Irregular RNA splicing curtails postsynaptic gephyrin in the cornu ammonis of patients with epilepsy

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Anomalous hippocampal inhibition is involved in temporal lobe epilepsy, and reduced gephyrin immunoreactivity in the temporal lobe epilepsy hippocampus has been reported recently. However, the mechanisms responsible for curtailing postsynaptic gephyrin scaffolds are poorly understood. Here, we have investigated gephyrin expression in the hippocampus of patients with intractable temporal lobe epilepsy. Immunohistochemical and western blot analyses revealed irregular gephyrin expression in the cornu ammonis of patients with temporal lobe epilepsy and four abnormally spliced gephyrins lacking several exons in their G-domains were isolated. Identified temporal lobe epilepsy gephyrins have oligomerization deficits and they curtail hippocampal postsynaptic gephyrin and GABA\textsubscript{A} receptor \(\alpha_2\) while interacting with regularly spliced gephyrins. We found that cellular stress (alkalosis and hyperthermia) induces exon skipping in gephyrin messenger RNA, which is responsible for curtailed postsynaptic gephyrin and GABA\textsubscript{A} receptor \(\alpha_2\) scaffolds. Accordingly, we did not obtain evidence for gephyrin gene mutations in patients with temporal lobe epilepsy. Cellular stress such as alkalosis, for example arising from seizure activity, could thus facilitate the development of temporal lobe epilepsy by reducing GABA\textsubscript{A} receptor \(\alpha_2\)-mediated hippocampal synaptic transmission selectively in the cornu ammonis.

Keywords: epilepsy; hippocampus; gephyrin; GABA(A) receptor; RNA splicing; exon skipping; cellular stress; molybdenum cofactor

Abbreviations: EGFP = enhanced green fluorescent protein; HEK = human embryonic kidney cells; NeuN = neuronal nuclei; PCR = polymerase chain reaction; TLE = temporal lobe epilepsy

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Introduction

Worldwide, over 50 million individuals are adversely affected by temporal lobe epilepsy (TLE) (Hauser, 1997), a neurological disorder that leads to serious medical illness. About 2.4 million new cases per year are reported globally, and in at least 50% of the cases, first signs of the disease develop in childhood or adolescence. Deficits in GABAergic synaptic transmission have been related to epilepsies (Kumar and Buckmaster, 2006; Stief et al., 2007; Eichler and Meier, 2008), and even a slight decrease (~20%) of the amplitude and decay time constant of inhibitory synaptic potentials might be sufficient for the onset of epileptic seizures (Eugene et al., 2007).

The postsynaptic scaffolding protein gephyrin is involved in anchoring, stabilization and enrichment of GABAergic synapses with GABA receptors. Accordingly, gephyrin gene (GPHN) knockout mice display reduced amplitudes of inhibitory synaptic potentials (Levi et al., 2004; Jacob et al., 2008). Because gephyrin controls the number of postsynaptic GABA receptors, particularly of those containing the α2 subunit (Jacob et al., 2005; Trettter et al., 2008), it is not surprising that a decrease of gephyrin immunoreactivity in the cornu ammonis of epileptic hippocampi mirrors reduced GABA receptor immunoreactivity (Bouilleret et al., 2000; Knuesel et al., 2001; Kumar and Buckmaster, 2006). However, the molecular basis of curtailed postsynaptic gephyrin in the epileptic hippocampus is unknown.

We analysed gephyrin splicing and expression in the hippocampus of patients with intractable TLE since alternative splicing of GPHN transcripts produces the G2 gephyrin splice variant, which interferes with postsynaptic gephyrin recruitment and receptor stabilization (Meier et al., 2000; Meier and Grantyn, 2004; Bedet et al., 2006; Fritschy et al., 2008). Contrary to our expectations, we did not obtain evidence for up-regulation of this variant. Instead, we have isolated four novel gephyrin splice variants lacking several exons in their G-domains, and functional characterization of these variants revealed their pathological dominant negative effects on regular hippocampal gephyrin and GABA receptor α2. While ruling out GPHN gene mutations in our patients with TLE, this study demonstrates that cellular stress such as alakosis, for example resulting from seizure activity or hyperthermia, is sufficient to trigger exon skipping in gephyrin mRNA. Furthermore, in patients with TLE, considerable amounts of gephyrin and the neuronal splice factor ‘neuronal nuclei’ (Neun) (Kim et al., 2009) are associated with the ubiquitin-proteasome system selectively in the hippocampal cornu ammonis, which is not dependent on the degree of hippocampal sclerosis. Consequently, this study reveals a novel postsynaptic mechanism of cellular stress that could facilitate the development of TLE, for example secondary to seizure activity, by reducing GABA receptor α2-mediated hippocampal synaptic transmission in the cornu ammonis.

Materials and methods

All animals were sacrificed according to the permit (LaGeSo, 0122/07) given by the Office for Health Protection and Technical Safety of the regional government of Berlin and in compliance with regulations down in the European Community Directive.

Patients with temporal lobe epilepsy

Patients were classified pharmaco-resistant if at least two medications failed. Analysis of resected TLE hippocampi was performed according to the rules laid down by the Ethical Committee (Charité, EA1/142/05), and informed consent was obtained according to the Declaration of Helsinki (BMJ, 1991; 302: 1194). The presurgical evaluation was performed at the Epilepsy Centre Berlin-Brandenburg. The ‘epileptogenic zone’, namely the area of the brain that is necessary and sufficient for habitual seizure generation, and whose removal or disconnection will effect a cure (Lüders, 1993), the type of seizure, the ictal onset zone and the irritative zone were identified by the use of MRI, neuropsychological diagnostic and video-EEG-monitoring (Supplementary Table 1). If required, invasive intracranial recordings (e.g. TLE Patient #2.47.5, Supplementary Fig. 1; Supplementary Table 1, Electrocorctogram) were performed.

The standard operative procedure at the Epilepsy Centre Berlin-Brandenburg in the case of isolated mesial TLE consists of the resection of the temporal pole with an amygdala-lateralh hippocampectomy. If an epileptogenic lesion was shown inside the temporal lobe outside the mesial structures, the mesial temporal structures and the lesion were removed if the presurgical diagnostic showed that both structures where part of the epileptogenic zone. In all cases it was shown that the mesial temporal lobe structures were part of the epileptogenic zone. It is important to note that seizures may initially only spread to the hippocampus, but later in the course of the epilepsy the hippocampus can generate seizures itself.

Isolation of irregularly spliced GPHN transcripts

Total RNA was isolated from whole hippocampipectomies of patients with intractable TLE or primary rat hippocampal neurons using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). For polymerase chain reaction (PCR) control purposes, post-mortem hippocampal RNA (pool of 20 healthy Caucasians) was purchased from Clontech (Palo Alto, CA, USA). Complementary DNA was obtained by reverse transcription (Superscript II, Invitrogen) of 2 μg RNA with an equimolar mixture of 3’-anchored poly-T oligonucleotides (T18V, T15V, T13V). For screening of cDNAs, oligonucleotides spanning the alternatively spliced regions of the G-domain (exon 1–9, 5’-GGAGGTTCCATGATGAACTTGA-3’ and 5’-TGCTGGCACTGCTGG ACTG-3’), the C-domain (exon 9–16, 5’-GAGTCCTCACGTGACT GATA-3’ and 5’-CAGTCTCCACATGCACCTCC-3’) and the E-domain (exon 18–24, 5’-GGATCGAAAGAGGATGCCTGATGAT-3’ and 5’-GGTCAT CTCAGGAATTAGTAG-3’) were used with RedEq DNA polymerase (Sigma-Aldrich, Deisenhofen, Germany) and manual hot start. Forty-five cycles (1 min annealing at 50°C and 1 min elongation at 72°C) were run and the agarose gel regions containing unusually sized G-domain PCR products were purified and ligated with an in-house TA cloning vector (derived from pBluescript). As described (Eichler et al., 2009), PCR product intensities were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Our low stringency PCR protocol is suitable for screening patients with TLE because amplification of post-mortem control hippocampus cDNA produced the well known PCR band pattern (Meier and Grantyn, 2004; Smolinsky et al., 2008) including regular (Δ6) gephyrin and the G2 splice variant (Fritschy et al., 2008). A non-specific band
Figure 1 Confocal laser scanning microscopy reveals curtailed postsynaptic gephyrin scaffolds in CA3 regions of TLE hippocampectomies. (A–C) Immunoreactivities of vesicular inhibitory amino acid transporter (VIAAT) and gephyrin (mAb7a) are shown in representative images of the hippocampal CA3 region of three patients with TLE. Note that a large fraction of vesicular inhibitory amino acid transporter-positive nerve endings lacks gephyrin. Also note the presence of filamentous and diffuse gephyrin. Also note the presence of gephyrin-apposed vesicular inhibitory amino acid transporter (VIAAT) and gephyrin (mAb7a) are shown in representative images of the hippocampal CA3 region of three patients with TLE. Note that a large fraction of vesicular inhibitory amino acid transporter-negative nerve endings lacks gephyrin. Also note the presence of filamentous and diffuse gephyrin. Also note the presence of gephyrin-apposed vesicular inhibitory amino acid transporter (VIAAT) and gephyrin (mAb7a) are shown in representative images of the hippocampal CA3 region of three patients with TLE. Note that a large fraction of vesicular inhibitory amino acid transporter-negative nerve endings lacks gephyrin. Also note the presence of filamentous and diffuse gephyrin. (arrows) as well as unusually large gephyrin aggregates (arrows). Postsynaptic gephyrin in the dentate gyrus (DG) of these patients appears normal, example given for Patient #240708 (D). Quantification of the fraction of gephyrin-apposed vesicular inhibitory amino acid transporter nerve endings in six patients with TLE is shown in Table 1. Scale bar: 10 μm, high power views: 3 μm.

was observed (Fig. 1B). A total of 25 clones were DNA sequenced and four different TLE-specific gephyrin G-domains could be isolated.

Enhanced green fluorescent protein and DsRed-Express-tagged gephyrin expression constructs

Regular enhanced green fluorescent protein (EGFP)-tagged Δ6 gephyrin (Lardi-Studer et al., 2007) was used as a control and served as the basis for all TLE gephyrin expression constructs. The expression construct (Δ6–8) was obtained by fusion PCR on EGFP-tagged Δ6 gephyrin, using oligonucleotides 5′-GGGTTCGGGAG GAAATCTTCCAGTTAT-3′ and 5′-CCCTCCGGAAGTGACATCTCCTG TCTGCAAGATTC-3′ (BspEI sites are underlined) and Phusion Hot Start High-Fidelity DNA polymerase (New England Biolabs, Hertfordshire, UK) in the presence of dimethylformamide (5%). A touch-down PCR protocol was followed and five cycles were run at 53°C annealing, followed by five cycles at 49°C and 32 cycles at 45°C annealing, respectively. Elongation was carried out for 7 min at 68°C. Subsequent digestion with BspEI followed by ligation ensured in-frame excision of the TLE-specific region spanning exons 4–8. The introduced BspEI site is silent with respect to the deduced amino acid sequence.

To obtain expression constructs for the remaining three irregularly spliced gephyrin, the DNA sequences of G-domains were amplified by PCR (5′-AGAAGACACATCTCGCCGAAATACAATCTCAG-3′ and 5′-CAAT GGCATCACTTGAAGGTCAATGG-3′) using Phusion polymerase. Thirty-two cycles were run at 58°C annealing temperature. Following restriction digest of PCR products with BspEI and AflII (underlined in oligonucleotides) ligation with EGFP-tagged Δ6 gephyrin was carried out. To this purpose, BspEI and AflII sites were introduced by PCR-based silent mutagenesis (as described above, fusion PCR) in two subsequent rounds. For consecutive insertions of BspEI and AflII restriction sites oligonucleotide combinations 5′-GGGTCC CGGAATAAATCTCCTAAAGATCGTTTCGTCCTGCAAGATTC-3′/5′-GGGTTCGGGAGGGCGTCCTGCAAGATTC-3′ were used. All expression constructs were verified by DNA sequencing.

To obtain DsRed-Express-tagged gephyrin exon expression constructs, the EGFP-tagged Δ6 gephyrin was amplified by PCR. Corresponding oligonucleotides containing XhoI and BamHI restriction sites for in-frame insertion into pDsRed-Express C1 (Clontech) were used. Exon 1–3: 5′-GGGTTCGGGAGGGGTCATCTCCTGCAAGATTC-3′ and 5′-GGGTTCGGGAGGGCGTCCTGCAAGATTC-3′ were used as the basis for all TLE gephyrin expression constructs. Expression constructs were verified with DNA sequencing.

DNA sequencing of genomic regions

Genomic DNA was isolated from TLE hippocampectomies using Inviror Spin Tissue Midi Kit (Invitek, Berlin, Germany). Exons with flanking intron sequences (~200bp up- and downstream of each exons for verification of consensus sequences required for splice 'lariat' formation) were amplified by PCR using oligonucleotides
Experimental cellular stress procedures

Two cellular stress protocols were applied to cultures of rat hippocampal neurons during the period of 8–9 days in vitro. For high-temperature cellular stress, the incubator temperature was set to 39°C for 16 h, which minimized cell death (pyknotic nuclei). In another part of the experiment, hippocampal neurons were alkalinized by setting the incubator CO₂ level to 0%. The change of pH in the extracellular milieu was monitored over 16 h (Fig. 9A). The pH of the extracellular milieu (pH(e)) reached a value of 7.94 ± 0.06 at the end of the alkalosis stress period. Control and stressed neurons were then processed for RNA isolation (above) and immunocytochemistry (below). DAPI (4′,6-diamidino-2-phenylindole) staining allowed evaluation of cell death according to the appearance of pyknotic nuclei. Neurons with pyknotic nuclei were excluded from analysis.

Human embryonic kidney cell culture and transfection

Human embryonic kidney (HEK293) cells were grown in minimum essential medium supplemented with 10% foetal calf serum, 30 mM sucrose, 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid) (HEPES), 0.25 mM L-glutamine and 230 μM sodium pyruvate. For transfection, a standard Ca²⁺/phosphate protocol was followed and cells were analysed 4 days after transfection.

Antibodies

Hippocampal endogenous gephyrin was visualized using a mouse monoclonal antibody (mAb7a, 1:50, Synaptic Systems GmbH, Goettingen, Germany). An antibody specific to the E-domain (mAb 3811) (Smolinsky et al., 2008) was used in western blot analysis. Furthermore, a rabbit polyclonal gephyrin antibody (1:500, Synaptic Systems GmbH) was used in experiments involving NeuN staining. The vesicular inhibitory amino acid transporter was detected with a rabbit polyclonal antibody (1:200, Synaptic Systems GmbH) and transfected N-terminally EGFP-tagged gephyrins with an EGFP-specific antibody made in chicken (1:300, Chemicon, Temecula, CA, USA). Ubiquitin was visualized using a polyclonal antibody (UB N-19) made in goat (Santa Cruz Biotechnology Inc., Heidelberg, Germany). The neuronal splice factor NeuN (Kim et al., 2009) was detected using a mouse monoclonal antibody (1:200, Chemicon). Finally, the well-characterized guinea pig GABA_A receptor α2 antibody (Loup et al., 2000) made by Dr Jean-Marc Fritschy (University of Zurich, Switzerland), was used as a control for NeuN expression. Secondary antibodies were all made in donkey (affinity purified and multi-labelling declared), coupled to carboxymethyl indocyanine (Cy5), fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Immunofluorescence and co-localization analysis

Transfected HEK293 cells and neurons were fixed using an ice-cold mixture of 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (15 min at room temperature) and stained as described previously (Meier and Grantyn, 2004; Eichler et al., 2008) with antibodies at the indicated dilutions (above). Coverslips were mounted on microscope slides using Vectashield medium with DAPI (Vector Laboratories, Burlingame, CA, USA).

Temporal lobe epilepsy gephyrin RNA splice reporter

The fluorescence reporter consists of two fluorescent proteins (tdTomato and EGFP) separated by gephyrin exons 3–5 including flanking introns, which were DNA sequenced. This reporter monitors skipping of exon 4 during gephyrin RNA splicing. See Supplementary Material for details.

Semi-quantitative PCR amplification of GABA_A receptor subunits

For semi-quantitative reverse transcription PCR, the amplification of GABA_A receptor subunits and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Supplementary Fig. 7) was performed in the same reaction tubes. Oligonucleotides specific to GABAA receptors α1, α2, α3, β2, β3 and γ2 were published previously (Meier et al., 2002). For amplification of GABA_A receptor α5, oligonucleotides 5’-AATCTGTCCCACCTGACG-3’ and 5’-TGGGATGGTGAGGATGG-3’ were used. Glyceraldehyde 3-phosphate dehydrogenase was amplified using 5’-CAGTAGCTACCACCG-3’ and 5’-CTCAGTGACCCGAG-3’. Manual hot-start was performed and the annealing temperature was set to 58°C. Thirty cycles were run in order to keep the PCR in the linear range of amplification (i.e. semi-quantitative).

Hippocampal cell culture and transfection

Hippocampal cultures from E19 Wistar rats were prepared as previously described (Meier and Grantyn, 2004) and maintained in B27- and 1% foetal calf serum-supplemented neurobasal medium (Brewer and Cotman, 1989). The initial cell density was 68 000/cm². Transfection and protein expression were carried out as described (Meier et al., 2005) on in vitro Days 6–8 (in vitro Day 6 for Δ6–8 and Δ4–8, in vitro Day 7 for Δ6 control and in vitro Day 8 for Δ5–7* and Δ4*). For transfection, coverslips were transferred to wells containing transfection medium (neurobasal supplemented with 0.25 mM glutamine) and were incubated with complexes formed with 5 μl of electro transfection reagent (Qiagen, Hilden, Germany) and 300 ng of DNA. The Qiangen transfection protocol was followed, except that the incubation time was reduced to 30 min for Δ6 control, 1 h for Δ4–8 and Δ5–7* and 90 min for Δ4* and Δ6–8. This protocol ensured moderate protein expression in ~1% of hippocampal neurons at in vitro Day 9.

5′-AAATAAATCAATGTAATGTTGAGG-3′/5′-AATATGTGAAAACGCGATTATAAG-3′ (E3), 5′-CTACCTCTTTAACAATACTCATG-3′/5′-ATCTGATGCTTATCGTATA (E4), 5′-GATCAAGTGAAAATGCACTT-3′/5′-CAAAGCTTTGTTATGCCCTAAG-3′ (E5), 5′-TTCCTATTTTGCAATACCACT-3′/5′-TCATGGAATTCTGACTGACC-3′ (E6), 5′-AACATGGTCGGCGACTGACC-3′/5′-TGCAGTGATACAATTTGCCATA-3′ (E8), and 5′-AACATGGTGAACGTAGTGTTCC-3′/5′-CGAGTGATACAATTTGCCATA-3′ (E9). Introns 8 was amplified using oligonucleotides 5′-GAGTCTCACTGTGTCACCCTG-3′ and 5′-AACATTGTAGCACTGTAGTTCC-3′. DNA sequences were obtained by direct sequencing of purified (Invinsorb Spin DNA Extraction Kit, Invitrogen) PCR products using the above mentioned oligonucleotides. In case composite sequences were detected, corresponding PCR products were ligated with in-house TA cloning vector and 4–6 recombinant clones were sequenced.
Labeled neurons and HEK293 cells were visualized with a standard epifluorescence microscope (Olympus BX51, Olympus Deutschland GmbH, Hamburg, Germany) under U Plan Apo ×40 oil objective with a numerical aperture of 1.00 (Olympus). Appropriate filters (U-MSP100v2 MFISH DAPI, U-MSP101v1 MFISH FITC, U-MSP102v1 MFISH Cy3 and U-MSP104v1 MFISH Cy5; Olympus GmbH, Germany) allowed the detection and separation of fluorescent signals. Images were acquired using a 14-bit cooled charge couple device (CCD) camera (Spot PURSUIT, Visitron Systems GmbH, Puchheim, Germany) and the Metamorph software (Universal Imaging Corp., Downingtown, PA, USA). In hippocampal cell cultures, fluorescent signals were acquired along proximal dendrites and co-localization between vesicular inhibitory amino acid transporter, gephyrin and GABA_A receptor α2 was determined manually on minimum-maximum threshold images. The number of GABAergic synapses was quantified within a circular region of interest (diameter: 100 μm) projected on the centre of the neuron soma. Vesicular inhibitory amino acid transporter puncta were counted manually, as described previously (Singh et al., 2006; Eichler et al., 2008). Vesicular inhibitory amino acid transporter counts were performed on in vitro Day 9 on the cell culture batches that were also analysed with electrophysiology.

Confocal laser scanning microscopy was utilized for analysis of TLE hippocampectomies. For this purpose, 3–5 mm thick hippocampal cross sections were immersed in an ice-cold mixture of 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline, immediately after resection in the operation theatre. After fixation (1 h on ice) slices were washed three times with phosphate-buffered saline at room temperature and cryoprotected overnight at 4°C in phosphate-buffered saline supplemented with 8% sucrose. Sections were embedded in O.C.T TissueTek (Sakura Finetec, USA) and 12 μm cryosections were obtained (CM1850, Leica Microsystems, Wetzlar, Germany) and mounted on Superfrost Plus microscope slides (Menzel GmbH, Braunschweig, Germany). Prior to immunofluorescence staining, sections were post-fixed for 5 min with ice-cold paraformaldehyde/sucrose, washed in phosphate-buffered saline and incubated with 50 mM ammonium chloride for 15 min at room temperature (to eliminate residual paraformaldehyde). After incubation with antibodies (above), sections were mounted on microscope slides using Vectashield medium with DAPI (Vector Laboratories). Fluorescent signals in granule and pyramidal cell layers of hippocampal dentate gyrus and CA3 region were acquired using the laser-scanning microscope DM TCS SP2 (Leica Microsystems, Wetzlar, Germany). Fluorochromes were excited sequentially to minimize cross-talk between fluorescent signals. Fluorescence was acquired using a HCX PL APO ×40 UV oil objective with a numerical aperture of 1.25 (Leica Microsystems). Images were obtained using LCS software (Leica Microsystems) by multiple scanning and averaging of frames (six times each) in order to reduce noise. Quantification of co-localization indices was performed as described above.

**Determination of molybdopterin and molybdenum cofactor content**

EGFP-tagged TLE gephyrins as well as regular gephyrin (Δ6) were expressed in HEK293 cells, as described above. Cells were harvested after 48 h, washed twice in phosphate-buffered saline and sonicated in 100–200 μl nit-1-buffer (50 mM sodium phosphate, 200 mM NaCl, 5 mM EDTA). To determine molybdopterin and molybdenum cofactor content by the nit-1 reconstitution assay, 1–20 μl of crude extracts were incubated with 20 μl Neurospora crassa nit-1 extract supplemented with 2 mM reduced glutathione. Molybdopterin and molybdenum cofactor were determined by reconstitution (overnight) of nit-1 nitrate reductase in the presence and absence of 5 mM sodium molybdate, respectively, as described (Reiss et al., 2001).

**Western blot**

Hippocampus extracts from patients with TLE were re-suspended and sonicated in 100–200 μl of phosphate-buffered saline. Aliquots of 100 μg of the prepared proteins were run on a 8% sodium dodecyl sulphate polyacrylamide electrophoresis gel together with a PageRuler™ Prestained Protein Ladder (Fermentas GmbH, St. Leon-Rot, Germany). Subsequently, proteins were transferred onto a polyvinylidene difluoride membrane (PVDF) at 75 mA for 1 h. Non-specific binding sites were blocked by immersing the membrane in 5% blocking buffer (100 mM Tris/HCl pH 7.4, 154 mM NaCl, 0.05% Tween-20, 5% milk powder) for 1 h. Monoclonal anti-gephyrin E-domain antibody 3811 was diluted 1:1000 and incubated with the membrane for 1 h at room temperature. Furthermore, the membrane was washed three times with phosphate-buffered saline, once with wash-buffer (15 mM sodium phosphate, 200 mM NaCl, 2.5% (v/v) Tween-20) and once with phosphate-buffered saline for 10 min. Mouse IgG horseradish peroxidase-conjugated antibody was diluted 1:10000 and incubated with the membrane for 1 h at room temperature and washed again with phosphate-buffered saline, wash-buffer and phosphate-buffered saline. Afterwards, the membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) for 2 min and analysed in a Chemolumineszenz DeVision R2000 (Decon Science Tec GmbH, Hohengardern, Germany). The quantification of proteins was finally achieved with Gel-Pro Analyser (MediaCybernetics, Bethesda, MD, USA).

**Electrophysiology**

Electrophysiological analysis was performed on cell culture batches that were also analysed with immunocytochemistry. For electrophysiology the hippocampal cultures were mounted in a recording chamber with a solution containing: 105 mM NaCl, 3 mM KCl, 10 mM HEPES, 5 mM glucose, 2 mM CaCl_2 and 1 mM MgCl_2. The osmolality was adjusted to that of the culture medium (240 mosmol/kg). The recording pipette solution contained: 90 mM KCl, 3 mM NaCl, 5 mM HEPES, 5 mM glucose, 0.5 mM CaCl_2 and 4 mM MgCl_2 and was buffered to pH 7.3 (210 mosmol/kg). Recordings of postsynaptic currents were obtained at in vitro Day 9 from untransfected neurons and from neurons expressing EGFP or Δ4° TLE gephrin. Recordings were performed at a holding potential of −70 mV at room temperature. Series resistance (5–20 MOhm) was compensated up to 70% and was monitored throughout the whole experiment. Miniature GABAergic postsynaptic currents were isolated pharmacologically by blocking glutamatergic synaptic transmission with 6,7-dinitroquinoxaline-2,3-dione (DNQX) (20 μM) and DL-2-Amino-5-phosphonopentanoic acid (DL-APV) (50 μM) as well as action potential generation with tetrodotoxin (TTX) (1 μM) and recorded for at least 300 s. Recordings were made using an EPC-9 (HEKA Electronics, Lambrecht/Pfalz, Germany). Signals were sampled at a rate of 10 kHz and analysed off-line using WinTida 5.0 (HEKA Electronics, Lambrecht/Pfalz, Germany). Postsynaptic currents were filtered at 3 kHz and analysed off-line using MiniAnalysis (Synaptosoft Inc., Decatur, GA, USA).
Statistics

Numerical data are reported as mean ± standard deviation. In hippocampal cell culture experiments neurons were sampled from at least two different cultures. If not stated otherwise, statistical analysis (unpaired Student’s t-test or ANOVA, followed by post hoc Bonferroni’s test) was performed using the software Origin (Microcal, Northampton, MA, USA). Significance levels are indicated in tables and figures as *P < 0.05, **P < 0.01, ***P < 0.001.

Results

Reduced gephyrin immunoreactivity in the hippocampal cornu ammonis has been associated with epileptogenesis in the kainate model of TLE (Knuesel et al., 2001). Therefore, we investigated gephyrin expression in the hippocampus of patients with intractable TLE (Figs 1–3, Supplementary Table 1 for history of patients with TLE and neurosurgical outcome). First, we analysed the percent fraction of GABAergic synapses (vesicular inhibitory amino acid transporter) with postsynaptic gephyrin (mAb7a antibody). Compared with the dentate gyrus of these patients (Fig. 1D), postsynaptic gephyrin was significantly reduced in CA3 regions of TLE hippocampi (Fig. 1A–C, Table 1 for values obtained from six patients). Furthermore, unusual gephyrin immunoreactivity [Fig. 1, filamentous and diffuse (arrowheads) and non-synaptic large aggregates (arrows)] suggested that gephyrin expression was impaired in CA3. For control purposes, we analysed post-mortem human hippocampus obtained from a 23-year-old healthy male (Supplementary Fig. 2). This control CA3 region did not exhibit unusual gephyrin immunoreactivity, and the fraction of GABAergic synapses with gephyrin amounted to 77.2 ± 5.4% (P < 0.001 compared with TLE CA3 areas).

To investigate unusual gephyrin expression in CA3 areas of TLE hippocampi further, we performed western blot analysis (Fig. 2). In patients with TLE, reduced postsynaptic gephyrin was accompanied by the occurrence of gephyrin fragments smaller than regular gephyrin (Fig. 2A, ‘B’ versus ‘A’). Gephyrin contains proline (P), glutamic acid (E), serine (S), and threonine (T) rich peptide (PEST) sequences (Prior et al., 1992) involved in protein degradation (Dixon, 1987; Belizario et al., 2008) and the 51 kDa gephyrin fragment (Fig. 2A, band ‘B’) corresponds to the calculated molecular mass (51.4 kDa) of PEST sequence DTASLSTTPSEP-cleaved C-terminal gephyrin E-domains. Therefore, we analysed whether gephyrin was associated with the ubiquitin-proteasome system, where misfolded proteins are degraded (Lehman, 2009). To this end, ubiquitin was used as immunochemical marker of this compartment and gephyrin was indeed found to be embedded in a matrix of ubiquitin immunoreactivity (Fig. 2B, for overview of a cross-section of the hippocampus of TLE Patient #250309 see Supplementary Fig. 3).

Gephyrin is expressed in both glial cells and neurons (Smolinsky et al., 2008), and therefore we had to determine whether gephyrin degradation actually occurred in neurons. For this purpose, the neuronal marker NeuN was used in combination with a rabbit polyclonal gephyrin antiserum and the UB N-19 antibody in immunohistochemical analysis of three more TLE hippocampi (Fig. 3, for overview of a cross-section of the hippocampus of TLE Patient #030310 see Supplementary Fig. 4). The pronounced overlap between gephyrin, ubiquitin and the neuronal RNA splice factor NeuN (Kim et al., 2009) in CA2 and CA3 regions (co-localization of all three proteins appears in white, Fig. 3A–C, lower panels) suggested that abnormal gephyrin RNA splicing had occurred in the cornu ammonis of TLE hippocampi, but not in dentate gyri. It should be noted here that unusual gephyrin expression obviously occurred irrespective of hippocampal sclerosis because CA2 generally is less affected by neuronal dropout. Next, we investigated gephyrin mRNA by reverse transcription PCR. For control purposes, human post-mortem hippocampus RNA obtained from 20 non-affected Caucasians was included (Fig. 4, Supplementary Fig. 5). For amplification of gephyrin G-, C- and E-domains, PCR oligonucleotides were chosen according to splice hotspots [Fig. 4A, spliced exons in gephyrin splice variants G2, C3/4 and E1/2 (Meier and Grantyn, 2004; Fritschy et al., 2008; Smolinsky et al., 2008) are highlighted grey]. This approach revealed existence of multiple PCR products of G-domain coding sequences in seven out of eight TLE hippocampi (Fig. 4B, ‘G-domain’), while amplification of hippocampal post-mortem control cDNA produced DNA bands that correspond to regular gephyrin (Fig. 4B, ‘Δ6’, arrow) and the G2 splice variant (Fig. 4B, ‘G2’, arrow). In patients with anomalous G-domain splicing, regularly spliced gephyrin was reduced and reached maximally 50% of the intensity of the sum of PCR products with lower molecular weights. Gephyrin C- and E-domains did not exhibit irregularities (Supplementary Fig. 5, ‘C-domain’ and ‘E-domain’).

Gephyrin G-domain amplification products with a molecular weight lower than control (Δ6) were excised, purified and cloned. Sequencing of a total of 45 cDNA clones revealed the presence of mRNAs coding for four different novel gephyrin G-domains (Fig. 4B and C). Two of them contained premature stop codons (Fig. 4B and C, asterisks), and all four TLE gephyrins

<table>
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n = number of analysed fields of view; DG = dentate gyrus; SD = standard deviation.

***P < 0.001, **P < 0.01.
lacked exons 6 and 7. To obtain expression constructs for analysis of cellular effects, the isolated and purified TLE gephyrin G-domain coding sequences were inserted into N-terminally EGFP-tagged gephyrin (Prior et al., 1992; Lardi-Studler et al., 2007) (Fig. 4D).

Because the cornu ammonis of epileptic hippocampi is prone to down-regulation of mAb7a (gephyrin) immunoreactivity (Pfeiffer et al., 1984; Knuesel et al., 2001) we addressed the question whether TLE gephyrins are mAb7a immunoreactive. In addition, we asked whether TLE gephyrins are able to form intracellular aggregates, similarly to regular gephyrin. For these purposes, the widely used gephyrin ‘blob’- assay (Kirsch et al., 1995; Meyer et al., 1995; Meier et al., 2000; Lardi-Studler et al., 2007; Smolinsky et al., 2008) was utilized (Supplementary Fig. 6). None of the TLE gephyrins produced globular aggregates as seen with regular gephyrin (Supplementary Fig. 6A). Three variants (Δ6–8, Δ5–7* and Δ4*) exhibited a diffuse distribution, while Δ4–8 gephyrin produced filamentous aggregates, highly similar to the G2 gephyrin splice variant. None of the TLE gephyrins, except Δ4–8, could be visualized with mAb7a antibody (Supplementary Fig. 6B-E).

According to their deficits in aggregate formation, potential dominant negative effects of TLE gephyrins were considered and analyzed. Dominant negative effects of TLE gephyrins require interaction with regular gephyrin. To investigate this possibility, we first analyzed the catalytic function of gephyrin in HEK293 cells (Fig. 5). It is important to note that gephyrin catalyses biosynthesis of the molybdenum cofactor, which is required for the activity of four human metabolic enzymes (Schwarz et al., 2009).

In comparison to EGFP expressing HEK293 cells, the expression of regular gephyrin (Δ6) resulted in increased production of both molybdenum cofactor as well as molybdopterin, the metal-free precursor of the cofactor (Fig. 5A). As all TLE gephyrin variants lack catalytically relevant parts of the N-terminal gephyrin G-domain (Smolinsky et al., 2008), it is not surprising that they are unable to increase G-domain-mediated molybdopterin synthesis (Fig. 5A). They showed different degrees of reduced molybdopterin levels, as is the case with the well known G2 gephyrin splice variant (Bedet et al., 2006; Smolinsky et al., 2008) (Fig. 5A). The fact that E-domain mediated molybdenum cofactor synthesis was also reduced (Fig. 5A), even upon expression of truncated TLE gephyrins without E-domain, indicated that they had interacted with HEK293 endogenous gephyrin. To further address this possibility, HEK293 cells were co-transfected with regular gephyrin and TLE gephyrins. Against the increased level of molybdenum cofactor synthesis through regular gephyrin, all TLE gephyrins reduced both molybdopterin and molybdenum cofactor contents (Fig. 5B). As all substrates were in excess, TLE gephyrins are concluded to render regular gephyrin less catalytically active, in fact as a result of their interaction with, and presumably conformational re-arrangement of, regular gephyrin. Furthermore, to find out which of the remaining parts of TLE gephyrin G-domains could interact with regular gephyrin, we expressed N-terminally DsRed-express-tagged gephyrin G-domain parts together with EGFP-tagged regular gephyrin (Fig. 5C). This approach revealed co-localization of EGFP-tagged regular gephyrin with DsRed-Express-tagged exon 1–3, exon 7 and exon 8, respectively.
Exon 7 was expected to co-localize with regular gephyrin, because it is the integral part of the G-domain trimer interface (Schwarz et al., 2001; Sola et al., 2001). Most importantly, expression of exon 1–3 dissolved globular aggregates formed by regular gephyrin (Fig. 5C).

As a consequence of their dominant negative effects on regular gephyrin, TLE gephyrins could deplete GABAergic postsynaptic domains from hippocampal endogenous gephyrin, similarly to what was observed with the G2 splice variant (Bedet et al., 2006; Smolinsky et al., 2008). To test this hypothesis, we analysed their potential to interfere with regular hippocampal endogenous postsynaptic gephyrin. Primary hippocampal neurons were transfected with EGFP-tagged TLE gephyrins or regular gephyrin, and hippocampal endogenous gephyrin and GABAergic synapses were visualized using mAb7a in combination with an antibody specific to the vesicular inhibitory amino acid transporter (Fig. 6). The mAb7a antibody can be used here for visualization of hippocampal endogenous gephyrin as it does not react with TLE gephyrins, except Δ4–8 (above). Compared with the expression of EGFP-tagged regular gephyrin, all TLE gephyrins exhibited dominant negative effects on hippocampal endogenous gephyrin, as they curtailed postsynaptic gephyrin scaffolds at GABAergic synapses (Fig. 6B–E, Table 2).

To determine whether curtailed postsynaptic gephyrin level involves changes in the number of GABAergic synapses, we quantified the number of vesicular inhibitory amino acid transporter-positive nerve endings in contact with neurons expressing TLE gephyrins, and performed electrophysiological analyses on the same culture batches, both at *in vitro* Day 9. While the number of vesicular inhibitory amino acid transporter-positive

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**Figure 3** Confocal laser scanning microscopy reveals co-localization of gephyrin, ubiquitin and the neuronal splice factor NeuN in CA2 and CA3 regions of TLE hippocampectomies. Three patients were analysed (A–C). Compared with the dentate gyrus (DG), extensive co-localization of gephyrin (polyclonal antibody), ubiquitin and NeuN in the perinuclear compartment of CA2 and CA3 neurons is obvious (white, arrows). For an overview of the hippocampus of TLE Patient #030310 see Supplementary Fig. 4. Scale bars: 10 μm.
nerve terminals remained constant (Table 3), the mean frequency of miniature postsynaptic GABAergic currents was reduced in neurons expressing TLE gephyrins (Fig. 7A and B, example given for Δ4* TLE gephyrin). In addition, the Kolmogorov–Smirnov test revealed significant reduction of large scale miniature GABAergic postsynaptic currents in Δ4* TLE gephyrin expressing neurons (Fig. 7D–E, \( P < 0.0001 \)). GABA\(_A\) receptor \( \alpha_2 \) binding to gephyrin facilitates postsynaptic receptor accumulation (Tretter et al., 2008), and because of preponderant GABA\(_A\) receptor \( \alpha_2 \) expression in the hippocampal cell cultures at in vitro Day 9 (Supplementary Fig. 7) we conclude that reduction of miniature GABAergic postsynaptic current frequency reflects depletion of postsynaptic GABAergic domains from GABA\(_A\) receptors upon Δ4* TLE gephyrin expression. We have thus focused on GABA\(_A\) receptor \( \alpha_2 \) distribution in control (EGFP) and Δ4* TLE gephyrin expressing neurons (Fig. 8A and B). Again, our experiments reveal a significant Δ4* TLE gephyrin-mediated reduction of the fraction of GABAergic synapses with postsynaptic gephyrin (Fig. 8C, vesicular inhibitory amino acid transporter with gephyrin (%), EGFP: 79.2 ± 3.5; Δ4*: 38.5 ± 11.5, \( P < 0.001 \)). Notably, postsynaptic loss of gephyrin was mirrored by loss of GABA\(_A\) receptor \( \alpha_2 \) (Fig. 8B, arrows; Fig. 8D, vesicular inhibitory amino acid transporter with GABA\(_A\) receptor \( \alpha_2 \) (%), EGFP: 85.5 ± 4.5; Δ4*: 42.3 ± 9.7, \( P < 0.001 \)), as was the case in CA3 regions of TLE hippocampectomies (Fig. 8E–G, arrows). Therefore, depletion of both postsynaptic gephyrin and GABA\(_A\) receptor \( \alpha_2 \) had occurred in a subset of GABAergic synapses in contact with TLE gephyrin expressing neurons.

To determine a potential genetic basis of abnormal gephyrin expression in TLE hippocampus, we sequenced genomic DNA of the patients with TLE from which irregularly spliced gephyrins were isolated. However, neither the genomic DNA sequences (Supplementary Fig. 8) of exons (E3, 4, 5, 8 and 9) nor of adjacent introns (up to 200 bp up- and down-stream of the respective exon) differed from published sequences available at the National Centre for Biotechnology Information. A genetic basis of deficiency in Nova-2 signalling could also be ruled out because the Nova CLIP tag sequence in intron 8 was conserved (Supplementary Fig. 8B).

Therefore, and because the neuronal splice factor NeuN was associated with ubiquitin in TLE hippocampectomies, we considered the possibility that inhibition of gephyrin RNA splicing (exon skipping) occurs in response to cellular stress, thereby presenting a mechanism underlying the formation of TLE gephyrins. In fact, due to unusual positioning of splice donor and acceptor sites, skipping of E4 and E5-7 inevitably leads to frame shifts and
premature stop codons, resulting in the truncated TLE gephyrins Δ4* and Δ5–7*, respectively (Supplementary Fig. 8C–D). Similarly, skipping of exons 4–8 is expected to result in protein truncation, but unusual assembly of exons 3 and 9 actually circumvented this (Supplementary Fig. 8E). Post-transcriptional processing is known to be disturbed in the TLE hippocampus (Vollmar et al., 2004; Eichler et al., 2008, 2009; Legendre et al., 2009) and cellular stress (alkalosis and high temperature).
is known to be involved in epileptogenesis (Church and Baimbridge, 1991; Hentschke et al., 2006; Schuchmann et al., 2006; Qu et al., 2007). Most importantly, hyperthermia was shown to decrease GABAergic synaptic transmission in cornu ammonis pyramidal cells, but not in dentate gyrus granule cells (Qu and Leung, 2009). Therefore, we finally addressed the possibility that experimentally induced alkalosis and hyperthermia (Fig. 9A) are sufficient to provoke exon skipping in GPHN gene transcripts. For this purpose, the capacity of primary hippocampal neurons to maintain regular gephyrin splicing upon alkalosis and hyperthermia was analysed (Fig. 9B and C). Both alkalization and hyperthermia reduced the amount of regularly spliced gephyrin down to 52 ± 5% (alkalosis) and 30 ± 2% (hyperthermia) of control (Fig. 9B and C), and molecular cloning and sequencing confirmed the TLE identity of PCR products obtained from stressed neurons (Fig. 9B, arrows). Alkalosis and hyperthermia increased the fraction of neurons with overlapping gephyrin, ubiquitin and NeuN immunoreactivities (control: 3.4 ± 1.1%, alkalosis: 49.0 ± 5.0%, hyperthermia: 60.1 ± 3.9%, P < 0.001; Fig. 9D, arrowhead and arrow, E). Moreover, in control neurons (Fig. 9D, left lower panel), 80.1 ± 2.6% of GABAergic synapses (vesicular inhibitory amino acid transporter) contained gephyrin and GABAA receptor α2 (Fig. 9F), while in neurons challenged with alkalosis or hyperthermia (Fig. 9D, right lower panel, arrows) significantly less synapses contained gephyrin and GABAA receptor α2 (alkalosis: 47.6 ± 8.5%, hyperthermia: 44.8 ± 10.5%, P < 0.001; Fig. 9F). Finally and most conclusively, a reporter construct consisting of red (tdTomato) and green (EGFP) fluorescent proteins separated by exons 3–5 (including the sequenced TLE intron fragments) reliably monitored skipping of exon 4 upon cellular stress (Supplementary Fig. 9, ‘EGFP ON’). Therefore, we conclude that cellular stress has induced exon

Figure 6  TLE gephyrins exert dominant negative effects on hippocampal endogenous postsynaptic gephyrin. Proximal dendrites of primary rat hippocampal neurons expressing EGFP-tagged regular (A, ∆6) or TLE gephyrins (B–E) are shown. GABAergic synapses and endogenous gephyrin were visualized with anti-vesicular inhibitory amino acid transporter (VIAAT) and mAb7a antibodies, respectively. Note that the fraction of GABAergic synapses (vesicular inhibitory amino acid transporter) with postsynaptic gephyrin (mAb7a) is reduced in cells expressing TLE gephyrins. Scale bar: 5 μm.
skipping in gephyrin mRNA and produced TLE gephyrins with dominant negative effects on GABAergic postsynaptic protein scaffolds (Fig. 9G).

### Discussion

Anomalous GABAergic inhibition is involved in TLE (Kumar and Buckmaster, 2006; Stief et al., 2007; Eichler and Meier, 2008) and corresponding reduction of gephyrin immunoreactivity was observed in the hippocampal cornu ammonis of kainate-injected epileptic mice (Kneussel et al., 2001). However, the mechanisms underlying curtained postsynaptic gephyrin remained unknown. This study identifies pathological gephyrin expression in the cornu ammonis of patients with TLE, irrespective of hippocampal sclerosis. Therefore, curtailing of postsynaptic gephyrin and GABA<sub>A</sub> receptor α2 scaffolds is not a consequence of neuronal dropout, but rather results from cellular stress-induced inhibition of gephyrin RNA splicing and the resulting synthesis of dominant negative gephyrins. Notably, cellular stress (alkalosis or hyperthermia) has been involved in epileptogenesis (Church and Hendry, 2001). Furthermore, the presence of filamentous and diffusely stained gephyrin in HEK293 cells, and third, the impact of the TLE gephyrins on hippocampal endogenous postsynaptic gephyrin and GABA<sub>A</sub> receptor α2 was obvious in vitro. Thus, dominant negative effects can be attributed to TLE gephyrin variants.

Gephyrin regulates the size of postsynaptic scaffolds and consequently their ability to locally stabilize and enrich postsynaptic domains with GABA<sub>A</sub> receptors (Kneussel et al., 1999; Levi et al., 2004) by providing binding sites for GABA<sub>A</sub> receptor α2 (Tretter et al., 2008). Its capacity to form oligomers is essential to this mechanism as it amplifies the number of postsynaptic GABA<sub>A</sub> receptor docking sites. Based on structural and functional data, gephyrin oligomerization involves both G-domain trimer and E-domain dimer formation, leading to the proposed hexagonal protein lattice (Schwarz et al., 2001; Sola et al., 2001, 2004; Schrader et al., 2004; Lardi-Studler et al., 2007). Thus, any process that interferes with G- or E-domain interactions will impact on available GABA<sub>A</sub> receptor α2 binding sites. Alternative splicing of gephyrin G-domains was already proposed to produce dominant negative gephyrins, since inclusion of exon 6 (G2 gephyrin splice variant (Fritschy et al., 2008)) was shown to disrupt the G-domain trimer interface (Schwarz et al., 2001). Consequently, exon 6-containing G-domains are no longer able to form trimers, and in conjunction with E-domain dimerization, filamentous gephyrin aggregates emerge (Meier et al., 2000; Smolinsky et al., 2008). We have also shown that the gephyrin G2 splice variant is catalytically inactive in synthesizing molybdenum cofactor (Smolinsky et al., 2008). Therefore, G2-gephyrin was a prime candidate for impairment of GABAergic synapses in TLE. However, we did not obtain evidence for up-regulation of G2-gephyrin in patients with TLE. Instead, we isolated four abnormally spliced G-domains that all abolished regular gephyrin aggregation. One TLE gephyrin (Δ4-8) produced filamentous aggregates, as was observed with the G2 splice variant (Meier et al., 2000; Bedet et al., 2006; Smolinsky et al., 2008), while the other three TLE variants produced a diffuse signal in fibroblasts. In all cases, deficits in gephyrin oligomerization most likely result from omission of exon 7 because these amino acids constitute the integral part of the trimer interface of gephyrin G-domains (Schwarz et al., 2001; Sola et al., 2001). Furthermore, the presence of filamentous and diffuse mAb7a immunoreactivities in combination with curtained postsynaptic gephyrin, gephyrin cleavage products in western blots as well as overlapping signals of gephyrin, ubiquitin and the neuronal splice factor NeuN indicates that abnormal gephyrin splicing and expression had occurred in the hippocampal cornu ammonis of patients with TLE.

Dominant negative effects of TLE gephyrins on hippocampal regular gephyrin were determined using three experimental strategies. First, gephyrin’s catalytic activity in synthesizing molybdenum cofactor was used as a measure for their ability to interact with regular gephyrin in HEK293 cells. As TLE gephyrin rendered catalytically active regular gephyrin non-functional both in terms of G-domain dependent molybdopterin and E-domain catalysed molybdenum cofactor synthesis, even upon co-expression of truncated variants with fragmental G-domains, interaction of TLE gephyrin with regular gephyrin must have occurred. Second, we could show that N- and C-terminal parts of TLE gephyrin G-domains co-localize with regular gephyrin in HEK293 cells, and third, the impact of the TLE gephyrins on hippocampal endogenous postsynaptic gephyrin and GABA<sub>A</sub> receptor α2 was obvious in vitro. Thus, dominant negative effects can be attributed to TLE gephyrin variants.

### Table 2

Percent fraction of GABAergic synapses (vesicular inhibitory amino acid transporter) with postsynaptic gephyrin (mAb7a)

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n = number of analysed neurons per culture; SD = standard deviation.

### Table 3

Number of GABAergic synapses (vesicular inhibitory amino acid transporter)

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n = number of analysed neurons per culture; VIAAT = vesicular inhibitory amino acid transporter; ns = not significant, P < 0.05.

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n = number of analysed neurons per culture; VIAAT = vesicular inhibitory amino acid transporter; ns = not significant, P < 0.05.
DNA sequencing of TLE gephyrin G-domain exons and their flanking intron fragments has indicated that gephyrin genome sequence modifications did not underlie abnormal gephyrin expression in TLE hippocampectomies. A novel fluorescence reporter construct of irregular gephyrin RNA splicing was generated to verify that the sequenced intron fragments were long enough to draw such a conclusion, by monitoring skipping of exon 4 upon cellular stress (Supplementary Fig. 9). Thus, cellular stress (alkalosis and hyperthermia) is sufficient to trigger exon skipping and to produce dominant negative TLE gephyrin, associated with overlapping signals of gephyrin, ubiquitin and NeuN in vitro and corresponding to our observations made in CA3 regions, but not in

Figure 7 TLE gephyrin expression in hippocampal neurons reduces the number of functional GABAergic synapses. (A) Representative traces of miniature GABAergic postsynaptic currents obtained from untransfected neurons (NT), from neurons expressing EGFP and from neurons expressing ∆4* TLE gephyrin are shown. (B) Quantitative analysis of the frequency of miniature GABAergic postsynaptic currents (mIPSC) reveals significant differences between control neurons (NT or EGFP) and TLE gephyrin expressing neurons (∆4*). (C) Mean amplitudes of miniature GABAergic postsynaptic currents are shown. (B and C) The number of investigated neurons is indicated. (D) The normalized histogram of miniature GABAergic postsynaptic current amplitude distributions illustrates under-representation of large miniature GABAergic postsynaptic currents (>70 pA) in ∆4* TLE gephyrin expressing neurons. The bin size was set to 10 pA, and 1571 (EGFP) and 657 (∆4* TLE gephyrin) events were included. (E) Cumulative probability distribution of miniature GABAergic postsynaptic current amplitudes and P-value obtained with statistical analysis using Kolmogorov-Smirnov test. **P < 0.01, *P < 0.05, ns = not significant (P = 0.8).
dentate gyri, of TLE hippocampectomies. In fact, hyperthermia was already shown to decrease GABAergic synaptic transmission to pyramidal cells of the cornu ammonis, but not to dentate gyrus granule cells (Qu and Leung, 2009). The rise of extracellular glutamate during epileptic seizures (During and Spencer, 1993) is another example of critical cellular stress, because seizures produce rebound alkalosis up to intracellular pH of 8.0 (Hartley and Dubinsky, 1993), also culminating in exon skipping (Supplementary Fig. 9) and hence the postsynaptic signature of inhibited gephyrin RNA splicing (Fig. 9G). In fact, a large body of evidence supports the involvement of rises in intracellular pH in increased neuronal excitability and epileptiform activity both in vitro and in vivo (Aram and Lodge, 1987; Balestrino and Somjen, 1988; Jarolimek et al., 1989; Lee et al., 1996; Kaila and Ransom, 1998).

Although the overall role of gephyrin in enrichment of postsynaptic sites with GABA_A receptors is a matter of controversy (Kneussel et al., 1999; Levi et al., 2004), this study and others (Fischer et al., 2000; Kneussel et al., 2001; Yu et al., 2007) support a subunit-specific role of gephyrin in postsynaptic GABA_A receptor enrichment. Direct binding of GABA_A receptor α2 to gephyrin was demonstrated recently (Tretter et al., 2008), while postsynaptic clustering of α1-GABA_A receptors seems to be independent of gephyrin (Kneussel et al., 2001; Levi et al., 2004; Yu et al., 2007). α1-GABA_A receptors are preferentially found at synapses of fast-spiking interneurons (involved in high frequency gamma network oscillatory activity), while α2-GABA_A receptors preferentially associate with synapses of regular, low frequency, spiking interneurons (Freund and Katona, 2007). Notably, a preponderance of high frequency hippocampal network oscillatory activity is known to precede seizures (Fisher et al., 1992; Bragin et al., 2007), and small changes in the overall activity in the hippocampal CA3 area can trigger the neuronal network to switch from gamma oscillations to epileptiform bursts (Fisahn, 2005). In fact, dentate gyrus granule cell to CA3 synapses are ‘conditional detonators’ (Henze et al., 2002), which can easily elicit recurrent CA3–CA3 activity if not controlled appropriately. Thus, exon skipping in gephyrin mRNA could reduce seizure threshold, for example secondary to seizure activity, due to cellular stress (e.g. alkalosis) and its impact on postsynaptic gephyrin and GABA_A receptor α2.

Figure 8 TLE gephyrin expression curtails postsynaptic GABA_A receptor α2 scaffolds. (A–D) Analysis of postsynaptic GABA_A receptor distribution in neurons with Δ4* TLE gephyrin reveals coherent depletion of postsynaptic endogenous gephyrin (Ge, mAb7a) and GABA_A receptor α2. Dendrites of neurons expressing EGFP (A, control) or Δ4* TLE gephyrin (B) are shown. Co-localization of vesicular inhibitory amino acid transporter (VIAAT), gephyrin and GABA_A receptor α2 appears in white. Synapses devoid of gephyrin also lack GABA_A receptor α2 (B, arrows). Thus, Δ4* TLE gephyrin expression reduces both postsynaptic gephyrin and GABA_A receptor α2 (C and D). (E–G) In CA3 regions of TLE hippocampectomies, vesicular inhibitory amino acid transporter-positive nerve endings without gephyrin (mAb7a) also lack GABA_A receptor α2 (arrows). Scale bars, A–B: 5 μm, E–G: 10 μm. ***P < 0.001.
Figure 9  Cellular stress (alkalosis and 39°C) triggers curtailing of postsynaptic gephyrin and GABA\(\alpha_2\) receptor \(\alpha_2\) scaffolds in hippocampal neurons. (A) High temperature (39°C) and alkalosis experimental paradigms. Change of the extracellular milieu pH (pH\([\text{e}]\)) over time is shown. (B) Reverse transcription PCR analysis of G-domain splicing in control condition and upon experimentally induced cellular stress. DNA sequencing confirmed the TLE identity of gephyrin amplification products (arrows). Note that splicing of regular gephyrin (\(\Delta6\)) is reduced in neurons challenged with alkalosis or 39°C (C). (D and E) Determination of the fraction of cells with co-localized gephyrin and ubiquitin in perinuclear (DAPI) compartments. Note that control neurons (NeuN) rarely display co-localized gephyrin and ubiquitin immunoreactivities, and the rare perinuclear aggregates do not contain NeuN (D, high power view, arrowhead). In contrast, cellular stress increases the fraction of neurons with perinuclear gephyrin and ubiquitin (E). These aggregates also contain the neuronal splice factor NeuN (D, high power view, right panel, arrow). (D and F) Neurons challenged with 39°C or alkalosis display coherent loss of postsynaptic gephyrin and GABA\(\alpha_2\) receptor \(\alpha_2\) (D, high power views of dendrites with mAb7a, \(\alpha_2\) and vesicular inhibitory amino acid transporter, arrows). Accordingly, cellular stress induces exon skipping during RNA splicing (Supplementary Fig. 9). (G) Scheme summarizing effects of cellular stress on gephyrin splicing and postsynaptic GABAergic domains. ***\(P<0.001\).

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Supplementary material

Supplementary material is available at Brain online.

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