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PROGRESSIVE LOSS OF PHASIC, BUT NOT TONIC, $GABA_A$ RECEPTOR-MEDIATED INHIBITION IN DENTATE GRANULE CELLS IN A MODEL OF POST-TRAUMATIC EPILEPSY IN RATS

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Abstract—Traumatic brain injury (TBI) is a risk factor for the development of epilepsy, which can occur months to years after the insult. The hippocampus is particularly vulnerable to the pathophysiological effects of TBI. Here, we determined whether there are long-term changes in inhibition in the dentate gyrus that could contribute to the progressive susceptibility to seizures after TBI. We used severe lateral-fluid percussion brain injury to induce TBI in rats. In this model, spontaneous seizure activity, which involves the hippocampus, appears after a long latent period, resembling the human condition. We demonstrate that synaptic $GABA_A$ receptor-mediated inhibition is profoundly reduced in ipsilateral dentate granule cells 1 month after TBI. Moreover, synaptic inhibition decreases over time, and by 6 months after TBI, it is also significantly decreased contralaterally. Progressive loss of synaptic inhibition is paralleled by a decline in the number of parvalbumin-positive interneurons, but, in contrast to status epilepticus models, $GABA_A$ receptor subunit expression is largely unaltered. At both time points, the magnitude of tonic $GABA_A$ receptor-mediated currents after TBI is maintained, indicating a preservation of the inhibitory constraint of granule cells through tonic inhibition. Our results extend the time window during which strategies to target epileptogenesis may be effective.

Key words: dentate gyrus, $GABA_A$ receptors, interneurons, parvalbumin, tonic inhibition, traumatic brain injury.

Traumatic brain injury (TBI) accounts for up to 20% of symptomatic epilepsies (Agrawal et al., 2006). Seizures can occur, however, after a considerable delay, often months to years after the insult (Annegers et al., 1998). Besides damage and seizures arising at the site of injury, TBI in many cases leads to hippocampal atrophy resulting in mesial temporal lobe seizures (Swartz et al., 2006). Alterations in neuronal excitability and neurotransmission have been proposed to contribute to post-traumatic epilepsy (Toth et al., 1997; D'Ambrosio et al., 2004; Hunt et al., 2009; Pitkanen et al., 2009), but the delay to seizure occurrence remains unexplained.

Dentate granule cells (DGCs) are the primary postsynaptic targets for cortical inputs to the hippocampus and form monosynaptic connections with CA3 pyramidal cells via mossy fibers. They also innervate glutamatergic hilar mossy cells, and in turn, receive excitatory drive from these cells. Due to their relatively low excitability, pronounced spike-frequency adaptation, and strong inhibition by interneurons predominantly located in the hilus, DGCs have been argued to act as a brake against seizure propagation through the limbic circuitry (Collins et al., 1983; Lothman and Bertram, 1993). Decreases in inhibitory postsynaptic currents (IPSCs) in DGCs have been described after status epilepticus (Kobayashi and Buckmaster, 2003; Shao and Dudek, 2005) but are less certain after TBI (Lowenstein et al., 1992; Reeves et al., 1997; Santhakumar et al., 2001; Mtchedlishvili et al., 2010; Hunt et al., 2011). Furthermore, a compensatory increase in the excitatory drive from hilar mossy cells onto interneurons may follow TBI (Santhakumar et al., 2001). Moreover, these studies have focused on phasic (synaptic) inhibition.

Tonic $GABA_A$ receptor-mediated signaling is the dominant contributor to the mean $GABA_A$ receptor-mediated conductance in specific cell types (Mody and Pearce, 2004) and can efficiently regulate cell excitability and oscillatory activity (Mitchell and Silver, 2003; Cavelier et al., 2005; Pavlov et al., 2009; Mann and Mody, 2010). Alterations in tonic inhibition have been implicated in the pathophysiology of several neurological disorders including epilepsy (e.g. Zhang et al., 2007; Cope et al., 2009).

Here, we used a rat model in which spontaneous seizures that involve hippocampus develop over a period of months after severe TBI (Kharatishvili et al., 2006). This provides an ideal opportunity to determine molecular and cellular changes associated with progressive increases in seizure occurrence (Englander et al., 2003; Christensen et al., 2009). This model captures the essential pathophysiological features of hippocampal sclerosis, including hilar cell loss and mossy fiber sprouting (Kharatishvili et al., 2006; Pitkanen et al., 2009).

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Abbreviations: DGC, dentate granule cell; IPSC, inhibitory postsynaptic current; KPBS, potassium phosphate buffered saline; mIPSC, miniature IPSC; NHS, normal horse serum; PARV, parvalbumin; PFA, paraformaldehyde; ROD, relative optic density; RT, room temperature; sIPSC, spontaneous IPSC; TBI, traumatic brain injury; WFA, wisteria floribunda agglutinin.

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Here, we show a progressive loss of phasic inhibition onto DGCs from 1 to 6 months after TBI, which is associated with a loss of parvalbumin (PARV)-positive interneurons. In contrast to studies in post-status epilepticus rats, $GABA_A$ receptor subunit expression remains largely unchanged. Importantly, there is persistent expression of the δ subunit, resulting in the maintenance of tonic $GABA_A$ receptor currents. We propose that preserved tonic $GABA_A$ receptor currents maintain the inhibitory constraint of DGCs, but that sufficient loss of synaptic inhibition after TBI can lead to a failure of inhibitory mechanisms as network activity increases.

EXPERIMENTAL PROCEDURES

Ethical approval

All animal procedures were carried out in accordance with the guidelines of the European Community Council Directives 86/609/EEC and the United Kingdom Animals (Scientific Procedures) Act 1986. The procedures were approved by the Animal Ethics Committee of the participating institutions.

Animals

Adult male Sprague–Dawley rats (Harlan Netherlands B.V, Horst, Netherlands) weighing 280–380 g were used in the study: 33 rats for PARV immunohistochemistry, 11 rats for $GABA_A$ receptor subunit *in situ* hybridization, and 46 rats for electrophysiology. Rats were housed individually under controlled conditions (12 h light/dark cycle, temperature 22±1 °C, humidity 50–60%, *ad libitum* access to food and water).

Induction of lateral-fluid percussion brain injury

The procedure of lateral-fluid percussion brain injury induction has been described previously (McIntosh et al., 1989; Kharatishvili et al., 2006). In brief, rats were anesthetized with an i.p. injection of a solution (6 ml/kg) containing sodium pentobarbital (58 mg/kg), chloral hydrate (60 mg/kg), magnesium sulfate (127.2 mg/kg), propylene glycol (42.8%), and absolute ethanol (11.6%), and were placed in a Kopf stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). The skull was exposed with a midline skin incision and periosteum was removed. The left temporal muscle was gently detached from the lateral ridge. A circular craniectomy (ø=5 mm) was made over the left parietal lobe midway between lambda and bregma so that the dura mater remained intact. Luer-Lock cap was placed into the craniectomy, its edges sealed with glue, and the cap that was filled with saline. Lateral-fluid percussion brain injury was induced 90 min after the administration of anesthsia by connecting the rat to a fluid-percussion device (AmScien Instruments, Richmond, VA, USA) via the female Luer-Lock fitting. The mean impact pressure, measured by an extracranial transducer, was 3.38±0.01 atm. The mortality within the first 72 h post TBI was 25%. Sham-operated control animals underwent all surgical procedures, except the fluid-percussion impact. All animals studied at different time points after the injury were compared with the age-matched sham controls that were operated at the same time as the experimental group and were drawn from the same batch of animals.

In vitro electrophysiology

In vitro electrophysiological recordings were performed in the dentate gyrus from rats 1 and 4–6 months after the surgery. Animals were killed according to the United Kingdom Animals (Scientific Procedures) Act 1986. After decapitation, the brains were rapidly removed and the hippocampi were dissected. Transverse hippocampal slices (350 μm thick) were cut with a vibratome (VT1200S, Leica, Germany) in an ice-cold sucrose-based solution containing (in mM) sucrose (70), NaCl (80), KCl (2.5), $MgCl_2$ (7), $CaCl_2$ (0.5), $NaHCO_3$ (25), NaH_2PO_4 (1.25), and glucose (22), bubbled continuously with 95% O_2 and 5% CO_2 to yield a pH of 7.4. The slices were then allowed to recover in sucrose-free solution (in mM) containing NaCl (119), KCl (2.5), $MgSO_4$ (1.3), $CaCl_2$ (2.5), $NaHCO_3$ (26.2), NaH_2PO_4 (1), and glucose (22), bubbled with 95% O_2 and 5% CO_2 in an interface chamber for at least 1 h at room temperature (RT) before being transferred to a submerged recording chamber, and perfused with the same solution.

Visualized whole-cell voltage-clamp recordings were performed from dentate gyrus granule cells using infrared differential interference contrast imaging system. All recordings were done at 32 °C. Recording pipettes were filled with Cs-based internal solution containing (in mM) CsCl (120), HEPES (10), EGTA (2), NaCl (8), $MgCl_2$ (0.2), Mg–ATP (2), Na-GTP (0.3), QX-314 Br^- salt (5), pH 7.2, 290 mOsm. For some experiments (Figs. 2 and 3C, D), we used a low Cl^- internal solution, containing (in mM) Cs-methanesulfonate (120), HEPES (10), EGTA (0.2), NaCl (8), $MgCl_2$ (0.2), Mg–ATP (2), Na-GTP (0.3), QX-314 Br^- salt (5), pH 7.2, 290 mOsm.

Pharmacologically isolated $GABA_A$ receptor-mediated IPSCs were recorded from granule cells voltage clamped at −70 mV in the presence of AMPA/kainate, NMDA and $GABA_B$ receptor blockers: NBQX (25 μM), D-AP5 (50 μM), and CGP52432 (5 μM). Action potential-independent miniature IPSCs (mIPSCs) were recorded with the Na^+ channel blocker tetrodotoxin (TTX, 1 μM) in the perfusion solution. In the absence of glutamate receptor blockers, IPSCs were recorded with low Cl^- Cs-methanesulfonate-based solution at 0 mV (close to the reversal of glutamatergic currents) as outward currents. Tonic $GABA_A$ receptor-mediated current was measured as a change in holding current after application of $GABA_A$ receptor blocker picrotoxin (PTX) (100 μM). Neurons were held either at −70 mV (with CsCl-based intracellular solution) or at 0 mV (with Cs-methanesulfonate-based intracellular solution). Series resistance was monitored throughout the experiment using a −5 mV step command. Cells showing a >20% change in series resistance, or a series resistance >25 MΩ, or an unstable holding current were rejected.

Recordings were obtained using a MultiClamp 700B amplifier (Axon Instruments, Foster City, CA, USA), filtered at 4 kHz, digitized at 10 kHz, and stored on a personal computer. Data acquisition and off-line analysis were performed using Strathclyde Electrophysiology Windows Electrophysiology Disk Recorder (WinEDR v3.0.1, Dr. John Dempster, University of Strathclyde, Glasgow, UK) and LabVIEW 8.0 (National Instruments, Austin, TX, USA) software. Spontaneous and mIPSCs were detected using a peak detection algorithm that measured peak amplitude, and rise and decay time. Undetected events and false-positives were corrected by visual inspection. Threshold values were set at three times the root mean square of the baseline noise amplitude. Frequency of events was measured over 240–360 s periods of continuous recordings.

Drugs were purchased from Tocris Cookson (Bristol, UK) and Ascent Scientific (North Somerset, UK) unless indicated otherwise.

Histology

Separate groups of animals were prepared for histological analysis that was performed at 1, 4, or 6 months post-TBI.

Fixation. Rats were deeply anesthetized and transcardially perfused according to the pH-shift perfusion protocol: 0.9% sodium chloride solution (+4 °C) for 2 min at the speed of 30–35 ml/min, followed by 4% paraformaldehyde (PFA) in 0.1 M sodium

acetate buffer, pH 6.5 (+4 °C), for 10 min, and 4% PFA in 0.1 M sodium borate, pH 9.5 (+4 °C), for 15 min. Brains were immediately removed from the skull and postfixed in 4% PFA in 0.1 M sodium borate, pH 9.5 (+4 °C), for 6 h. Subsequently, the brains were cryoprotected in 20% glycerol in 0.02 M potassium phosphate-buffered saline (KPBS, pH 7.4) for 36 h. They were then blocked, frozen in dry ice for 15 min, and stored at −70 °C until further processed.

Processing of tissue. Brains were sectioned in a coronal plane (1-in-8 series, 25 μm) with a sliding microtome (Leica SM 2000, Leica Microsystems Nussloch GmbH, Nussloch, Germany). The first series of sections was stored in 10% formalin at RT and the rest in cryoprotectant tissue-collecting solution (30% ethylene glycol, 25% glycerol in 0.05 M sodium phosphate buffer, pH 7.4) at −20 °C until processed. The first series was stained with thionin to localize the cytoarchitectonic fields of different brain areas.

PARV immunohistochemistry. For PARV immunohistochemistry, one series of free-floating sections was first washed three times in 0.02 M KPBS (pH 7.4). To remove endogenous peroxidase activity, sections were incubated in 1% H_2O_2 in 0.02 M KPBS at RT for 15 min. After washing six times in 0.02 M KPBS, nonspecific binding was blocked by incubating the sections in 10% normal horse serum (NHS) and 0.5% Triton X-100 (TX-100) in 0.02 M KPBS at RT for 2 h. Sections were washed in 2% NHS in 0.02 M KPBS and incubated for 2 days (+4 °C) in a primary antibody solution containing mouse polyclonal antibody raised against PARV (1:15,000, #235, Swant, Bellinzone, Switzerland), 1% NHS, and 0.5% TX-100 in 0.02 M KPBS. Sections were washed three times (2% NHS in 0.02 M KPBS) and incubated in biotinylated horse antimouse IgG (1:200, Vector BA-2000, Vector Laboratories, CA, USA), 1% NHS and 0.3% TX-100, in 0.02 M KPBS for 1 h at RT. Then, sections were washed three times (2% NHS in 0.02 M KPBS) and incubated in avidin-biotin (1:100, PK-4000, Pierce Chemical, Rockford, IL, USA) in 0.02 M KPBS for 45 min at RT. After three washes (2% NHS in 0.02 M KPBS), sections were recycled back to the secondary antibody solution for 45 min at RT, washed, and incubated in avidin-biotin solution for 30 min at RT. After three washes (2% NHS in 0.02 M KPBS), the secondary antibody was visualized with 0.05% 3′, 3′-diaminobenzidine (DAB, Pierce Chemicals, Rockford, IL, USA) and 0.04% H_2O_2 in 0.02 M KPBS. Sections were mounted on gelatin-coated microscope slides. Slides were dried overnight at +37 °C. Subsequently, the reaction product was intensified with osmium (OsO_4) thiocarbohydrate according to the method of Lewis et al (1986).

Estimation of the total number of PARV immunoreactive neurons in the dentate gyrus using unbiased stereology. Each PARV-positive neuron throughout the entire septotempotral axis of the hippocampus and dentate gyrus was plotted in a 1-in-16 series of 25 μm-thick sections (400 μm apart from each other) using the AccuStage MDPlot 5.3 graphical program and MD3 Microscope Digitizer (AccuStage, Shoreview, MN, USA) connected to a Leica DMRB microscope. Then, the cytoarchitectonic boundaries of different hippocampal subfields were drawn on computer-generated plots from the immunostained and/or thionin-stained sections using a camera lucida on a stereomicroscope equipped with a drawing tube. To calculate the total number of hilar cells, the following equation was used:

$$N_{tot} = \sum Q \times 1/ssf \times 1/asf \times t/h,$$

where section sampling fraction (ssf) is 1/16, area sampling fraction (asf) is 1, and tissue sampling fraction (t/h, the height of the mounted section thickness divided by the dissector height) is 1 (West et al., 1991).

In situ *hybridization.* For *in situ* hybridization, brains were rapidly removed and snap-frozen in −70 °C isopentane for 3 min. After evaporating the isopentane at −70 °C for 48 h, the brains were transferred to vials; these were tightly sealed and stored at −70 °C. *In situ* hybridization was performed on 20 μm-thick microtome sections mounted on chrome alum-coated slides. Synthetic oligonucleotides (Tsunashima et al., 1997) were labeled with [^{35}S] α-thio-dATP (1300 Ci/mmol; New England Nuclear, Boston, MA, USA) on the 3′ end by reaction with terminal deoxynucleotidyl transferase (Roche, Mannheim, Germany), and *in situ* hybridization was performed as described previously (Tsunashima et al., 1997).

Densitometric analysis of hybridization signals on autoradiography films was done over the granule cell layer using ImageJ 1.34 (NIH, USA). Standard curves based on relative optic density (ROD) values of the microscales were used for converting RODs of samples into nCi/g tissue (Tsunashima et al., 1997).

Statistical analysis

Data were analyzed using SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA). Difference in neuronal numbers between the groups was analyzed using Mann–Whitney *U*-test. Interhemispheric differences were assessed by Wilcoxon test.

Two-tailed Student's *t*-test or multifactorial ANOVA were used for statistical analysis of electrophysiological and *in situ* hybridization data. For analysis of changes in mIPSCs with time from TBI, we used a multifactorial ANOVA with between-subject effects of TBI and its interactions with side (ipsilateral or contralateral to injury) and time. All tests were powered to detect at least a two standard deviation difference between means at $P<0.05$ with a power of 80%.

Differences were considered significant when $P<0.05$. Data are shown as mean±SEM.

RESULTS

Phasic, but not tonic, inhibition is decreased ipsilaterally 1 month after TBI

One month after TBI, the frequency of spontaneous IPSCs (sIPSCs) recorded from DGCs was profoundly reduced ipsilateral to the injury (47.4±5.3% of control, $P<0.01$; Fig. 1A, B). Although reduced contralaterally, sIPSC frequency was not significantly different from control values (75.9±10%, $P=0.2$; Fig. 1A, B). There was no significant difference between hemispheres in sham control animals either in sIPSC frequency (ipsilateral: 3.2±0.7 Hz; contralateral: 2.8±0.4 Hz; $n=12$ for each hemisphere) or any other parameter studied. Therefore, the control data from both hemispheres were pooled.

Tetrodotoxin significantly reduced the frequency of IPSCs in all experimental groups ($P<0.05$). This reduction was similar in control animals and TBI rats (Fig. 1C) indicating that ipsilateral decrease in inhibition of DGCs was not due to lower excitability and/or less frequent spontaneous discharges of interneurons. Accordingly, the frequency of activity-independent mIPSCs in TBI rats was 43.4±6.2% of control ipsilaterally ($P<0.0001$). Contralaterally, the decrease in mIPSC frequency (76.0±10.7% of control) did not reach significance, and was significantly less than that of the ipsilateral side ($P<0.05$; Fig. 1D).

The reduction of mIPSC frequency indicates that there is a presynaptic decrease in inhibitory control of DGCs after brain injury. We next asked whether there were changes at the postsynaptic site. To address this, we compared the mIPSC properties in control and TBI rats.

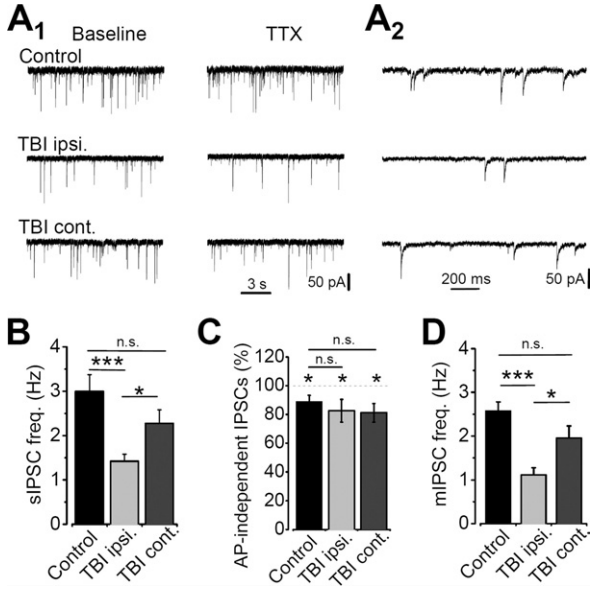

Fig. 1. Decrease in phasic inhibition of dentate granule cells after TBI. (A) Representative traces of sIPSCs (left) and mIPSCs (right) in sham control rats and animals after head trauma (A_1). Sample traces of mIPSCs with expanded timescale are shown in A_2. (B) The frequency of sIPSCs is reduced ipsilateral to TBI. The contralateral side is not significantly affected. (C) The proportion of action potential-independent spontaneous neurotransmission is similar in each experimental group. (D) The frequency of mIPSCs is similarly decreased on the ipsilateral side compared with both control and the contralateral side. Ipsi., ipsilateral; cont., contralateral; n.s., nonsignificant changes. * $P<0.05$, *** $P<0.001$.

Although the mIPSC amplitude was not affected, the decay time was significantly slower on the ipsilateral side post-TBI (Table 1). As a result, charge transfer mediated by the unitary $GABA_A$ receptor-mediated currents was significantly increased on the ipsilateral side (Table 1). However, these post-synaptic increases in inhibition do not compensate for the decrease in IPSC frequency; total charge transfer per time (phasic current) was significantly reduced ipsilaterally (60.6±11.9% of control, $P<0.02$), but not contralaterally (82±13.6% of control, $P=0.24$).

Table 1. Changes in the mIPSC parameters after TBI

	Sham control	TBI ipsilateral	TBI contralateral
1 month			
Amplitude (pA)	43.2±1.9	47.9±4.2	40.8±2.6
τ_{rise} (ms)	0.35±0.01	0.42±0.03*	0.38±0.02
τ_{decay} (ms)	14.53±0.51	17.91±1.42*	17.67±1.70*
Charge transfer (fC)	610.5±28.4	837.3±119.9*	653.4±43.1
n cells (animals)	26 (9)	14 (10)	13 (9)
6 months			
Amplitude (pA)	45.9±2.6	58.5±5.3*	38.5±3.9#
τ_{rise} (ms)	0.35±0.02	0.37±0.02	0.42±0.04
τ_{decay} (ms)	13.55±0.99	15.18±1.50	17.3±2.4
Charge transfer (fC)	597.8±37.0	838.4±88.3**	587.6±28.4#
n cells (animals)	18 (10)	11 (8)	6 (6)

* $P<0.05$, ** $P<0.01$ (*t*-test compared with sham-control group).
# $P<0.05$ (*t*-test contralateral compared with ipsilateral).

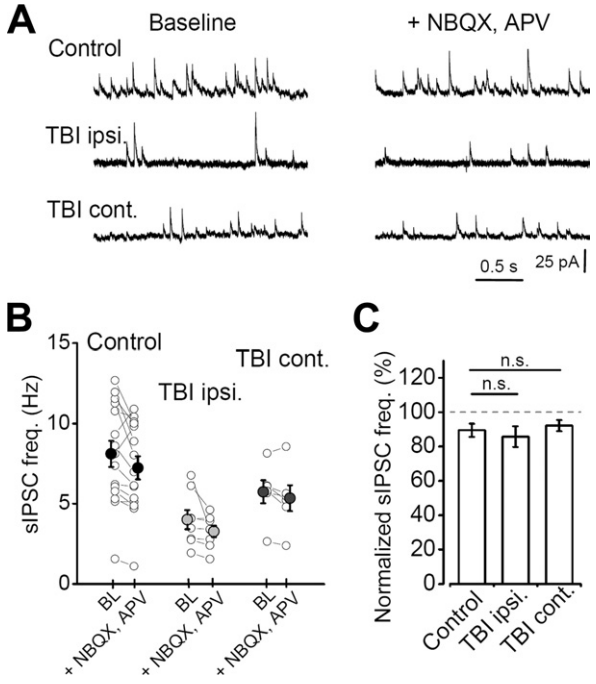

Fig. 2. The decrease in the frequency of sIPSCs is maintained in the presence of intact glutamatergic neurotransmission. (A) Sample traces of sIPSCs before (left) and after (right) application of AMPA/kainate and NMDA receptor blockers. Neurons were recorded using low Cl^- intracellular solution and voltage clamped at 0 mV (the reversal potential for glutamatergic transmission), hence the IPSCs are outward. (B) Blockade of ionotropic glutamate receptors by NBQX and APV has only a small effect on the frequency of sIPSCs. Open circles—data from individual cells; filled circles—averaged data. (C) The reduction of sIPSC frequency after the blockade of glutamatergic neurotransmission is similar in control and TBI rats. sIPSC frequencies in NBQX and APV are normalized to pretreatment values. BL, baseline.

Increased sIPSC frequency in TBI rats was reported previously when excitatory transmission was left intact (Santhakumar et al., 2001). Therefore, we asked whether this was the case in our model and performed experiments without inhibitors of excitatory transmission. A low [Cl^-] internal solution was used and neurons were held at 0 mV (the reversal potential for EPSCs) to isolate IPSCs. Similar to experiments with blocked glutamatergic neurotransmission, sIPSC frequency was decreased by 49.3±7.3% ipsilaterally and 70.7±8.9% contralaterally (Fig. 2A, B). Application of NBQX and APV reduced the frequency of sIPSCs to a similar extent in all experimental groups (Fig. 2C), indicating that the reduction of inhibition was not compensated by increased excitatory drive onto interneurons.

Unlike phasic inhibition, the density of tonic $GABA_A$ receptor-mediated currents were not decreased after TBI (ipsilaterally: 104.9±14.1% of control; contralaterally: 127.2±13.8% of control; Fig. 3A, B). The tonic current in DGCs is predominantly mediated by $GABA_A$ receptors sensitive to THIP (Glykys et al., 2008). After TBI, the sensitivity of tonic currents to THIP was unchanged, indicating a preservation of δ subunits (Fig. 3C, D).

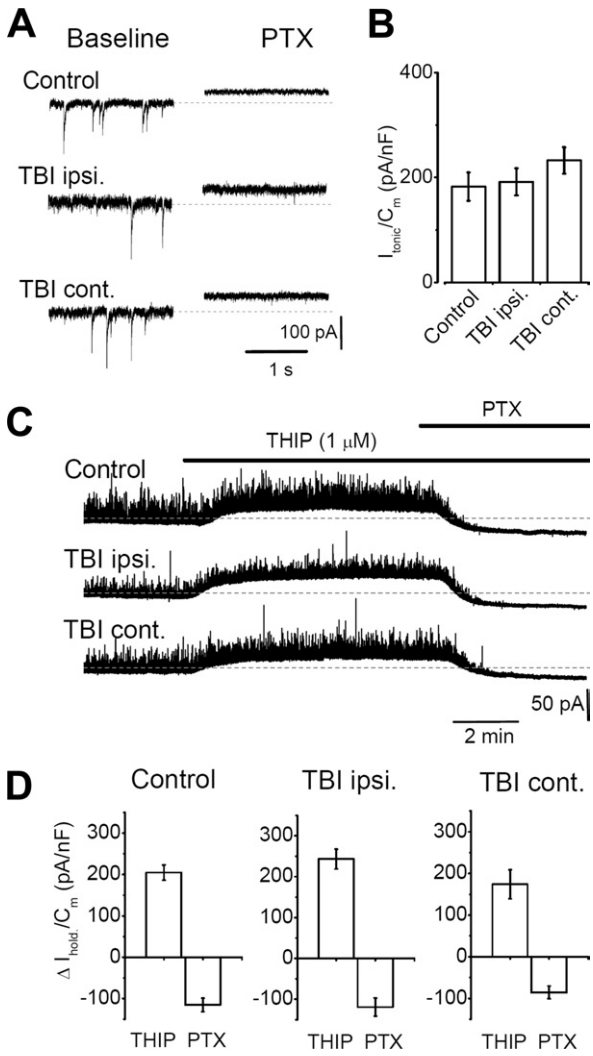

Fig. 3. Tonic $GABA_A$ receptor-mediated currents are maintained after TBI and retain their sensitivity to THIP. (A) Representative traces indicating the presence of tonic $GABA_A$ receptor-mediated currents after TBI. both ipsilaterally and contralaterally. Application of PTX abolished phasic IPSCs and produced an outward shift in the holding current in cells voltage clamped at −70 mV. Recordings were performed using high Cl^- intracellular solution. (B) The density of tonic current was similar in all experimental groups. (C) Tonic currents retain their sensitivity to THIP (1 μM) after TBI. Recordings were done using low Cl^- intracellular solution, and neurons were voltage-clamped at 0 mV. Therefore, an increase in tonic $GABA_A$ receptor-mediated currents produced an outward shift, whereas blockade of $GABA_A$ receptors produced an inward shift in holding current. (D) Summary data of holding currents after application of THIP and PTX in rats after TBI and in sham control animals.

Further loss of phasic inhibition occurs over time

The proportion of rats with epilepsy increases months after TBI (Kharatishvili et al., 2006), suggesting that injury-induced changes continue to develop long after the impact. We asked whether this was the case with inhibitory neurotransmission onto DGCs. We performed the aforementioned experiments 4–6 months after the injury. sIPSC and mIPSC frequency was now significantly reduced in both hemispheres of the TBI rats (ipsilaterally: sIPSC frequency 32.0±3.7% of control, $P<0.0001$; mIPSC frequency: 37.1±6.5% of control, $P<0.001$; contralaterally: sIPSC frequency 45.3±5.6% of control, $P<0.002$; mIPSC frequency: 48.3±7.4%, $P<0.02$; Fig. 4). We compared these mIPSC frequency data with the data at 1 month, using a multifactorial ANOVA. There was a significant effect of TBI on mIPSC frequency ($P<0.0001$) and a significant interaction of the effect of TBI and time ($P<0.03$), implying a significantly greater effect of TBI on inhibition at 4–6 months compared with 1 month. Similar to 1 month post-TBI, the mean charge transfer by an mIPSC was increased ipsilaterally, but not contralaterally (Table 1). However, the total phasic current was reduced on both sides (ipsilaterally: 47.6±5.9% of control, $P<0.01$; contralaterally: 47.9±8.1% of control, $P<0.05$).

Tonic $GABA_A$ receptor-mediated currents in both hemispheres of TBI rats were similar to those in control animals (Fig. 5). Therefore, these results indicate a progressive loss of phasic inhibition, which is not restricted to the side of the injury, with preservation of tonic inhibition in TBI animals.

Progressive loss of PARV-positive interneurons in the dentate gyrus after TBI

Consistent with the mIPSC frequency decrease, we found a marked decrease in the number of PARV-immunoreac-

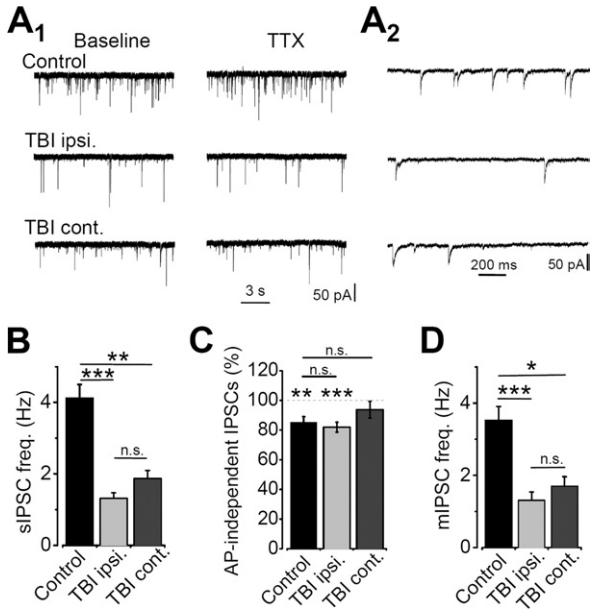

Fig. 4. Contralateral loss of phasic inhibition of DGCs 4–6 months after TBI. (A) Representative traces of sIPSCs (left) and mIPSCs (right) in sham control rats and animals 4–6 months after head trauma (A_1). Sample traces of mIPSCs with expanded timescale are shown in A_2. (B) The frequency of sIPSCs is reduced both ipsilaterally and contralaterally. No difference in sIPSC frequency was observed between contralateral and ipsilateral sides. (C) The proportion of action potential-independent spontaneous neurotransmission is similar in each experimental group. (D) The frequency of mIPSCs is similarly reduced on both the ipsilateral and contralateral sides. Ipsi., ipsilateral; cont., contralateral; n.s., nonsignificant changes. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

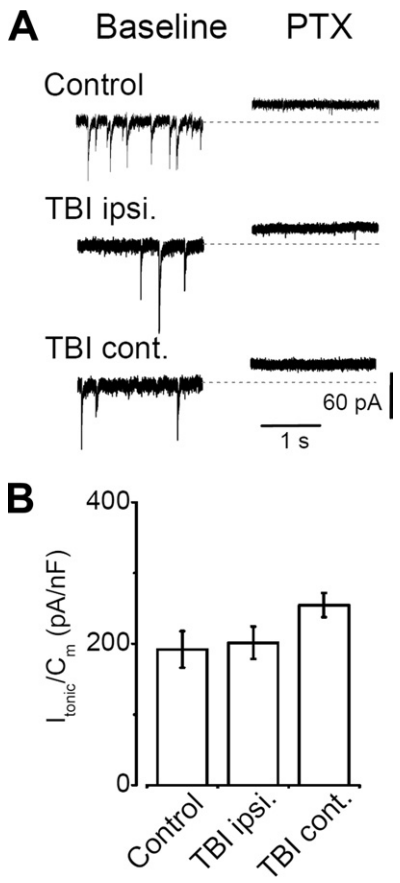

Fig. 5. Tonic $GABA_A$ receptor-mediated currents are preserved in the DGCs 4–6 months after the injury. (A) Representative traces of holding currents in cells voltage-clamped at −70 mV. (B) Summary data for density of tonic currents (control: $n=10$, TBI ipsilateral: $n=11$, TBI contralateral: $n=5$) showing that there is no significant change in tonic currents after TBI.

tive interneurons (the main interneuron subtype mediating perisomatic inhibition) ipsilaterally at 1 month postinjury (62% remaining, $P=0.001$; Figs. 6A and 7A, C). This decrease was even more pronounced at 6 months (38% remaining; $P=0.001$ for comparison with control and 1-month groups; Figs. 6C and 7A, E). Laminar analysis indicated that this was due to a reduction of PARV-positive cells in the granule cell layer and hilus, but not in the molecular layer (Fig. 6A, C).

Also in keeping with the electrophysiological results, the number of dentate PARV-positive neurons contralaterally 1 month postinjury was similar to that in control rats (94% remaining, $P>0.05$; Figs. 6B and 7B, D). However, after 6 months, only 57% of these interneurons were left (significantly less compared with the 1-month group, $P=0.001$; Figs. 6D and 7B, F). The results of the laminar analysis were similar to those in the ipsilateral side (Fig. 6B, D).

A comparison between hemispheres revealed that at 1 month post-TBI, the total number of PARV-positive neurons ipsilaterally was 61% of that contralaterally ($P<0.01$), with 68% in the granular cell layer ($P<0.01$) and 42% in the hilus ($P<0.01$). No differences were found in the molecular layer. A smaller difference was observed at 6 months post-TBI. The total number of PARV-positive neurons in the ipsilateral dentate gyrus was 69% of that contralaterally ($P<0.05$). This was due to the loss of interneurons in the hilus (49% of that contralaterally, $P<0.05$), as no interhemispheric differences were found in granule cell or molecular layers.

In addition, we noted that the density of immunopositive terminal labeling was markedly reduced in the granule cell layer ipsilaterally at both 1 and 6 months post-TBI, indicating a loss of perisomatic inhibition. At 6 months post-TBI, the reduction was evident also contralaterally (Fig. 8).

PARV-positive neurons are the primary cell population among hippocampal neurons that contain perineuronal nets on their surface (Hartig et al., 1992; Celio and Blumcke, 1994). Therefore, to confirm that the loss of PARV-positive neurons does not just reflect a reduction in PARV immunoreactivity, but rather the death of PARV-containing neurons, we stained perineuronal nets with Wisteria floribunda agglutinin (WFA) in the septal hippocampus of rats 2 weeks post-TBI (three successive sections, 150 μm apart). The number of WFA-positive neurons in injured rats was 48% of that in controls ($P<0.05$; not shown), suggesting that PARV staining reduction is due to degeneration of PARV-immunoreactive cells, and not the result of reduced PARV expression.

Changes in $GABA_A$ receptor subunit expression after TBI

Because previous poststatus epilepticus studies have demonstrated profound changes in $GABA_A$ receptor subunit expression, we asked whether this was the case at least 4 months after TBI. *In situ* hybridization was used to measure mRNA levels of $\alpha1$, $\alpha2$, $\alpha4$, $\alpha5$, $\beta1$, $\beta2$, $\beta3$, $\gamma2$, and δ subunits (Fig. 9). We used multifactorial ANOVA to assess the effect of TBI and side and their interaction on the expression levels of each subunit. TBI only had a statistically significant effect on the expression of $\alpha4$ and $\beta1$ subunits ($P<0.001$ and $P<0.01$, respectively; Table 2). This contrasts with studies in post-status epilepticus rats from our group and others where multiple $GABA_A$ receptor subunits are usually affected (e.g. Schwarzer et al., 1997; Tsunashima et al., 1997; Brooks-Kayal et al., 1998; Fritschy et al., 1999). Concordant with unchanged sensitivity of the tonic currents to THIP, we found no changes in the expression of δ subunit mRNA. There was also no statistically significant difference between the effects of TBI on either hemisphere at this time point (Table 2).

DISCUSSION

Here, we have shown a progressive loss of synaptic inhibition over a period of months in the DGCs in an animal model of post-traumatic epilepsy, associated with a loss of PARV-immunoreactive interneurons. Despite these profound changes in synaptic inhibition, tonic inhibition and the pattern of $GABA_A$ receptor expression are preserved throughout this period.

Reduced inhibition in DGCs after TBI

DGCs have been proposed to act as a brake against seizure propagation through the limbic circuitry, and

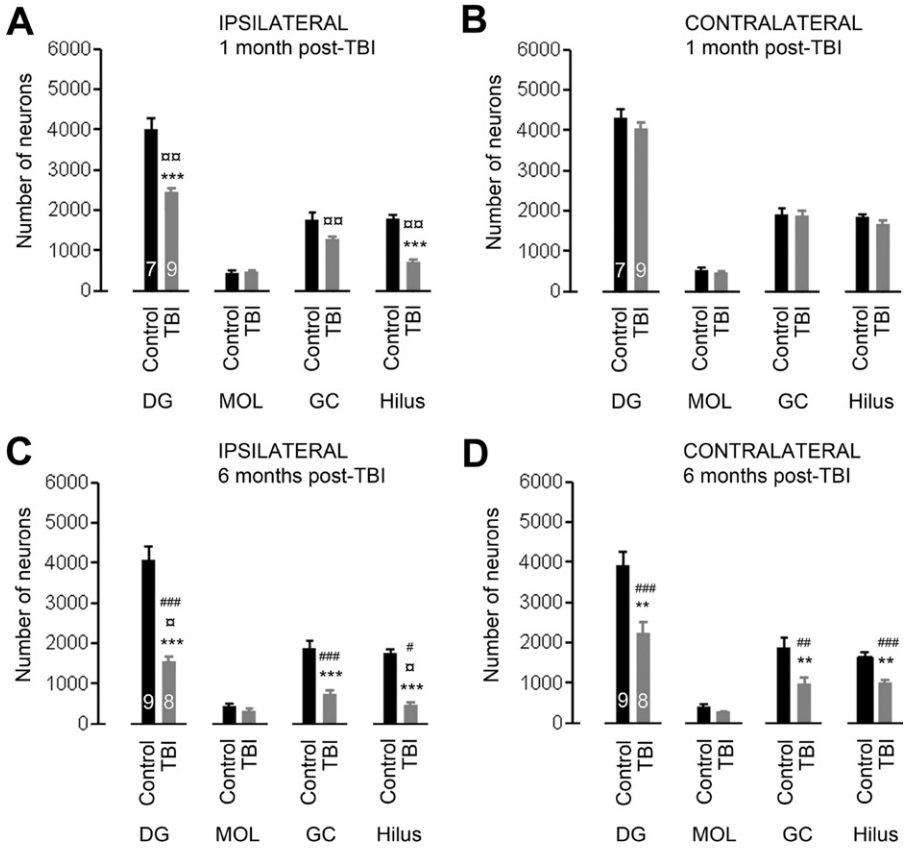

Fig. 6. Changes in the number of parvalbumin-immunoreactive (PARV-ir) interneurons in the dentate gyrus and in its different layers after TBI. (A) At 1 month postinjury, the total PARV-ir neuronal number in the dentate gyrus was significantly reduced in rats with TBI as compared with controls. In particular, the decrease in immunopositive cells was found in the hilus. (B) Contralaterally at 1 month post-TBI, there were no differences in PARV-ir cell numbers between the controls and injured rats. (C) At 6 months post-TBI, the total number of PARV-ir cells in the ipsilateral dentate gyrus was reduced as compared with controls. This reduction was due to the loss of cells in the granule cell layer and the hilus. (D) By 6 months post-TBI, the total number of PARV-ir neurons in the dentate gyrus was now significantly reduced at the contralateral side as well. Like ipsilaterally, the decrease was found both in granule cell layer and the hilus. No difference was observed in PARV-ir neuronal numbers in the molecular layer at any time point between the controls and injured rats. DG, dentate gyrus; MOL, molecular layer; GC, granule cell layer. The number of animals is shown at the bottom of the bars. Statistical significances: * $P<0.05$, ** $P<0.01$, *** $P\leq0.001$ (Mann–Whitney *U*-test, as compared with controls); $^{\#}$ $P<0.05$, $^{\#\#}$ $P<0.01$ (Mann–Whitney's *U*-test, as compared with 1-month group); ¤ $P<0.05$, ¤¤ $P<0.01$ (Wilcoxon test, as compared with the contralateral side).

changes in DGC excitability have been implicated in epileptogenesis. The gating properties of DGCs have been attributed to the large inhibitory drive onto these neurons—disruption of this results in a breakdown of this gate and an increase in signal propagation from the entorhinal cortex to the hippocampus proper (Coulter and Carlson, 2007).

The >50% reduction in $GABA_A$ receptor-mediated synaptic current frequency 1 month after TBI in the ipsilateral hippocampus is similar to or greater than that after status epilepticus (our own unpublished observation; and Kobayashi and Buckmaster, 2003; Shao and Dudek, 2005). Importantly, we and others (Kobayashi and Buckmaster, 2003) have sampled the dentate gyrus along its length and have not restricted ourselves to the septal hippocampus where there may be better preservation of inhibition—a possible confounder of other studies. However, we did not study this systematically. The decrease in mIPSC frequency could be secondary to a decrease in inhibitory synapse number or/and a decrease in release probability. Although the decrease in the number of PARV-immunoreactive interneurons does not rule out a reduction in release probability as a contributing factor, it suggests that a reduced number of presynaptic terminals contributes to the electrophysiological phenotype of injured rats. Despite the fact that a reduction of PARV-immunoreactivity can occur without degeneration of interneurons (Sloviter et al., 2003), PARV-immunoreactivity loss alone would not be expected to change the frequency of sIPSCs in DGCs (Lucas et al., 2010). Moreover, the decreased PARV immunoreactivity observed here was accompanied by a decrease in perineuronal net staining, suggesting a loss of these interneurons rather than change in the PARV expression level.

In these experiments, we blocked excitatory transmission, but a previous study suggested that when excitatory transmission is intact, TBI results in a compensatory increase in interneuronal recruitment and so sIPSC frequency (Santhakumar et al., 2001). We, in contrast, found reduced sIPSC frequency when excitatory transmission was intact. A possible explanation for this difference is that the TBI in our model is more severe and results in the occurrence of spontaneous seizures, not observed or documented in this previous study.

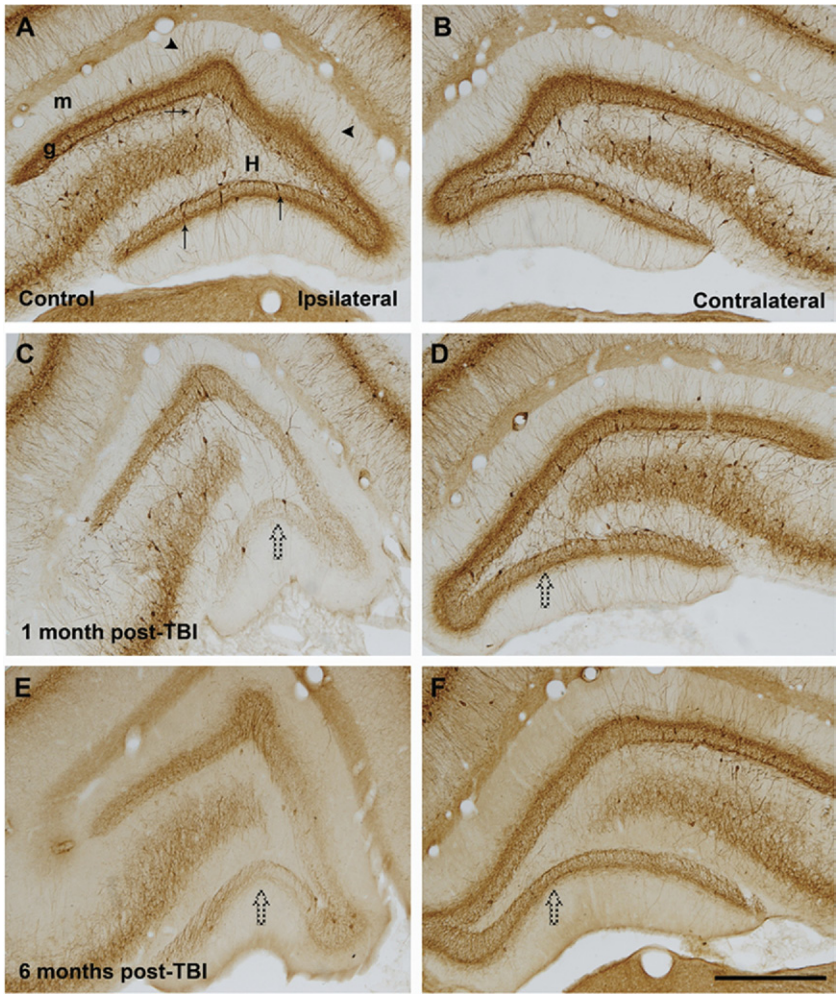

Fig. 7. Representative photomicrographs from coronal sections of the dentate gyrus in control and injured rats stained with an antibody raised against PARV. Both ipsilateral (panels A, C, and E) and contralateral (B, D, F) sides are shown. (A, B) Distribution of parvalbumin-immunoreactive (PARV-ir) cells (arrows), terminals, and dendrites (arrowheads) in a control animal. (C) At 1 month after TBI, there was a remarkable reduction in the number of PARV-ir cellular elements in the hilus. Also, the density of immunopositive dendrites was reduced in the molecular layer. Finally, the density of terminal labeling around the granule cells showed substantial reduction (open arrow). (D) Contralaterally, the density of terminal labeling appeared slightly reduced (open arrow). (E) At 6 months post-TBI, there was a substantial reduction both in the neuronal, dendritic, and terminal (open arrow) labeling in the ipsilateral dentate gyrus. (F) Contralaterally, the loss of neuronal, dendritic, and terminal (open arrow) labeling was less severe than ipsilaterally (panel E), but clearly more severe than seen contralaterally at 1 month post-TBI (compare D and F). g, granule cell layer; H, hilus; m, molecular layer. Scale bar: 500 μm.

Notwithstanding this, there is a compensatory increase in the total charge carried by mIPSCs, similar to that observed in kindling and post-status epilepticus studies (e.g. Shao and Dudek, 2005). This has previously been partly attributed to an increase in the number of $GABA_A$ receptors per synapse (Nusser et al., 1998), but our results of a change in mIPSC kinetics with preservation of amplitude at 1 month cannot be completely explained by this observation. Nevertheless, even allowing for these compensatory mechanisms, the total charge transfer per unit time is still significantly reduced in TBI rats, indicating a significant loss of synaptic inhibition.

In addition, the loss of interneurons observed here is not restricted to the PARV-containing cells, as a similar decrease in cell numbers was also found for other interneuronal subtypes (including somatostatin-, cholecystokinin-, neuropeptide Y-, and calretinin-positive interneurons) after TBI (Huusko et al., 2010). The loss of inhibition therefore cannot be ascribed completely to the loss of one class of interneuron group.

Progressive loss of inhibition

An important finding from our study is the progressive loss of synaptic inhibition from 1 to 4–6 months after TBI. Indeed, by 4–6 months, there is a significant reduction in synaptic inhibition contralaterally. A loss over this time course has not been previously described in other models of epilepsy. One major difference between other studies and ours is that the latent period in post-status epilepticus models ranges from days to 1–3 weeks, whereas in the model here, it lasts from several weeks to months, more akin to that observed after

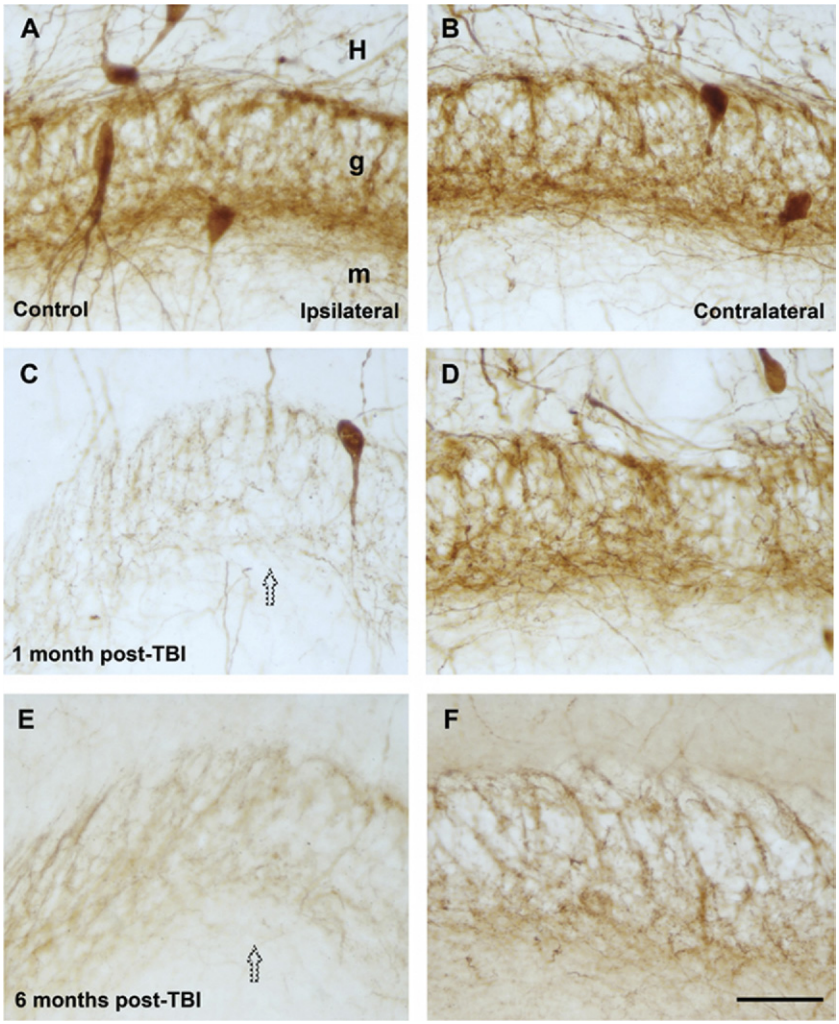

Fig. 8. High-magnification photomicrographs demonstrating the parvalbumin immunoreactivity (PARV-ir) in control and injured rats. (A) Ipsilateral and (B) contralateral granule cell layer in a control rat. (C) At 1 month after TBI, a clear reduction in the PARV-ir terminal labeling was seen in some parts of the ipsilateral granule cell layer like in this case, the infrapyramidal blade (open arrow). (D) Contralaterally, the density of terminal labeling was only slightly reduced. (E) At 6 months post-TBI, both the terminal and cellular labeling were even more remarkably reduced in the granule cell layer (open arrow). (F) Contralaterally, the reduction in immunoreactivity was apparent but less severe than ipsilaterally. g, granule cell layer; H, hilus; m, molecular layer. Scale bar: 50 μm.

TBI in humans (Englander et al., 2003; Christensen et al., 2009). This progressive change in synaptic inhibition may explain apparently contradicting results that have been recently reported in another model of brain injury. In one study, the contralateral side had a reduced frequency of mIPSCs (Mtchedlishvili et al., 2010), whereas in the other study, only the ipsilateral side was affected (Hunt et al., 2011). The mechanisms underlying progressive changes in inhibition are unclear, but age-related changes in inhibitory transmission have been well documented in primates (Luebke and Rosene, 2003) and so the progression observed may be due to an alteration in normal aging. This is supported by our observation of a tendency for mIPSC frequency to increase with age. An alternative explanation is that abnormal electrical activity in the damaged hippocampus promotes the further loss of inhibition through a form of kindling (Sloviter, 1987). Interestingly, a progressive development of seizures has been recently documented in another model of epilepsy (Kadam et al., 2010). It is appealing to speculate that progressive decreases in inhibition may contribute to the cumulative risk of epilepsy with time and may partly explain the long latency periods often observed in humans. Our finding has therefore important therapeutic implications, as it extends the period during which pharmacological interventions may modify the risk of epilepsy.

Changes in $GABA_A$ subunits and preservation of tonic inhibition

We have found that after TBI, the pattern of $GABA_A$ receptor subunit expression is largely preserved, except for increased levels of α4 and β1 subunit mRNA. This contrasts with studies following status epilepticus in which there is selective loss or upregulation of specific $GABA_A$ receptors (e.g. Schwarzer et al., 1997; Tsunashima et al., 1997; Brooks-Kayal et al., 1998; Fritschy et al., 1999).

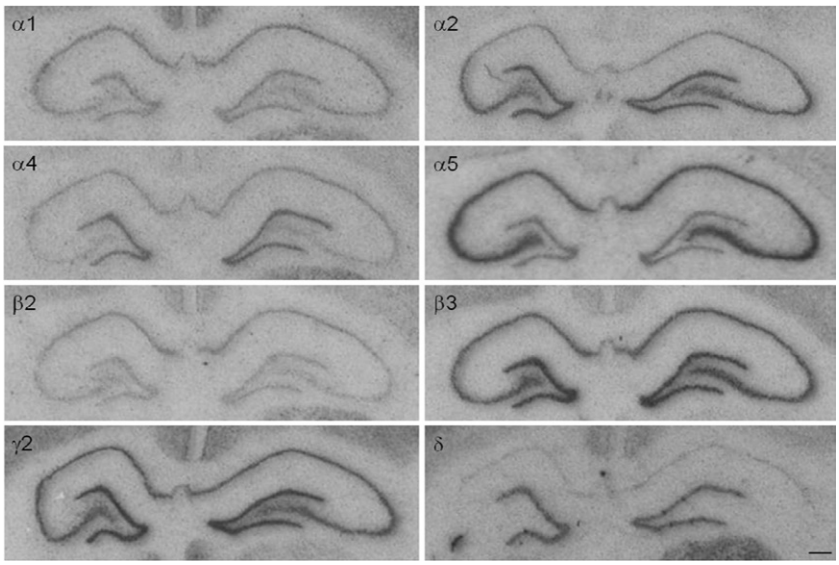

Fig. 9. $GABA_A$ receptor subunit mRNA expression ($\alpha1$, $\alpha2$, $\alpha4$, $\alpha5$, $\beta2$, $\beta3$, $\gamma2$, and δ) 4 months after TBI. Film autoradiograms of the hippocampus after *in situ* hybridization. Scale bar: 250 μm.

Although our analysis may not be sensitive enough to pick up relatively small changes in subunit expression, the electrophysiological data suggest that postsynaptic modifications in the $GABA_A$ receptor composition do not compensate for the presynaptic loss of inhibition.

Interestingly, the profound downregulation of δ subunits described after status epilepticus was not observed here (Schwarzer et al., 1997; Tsunashima et al., 1997; Peng et al., 2004; Nishimura et al., 2005; Zhang et al., 2007; Zhan and Nadler, 2009). The discrepancy may be partly attributed to the different time points at which the expression of $GABA_A$ receptor subunits was evaluated and to the speed of epileptogenesis in the status epilepticus models. One study reported increased expression of δ subunit and little changes in other $GABA_A$ receptor subunits contralaterally 3 months after brain injury induced by controlled cortical impact (Kharlamov et al., 2011). However, the lesion used in that study produced substantial damage to the brain to the extent that precluded investigation of slices from the ipsilateral hemisphere. It is also unclear whether this change is specific for the dentate gyrus, as the whole hippocampus was used for the analysis. Our present results further suggest that although subunit alterations may contribute to certain forms of epileptogenesis, they are not necessary, and that alterations in inhibitory drive may play a more important role by tipping the network into an hyperexcitable state. Why then is there a change in the kinetics of mIPSCs? Although we did not explore this here, there are a number of possible explanations. Changes in synaptic $GABA_A$ receptor subunit expression are likely to be reflected in the kinetics of postsynaptic currents. However, the interpretation of immunohistochemical and mRNA changes at the cellular level is far from straightforward because there could be alterations in $GABA_A$ receptor composition in individual synapses without alterations in the total pool. Further, changes in the phosphorylation state of synaptic receptors (e.g. Brandon et al., 2000) may also contribute. Another possibility is that apparent changes in the mIPSC kinetics observed in our study are due to the redistribution of inhibitory inputs along the somatodendritic axis of the granule cells as a result of heterogeneous and/or asynchronous loss of different interneuron populations, for example, dendritically targeting somatostatin-positive cells (Milner and Bacon, 1989; Leranth et al., 1990). Further, axonal sprouting has been reported as a compensatory mechanism for the interneuronal degeneration in epilepsy (Zhang et al., 2009) and may also contribute to the shifted balance of somatic versus dendritic inhibitory inputs.

In post-status epilepticus animals, the tonic current is preserved in DGCs but the extrasynaptic δ subunit is replaced by the γ subunit, changing the pharmacology, but

Table 2. Expression of $GABA_A$ receptor subunit mRNA in DGC layer 4 months after TBI

Subunit	Ipsilateral (% of control)	Contralateral (% of control)	Ipsilateral (% of contralateral)
$\alpha1$	101.2±12.5	116.7±10.4	86.7±10.7
$\alpha2$	104.5±8.2	106.1±10.2	98.5±7.7
$\alpha4$*	151.9±10.5	176.6±6.9	86.0±5.9
$\alpha5$	106.1±5.3	103.6±6.5	102.4±5.1
$\beta1$**	127.2±12.6	137.6±10.3	92.4±9.2
$\beta2$	94.91±7.9	107.8±8.0	88.1±7.3
$\beta3$	99.17±7.5	106.7±4.9	93.0±7.0
$\gamma2$	104.5±9.6	110.5±3.0	94.6±8.7
δ	102.2±6.4	107.7±4.3	94.9±6.0

Data represent relative optical density values ±SEM.

Multifactorial ANOVA analysis was used to evaluate the effect of TBI on $GABA_A$ receptor subunit expression in ipsilateral (n=6) and contralateral (n=5) sides.

Sham control rats: n=5.

* $P<0.001$.

** $P<0.01$.

not the magnitude of the tonic current (Zhang et al., 2007; Rajasekaran et al., 2010). In support of the preservation of the δ subunit in our model, we did not observe a change in the sensitivity of the tonic current to the δ subunit-selective agonist, THIP. Interestingly, a recent study has demonstrated an enhanced tonic inhibition in DGCs following controlled cortical impact (Mtchedlishvili et al., 2010). However, as this study used a different (and apparently more destructive) method to induce head trauma, direct comparison with our study is difficult. Preservation or increases in tonic currents in the hippocampus is, therefore, a consistent finding in CA1 pyramidal cell, CA1 interneurons, and DGCs in rat models of epilepsy, and in DGCs from humans with epilepsy (Naylor et al., 2005; Scimemi et al., 2005, 2006; Zhang et al., 2007; Zhan and Nadler, 2009; Mtchedlishvili et al., 2010), and here it occurs in the face of profound decreases in synaptic inhibition.

Functional implications

What are the functional implications of these changes in inhibition? Notwithstanding that TBI may lead to a highly heterogeneous condition with variable location of seizure onset, our and other group's findings indicate that the dentate gyrus is an area that can be significantly affected. The changes are mainly in synaptic, but not in tonic inhibition, and continue over an extended period. In DGCs from both healthy and epileptic animals, tonic currents provide the major inhibitory drive, representing approximately four- to fivefold the synaptic inhibitory currents. However, tonic currents by their nature cannot respond rapidly to changes in excitatory activity; in contrast, synaptic inhibition can be recruited through a feed-forward network on a millisecond time scale. In addition, phasic inhibition contributes significantly to synaptic noise, whereas tonic currents in the hippocampus have a minimal effect on synaptic noise (Pavlov et al., 2009). These have a number of important implications. First, tonic currents predominantly affect the offset of the input-output function of hippocampal neurons. Second, phasic inhibition can affect both offset and gain of the input-output function. Therefore, we speculate that loss of phasic inhibition but preservation of the dominant tonic inhibition will mainly affect the gain of neurons, not their offset. Further, the DGCs will be less able to respond rapidly to increases in excitatory drive. These two consequences may lead to an unstable network in which small increases in excitation result in large numbers of granule cells firing, promoting seizure generation. However, under resting conditions, the offset mediated by tonic inhibition is sufficient to maintain the gating properties of the dentate gyrus and prevents seizure generation and propagation. Our results suggest that intervention strategies that target tonic inhibition may be effective in controlling network excitability and preventing seizure generation in post-traumatic epilepsy.

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