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Glycine Transporter 1 is a Target for the Treatment of Epilepsy

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Abstract

Glycine is the major inhibitory neurotransmitter in brainstem and spinal cord, whereas in hippocampus glycine exerts dual modulatory roles on strychnine-sensitive glycine receptors and on the strychnine-insensitive glycine$_B$ site of the *N*-methyl-D-aspartate receptor (NMDAR). In hippocampus, the synaptic availability of glycine is largely under control of glycine transporter 1 (GlyT1). Since epilepsy is a disorder of disrupted network homeostasis affecting the equilibrium of various neurotransmitters and neuromodulators, we hypothesized that changes in hippocampal GlyT1 expression and resulting disruption of glycine homeostasis might be implicated in the pathophysiology of epilepsy. Using two different rodent models of temporal lobe epilepsy (TLE) – the intrahippocampal kainic acid model of TLE in mice, and the rat model of tetanic stimulation-induced TLE – we first demonstrated robust overexpression of GlyT1 in the hippocampal formation, suggesting dysfunctional glycine signaling in epilepsy. Overexpression of GlyT1 in the hippocampal formation was corroborated in human TLE samples by quantitative real time PCR. In support of a role of dysfunctional glycine signaling in the pathophysiology of epilepsy, both the genetic deletion of GlyT1 in hippocampus and the GlyT1 inhibitor LY2365109 increased seizure thresholds in mice. Importantly, chronic seizures in the mouse model of TLE were robustly suppressed by systemic administration of the GlyT1 inhibitor LY2365109. We conclude that GlyT1 overexpression in the epileptic brain constitutes a new target for therapeutic intervention, and that GlyT1 inhibitors constitute a new class of antiictogenic drugs. These findings are of translational value since GlyT1 inhibitors are already in clinical development to treat cognitive symptoms in schizophrenia.

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Keywords

temporal lobe epilepsy; seizures; glycine transporter 1; GlyT1; antiepileptic drugs; histopathology

1. Introduction

Epilepsy treatment is limited by poor response to available antiepileptic drugs and limited tolerability due to major cognitive side effects (Arif et al., 2009; Hirsch et al., 2003; Loscher and Schmidt, 2011; Ortinski and Meador, 2004). The development of therapies that combine anticonvulsant with pro-cognitive properties would therefore be a significant advancement. Glycine is the major inhibitory neurotransmitter in brainstem and spinal cord (Betz et al., 2001). In the hippocampus glycine has evolved into a homeostatic modulator of neuronal function by assuming potentially opposing activities that depend on the extracellular concentrations of glycine: *(i)* Glycine, but also D-serine, act as obligatory co-agonists of the N-methyl-D-aspartate receptor (NMDAR) by binding to its strychnine-insensitive glycine$_B$ site (Labrie and Roder, 2010). Since this binding site is normally not saturated (Bergeron et al., 1998), increases in extracellular glycine can potentiate impulse-dependent NMDAR activation, with resulting pro-cognitive effects (Black et al., 2009). The indirect modulation of NMDAR function via glycine is a promising strategy to improve cognition (Labrie and Roder, 2010; Mohler et al., 2008). Importantly, and in contrast to direct NMDAR agonism, the allosteric modulation of NMDARs through activation of the glycine$_B$ site does not bear risks for seizure generation or neurotoxicity (Yang and Svensson, 2008). *(ii)* Low concentrations of extracellular glycine (10 μM) activate presynaptic glycine receptors (GlyRs) and thereby promote pro-convulsant mechanisms (Chen et al., 2014; Kubota et al., 2010; Winkelmann et al., 2014). *(iii)* Under higher extracellular concentrations (100 μM) glycine binds to extrasynaptic GlyRs in the postsynaptic compartment providing tonic suppression of network excitability (Eichler et al., 2008; Kirchner et al., 2003). Together, those mechanisms implicate that low concentrations of extracellular glycine favor epileptiform activity and impairment of cognitive function. Consequently, the maintenance of glycine homeostasis plays a crucial role for the regulation of excitability in the hippocampal formation. Accordingly, *in vitro* studies demonstrated that exogenous glycine suppressed neuronal excitation in the dentate gyrus (Chattipakorn and McMahon, 2003) and reduced the firing of action potentials in hippocampal neurons (Song et al., 2006), whereas blockade of glycine-reuptake depressed excitatory postsynaptic potentials (Zhang et al., 2008).

Hippocampal glycine is largely regulated by its reuptake transporter GlyT1 found in both excitatory neurons and astrocytes (Aragon and Lopez-Corcuera, 2005; Betz et al., 2006; Cubelos et al., 2005; Eulenburg et al., 2005; Martina et al., 2005; Tsai et al., 2004). Consequently, the genetic deletion of GlyT1 increased synaptic glycine availability (Gomeza et al., 2003). Engineered mice with a genetic deletion of GlyT1 in forebrain were characterized by a decrease in hippocampal glycine uptake, an increase in hippocampal NMDAR function, and a wide spectrum of pro-cognitive effects (Mohler et al., 2011; Mohler et al., 2008; Yee et al., 2006). Therefore, GlyT1 has emerged as a promising target for the treatment of cognitive symptoms in schizophrenia and several compounds are

currently in phase II and III clinical trials (Black et al., 2009; Mohler et al., 2011; Singer et al., 2009).

Whereas the role of glycine regulation within the context of schizophrenia has received much attention, glycine may also play an underappreciated role in epilepsy. In patients with temporal lobe epilepsy changes in hippocampal glycine receptor expression have been reported, suggesting dysregulation of glycinergic signaling in epilepsy (Eichler et al., 2008). In line with those findings activation of glycine receptors modulated spontaneous epileptiform activity in the immature rat hippocampus (Chen et al., 2014), whereas GlyT1 inhibitors demonstrated anticonvulsant properties in a rat maximal electroshock test (Kalinichev et al., 2010). However, the role of GlyT1 in human epilepsy and in clinically relevant rodent models of chronic epilepsy has not been studied to date.

The present study was designed to investigate the role of GlyT1 in temporal lobe epilepsy and to evaluate whether GlyT1 inhibition might be a feasible strategy for seizure control in chronic epilepsy. Using two different rodent models of TLE and samples from human TLE patients, we demonstrate robust increases of GlyT1 in the epileptogenic hippocampus. Consequently, the genetic deletion or pharmacological inhibition of GlyT1 suppressed both induced and chronic seizures.

2. Materials & Methods

2.1. Studies in mice

All animal procedures were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Legacy Research Institute and the principles outlined by the National Institutes of Health (NIH). Eight to 10 week old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine) as well as CamKIIαCre:GlyT1$^{fl/fl}$ (“GlyT1-KO”) mice (Yee et al., 2006) and their wild-type littermates GlyT1$^{fl/fl}$ (“GlyT1-WT”) were used. All mutant animals were in an identical C57Bl/6 background. GlyT1-KO mice are characterized by a pro-cognitive phenotype as described previously (Yee et al., 2006). All animals were housed in temperature- and humidity-controlled rooms with a 12 h light/dark cycle (lights on at 6:30 AM).

2.2. Mouse model of temporal lobe epilepsy (TLE)

Chronic epilepsy in adult male C57Bl/6 mice was induced by intrahippocampal kainic acid (KA) injection according to our standard procedures (Gouder et al., 2003) with modifications. Briefly, under anesthesia with 68.5% N_2O, 30% O_2, and 1.5% isoflurane the animals received stereotactic injections into the right dorsal hippocampus (coordinates: AP = −2.10 mm; ML = ± 1.80 mm; DV = −1.70 mm to Bregma) with 400 ng of KA (K0250, Sigma, USA) in a volume of 200 nl 0.9% NaCl using a 1-μl microsyringe (Hamilton, Reno, NV, USA). Injections were performed over a period of 3 min. At the end of the injection, the cannula was left in place for an additional 3 min to limit reflux along the cannula track. Control mice received intrahippocampal injection of 200 nl saline. The KA injection triggered non-convulsive status epilepticus, which is the precipitating event for subsequent epileptogenesis. Four weeks after the injection of KA or vehicle, the animals were implanted

with bipolar coated stainless steel electrodes (80 μm in diameter; Plastics One) into the right dorsal hippocampus using the same coordinates as the previous KA injection. A cortical screw electrode was placed over the frontal cortex and a ground electrode over the cerebellum. All electrodes were secured to the skull with dental cement.

2.3. Electroencephalography and drug treatment

Electroencephalography (EEG) monitoring was performed according to our previous publications (Shen et al., 2014; Theofilas et al., 2011). Six weeks after KA-injection and two weeks after electrode implantation the animals were subjected to blocks of 24 hours of EEG monitoring (Grass Technologies). Electrical brain activity was amplified and digitized (PowerLab; AD Instruments). Quantification of EEG records was performed blinded to the experimental treatment. EEG seizure activity was defined as high-amplitude rhythmic discharges that clearly represented a new pattern of tracing lasting for >5 seconds (repetitive spikes, spike-and-wave discharges, or slow waves). Epileptic events occurring with an interval <5 seconds without the EEG returning to baseline were defined as belonging to the same seizure. Seizures were primarily electrographic in nature, and only occasionally accompanied by arrest or staring episodes; thus seizure quantification was performed exclusively by intrahippocampal EEG recordings. Before each recording session the animals were habituated in the recording cage for 4 hours. After habituation the EEGs were recorded for 24 h. To assess the antiepileptic effect of GlyT1 inhibition the animals received daily injections with the GlyT1 inhibitor [2-(4-benzo[1,3]dioxol-5-yl-2-tert-butylphenoxy)ethyl]-methylamino-acetic acid LY2365109 (Tocris Bioscience, Bristol, UK) (3 – 30 mg/kg i.p, dissolved in 5% DMSO), or vehicle.

2.4. Chemoconvulsant seizure tests

To quantify the anticonvulsive effect of pharmacological inhibition or genetic disruption of GlyT1 in an acute seizure model we used standard pentylenetetrazole (PTZ) seizure tests (White, 2003; White et al., 2007) in WT and GlyT1-KO mice. PTZ (P6500, Sigma, USA) was freshly dissolved daily in sterile saline. Mice were subjected repeated PTZ-injections (10 mg/kg, i.p.) every 10 minutes until they reached a behavioral seizure score of 5 according to Racine (Racine, 1978). The cumulative PTZ dose (in mg PTZ per kg body weight) needed to trigger stage 5 seizures was considered the convulsant threshold dose. To quantify the anticonvulsant efficacy of GlyT1 inhibition, the GlyT1 inhibitor LY2365109 (10 mg/kg, single i.p.) was administered 30 seconds prior to the first PTZ injection, whereas 5% DMSO in saline was used as vehicle control.

2.5. Maximal electroshock seizure (MES) test

To evaluate the anticonvulsive property of pharmacological GlyT1 inhibition in a mechanistically different acute seizure test we chose the MES test, a standard seizure test for pre-clinical drug development (Giardina and Gasior, 2009; Loscher, 2011). LY2365109 (10 – 60 mg/kg i.p. in 5% DMSO), valproic acid (169 mg/kg i.p. in saline), or vehicle (5% DMSO) was administered 30 to 120 minutes prior to each MES test. Animals were restrained and a drop of ophthalmic solution (Proparcaine Hydrochloride 0.5%) was placed in each eye, after which electroshock stimulation was induced via corneal electrodes. Stimulation parameters consisted of a 50-mA stimulus at 60Hz, for a duration of 0.2

seconds, delivered by a Hugo Sachs Elektronik Rodent Shocker-Type 221 (March-Hugstetten, Germany). Abrogation of tonic hind limb extension (THLE) was used as the index for therapeutic efficacy of each compound. Animals failing to show THLE were scored as being protected from seizure activity.

2.6. Rat model of temporal lobe epilepsy

Adult male Sprague Dawley rats (Harlan Netherlands, Horst, The Netherlands) weighing 400–600 grams were used in this study, which was approved by the University of Amsterdam Animal Welfare committee. The rats were housed individually in a controlled environment (21±1°C; humidity 60%; lights on 08:00 AM – 8:00 PM; food and water available *ad libitum)*. Rats were anaesthetized with an intramuscular injection of ketamine (57 mg/kg; Alfasan, Woerden, The Netherlands) and xylazine (9 mg/kg; Bayer AG, Leverkusen, Germany) and placed in a stereotactic apparatus. In order to record hippocampal EEGs, a pair of insulated stainless steel electrodes (70 μm wire diameter, tips 80 μm apart) were implanted into the left dentate gyrus (DG) under electrophysiological control as previously described (Gorter et al., 2001). A pair of stimulation electrodes was implanted in the angular bundle. Two weeks after recovery from the operation, each rat was transferred to a recording cage (40×40×80 cm) and connected to a recording and stimulation system (NeuroData Digital Stimulator, Cygnus Technology Inc, USA) with a shielded multi-strand cable and electrical swivel (Air Precision, Le Plessis Robinson, France). After habituation to the new condition, rats underwent tetanic stimulations (50 Hz) of the hippocampus in the form of a succession of trains of pulses every 13 seconds. Each train lasted 10 seconds and consisted of biphasic pulses (pulse duration 0.5 ms, maximal intensity 500 μA). Stimulation was stopped when the rats displayed sustained forelimb clonus and salivation for minutes, which usually occurred within 1 hour. However, stimulation never lasted longer than 90 minutes. Immediately after termination of the stimulation, periodic epileptiform discharges (PEDs) occurred at a frequency of 1–2 Hz which lasted several hours (status epilepticus; SE). Differential EEG signals were amplified (10x) via a FET transistor that connected the headset to a differential amplifier (20x; CyberAmp, Axon Instruments, Burlingame, CA, USA), filtered (1–60 Hz), and digitized by a computer. A seizure detection program (Harmonie, Stellate Systems, Montreal, Canada) sampled the incoming signal at a frequency of 200 Hz per channel. EEG recordings were monitored also visually and screened for seizure activity. Behavior was observed during electrical stimulation and several hours thereafter. Immediately after termination of the stimulation, periodic epileptiform discharges (PEDs) occurred at a frequency of 1–2 Hz and they were accompanied by behavioral and EEG seizures (SE). Most rats were monitored continuously from the cessation of SE to the time of sacrifice. The chronic epileptic group (6 months after SE) was monitored during and shortly after SE and for a period of 4 weeks before sacrifice in order to determine the frequency of spontaneous seizures. Sham-operated control rats were handled and recorded identically, but did not receive electrical stimulation. None of these rats needed to be reimplanted. Chronic epileptic rats had frequent daily seizures (range, 5–12). The time between the last spontaneous seizure and sacrifice was < 5 h.

2.7. Immunohistochemistry

To determine changes in GlyT1 expression a polyclonal rabbit antiserum was raised against the C-terminal peptide (C)AQIPIVGSNGSSRFQDSRI (Gabernet et al., 2005). Specificity of the antibody was validated in peptide competition assays and in samples derived from GlyT1 knockout animals (Gabernet et al., 2005; Yee et al., 2006). Rats were sacrificed 24 hours (acute phase, n=5), 1 week (latent phase, n=6) and 6 months (chronic phase, progressive n=6; non-progressive n=4) after electrically induced SE for immunohistochemical analysis. After the rats were deeply anesthetized with pentobarbital (Nembutal, intraperitoneally, 60 mg/kg), they were perfused through the ascending aorta with 300 ml 0.37% Na_2S solution followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were post-fixed *in situ* overnight at 4°C, dissected and cryoprotected in 30% phosphate-buffered sucrose solution (pH 7.4). After overnight incubation at 4°C, the brains were frozen in isopentane (−25°C) and stored at −80°C until sectioning. The brains were cut on a sliding microtome and 40 μm horizontal sections were collected in 0.1 M phosphate buffer. Horizontal sections between 5100–5600 μm below the cortex surface of the contralateral brain part of control and post-SE rats were used for immunohistochemistry. Sections were washed in 0.05 M phosphate buffered saline (PBS), pH 7.4 and incubated for 30 minutes in 0.3% hydrogen peroxide in PBS to inactivate endogenous peroxidase. Sections were then washed (2 x 10 minutes) in 0.05 M PBS, followed by washing (1 × 60 min) in PBS +0.5% Triton X-100+0.4% Bovine Serum Albumin (BSA). Sections were incubated with affinity purified GlyT1 antiserum (1:300) (Gabernet et al., 2005) in PBS+0.1% Triton X-100+0.4% BSA at 4°C. Twenty-four hours after the incubation with the primary antibody, the sections were washed in PBS (3×10 minutes) and then incubated for 1.5 hours in biotinylated sheep anti-rabbit Ig (GE Healthcare, Diegem, Belgium), diluted 1:200 in PBS+0.1% Triton X-100. This was followed by incubation for 60 minutes in AB-mix (Vectastain ABC kit, Peroxidase Standard pk-4000, Vector Laboratories, Burlingame, CA, USA). After washing (3×10 minutes) in 0.05 M Tris-HCl, pH 7.9, the sections were stained with 3,3′-diaminobenzidin tetrahydrochloride (50 mg DAB, Sigma-Aldrich, Zwijndrecht, The Netherlands) and 5 μl 30% hydrogen peroxide in a 10 ml solution of Tris-HCl. The staining reaction was followed under the microscope and stopped by washing the sections in Tris-HCl. After mounting on superfrost plus slides, the sections were air dried, dehydrated in alcohol and xylene and coverslipped with Entellan (Merck, Darmstadt, Germany). All procedures were carried out so that all sections were processed in parallel in the same solutions using identical incubation times. Digital images of GlyT1 immunohistochemistry were acquired using an Olympus BX51 microscope equipped with an Olympus DP70 camera (Olympus Nederland, Zoeterwoude, The Netherlands) and used for semiquantitative analysis. Immunodensities of hippocampal subfields were scanned and translated into mean pixel intensities using Adobe Photoshop.

To determine GlyT1 expression profiles in epileptic mice, the animals were sacrificed for immunohistochemical analysis after the completion of seizure assessment. Similar procedures as described above were used with the following modifications: The animals were transcardially perfused with saline followed by 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde followed by 30% sucrose, and then

sectioned into 403μm coronal sections using a cryostat (VT 10003S, Leica, Bannockburn, IL, USA). Immunoperoxidase staining was used for the detection of GlyT1. Brain slices were incubated overnight at 4°C with affinity purified GlyT1 antiserum (1:500) (Gabernet et al., 2005). After washing in TBS, slices were incubated with biotinylated goat anti-rabbit secondary antibody (1:200) for 2 hours. Then the slices were incubated with an avidin–biotin enzyme complex (Vectastain Kit, PK-6100, Vector Labs, CA, USA) for 1 hour, followed by incubation in hydrogen peroxide and 3, 3-diaminobenzidine hydrochloride (SK-4100, Vector Labs, CA, USA). All sections were processed in parallel using identical solutions and identical incubation times. All images were acquired under identical conditions and all image processing was applied identically across different experimental groups.

2.8. Western blot analysis

Mouse hippocampi were processed for the extraction of aqueous membrane proteins. 403μg of protein from each sample was loaded and electrophoresed on a 10% Tris-glycine gel. After the transfer of the proteins onto nitrocellulose membranes the blots were blocked for 1 hour in TBSTM (10 mM Tris/HCl, pH 8, 0.15 M NaCl, 0.05 % Tween 20, containing 5% nonfat milk) at room temperature, followed by incubation with affinity purified GlyT1 antiserum (1:1,000) (Gabernet et al., 2005) overnight at 4°C in TBSTM, three washes with TBST, and incubation with peroxidase-conjugated anti-rabbit antibody (#7074, 1:8,000, Cell Signaling, Boston, MA, USA) at RT for 1 hour. After extensive washing, immunoreactivity was detected by chemiluminescence (#34087, Pierce, IL, USA). Immunoblots were quantified using a Kodak Scientific Imaging System (v3.6.5.k2, Kodak, Rochester, NY, USA). To normalize ADK immunoreactivity to protein loading, a mouse monoclonal anti-α-tubulin antibody (# sc-8035, 1:5,000, Santa Cruz Biotechnology, CA, USA) was used to re-probe the same blot and the OD ratio of ADK to α-tubulin was calculated. Samples from brainstem (highly enriched in GlyT1) were added as positive control.

2.9. Real-time quantitative PCR

The clinical cases and controls included in this study (Table 1) were selected from the files of the departments of neuropathology of the Academic Medical Center (AMC, University of Amsterdam) and the VU University Medical Center (VUMC). Tissue was obtained and used in accordance with the Declaration of Helsinki and the AMC Research Code provided by the Medical Ethics Committee and approved by the science committee of the VUMC Biobank. The clinical characteristics derived from the patient's medical records are summarized in Table 1.

Hippocampi from control autopsy patients (n=5) and resected hippocampi from patients with drug-resistant temporal lobe epilepsy (n=9) were snap frozen in liquid nitrogen and stored at −80°C until further use. Total RNA was isolated using Qiazol Lysis Reagent (Qiagen Benelux, Venlo, The Netherlands) and an RNeasy Mini kit (Qiagen Benelux, Venlo, the Netherlands) according to the manufacturer's instructions. The concentration and purity of RNA was determined at 260/280 nm using a Nanodrop spectrophotometer (Ocean Optics, Dunedin, FL, USA). Five micrograms of total RNA were reverse-transcribed into cDNA using oligo dT primers. Five nmol oligo dT primers were annealed to 5 μg total RNA in a

total volume of 25 μl, by incubation at 72 °C for 10 min, and cooled to 4°C. Reverse transcription was performed by the addition of 25 μl RT-mix, containing: First Strand Buffer (Invitrogen-Life Technologies), 2 mM dNTPs (Pharmacia, Germany), 30 U RNAse inhibitor (Roche Applied Science, Indianapolis, IN, USA) and 400 U M-MLV reverse transcriptase (Invitrogen - Life Technologies, The Netherlands). The total reaction mix (50 μl) was incubated at 37 °C for 60 min, heated to 95 °C for 10 min and stored at −20°C until use. For each PCR, a mastermix was prepared on ice, containing per sample: 1 μl cDNA, 2.5 μl of SensiFAST SYBR no-ROX mix (Bioline Reagents Ltd, London, UK), 0.4 μM of both forward and reverse primers for GlyT1 (forward: CCATGTTCAAAGGAGTGGGCTA; reverse: TGACCACATTGTAGTAGATGCCG) and the house keeping reference gene Elongation Factor 1α (EF1α) (Iyer et al., 2012) (forward: ATCCACCTTTGGGTCGCTTT; reverse: CCGCAACTGTCTGTCTCATATCAC). The final volume was adjusted to 5 μl with PCR grade water. The LightCycler® 480 Real-Time PCR System (Roche Applied Science, Basel, Switzerland) was used with a 384-multiwell plate format. The cycling conditions were carried out as follows: initial denaturation at 95°C for 2 min, followed by 55 cycles of denaturation at 95°C for 5 s, annealing at 65°C for 10 s and elongation at 72°C for 15 s. The fluorescent product was measured by a single acquisition mode at 72°C after each cycle. For distinguishing specific from non-specific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 65°C for 1 min followed by a gradual increase in temperature to 95°C at a rate of 2.5°C/s, with the signal acquisition mode set to continuous. Quantification of data was performed using the computer program LinRegPCR in which linear regression on the Log(fluorescence) per cycle number data is applied to determine the amplification efficiency per sample (Ruijter et al., 2009). The starting concentration of GlyT1 was divided by the starting concentration of the reference gene Elongation Factor 1α (EF1α) and this ratio was compared between patient/control groups.

2.10. Statistical analysis

Data are expressed as means ± standard error of mean (SEM). Statistical analysis was performed by a one way analysis of variance (ANOVA) followed by Bonferroni post-hoc analysis or student t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Dysregulation of GlyT1 expression in the epileptogenic hippocampus of mice

Glycine in the hippocampus combines unique properties to regulate network excitability through opposing roles of pre- and postsynaptic GlyRs (Chen et al., 2014; Eichler et al., 2008; Kirchner et al., 2003; Kubota et al., 2010; Winkelmann et al., 2014), and through activation of the glycine$_B$ site of the NMDAR (Black et al., 2009; Labrie and Roder, 2010; Mohler et al., 2008). Therefore, maintenance of glycine homeostasis might play a critical role in epilepsy. Because glycine homeostasis in the hippocampus is largely under the control of GlyT1 (Pinto et al., 2015; Yee et al., 2006), we predicted that GlyT1 dysregulation might be associated with chronic epilepsy. We therefore evaluated the GlyT1 expression profile in samples derived from two mechanistically different rodent models of temporal lobe epilepsy (TLE) and in specimen from surgically resected human epileptogenic

hippocampus. We first generated a cohort of epileptic mice by intrahippocampal injection of KA (n=12), whereas control animals received an intrahippocampal injection of saline. Development of epilepsy was confirmed via EEG recordings and histopathological analysis. Six weeks after the injections, only the KA injected animals (12 out of 12 animals) developed spontaneous electrographic seizures at a rate of 18.7 ± 1.9 seizures per hour that originated from the epileptogenic hippocampus in accordance to our prior work (Gouder et al., 2003; Gouder et al., 2004). After completion of EEG monitoring all animals were sacrificed and processed for immunohistochemical (n=8 per group) or biochemical (n=4 per group) analysis. Nissl staining confirmed typical histopathology reminiscent of human TLE in the epileptogenic KA-injected hippocampus. Major morphological features of the epileptogenic hippocampus included loss of CA1 and hilar neurons and granule cell dispersion, whereas saline-injected control hippocampi or hippocampi contralateral to the KA injection were not affected (Fig. 1A). Staining for GlyT1 immunoreactivity in control animals revealed a typical pattern of low GlyT1 expression levels in the hippocampal formation and adjacent cortex, whereas thalamus (as well as all mid-brain and brain-stem structures, not shown) displayed high levels of GlyT1 expression according to published data (Cubelos et al., 2005; Mohler et al., 2011). In contrast, we found a major dysregulation of GlyT1 expression specific to the epileptogenic hippocampus of KA-injected animals, whereas other brain areas were not affected. Increased GlyT1 expression was associated with most areas of the hippocampal formation and closely linked to dispersed granule cells (Fig. 1A). To quantify the extent of GlyT1 dysregulation in the epileptogenic hippocampus we performed Western blot analysis using homogenates of dissected KA-injected or saline-injected hippocampi (n=4, each). Quantification of immunodensities and normalization to α-tubulin revealed a 2.95 ± 0.47-fold increase in GlyT1 expression in KA-injected versus vehicle-injected hippocampus (Figure 1B, $p<0.01$, t-test). To the best of our knowledge, these data are the first demonstration that GlyT1 expression is dysregulated in the epileptogenic hippocampus.

3.2. Dysregulation of GlyT1 expression is associated with a progressive form of epilepsy in rats

To assess whether overexpression of GlyT1 is unique to the intrahippocampal KA model of TLE or a general feature of TLE, we evaluated GlyT1 expression in the hippocampus of the tetanic stimulation model of TLE in rats (Aronica et al., 2011; Gorter et al., 2001; van Vliet et al., 2012). This model offers the unique opportunity to study GlyT1 expression changes during epileptogenesis and within the context of progressive versus non-progressive forms of epilepsy (Gorter et al., 2001). Twenty-one rats were subjected to tetanic stimulation to trigger epileptogenesis. Rats were perfused and processed for histopathological analysis at different time-points following the tetanic stimulation-induced status epilepticus, i.e., 24 hours (acute phase, n=5), 1 week (latent phase, n=6) and 6 months (chronic phase, progressive n=6; non-progressive n=4). Rats with a progressive type of epilepsy had on average 9 seizures per day in the week before they were sacrificed, whereas rats with a non-progressive type of epilepsy exhibited less than 1 seizure per day. Similarly as observed in mice, the expression of GlyT1 in control rats (n=5) was low in the hippocampal formation (Figure 2A) and adjacent cortex, whereas thalamus (as well as all mid-brain and brain-stem structures, not shown) displayed high levels of GlyT1. Interestingly, GlyT1

immunoreactivity in the hippocampal formation significantly decreased during the acute (24 hours after stimulation) and latent phase of epileptogenesis (1 week after stimulation) compared to controls ($p<0.05$, one-way ANOVA), in the dentate gyrus (molecular layer, granule cell layer and hilus) as well as in CA3 (stratum radiatum and pyramidal cell layer) (Figure 2B). Low levels of hippocampal GlyT1 expression were maintained in those animals that developed a non-progressive form of epilepsy, whereas animals with a chronic progressive form of epilepsy developed robust overexpression of GlyT1 in all layers of the dentate gyrus, CA3 and CA1 throughout the hippocampal formation compared to control ($p<0.05$, one-way ANOVA) (Figure 2B). These findings demonstrate that epileptogenesis is associated with dynamic GlyT1 expression changes and that only the chronic progressive form of epilepsy was associated with increased levels of GlyT1 expression.

3.3. Overexpression of GlyT1 in the hippocampus of TLE patients

To assess whether GlyT1 dysregulation is likewise associated with human TLE, we used quantitative real time PCR to quantify GlyT1 mRNA expression from hippocampal resection material derived from patients with TLE who underwent resective epilepsy surgery. Our findings show that the ratio between GlyT1 and the housekeeping gene EF1α increased 2.17 ± 0.32-fold in resected hippocampi from patients with drug-resistant temporal lobe epilepsy (n=9) as compared to control specimens (n=5, $p<0.05$ t-test) (Figure 3). We conclude that overexpression of GlyT1 is also associated with human TLE.

3.4. Pharmacological inhibition of GlyT1 prevents acute seizures in mice

If overexpression of GlyT1 in epilepsy is functionally related to seizure generation, then pharmacological blockade of GlyT1 should exert anticonvulsive effects. We chose the selective GlyT1 inhibitor LY2365109, which is known to robustly increase glycine levels in the brain (Perry et al., 2008). Using the maximal electroshock seizure test (Giardina and Gasior, 2009; Loscher, 2011; White, 2003) we first show that a standard antiepileptic drug valproic acid delivered at a dose of 169 mg/kg prevented tonic hind limb extension in 50% of the animals (positive control) whereas none of the naïve (untreated) or vehicle injected animals (negative control) were protected from (THLE) (n=10, each) (Figure 4A). To test the therapeutic efficacy of LY2365109, the drug was administered intraperitoneally at concentrations of 10, 30, and 60 mg/kg 30 min prior to the electrical stimulation (n = 10, each). The dose of 30 mg/kg LY2365109 effectively prevented THLE in 100% of the animals whereas both the lower as well as the higher dose showed reduced efficacy (Figure 4A). To determine the time of peak effect, we chose the lower dose of 10 mg/kg LY2365109, but varied the time interval between drug application and electrical stimulation. We demonstrated that pretreatment with 10 mg/kg LY2365109 one hour prior to the stimulation prevented THLE in 100% of the animals indicating relatively slow drug kinetics (Figure 4A). In line with those findings a dose of 10 mg/kg LY2365109 significantly enhanced PTZ seizure thresholds (57.5 ± 3.7 mg/kg PTZ, n = 8) compared to a vehicle-treated control group (45.7 ± 3.0 mg/kg PTZ, n=7, $p<0.05$) (Figure 4B). Likewise, the PTZ seizure-threshold in GlyT1-KO mice (73.3 ± 8.0 mg/kg, n=6) was significantly higher (26.3% increase) than in GlyT1-WT littermates (58.0 ± 2.0 mg/kg, n=10, $p<0.05$) (Figure 4C). Together, these data demonstrate that pharmacological blockade of GlyT1 effectively reduces acute seizure susceptibility.

3.5. Pharmacological inhibition of GlyT1 prevents chronic seizures in a mouse model of temporal lobe epilepsy

To assess whether GlyT1 inhibition might likewise be effective to suppress chronic seizures in a model of TLE, we induced chronic epilepsy in adult male C57Bl/6 WT mice via intrahippocampal injection of KA. Five weeks later, according to our prior study (Gouder et al., 2003) only the KA-injected animals developed chronic recurrent seizures at a rate of 19.7 ± 1.1 seizures per hour and an average duration of 17.8 ± 3.6 seconds (Figure 5A, B). After completion of the baseline recordings, the epileptic animals received intraperitoneal injections with the GlyT1 inhibitor LY2365109 (3, 5, 7.5, 10, or 30 mg/kg), or vehicle (n=8–12 per group) followed by 24 hours of continuous monitoring of the EEG. After a delay of one hour LY2365109 at doses of 7.5 and 10mg/kg provided >50% seizure reduction in all epileptic animals ($p < 0.05$, one-way ANOVA) (Figure 5C). The therapeutic effects of 10 mg/kg LY2365109 lasted for ~16 hours with a maximum of protection at 4 hours following the drug injection (Figure 5D). A dose of 30 mg/kg was found to trigger lethal respiratory arrest within 3 to 4 hours in all epileptic animals. Lower doses of LY2365109 (3 mg/kg and 5 mg/kg) were either without effect or displayed reduced efficacy with average hourly seizure numbers of 23.2 ± 2.6 (3 mg/kg) and 19.5 ± 1.3 (LY, 5mg/kg) compared the vehicle treated control group (23.1 ± 1.2 seizures per hour, $p>0.05$). We conclude that a dose range of 7.5–10 mg/kg LY2365109 effectively reduces seizures in a mouse model of chronic TLE.

4. Discussion

This is the first comprehensive analysis of GlyT1 regulation in chronic epilepsy. We demonstrate a biphasic response of GlyT1 expression during epileptogenesis with initial downregulation of GlyT1 after epileptogenesis-precipitating seizures followed by sustained pathological overexpression of GlyT1 in chronic epilepsy as demonstrated in two mechanistically different models of TLE in mice and rats. Further, we show that human TLE is likewise associated with increased levels of GlyT1. Conversely, the pharmacological suppression of GlyT1 or the genetic ablation of GlyT1 in the hippocampus provides robust reduction of both acute as well as chronic seizure activity in three different model systems. We conclude that glycinergic regulation of network excitability is altered in epilepsy and that GlyT1 therefore presents a rational therapeutic target for the treatment of epilepsy. Our findings warrant further discussion.

4.1. Dynamic changes of GlyT1 regulation during epileptogenesis

We have previously shown that reduced levels of hippocampal GlyT1 expression lead to a decrease in the N-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)-propyl] sarcosine (NFPS) specific [3H]glycine uptake (Yee et al., 2006). Further, experimental overexpression of GlyT1 led to a reduction in the extracellular glycine tone (Supplisson and Bergman, 1997). These findings directly demonstrate that GlyT1 expression determines the extracellular tone of glycine. Our findings reported here show rapid decreases of hippocampal GlyT1 immunoreactivity during the acute and latent phases of epileptogenesis. The relevance of reduced GlyT1 expression during the early stages of epilepsy development is currently unknown. GlyT1 is a symporter that co-transports 1 glycine with 2 Na^+ and 1 Cl^-

(Eulenburg et al., 2005). Thus, the direction of glycine transport depends on ionic concentration gradients across the plasma membrane. Whereas hyperpolarization promotes the reuptake of glycine into the cell, depolarization of the membrane induces the cellular release of glycine. Consequently, an epileptogenic event that induces SE would trigger the cellular release of glycine, which, in light with our current findings, might be an endogenous mechanism to limit seizure activity. Likewise, the rapid reduction of GlyT1 expression after an acute seizure would limit the reuptake of glycine and thereby boost the anticonvulsive functions of glycine. Interestingly, reduced GlyT1 is maintained in a non-progressive form of epilepsy (Figure 2), whereas only progressive forms of epilepsy with frequent spontaneous seizure activity are characterized by increased levels of GlyT1 (Figures 1–3). Thus, the transition from low to high levels of GlyT1 expression might be implicated in the transition from the latent phase to the progressive chronic phase of epilepsy. The recent development of a PET tracer for GlyT1 in humans (Joshi et al., 2015) may offer unique diagnostic opportunities to distinguish patients with a progressive course of epilepsy from those with a non-progressive course.

Epileptogenesis is associated with inflammatory processes and glial activation, which are both thought to play a functional role in the pathogenesis and disease progression of epilepsy (Aronica et al., 2012; Vezzani et al., 2011). In our tetanic stimulation model the progressive form of epilepsy is associated with massive astrogliosis in the hippocampal formation (Aronica et al., 2012; Aronica et al., 2011; Gorter et al., 2001). Likewise, astrogliosis is a major pathological hallmark of the intrahippocampal KA model of epilepsy (Gouder et al., 2004). Since GlyT1 is expressed in both neurons as well as in glia (Cubelos et al., 2005; Zafra et al., 1995), glial activation in the epileptic brain might play a significant role in the induction of GlyT1 overexpression in the chronically epileptic brain. Interestingly, the biphasic response of GlyT1 expression changes reported here closely mimic changes in the expression of the adenosine regulating enzyme adenosine kinase (Aronica et al., 2011; Boison, 2012). Thus, in both rodent models of epilepsy as well as in human epilepsy astroglial activation is associated with molecular changes leading to dysregulation of the two fundamental metabolites glycine and adenosine. Future studies to determine the cell-type selectivity of GlyT1 expression changes during epileptogenesis will elucidate the involvement of different cell types to the biphasic changes in GlyT1 expression during epileptogenesis described here.

4.2. Functional significance

In the hippocampal formation, glycine can exert opposing effects that depend on the activation of presynaptic (Kubota et al., 2010; Winkelmann et al., 2014) versus postsynaptic glycine receptors (Aroeira et al., 2011). A recent study (Chen et al., 2014) demonstrated that glycine at low concentrations (10 μM) had pro-convulsive effects whereas higher glycine concentrations (100 μM) attenuated recurrent epileptiform discharges. This pro-convulsive effect of glycine was blocked by the loop diuretic NKCC1 inhibitor bumetanide, a finding which supports a presynaptic mechanism and is in agreement with activation of presynaptic glycine receptors on glutamatergic synapses (Chen et al., 2014; Winkelmann et al., 2014). The pro-convulsive actions of presynaptic glycine receptors expressed on glutamatergic synapses (Winkelmann et al., 2014) is further supported by findings showing that the

expression of edited glycine receptor coding mRNA is increased in the human epileptic hippocampus (Eichler et al., 2008) and that glycine receptor RNA editing regulates glycine affinity (Meier et al., 2005). These findings suggest that glycine homeostasis plays a crucial role in maintaining the balance between increased and decreased neuronal excitability. Consequently, as documented here, disruption of glycine homeostasis via increased expression of GlyT1 in the epileptic brain (Fig. 1–3) is likely to affect hippocampal excitability.

Although glycine was not quantified directly in the present study, previous findings from us and others support an inverse relationship between GlyT1 expression levels and extracellular glycine (Supplisson and Bergman, 1997; Yee et al., 2006). We therefore hypothesize that the increased expression of GlyT1 in the epileptogenic hippocampus leads to reduced extracellular glycine during the interictal phase. Under those conditions preferential activation of pro-convulsive presynaptic glycine receptors would contribute to seizure generation in chronic epilepsy. During ictal discharges, however, the reversal of the ion gradient across the plasma membrane would enable a more efficient release of glycine, which might be an endogenous mechanism to limit seizure extent. Whether increased GlyT1 in the epileptic hippocampus is cause or (adaptive) response to the epileptic state is currently unclear and warrants further investigation.

Decreased extracellular glycine might also contribute to cognitive and psychiatric impairments, major comorbidities of epilepsy (Dalmagro et al., 2012; Lin et al., 2012; Rudzinski and Meador, 2013), via reduced activation of the $glycine_B$ site of the NMDAR. Reduced activation of the $glycine_B$ site of the NMDAR has indeed been associated with cognitive impairment and psychosis (Javitt and Coyle, 2004; Mohler et al., 2011; Tsai and Coyle, 2002). Thus, overexpression of GlyT1 and resulting disruption of glycine homeostasis in the epileptic hippocampus might contribute to the comorbid spectrum of seizures, cognitive impairment and psychiatric disruptions in epilepsy.

The recent discovery of glycine-N-methyltransferase in the hippocampus (Carrasco et al., 2014) suggests that the availability of hippocampal glycine also controls the S-adenosylmethionine (SAM) -dependent transmethylation pathway that provides the substrate for DNA- and histone- methylation. Since epigenetic mechanisms are implicated in epileptogenesis (Kobow and Blumcke, 2011; Kobow et al., 2013; Williams-Karnesky et al., 2013), dysregulation of GlyT1 might influence epilepsy development through an epigenetic mechanism, an exciting possibility that warrants further investigation. Increased GlyT1 and decreased glycine would shift the biochemical equilibrium to increased SAM, thus favoring increased DNA methylation; this mechanism would be in line with hypermethylation of the epileptogenic hippocampus (Kobow and Blumcke, 2011; Kobow et al., 2013; Williams-Karnesky et al., 2013).

4.3. Translational significance

Intense research and drug development efforts have focused on GlyT1 inhibitors as a new class of drugs to ameliorate the negative and cognitive symptoms of schizophrenia, which have been attributed to hypofunction of NMDARs (Coyle and Tsai, 2004; Goff and Coyle, 2001; Javitt, 2012). In contrast to the use of direct NMDAR agonists, allosteric modulation

of the NMDAR by its co-agonist glycine is not associated with any known risks of seizure generation (Coyle and Tsai, 2004; Mohler et al., 2011; Tsai et al., 2004). A 320 patient phase II proof-of-concept study and a recent phase III study have identified no safety or tolerability concerns with the GlyT1 antagonist bitopertin (RG1678) at doses of 30 and 175 mg/kg (Hofmann et al., 2012; Hopkins, 2011). These studies demonstrated general safety of systemic GlyT1 inhibition and, most importantly, rule out adverse brainstem effects of GlyT1 inhibitors (Hofmann et al., 2012).

To be of therapeutic value for the therapy of epilepsy, GlyT1 inhibitors need to raise extracellular glycine levels sufficiently in order to suppress epileptiform activity through activation of extrasynaptic glycine receptors in the postsynaptic compartment (Chen et al., 2014). Tonic inhibition of neuronal excitation through this mechanism may however bear risks for neurodegeneration under conditions of reduced expression levels of the chloride transporter KCC2 as is the case in chronic adulthood epilepsy (Eichler et al., 2008; Legendre et al., 2009; Rivera et al., 2002; Rivera et al., 2004; Winkelmann et al., 2015). Although neurodegeneration was not assessed in our studies with acute administration of the GlyT1 inhibitor LY2365109, the chronic deletion of GlyT1 from hippocampal neurons in mice was not associated with any signs of neurodegeneration (Yee et al., 2006). Our data show that acute doses of 7.5 and 10 mg/kg of the GlyT1 inhibitor LY2365109 effectively prevent acute and chronic seizures (Figure 4 and 5), whereas higher doses (30 mg/kg) of LY2365109 are associated with excessive suppression of respiratory functions in epileptic animals. These findings are in line with the study of homozygous GlyT1 knockout mice, which die perinatally due to suppression of respiratory functions in brainstem (Gomeza et al., 2003), which, in contrast to the forebrain, is characterized by high levels of GlyT1 expression (Mohler et al., 2011; Zafra et al., 1995). Given the antiepileptic efficacy of LY2365109 in a dose range of 7,5 to 10 mg/kg, the therapeutic index for GlyT1 inhibitors for the treatment of epilepsy might be relatively narrow. Excessive blockade of GlyT1 transporters in the brainstem might exacerbate the risk for sudden unexpected death in epilepsy (SUDEP), a major lethal risk factor for patients with chronic epilepsy that involves suppression of respiratory and cardiac function (Hirsch, 2010; Richerson and Buchanan, 2011; Shen et al., 2010). Strategies for local glycine augmentation might therefore constitute an alternative to limit the beneficial effects of glycine to the hippocampal formation while sparing GlyT1 rich midbrain and brainstem areas. Since stem cells can be engineered to secrete endogenous therapeutically beneficial metabolites (Fedele et al., 2004), it might be feasible to develop stem cell derived brain implants to augment glycine signaling in the hippocampal formation. Cell therapies for epilepsy constitute a promising avenue for the local restriction of treatment to a brain area of increased epileptogenicity (Boison, 2007; Sebe and Baraban, 2011; Shetty and Hattiangady, 2007). Our findings with the CamKIIαCre:GlyT1$^{fl/fl}$ mice (Figure 4C), which are characterized by the deletion of GlyT1 in forebrain neurons only (Mohler et al., 2011; Yee et al., 2006), demonstrate that local glycine augmentation therapy restricted to the forebrain might be a feasible strategy to increase thresholds for seizure induction.

Despite the challenges for future clinical translation we demonstrate here innovative use of the experimental GlyT1 inhibitor LY2365109 and document its therapeutic potential for seizure control in epilepsy. Importantly, our data presented here suggest that GlyT1

inhibitors represent a class of drugs with the potential to reduce the seizure burden in chronic epilepsy. Since GlyT1 antagonists have proven pro-cognitive activities in models of schizophrenia, autism, and Alzheimer's disease (Burket et al., 2015; Chaki et al., 2015; Harada et al., 2012), GlyT1 inhibitors would differ from conventional antiepileptic drugs, which generally impede cognitive function (Arif et al., 2009; Ortinski and Meador, 2004; Vajda, 2007). While we currently do not propose to use GlyT1 inhibitors in lieu of conventional epilepsy therapy, the new agents, in particular at lower doses, might yield highly desirable outcome as add-on to existing treatments.

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Highlights

- Glycine transporter 1 (GlyT1) is pathologically overexpressed in two rodent models and human patients of temporal lobe epilepsy.
- Pharmacological inhibition of GlyT1 attenuates acute and chronic seizures.
- GlyT1 is a rational therapeutic target for the treatment of epilepsy.

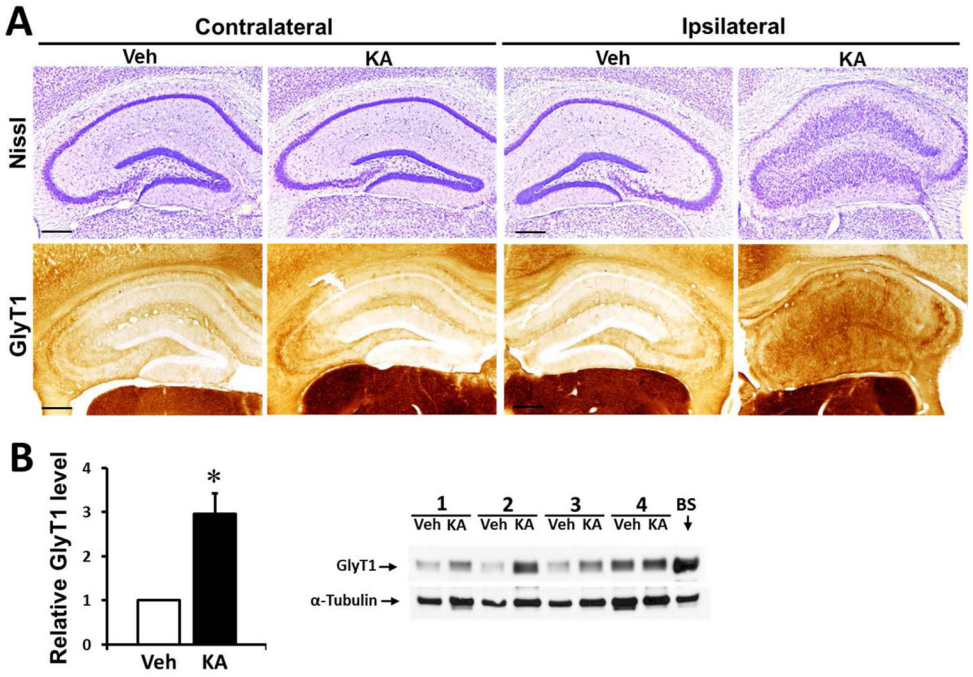

Figure 1. Dysregulation of GlyT1 expression in the epileptogenic hippocampus of mice

(A) Representative Nissl staining (upper panel) and immunohistochemical staining for GlyT1 (lower panel) of contralateral non-epileptic, and ipsilateral epileptic hippocampi from C57Bl/6 wild-type mice, 6 weeks after the intrahippocampal injection of kainic acid (KA) into the ipsilateral hippocampus. Control mice (VEH) were received intrahippocampal injections with the same volume (200nl) of vehicle. Note profound histopathology including CA1 and hilar neuronal cell loss with granule cell dispersion and overexpression of GlyT1 only in the KA-injected epileptogenic hippocampus. Data are representative images from n=8 animals per group. **(B)** Representative Western blot showing expression levels of GlyT1 (upper panel) and α-tubulin (as internal control) from homogenates of resected epileptogenic hippocampi (KA) and vehicle injected control hippocampi 6 weeks following the KA injection. As sample from brainstem (highly enriched in GlyT1) has been added as positive control. Quantification of immunodensities and normalization to α-tubulin revealed a 2.95 ± 0.47-fold increase in GlyT1 expression in KA-injected versus vehicle-injected hippocampus (n=4, each). Scale bar = 500 μm. Data are displayed as mean ± SEM. *p <0.05 KA versus VEH.

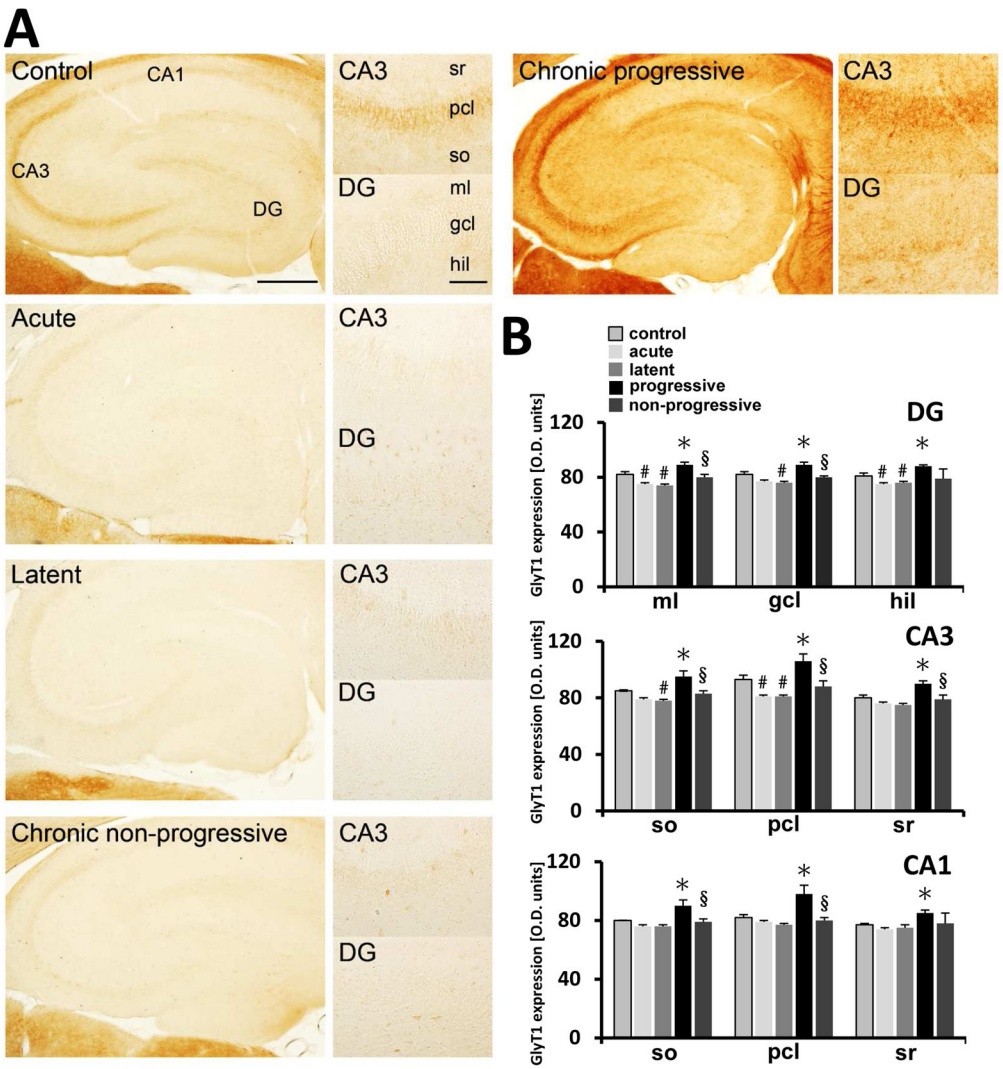

Figure 2. Dysregulation of GlyT1 expression is associated with a progressive form of epilepsy in rats

(A) Typical examples of GlyT1 expression in the hippocampus of a control rat and in rats that were sacrificed after SE during the acute, latent and chronic epileptic phase. (B) Semiquantitative analysis of GlyT1 expression in the dentate gyrus (DG), CA3 and CA1 at different timepoints after SE. GlyT1 expression levels are presented in arbitrary optical density (O.D.) units. GlyT1 expression was low in the hippocampus of control rats. During the acute (n=5) and latent (n=6) phase, the expression of GlyT1 was lower compared to controls in the dentate gyrus, i.e., molecular layer (ml), granule cell layer (gcl) and hilus (hil) as well as in CA3, i.e., the stratum oriens (so), stratum radiatum (sr) and pyramidal cell layer (pcl). During the chronic phase, the expression of GlyT1 was higher in rats with a progressive form of epilepsy (n=6) in all layers of the dentate gyrus, CA3 and CA1 as compared to control. In contrast, the expression of GlyT1 in rats with a non-progressive form of epilepsy (n=4) did not differ from controls. DG, dentate gyrus; ml, molecular layer; gcl, granule cell layer; sr, stratum radiatum; pcl, pyramidal cell layer; so, stratum oriens. Scale bar in A= 500 μm, scale bar in inset of A= 100 μm. # lower compared to control,

p<0.05; * higher compared to control, p<0.05); § lower compared to progressive epilepsy, p<0.05, one-way ANOVA.

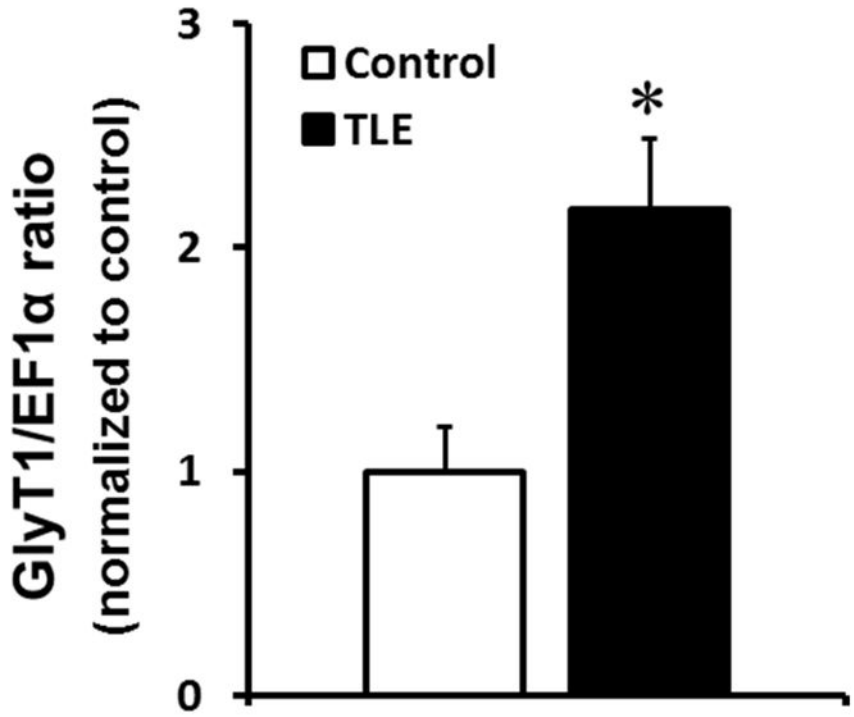

Figure 3. Overexpression of GlyT1 in the hippocampus of TLE patients

The expression of GlyT1 in the human brain was studied using real-time quantitative PCR. The ratio between GlyT1 and the housekeeping gene EF1α increased 2.17 ± 0.32-fold ($p<0.05$) in resected hippocampi from patients with drug-resistant temporal lobe epilepsy (n=9) as compared to autopsy control tissue (n=5). *$p<0.05$, t-test.

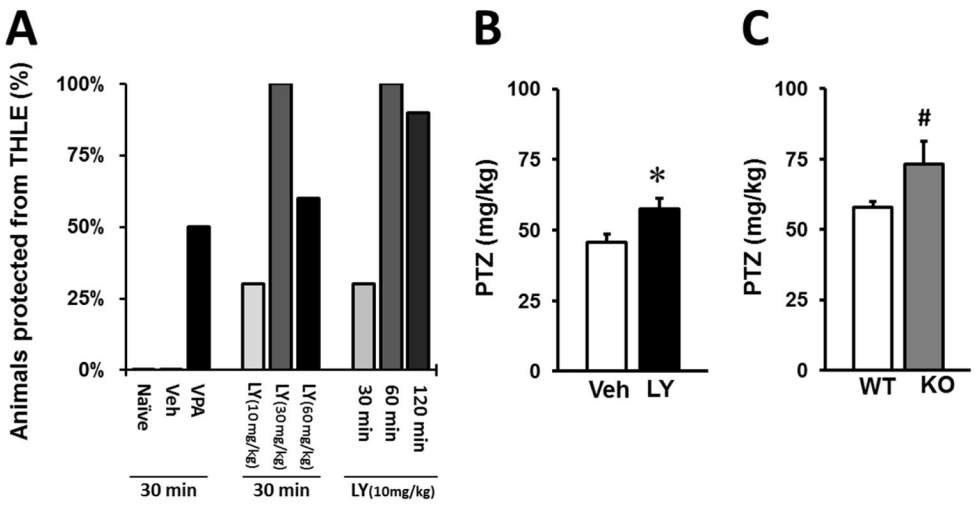

Figure 4. Pharmacological inhibition of GlyT1 prevents acute seizures in mice

(A) The anticonvulsant activity of the GlyT1 inhibitor, LY2365109 (LY) was quantified using the maximal electroshock (MES) test in mice. The anticonvulsant activity is indicated as percentage of mice fully protected from tonic hind-limb extension (THLE). The conventional antiepileptic drug valproic acid (VPA) at a dose of 169 mg/kg was used as a positive control and both treatment-naïve and vehicle-treated animals were used as negative controls. The anticonvulsant efficacy of LY2365109 was assessed at different doses (10 to 60 mg/kg, i.p.) injected 30 minutes prior to the MES, and at a constant dose (10 mg/kg, i.p.) delivered at different time-points prior to the MES. Data are based on n=10 animals per treatment. **(B, C)**. A pentylenetetrazole (PTZ) seizure threshold test demonstrated that both the pharmacological blockade of GlyT1 via LY2365109 (10mg/kg, i.p., **B**), and the genetic deletion of neuronal GlyT1 in the forebrain of CamKIIαCre:GlyT1$^{fl/fl}$ (KO) mice (**C**) significantly increased seizure thresholds. Mice were subjected to repeated PTZ-injections (10 mg/kg, i.p.) every 10 minutes until they reached a behavioral seizure score of 5 according to Racine's scale. The cumulative PTZ dose needed to trigger stage 5 seizures was considered the convulsant threshold dose. Data are displayed as mean ± SEM. *$p < 0.05$ LY versus vehicle, n=7–8; # $p < 0.05$ KO versus wild-type littermates (WT), n=6–10).

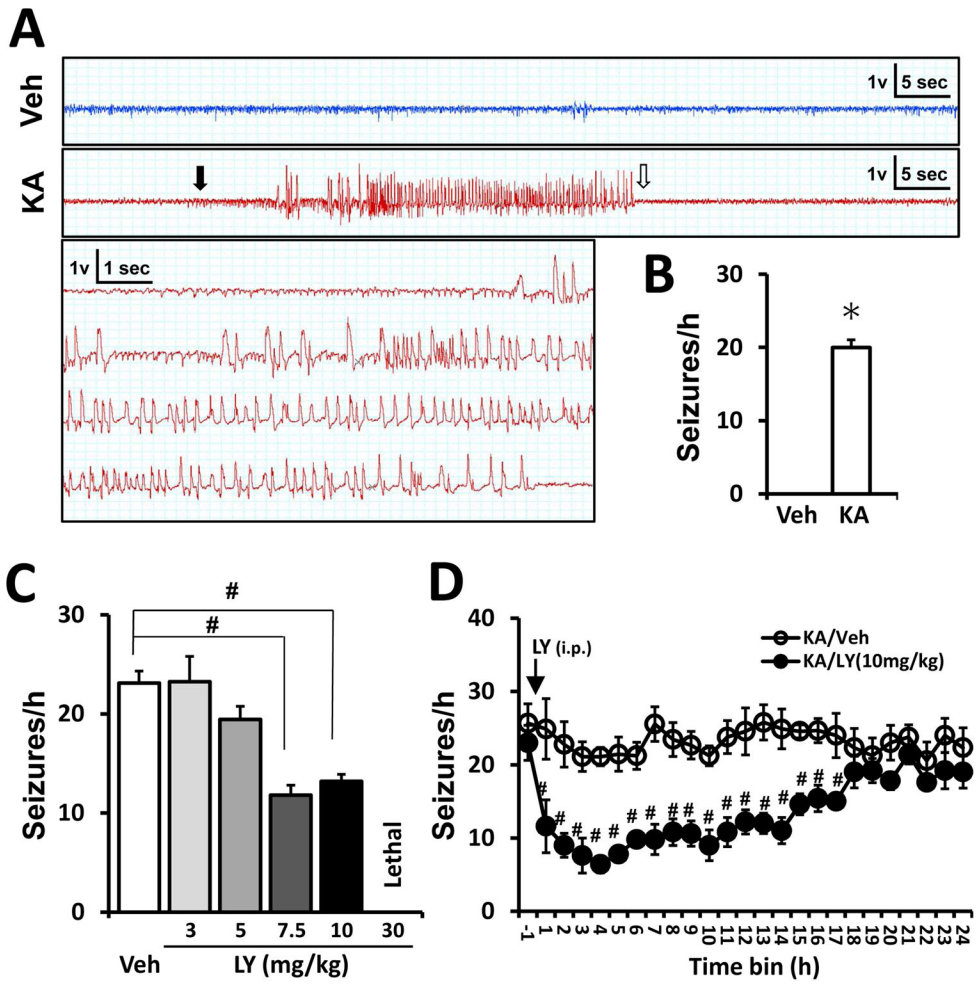

Figure 5. Pharmacological inhibition of GlyT1 suppresses seizures in chronic epilepsy

(A) Representative intrahippocampal EEG traces from mice injected with vehicle (Veh, upper panel) or KA (lower panel) 6 weeks after the intrahippocampal injection. Seizure onset and end are represented by closed and open arrows, respectively. A representative seizure is also shown at higher resolution. **(B)** Quantification of chronic recurrent seizure activity revealed a rate of 19.7 ± 1.1 seizures per hour with an average duration of 17.8 ± 3.6 seconds in the KA-injected epileptic mice (n=12) whereas vehicle-injected mice (n=12) were devoid of any seizures. **(C)** The average number of seizures per hour was significantly reduced in the epileptic mice treated with LY2365109 (LY) (n=8–12 per dose) at doses of 7.5 and 10 mg/kg i.p., providing >50% seizure reduction compared to vehicle-treated controls. Lower doses of 3 and 5 mg/kg were not effective, whereas a higher dose (30 mg/kg, i.p) triggered lethal respiratory arrest within 4 hours. **(D)** 24 hour EEG data from the 10 mg/kg LY2365109 group demonstrates the time course of therapeutic effects that commenced one hour after drug injections and that lasted for ~16 hours, with a maximum of protection at 4 hours following the drug injection. Data are displayed as mean ± SEM. *p < 0.05 treatment versus vehicle.

Table 1

Clinical characteristics of patients

Patient	age	gender	age onset	duration epilepsy	seizure type	seizures/month	AEDs
1	81	f	-	-	-	-	-
2	68	f	-	-	-	-	-
3	86	m	-	-	-	-	-
4	30	m	-	-	-	-	-
5	71	f	-	-	-	-	-
6	26	m	6	25	CPS	5	LEV, TPM
7	28	f	14	14	SPS	120	CBZ, LMT
8	29	f	13	16	SPS/GTCS	32	LMT, TPM
9	39	m	33	6	CPS	32	CBZ
10	29	m	18	11	CPS	3	CBZ
11	42	f	4	28	CPS/GTCS	1	PHT, OXC
12	29	m	15	14	CPS	36	CBZ, CLB
13	66	f	10	56	CPS	8	CBZ, PB, PHT
14	51	m	28	20	CPS	4	PHT, OXC

Patients 1–5: autopsy controls, patients 6–14: temporal lobe epilepsy patients with hippocampal sclerosis.

Seizure type: SPS, simple partial seizure; CPS, complex partial seizure; GTCS, generalized tonic/clonic seizures, AEDs, anti-epileptic drugs: CLB, Clobazam ; CBZ, Carbamazepine; LEV, Levetiracetam; OXC, Oxcabazepine; PB, Phenobarbital; PHT, Phenytoin; LMT, Lamotrigine; TPM, Topiramate.