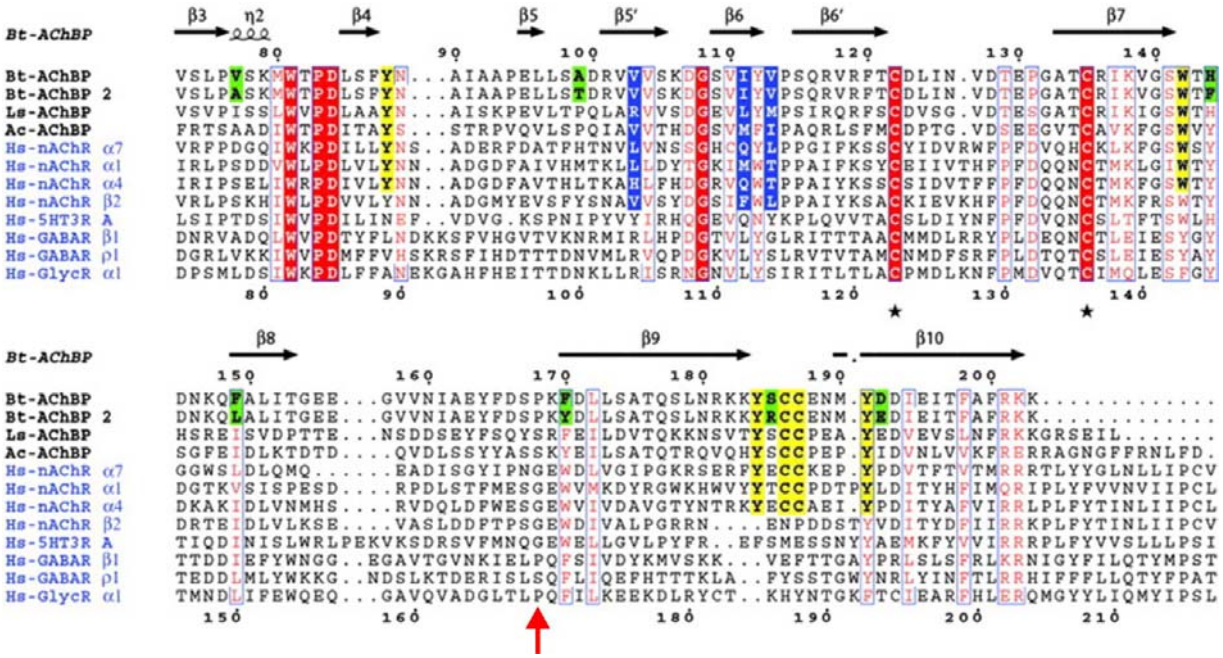

Figure 7: Protein sequence alignment of acetylcholine binding domain (AChBP) and some subunits of LGIC superfamily members, including nicotinic acetylcholine receptor (nAChR), serotonin receptor (5-HT_3R), $GABA_{A/C}$R and GlyR. BT = *Bulinus truncatus*, Ls= *Lymnaea stagnalis*, Ac= *Aplysia californica*, Hs= *Homo sapiens*. The red arrow indicates GlyR RNA editing site. Asterisks mark the cystidine residues of the disulfide bridge, which is characteristic for the LGIC superfamily. The figure is adapted from the original publication (Celie et al., 2005).

The crystal structure of the acetylcholine binding protein was established as a model system for ligand binding domains of the LGIC superfamily. The disulfide bridge between the end of β-sheet 6' and β-sheet 7 is highly conserved across all family members. Proline and glycine are unique amino acids in that they appear to influence the conformation of the polypeptide. Glycine essentially lacks a side chain and therefore can adopt conformations which are sterically forbidden for other amino acids. This confers a high degree of local flexibility on the polypeptide. Hence, proline and glycine residues are frequently found in turn regions of proteins where the backbone has to make a sharp turn. The conserved proline residue of $GABA_A$R and GlyR is located directly at the beginning of β-sheet 9, a region that should be highly variable. Accordingly, nAChR have a glycine residue at this position (figure 7). Furthermore, this position is close to the plasma membrane (figure 8) where these receptors enter the cytosol through transmembrane domain 1. In contrast, leucine contains bifurcated side chains that are non-polar. Leucine is thus usually located on the interior of the protein. The bifurcation is close to the main chain and can therefore restrict the conformation of the polypeptide by steric hindrance.

Due to the dichotomous nature of proline and leucine with respect to conformation, the amino acid substitution proline (hydrophobic neutral) to leucine (aliphatic hydrophobic neutral) is

expected to cause structural rearrangement of the ligand binding domain tertiary structure (figure 8).

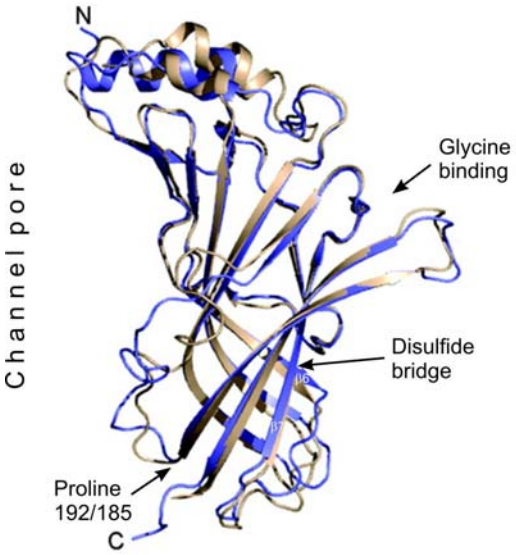

Figure 8: Tertiary structure of AChBP. According to this structure, the glycine binding site, conserved disulfide bridge as well as P192 (α2) and P185 (α3) are assumed to be located at the indicated positions.
Yellow: AChBP of *Bulinus truncatus*, containes proline at the position homologous to P185 and P192, respectively.
Blue: AChBP of *Lymnaea stagnalis*, containes serine at the position homologous to P185 and P192, respectively.
Picture was adapted to (Celie et al., 2005).

This may cause structural rearrangement of β-sheet 8, which is located between proline 185 and the glycine binding motif FGY (Legendre, 2001). The stabilizing disulfide bridge between β-sheets 6' and 7 could serve as a hinge. Further, a conformational rearrangement by proline to leucine substitution is supported by the observation that RNA-edited GlyR also bind GABA (Meier et al., 2005), which requires more space than glycine. Therefore, detailed structural analysis has to be performed in order to address this question directly.

4.1.3 Activation of RNA-edited high affinity GlyR by hippocampal ambient glycine silences neuronal output through shunt inhibition

Non-synaptic and synaptic receptor activations have different impact on the neuronal excitability because charge transfer by tonic inhibition can be more than three times larger than by phasic inhibition, even when the frequency of synaptic currents is large (Mody and Pearce, 2004). Recently, it was suggested that synaptic $GABA_AR$-mediated inhibition is not hyperpolarizing, but shunting in some types of neurons, including GABAergic interneurons (Bartos et al., 2007). This is supported by measurements of single evoked IPSCs in $GABA_AR$-containing basket cells of the hippocampus, where the reversal potential (-52mV) is between the resting membrane potential (-59 mV) and the threshold for AP generation (-40 mV) (Bartos et al., 2007). In general, shunt inhibition is likely to occur when the reversal potential is close to the resting membrane potential and lies between the latter and the threshold for AP generation.

In the present work it has been shown that GlyRα3^{185L}-expressing neurons depress $GABA_AR$-mediated spontaneous calcium transients as well as AP generation (Eichler et al., 2008). More detailed analysis of GlyRα3^{185L}-expressing neurons revealed that the chloride equilibrium potential in these neurons (values in (Eichler et al., 2008)) was between the resting membrane potential (figure 9, values in (Eichler et al., 2008)) and threshold for AP generation (values in (Eichler et al., 2008)). Therefore, a shunt mechanism of GlyRα3^{185L}-mediated inhibition is likely to account for suppression of $GABA_AR$-mediated spontaneous calcium transients and AP generation. In addition, the resting membrane potential and, most importantly, the membrane resistance were markedly reduced in GlyRα3^{185L}-expressing neurons recorded in the whole cell configuration (figure 9, values in (Eichler et al., 2008)).

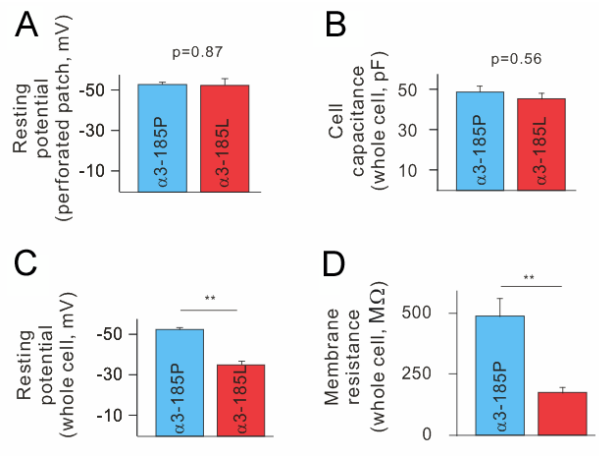

Figure 9: Patch clamp analysis of hippocampal neurons expressing either GlyRα3^{185P} or GlyRα3^{185L} *in vitro*. A: Analysis of the resting membrane potential recorded in the perforated patch configuration. Note that there is no difference between GlyRα3^{185P}- and GlyRα3^{185L}-expressing neurons. B – D: Cell capacitance, resting membrane potential and membrane resistance determined by the whole cell configuration. Note that the cell capacitance is not dependent on expression of GlyRα3^{185P} or GlyRα3^{185L}, but a decrease in the resting membrane potential and the membrane resistance in GlyRα3^{185L}-expressing neurons reveals a steady-state leak conductance and an increased permeability of the membrane to chloride ions, respectively.

Comparison of resting membrane potentials recorded in whole cell and perforated patch configurations (figure 9) reveals that neurons with an intact cytosol (perforated patch) are apparently capable of compensating a steady-state loss of chloride ions due to tonic GlyRα3^{185L} activation (also see 4.1.8). Accordingly, neurons with a fixed intracellular chloride concentration as imposed by the intracellular pipette solution (whole cell) cannot stabilize their resting membrane potential at the level of GlyRα3^{185P}-expressing ones, where these receptors are not activated due to their low affinity. That spontaneous calcium transients arise from synaptic $GABA_AR$ activation is unexpected at a first glance, but can be explained by the much stronger charge transfer within time due to pulsatory synaptic transmitter release, which might transiently surpass the steady-state capacity of chloride intrusion mechanisms, such as those mediated by NKCC1 chloride transport (*quot erat demonstradum*).

4.1.4 Silencing of neurons with reduced KCC2 expression and a high chloride equilibrium potential elicits excitotoxicity

Neuron silencing cannot only occur in response to activation of $GABA_AR$ and GlyR, but also in response to activation of potassium channels for example (Nadeau et al., 2000; Pal et al., 2003). A pathophysiological activation of potassium channels was reported e.g. in Parkinson's disease (Liss et al., 2005). Cell death of dopaminergic neurons was suggested to be induced by mitochondrial ROS, which trigger activation of hyperpolarizing ATP-sensitive potassium (K-ATP) channels in the degenerating neurons (Liss et al., 2005). However, in case of abnormal potassium channel activation neurodegeneration resulted from apoptosis, which could be prevented by raising extracellular potassium levels (Nadeau et al., 2000; Pal et al., 2003).

In this study I have used RNA-edited high affinity GlyR to investigate the consequences of neuron silencing against varying intracellular potassium and chloride levels. To this purpose, a cell culture system was established, in which the time window for analysis was chosen according to a specific chloride equilibrium potential: Hippocampal neurons with high chloride equilibrium potential were used (see 4.1.3) because KCC2 expression was an experimental option for modulation of intracellular potassium and chloride and because KCC2 expression is reduced in the hippocampus of TLE patients (Palma et al., 2006).

Under these experimental conditions neuron silencing also resulted in an increased fraction of degenerated neurons (Eichler et al., 2008). According to results of others (Nadeau et al., 2000; Pal et al., 2003; Liss et al., 2005) potential effects of altered potassium conductances were first investigated. On the one hand, tetraethylammonium (TEA) primarily acts by blocking calcium-activated and K-ATP channels (Fatherazi and Cook, 1991), which mediate intracellular potassium accumulation. On the other hand, KCl application mimics increased extracellular potassium levels. Unexpectedly, both KCl and TEA failed to rescue neurons (figure 10).

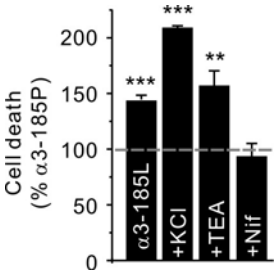

Figure 10: Effects of TEA, KCl and nifedipine (Nif) on neuron survival. Only nifedipine, but neither KCl nor TEA, were effective in preventing $GlyR\alpha3^{185L}$-mediated cell death.

However, block of voltage-sensitive calcium channels by nifedipine was effective in rescuing neurons. This gave me the first hint to excitotoxicity as an executive mechanism of neuron silencing. Further analysis then revealed blockade of both AMPAR and NMDAR was effective in preventing high affinity GlyR-mediated cell death (Eichler et al., 2008).

Although different pathways mediate apoptosis and necrosis, the same insult can induce both processes depending on its intensity; and *in vitro* excitotoxic cell death was shown to result in a mixture of apoptosis and necrosis, as it is the case *in vivo* due to ischemic brain injury (Yuan et al., 2003), which often occurs during a seizure in TLE patients. Also individual cells may differ in their determination to undergo apoptosis or necrosis (Yuan et al., 2003). However, analysis of apoptosis induction in silenced hippocampal neurons by measuring caspase-3 activation (pCaspase3-sensor) failed to reveal differences in the fractions of apoptotic GlyR$\alpha3^{185P}$- and GlyR$\alpha3^{185L}$-expressing neurons, respectively (Eichler et al., 2008). Presumably, at a later time point of investigation excitotoxicity may have ended in apoptosis or necrosis. Nonetheless, excitotoxicity is concluded to represent the executive mechanism of degeneration of GlyR$\alpha3^{185L}$-silenced neurons (see also 4.1.3).

4.1.5 KCC2 expression decreases chloride equilibrium potential and rescues neurons from excitotoxicity

In silenced neurons, loss of GABAergic synapses was suggested to reflect failure of synchronization of glutamatergic and GABAergic postsynaptic currents in the developing brain (Tao and Poo, 2005). In my experimental system silenced hippocampal neurons (GlyR$\alpha3^{185L}$) were found to lose GABAergic synapses (Eichler et al., 2008), and this is in agreement with the proposal that appropriate GABAergic activity is essential for the coordinated refinement of excitatory and inhibitory connections (Tao and Poo, 2005). BDNF was recently involved in control of GABAergic synapse development (Singh et al., 2006), and in this study block of TrkB receptors with K252a was shown to override BDNF-mediated loss of GABAergic synapses. Therefore, the capacity of K252a to prevent neuronal cell death was investigated here. K252a did not save silenced hippocampal neurons, but rescued or even increased GABAergic synapses, ruling out the possibility that loss of GABAergic synapses *per se* was responsible for neurodegeneration.

In the mature nervous system, upregulation of GABAergic synapses occurs in response to neuron silencing (Burrone et al., 2002). As the chloride equilibrium potential makes the only

apparent difference between my experimental system and the situation in the adult the potential of KCC2 expression to rescue silenced neurons was investigated. In fact, KCC2 expression restored silenced hippocampal neurons in all concerns, except altered dendrite morphology (see below, 4.1.6) (Eichler et al., 2008), but the exact mechanism by which decreased chloride equilibrium potential rescues hippocampal neurons is still elusive. As discussed in the introduction (see 1.1.3.2) two possibilities are available to explain KCC2-mediated neuroprotective effects. Briefly, either non-synaptic NMDAR activation or coincident synaptic NMDAR and nNOS activation constitute potential degenerative mechanisms. Non-synaptic NMDAR are frequently associated with immature neuronal systems and degenerative mechanism in TLE patients (Sierra-Paredes and Sierra-Marcuno, 2007) and are therefore, at a first glance, prime candidates for neurodegeneration. Also the participation of the NR2B subunit was assumed (King et al., 2006). At the moment, it is not clear which of the both excitotoxicity models would fit my results best. To investigate this further it is planned to treat silenced KCC2-expressing neurons with Tat-NR2B9c-peptide (Aarts et al., 2002), nNOS inhibitors and to evaluate the fractions of synaptic and/or non-synaptic NR2B-containing receptors.

4.1.6 In addition to excitotoxicity, abnormal dendrite morphology of, and increased Glu/GABA ratio of synaptic input to, silenced hippocampal neurons is reminiscent of TLE histopathology

An adequate balance between inhibition and excitation is necessary for information transfer in the brain. Deregulated inhibition or excitation is frequently associated with neurological diseases (Eichler and Meier, 2008).

Increased Glu/GABA ratios of synaptic input were associated with the pathophysiology of TLE, as for example feed-forward inhibiting interneurons were shown to receive more glutamatergic and less GABAergic synapses (Stief et al., 2007). However, although the experiments by Gloveli and colleagues (Dugladze et al., 2007) have demonstrated a net increase in glutamatergic transmission (due to increased sEPSC frequency) to another type of hippocampal GABAergic interneuron (OL-M cells, see 1.1.5.4) these neurons were shown to receive less glutamatergic terminals (according to reduced mEPSC frequency). It is conceivable that such a net increase in glutamatergic drive results from increased firing frequencies of presynaptically connected neurons, which could be disinhibited due to silencing of inhibitory interneurons that innervate them. A permanent trigger of

pathophysiological network activity could then emerge if these interneurons degenerate due to activity deprivation (see 4.1.4 and 4.1.5, also see below, 4.1.7). Today, it is known that pyramidal as well as granule cells express functional GlyR (Chattipakorn and McMahon, 2003). In addition, hippocampal interneurons express functional GlyR (Song et al., 2006), but it is not clear which type of interneuron is concerned. Therefore, prior to establishment of a direct link between high affinity GlyR and the TLE pathophysiology, it is necessary to determine a cell type-specific expression pattern of these particular receptors.

Although GlyR were reported to have anticonvulsant effects (Kirchner et al., 2003; Song et al., 2006) the results obtained in this work point to a pathophysiological role of enhanced GlyR-mediated signaling, particularly in the context of a high chloride equilibrium potential (see 4.1.4 and 4.1.5), as is the case in the hippocampus of temporal lobe epilepsy patients (Palma et al., 2006). In this study, neuron silencing was revealed as a trigger for upregulation of the Glu/GABA ratio of synaptic input, which in agreement with others is responsible for reduced dendrite length (Lohmann et al., 2005; Eichler et al., 2008), both of which phenomena are highly reminiscent of the TLE histopathology (Loup et al., 2000; Stief et al., 2007). My results thus have identified a cellular mechanism governing increases in Glu/GABA ratios of synaptic input. As large scale rhythmic burst activity frequently showed up in silenced neurons after glycine withdrawal (Igor Melnick, unpublished data) it is also conceivable that neuron silencing directly contributes to increased excitatory drive by burst activity and thus to epileptogenesis. For example, alterations in the phosphorylation state (see also 4.1.5) of GlyR, particularly the α3 subunit (Harvey et al., 2004) by BDNF signaling through PKA (see also 1.1.4.4, (Gallo et al., 2002), could indeed render this scenario possible.

4.1.7 GlyR C-to-U RNA editing is not species-specific and occurs in humans, and both high affinity GlyRα2 and α3 is upregulated in the hippocampus of TLE patients with a severe course of disease

The hippocampus, particularly the dentate gyrus, is highly susceptible to seizures (Sloviter et al., 2003; Frotscher et al., 2006), and granule cells for example shape activity patterns of CA3 pyramidal cells and can repress seizure activity as they also innervate at least ten inhibitory neurons per CA3 pyramidal neuron (Gibb and McNamara, 2006). With regard to the relevance of individual interneuron types in epileptogenesis (see 4.1.6, 1.1.5.4 and 1.1.5.5) it is noteworthy that novel concepts of TLE mechanisms discuss decrease in granule cell excitability, rather than hyperexcitability, as a source of spontaneous seizures (Harvey and

Sloviter, 2005). In fact, it was recently demonstrated that activity deprivation leads to seizures in hippocampal slice cultures (Trasande and Ramirez, 2007). The results in the present work support this novel concept as they revealed homeostatic, but pathophysiological, involvement of neuron silencing in structural reorganization reminiscent of TLE histopathology (Eichler et al., 2008), provided that high affinity GlyR expression occurs in TLE patients. Therefore expression of high affinity GlyRα2 and α3 was analyzed in hippocampectomies of TLE patients.

There is indeed evidence for upregulation of high affinity GlyR expression in TLE patients, especially in patients with a strong hippocampal damage or a severe course of disease (Eichler et al., 2008). High affinity GlyR of both α2 and α3 were found, and the majority of W2-4 patients expressed both high affinity GlyR variants. Considering decreased KCC2 expression in TLE (Rivera et al., 2002; Rivera et al., 2004; Rivera et al., 2005; Palma et al., 2006) downregulation of KCC2 expression also occurred in patients analyzed here and, most importantly, inversely correlated with high affinity GlyR expression (Eichler et al., 2008). This *in vivo* data fits in with the cellular *in vitro* study (see 4.1.4 and 4.1.5) where excitotoxic cell death was conditional upon low KCC2 expression and high intracellular chloride equilibrium potential. Again, it is necessary to determine a cell type specific expression pattern of hippocampal high affinity GlyR in order to strengthen this conclusion.

4.1.8 Alternative splicing of both GlyRα2 and α3 is altered in severely damaged TLE hippocampi, but concerns a non-overlapping group of patients

Besides C-to-U RNA editing of *GLRA* transcripts alternative splicing of *GLRA2* and *GLRA3* messengers are well known posttranscriptional processes (Legendre, 2001), and synthesis of GlyR with increased affinity can also result from alternative splicing (see 1.1.4.1), whereby α2B is about twice as sensitive to glycine as α2A (Miller et al., 2004). Analysis of W0-1 classified TLE patients mostly expressed the α2A splice variant (Eichler et al., 2008). In contrast, W2-4 patients showed a reduced fraction of α2A expression, and in 40% of these patients prevailing α2B expression was found. Therefore epileptic seizure activity may involve expression of more sensitive GlyR, either produced by editing or splicing.

Alternative splicing of *GLRA2* and *GLRA3* transcripts concerns non-overlapping groups of TLE patients, but in this study only the role of α3 splicing was investigated in more detail. In TLE patients with strong hippocampal damage (W2-4) GlyRα3L expression was

downregulated. Most importantly, 57% of severely damaged hippocampi (Wyler 3-4 classification of hippocampal sclerosis) were affected while post-mortem and Wyler 0-1 TLE hippocampi showed homogenous predominance of GlyRα3L (Eichler et al., submitted). Furthermore, these patients were afflicted with reduced GlyRβ expression, which is known to occur in hippocampal principal cell layers (Malosio et al., 1991b). As *GLRA3L* transcripts outnumber *GLRA3K* messengers five times, my data again suggests a relationship between GlyRα3 expression and neurodegeneration (see 4.1.4 and 4.1.5).

To carry out a more detailed analysis of potential splice specific roles of GlyRα3, mouse hippocampus cryosections and primary hippocampal neurons were used as experimental system (Eichler et al., submitted). Irrespective of the splice variant analyzed most of GlyRα3 immunoreactivity was non-synaptic, with α3K displaying a diffuse distribution at the neuronal surface and α3L appearing as clusters. However, when localized at hippocampal synapses GlyRα3L is associated preferentially with glutamatergic nerve endings and NMDAR. As GlyR activation requires glycine and because GlyT1 was shown to be present at hippocampal glutamatergic presynaptic terminals (Cubelos et al., 2005), a modulatory pathway of glutamatergic transmission by GlyR is conceivable, particularly in case of high affinity GlyRα3L expression (Eichler et al., submitted, supplementary figure 2). On the other hand, GlyRβ promoted association of both GlyRα3 splice variants with hippocampal GABAergic terminals in a gephyrin dependent way, while maintaining the preference of GlyRα3L for glutamatergic synapses. Again, these results support a role of GlyRα3 in tonic hippocampal inhibition, particularly in W2-4 TLE patients with reduced α3L and β expression.

4.2 Starter to other projects (non-neuronal systems)

4.2.1 Cortical glia cells and neurons express different sets of gephyrin splice variants, and glia cells, but not neurons, synthesize molybdenum cofactor

Human Moco deficiency is a hereditary metabolic disorder characterized by severe neurodegeneration resulting in early childhood death (Schwarz, 2005). So far, disease-causing mutations have been identified in three of the four known Moco-synthetic human genes: *MOCS1*, *MOCS2* and *GPHN* (Reiss and Johnson, 2003). The severe phenotype of Moco-deficient patients is characterized by progressive neurological damage due to deficiency of sulfite oxidase that is normally expressed in liver tissue and protects the organism against toxic sulfite (Schwarz, 2005).

Several studies have unequivocally demonstrated that gephyrin is a multifunctional protein catalyzing Moco biosynthesis, in addition to its brain-specific function (Schwarz, 2005). As 50% of Moco-deficient patients have severe epilepsy the question whether Moco synthesis occurs in the brain and whether it is regulated by alternative splicing was addressed in this study (Smolinsky et al., 2008). Analysis of cultured cortical neurons and glia cells revealed that Moco synthesis is carried out by cortical glial cells, but not by cortical neurons. Furthermore, *GPHN* exon 6 (Paarmann et al., 2006) was identified as a negative regulator of Moco synthesis. However, as both cortical glia cells and neurons express similar amounts of gephyrins with exon 6 alternative splicing as a cause for selective glial Moco synthesis can be ruled out. The study by Smolinsky et al. (2008) also identified glia- and neuron-specific gephyrin splice variants. Glia cells alternatively splice exon 10 (Paarmann et al., 2006), whereas neurons alternatively splice exons 12-15 (Paarmann et al., 2006). Again, as all of these gephyrin splice variants *in vitro* are capable of Moco synthesis the glial cell-specific brain Moco synthesis is not regulated by alternative splicing. Nonetheless, these experiments (Smolinsky et al., 2008) provided the first experimental demonstration of brain-specific Moco synthesis, beyond the well known roles of Moco at a systemic level of investigation.

4.2.2 Glioblastoma elicits excitotoxicity through glutamate release and neuronal NMDAR activation

Glioblastoma can trigger epilepsy (Senner et al., 2004; Kohling et al., 2006), and these tumors differ from other solid tumors because growth is limited by the skull and therefore the tumor cells must have means to reduce brain mass. Glioblastoma cells are known to release high

amounts of glutamate (Takano et al., 2001; Sontheimer, 2003), which *per se* could trigger epileptiform activity. Otherwise, glutamate release by tumor cells could elicit excitotoxicity, and cell death could contribute to epileptiform activity. Briefly, cocultivation of tumor cells and hippocampal neurons was used here as an experimental model system. Conditional upon NMDAR block by MK801, the majority (> 80%) of hippocampal neurons were lost within 24h, and the remaining neurons displayed drastically shortened or even fragmented dendrites, morphologically reminiscent of neurons expressing GlyR$\alpha 3^{185L}$ (Eichler et al., 2008). As the remaining neurons displayed a faint unusually homogenous DAPI signal as if the nuclear compartment was dissolving it was concluded that karyolysis occurred which is typical for necrosis (Segerer et al., 2002). Although the experiments in this work do not allow to draw conclusions on the mechanism of epileptogenesis by glioma expansion they identify excitotoxicity as an executive mechanism for tumor space demands (Chirasani et al., 2009). As concomitant up- and downregulation of neuronal NKCC1 and KCC2, respectively, were shown to accompany glioma growth (Aronica et al., 2007) the results of the present work predicts a neuroprotective effect of KCC2 in the tumor surrounding tissue (see 4.1.5).

4.3 General conclusions

The main outcome of this work can be summarized as follows. Activity deprivation of neurons with a high chloride equilibrium potential, as is the case with epilepsy patients or in the immature brain, represents a pathophysiological mechanism. Most importantly, KCC2 expression was identified as therapeutic option against excitotoxic neurodegeneration. Altogether, GlyR posttranscriptional mechanisms were identified as homeostatic, but pathophysiological, executive cellular processes within a deregulated neuronal network.

5. Appendix

5.1 Abbreviations

AChBP	acetylcholine binding domain
AMCA	7-amino-4-methylcumarin-3-acetic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid
AMPAR	AMPA receptor
ANOVA	analysis of variance
AOTF	acousto-optical beam-splitter
AP	action potential
AraC	cytosine β-D-arabinofuranoside hydrochloride
BDNF	brain-derived neurotrophic factor
CA	*Cornu Ammonis*
CaMKII	calcium/calmodulin-dependent protein kinase
CCK	cholecystokinin
cDNA	complementary DNA
cfu	colony forming unit
CNS	central nervous system
Cy	carboxymethyl-indocyanine
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethyl pyrocarbonate
div	days in vitro
DMEM	Dulbecco's modified eagle medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNQX	6,7-Dinitroquinoxaline-2,3-dione
DTT	dithiothreitol

EAAT	excitatory amino acid transporter
EDTA	ethylenediamine tetraaceticacid
EAAT	excitatory amino acid transporters
EEG	electroencephalography
EGFP	enhanced green fluorescent protein
EPSC	excitatory postsynaptic current
EYFP	enhanced yellow fluorescent protein
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GAT	GABA transporter
GDP	giant depolarizing potential
Glu	glutamate
GlyR	glycine receptor
GlyT	glycine transporter
HA	hemagglutinin
HBS	HEPES buffered saline
HEK	human embryonic kidney
HEPES	*N*-2-Hydroxyethylpiperazin-*N'*2-ethansulphonic acid
hGlyR	human glycine receptor
HoS	horse serum
5-HT_3R	serotonin receptor
i.a.	inter alia
i.e.	id est
IPSC	inhibitory postsynaptic current
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KCC2	potassium/chloride-cotransporter 2

LGIC	ligand-gated ion channel
LTD	long term depression
LTP	long term potentiation
MAPK	mitogen-activated protein kinase
MEM	minimum essential medium
mIPSC	miniature IPSC
mEPSC	miniature EPSC
MK801	dizocilpine
M-MLV	Moloney Murine Leukemia Virus
Moco	molybdenum cofactor
nAChR	nicotinic acetylcholine receptor
NB	Neurobasal medium
NB-B27	Neurobasal medium supplemented with B27-Supplement
NES	nuclear export sequence
Nif	nifedipine
NKCC1	sodium/potassium/chloride-cotransporter 1
NLS	nuclear localization sequence
NMDA	*N*-methyl-*D*-aspartic acid
NMDAR	NMDA receptor
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
O-LM	oriens-lacunosum moleculare
ORF	open reading frame
PBS	phosphate buffered saline
PBS-CMF	phosphate buffered saline, calcium- and magnesium-free
pBSK	pBluescript vector
PFA	paraformaldehyde
PKA	protein kinase A
PO	polyornithine

PSD-95	postsynaptic density protein
r.o.i.	region of interest
ROS	reactive oxygen species
s.e.m.	standard error of the mean
SGTCS	secondarily generalized bilateral tonic-clonic seizures
SHMT	serine hydroxymethyltransferase
SSS	standard salt solution
TEA	tetraethylammonium
TLE	temporal lobe epilepsy
TRIS	tris(hydroxymethyl)aminomethane
TRITC	tetramethyl rhodamine isothiocyanate
TrkB	tyrosine kinase B
U	Unit, enzyme
VGluT	vesicular glutamate transporter
VIAAT	vesicular inhibitory amino acid transporter
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

5.2 Declaration to the manuscripts

Publication 1:

Literature screen and selection of cited publications, first and final drafts of manuscript, figure design.

Publication 2:

Conception: RNA editing is a neuronal mechanism for modulation of GlyR agonist affinity. RNA-edited GlyRs strongly inhibit neuronal AP firing. Therefore, it was meaningful to screen patients with hyperexcitability disorders (TLE) for the expression of these receptor variants. As RNA-edited GlyR were more abundant in TLE patients with a severe course of disease it was necessary to characterize a potential pathophysiological role of these receptors at a cellular level, i.e. in primary hippocampal neurons. Furthermore, as it was known already that KCC2 expression is decreased in TLE hippocampus the culture system was adapted accordingly in terms of chloride equilibrium potential. This furthermore provided me with the possibility to characterize the role of KCC2 expression in neurons with increased expression of RNA-edited GlyR.

Execution: Experiments and data analysis was carried out according to following figures. Figure 1: All, except panel B; Figure 2: all, except panels D-F; Figure 3: none; Figure 4: all, except panels G-H; Figure 5: all; Figure 6: all; Figure 7: all; Figure 8: all; Figure 9: all. Suppl. Figure 1: all, except panels B, D.

Manuscript: Literature screen and selection of cited publications, first and final drafts of manuscript, figure design.

Manuscript 3:

Conception: As a second step towards the characterization of the role of RNA-edited GlyR in the hippocampus it was necessary to determine their (sub-)cellular distribution. However, because antibodies specific to RNA-edited GlyR were not available the hippocampal expression of GlyR was analyzed irrespectively of proline-to-leucine substitution.

Execution: Experiments and data analysis was carried out according to following figures. Figure 1: All, except panel A; Figures 2-7: all; Figure 8: all, except panel A; Suppl. Figure 1: all; Suppl. Figure 2: none.

Manuscript: Literature screen and selection of cited publications, first and final drafts of manuscript, figure design.

Publication 4:

Conception: It was known that Molybdenum cofactor (Moco) deficiency results in severe epilepsy and early childhood death. However, the role of posttranscriptional processing of messengers coding for the key enzyme in Moco synthesis, namely gephyrin, was not known. Therefore, a fruitful collaboration with the key personality in Moco research, namely Günter Schwarz, was established.

Execution: Experiments and data analysis was carried out according to following figures. Figures 1-2: none; Figure 3: panel A; Figures 4-6: none; Figure 7: panel B; Figure 8: none.

Manuscript: Participation in literature screen and selection of cited publications (~25%), participation in first and final drafts of manuscript (~35%), design of above mentioned figures.

Publication 5:

Conception: Glioblastoma is known to release glutamate, and because the lab has expertise in excitotoxicity mechanisms a fruitful collaboration with the group of Helmut Kettenmann was established.

Execution: Experiments and data analysis was carried out according to following figures. Figures 1-5: none; Figure 6: all, except panel D, E; Suppl. Figures 1-2: none.

Manuscript: Participation in literature screen and selection of cited publications to be included in revision (~5%), participation in draft of revision (~5-10%), design of above mentioned figures.

5.2 Summary

Inhibition is crucial for control of hippocampal network activity, and deregulated inhibition is associated with TLE. GlyR were suggested to contribute to hippocampal tonic inhibition, but the molecular and cellular requirements for such a role are controversial or unknown. On the one hand, the affinity of GlyR is too low for a response to hippocampal ambient glycine; on the other hand, their (sub)-cellular localization in the hippocampus is totally unknown. The distribution of hippocampal GlyR was therefore investigated first, and it was found that the long splice variant α3L is the predominant one in mouse hippocampus and the majority of TLE patients. In a subset of TLE patients with a high degree of hippocampal cell loss GlyRα3L and β expression was decreased. This supports preferential GlyRα3L immunoreactivity observed in mouse dentate gyrus and *Cornu Ammonis* regions because TLE-associated hippocampal cell loss mostly concerns principal cell layers. In addition, GlyRα3L immunoreactive signals were clustered (independently of GlyRβ and gephyrin), and GlyRα3L clusters were present mostly at non-synaptic sites; thus fulfilling the first requirement for their involvement in tonic inhibition.

The second requirement for tonic inhibition is the presence of GlyR with a high affinity to glycine. In fact, the recently discovered RNA-edited GlyR could fulfill this task as their affinity approximates hippocampal ambient glycine, but their expression in humans was not investigated. The second major finding of this study therefore is the detection of edited GlyRα2 and α3 mRNA in the hippocampus of TLE patients. Thus, GlyR RNA editing is both a cross-subunit and a cross-species posttranscriptional mechanism for modulation of receptor agonist affinity.

That expression of high affinity GlyR (either produced by RNA editing or by alternative splicing, i.e. GlyRα2B) was found to be increased in TLE patients with a high degree of hippocampal damage suggested a pathophysiological role of GlyR posttranscriptional processing. Analysis of RNA-edited GlyRα3 at a cellular level in primary hippocampal neurons indeed corroborated this theory because neurons expressing the high affinity receptor variant resembled TLE histopathology. In detail, these neurons exhibited abnormal dendrite morphology and upregulated Glu/GABA ratios of synaptic input; the latter resulting from loss

of GABAergic synaptic contacts and increased glutamatergic innervation. Consequently, they were more susceptible to excitotoxicity.

The last major result of this study is the finding that restoration of a "physiological" chloride equilibrium potential by KCC2 expression is neuroprotective. Prevention of KCC2 downregulation in TLE hippocampus could therefore open avenues for new therapeutic strategies. Altogether, GlyR posttranscriptional mechanisms were identified as homeostatic, but pathophysiological, executive cellular processes within a deregulated neuronal network.

5.4 Zusammenfassung

Neuronale Hemmung trägt wesentlich zur Kontrolle der Netzwerkaktivität im Hippocampus bei. Eine deregulierte Hemmung wird oft als ursächlich für TLE angesehen. GlyR wurden bereits hinsichtlich ihrer Beteilgung an hippocampaler tonischer Hemmung diskutiert; allerdings sind die zellulären und molekularen Voraussetzungen für eine solche Aufgabe unbekannt, denn die Affinität der Rezeptoren reicht für eine Antwort auf hippocampales Umgebungsglycin nicht aus. Zudem ist die (sub)zelluläre Verteilung der Rezeptoren im Hippocampus gänzlich unbekannt. Aus diesem Grund wurde zuerst die GlyR-Verteilung mittels Immunhistochemie im Hippocampus der Maus ermittelt. Sowohl im Maus-Hippocampus als auch bei der Mehrheit der TLE Patienten wurde die lange Spleißvariante α3L als die majoritäre Rezeptorform identifiziert. Bei Patienten mit ausgeprägten Gewebeschädigungen war die α3L-Expression (zusammen mit GlyRβ) verringert. Da Prinzipalzellschichten hauptsächlich von Gewebeschädigungen betroffen sind, bekräftigt dieser Befund die immunhistochemische Lokalisierung von α3L in Prinzipalzellschichten (*Gyrus dentatus* und *Cornu Ammonis*) des Maus-Hippocampus. Zudem wurde die α3L-Immunreaktivität in Aggregatform festgestellt, die ohne GlyRβ und Gephyrin zustande kommt. GlyRα3L-Aggregate waren größtenteils an nicht-synaptischen Stellen der Plasmamembran lokalisiert, wodurch die erste Voraussetzung zur Vermittlung von tonischer Hemmung erfüllt ist.

Die zweite Voraussetzung für eine Beteiligung von GlyR an tonischer hippocampaler Hemmung betrifft die Affinität der Rezeptoren. Die neuerdings entdeckten RNA-editierten Rezeptoren wären dafür sehr gut geeignet, denn ihre Affinität entspricht annähernd dem hippocampalen Umgebungsglycin. Das zweite hervorzuhebende Ergebnis dieser Arbeit ist demnach der Nachweis von editierten *GLRA2*- und *GLRA3*-Transkripten im Hippocampus von TLE-Patienten. RNA-Editierung findet also Spezies- und Untereinheiten-übergreifend statt.

Interessanterweise war die Expression von hochaffinen Rezeptorvarianten (entweder durch RNA-Editierung oder aber alternatives Spleißen, i.e. GlyRα2B) im Hippocampus derjenigen TLE-Patienten erhöht, bei denen der Schweregrad der Erkrankung sehr hoch war, wodurch diesen posttranskrpionalen Mechanismen eine pathophysiologische Rolle zugesprochen werden kann. Die anschließende Charakterisierung der hochaffinen Rezeptoren in neuronalen Primärkulturen aus Rattenhippocampus bekräftigte diese Annahme insofern als die Neurone verkürzte Dendriten und ein erhöhtes Glu/GABA-Verhältnis der synaptischen Eingänge aufwiesen. Letzteres erklärt sich aus dem Verlust GABAerger Synapsen begleitet von einer Hochregulierung glutamaterger synaptischer Eingänge, wodurch diese Neurone besonders empfindlich für Glutamat-Excitotoxizität wurden. Alles in allem erinnern diese Beobachtungen stark an die TLE-Histopathologie.

Schließlich ist hervorzuheben, dass das Wiederherstellen eines „physiologischen“ Chloridequilibriumpotentials durch KCC2-Expression neuroprotektiv wirkt. Somit eröffnet die Verhinderung der KCC2-Abregulierung in TLE-Hippocampi neue therapeutische Möglichkeiten. Zusammenfassend wurden posttranskriptionale Mechanismen an GlyR-Transkripten als homeostatische ausführende Mechanismen eines deregulierten neuronalen Netzwerks mit pathophysiologischen Konsequenzen identifiziert.

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5.6 Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel „ Disease-related posttranscriptional modifications of glycine receptor mRNA under physiological and pathophysiological conditions“ selbstständig und nur mit den erlaubten Hilfsmitteln angefertigt habe. Alle verwendeten Hilfsmittel und Quellen wurden angegeben. Abbildungen, die aus anderen Arbeiten entnommen wurden, sind als solche gekennzeichnet.

Des Weiteren erkläre ich, dass die vorliegende Dissertation in keiner anderen Fakultät oder Universität vorgelegt oder veröffentlicht wurde, mit Ausnahme der in PubMed indizierten Publikationen..

Die Promotionsordnung des Fachbereichs Biologie, Chemie und Pharmazie der Freien Universität Berlin ist mir bekannt.

Sabrina Eichler

Berlin, den 6.04.2009

6. Reference List

1. Aarts M, Liu Y, Liu L, Besshoh S, Arundine M, Gurd JW, Wang YT, Salter MW, Tymianski M (2002) Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions. Science 298: 846-850.

2. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2008) Cancer. In: Molecular Biology of the Cell (Anderson M, Granum S, eds), pp 1205-1268. New York: Garland Science.

3. Aronica E, Boer K, Redeker S, Spliet WG, van Rijen PC, Troost D, Gorter JA (2007) Differential expression patterns of chloride transporters, Na+-K+-2Cl--cotransporter and K+-Cl--cotransporter, in epilepsy-associated malformations of cortical development. Neuroscience 145: 185-196.

4. Bartos M, Vida I, Jonas P (2007) Synaptic mechanisms of synchronized gamma oscillations in inhibitory interneuron networks. Nat Rev Neurosci 8: 45-56.

5. Baulac S, Huberfeld G, Gourfinkel-An I, Mitropoulou G, Beranger A, Prud'homme JF, Baulac M, Brice A, Bruzzone R, LeGuern E (2001) First genetic evidence of GABA(A) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene. Nat Genet 28: 46-48.

6. Ben Ari Y (2001) Developing networks play a similar melody. Trends Neurosci 24: 353-360.

7. Blum BP, Mann JJ (2002) The GABAergic system in schizophrenia. Int J Neuropsychopharmacol 5: 159-179.

8. Blumcke I, Pauli E, Clusmann H, Schramm J, Becker A, Elger C, Merschhemke M, Meencke HJ, Lehmann T, von Deimling A, Scheiwe C, Zentner J, Volk B, Romstock J, Stefan H, Hildebrandt M (2007) A new clinico-pathological classification system for mesial temporal sclerosis. Acta Neuropathol 113: 235-244.

9. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A 92: 7162-7166.

10. Bouilleret V, Loup F, Kiener T, Marescaux C, Fritschy JM (2000) Early loss of interneurons and delayed subunit-specific changes in GABA(A)-receptor expression in a mouse model of mesial temporal lobe epilepsy. Hippocampus 10: 305-324.

11. Bragin A, Claeys P, Vonck K, Van Roost D, Wilson C, Boon P, Engel J, Jr. (2007) Analysis of initial slow waves (ISWs) at the seizure onset in patients with drug resistant temporal lobe epilepsy. Epilepsia 48: 1883-1894.

12. Breitinger HG, Becker CM (2002) The inhibitory glycine receptor-simple views of a complicated channel. Chembiochem 3: 1042-1052.

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

14. Brown N, Kerby J, Bonnert TP, Whiting PJ, Wafford KA (2002) Pharmacological characterization of a novel cell line expressing human alpha(4)beta(3)delta GABA(A) receptors. Br J Pharmacol 136: 965-974.

15. Burrone J, O'Byrne M, Murthy VN (2002) Multiple forms of synaptic plasticity triggered by selective suppression of activity in individual neurons. Nature 420: 414-418.

16. Cao J, Viholainen JI, Dart C, Warwick HK, Leyland ML, Courtney MJ (2005) The PSD95-nNOS interface: a target for inhibition of excitotoxic p38 stress-activated protein kinase activation and cell death. J Cell Biol 168: 117-126.

17. Caraiscos VB, Elliott EM, You T, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 101: 3662-3667.

18. Celie PH, Klaassen RV, Rossum-Fikkert SE, van Elk R, van Nierop P, Smit AB, Sixma TK (2005) Crystal structure of acetylcholine-binding protein from Bulinus truncatus reveals the conserved structural scaffold and sites of variation in nicotinic acetylcholine receptors. J Biol Chem 280: 26457-26466.

19. Chattipakorn SC, McMahon LL (2002) Pharmacological characterization of glycine-gated chloride currents recorded in rat hippocampal slices. J Neurophysiol 87: 1515-1525.

20. Chattipakorn SC, McMahon LL (2003) Strychnine-sensitive glycine receptors depress hyperexcitability in rat dentate gyrus. J Neurophysiol 89: 1339-1342.

21. Chen HS, Lipton SA (2006) The chemical biology of clinically tolerated NMDA receptor antagonists. J Neurochem 97: 1611-1626.

22. Chirasani SR, Markovic DS, Synowitz M, Eichler SA, Wisniewski P, Kaminska B, Otto A, Wanker E, Schafer M, Chiarugi P, Meier JC, Kettenmann H, Glass R (2009) Transferrin-receptor-mediated iron accumulation controls proliferation and glutamate release in glioma cells. J Mol Med 87: 153-167.

23. Cossart R, Bernard C, Ben Ari Y (2005) Multiple facets of GABAergic neurons and synapses: multiple fates of GABA signalling in epilepsies. Trends Neurosci 28: 108-115.

24. Cubelos B, Gimenez C, Zafra F (2005) Localization of the GLYT1 glycine transporter at glutamatergic synapses in the rat brain. Cereb Cortex 15: 448-459.

25. Dlugaiczyk J, Singer W, Schick B, Iro H, Becker K, Becker CM, Zimmermann U, Rohbock K, Knipper M (2008) Expression of glycine receptors and gephyrin in the rat cochlea. Histochem Cell Biol.

26. Dugladze T, Vida I, Tort AB, Gross A, Otahal J, Heinemann U, Kopell NJ, Gloveli T (2007) Impaired hippocampal rhythmogenesis in a mouse model of mesial temporal lobe epilepsy. Proc Natl Acad Sci U S A 104: 17530-17535.

27. Eichler SA, Kirischuk S, Juttner R, Schafermeier PK, Legendre P, Lehmann TN, Gloveli T, Grantyn R, Meier JC (2008) Glycinergic tonic inhibition of hippocampal neurons with depolarizing GABAergic transmission elicits histopathological signs of temporal lobe epilepsy. J Cell Mol Med 12: 2848-2866.

28. Eichler SA, Meier JC (2008) E-I balance and human diseases - from molecules to networking. Front Mol Neurosci 1: 2.

29. El Hassar L, Milh M, Wendling F, Ferrand N, Esclapez M, Bernard C (2007) Cell domain-dependent changes in the glutamatergic and GABAergic drives during epileptogenesis in the rat CA1 region. J Physiol 578: 193-211.

30. Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B (1998) Postsynaptic clustering of major GABAA receptor subtypes requires the gamma 2 subunit and gephyrin. Nat Neurosci 1: 563-571.

31. Eugene E, Depienne C, Baulac S, Baulac M, Fritschy JM, Le Guern E, Miles R, Poncer JC (2007) GABA(A) receptor gamma 2 subunit mutations linked to human epileptic syndromes differentially affect phasic and tonic inhibition. J Neurosci 27: 14108-14116.

32. Fatherazi S, Cook DL (1991) Specificity of tetraethylammonium and quinine for three K channels in insulin-secreting cells. J Membr Biol 120: 105-114.

33. Feng G, Tintrup H, Kirsch J, Nichol MC, Kuhse J, Betz H, Sanes JR (1998) Dual requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity. Science 282: 1321-1324.

34. Fisher RS, Webber WR, Lesser RP, Arroyo S, Uematsu S (1992) High-frequency EEG activity at the start of seizures. J Clin Neurophysiol 9: 441-448.

35. Fritschy JM, Harvey RJ, Schwarz G (2008) Gephyrin: where do we stand, where do we go? Trends Neurosci 31: 257-264.

36. Frotscher M, Jonas P, Sloviter RS (2006) Synapses formed by normal and abnormal hippocampal mossy fibers. Cell Tissue Res 326: 361-367.

37. Gall D, Prestori F, Sola E, D'Errico A, Roussel C, Forti L, Rossi P, D'Angelo E (2005) Intracellular calcium regulation by burst discharge determines bidirectional long-term synaptic plasticity at the cerebellum input stage. J Neurosci 25: 4813-4822.

38. Gallo G, Ernst AF, McLoon SC, Letourneau PC (2002) Transient PKA activity is required for initiation but not maintenance of BDNF-mediated protection from nitric oxide-induced growth-cone collapse. J Neurosci 22: 5016-5023.

39. Ganguly K, Schinder AF, Wong ST, Poo M (2001) GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell 105: 521-532.

40. Gibb JW, McNamara JO (2006) The Epilepsies: Phenotype and Mechanisms. In: Basic Neurochemistry - Molecular, Cellular, and Medical Aspects (Siegel GJ, Albers RW, Brady ST, Price DL, eds), pp 629-638. Burlington, San Diego, London: Elsevier Academic Press.

41. Gillessen T, Budd SL, Lippton SA (2003) Excitatory Amino Acid Neurotoxicity. In: Molecular and Cellular Biology of Neuroprotection in the CNS (Alzheimer C, ed), pp 3-40. Kiel: Springer Verlag.

42. Gloveli T, Dugladze T, Rotstein HG, Traub RD, Monyer H, Heinemann U, Whittington MA, Kopell NJ (2005a) Orthogonal arrangement of rhythm-generating microcircuits in the hippocampus. Proc Natl Acad Sci U S A 102: 13295-13300.

43. Gloveli T, Dugladze T, Saha S, Monyer H, Heinemann U, Traub RD, Whittington MA, Buhl EH (2005b) Differential involvement of oriens/pyramidale interneurones in hippocampal network oscillations in vitro. J Physiol 562: 131-147.

44. Gomeza J, Hulsmann S, Ohno K, Eulenburg V, Szoke K, Richter D, Betz H (2003) Inactivation of the glycine transporter 1 gene discloses vital role of glial glycine uptake in glycinergic inhibition. Neuron 40: 785-796.

45. Grudzinska J, Schemm R, Haeger S, Nicke A, Schmalzing G, Betz H, Laube B (2005) The beta subunit determines the ligand binding properties of synaptic glycine receptors. Neuron 45: 727-739.

46. Hammer O, Harper D, Ryan P (2001) Palaeontological Statistics software Package for Education and Data Analysis. Palaeontologia Electronica 4(1): 9pp.

47. Harvey BD, Sloviter RS (2005) Hippocampal granule cell activity and c-Fos expression during spontaneous seizures in awake, chronically epileptic, pilocarpine-treated rats: implications for hippocampal epileptogenesis. J Comp Neurol 488: 442-463.

48. Harvey RJ, Carta E, Pearce BR, Chung SK, Supplisson S, Rees MI, Harvey K (2008) A critical role for glycine transporters in hyperexcitability disorders. Front Mol Neurosci 1: 1.

49. Harvey RJ, Depner UB, Wassle H, Ahmadi S, Heindl C, Reinold H, Smart TG, Harvey K, Schutz B, Abo-Salem OM, Zimmer A, Poisbeau P, Welzl H, Wolfer DP, Betz H, Zeilhofer HU, Muller U (2004) GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. Science 304: 884-887.

50. Hassel B, Dingledine R (2006) Glutamate. In: Basic Neurochemistry - Molecular, Cellular, and Medical Aspects (Siegel GJ, Albers RW, Brady ST, Price DL, eds), pp 267-290. Burlington, San Diego, London: Elsevier Academic Press.

51. Haverkamp S, Muller U, Harvey K, Harvey RJ, Betz H, Wassle H (2003) Diversity of glycine receptors in the mouse retina: localization of the alpha3 subunit. J Comp Neurol 465: 524-539.

52. Herzog E, Bellenchi GC, Gras C, Bernard V, Ravassard P, Bedet C, Gasnier B, Giros B, El Mestikawy S (2001) The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. J Neurosci 21: RC181.

53. Holland EC (2000) Glioblastoma multiforme: the terminator. Proc Natl Acad Sci U S A 97: 6242-6244.

54. Holland EC (2001) Gliomagenesis: genetic alterations and mouse models. Nat Rev Genet 2: 120-129.

55. Huntsman MM, Tran BV, Potkin SG, Bunney WE, Jr., Jones EG (1998) Altered ratios of alternatively spliced long and short gamma2 subunit mRNAs of the gamma-amino butyrate type A receptor in prefrontal cortex of schizophrenics. Proc Natl Acad Sci U S A 95: 15066-15071.

56. Janeway CAJr, Travers P, Walport M, Shlomchik KJ (2001) T Cell-mediated Immunity. In: Immunobiology: The immun system in health and disease (Austin P, Lawrence E, eds), pp 328-333. New York: Garland Publishing.

57. Jonas P, Bischofberger J, Sandkuhler J (1998) Corelease of two fast neurotransmitters at a central synapse. Science 281: 419-424.

58. Kim E, Sheng M (2004) PDZ domain proteins of synapses. Nat Rev Neurosci 5: 771-781.

59. King AE, Chung RS, Vickers JC, Dickson TC (2006) Localization of glutamate receptors in developing cortical neurons in culture and relationship to susceptibility to excitotoxicity. J Comp Neurol 498: 277-294.

60. Kirchner A, Breustedt J, Rosche B, Heinemann UF, Schmieden V (2003) Effects of taurine and glycine on epileptiform activity induced by removal of Mg2+ in combined rat entorhinal cortex-hippocampal slices. Epilepsia 44: 1145-1152.

61. Kohling R, Senner V, Paulus W, Speckmann EJ (2006) Epileptiform activity preferentially arises outside tumor invasion zone in glioma xenotransplants. Neurobiol Dis 22: 64-75.

62. Krampfl K, Schlesinger F, Zorner A, Kappler M, Dengler R, Bufler J (2002) Control of kinetic properties of GluR2 flop AMPA-type channels: impact of R/G nuclear editing. Eur J Neurosci 15: 51-62.

63. Kuhse J, Kuryatov A, Maulet Y, Malosio ML, Schmieden V, Betz H (1991) Alternative splicing generates two isoforms of the alpha 2 subunit of the inhibitory glycine receptor. FEBS Lett 283: 73-77.

64. Lardi-Studler B, Smolinsky B, Petitjean CM, Koenig F, Sidler C, Meier JC, Fritschy JM, Schwarz G (2007) Vertebrate-specific sequences in the gephyrin E-domain regulate cytosolic aggregation and postsynaptic clustering. J Cell Sci 120: 1371-1382.

65. Lau CG, Zukin RS (2007) NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. Nat Rev Neurosci 8: 413-426.

66. Lee H, Chen CX, Liu YJ, Aizenman E, Kandler K (2005) KCC2 expression in immature rat cortical neurons is sufficient to switch the polarity of GABA responses. Eur J Neurosci 21: 2593-2599.

67. Lee HJ, Adham IM, Schwarz G, Kneussel M, Sass JO, Engel W, Reiss J (2002) Molybdenum cofactor-deficient mice resemble the phenotype of human patients. Hum Mol Genet 11: 3309-3317.

68. Legendre P (2001) The glycinergic inhibitory synapse. Cell Mol Life Sci 58: 760-793.

69. Levi S, Logan SM, Tovar KR, Craig AM (2004) Gephyrin is critical for glycine receptor clustering but not for the formation of functional GABAergic synapses in hippocampal neurons. J Neurosci 24: 207-217.

70. Liss B, Haeckel O, Wildmann J, Miki T, Seino S, Roeper J (2005) K-ATP channels promote the differential degeneration of dopaminergic midbrain neurons. Nat Neurosci 8: 1742-1751.

71. Lohmann C, Finski A, Bonhoeffer T (2005) Local calcium transients regulate the spontaneous motility of dendritic filopodia. Nat Neurosci 8: 305-312.

72. Loup F, Wieser HG, Yonekawa Y, Aguzzi A, Fritschy JM (2000) Selective alterations in GABAA receptor subtypes in human temporal lobe epilepsy. J Neurosci 20: 5401-5419.

73. Lurton D, El Bahh B, Sundstrom L, Rougier A (1998) Granule cell dispersion is correlated with early epileptic events in human temporal lobe epilepsy. J Neurol Sci 154: 133-136.

74. Malosio ML, Grenningloh G, Kuhse J, Schmieden V, Schmitt B, Prior P, Betz H (1991a) Alternative splicing generates two variants of the alpha 1 subunit of the inhibitory glycine receptor. J Biol Chem 266: 2048-2053.

75. Malosio ML, Marqueze-Pouey B, Kuhse J, Betz H (1991b) Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. EMBO J 10: 2401-2409.

76. Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, Bashir ZI (2004) Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. J Neurosci 24: 7821-7828.

77. Mattson MP, Bazan NG (2006) Apoptosis and Necrosis. In: Basic Neurochemistry - Molecular, Cellular, and Medical Aspects (Siegel GJ, Albers RW, Brady ST, Price DL, eds), pp 603-616. Burlington, San Diego, London: Elsevier Academic Press.

78. Meier J (2003) The enigma of transmitter-selective receptor accumulation at developing inhibitory synapses. Cell Tissue Res 311: 271-276.

79. Meier J, Grantyn R (2004a) A gephyrin-related mechanism restraining glycine receptor anchoring at GABAergic synapses. J Neurosci 24: 1398-1405.

80. Meier J, Grantyn R (2004b) Preferential accumulation of GABAA receptor gamma 2L, not gamma 2S, cytoplasmic loops at rat spinal cord inhibitory synapses. J Physiol 559: 355-365.

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

82. Meier JC, Henneberger C, Melnick I, Racca C, Harvey RJ, Heinemann U, Schmieden V, Grantyn R (2005) RNA editing produces glycine receptor alpha3(P185L), resulting in high agonist potency. Nat Neurosci 8: 736-744.

83. Meyer G, Kirsch J, Betz H, Langosch D (1995) Identification of a gephyrin binding motif on the glycine receptor beta subunit. Neuron 15: 563-572.

84. Michel PP, Alvarez-Fischer D, Guerreiro S, Hild A, Hartmann A, Hirsch EC (2007) Role of activity-dependent mechanisms in the control of dopaminergic neuron survival. J Neurochem 101: 289-297.

85. Miller PS, Harvey RJ, Smart TG (2004) Differential agonist sensitivity of glycine receptor alpha2 subunit splice variants. Br J Pharmacol 143: 19-26.

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

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

88. Mody I, Pearce RA (2004) Diversity of inhibitory neurotransmission through GABA(A) receptors. Trends Neurosci 27: 569-575.

89. Mohn AR, Gainetdinov RR, Caron MG, Koller BH (1999) Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. Cell 98: 427-436.

90. Monyer H, Markram H (2004) Interneuron Diversity series: Molecular and genetic tools to study GABAergic interneuron diversity and function. Trends Neurosci 27: 90-97.

91. Moss SJ, Smart TG (2001) Constructing inhibitory synapses. Nat Rev Neurosci 2: 240-250.

92. Nadeau H, McKinney S, Anderson DJ, Lester HA (2000) ROMK1 (Kir1.1) causes apoptosis and chronic silencing of hippocampal neurons. J Neurophysiol 84: 1062-1075.

93. Nikolic Z, Laube B, Weber RG, Lichter P, Kioschis P, Poustka A, Mulhardt C, Becker CM (1998) The human glycine receptor subunit alpha3. Glra3 gene structure, chromosomal localization, and functional characterization of alternative transcripts. J Biol Chem 273: 19708-19714.

94. Olsen RW, Betz H (2006) GABA and Glycine. In: Basic Neurochemistry - Molecular, Cellular, and Medical Aspects (Siegel GJ, Albers RW, Brady ST, Price DL, eds), pp 291-302. Burlington, San Diego, London: Elsevier Academic Press.

95. Paarmann I, Schmitt B, Meyer B, Karas M, Betz H (2006) Mass spectrometric analysis of glycine receptor-associated gephyrin splice variants. J Biol Chem 281: 34918-34925.

96. Pal S, Hartnett KA, Nerbonne JM, Levitan ES, Aizenman E (2003) Mediation of neuronal apoptosis by Kv2.1-encoded potassium channels. J Neurosci 23: 4798-4802.

97. Palma E, Amici M, Sobrero F, Spinelli G, Di Angelantonio S, Ragozzino D, Mascia A, Scoppetta C, Esposito V, Miledi R, Eusebi F (2006) Anomalous levels of Cl- transporters in the hippocampal subiculum from temporal lobe epilepsy patients make GABA excitatory. Proc Natl Acad Sci U S A 103: 8465-8468.

98. Palma E, Spinelli G, Torchia G, Martinez-Torres A, Ragozzino D, Miledi R, Eusebi F (2005) Abnormal GABAA receptors from the human epileptic hippocampal subiculum microtransplanted to Xenopus oocytes. Proc Natl Acad Sci U S A 102: 2514-2518.

99. Perez-Ortiz L, Zaldivar-Vaillant T, Tamayo-Suarez JD (2000) [Malignant gliomas. Oncogenic considerations]. Rev Neurol 31: 49-52.

100. Prange O, Wong TP, Gerrow K, Wang YT, El Husseini A (2004) A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. Proc Natl Acad Sci U S A 101: 13915-13920.

101. Purves D, Augustine GJ, Fitzpatrick D, Hall WC, LaMantia A-S, McNamara JO, White LE (2007) Neuroscience. Sunderland: Sinauer Associates, Inc.

102. Raine CS (2006) Neurocellular Anatomy. In: Basic Neurochemistry - Molecular, Cellular, and Medical Aspects (Siegel GJ, Albers RW, Brady ST, Price DL, eds), pp 3-20. Burlington, San Diego, London: Elsevier Academic Press.

103. Ramakers GJ, Corner MA, Habets AM (1990) Development in the absence of spontaneous bioelectric activity results in increased stereotyped burst firing in cultures of dissociated cerebral cortex. Exp Brain Res 79: 157-166.

104. Ramakers GJ, van Galen H, Feenstra MG, Corner MA, Boer GJ (1994) Activity-dependent plasticity of inhibitory and excitatory amino acid transmitter systems in cultured rat cerebral cortex. Int J Dev Neurosci 12: 611-621.

105. Rees MI, Lewis TM, Kwok JB, Mortier GR, Govaert P, Snell RG, Schofield PR, Owen MJ (2002) Hyperekplexia associated with compound heterozygote mutations in the beta-subunit of the human inhibitory glycine receptor (GLRB). Hum Mol Genet 11: 853-860.

106. Rees MI, Lewis TM, Vafa B, Ferrie C, Corry P, Muntoni F, Jungbluth H, Stephenson JB, Kerr M, Snell RG, Schofield PR, Owen MJ (2001) Compound heterozygosity and nonsense mutations in the alpha(1)-subunit of the inhibitory glycine receptor in hyperekplexia. Hum Genet 109: 267-270.

107. Reiss J, Johnson JL (2003) Mutations in the molybdenum cofactor biosynthetic genes MOCS1, MOCS2, and GEPH. Hum Mutat 21: 569-576.

108. Rivera C, Li H, Thomas-Crusells J, Lahtinen H, Viitanen T, Nanobashvili A, Kokaia Z, Airaksinen MS, Voipio J, Kaila K, Saarma M (2002) BDNF-induced TrkB activation down-regulates the K+-Cl- cotransporter KCC2 and impairs neuronal Cl- extrusion. J Cell Biol 159: 747-752.

109. Rivera C, Voipio J, Kaila K (2005) Two developmental switches in GABAergic signalling: the K+-Cl- cotransporter KCC2 and carbonic anhydrase CAVII. J Physiol 562: 27-36.

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

111. Rivera C, Voipio J, Thomas-Crusells J, Li H, Emri Z, Sipila S, Payne JA, Minichiello L, Saarma M, Kaila K (2004) Mechanism of activity-dependent downregulation of the neuron-specific K-Cl cotransporter KCC2. J Neurosci 24: 4683-4691.

112. Rivera-Cervantes MC, Torres JS, Feria-Velasco A, Armendariz-Borunda J, Beas-Zarate C (2004) NMDA and AMPA receptor expression and cortical neuronal death are associated with p38 in glutamate-induced excitotoxicity in vivo. J Neurosci Res 76: 678-687.

113. Salama-Cohen P, Arevalo MA, Meier J, Grantyn R, Rodriguez-Tebar A (2005) NGF controls dendrite development in hippocampal neurons by binding to p75NTR and modulating the cellular targets of Notch. Mol Biol Cell 16: 339-347.

114. Sanes JR, Lichtman JW (1999) Can molecules explain long-term potentiation? Nat Neurosci 2: 597-604.

115. Schwarz G (2005) Molybdenum cofactor biosynthesis and deficiency. Cell Mol Life Sci 62: 2792-2810.

116. Seeburg PH, Hartner J (2003) Regulation of ion channel/neurotransmitter receptor function by RNA editing. Curr Opin Neurobiol 13: 279-283.

117. Segerer S, Eitner F, Cui Y, Hudkins KL, Alpers CE (2002) Cellular injury associated with renal thrombotic microangiopathy in human immunodeficiency virus-infected macaques. J Am Soc Nephrol 13: 370-378.

118. Senner V, Kohling R, Puttmann-Cyrus S, Straub H, Paulus W, Speckmann EJ (2004) A new neurophysiological/neuropathological ex vivo model localizes the origin of glioma-associated epileptogenesis in the invasion area. Acta Neuropathol 107: 1-7.

119. Shiang R, Ryan SG, Zhu YZ, Fielder TJ, Allen RJ, Fryer A, Yamashita S, O'Connell P, Wasmuth JJ (1995) Mutational analysis of familial and sporadic hyperekplexia. Ann Neurol 38: 85-91.

120. Shiang R, Ryan SG, Zhu YZ, Hahn AF, O'Connell P, Wasmuth JJ (1993) Mutations in the alpha 1 subunit of the inhibitory glycine receptor cause the dominant neurologic disorder, hyperekplexia. Nat Genet 5: 351-358.

121. Sierra-Paredes G, Sierra-Marcuno G (2007) Extrasynaptic GABA and glutamate receptors in epilepsy. CNS Neurol Disord Drug Targets 6: 288-300.

122. Singh B, Henneberger C, Betances D, Arevalo MA, Rodriguez-Tebar A, Meier JC, Grantyn R (2006) Altered balance of glutamatergic/GABAergic synaptic input and associated changes in dendrite morphology after BDNF expression in BDNF-deficient hippocampal neurons. J Neurosci 26: 7189-7200.

123. Sloviter RS, Zappone CA, Harvey BD, Bumanglag AV, Bender RA, Frotscher M (2003) "Dormant basket cell" hypothesis revisited: relative vulnerabilities of dentate gyrus mossy cells and inhibitory interneurons after hippocampal status epilepticus in the rat. J Comp Neurol 459: 44-76.

124. Sloviter RS, Zappone CA, Harvey BD, Frotscher M (2006) Kainic acid-induced recurrent mossy fiber innervation of dentate gyrus inhibitory interneurons: possible anatomical substrate of granule cell hyper-inhibition in chronically epileptic rats. J Comp Neurol 494: 944-960.

125. Smolinsky B, Eichler SA, Buchmeier S, Meier JC, Schwarz G (2008) Splice-specific functions of gephyrin in molybdenum cofactor biosynthesis. J Biol Chem 283: 17370-17379.

126. Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK, Greengard P (2005) Regulation of NMDA receptor trafficking by amyloid-beta. Nat Neurosci 8: 1051-1058.

127. Song W, Chattipakorn SC, McMahon LL (2006) Glycine-gated chloride channels depress synaptic transmission in rat hippocampus. J Neurophysiol 95: 2366-2379.

128. Sontheimer H (2003) Malignant gliomas: perverting glutamate and ion homeostasis for selective advantage. Trends Neurosci 26: 543-549.

129. Stallmeyer B, Schwarz G, Schulze J, Nerlich A, Reiss J, Kirsch J, Mendel RR (1999) The neurotransmitter receptor-anchoring protein gephyrin reconstitutes molybdenum cofactor biosynthesis in bacteria, plants, and mammalian cells. Proc Natl Acad Sci U S A 96: 1333-1338.

130. Steinhauser C, Seifert G (2002) Glial membrane channels and receptors in epilepsy: impact for generation and spread of seizure activity. Eur J Pharmacol 447: 227-237.

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

132. Stief F, Zuschratter W, Hartmann K, Schmitz D, Draguhn A (2007) Enhanced synaptic excitation-inhibition ratio in hippocampal interneurons of rats with temporal lobe epilepsy. Eur J Neurosci 25: 519-528.

133. Swartz ME, Eberhart J, Pasquale EB, Krull CE (2001) EphA4/ephrin-A5 interactions in muscle precursor cell migration in the avian forelimb. Development 128: 4669-4680.

134. Synowitz M, Ahmann P, Matyash M, Kuhn SA, Hofmann B, Zimmer C, Kirchhoff F, Kiwit JC, Kettenmann H (2001) GABA(A)-receptor expression in glioma cells is triggered by contact with neuronal cells. Eur J Neurosci 14: 1294-1302.

135. Takano T, Lin JH, Arcuino G, Gao Q, Yang J, Nedergaard M (2001) Glutamate release promotes growth of malignant gliomas. Nat Med 7: 1010-1015.

136. Tao HW, Poo MM (2005) Activity-dependent matching of excitatory and inhibitory inputs during refinement of visual receptive fields. Neuron 45: 829-836.

137. Titz S, Hans M, Kelsch W, Lewen A, Swandulla D, Misgeld U (2003) Hyperpolarizing inhibition develops without trophic support by GABA in cultured rat midbrain neurons. J Physiol 550: 719-730.

138. Trasande CA, Ramirez JM (2007) Activity deprivation leads to seizures in hippocampal slice cultures: is epilepsy the consequence of homeostatic plasticity? J Clin Neurophysiol 24: 154-164.

139. Tretter V, Jacob TC, Mukherjee J, Fritschy JM, Pangalos MN, Moss SJ (2008) The clustering of GABA(A) receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor alpha 2 subunits to gephyrin. J Neurosci 28: 1356-1365.

140. Tsien JZ (2006) Learning and Memory. In: Basic Neurochemistry - Molecular, Cellular, and Medical Aspects (Siegel GJ, Albers RW, Brady ST, Price DL, eds), pp 859-874. Burlington, San Diego, London: Elsevier Academic Press.

141. Turrigiano GG, Nelson SB (2004) Homeostatic plasticity in the developing nervous system. Nat Rev Neurosci 5: 97-107.

142. Vollmar W, Gloger J, Berger E, Kortenbruck G, Kohling R, Speckmann EJ, Musshoff U (2004) RNA editing (R/G site) and flip-flop splicing of the AMPA receptor subunit GluR2 in nervous tissue of epilepsy patients. Neurobiol Dis 15: 371-379.

143. Wake H, Watanabe M, Moorhouse AJ, Kanematsu T, Horibe S, Matsukawa N, Asai K, Ojika K, Hirata M, Nabekura J (2007) Early changes in KCC2 phosphorylation in response to neuronal stress result in functional downregulation. J Neurosci 27: 1642-1650.

144. Wassle H (2004) Parallel processing in the mammalian retina. Nat Rev Neurosci 5: 747-757.

145. Wisden W, Cope D, Klausberger T, Hauer B, Sinkkonen ST, Tretter V, Lujan R, Jones A, Korpi ER, Mody I, Sieghart W, Somogyi P (2002) Ectopic expression of the GABA(A) receptor alpha6 subunit in hippocampal pyramidal neurons produces extrasynaptic receptors and an increased tonic inhibition. Neuropharmacology 43: 530-549.

146. Wyler AR, Dohan JrFC, Schweitzer JB, Berry III AD (1992) A Grading System for Mesial Temporal Pathology (Hippocampal Sclerosis) from Anterior Temporal Lobectomy. J Epilepsy 5: 220-225.

147. Xiang S, Nichols J, Rajagopalan KV, Schindelin H (2001) The crystal structure of Escherichia coli MoeA and its relationship to the multifunctional protein gephyrin. Structure 9: 299-310.

148. Yeh JH, Jeng CJ, Chen YW, Lin HM, Wu YS, Tang CY (2005) Selective enhancement of tonic inhibition by increasing ambient GABA is insufficient to suppress excitotoxicity in hippocampal neurons. Biochem Biophys Res Commun 338: 1417-1425.

149. Yuan J, Lipinski M, Degterev A (2003) Diversity in the mechanisms of neuronal cell death. Neuron 40: 401-413.

150. Zhang SJ, Steijaert MN, Lau D, Schutz G, Delucinge-Vivier C, Descombes P, Bading H (2007) Decoding NMDA Receptor Signaling: Identification of Genomic Programs Specifying Neuronal Survival and Death. Neuron 53: 549-562.