Modulation of Network Excitability and Epileptiform Activity in the Hippocampus of Immature Rats by the Activation of GlyRs

Dissertation

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Rongqing Chen

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List of symbols and abbreviations

ACSF	Artificial cerebrospinal fluid
AD/DA	Analogue-to-digital conversion
AED	Anti-epileptic drug
ALX 1393	O-[(2-Benzyloxyphenyl-3-flurophenyl)methyl]-L-serine
ALX 5407	N-[3-(4'-Fluorophenyl)-3-(4'-phenylphenoxy)propyl]-sarcosine hydrochloride
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV	(2*R*)-amino-5-phosphonovaleric acid
CA	Cornu Ammonis area
CHF	Corticohippocampal formation
CNS	Central nervous system
DG	Dentate gyrus
DMSO	Dimethyl sulfoxide
E	Embryonic day
EC	Entorhinal cortex
E_{Cl}	Chloride reversal potential
Fig.	Figure
Freq	Frequency
GABA	γ-Aminobutyric acid
GABA-PSC	GABAergic postsynaptic current

$GABA_AR$	$GABA_A$ receptor
GBZ	Gabazine
GES	Guanidinoethanesulfonic acid
GlyR	Glycine receptor
GlyT	Glycine transporter
Hz	Hertz
KCC2	Potassium chloride cotransporter 2
ILAE	International League against Epilepsy
MΩ	Megaohm
NBQX	1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt hydrate
NKCC1	Isoform 1 of Na^+-K^+-$2Cl^-$ cotransporter
NMDA	N-methyl-D-aspartic acid
P	Postnatal days
Rel.	Relative
SEM	Standard error of the mean
sPSC	Spontaneous postsynaptic current
stry	Strychnine
TauT	Taurine transporter
4-AP	4-Aminopyridine

1 Introduction

1.1 Neurogenesis and early development in the central nervous system (CNS)

Despite great variety of brain growth among species, evolutionary conservatism allows comparison of brain development between human beings and small rodents to be possible (Andersen, 2003; Bayer *et al.*, 1993; Passingham, 1985). Anatomically the 2nd month of gestation in humans is corresponding to around embryonic days (E) 18 of small rodents, such as rats and mice. The brain structure at the 5th gestational month of humans is comparable to that of small rodents at its birth (Table 1, Ikonomidou & Turski, 2010; Rice & Barone, 2000).

The development of CNS starts with the neural plate, which invaginates along its central axis to make a specialized folding to a special formation called neural groove and later fusing to neural tube (Lenroot & Giedd, 2006; Victor *et al.*, 2001). Neural tube completely forms by around 3-4 weeks of gestation in human beings, or approximately E10.8 in rats, and differentiates to various structures of the CNS from 4-12 weeks of gestation in human beings (Lenroot & Giedd, 2006; Victor *et al.*, 2001). The forebrain, midbrain and hindbrain derive from the anterior portion of neural tube, the most anterior of which gives rise of telencephalon (cerebral cortex) and diencephalon (thalamus, subthalamus, hypothalamus and epithalamus). The spinal cord develops from the posterior portion of neural tube. Afterwards neural tube starts to close following a caudal-to-rostral trajectory. Meanwhile various specific regions of the CNS start to form by caudal-to-rostral sequence as well (Table 1). Part of center of neural tube remains and becomes ventricles.

The development of anatomical structure is built and refined by processes of functional emergence and maturation, such as neuronal proliferation, migration, apoptosis and differentiation (Andersen, 2003; Rodier, 1980). These processes are also comparable among species (Table 2; DeSesso, 1997; Ikonomidou & Turski, 2010; Rice & Barone, 2000). Neuronal precursors or neuroblasts within proliferative zone, which is near ventricles, start to proliferate rapidly to neurons by 5-6 weeks of gestation in humans, or E11-13 in rats. From 8 weeks of gestation in humans, these young neurons multiply, differentiate and migrate to fill or form specific structures and nuclei with

specific cell types, such as glias, principle neurons, granule cells, interneurons, olfactory bulb cells, sensory or motor neurons in nuclei (Gilber, 2000). Different cell types may follow different patterns of migration, e.g. cortical principle pyramidal neurons primarily migrate radially to form typical cortical layers (Kwan *et al.*, 2012; Nadarajah *et al.*, 2001), while cortical interneurons primarily migrate tangentially (Faux *et al.*, 2012; Polleux *et al.*, 2002).

Neuronal migration completes mostly by 26-29 weeks of gestation in humans. Thus specific structures like pons, medulla, tectum, tegmentum, thalamus, hypothalamus, amygdale, cerebral cortex, entorhinal cortex, hippocampus, etc, gradually mature with corresponding cell types or nuclei, by a gradient from hindbrain to forebrain in general.

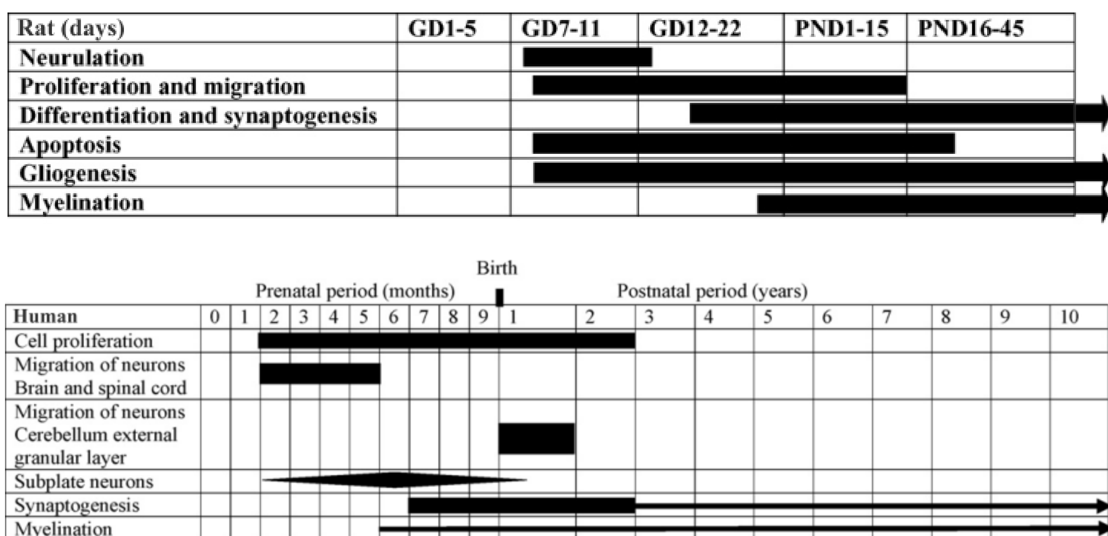
Table 1. Estimated timeline of neurogenesis for anatomic structures of humans and rats. Adapted from Ikonomidou & Turski, 2010.

Human (weeks)	**3.5-4.0**	**4.1-5.2**	**5.3-5.7**	**5.8-6.6**	**6.7-7.0**	**7.1-7.4**	**7.5-7.9**	**8.0-9.9**	**10.0-11.9**	**12.0-14.9**	**15.0-18.9**	**19.0-23.9**	**24.0-27.9**	**28.0-31.9**	**32.0-35.9**	**36.0-40.0**
Rat (days)	**GD 11**	**GD 12**	**GD 13**	**GD 14**	**GD 15**	**GD 16**	**GD 17**	**GD 18**	**GD 19**	**GD 20**	**GD 21-23**	**PND 0-3**	**PND 4-7**	**PND 8-11**	**PND 12-15**	**PND 16-19**
spinal cord																
cerebellum																
mesencephalic tegmentum																
mesencephalic tectum																
thalamus																
hypothalamus																
amygdala																
neocortex and limbic system																
entorhinal cortex																
hippocampal CA1-3																
hippocampal dentate gyrus																

1.2 Synaptogenesis and synaptic refinement of the CNS

Another critically important developmental event is synaptogenesis that establishes complex brain circuits and enables nervous integral functions (Andersen, 2003; Lenroot & Giedd, 2006). Synaptogenesis begins right after the migration of neurons to its given locations (Rakic, 1990). Under control of molecular and cellular processes, functional and morphologic pre- and postsynaptic components differentiate (Lardi-Studler & Fritschy, 2007). Synaptic assemblies come into formation while presynaptic adhension proteins like neurexin and neuroligin guide presynaptic axons to make precise contact and interaction with dendritic or somatic counterparts of postsynaptic cells (Dean *et al.*, 2003; Scheiffele *et al.*, 2000).

Table 2. Timeline of neurogenesis for cellular generation and maturation of rats (up) and humans (down). *Adopted from Ikonomidou & Turski, 2010.*



At chemical synapses, immediately after the initial contact between two cells forms, synaptic vesicles containing transmitters accumulate at presynaptic sites (Ahmari *et al.*, 2000; Friedman *et al.*, 2000). Correspondingly, specific receptors for neurotransmitters, scaffolding proteins, such as PSD-95 and microtubule-associated protein, and signal transduction molecules aggregate at postsynaptic sites (Ahmari *et al.*, 2000; Barrow *et al.*, 2009; Elferink & Scheller, 1995; Han & Kim, 2008). The process of synaptic assembly is thought to be dynamic and interactive between pre- and postsynaptic components (Lardi-Studler & Fritschy, 2007). The density and function of synaptic

connections reach maturation over a long developmental timeline until postnatal days 21 in rats, or adolescence in humans (Bourgeois *et al.*, 1994; Jacobson, 1991; Uylings & Vaneden, 1990).

During synaptogenesis, expression and release of neurotransmitters (Antal *et al.*, 1994; Lauder *et al.*, 1986; Root *et al.*, 2008) precede expression of receptors, such as glutamate receptor (Martin *et al.*, 1998; Root *et al.*, 2008; van den Pol *et al.*, 1995), GABA receptor (Root *et al.*, 2008) and glycine receptor (GlyR) (Aguayo *et al.*, 2004; Avila *et al.*, 2013a; Flint *et al.*, 1998; Malosio *et al.*, 1991). The neurotransmitters may be released as early as late neural plate stage, while the receptors can be detected at around E13 for small rodents. With the maturation of synaptic formation and function in the mammalian CNS, two functionally balanced major neurotransmitter systems, excitatory and inhibitory synaptic transmissions, are established (Lardi-Studler & Fritschy, 2007; Turrigiano & Nelson, 2004). Glutamatergic system mediates excitatory neurotransmission via three subtypes of ionotropic glutamate receptors: NMDA receptor, AMPA receptor and kainite (KA) receptor. GABAergic and glycinergic systems mediate fast inhibitory neurotransmission via $GABA_A$ receptor ($GABA_AR$) and GlyR, respectively, and slow inhibitory neurotransmission via $GABA_B$ receptor. A balance between excitation and inhibition is fundamentally crucial for normal brain function (Turrigiano & Nelson, 2004).

1.3 GlyR in the CNS

GABA is the primary inhibitory neurotransmitter in the whole CNS. In the brain stem and spinal cord, glycine, in addition to GABA, was identified as inhibitory neurotransmitter which activates GlyR (Aprison & Werman, 1965; Curtis *et al.*, 1968; Spencer *et al.*, 1989). Besides GlyR, glycine also binds to NMDA receptor as a coagonist (Chen *et al.*, 2011; Johnson & Ascher, 1987; Wroblewski *et al.*, 1989). Apart from glycine, taurine and β-alanine are also endogenous ligands with less affinity to GlyR (Mori *et al.*, 2002). Concentration of these amino acids at extracellular space is modulated by either synaptic release or corresponding transporters (Liu *et al.*, 1993; Mori *et al.*, 2002; Smith *et al.*, 1992). All the three corresponding transporters for glycine (Eulenburg *et al.*, 2005; Harvey & Yee, 2013), taurine (Han *et al.*, 2006; Lambert, 2004) and β-alanine (Jessen, 1994; Komura *et al.*, 1996) belong to the family of Na^+/Cl^--dependent transporters. Glycine transporters (GlyTs) consist of two subtypes,

glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2). GlyT1 are mainly expressed at excitatory synapse and glial cells, while GlyT2 are mainly expressed at inhibitory synapse (Eulenburg *et al.*, 2005; Harvey & Yee, 2013). Expression of taurine transporters (TauTs) has been detected in a variety of organs, including abundantly in the rat brain (Smith *et al.*, 1992).

Glycinergic synaptic transmission is believed to be confined in the brain stem, cerebellum, spinal cord and other lower portions of mammalian CNS. In contrast, studies using the methods of *in suit* hybridization, immunochemistry and electrophysiology have reported the wide expression of GlyRs in the CNS of rats or mice of around E14 (Avila *et al.*, 2013b; Flint *et al.*, 1998; Malosio *et al.*, 1991), earlier postnatal days (Flint *et al.*, 1998; Kilb *et al.*, 2002; Kuhse *et al.*, 1991; Malosio *et al.*, 1991) until mature ages (Chattipakorn & McMahon, 2002; Malosio *et al.*, 1991). In the hippocampus, GlyRs are expressed both on principal neurons and interneurons (Chattipakorn & McMahon, 2002), both presynaptically and postsynaptically (Brackmann *et al.*, 2004; Kubota *et al.*, 2010; Lee *et al.*, 2009). Similarly to small rodents, human GlyRs also have been detected in wide regions of the human brain including spinal cord, brain stem and forebrain (Baer *et al.*, 2003; Baer *et al.*, 2009; Waldvogel *et al.*, 2007; Waldvogel *et al.*, 2009).

Like $GABA_A$Rs, GlyRs are pentameric transmembrane receptors. GlyRs can be either homomeric receptors with 5 α units or heteromeric receptors with a stoichiometry of 2 α : 3 β or 3 α : 2 β subunits (Fig. 1; Grudzinska *et al.*, 2005; Laube, 2002; Legendre, 2001). To date, four genes encoding α1-4 subunits and one gene encoding single β subunit have been identified in the mammalian brain (Laube, 2002; Lynch, 2004; Matzenbach *et al.*, 1994). The β subunit is important for GlyRs in the modulation of ligand binding and synaptic anchoring through postsynaptic scaffolding protein gephyrin (Grudzinska *et al.*, 2005; Kirsch, 2006; Meyer *et al.*, 1995; Sola *et al.*, 2004), while α subunits are functionally necessary for the binding of ligands and gating of Cl^- channel, and largely determine heterogenic functional characteristics of GlyRs (Betz & Laube, 2006; Graham *et al.*, 1983). Heterogeneity of GlyRs is further expressed by alternative splice variants of α subunits (Betz, 1991; Kuhse *et al.*, 1995). For example, rat α2 subunit has α2A and α2B isoforms as a result of two alternative splicing of α2 messengers (Kuhse *et al.*, 1991). In human beings, expression of α1-3 and β subunits has been identified (Lynch, 2004). Human and rat α3 subunit consists of α3L and α3K

isoforms due to alternative splice variants (Eichler *et al.*, 2009; Meier *et al.*, 2005; Nikolic *et al.*, 1998).

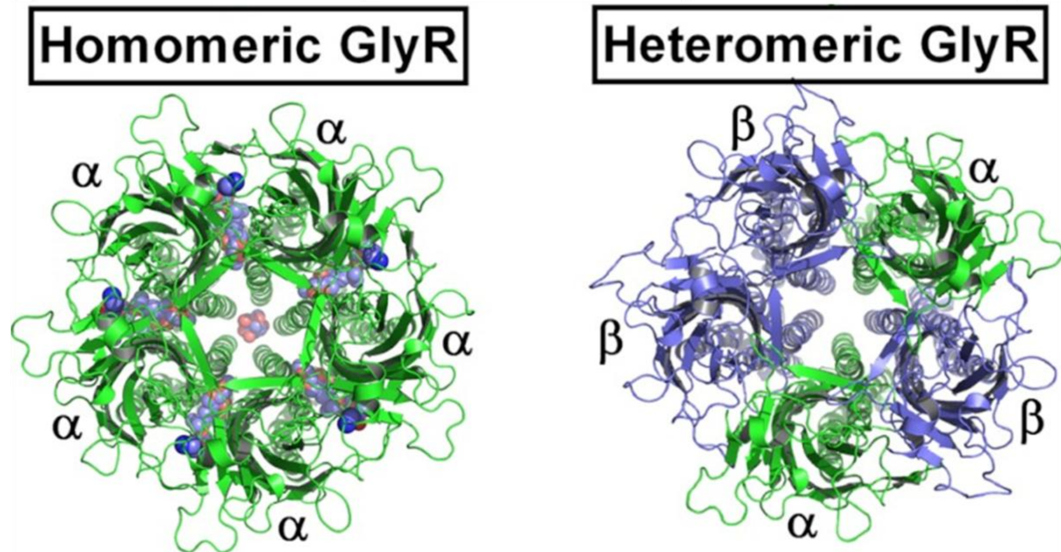


Figure 1. Schematic graphs (from Dutertre et al., 2012) showing structures of homomeric (left) and heteromeric (right) GlyRs. Spheres in the left graph represent binding of ligands to the interfaces of subunits.

Although there is > 80% sequence homology between α subunits (Betz & Laube, 2006), composition in α subunit or isoform of GlyRs largely determines its characteristics, such as regional and subcellular distribution (Deleuze *et al.*, 2005; Laube, 2002; Mangin *et al.*, 2003; Racca *et al.*, 1998), gating kinetics (Deleuze *et al.*, 2005; Laube, 2002; Mangin *et al.*, 2003), affinity for ligands (Chen *et al.*, 2009; Kuhse *et al.*, 1990), or efficacy of antagonist (Han *et al.*, 2004; Pribilla *et al.*, 1992). Homomeric GlyRs are extrasynaptically distributed, whereas heteromeric GlyRs are found within synapse (Deleuze *et al.*, 2005). Studies using *in suit* hybridization (Malosio *et al.*, 1991) and ^{3}H-strychnine binding analysis (White *et al.*, 1990) in the brain revealed that α1 subunit was mainly expressed in spinal cord, brain stem and midbrain, while α2,3 subunits displayed widely in the CNS. In contrast, expression of α4 subunit is confined in the retina (Heinze *et al.*, 2007). In the rat hippocampus, research via *in suit* hybridization indicated that expression of α2 and β subunits are higher relatively to α3 subunits (Racca *et al.*, 1998).

1.4 Developmental changes of GlyRs

As pentameric transmembrane receptors, both $GABA_A$Rs and GlyRs are formed by 5 subunits with an anion channel in the center. The anion channels of both $GABA_A$Rs and GlyRs are mainly permeable to Cl^-, so that commonly they are mentioned in most literature as Cl^- channels. Activating $GABA_A$Rs and GlyRs induces Cl^- influx or efflux depending on the electromotive force, which is determined by the difference between Cl^- equilibrium potential and the resting membrane potential. Due to developmental or plastic change in the driving force of Cl^-, activation of $GABA_A$Rs and GlyRs subjects to a developmental or plastic change.

Developmental changes in the properties of GlyRs are first attributed to the developmental shift of intracellular Cl^- concentration. Neuronal Cl^- concentration is mainly determined by relative contribution of two Cl^- transporters, NKCC1 (isoform 1 of Na^+-K^+-$2Cl^-$ cotransporter) and KCC2 (K^+-Cl^- cotransporter 2). Distinct expression patterns of NKCC1 and KCC2 at different developmental stages underlie developmental shift of Cl^- hemostasis (Fig. 2). NKCC1 is expressed at high level during embryonic and developmental stages (Plotkin *et al.*, 1997; Wang *et al.*, 2002) when KCC2 expression is very low (Rivera *et al.*, 1999; Wang *et al.*, 2002), while KCC2 is expressed at high level in mature stage (Wang *et al.*, 2002) when NKCC1 expression in neurons is faint (Plotkin *et al.*, 1997; Wang *et al.*, 2002).

As is shown in Fig. 2, during developmental phase, highly-expressed NKCC1 raises intracellular Cl^- by importing Na^+, K^+ and Cl^- (Delpire, 2000; Farrant & Kaila, 2007; Marty *et al.*, 2002; Rivera *et al.*, 1999; Sipila *et al.*, 2006b; Wang *et al.*, 2002; Yamada *et al.*, 2004). Elevated intracellular Cl^- sets Cl^- equilibrium potential positive to resting membrane potential. Thereby Cl^- effluxes, membrane depolarizes and consequently neuronal excitation increases upon the activation of $GABA_A$Rs and GlyRs in immature neurons (Ehrlich *et al.*, 1999; Kang *et al.*, 2002; Luhmann & Prince, 1991; Plotkin *et al.*, 1997). In contrast, in the adult CNS, intracellular Cl^- is maintained at low concentration level by the KCC2 which extrudes Cl^- from neurons using K^+ gradient as driving force (Balakrishnan *et al.*, 2003; Delpire, 2000; Hubner *et al.*, 2001; Rivera *et al.*, 1999). Low intracellular Cl^- sets Cl^- equilibrium potential negative to resting membrane potential. Thus ligand-gated opening of $GABA_A$Rs and GlyRs leads to influx of Cl^- and results in hyperpolarization of membrane potential and down-

regulation of excitation (Ehrlich *et al.*, 1999; Redecker *et al.*, 2002; Rivera *et al.*, 1999; Yamada *et al.*, 2004).

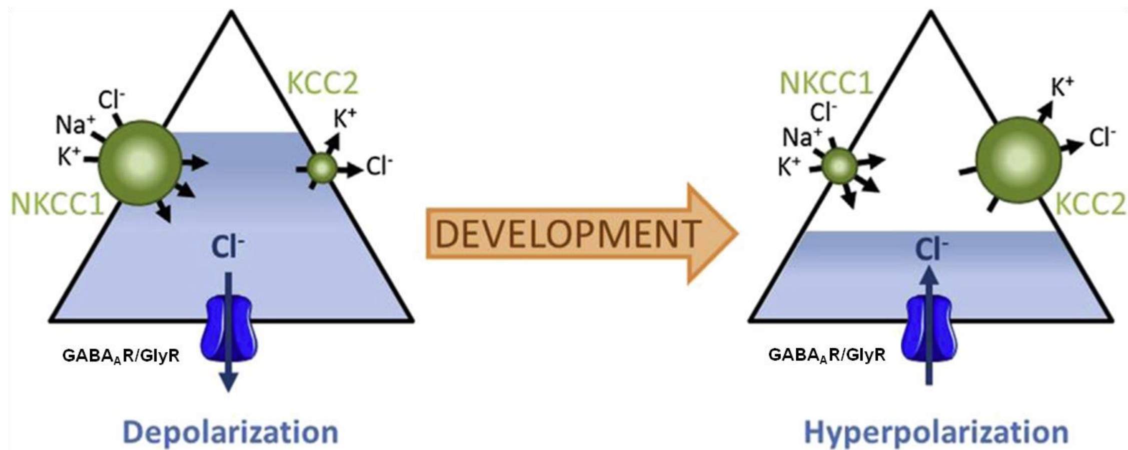


Figure 2. Schematic graph (adapted from Nardou et al., 2013) illustrating developmental changes in relative contribution of NKCC1 and KCC2 on chloride homeostasis. Left: In immature neurons, intracellular chloride accumulates due to higher expression of NKCC1 and low expression KCC2. As a result, activation of $GABA_A$Rs and GlyRs is depolarizing. Right: In mature neurons, intracellular chloride is maintained at low level due to higher expression of KCC2 and low expression NKCC1. Thus, activation of $GABA_A$Rs and GlyRs is hyperpolarizing.

Synaptic $GABA_A$Rs and GlyRs exert short-lasting, so-called phasic action, which can be either hyperpolarizing or depolarizing depending on directions of Cl^- currents (Farrant & Nusser, 2005). However extrasynaptic $GABA_A$Rs and GlyRs activated by ambient GABA or glycine, respectively, mediate a relatively long-lasting effect which is termed tonic action (Farrant & Nusser, 2005). Tonic action leads to inhibitory effect on membrane potential via a mechanism called 'shunting' inhibition in both mature and immature CNS (Farrant & Nusser, 2005; Mitchell & Silver, 2003). Shunting inhibition is mediated by increased membrane conductance toward opening of $GABA_A$Rs and GlyRs. Increased membrane conductance shunts excitatory inputs if the reversal potential is below action potential threshold (Chao *et al.*, 2010; Jean-Xavier *et al.*, 2007; Kilb, 2012; Lamsa *et al.*, 2000; Qian & Sejnowski, 1990; Staley & Mody, 1992). By shunting inhibition, $GABA_A$Rs and GlyRs suppress glutamatergic input because reversal potential of $GABA_A$Rs and GlyRs in immature CNS is still negative to that of

glutamate receptors. So $GABA_A$Rs and GlyRs mediate stringent inhibitory effects in the mature CNS, but contribute to either excitation or inhibition in immature CNS.

In addition, subunit composition of GlyR changes with development. Transcripts of α1 subunits of GlyRs increase strongly after birth and dominate in the adult rat CNS. In contrast to α1 subunit, α2 subunit is widely expressed at early embryonic stage and diminishes postnatally. Expression of α3 and α4 subunits is overall very lower and regionally confined. Similar to α1 subunit, there is increment in the expression of α3 subunit after late developmental stage (Meyer *et al.*, 1995; Racca *et al.*, 1998). In accordance with the expression pattern of GlyR, physiological studies showed that affinity to the ligands, Cl^- permeability and phosphorylation sites of GlyR change during development (Betz & Laube, 2006). The developmental shift of subunit composition of GlyRs possibly coincides mutually with its shift from depolarizing to hyperpolarizing actions, as well as its functional roles (Garcia-Alcocer *et al.*, 2008).

Developmental changes in the properties of GlyR may correspondingly contribute to the control of sequential developmental processes of the brain (Fig. 3, Avila *et al.*, 2013a). During embryonic and earlier postnatal stages, there is evidence that GlyRs participate in developmental processes such as neurotransmitter release (Kawa, 2003; Platel *et al.*, 2005), cell differentiation (McDearmid *et al.*, 2006) and cell migration (Avila *et al.*, 2013b; Nimmervoll *et al.*, 2011). At postnatal development and adult phases, GlyRs are involved mainly in modulating neuronal excitability as excitatory or inhibitory receptors depending on depolarizing or hyperpolarizing action.

1.5 Epilepsy and seizure

Seizure represents clinic manifestation of the rapid, disordered, excessive, hypersynchronized electrophysiological activity of a large population of neurons in the CNS (Fisher *et al.*, 2005). Epilepsy is a major chronic neurologic disorder characterized by unprovoked, repetitive seizures (Berg *et al.*, 2010; Chang & Lowenstein, 2003). Thus at least two unprovoked seizures are necessary for the definition of epilepsy. More than half of epileptic seizures occur for predisposing or idiopathic causes and probably are mainly acquired form heredity (Johnson & Shorvon, 2011; Shorvon, 2011). The causes of other epileptic seizures include a variety of diseases, especially neurologic disorders such as stroke, brain tumor, neuroinflammation, hypoxia-ischemia, infection,

misuse of drugs, etc (Johnson & Shorvon, 2011; Shorvon, 2011). A basic mechanism underlying epileptic seizures is probably an impaired balance between excitation and inhibition toward overexcitation (Avoli, 1983; Mody *et al.*, 1992).

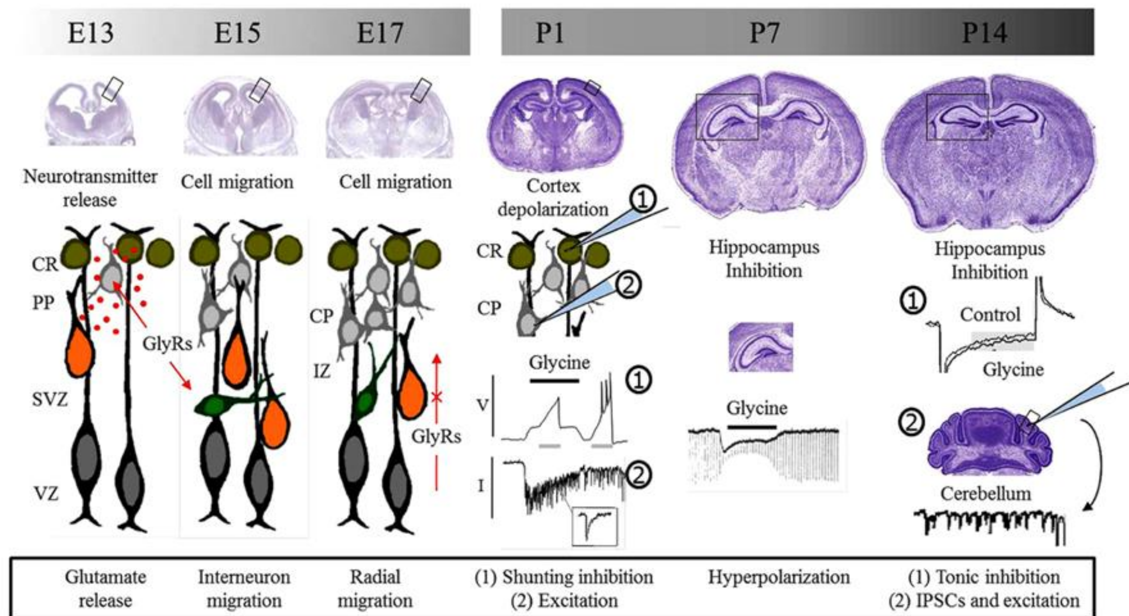


Figure 3. Schematic graph (adopted from Avila et al., 2013a) illustrating contribution of GlyR to sequential developmental processes. Left: During prenatal development of the brain, GlyRs are involved in the modulation of neurotransmitter release and control of cell migration (green cells represent migrating interneurons; orange cells represent radially migrating cells). Right: during postnatal development of the brain, GlyRs control neuronal excitability depending on the depolarizing or hyperpolarizing action. E, embryonic day; P, postnatal day; CR, Cajal–Retzius cells; PP, pre-plate; CP, cortical plate; SVZ, sub-ventricular zone; VZ, ventricular zone; IZ, intermediate zone; GlyRs, glycine receptors; V, voltage; I, current.

Clinic manifestation of seizures varies in behaviour, subjective sense and consciousness, depending on the basic function of the involved neuronal population. According to various manifestations, epileptic seizures can be categorized into 3 types: partial seizures, generalized seizures and unclassified seizures (table 3, Dreifuss, 1989). Partial seizures happen focally within one brain hemisphere. Depending on whether consciousness is kept during seizures, partial seizures are divided to simple partial seizures if consciousness is kept and complex partial seizures if consciousness is lost. Generalized seizures refer to seizures occurring simultaneously at both hemisphere

without identifiable etiology and onset. Generalized seizures may or may not preserve consciousness and may or may not produce muscular convulsion. A generalized seizure is sub-classified into six categories (table 3).

Epilepsy expresses in various syndromes, in which general constituents are the neuronal overexcitation and seizure generation, based on etiology, pathological location, symptoms, family history, electroencephalogram (EEG) pattern, onset age, etc. They can be defined by the International Classification of Epilepsies and Epileptic Syndromes scheme (table 4).

Table 3. The International Classification of Epileptic Seizures (Dreifuss, 1989).

Partial seizures
Simple partial seizures
With motor symptoms
With somatosensory or special sensory symptoms
With autonomic symptoms
With psychic symptoms
Complex partial seizures
Beginning as simple partial seizures
Impairment of consciousness at the onset
Partial seizure becoming secondarily generalized
Generalized seizures
Absent seizures
Typical absent seizures
Atypical absent seizures
Myoclonic seizures
Clonic seizures
Tonic seizures
Tonic-clonic seizures
Atonic seizures
Unclassified seizures

1.6 Epilepsy and seizure in the developing brain

While epileptic seizures are the major neurologic problem, it has higher incidence in children. Approximately 45 out of 100,000 children are suffering from epilepsy every year (Camfield & Camfield, 1996; Wirrell *et al.*, 2011). In particular, in the first one year of human life, epilepsy occurs at its highest incidence (Hauser, 1994; Moshe *et al.*, 1983). Neonatal seizures, which occur in the neonatal period of humans, are the most common epileptic seizures in childhood (Hallberg & Blennow, 2013; Lanska *et al.*, 1995; Mizrahi & Clancy, 2000). Due to the fragility in molecular, cellular and network

levels during development, uncontrolled seizures and epilepsy in children often result in a series of physical, mental, psychiatric dysfunction and social outcomes (Wirrell, 2013).

The high incidence and susceptibility to epileptic seizures for children are primarily due to high exposure to numerous developmental malfunctions and many neurologic insults such as hypoxia, traumatic brain injury, infectious encephalopathy, metabolic disorders, nutrition deficiency (Hauser, 1994; Holmes & Ben-Ari, 2001; Scher *et al.*, 1993; Silverstein & Jensen, 2007). Besides, higher neuronal excitability due to depolarizing action of $GABA_A$Rs in the immature brain is believed to make large contribution to the susceptibility to childhood epileptic seizures (Ben-Ari *et al.*, 1989; Ben-Ari, 2002; Ben-Ari *et al.*, 2007).

One critic issue in the treatment with childhood seizures and epilepsy is that, approximately 20 percent of children are pharmacoresistant to anti-epileptic drugs (AEDs) available in pharmacy (Jarrar & Buchhalter, 2003; Wirrell, 2013). Although other therapeutic options such as surgery and dietary control may be suitable to some pharmacoresistant cases, a large population of children with epileptic seizures undergo poor control on their situation and are associated with worse comorbidities (Baca *et al.*, 2011; Johnson *et al.*, 2004; Tellez-Zenteno *et al.*, 2007).

1.7 GlyR and epileptic seizures

The balance between excitation and inhibition is crucial for normal brain function. Epileptic seizures are thought to be a result of unbalanced runaway of excitation in network (Liu *et al.*, 2007; Parpura *et al.*, 1994; Turrigiano, 1999). As glutamate and GABA are the main excitatory and inhibitory neurotransmitters in the CNS, balance between excitation and inhibition mainly relies on relative weight of glutamatergic and GABAergic activation (Dichter & Ayala, 1987; Le Roux *et al.*, 2008; Liu *et al.*, 2007; Morimoto, 1989). On the basis this notion, large amount of studies on epileptic seizures centre around roles of glutamatergic and GABAergic activation in the mechanism and medication of epilepsy (Babb *et al.*, 1998; Bradford, 1995; Houser & Esclapez, 1996; Loup *et al.*, 2000; McNamara, 1994; Meldrum *et al.*, 1999; Perreault & Avoli, 1992). Most of the currently available AEDs are designed effecting on

Table 4: International Classification of Epilepsies and Epileptic Syndromes (International League Against Epilepsy, 1989).

- *Localization-related (focal, local, partial) epilepsies and syndromes*
  - *Idiopathic with age-related onset*
    - *Benign childhood epilepsy with centrotemporal spikes*
    - *Childhood epilepsy with occipital paroxysms*
  - *Symptomatic*
    - *Chronic progressive epilepsia partialis continua of childhood*
    - *Syndromes characterized by seizures with specific modes of precipitation*
    - *Temporal lobe epilepsies*
    - *Frontal lobe epilepsies*
    - *Parietal lobe epilepsies*
    - *Occipital lobe epilepsies*
  - *Cryptopgenic*
- *Generalized epilepsies and syndromes*
  - *Idiopathic, with age-related onset (listed in order of age)*
    - *Benign neonatal familial convulsions*
    - *Benign neonatal convulsions*
    - *Benign myoclonic epilepsy in infancy*
    - *Childhood absence epilepsy (pyknolepsy)*
    - *Juvenile absence epilepsy*
    - *Juvenile myoclonic epilepsy (impulsive petit mal)*
    - *Epilepsy with grand mal seizures on awakening*
    - *Other generalized idiopathic epilepsies not defined above*
    - *Epilepsies with seizures precipitated by specific modes of activation*
  - *Cryptogenic and/or symptomatic (listed in order of age)*
    - *West syndrome (infantile spasms)*
    - *Lennox-Gastaut syndrome*
    - *Epilepsy with myoclonic-astatic seizures*
    - *Epilepsy with myoclonic absences*
  - *Symptomatic*
    - *Nonspecific etiology*
      - *Early myoclonic encephalopathy*
      - *Early infantile epileptic encephalopathy with suppression burst*
      - *Other symptomatic generalized epilepsies not defined above*
    - *Specific etiology*
      - *Epileptic seizures may complicate many disease states*
- *Epilepsies and syndromes undetermined as to whether they are focal or generalized*
  - *With both generalized and focal seizures*
    - *Neonatal seizures*
    - *Severe myoclonic epilepsy in infancy*
    - *Epilepsy with continuous spike waves during slow-wave sleep*
    - *Acquired epileptic aphasia (Landau-Kleffner syndrome)*
    - *Other undetermined epilepsies not defined above*
  - *Without unequivocal generalized or focal features*
- *Special syndromes*
  - *Situation-related seizures*
    - *Febrile convulsions*
    - *Isolated, apparently unprovoked epileptic events*
    - *Seizures occurring only when there is an acute metabolic or toxic event (alcohol, drugs, eclampsia, nonketotic hyperglycemia)*

glutamatergic or GABAergic system to lower the weight of glutamatergic activity or enhance the weight of GABAergic activity, while other agents mainly work on voltage-dependent ionic channels to suppress neuronal excitability and electrical conductivity (Bohme & Luddens, 2001; Kirchner *et al.*, 2003; Schmidt, 2009).

In contrast, the role of glycinergic system in epileptic seizures is less documented. The fact that studies of GlyRs in epilepsy have been less underlined could be due to two reasons. One reason is the absent glycinergic synaptic transmission but only presence of 'extrasynaptic' GlyRs in the higher brain (Betz *et al.*, 2006; Legendre, 2001). The other reason is that, most of electrophysiological researches support the notion that glycinergic system, comparing with GABAergic system, seems to play a less important role in inhibitory tone in the CNS (Curtis *et al.*, 1970; Trombley *et al.*, 1999).

Since strychnine, the active component of plant strychnos (Philippe *et al.*, 2004) which is known to be epileptogenic since ancient times, was identified as an antagonist of GlyR about 40 years ago (Young & Snyder, 1973), association of GlyRs with epilepsy was investigated both on human beings and animals. Application of GlyR agonists taurine and glycine has been proved to be beneficial to calm down convulsion in adult rodent brain (Chattipakorn & McMahon, 2003; Cherubini *et al.*, 1981; Durelli *et al.*, 1976; El Idrissi *et al.*, 2003; Halsey *et al.*, 1989; Peterson, 1986; Seiler & Sarhan, 1984). Inhibitory effect of TauT and GlyT blockers on epilepsy has been also reported in the mature CNS (Bonhaus *et al.*, 1985; Cherubini *et al.*, 1981; Goodman *et al.*, 1980; Harvey & Yee, 2013; Zhang *et al.*, 2008a). However, in the immature CNS so far there is no available information about the influence of the glycinergic system on epileptiform activity.

1.8 Aims of this study

Along with $GABA_A$Rs, GlyRs provide fast inhibitory synaptic transmission in the spinal cord and brain stem. However, in the forebrain fast inhibitory synaptic transmission is completely abolished by antagonist of $GABA_A$R, indicating $GABA_A$R is exclusively involved in fast inhibitory synaptic transmission in forebrain. On the hand, numerous investigations pointed out that expression of GlyRs in the higher portion of brain is wide and abundant. Moreover, GlyRs in the higher brain are functionally coupled to the structural and functional development since middle embryonic days,

being involved in several important developmental processes, such as neurotransmitter release, cell differentiation, cell migration, network activity as well as neuronal excitability.

As epileptic seizures have a particularly high incidence during early life and childhood epileptic seizures show a poor responsiveness to traditional medication by AEDs, which mainly target on glutamatergic and GABAergic systems, in particular for these cases alternative pharmacological targets are required. So far no AED is designed on the basis of glycinergic system despite that GlyRs are functionally expressed throughout the brain. Thus, alternatively glycinergic system could potentially be a target for the control of epileptic seizures, especially pharmacoresistant ones. Researches performed on the adult rodent brains have promisingly proved that activation of GlyRs is capable of suppressing epileptiform activity.

However, no study has been done to reveal whether activation of GlyRs modulates epileptiform activities in the immature CNS. Therefore the studies summarized in this thesis address the role of GlyRs in the modulation of neuronal excitability and epileptiform activity in the immature hippocampus.

2 Material and Methods

2.1 Solutions and drugs

Standard artificial cerebrospinal fluid (ACSF) used during preparation, incubation and recording consisted of (in mM) 126 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 2.5 KCl and 10 glucose, with pH 7.4 after being equilibrated with carbogen (95% O_2 and 5% CO_2) at least one hour before use. To make low-Mg^{2+} solutions, $MgCl_2$ was deleted from and additional 1 mM $CaCl_2$ was added to standard ACSF. Low-Mg^{2+} solutions were also equilibrated with carbogen at least one hour before use. Strychnine, taurine, glycine, muscimol, γ-aminobutyric acid (GABA), 4-Aminopyridine (4-AP), bumetanide, D-Serine, N-[3-(4'-Fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine hydrochloride (ALX 5407), O-[(2-Benzyloxyphenyl-3-flurophenyl)methyl]-L-serine (ALX-1393) and 1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt hydrate (NBQX) were purchased from Sigma (Taufkirchen, Germany). DL-2-Amino-5-phosphonopentanoic acid (APV) and gabazine were purchased from Biotrend (Cologne, Germany), and guanidinoethanesulfonic acid (GES) from TRC (North York, Canada). Bumetanide, 4-AP, strychnine, gabazine, NBQX, APV, ALX 5407 and ALX 1393 were dissolved in dimethylsulfoxide (DMSO) to make stock solution, while GABA, Glycine, Taurine, D-Serine and GES were dissolved in distilled water. All substances were diluted to final concentrations in recording solutions form substances stock solutions shortly before the experiments. The DMSO concentration of the final solution was no more than 0.2%.

2.2 Preparation

2.2.1 Ethical approval for animal use

All experimental procedures were performed in accordance with EU directive 86/609/EEC for the animal use in research and the National Institutes of Health (NIH) Guidelines for the care and use of laboratory animals and with approval of the local ethical committee (Landesuntersuchungsanstalt RLP, Koblenz, Germany). All the animals were obtained from the institute's animal facility. Maximal efforts were done to minimize the number of animals and their suffering.

2.2.2 Preparation of hippocampal slice

Wistar rat pups of P (postnatal days) 4–7 were deeply anesthetized with enflurane (Ethrane; Abbot Laboratories, Wiesbaden, Germany). The animals were quickly decapitated after anesthesia, and the brains were rapidly removed and immersed for 2–3 min in ice-cold standard ACSF saturated with carbogen. Then the brains were trimmed according to requirement of semicoronal slice (tilted between 10° and 45° in medial direction). The trimmed brains were fixed on the platform of microtome vibrating slicer (HR2, Sigmann Elektronik, Hüffenhardt, Germany) where 400 µm thick semicoronal slices (Canto & Witter, 2012), including hippocampi were cut in ice-cold ACSF. All cut slices were transferred to an incubating chamber filled with constantly carbogenated ACSF at room temperature. Slices were incubated at least one hour before being moved to recording chamber.

2.3 Preparation of intact corticohippocampal formation (CHF)

For preparation of the intact CHF (Khalilov *et al.*, 1997; Kilb *et al.*, 2007; Moser *et al.*, 2006; Sharopov *et al.*, 2012) neonatal rat pups of P4–7 were also used. After anesthesia and subsequent decapitation, the brains were quickly removed and immersed for 2–3 min in the continuously carbogenated ice-cold ACSF, the cerebellum was removed and the two hemispheres were separated by a scalpel cut through the midline. The frontal cortex, brain stem and all diencephalic structures were removed from separated hemispheres to isolate intact CHF from which the pial membranes were carefully stripped. The intact CHF includes whole hippocampus with connection to entorhinal and temporal cortex (Fig. 6). Isolated CHF were transferred to a fully submerged chamber and constantly superfused with carbogenated ACSF at a flow rate of ~5 ml/min (Khalilov *et al.*, 1997; Sharopov *et al.*, 2012) at 30 ± 1°C. Preparations were incubated more than 45 min before recording in the same chamber (Sharopov *et al.*, 2012).

2.4 Data acquisition and analysis

2.4.1 Data acquisition and analysis for whole-cell recordings in the slice

Whole-cell patch-clamp recordings from CA3 pyramidal neurons of hippocampal slices were performed at 30 ± 1 °C in a submerged recording chamber on the fixed stage of microscope (BX51 WI, Olympus). The recording temperature was manipulated by a peltier-element based temperature controller. Slices in the recording chamber were continuous superfused with carbogenated ACSF at a rate of about 2 ml/min. Under infrared differential interference contrast optics (C5405, Hamamatsu, Japan) and CCD-camera, pyramidal neurons were recognized according to their morphological appearance and location. All recorded cells were filled with 0.5% biocytin through recording pipettes for morphological staining for post-hoc confirmation on appearance and location. Signal data were acquired with a discontinuous voltage-clamp/current-clamp amplifier (SEC05L, NPI, Tamm, Germany), low-pass filtered at 3 kHz. Acquired data were stored and processed on a personal computer through an AD/DA board (ITC-16, HEKA, Lamprecht, Germany) and TIDA software (Tida 4.11; Heka, Lambrecht, Germany).

Patch pipettes were made from borosilicate glass capillaries (2.0 mm outside and 1.16 mm inside diameters, Science Products, Hofheim, Germany) and pulled with a vertical puller (PP-830, Narishige) to have an impedance of 5-12 MΩ when filled with pipette solution. Pipette solution contains (in mM) 80 K-Gluconate, 44 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 10 HEPES, 2 Na_2-ATP, 0.5 Na-GTP (pH was 7.4 adjusted with KOH and osmolarity was 306 adjusted with sucrose).

Spontaneous postsynaptic currents or focal pressure application of substances-induced currents were recorded in whole-cell voltage-clamp model at holding potentials of -60 mV. To monitor the quality of patched cells before voltage-clamp recordings, resting membrane potentials were recorded immediately after establishment of whole-cell configuration and membrane input resistance were determined in current-clamp or voltage-clamp models. The averaged resting membrane potential was -65.6 ± 1 mV after a correction of liquid junction potential of 9.6 mV and membrane input resistance 1.4 ± 0.1 GΩ, in agreement with previous study (Tyzio *et al.*, 2003). For focal pressure application experiments, substances such as glycine, GABA and taurine were loaded in a patch pipette and applied directly to the soma for 2-100 ms by a pressure of 0.4 bar

through a pressure application system (PDES 02T, NPI or LHDA0533115H, USA). Minianalysis program (Synaptosoft, USA) was used to analyze the frequency and amplitude of spontaneous postsynaptic currents with at least 3 min recording for each condition like baseline and drug wash-in. Either Minianalysis or Tida program was used for the analysis of the amplitude of focal pressure application of substance-induced responses.

2.4.2 Data acquisition and analysis for field potential recordings in the CHF

Extracellular field potential of population spikes in epileptiform discharges was recorded in the stratum radiatum of hippocampal CA3 region of the intact CHF using tungsten microelectrodes (4–5-MΩ, FHC, Bowdoinham, ME). Signals were recorded with a purpose built amplifier with maximal 8 channels, low-pass filtered at 3 kHz. Acquired data were stored on a person computer through an AD/DA board (ITC-16, HEKA, Lamprecht, Germany) and 8 channels software (Tida 4.11; Heka, Lambrecht, Germany). Up to four separate CHFs were recorded simultaneously by independent 4 channels. Data were analyzed by either MiniAnlysis program or threshold crossing algorithms developed on the basis of Matlab environment (Matlab R2006a; Mathworks, Natick, MA). Data of the identified population spikes in epileptiform discharges were extracted to an excel-script where data were classified and grouped into ictal-like, interictal and intermediate events according to discharge properties. Parameters or properties for the classification and quantification of epileptiform discharges such as amplitude of discharges, occurrence (events per time window) or frequency of discharges, duration of discharge, interval between discharges, and number of spikes within a discharge were analyzed.

2.4.3 Post-hoc morphological staining of pyramidal cells

In all whole-cell experiments, for post-hoc confirmation on appearance and location of recorded cells, all recorded cells were filled with 0.5% biocytin (Sigma, Taufkirchen, Germany) through recording pipettes for morphological staining and reconstricution (Achilles *et al.*, 2007). Immediately after recording, slices were fixed in a 4% parafolmaldehyde solution overnight. Subsequentlty slices were washed in with phosphate buffer and incubated in blocking solution. Afterwards slices were stained with streptavidin-coupled Cy-3 (Dianova) fluorophore (Dianova, Hamburg, Germany).

For resconstruction, fluorescence was excited with the 568 nm line of a Kr/Ar laser (Laser Physics, Malpas, Uk).

2.4.4 Statistical analysis

Statistical data were displayed as mean ± SEM (standard error of the mean). The sign test and Mann-Whitney U-test (Systat 11 and SPSS 13.0) were employed for the tests of statistical significance. Significance levels were represented as * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.

3 Results

3.1 Pharmacologic properties of GlyR-mediated currents

To investigate the potential action of GlyRs on epileptiform activity in immature rodents, I first clarified the pharmacologic properties of GlyR-mediated currents of pyramidal neurons (Fig. 4A) in the P4-7 rat hippocampal CA3 region, which plays a central role in the initiation and propagation of epileptiform activity in some in vitro models of epilepsy (Barbarosie & Avoli, 1997; D'Arcangelo *et al.*, 2005; Ishizuka *et al.*, 1990; Kohling *et al.*, 1995; Luhmann *et al.*, 2000; Nagao *et al.*, 1994; Wheal *et al.*, 1998).

Previous study has shown that the affinity of GlyRs in the spinal cord to its agonist, glycine, is similar between earlier developmental and mature stages (Tapia & Aguayo, 1998), while some other pharmacologic properties of GlyRs, e.g. sensitivity to antagonist, change during development (Aguayo *et al.*, 2004; Tapia & Aguayo, 1998). Therefore I analyzed sensitivity of GlyRs in the CA3 region of immature rat hippocampus to its antagonist, strychnine, using whole-cell patch clamp recordings with 50 mM Cl^- in recording pipette solution to mimic high Cl^- concentration in immature neurons (Ben-Ari, 2002; Kahle & Staley, 2008). Under voltage-clamp conditions, focal application of 20 µM glycine to the soma of P4-7 hippocampal CA3 pyramidal neuron induced an inward current of 53.2 $\pm$ 7.1 pA (n=15. Fig. 4B1). Dose response experiments revealed that the glycinergic current was rather sensitive to strychnine. Strychnine at concentrations of 0.3 µM and 1 µM has already reduced the current by 96.7 $\pm$ 3.9% and 98.4 $\pm$ 1.3%, respectively. At 3 µM, strychnine completely blocked glycinergic currents (n=11. Fig. 4B1, C).

There have been reports that strychnine also antagonizes $GABA_AR$ (Oja *et al.*, 1990; Puka & Lazarewicz, 1993; Shirasaki *et al.*, 1991). In order to validate the pharmacological distiction between GlyR and $GABA_AR$ by the GlyR antagonist strychnine or the $GABA_AR$ antagonist gabazine, I checked the sensitivity of $GABA_AR$ to strychnine and GlyR to gabazine. Focal application of 20 µM GABA produced much larger inward current of 267.4 $\pm$ 81.1 pA (n=6. Fig. 4B2), which was relatively insensitive to strychnine. When the concentration of strychnine is lower than 1 µM, it exerted no obvious effect on GABAergic currents. At 3 µM, strychnine slightly but not

significantly attenuated the GABAergic current by 4.5 ± 10.4%. It did significantly inhibit GABAergic current by 59.4 ± 21.6% when the concentration was up to 30 μM (Fig. 4B2, C. n=5-6).

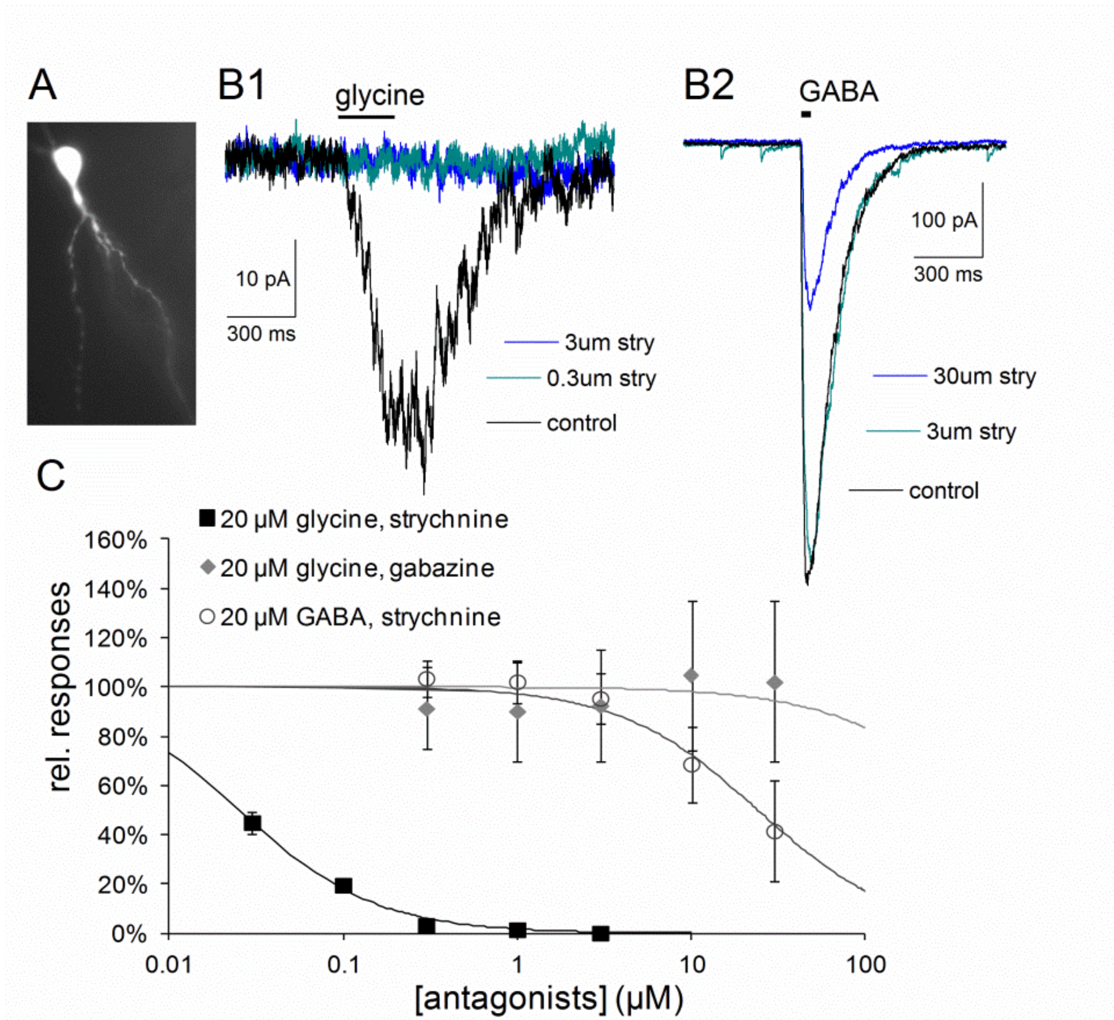


Figure 4. Effects of GlyR antagonist strychnine and $GABA_A$R antagonist gabazine on focally-applied glycine- or GABA-induced currents. A: Photomicrograph of a typical CA3 pyramidal neuron labeled with biocytin through recording pipette electrode. B1: Representative traces illustrating strychnine (stry) sensitivity of focal application of 20 μM glycine-evoked inward currents. B2: Representative traces illustrating strychnine (stry) sensitivity of focal application of 20 μM GABA-evoked inward currents. C: Dose-response curve showing high sensitivity of glycine-evoked currents to strychnine, relative insensitivity of GABA-evoked currents to strychnine, and rather insensitivity of glycine-evoked currents to gabazine. ('rel.' in the title of the vertical axis of this and below figures means relative value to the value of baseline or control).

Many studies have shown that gabazine is a specific and effective antagonist of $GABA_AR$ both in mature and immature neurons (Hussy *et al.*, 1997; Kilb *et al.*, 2008; Kolbaev *et al.*, 2012; Ueno *et al.*, 1997). Consistent with these studies, my experiments showed that gabazine had virtually no obviously observable effect on glycinergic current induced by focally applied 20 μM glycine even at a very high concentration of 30 μM (n=4. Fig. 4C). Thus specificity of gabazine on $GABA_AR$ was confirmed. Comparable investigations on the properties of GlyR-mediated currents in CA3 of P4-7 rat hippocampus have been performed in our lab by Akihito Okabe, whose data were virtually identical with mine.

Subsequently, I analyzed the strychnine sensitivity of pharmacologically isolated spontaneous GABAergic postsynaptic currents (GABA-PSCs). Spontaneous GABA-PSCs were recorded in the continuous presence of 10 μM NBQX and 60 μM APV to block synaptic glutamatergic events. The averaged amplitude and frequency of spontaneous GABA-PSCs were -8.7 ± 0.7 pA and 1.6 ± 0.4 Hz (n=18. Fig. 5A), respectively. All the events were completely suppressed by 1-3 μM gabazine (n=5), confirming that they were exclusively fast GABAergic synaptic events and consistent with previous finding that no glycinergic synaptic event could be detected in this region (Freund & Buzsaki, 1996; Mody *et al.*, 1994; Mori *et al.*, 2002). While application of 0.03 μM, 0.1 μM, 0.3 μM strychnine had no significant effect, 1 μM, 3 μM, 10 μM strychnine significantly reduced the spontaneous GABA-PSCs amplitude by 26.7 ± 5.5%, 53.2 ± 6.3%, 79.1 ± 5.5% (n=7 for each concentration. Fig. 5A, B), respectively, demonstrating a dose dependent reduction of amplitude of spontaneous GABA-PSCs by strychnine. In agreement with the change of amplitude, the spontaneous GABA-PSCs charge transfer diminished in the same dose dependent manner (Fig. 5B), indicating that strychnine suppressed spontaneous GABA-PSCs via acting on postsynaptic site, most likely non-specifically on $GABA_ARs$ (Mori *et al.*, 2002).

Taken together, these data indicate that GlyRs do not contribute to fast PSCs of pyramidal neurons at hippocampal CA3 region, and that strychnine and gabazine are capable of relatively identifying currents mediated by GlyR and $GABA_AR$. At concentration of 0.3 μM, strychnine can have already largely attenuated glycine-evoked currents, but has no effects on both GABA-induced currents and phasic GABAergic PSCs. At 3 μM, strychnine completely abolishes glycine-evoked currents, but meanwhile slightly reduces GABA-induced currents and significantly reduces phasic

GABAergic PSCs. While gabazine, at 3 μM, has no any effect on glycine-evoked currents, but completely abolishes all GABAergic currents.

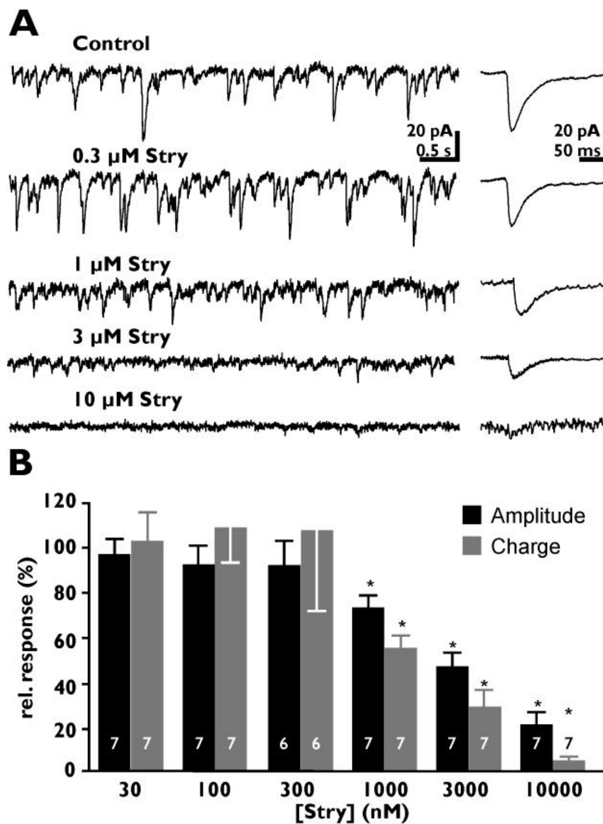


Figure 5. Effects of glycine receptor antagonist strychnine on isolated spontaneous GABA-PSCs in the presence of glutamatergic blockers NBQX and APV. A: Representative traces (left, original traces; right, average of spontaneous GABA-PSCs in the left traces) illustrating effects of strychnine on spontaneous GABA-PSCs. B: Statistical analysis showing that < 1 μM strychnine had no effect on spontaneous GABA-PSCs, while ≥ 1 μM strychnine reduced the amplitude and charge transfer of spontaneous GABA-PSCs.

3.2 In vitro intact CHF for the study of epileptiform activity

In vitro intact CHF (Fig. 6) contains brain structures including intact hippocampus, entorhinal cortex, partial temporal cortex, and preserves native connection between these structures (Figueroa *et al.*, 2002). Isolated CHF from newborn rodent's brain is highly viable and thus offers unique advantages of stable, long-term recording (Moser *et al.*, 2006). If necessary for the study, the preparation is compatible for multi-site recordings at visually-identified sub-structures especially for epileptiform activities which are highly synchronized within formation, with highest amplitudes at CA3 (Luhmann *et al.*, 2000). Additionally, as blood-brain barrier is taken off, drug application to this preparation is as rapid as many other in vitro tissue. Thus isolated CHF has been accepted as a useful model for the study of epileptiform activity

(Khalilov *et al.*, 1997; Moser *et al.*, 2006; Quilichini *et al.*, 2002; Sharopov *et al.*, 2012).

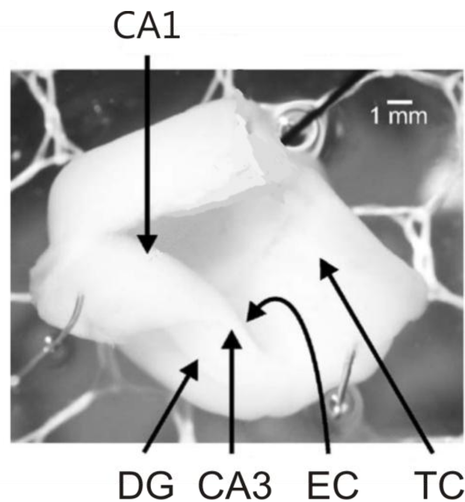


Figure 6. Photograph (adapted from Moser et al. 2006) indicating the structure of in vitro intact CHF. CA: *Cornu Ammonis area. DG: dentate gyrus. EC: entorhinal cortex. TC: temporal cortex.*

In my study, electrodes for the recording of epileptiform activity were precisely located at striatum of hippocampal CA3 region, according to the previous reports that CA3 plays a critic role in the generation and propagation of epileptiform activity (Barbarosie & Avoli, 1997; D'Arcangelo *et al.*, 2005; Ishizuka *et al.*, 1990; Kohling *et al.*, 1995; Luhmann *et al.*, 2000; Nagao *et al.*, 1994; Wheal *et al.*, 1998).

3.3 In vitro model of epileptiform activity induced by low Mg^{2+}/4-AP

In low Mg^{2+} ACSF, bath application of 20 µM 4-AP reliably induced highly stable and repetitive epileptiform discharges in CA3 of CHF isolated form P4-7 rats (Kilb *et al.*, 2006; Kilb *et al.*, 2007; Sharopov *et al.*, 2012). Epileptiform activity induced by low Mg^{2+}/4-AP started with one or few ictal-like discharges which were converted to short repetitive interictal discharges (Fig. 7A, B). These repetitive discharges occurred at a frequency of 0.33 ± 0.02 Hz, had an average of amplitude of 981.9 ± 69.1 µV. Each discharge lasted with duration of 0.61 ± 0.04 ms and consisted of 8.1 ± 0.3 single spikes. The frequency of spikes within a discharge was 20.7 ± 0.4 Hz (n=85. Fig. 7A, B). Among those parameters, occurrence or frequency of repetitive discharges (occurrence or frequency), spike amplitude (amplitude), frequency of spikes within discharges (freq of spikes) and number of spikes per discharge (# of spikes) are the most important

indicators of neuronal excitability. Thus in the study I mainly analyzed these four parameters of epileptiform discharges. Such repetitive discharges display very uniform pattern, temporal precision and stability which enable the precise detection of pro- and anticonvulsive effects (Kilb *et al.*, 2006; Kilb *et al.*, 2007; Sharopov *et al.*, 2012).

Figure 7. In vitro epileptiform activity in low Mg^{2+}/4-AP model. A Typical field potential recording of epileptiform activity induced by low-Mg^{2+}/20 µM 4-AP solution, showing that epileptiform activity started with ictal-like discharges which were converted to stable short repetitive discharges. B: Typical repetitive discharge as identified in A at higher temporal resolution.

3.4 Effect of strychnine on intrinsic network excitability and epileptogenesis

To investigate the potential action of GlyRs on epileptic seizures, I first I investigated the contribution of GlyRs on the intrinsic overall network excitability of immature CNS. To this end, field potential was recorded in the CA3 region of CHF and strychnine was applied in the bath ACSF to fully inactivate endogenously intrinsic activation of GlyRs. Bath application of 3 µM strychnine induced epileptiform discharges composed of interictal events in 12 out of 16 preparations. The epileptiform discharges induded by 3 µM strychnine occurred 30.7 ± 5.5 min after wash-in at a frequency of 0.014 ± 0.002 Hz and consisted of short bursts of 4 ± 0.5 spikes per discharge with an average spike amplitude of 1089.4 ± 143.8 µV and a spike frequency of 18.8 ± 1.4 Hz (n=12. Fig. 8A, D).

In contrast, inhibition of $GABA_AR$ by bath application of 3 µM gabazine induced considerably stronger epileptiform activity. The gabazine induced epileptiform discharges in significantly faster time of 3.3 ± 0.5 min ($P<0.0001$ by U-test) after wash-in. The epileptiform discharges induced by gabazine had a frequency of 0.04 ± 0.01 Hz

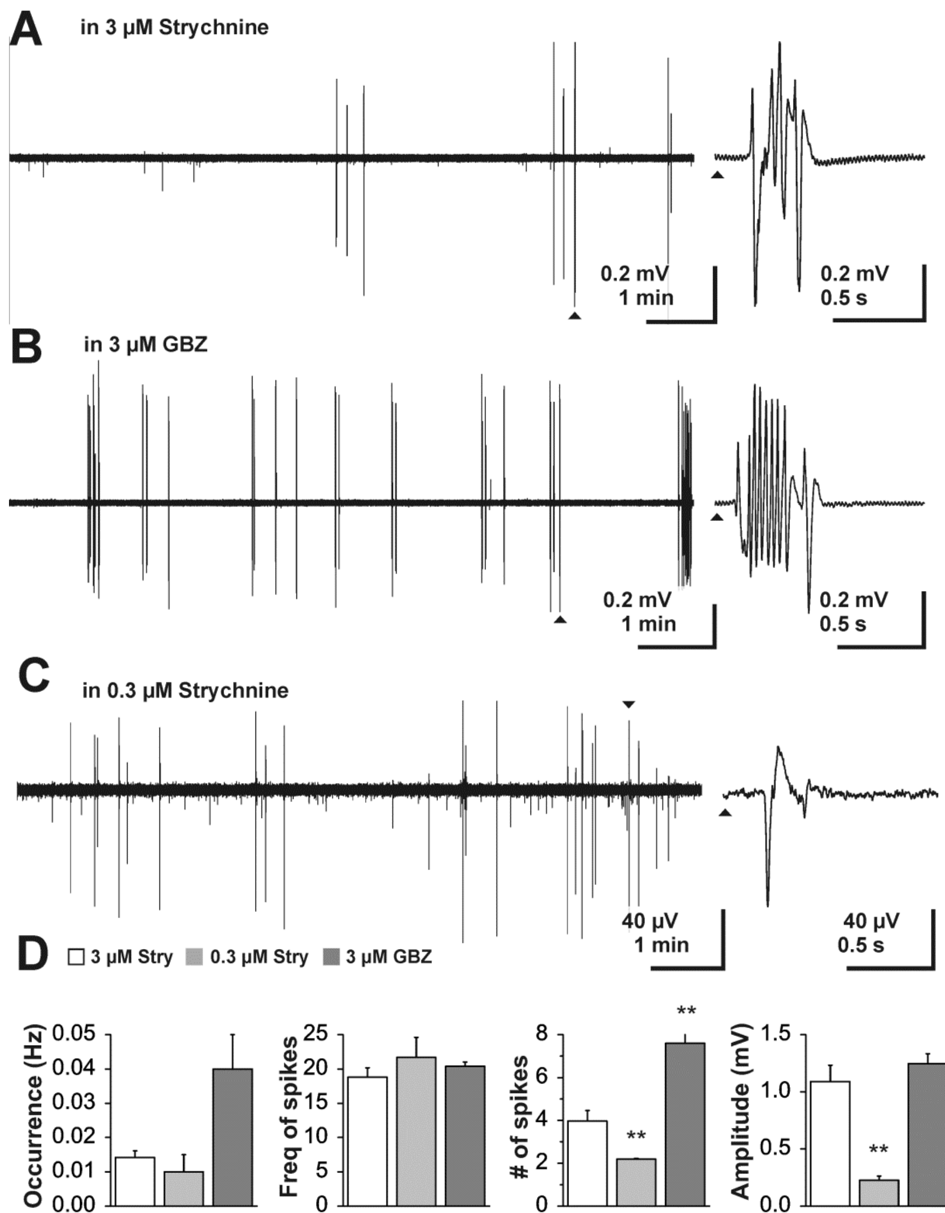


*Figure 8. Effect of glycinergic antagonist strychnine on intrinsic network excitability and epileptogensis. A: Representative field potential recording illustrating that bath application of 3 μM strychnine provoked epileptiform discharges. B: Representative field potential recording illustrating that bath application of 3 μM gabazine (GBZ) provoked stronger epileptiform discharges. C: Representative field potential recording illustrating that bath application of 0.3 μM strychnine provoked less complex epileptiform discharges with lower spike amplitude and less spikes per discharge. The discharges indicated by the arrowheads were displayed at larger temporal resolution on the right side. D: Statistical analysis of occurrence, frequency of spikes, number of spikes and spike amplitude in each epileptiform event of epileptiform discharges provoked by 3 μM strychnine (stry, open bars, n=12), 0.3 μM strychnine (light gray bars, n=5) and 3 μM gabazine (dark gray bars, n=47). * * represents $p<0.01$ by U-test.*

and a spike amplitude of 1246 ± 87 μV, consisting of significantly more number of spikes of 7.6 ± 0.6 per discharge (p=0.002 by U-test) but comparable spike frequency of 20.4 ± 0.6 Hz (n=47. Fig. 8B, D).

A possibility is that 3 μM strychnine induced epileptiform activity by inhibiting a portion of $GABA_A$Rs as shown in Fig 4 & 5. To examine this possibility, I lowered the concentration of strychnine to 0.3 μM to avoid effect of strychnine on $GABA_A$R. Bath application of 0.3 μM strychnine was also capable of inducing epileptiform activity in 5 out of 20 preparations. The epileptiform discharges induded by 0.3 μM strychnine occurred in 29 ± 8 min after wash-in at a frequency of 0.01 ± 0.005 Hz and each discharge consisted of significantly less spikes (2.2 ± 0.03, p=0.0016 by U-test) with significantly lower spike amplitude of 226 ± 37 μV (p=0.0044 by U-test) at a comparable spike frequency of 21.7 ± 2.9 Hz (n=5. Fig. 8C, D).

These results suggest that activation of intrinsic GlyRs in immature rat hippocanpus exerts a persistent inhibitory tone that is required to prevent epileptiform activity, although the inhibitory potency mediated by GlyRs is considerably weaker than that mediated by $GABA_A$Rs.

3.5 Effect of strychnine on epileptiform activity

Next, I investigated how inactivation of GlyRs might interfere with epileptiform activity in the immature CNS, as GlyRs mediate both membrane depolarization and shunting inhibition at the developmental stage. For this purpose, I utilized low Mg^{2+}/4-AP model of in vitro epileptiform activity in the CHF isolated from P4-7 rats. Epileptiform discharges in control condition were recorded after short repetitive discharges were obtained stably. However, bath application of 3 μM strychnine to fully inactivate GlyRs only showed slight effect on epileptiform discharges by increasing the spike amplitude of epileptiform discharges significantly to 123 ± 8.4% (p=0.038 by sign test), but keeping the occurrence (94.2 ± 4.7%, p=0.388 by sign test), frequency of spikes (101.6 ± 3.5%, p=0.774 by sign test), the number of spikes per discharge (123.3 ± 8.6%, p=0.388 by sign test. n=12. Fig. 9A, B, G) undisturbed.

For comparison, bath application of 3 μM gabazine substantially interfered with low Mg^{2+}/4-AP-induced epileptiform discharges. The occurrence of epileptiform discharges significantly decreased to 50.4 ± 4.8% ($p<0.001$ by sign test) and frequency

of spikes within discharges to 90.2 ± 2.5%, $p=0.003$ by sign test). The number of spikes per discharge significantly increased to 155.3 ± 9.6% ($p<0.001$ by sign test) and the spike amplitude to 132.6 ± 8.3% ($p=0.022$ by sign test, n=32. Fig. 9C, D & G). Co-application of 3 µM strychnine and 3 µM gabazine (n=48) showed same effects on epileptiform discharges as application of 3 µM gabazine alone.

To uncover possible additional proconvulsive effect of GlyR inhibition, I applied 3 µM strychnine to the preparations showing 3 µM gabazine-induced epileptiform discharges. These experiments revealed that strychnine did not affect the properties of the gabazine-induced epileptiform discharges (Fig. 9E, F & G). Neither occurrence (98.3 ± 6%, n=22), nor spike amplitude (99.8 ± 2.7%), frequency of spikes (98.4 ± 0.02%) or number of spikes per discharge (101.1 ± 4%, n=20) was significantly altered by 3 µM strychnine.

These results suggest that inhibitory tone of GlyR is less profound than that of $GABA_AR$, and that an inhibition of GlyR is not sufficient to modulate existing epileptiform discharges.

3.6 Effect of taurine on epileptiform activity

After establishing that activation of GlyRs contributes to an inhibitory tone on intrinsic network which prevents spontaneous epileptogenesis, and that inhibition of GlyRs is not sufficient to modulate existing epileptiform discharges, next I elucidated to know how taurine, which has been proposed to be the major endogenous agonist of GlyRs in immature CNS (Flint *et al.*, 1998), might affect neuronal excitability and epileptiform activity.

It has been reported that taurine at concentrations of 0.5-5 mM, but not 0.2 mM, increasingly suppressed seizure-like events in CA1 region of entorhinal-hippocampal slices of adult wistar rats (Kirchner *et al.*, 2003). Similar results were obtained by Akihito Okabe in our lab in CA3 region of isolated CHF of P4-7 wistar rats. In this preparation taurine at concentrations of 0.2-5 mM also had dose-dependent effects on epileptiform discharges induced by low Mg^{2+}/20 µM 4-AP. Bath application of 0.2 and 0.5 mM taurine had no effect on epileptiform activity; 1 mM taurine reduced the occurrence of epileptiform discharges by 29.3 ± 13%. 2 mM taurine blocked

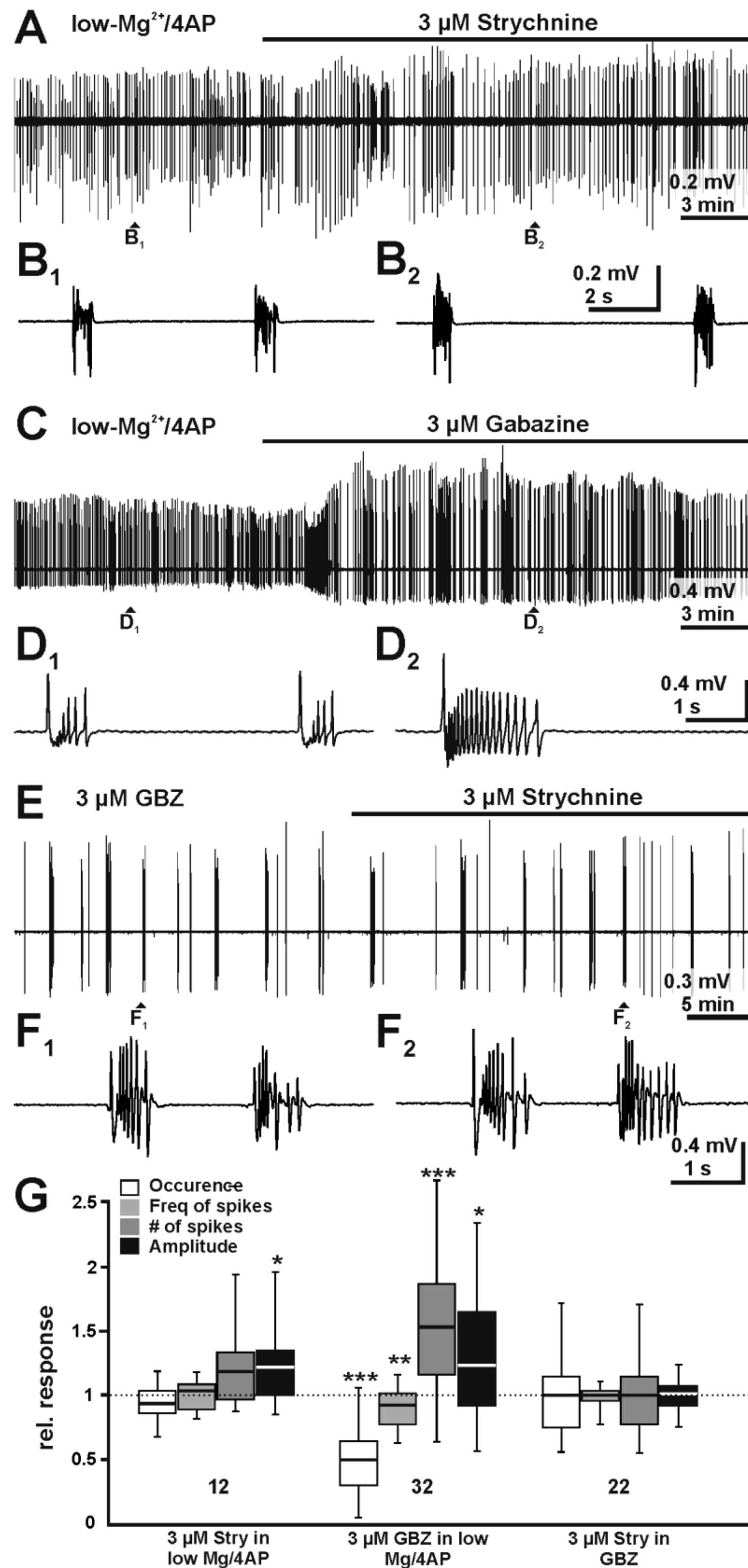


Figure 9. Effect of glycinergic antagonist strychnine on low Mg^{2+}/4-AP-induced epileptiform discharges. A: Bath application of 3 µM strychnine only slightly increased the spike amplitude of low Mg^{2+}/4-AP-induced epileptiform discharges, but had no effect on other parameters. B: Typical discharges under control (B1) and strychnine (B2) conditions as

*identified by arrows in A were displayed at higher temporal resolution. C: Bath application of 3 µM gabazine strongly altered properities of low-Mg^{2+}/4-AP-induced epileptiform discharges. D: Typical discharges under control (D1) and gabazine (D2) conditions as identified by arrows in C were displayed at higher temporal resolution. E: Bath application of 3 µM strychnine had no effect on gabazine (GBZ)-induced epileptiform discharges. F: Typical discharges under control (F1) and strychnine (F2) conditions as identified by arrows in E were displayed at higher temporal resolution. G: Statistical analysis of the effects of strychnine and gabazine on epileptiform discharges induced by low Mg^{2+}/4-AP or 3 µM GBZ. Box plots represent upper/lower quartile and median (bold line); whiskers indicate maximal and minimal values. Numbers of experiments are indicated below the boxes. * represents $p<0.05$; ** represents $p<0.01$; *** represents $p<0.001$ by sign-test.*

epileptiform activity in 6 out of 9 investigated CHF and reduced the occurrence by 85 ± 5.1% in the remaining preparations, while 5 mM taurine completely abolished epileptiform discharges in all 9 investigated preparations. I repeated 6 experiments with 1 mM taurine. Consistent with Akihito Okabe's data, in my hand 1 mM taurine suppressed epileptiform discharges by completely abolishing epileptiform discharges in 1 out of 6 experiments, and attenuating the occurrence of epileptiform discharges in the rest of 5 experiments by 56.6 ± 18.5%, spike amplitude by 22.3 ± 7.3% and number of spikes per discharge by 26.8 ± 14.9% (Fig. 10A, C). Most possibly, suppressing effect of GlyRs on epileptiform discharges was fulfilled through shunting inhibition (Ben-Ari, 2002; Meyer *et al.*, 1995; Pusch *et al.*, 1995).

During early postnatal development activation of GlyRs shows not only shunting inhibition (Ben-Ari, 2002; Meyer *et al.*, 1995) but also excitatory depolarization to the neurons (Ito & Cherubini, 1991; Kilb *et al.*, 2002; Wu & Xu, 2003). Thus I wondered whether taurine could promote epileptiform activity via its excitatory action of GlyRs. A strategy to manifest excitatory action of GlyRs could be applying lower concentration of agonist to avoid strong shunting inhibition of GlyRs produced by higher concentration of agonist. So I lowered the concentration of taurine to 0.1 mM. Actually, bath application of 0.1 mM taurine mildly but significantly promoted epileptiform discharges induced by low Mg^{2+}/4-AP in CA3 region of isolated CHF of P4-7 wistar rats. The occurrence of repetitive epileptiform discharges was promoted by 0.1 mM taurine to 112 ± 3.2% ($p<0.001$ by sign-test), and the frequency of the spikes in a

discharge to 107 ± 2.9% (p=0.015 by sign-test), while the number of spikes per discharge (97 ± 4%) and their amplitude (96.6 ± 3.4) were not significantly altered (n=37. Fig. 10B, C & 13A).

In summary, taurine has both anticonvulsive effect at high concentrations and proconvulsive effect at low concentration.

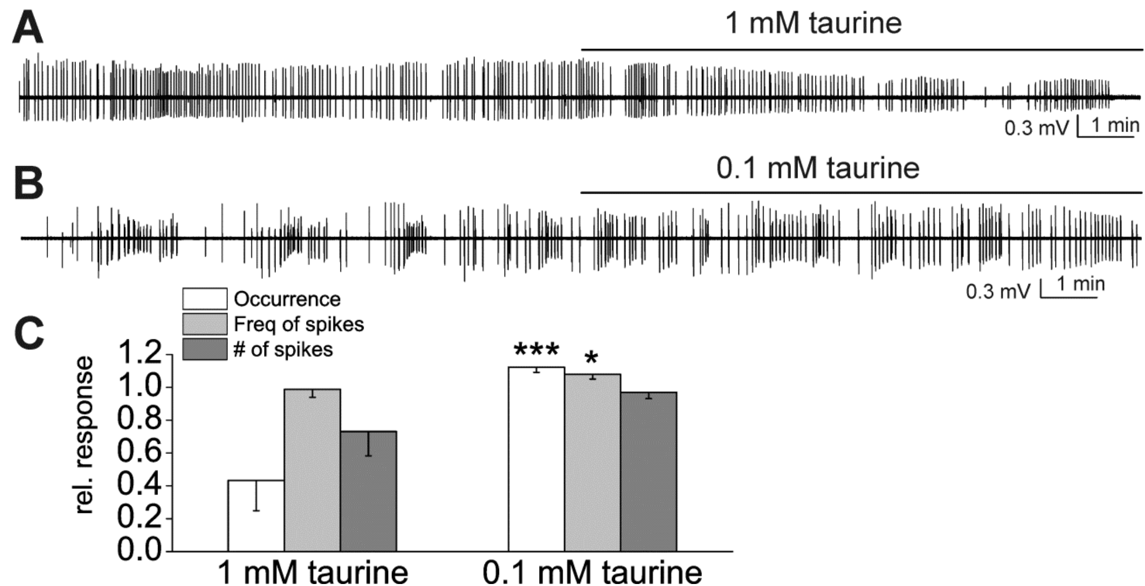


*Figure 10. Effect of glycinergic agonist taurine on low Mg^{2+}/4-AP-induced epileptiform discharges. A: Representative field potential recording showing that 1 mM taurine suppressed low Mg^{2+}/4-AP-induced epileptiform discharges. B: Representative field potential recording showing that 0.1 mM taurine promoted low Mg^{2+}/4-AP-induced epileptiform discharges. C: Statistical analysis of effect of two concentrations of taurine on low Mg^{2+}/4-AP-induced on epileptiform discharges. * represents p<0.05; *** represents p< 0.001 by sign-test.*

3.7 Contribution of GlyR to the effect of taurine on epileptiform activity

3.7.1 Taurine activates both $GABA_AR$ and GlyR

Taurine has been shown to be a full agonist of GlyR, but a weak agonist of $GABA_AR$ as well (Albrecht & Schousboe, 2005). For example, lower than 1 mM taurine activates GlyRs in the basolateral amygdala (McCool & Botting, 2000) and supraoptic nucleus (Hussy *et al.*, 1997) of adult rats, hippocampal CA1 (Wu & Xu, 2003) and inferior colliculus (Xu *et al.*, 2004) of immature rats, nucleus accumbens

(Jiang *et al.*, 2004) of young rats, whereas in the same brain regions taurine at concentrations higher than 1 mM also activates $GABA_A$Rs. In the thalamus of both mature and immature mice, taurine at low concentration can potently activate extrasynaptic $GABA_A$Rs (Jia *et al.*, 2008).

Therefore I validated that taurine activated both GlyR and $GABA_A$R in the CA3 region of immature rat hippocampus under whole-cell voltage-clamp conditions. Focal application of 5 mM taurine to the soma of CA3 pyramidal neuron induced an inward current of 418.6 ± 57.8 pA (Fig. 11A, B. n=15). In the presence of 3 μM strychnine taurinergic current was diminished by 61.7 ± 8.9% (Fig. 11A, C. n=9), and in the presence of 3 μM gabazine by 33.2 ± 9.1% (Fig. 11B, C. n=6). Combined application of 3 μM strychnine and 3 μM gabazine virtually abolished taurinergic responses (Fig. 11A-C. n=9). Focal application of 0.1 mM taurine induced inward taurinergic current of 11.6 ± 1.5 pA (n=15). Similarly, taurinergic current induced by 0.1 mM taurine was attenuated by 56.8 ± 9.7% by 3 μM strychnine (Fig. 11C. n=9) and 53.4 ± 10.9% by 3 μM gabazine (Fig. 11C. n=6). These results suggest that taurine preferentially activates GlyR, but also $GABA_A$R on the immature hippocampal CA3 pyramidal neurons.

3.7.2 Contribution of GlyR to the effect of high concentration of taurine

Then I investigated which receptor, GlyR or $GABA_A$R, mediates the anticonvulsive effect of high concentration of taurine, since taurine activates both GlyR and $GABA_A$R. As mentioned above, in our lab Akihito Okabe's data showed 5 mM taurine abolished all epileptiform activities in all preparation. Akihito Okabe's data also showed that in the presence of 3 μM strychnine, bath application of 5 mM taurine abolished epileptiform activity only in 1 out of 6 preparations, indicating that activation of GlyRs might partially contribute to the anticonvulsive effect of high concentration of taurine.

To clarify the role of GlyRs in the anticonvulsive effect of high concentration of taurine in immature CHF, first I analyzed the effect of 5 mM taurine on low Mg^{2+}/4-AP-induced epileptiform activities in the presence of 3 μM gabazine to block the involvement of activation of $GABA_A$Rs. In the absence of activation of $GABA_A$Rs, 5mM taurine significantly attenuated occurrence of epileptiform discharges by 38.1 ± 8% (n=12, p=0.012 by sign-test), but not significantly changed frequency of spikes within discharges and number of spikes per discharge (Fig. 12A, D). Secondly I analyzed the effect of 5 mM taurine on 3 μM gabazine-induced epileptiform activities

in ACSF. 5 mM taurine also significantly attenuated occurrence of gabazine-induced epileptiform discharges by 37.2 ± 9% (n=12, p=0.007 by sign-test. Fig. 12E). These results support the conclusion that activation of GlyRs contributes to the anticonvulsive effect of high concentration of taurine.

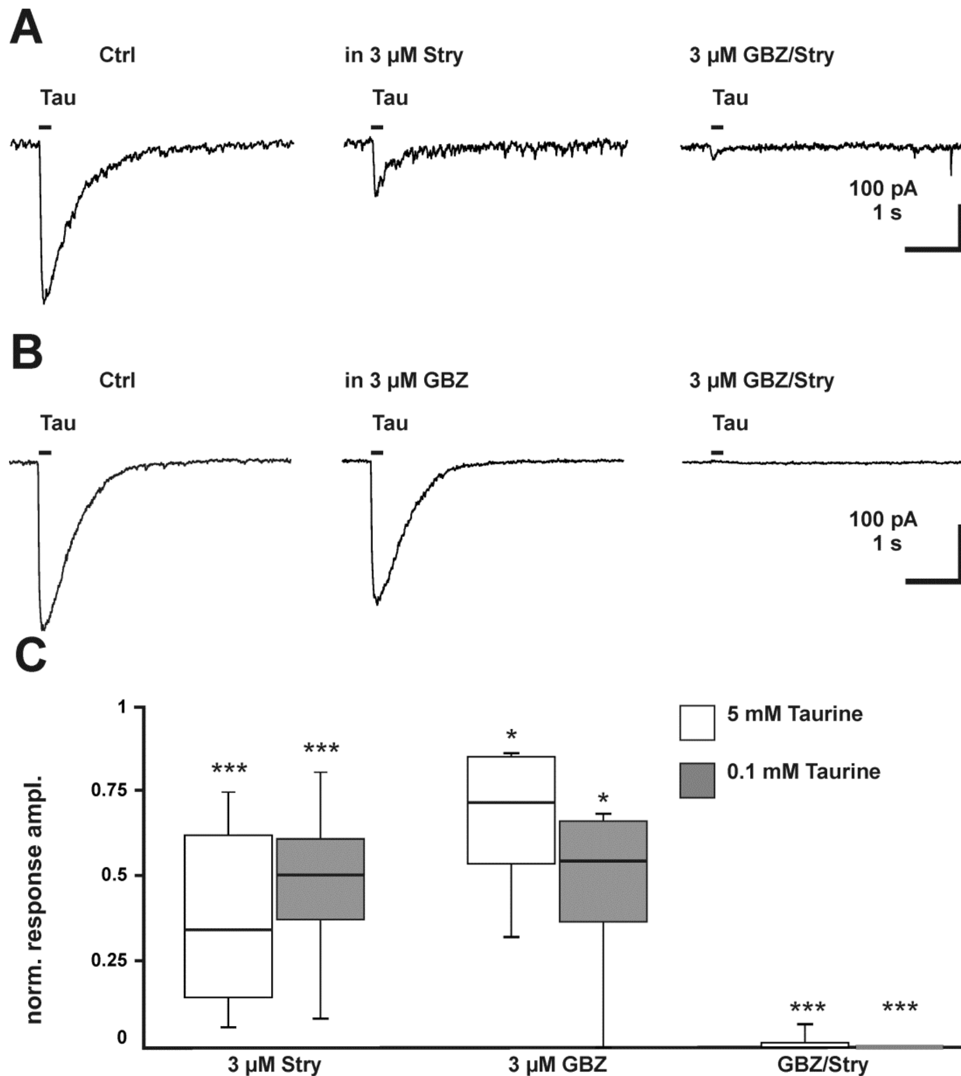


*Figure 11. Effect of glycinergic and GABAergic antagonists on taurine-induced currents. A: Whole-cell voltage-clamp recording showing that focal application of 5 mM taurine induced inward current, which was massively attenuated by bath application of 3 µM strychnine (Stry) and virtually abolished by the combined presence of 3 µM strychnine and 3 µM gabazine (GBZ). B: Whole-cell voltage-clamp recording showing that 3 µM gabazine had less effect on the taurine-induced current. C: Statistical analysis showing the pharmacology of taurinergic currents. Box plots represent upper/lower quartile and median (bold line); whiskers indicate maximal and minimal values. * represents p<0.05; *** represents p<0.001 by sign-test.*

Interestingly, 5 mM taurine induced an obvious proconvulsive effect when gabazine and strychnine were applied together to block both $GABA_A$Rs and GlyRs. Bath application of 5 mM taurine significantly increased the occurrence of low Mg^{2+}/4-AP-induced epileptiform discharges, in the presence of both 3 μM strychnine and 3 μM

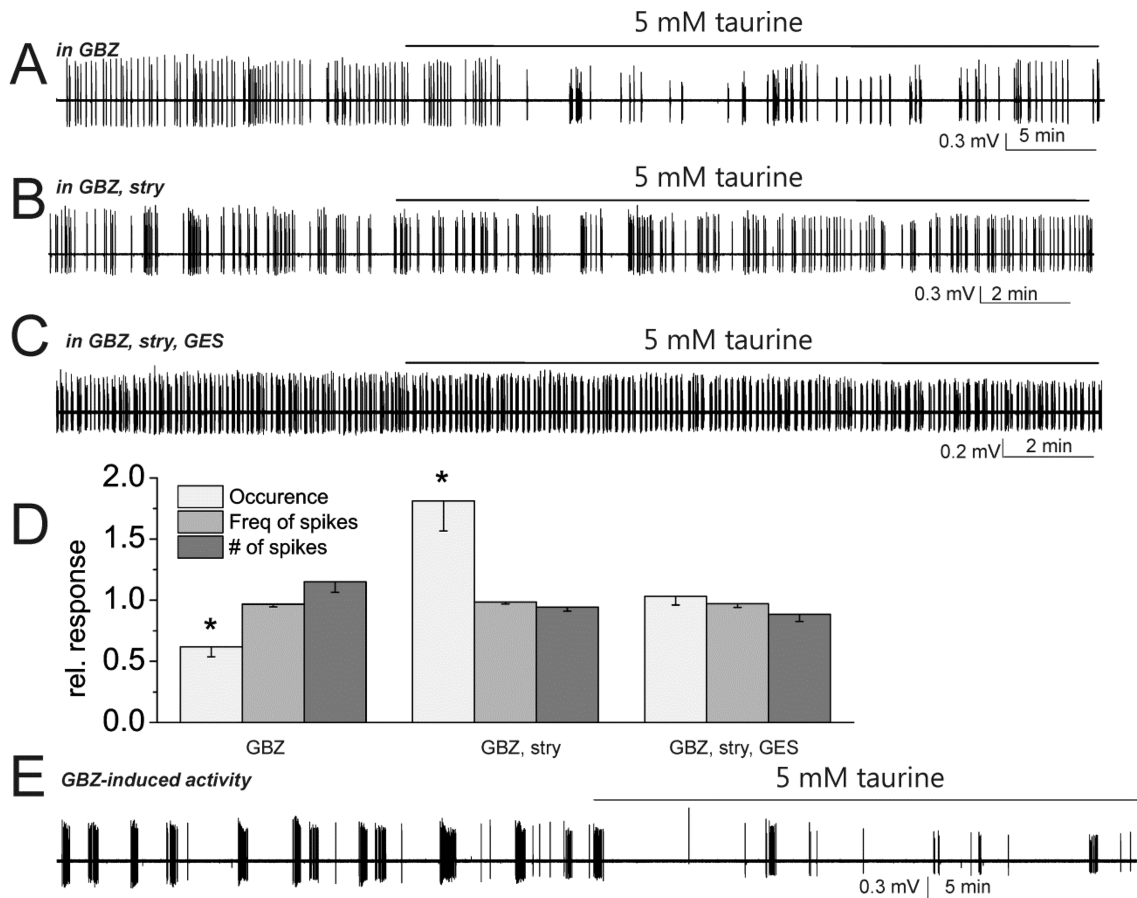


Figure 12. Pharmacology underlying the anticonvulsive effect of high concentration of taurine. A: Representative field potential recording showing that in the presence of 3 μM gabazine (GBZ), bath application of 5 mM taurine attenuated low-Mg^{2+}/4-AP-induced epileptiform discharges. B: Representative field potential recording showing that in the presence of 3 μM gabazine and 3 μM strychnine (stry), bath application of 5 mM taurine promoted low-Mg^{2+}/4-AP-induced epileptiform discharges. C: Representative field potential recording showing that in the presence of 3 μM gabazine, 3 μM strychnine and 300 μM GES, bath application of 5 mM taurine had no effect on low-Mg^{2+}/4-AP-induced epileptiform discharges. D: Statistical analysis showing combined application of 3 μM strychnine, 3 μM gabazine and 300 μM GES abolished effects of 5 mM taurine on low-Mg^{2+}/4-AP-induced epileptiform discharges. E: Representative field potential recording showing that 5 mM taurine attenuated 3 μM gabazine-induced epileptiform discharges.

gabazine, to 181 ± 24.4% (n=17, p=0.013 by sign-test. Fig. 12B, D). In order to elucidate this paradoxical action, I further performed experiments using guanidinoethanesulfonic acid (GES), an inhibitor of taurine transport, to see whether an uptake of taurine via the TauT is involved. The addition of 300 µM GES to 3 µM strychnine and 3 µM gabazine containing solutions virtually eliminated the paradoxical proconvulsive effect of 5 mM taurine (103 ± 7%, n=9. Fig. 12C, D).

These results demonstrate that both $GABA_A$Rs and GlyRs contribute to the anticonvulsive effect of high concentration of taurine, in agreement with my whole-cell data that showed taurine activated both $GABA_A$Rs and GlyRs in CA3 of immature rat hippocampus (Fig. 11).

3.7.3 Contribution of GlyR to the effect of low concentration of taurine

Next I investigated which receptor, GlyR or $GABA_A$R mediates the proconvulsive effect of low concentration of taurine. In the presence of 3 µM gabazine, 0.1 mM taurine exerted only a slight tendency to promote the occurrence of low Mg^{2+}/4-AP induced epileptiform discharges (107 ± 2.6%, n=10, p=0.11 by sign test. Fig. 13B, D). While in the presence of 3 µM strychnine, the proconvulsive effect of 0.1 mM taurine was completely abolished. Upon application of 0.1 mM taurine in the presence of 3 µM strychnine, the occurrence of epileptiform discharges (101.8 ± 5.1%), frequency of spikes (100 ± 1.8%) and number of spikes (106 ± 4.2%) remain the same (n=12. Fig. 13C, D).

These results suggest that the proconvulsive effect of low concentration of taurine depends on activation of GlyRs and might partially on $GABA_A$Rs as well.

3.8 Effect of TauT inhibitor on epileptiform activity

To determine whether endogenous taurine can replicate the effects of exogenously applied taurine on epileptiform activity, I assessed epileptiform activity induced by low Mg^{2+}/4-AP in the absence or presence of taurine uptake blocker GES (Molchanova *et al.*, 2004; Olive *et al.*, 2000). In the presence of 300 µM GES, the occurrence of epileptiform discharges was significantly reduced by 48 ± 11.8% (n=12, p=0.006 by

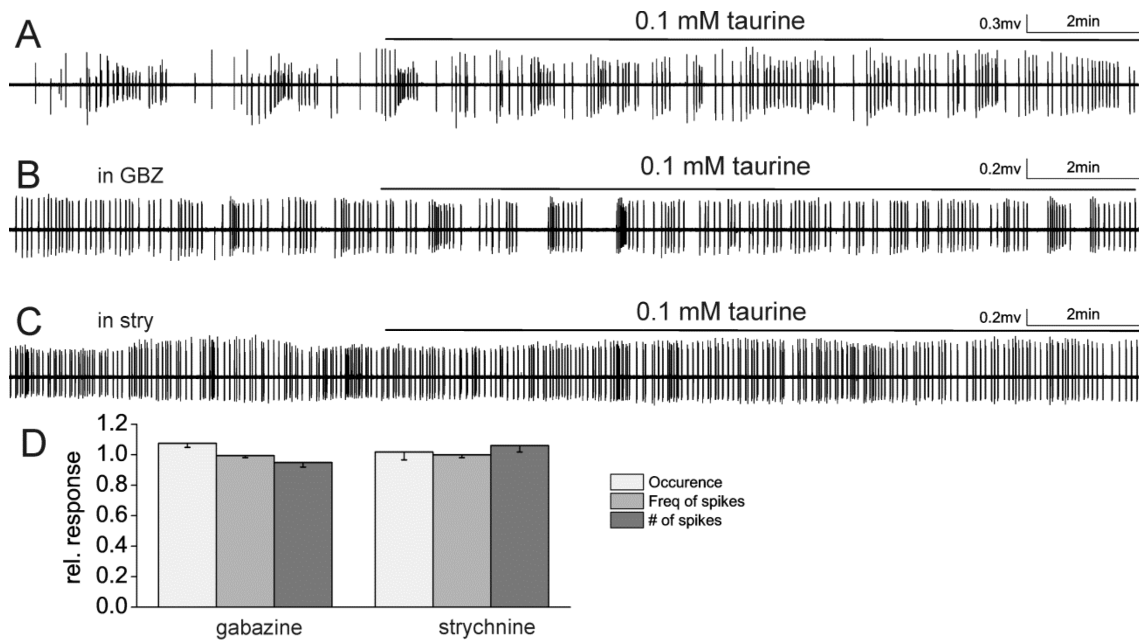


Figure 13. Pharmacology underlying the proconvulsive effect of low concentration of taurine. A: Representative field potential recording showing that 0.1 mM taurine promoted low-Mg^{2+}/4-AP-induced epileptiform discharges. B: Representative field potential recording showing that in the presence of 3 μM gabazine (GBZ), promotional effect of 0.1 mM taurine on low-Mg^{2+}/4-AP-induced epileptiform discharges was attenuated. C: Representative field potential recording showing that in the presence of 3 μM strychnine (stry), promotional effect of 0.1 mM taurine on low-Mg^{2+}/4-AP-induced epileptiform discharges was abolished. D: Statistical analysis showing the influence of 3 μM gabazine and 3 μM strychnine on the effects of 0.1 mM taurine on low-Mg^{2+}/4-AP-induced epileptiform discharges.

sign test), while the spike amplitude and number of spikes per discharge were not significantly changed (Fig. 14A). Thus 300 µM GES is anticonvulsive.

It has reported that GES is an agonist of $GABA_AR$ (Mellor *et al.*, 2000; Sergeeva *et al.*, 2002). To confirm the agonistic action of GES on $GABA_ARs$ in CA3 pyramidal neurons of P4-7 rats, I made experiments with whole-cell recordings which showed that focal application of 300 µM GES induced an inward current of 18 $\pm$ 4 pA (n=17). This inward current was virtually, completely and reversibly abolished by 3 µM gabazine (0.3 ± 0.3 pA, n=7. Fig. 14B1-3). Therefore GES-induced inward currents on CA3 pyramidal neurons of P4-7 rats were fully mediated by $GABA_ARs$.

Since GES is also an agonist at $GABA_AR$, the anticonvulsive effect of 300 µM GES might be mediated by the activation of $GABA_ARs$. In order to examine this

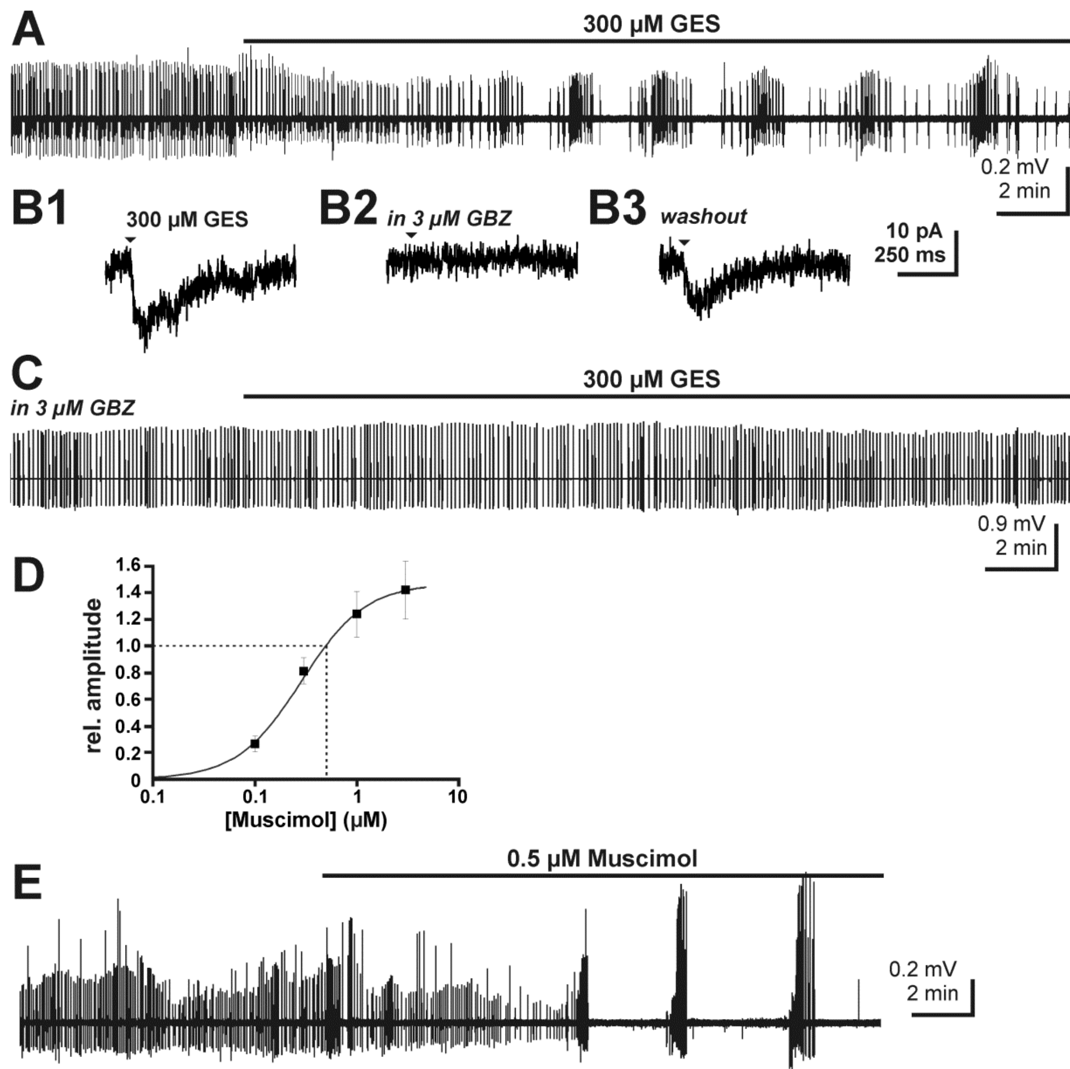


Figure 14. Effect of the TauT inhibitor GES on low Mg^{2+}/4-AP-induced epileptiform discharges. A: Representative field potential recording showing that 300 µM GES diminished low-Mg^{2+}/4-AP-induced epileptiform discharges. B1-3: Whole-cell voltage-clamp recordings showing that focal application of 300 µM GES evoked an inward current which was reversibly blocked by the $GABA_AR$ antagonist gabazine (GBZ). C: Representative field potential recording showing that in the presence of 3 µM gabazine, 300 µM GES failed to diminish epileptiform discharges. D: Dose-response curve of inward currents induced by bath application of the $GABA_AR$ agonist muscimol at concentrations of 0,1, 0.3, 1, 3 µM. The muscimol-induced currents were normalized to 300 µM GES-induced inward current in the same neuron. Dash line noting that 300 µM GES evoked a similar current amplitude as approximately 0.5 µM muscimol. E: Representative field potential recording showing that bath application of 0.5 µM muscimol diminished epileptiform discharges comparably to 300 µM GES.

possibility, first, I evaluated the effect of GES on epileptiform activity induced by low Mg^{2+}/4-AP in the presence of gabazine. Accordingly while activation of $GABA_AR$ was muted by 3 µM gabazine, 300 µM GES exhibited no significant effect on the occurrence of epileptiform discharges (111 $\pm$ 7.8%, p=1 by sign test, n=28. Fig 14C) as well as spike amplitude, frequency of spikes within discharges and number of spikes per discharge of epileptiform activities (Fig. 14C). Secondly, to further substantiate effect of GES on epileptiform activity is mediated by $GABA_ARs$, I used the specific GABAergic agonist muscimol to determine whether it could mimic the anticonvulsive effect of 300 µM GES. In whole–cell recordings, bath application of 0.1, 0.3, 1, 3 µM muscimol induced dose dependent inward currents of 13.6 $\pm$ 4, 41.8 $\pm$ 10.6, 64.2 $\pm$ 17.3, 70.3 $\pm$ 18.9 pA, respectively (n=8 each). Bath application of 300 µM GES elicited an inward current of 46 $\pm$ 7.1 pA (n=9). According to the dose-response relation curve, 0.52 µM muscimol and 300 µM GES would produce equivalent currents (Fig 14D). Subsequent field potential recordings on CHF revealed that 0.5 µM muscimol attenuated the occurrence of epileptiform discharges by 70 + 6.4% (n=15), which was not significantly different from effect of 300 µM GES (p=0.097 by U-test. Fig. 14A, E).

These results demonstrate that the TauT inhibitor GES is anticonvulsive. The anticonvulsive effect of GES, however, is expressed fully by its agonistic action on $GABA_AR$.

3.9 Effect of glycine on the epileptiform activity

Albeit taurine is the major endogenous agonist of GlyRs, the main agonist, glycine, acts more potently on GlyRs in the hippocampus (Mori *et al.*, 2002). To further clarify action of glycinergic system on epileptiform activity, I investigated effects of GlyR on epileptiform activity using glycine.

To avoid glycine being removed from interstitial space by GlyTs and isolate the influence of glycine on GlyRs from NMDA receptors (Johnson & Ascher, 1987), I performed experiments designated to explore effects of glycine via GlyR on epileptiform activity in the presence of 0.1 µM ALX 5407 and 0.5 µM ALX 1393 to block GlyT1 and GlyT2 respectively (Harvey & Yee, 2013), and 200 µM D-serine to saturate glycine binding site of NMDA receptors (Wroblewski *et al.*, 1989). Bath application of 200 µM D-serine had no significant effect on the occurrence of

epileptiform discharges (99 ± 2.4%), number of spikes per discharge (102 ± 2.7%) and spike amplitude (96.1 ± 3.3%, n=30. Fig. 15C) of epileptiform activities induced under low Mg^{2+}/4-AP conditions. Addition of ALX 5407 and ALX 1393 to D-serine containing solution also had no significant effect on the properties of epileptiform discharges. In the presence of D-serine, ALX 5407 and ALX 1393, occurrence of epileptiform discharges was 104 ± 2.1%, frequency of spikes within discharges 100.2 ± 1.4%, number of spikes per discharge 96.5 ± 3.7%, and spike amplitude 93.1 ± 3.3% of control (n=30. Fig. 15C).

In the continuous presence of D-serine, ALX 5407 and ALX 1393, bath application of glycine showed a dose dependent effect on low Mg^{2+}/4-AP-induced epileptiform activity. Glycine at concentrations of 0.1, 1 and 3 µM (n=18, 10 and 8, respectively) had no significant effects with respect to the occurrence of repetitive discharges,

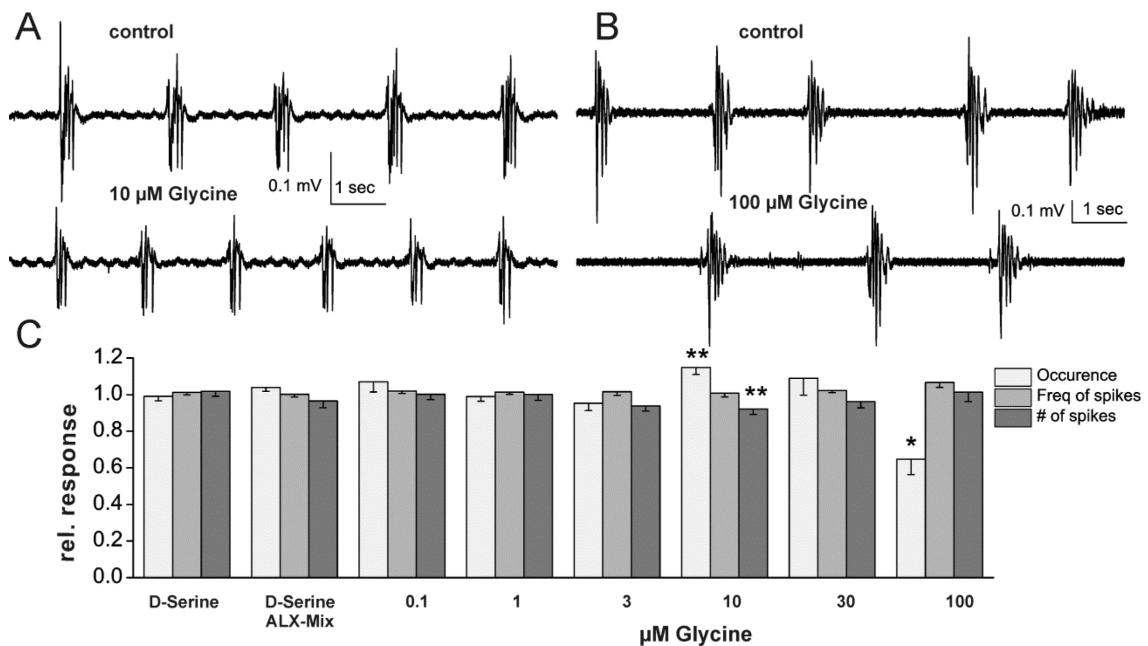


Figure 15. Effect of glycinergic agonist glycine on low Mg^{2+}/4-AP-induced epileptiform discharges. A: Representative field potential recording showing that 10 µM glycine, in the presence of D-serine, ALX 5407 and ALX 1393, promoted epileptiform activity. B: Representative field potential recording showing that 100 µM glycine, in the presence of D-serine, ALX 5407 and ALX 1393, decreased epileptiform activity. C: Statistical analysis showing effects of D-serine, ALX-Mix (ALX 5407 and ALX 1393) and a series of concentrations of glycine in the presence of D-serine and ALX-Mix on low Mg^{2+}/4-AP-induced epileptiform activity.

frequency of spikes within discharges, number of spikes per discharge and spike amplitude of epileptiform discharges (Fig. 15C). At 10 µM, glycine significantly enhanced the occurrence of repetitive discharges to 115 $\pm$ 3.8% (p=0.001 by sign test), but reduced significantly the number of spikes per discharge to 92 $\pm$ 2.9% (p=0.007 by sign test) and the amplitude to 94.2 $\pm$ 3.3% (n=15, p=0.035 by sign test. Fig. 15A, C). At 30 µM, glycine had no significant effects on the occurrence of repetitive discharges, frequency of spikes within discharges and number of spikes per discharge, but only reduced amplitude to 91.7 $\pm$ 4.6% (n=10, p=0.022 by sign test. Fig. 15C). At 100 µM glycine significantly attenuated the occurrence of repetitive discharges to 64.7 $\pm$ 8.2% (p=0.012 by sign test), but did not alter the frequency of spikes within discharges, number of spikes per discharge and spike amplitude (n=11. Fig. 15B, C).

These experiments using glycine strongly solidify previous results obtained by using taurine, that activation of GlyRs mediates both pro- and anticonvulsive effects in immature rat CHF, depending on the concentration of agonists.

3.10 Effect of low concentration of taurine and glycine on intrinsic network excitability and epileptogenesis

Since activation of GlyRs activated by lower concentrations of taurine and glycine mediated the proconvulsive effects, I investigated whether the proconvulsive effect of taurine or glycine was sufficient to influence intrinsic network excitability and thus to be epileptogenic. Bath application of 0.1 mM taurine in ACSF proved to be insufficient to induce reliable epileptiform activity. In only 1 out of 14 preparations, weak epileptiform activity was observed. Likewise, application of 10 µM glycine in ACSF containing D-serine, ALX 5407 and ALX 1393 evoked epileptiform activity in none of 10 preparations.

These results indicate that, although activation of GlyRs activated by low concentrations of taurine or glycine promotes ongoing epileptiform activity, it is not sufficient to promote the network excitability and impel epileptogenic outcome in immature rat hippocampus.

3.11 Contribution of NKCC1 to the effect of low concentration of taurine and glycine

In immature neurons, activation of NKCC1 maintains an accumulation of intracellular Cl^-, which enables neuronal depolarizing upon activation of GlyRs or $GABA_A$Rs. To analyze whether intracellular Cl^- accumulation contributes to the proconvulsive effect of low concentration of taurine (Russell, 2000), I blocked NKCC1 with 10 µM bumetanide (Kilb *et al.*, 2007). Bath application of 10 µM bumetanide had no significant effect on low-Mg^{2+}/4-AP-induced epileptiform discharges, as the occurrence of epileptiform discharges, frequency of spikes within discharges and number of spikes per discharge were not significantly altered by bumetanide (97.3 ± 9.2, 109.5 ± 4.8% and 97.2 ± 6.6%, respectively; n=17. Fig. 16C), in consistency with previous study (Kilb *et al.*, 2007). However, 10 µM bumetanide completely abolished the proconvulsive effect of 0.1 mM taurine (n=12. Fig. 16A, C). Upon application of 0.1 mM taurine in the presence of bumetanide, neither the occurrence of epileptiform discharges (99 ± 6%), nor frequency of spikes within discharges (100.9 ± 1.4%) or number of spikes per discharge (100.8 ± 5.4%.) was significantly different from the control level without bumetanide. This experiment demonstrates that the proconvulsive effect of low concentration of taurine depends on intracellular accumulation of Cl^- by the activation of NKCC1.

Additionally I examined whether the proconvulsive effect of 10 µM glycine also depends on intracellular accumulation of Cl^-. Similarly, when 10 µM bumetanide was applied in the bath solution to block activation of NKCC1, the proconvulsive effect of 10 µM glycine was completely abolished. In the presence of bumetanide, no significant change was observed in the occurrence of epileptiform discharges (99.9 ± 4.5%), frequency of spikes within discharges (99.7 ± 1.4%) and the number of spikes per discharge (101.2 ± 2.0%. Fig. 16B, C. n=16) of low-Mg^{2+}/4-AP-induced epileptiform discharges.

These results suggest that the proconvulsive effect of lower concentration of taurine and glycine depends on intracellular accumulation of Cl^- via NKCC1 which establishes depolarizing action of activation of GlyRs.

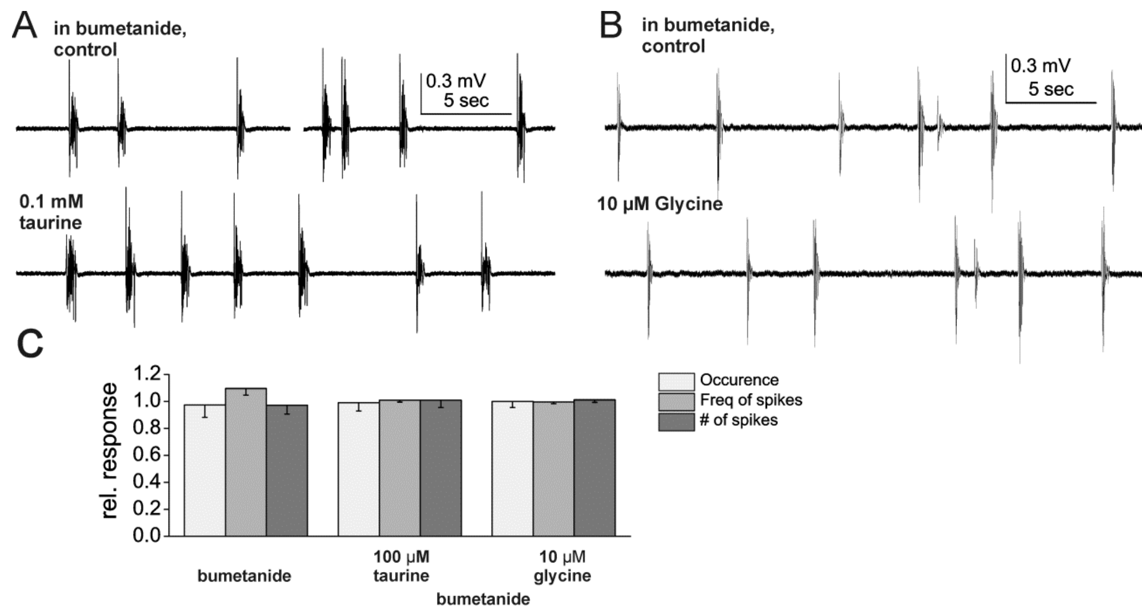


Figure 16. Proconvulsive effects of low concentrations of taurine and glycine were prohibited by NKCC1 blocker bumetanide. A: Representative field potential recording showing that in the presence of 10 µ M bumetanide, 0.1 mM taurine failed to promote epileptiform activity. B: Representative field potential recording showing that in the presence of 10 µM bumetanide, 10 µM glycine failed to promote epileptiform activity. C: Statistical analysis showing effects of bumetanide, 0.1 mM taurine or 10 µM glycine in the presence of 10 µM bumetanide on low Mg^{2+}/4-AP-induced epileptiform activity.

4 Discussion

By investigating actions of glycinergic system on both epileptogenesis and epileptiform activity of in vitro intact CHF of rats at P4-7, the data in this thesis uncover the following main findings: i) Inactivation of GlyRs by 3 μM or even 0.3 μM strychnine triggers epileptiform activity on immature CHF. ii) High concentration (Bujack, 1982) of glycine is anticonvulsive. This is in agreement with previous data from Akihito Okabe, which showed that another glycinergic ligand, taurine, at higher concentrations of $\geq$ 1 mM is anticonvulsive. But iii) lower concentrations of taurine and glycine, i.e. 100 μM taurine and 10 μM glycine, are proconvulsive. iiii) Promotive effects of taurine and glycine on epileptiform activity depend on the function of NKCC1 which maintains high intracellular Cl^- in the immature neurons. These novel findings make contribution to reveal how activation of GlyRs modulates network excitability and epileptiform activity in the immature CNS, and might guide clinic searching for new medications that are designed to prevent epileptic seizures, in particular pharmacoresistant seizures of young children.

4.1 Dual faces of GlyR on neuronal excitability in immature hippocampus

Although glycinergic synaptic transmission was initially detected at spinal cord, brain stem and some other lower brain regions (Betz & Laube, 2006), functional expression of GlyRs has been detected abundantly in a wide range of brain areas including cortex, hippocampus, amygdala, periaqueductal gray, cerebellum, substantial nigra, inferior colliculus, and ventral tegmental area (Chattipakorn & McMahon, 2003; Dumoulin *et al.*, 2001; Hussy *et al.*, 2001; Karkar *et al.*, 2004; Mangin *et al.*, 2002; McCool & Farroni, 2001; Min *et al.*, 1996; Sergeeva & Haas, 2001; Wang *et al.*, 2005; Xu *et al.*, 2004; Zhang *et al.*, 2008a). The detailed function of these GlyRs in the higher brain has not yet been illustrated.

Both $GABA_A$Rs and GlyRs are transmembrane anion channels mainly permeable to Cl^- and less permeable to HCO_3^- (Farrant & Kaila, 2007). In the adult brain, $GABA_A$Rs make a major inhibitory contribution in controlling the neural excitability (Farrant & Kaila, 2007), but GlyRs also have an important inhibitory function (Ye,

2008). In the immature brain, however, both $GABA_A$Rs and GlyRs mediate either excitatory depolarizing or inhibitory action (Ben-Ari, 2002; Meyer *et al.*, 1995). Depolarizing effects of GlyRs and $GABA_A$Rs rely on Cl^- efflux upon opening of the channels as a consequence of high intracellular Cl^- accumulation via NKCC1, which is the dominant Cl^- transporter in the developing brain (Farrant and Kaila, 2007; Rivera et al., 1999; Yamada et al., 2004). However, Cl^- efflux-associated depolarization can also leads to inhibitory consequence by decreasing the membrane input resistance and resultantly shunting membrane response (Edwards, 1990b; Kolbaev et al., 2011; Staley and Mody, 1992).

Except for Cl^--dependency, another mechanism underlying the dual effects of GlyRs and $GABA_A$Rs on neuronal excitability of the immature neurons could be special structural and physiological properties of the receptors. For example, expression of β and α1 subunits of GlyRs increases during development and is abundant at adult brain (Meyer *et al.*, 1995), while α2 and α3 subunit exhibits an opposite expression pattern by dominating in the first postnatal week (Hoch *et al.*, 1989; Malosio et al. 1991; Sato et al. 1992). Physiological studies showed that GlyR sensitivity to ligands, permeability to Cl^- and regulation by kinase change during development (Betz & Laube, 2006).

In my study, I employed 50 mM Cl^- in pipette solution for whole-cell patch-clamp recording. This concentration of Cl^- imitates high intracellular Cl^- concentration of immature neurons (Ben-Ari, 2002; Kahle & Staley, 2008). Under this condition, both agonists of GlyRs, taurine and glycine evoked inward currents being blocked by strychnine, evidencing an excitatory action of GlyRs (Kilb *et al.*, 2008; Xu & Gong, 2010). While under field potential recording condition, inhibition of GlyRs by strychnine leads to epileptiform runaway excitation, indicating an inhibitory action of GlyRs.

4.2 Intrinsic inhibitory action of GlyRs is required to prevent spontaneous epileptic seizures

GlyR activation induced depolarizing membrane response on immature hippocampal neurons (Xu & Gong, 2010). Paradoxically bath application of 3 μM strychnine in ACSF to CHF proved to be epileptogenic. As 3 μM concentration,

strychnine inhibits a proportion of $GABA_A$Rs, it cannot be excluded $GABA_A$R is the mediator of the epileptiform activity induced by 3 μM strychnine. Subsequent experiments showed that only 0.3 μM strychnine was still capable of provoking epileptiform activity. As 0.3 μM strychnine inhibited considerable GlyR-mediated currents, but had no obvious effect on $GABA_A$R-mediated currents, it is reasonable to conclude that the epileptogenic effect of 0.3 μM strychnine is a consequence of direct inhibition of GlyRs. Hence, intrinsic action of GlyRs is inhibitory, and this intrinsic inhibitory action is required to prevent spontaneous epileptic seizures.

Comparing with epileptiform discharges induced by strychnine and mediated GlyRs, inhibition of $GABA_A$R by gabazine initiated epileptiform discharges in substantially shorter latency and with more pronounced parameters, in accordance with previous studies (Kilb *et al.*, 2007; Wells *et al.*, 2000). This indicates a fact that GlyRs have a less inhibitory tone in neural intrinsic excitability. The fact that GlyRs have weaker inhibitory tone was further confirmed by experiments showing failure of strychnine to promote gabazine-induced epileptiform activity and efficiency of gabazine to enhance strychnine-induced epileptiform activity. Thus it can be concluded that the GABAergic system dominates intrinsic inhibitory control, while glycinergic system represents another important element for the control of neural excitability in the immature rat hippocampus.

The inhibitory action of GlyRs in the immature hippocampus is most probably fulfilled by shunting inhibition. It has been demonstrated that opening of GlyRs decrease the membrane input resistance of neurons (Kilb *et al.*, 2002), and the reduced membrane input resistance shunts the excitatory inputs (Kolbaev *et al.*, 2011; Lamsa *et al.*, 2000; Staley & Mody, 1992). Because the intrinsic overall activation of GlyRs provides an inhibitory tone, a conclusion could be that in the intrinsic situation depolarizing, excitatory aspect of GlyRs is weaker than its sustained shunting inhibitory aspect.

4.3 Low concentrations of exogenous agonists are proconvulsive and potentially epileptogenic

In my study epileptiform discharges induced by low Mg^{2+}/4-AP conditions on immature rat CHF moderately but significantly augmented when either taurine or

glycine was applied exogenously at low concentrations. Since strychnine completely abolished the proconvulsive effect of 0.1 mM taurine, this proconvulsive effect of taurine is most probably mediated by GlyRs. Activation of the glycine binding site of NMDA receptors probably does not contribute to the proconvulsive effect of 10 µM glycine, as in this experiment effects of glycine on glycine-binding site of NMDA receptors were occluded by saturating concentration of D-serine, which is another potent ligand to glycine-binding site of NMDA receptors.

In addition, the experiments showed that, in the absence of other proepileptic stimuli, 0.1 mM taurine applied in ACSF induced epileptiform activity in only 1 out of 14 preparations, while 10 µM glycine applied in the same conditions failed to produce epileptiform activity in all of 10 preparations. These experiments indicate that the proconvulsive effect of low concentrations of exogenous agonists of GlyRs is not sufficient to initiate epileptiform activity by itself, but is only potentially epileptogenic.

Most likely the proconvulsive effects of low concentrations of taurine and glycine reflect moderate GlyR activation resulting in depolarized membrane potential that causes enhanced neuronal excitability (Ehrlich *et al.*, 1999; Flint *et al.*, 1998; Ito & Cherubini, 1991; Kilb *et al.*, 2002; Kilb *et al.*, 2008), but weak enough to avoid overwhelming shunting inhibition. Membrane depolarization following opening of GlyRs in immature CNS is believed to be carried out by Cl^- efflux due to intracellular Cl^- accumulation mediated by active Cl^- uptake via NKCC1 (Achilles *et al.*, 2007; Russell, 2000; Yamada *et al.*, 2004). Supportively, my experiments revealed that preincubation with NKCC1 blocker bumetanide completely erased the proconvulsive effects of low concentrations of taurine and glycine. While in my experiment bumetanide itself did not affect epileptiform activity, in accordance with previous studies that showed bumetanide had no effect on epileptiform activity on intact hippocampal preparations (Kilb *et al.*, 2007), it has been reported that bumetanide produced an anticonvulsive effect in organotypic slices (Wahab *et al.*, 2011). Possibly alteration in Cl^- concentrations and/or extracellular levels of GABAergic agonists in the latter preparation might account for the discrepancy.

It is not known whether excitatory and proconvulsive effect of GlyRs in immature CNS is linked to specific subtypes or cellular location of receptors. During the first postnatal week, GlyRs in the hippocampus have low glycine affinity due to lower expression of α1 subunit (Aroeira *et al.*, 2011), but higher expression of α2 and α3

subunits. Expression of α3 splicing variants, especially α3L, can dramatically increase glycine affinity for GlyRs (Eichler *et al.*, 2009; Meier *et al.*, 2005). Such splicing variants possibly mediate the proconvulsive effect of low concentration of glycine. In the hippocampal hilar region and brainstem presynaptic GlyRs facilitate glutamatergic transmitter release and enhance postsynaptic neuronal excitability (Lee *et al.*, 2009; Turecek & Trussell, 2001). It cannot be ruled out that presynaptic GlyRs are responsible for the proconvulsive effect of low concentration of glycine shown in my experiment, although as to my knowledge so far no data indicates function of presynaptic GlyRs in hippocampal CA3 of early postnatal rats.

4.4 High concentrations of exogenous agonists are anticonvulsive

My experiments clearly demonstrated that application of 100 µM glycine attenuated epileptiform discharges induced by low Mg^{2+}/4-AP conditions. Meanwhile my experiments using 1 mM taurine showed also suppressive action on epileptiform discharges. Consistently, Akihito Okabe has previously demonstrated suppressive effects of taurine at concentrations of 1, 2 and 5 mM. These data indicate that in the immature hippocampus, high concentrations of exogenous agonists of GlyRs are anticonvulsive, in contrast to the proconvulsive effect of low concentrations of glycine and taurine.

Taurine binds to not only GlyRs, but also to $GABA_A$Rs and activates TauTs (Han *et al.*, 2006; Jia *et al.*, 2008). Akihito Okabe's data showed 5 mM taurine completely eliminated epileptiform discharges in all investigated preparations. Since my experiment showed that 5 mM taurine still exhibited considerably anticonvulsive action on epileptiform activity induced by low Mg^{2+}/4-AP conditions in the presence of 3 µM gabazine or epileptiform activity induced by 3 µM gabazine in ACSF, either GlyRs or TauTs mediates the remaining anticonvulsive effect of 5 mM taurine. Interestingly, when both GlyRs and $GABA_A$Rs were inhibited by a combined application of strychnine and gabazine, 5 mM taurine turned to be proconvulsive. These results suggest that the anticonvulsive effect of high concentrations of taurine is mediated by mainly $GABA_A$Rs but also considerably by GlyRs, and that taurine uptake via TauTs is virtually proconvulsive. The GlyR-mediated component of anticonvulsive effect was

confirmed by Akihito Okabe's experiments which showed in the presence of 3 μM strychnine, 5 mM taurine failed to completely abolish all epileptiform discharges but still largely attenuated epileptiform discharges.

In the immature hippocampus, where my recordings were performed, the anticonvulsive effect of high concentrations of glycine and taurine were mirrored by the epileptogenic action of strychnine. Shunting inhibition could be a feasible explanation for the anticonvulsive effects of high concentrations of GlyRs agonists. As activation of GlyRs opens anion conductance, outward flux of Cl^- mediates not only a fast excitatory action but also shunting inhibition by reducing the membrane input resistance (Kolbaev *et al.*, 2011; Lamsa *et al.*, 2000; Staley & Mody, 1992). The shunting inhibition by activation of both GlyRs and $GABA_A$Rs has already been showed in immature neocortical neurons (Kilb *et al.*, 2002).

In the GABAergic system, it's generally assumed that shunting inhibition is mainly produced by tonic activation of extrasynaptic $GABA_A$Rs, which are activated by ambient GABA, while phasic action is mediated by activation of synaptic $GABA_A$Rs (Farrant & Nusser, 2005). By comparison, it's less clear for glycinergic system which subpopulation of GlyRs is responsible for shunting inhibition. As glycinergic synaptic events in the hippocampus were not observed in my experiment and have not been reported previously, it is likely that hippocampal GlyRs are extrasynaptically located (Xu & Gong, 2010). Presumably these 'extrasynaptic' GlyRs are responsible for both excitatory depolarization and shunting inhibition in the immature hippocampus.

Although similar dual effects of activation of $GABA_A$Rs on epileptiform activity in the hippocampal CA3 region of same age rats were reported (Kolbaev et al., 2012), the mechanism underlying dual effect of $GABA_A$Rs seemed to be different from that of GlyRs. Pro- and anticonvulsive actions of $GABA_A$Rs were mediated by tonic $GABA_A$Rs and phasic $GABA_A$Rs, respectively (Kolbaev et al., 2012). While in my study, pro- and anticonvulsive actions of GlyRs were most probably mediated by extrasynaptic receptors via tonic activation and depend on the taurine or glycine concentrations.

4.5 Endogenously accumulated agonists are lacking an effect on epileptiform activity

It is assumed that glycine is an endogenous agonist of GlyR in the hippocampus, as evidence showed that in the hippocampus blockage of GlyTs reduced neuronal excitability through activation of GlyRs (Song *et al.*, 2006; Zhang *et al.*, 2008b). Other evidence showed blockage of GlyTs failed to induce GlyR-mediated currents in the hippocampus (Mori *et al.*, 2002), prompting a controversy whether in the hippocampus glycine is an endogenous agonist of GlyR. Besides glycine, taurine and β-alanine which share considerable structural similarity with glycine are considered to be endogenous agonists of GlyR in the hippocampus (Mori *et al.*, 2002). In particular, taurine which is one of the most abundant free amino acid in mammals, has been contemplated as the major endogenous agonist for hippocampal and neocortical GlyRs (Flint *et al.*, 1998; Huxtable, 1992; Mori *et al.*, 2002; Wu & Prentice, 2010).

In my experiments performed on the newborn rat hippocampus, D-serine did not show effect on epileptiform activity, indicating that an increase in extracellular glycine concentration cannot augment excitatory, NMDA receptor-dependent transmission. In adult and adolescent rodents, previous studies showed antiepileptic effect of inhibitors of GlyTs. It's not demonstrated in these studies which binding site between NMDA receptor or GlyR, mediated the anticonvulsive effect of endogenous glycine accumulated by inhibition of GlyTs (Harvey & Yee, 2013; Kalinichev *et al.*, 2010). It's documented that sarcosine, a GlyT1 inhibitor, had weak anticonvulsive activity through activation of GlyRs (Freed, 1985; Socala *et al.*, 2010). On the other hand, there are evidence showing glycine sites of NMDA receptors can mediate both anti- and proconvulsive actions. For example, in juvenile rats by agonizing glycine site of NMDA receptor D-cycloserine and D-serine provided anticonvulsive actions (Kalinichev *et al.*, 2010; Peterson & Schwade, 1993). Meanwhile D-serine was reported to be capable of potentiating NMDA-induced convulsions (Singh *et al.*, 1991). Specific model of in vitro epilepsy or immaturity of rats used in my experiments may account for my failure to observe effects of GlyTs inhibitors and D-serine on epileptiform activity.

In the presence of D-serine to saturate glycine biding site on NMDA receptors, blockade of two subtypes of GlyTs with ALX 1393 and ALX 5407 failed to present

effects of endogenously released glycine on epileptiform activity induced by low Mg^{2+}/4-AP conditions. One possible reason for the failure of GlyT blockers to exert influence on epileptiform activity is that glycine is indeed not the endogenous ligand of GlyRs, since blockage of GlyTs could not induce GlyR-mediated currents in the hippocampus (Mori *et al.*, 2002). Alternative explanations could be that endogenous glycine accumulation by blockage of GlyTs either is too low to adequately activate GlyRs or induced moderate, between depolarizing and shunting actions of GlyRs.

Taurine is proposed as the principal endogenous ligand of GlyRs and is maintained at a given concentration range at cytoplasma or interstitial (Lambert & Hansen, 2011). While TauT inhibitor GES did attenuate epileptiform activity, my experimental evidence suggested that the anticonvulsive effect of GES was actually mediated by $GABA_A$R, as suppressive effect of GES on epileptiform activity was prevented by gabazine and mimicked by equivalent concentration of muscimol. This result indicates that GES implements its anticonvulsive effect by an agonistic action of $GABA_A$Rs, and that effect of endogenous taurine on epileptiform activity cannot be manifested by TauT inhibitor. On the other hand, anticonvulsive action of high concentration of taurine was converted to proconvulsive action when both $GABA_A$Rs and GlyRs were blocked. This proconvulsive effect is probably caused by taurine uptake. As expected, application of GES in addition to $GABA_A$Rs and GlyRs blockers, high concentration of taurine actually had no more effect on epileptiform activity, indicative a proconvulsive action of TauT masked by $GABA_A$Rs and GlyRs. Most probably the hidden proconvulsive effect mediated by taurine uptake is caused by intracellular taurine-induced cell swelling (Oja & Saransaari, 1996), as cell swelling and the resulting decrease in extracellular volume fraction has been shown to decrease seizure thresholds in the immature hippocampus (Kilb et al. 2006).

4.6 NKCC1 shapes the proconvulsive effect of low concentrations of glycine and taurine

In the developing brain, intracellular Cl^- is elevated by the activation of NKCC1 (Delpire, 2000; Payne *et al.*, 2003; Sipila *et al.*, 2006a; Wang *et al.*, 2002; Yamada *et al.*, 2004). High intracellular Cl^- leads to a depolarizing Cl^- equilibrium potential and therefore a depolarizing action of $GABA_A$Rs and GlyRs in the immature neurons

(Ehrlich *et al.*, 1999; Kang *et al.*, 2002; Luhmann & Prince, 1991; Plotkin *et al.*, 1997). This situation of $GABA_A$Rs and GlyRs in the immature neurons is supposed to be one of the major reasons for high incidence of childhood epileptic seizures (Ben-Ari *et al.*, 1989; Ben-Ari, 2002; Ben-Ari *et al.*, 2007). Theoretically a reduction of intracellular Cl^- by inhibition of NKCC1 might negatively shift Cl^- equilibrium potential and prohibit excitatory action of $GABA_A$Rs and GlyRs in the immature neurons. As is evidenced, researches have revealed that activation of NKCC1 facilitated seizures in the developing brain (Dzhala *et al.*, 2005), whereas inactivation of NKCC1 alleviated neonatal seizure activity (Kahle *et al.*, 2009).

The data in this thesis does not support effectiveness of NKCC1 blocker bumetanide on epileptiform activity induced by low Mg^{2+} /4-AP conditions. This is consistent with some previous finding (Nardou *et al.*, 2009), but contrast to the finding that inactivation of NKCC1 by bumetanide alleviated neonatal seizure activity (Kahle *et al.*, 2009). The discrepancy could be that effect of bumetanide on epileptiform activity is mode-specific (Kilb *et al.*, 2007). Despite lack of effect of bumetanide itself, bumetanide completely abolished the proconvulsive action of low concentrations of glycine and taurine, indicating that the proconvulsive effect is NKCC1-dependent. This is in consistency with the finding that activation of NKCC1 facilitated seizures in the developing brain (Dzhala *et al.*, 2005).

4.7 Implication of this study in epileptic medication

Recurrent epileptic seizures are the characteristic manifestation of clinic epilepsy with a high incidence in children population (Camfield & Camfield, 1996; Wirrell *et al.*, 2011). Uncontrolled seizures and epilepsy in children can lead to a series of physical, mental, psychiatric and social sequences (Wirrell, 2013). Available AEDs mainly target on glutamatergic system, GABAergic system and some voltage-dependent ionic channels (Schmidt, 2009). However, about 20% of children with epilepsy are pharmacoresistant to these AEDs (Jarrar & Buchhalter, 2003; Wirrell, 2013). Moreover, some of classic AEDs have serious adverse effects, especially when they are used in a combined way (Schmidt, 2009).

In this study I report dual actions mediated by GlyRs on epileptiform activity of immature rat CHF. Activation of GlyRs is intrinsically required to prohibit spontaneous

epileptiform activity. High concentrations of exogenously-applied taurine and glycine are anticonvulsive, while low concentrations of exogenously-applied taurine and glycine are proconvulsive. Proconvulsive action of low concentrations of taurine and glycine is NKCC1-dependent. Meanwhile inhibition of TauT and GlyT to accumulate endogenous ligands has negligible effect.

Based on these novel findings, this study elicits the following medical implications.

First, for newborn babies and young children, accidental intake of strychnine-contaminated food and deficiency of taurine in nutrition can be a risk factor of epileptic seizures. Such cases have been reported (Jett, 2012; Philippe *et al.*, 2004).

Second, clinic trials using optimal doses of taurine or glycine or its analogues might be called for to medicate newborn babies and young children with epileptic seizures. A number of in vivo and in vitro post-developmental animal studies have shown consistently that taurine were capable of suppressing epileptic seizures or epileptiform activity (Kirchner *et al.*, 2003; Uemura *et al.*, 1991). In clinic studies, both alleviating and blank effects of taurine administrated orally or intravenously on epilepsy have been documented (Barbeau & Donaldson, 1974; Bergamini *et al.*, 1974; Konig *et al.*, 1977; Mantovani & DeVivo, 1979; Oja & Saransaari, 2013). One explanation for the lack of efficacy of taurine could be that taurine did not reach an optimal concentration in the brain due to metabolic degradation or low diffusibility across the blood-brain barrier. It is not known whether it is a different scenario for taurine treatment in the young brain. Since this study clearly showed strong anticonvulsive effect of high concentrations of taurine and glycine in immature hippocampus, further relevant in vivo animal studies and clinic studies need to be followed.

Third, inhibition of NKCC1 might be an auxiliary strategy to boost effects of $GABA_A$R- or GlyR-targeted AEDs for children epileptic seizures. Activation of NKCC1 during development provides $GABA_A$Rs and GlyRs with depolarizing action, as a consequence of which, GlyRs of immature neurons mediate the proconvulsive effect of low taurine and glycine concentrations. Inactivation of NKCC1 abolishes the proconvulsive aspect of taurine and glycine. Similarly, $GABA_A$Rs of immature neurons also can mediate proconvulsive effect which also can be blocked by bumetanide (Sharopov *et al.*, 2012). Thus, presumably in inactivation of NKCC1 might boost the anticonvulsive aspect of taurine and glycine or GABAergic agonists. Supportively to

this notion, some studies on animal model have suggested that NKCC1 could be a potential therapeutic target for neonatal seizures as inactivation of NKCC1 by bumetanide could decrease seizures (Kahle *et al.*, 2009; Kahle & Staley, 2008), and enhanced antiepileptic efficacy of phenobarbital which acts on $GABA_A$Rs (Cleary *et al.*, 2013; Dzhala *et al.*, 2008).

5 Summary

Glycine receptor (GlyR) is ionotrpic neurotransmitter receptor that upon binding of glycine opens an anion pore and mediates in the adult nervous system a consistent inhibitory action. While previously it was assumed that GlyRs mediate inhibition mainly in the brain stem and spinal cord, recent studies reported the abundant expression of GlyRs throughout the brain, in particular during neuronal development. GlyRs in the higher brain regions are involved in several important processes, such as neurotransmitter release, cell differentiation, cell migration and network recruitment. At prenatal and early postnatal stages activation of GlyRs mediates a membrane depolarization, as has been shown for the neurotransmitter GABA, which also opens a ligand-gated anion channel.

Epileptic seizures are the manifestations of epilepsy, which is a major neurological disorder. Epileptic seizures occur with a high incidence during early childhood. Uncontrolled epileptic seizures in the childhood often result in severe neurological complications and permanent damages of brain function. However, childhood epileptic seizures are considerably refractory to pharmacological therapies by traditional anti-epileptc drugs (AEDs) which mostly effect on glutamatergic and GABAergic receptors. No AED acts on glycinergic receptors so far.

Thus, the glycinergic system could potentially be a target for the control of epileptic seizures, especially pharmacoresistant ones. Interestingly, some studies have been directed to the role of GlyRs in epilepsy of mature rodents’ brain and provided promising evidence showing that GlyRs mediated inhibitory effects on epileptiform activities. However, thus far no study has been performed to reveal whether activation of GlyRs modulate epileptiform activities in the immature central nervous system (CNS). Therefore the study in this thesis addresses the role of GlyRs in the modulation of neuronal excitability and epileptiform activity in the immature rat brain.

By using in vitro intact corticohippocampal formations (CHF) of rats at postnatal days 4-7 and electrophysiological methods, a series of pharmacological examinations reveal that GlyRs are directly implicated in the control of hippocampal excitation levels at this age. In this thesis I am able to show that GlyRs are functionally expressed in the immature hippocampus and exhibit the classical pharmacology of immature GlyR, which can be activated by both glycine and the presumed endogenous agonist taurine.

This study also reveals that high concentration of taurine is anticonvulsive, but lower concentration of taurine is proconvulsive. A substantial fraction of both the pro- and anticonvulsive taurine effects is mediated via GlyRs, although activation of $GABA_A$ receptors also considerably contributes to the taurine effects. Similarly, glycine exerts both pro- and anticonvulsive effects at low and high concentrations, respectively. The proconvulsive effects of taurine and glycine depend on NKCC1-mediated Cl^- accumulation, as bath application of NKCC1 inhibitor bumetanide completely abolishes proconvulsive effects of low taurine and glycine concentrations. Inhibition of GlyRs with low strychnine concentration triggers epileptiform activity in the CA3 region of immature CHF, indicating that intrinsically an inhibitory action of GlyRs overwhelms its depolarizing action in the immature hippocampus. Additionally, my study indicates that blocking taurine transporters (TauTs) to accumulate endogenous taurine reduces epileptiform activity via activation of $GABA_A$Rs, but not GlyRs, while blocking glycine tansporters (GlyTs) has no observable effect on epileptiform activity.

From the main results of this study it can be concluded that in the immature rat hippocampus, activation of GlyRs mediates both pro- and anticonvulsive effects, but that a persistent activation of GlyRs is required to suppress neuronal overexcitability. In summary, this study uncovers an important role of GlyRs in the modulation of neuronal excitability and epileptiform activity in the immature rat hippocampus, and indicates that glycinergic system can potentially be a new therapeutic target against epileptic seizures of children.

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