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Alterations of $GABA_A$ and glutamate receptor subunits and heat shock protein in rat hippocampus following traumatic brain injury and in posttraumatic epilepsy

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Summary Traumatic brain injury (TBI) can result in the development of posttraumatic epilepsy (PTE). Recently, we reported differential alterations in tonic and phasic $GABA_A$ receptor ($GABA_AR$) currents in hippocampal dentate granule cells 90 days after controlled cortical impact (CCI) (Mtchedlishvili et al., 2010). In the present study, we investigated long-term changes in the protein expression of $GABA_AR$ $\alpha1$, $\alpha4$, $\gamma2$, and δ subunits, NMDA (NR2B) and AMPA (GluR1) receptor subunits, and heat shock proteins (HSP70 and HSP90) in the hippocampus of Sprague—Dawley rats evaluated by Western blotting in controls, CCI-injured animals without PTE (CCI group), and CCI-injured animals with PTE (PTE group). No differences were found among all three groups for $\alpha1$ and $\alpha4$ subunits. Significant reduction of $\gamma2$ protein was observed in the PTE group compared to control. CCI caused a 194% and 127% increase of δ protein in the CCI group compared to control ($p < 0.0001$), and PTE ($p < 0.0001$) groups, respectively. NR2B protein was increased in CCI and PTE groups compared to control ($p = 0.0001$, and $p = 0.011$, respectively).

Abbreviations: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; CCI, controlled cortical impact; DGCs, dentate granule cells; $GABA_ARs$, gamma-aminobutyric acid subtype A receptors; GluR1, glutamate receptor 1 of AMPA subtype; HSPs, heat shock proteins; NR2B, N-methyl-D-aspartate receptor 2B subunit; TBI, traumatic brain injury.

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GluR1 protein was significantly decreased in CCI and PTE groups compared to control ($p = 0.003$, and $p = 0.001$, respectively), and in the PTE group compared to the CCI group ($p = 0.036$). HSP70 was increased in CCI and PTE groups compared to control ($p = 0.014$, and $p = 0.005$, respectively); no changes were found in HSP90 expression. These results provide for the first time evidence of long-term alterations of $GABA_A$ and glutamate receptor subunits and a HSP following CCI.
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Introduction

Traumatic brain injury (TBI) is one of the leading causes of death and disability in children and young adults and is a major risk factor for the development of posttraumatic epilepsy (PTE). Recent studies have provided significant insight into the pathophysiological mechanisms underlying the development of epilepsy in the most widely employed TBI models, such as fluid percussion injury (FPI) and controlled cortical impact (CCI) (Lowenstein et al., 1992; D'Ambrosio et al., 1998, 2004; D'Ambrosio and Perucca, 2004; Garga and Lowenstein, 2006; Pitkänen and McIntosh, 2006; Hunt et al., 2009, 2010; Statler et al., 2008, 2009; Norris and Scheff, 2009; Pitkänen et al., 2009; Kharatishvili and Pitkänen, 2010a,b; Yang et al., 2010). Although the link between brain trauma and epilepsy is well recognized, the complex biological mechanisms that result in PTE following TBI have not been fully elucidated. Following TBI, there is an immediate increase in excitability and a long-term hyperexcitability in affected neurons due to changes in inhibitory GABAergic and excitatory ionotropic glutamatergic neurotransmission, which may play a causative or contributing role in the development of PTE (Reeves et al., 1995; Coulter et al., 1996; Bush et al., 1999; Golarai et al., 2001; Graber and Prince, 2004; Prince et al., 2009).

The diversity of $GABA_A$ receptor ($GABA_AR$) subunits (Sieghart and Sperk, 2002) allows for a variety of receptor subunit assemblies associated with specific pharmacological properties and receptor function, including maintenance of phasic and tonic inhibition, and modulation of neuronal excitability (Korpi et al., 2002; Sieghart, 2006; Fritschy, 2008). Several studies have evaluated $GABA_AR$-mediated changes of inhibition after TBI (Reeves et al., 1997; Sihver et al., 2001; Witgen et al., 2005; Gibson et al., 2010), but long-term alterations in $GABA_AR$s associated with PTE have not been studied. Recently, we reported differential alterations of phasic and tonic $GABA_AR$ currents in dentate granule cells (DGCs) 90 days following CCI in young adult rats (Mtchedlishvili et al., 2010). We hypothesized that these differential alterations in CCI DGCs could be caused by altered expression of $GABA_AR$ subunits located at synaptic and extrasynaptic sites in DGCs, respectively. In the present study, a subset of CCI-injured animals demonstrated convulsive posttraumatic seizures following a period of long-term survival. We hypothesized that TBI and PTE may alter phasic and tonic inhibition in the hippocampus. We sought to determine whether the expression and potential alteration of $GABA_AR$ subunits that mediate these two forms of inhibition were TBI- or PTE-associated and/or dependent. Because $\alpha1$ and $\gamma2$ subunits are found in synapses (Sun et al., 2004; Mangan et al., 2005) and participate in phasic inhibition, whereas $\alpha4$ and δ subunits are crucial for tonic inhibition in DGCs and are predominantly expressed in extrasynaptic locations (Peng et al., 2002; Wei et al., 2003), we focused on $GABA_AR$ $\alpha1$, $\alpha4$, $\gamma2$, and δ subunits in this study.

Glutamate acting on N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) can be involved in excitotoxicity following TBI and can play an essential role in the establishment of PTE. In the brain, NMDARs and AMPARs have different pharmacologic and kinetic properties, largely dependent on receptor subunit composition. The NMDAR 2B (NR2B) subunit and the glutamate receptor subunit 1 (GluR1) of the AMPA subtype are abundantly distributed in the hippocampus (Rogers et al., 1991; Martin et al., 1993; Monyer et al., 1994) and are critically important in synaptic transmission, plasticity, and protein—protein interactions (MacDonald et al., 2006; Martel et al., 2009). Several studies have demonstrated NR2B and GluR1 changes after TBI and in epilepsy (Loftis and Janowsky, 2003; Biegon et al., 2004; Atkins et al., 2006; Bigford et al., 2009). In the present study, potential alterations of NR2B and GluR1 following CCI and in PTE were investigated.

In addition to changes in selected $GABA_A$ and glutamate receptor subunits, we investigated the expression of specific heat shock proteins (HSPs). HSPs are a family of molecular chaperone proteins of various molecular sizes and/or multiple functions, which provide neuroprotection, have anti-apoptotic properties, and participate in downstream signaling, synthesis, transportation, and degradation of proteins (Yenari, 2002; Yenari et al., 2005; Powers et al., 2009, 2010; Luo et al., 2010). Previous reports have revealed upregulation of HSPs following excitotoxicity, ischemia, TBI, and epilepsy (Giffard and Yenari, 2004; Lai et al., 2004; Kochanek et al., 2006; Zhan et al., 2008; Zheng et al., 2008). Because HSP70 and HSP90 are involved in the regulation of trafficking of a large number of ionotropic and metabotropic receptor proteins, including AMPAR subunits (Gerges et al., 2004) and heterotrimeric G protein alpha (12) subunits (Waheed and Jones, 2002), we studied these HSPs to determine whether or not their expression was altered following CCI and in PTE.

Methods

CCI model of TBI and surgery

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Allegheny-Singer Research Institute and were carried out according to NIH guidelines and regulations. Animals were housed individually, maintained in a 12 h light/12 h dark cycle environment with controlled temperature (23 ± 2 °C), and food and water were provided *ad libitum*. The CCI pro-

cedure was performed according to Dixon et al. (1991) with modifications described previously (Mtchedlishvili et al., 2010). Male Sprague—Dawley (SD) rats (2—3-month old) were anesthetized with isoflurane using a Surgivet vaporizer. After reaching a surgical level of anesthesia (complete loss of response to painful stimuli), the animals were positioned and secured in a stereotactic frame (David Kopf Instruments, Tujunga, CA). Body temperature was monitored throughout the procedure using a rectal probe and maintained at $37 \pm 2\,^{\circ}C$ with a heating pad (Harvard Apparatus). Utilizing aseptic techniques, a midline scalp incision was made, and the skin and fascia were reflected to expose the skull. A craniectomy was performed over the right parietal cortex and the bone flap was removed. CCI was performed with a pneumatic impactor (Pittsburgh Precision Instruments, Pittsburgh, PA) with a 5.5-mm tip impounder, velocity of 4 m/s, depth of 2.8 mm, and impact duration of 100 ms. After the impact, the skin incision was closed with nylon sutures, a 2% lidocaine gel was applied topically, and the animal was returned to the cage for post-surgical recovery.

Video monitoring

Animals were housed individually in 12 monitoring chambers in a satellite vivarium of the Neurophysiology Laboratory and maintained on the 12 h light/12 h dark cycle. Animal behavior was monitored by closed-circuit television cameras, which were connected to video splitter units (Advanced Technology Video, Inc., Redmond, Washington). Digital video files (Diva, Stellate Systems) were recorded directly to high capacity hard disk drives using removable hard drive bays. Video recordings from 06:00 to 18:00 were reviewed offline and played at 2× speed to detect any behavioral seizure activity according to a modified classification scale (Racine, 1972), including forelimb clonus (class 3); running and rearing (class 4); and jumping and falling (class 5). Class 1 and 2 seizures were not scored in this study because their similarity to normal behaviors made their identification as ictal events very difficult when not observed in the expected progression of ictal severity as demonstrated in standard models of limbic status epilepticus. Immediately after CCI, animals were monitored for acute posttraumatic seizures for up to 7 days. For chronic monitoring, animals were monitored continuously for 7 days for each subsequent 30-day period. All animals continued to be video-monitored after the first recognized class 3—5 seizures and all were monitored for 1 week prior to sacrifice. To minimize the possibility of potential effects of acute seizures on protein expression, no animal was sacrificed within 24 h of identified convulsive activity. Animals were divided into three groups for protein analysis by Western blot: (1) naïve controls ($n = 5$, at ∼5—9 months of age); (2) CCI-injured rats without convulsive seizures ($n = 4$; non-epileptic, CCI group, at ∼11 months of age); and (3) CCI-injured animals with convulsive seizures ($n = 6$; PTE group, at ∼7 months of age).

Tissue sampling, gel electrophoresis and immunoblotting

After video monitoring, animals were decapitated under isoflurane anesthesia. Brains were quickly removed and dissected, and the contralateral hippocampus was separated from neocortex and subcortical structures. Collected hippocampal tissue from each animal formed a single sample that was immediately frozen in liquid nitrogen, weighed, and kept at $-80\,^{\circ}C$ until subsequent treatment. Tissue samples were homogenized in 20 mM Tris—HCl (pH 7.4), 0.32 M sucrose, 1.0 mM ethylenediaminetetraacetic acid solution containing a cocktail of protease inhibitors (Sigma; P8340). The homogenate was centrifuged at $1000 \times g$ for 10 min and an equal volume of 10% sodium dodecyl sulfate solution was added to the supernatant (nuclear-free homogenate), which was used in all subsequent experiments. Protein content was determined in quadruplicate using a micro bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Aliquots containing 30 μg of protein and of equal volume were applied to the gels. SDS gel electrophoresis and Western blotting were carried out using an electroblotting apparatus with plate electrodes according to the manufacturer's protocols as previously described (Solomonia et al., 2010), and each batch of samples was run in duplicate. The proteins were stained with Ponceau S solution to confirm transfer and uniform loading onto nitrocellulose membranes (Gallagher et al., 2008). The membranes were washed with phosphate-buffered saline (PBS) solution containing 0.05% Tween 20 (PBST) and incubated in blocking buffer (2% bovine serum albumin) for 2 h at room temperature followed by incubation with the primary antibodies diluted in PBST containing 1% serum albumin overnight at $4\,^{\circ}C$. After incubation, the membranes were washed in PBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody diluted in PBST for 1 h at room temperature, washed five times with PBST, and developed using Super-Signal West Pico chemiluminescent substrate (Pierce). To detect the expression of total actin, an internal loading control, membranes were stripped, reblocked, and reblotted with a primary monoclonal anti-actin antibody (1:1000; Cat # sc-81760, Santa Cruz Biotechnology Inc.) and horseradish peroxidase-conjugated secondary antibody (1:5000; Bio-Rad). The signals from the immunoreactive bands, as well as from total actin, were detected using intensifying screens to X-ray films pre-flashed with Sensitize (Amersham).

Polyclonal anti-$GABA_AR$ α1 (1:1000; Cat. # ab33299), anti-$GABA_AR$ α4 (1:1000; Cat. # ab7387), and anti-NR2B (1:500; Cat. # ab73001) were purchased from Abcam; anti-$GABA_AR$ γ2 was purchased from Novus Biologicals, LLC (1:500; Cat. # NB300—190); and anti-GluR1 was purchased from Millipore (1:100; Cat. # AB1504). Monoclonal anti-$GABA_AR$ δ was kindly provided by Dr. Jaideep Kapur from the University of Virginia; the antibody was raised in mouse against the specific synthetic peptide amino acids 15—34 of the rat $GABA_AR$ δ subunit (CTQPHHGARAM-NDIGDYVGS, extracellular N-terminus; NeuroMab, clone N151/3). Monoclonal anti-HSP70 was purchased from Sigma (1:4000; Cat. # H5147) and anti-HSP90 was purchased from ABR-Affinity Bioreagents Inc. (1:1000; Cat. # MA3-011). Secondary peroxidase-conjugated antibodies were obtained from Millipore. Several exposure times were tested to determine the best time for visualization of the investigated proteins. No protein band was visible on any gel when primary or secondary antibodies were omitted (negative controls).

It needs to be emphasized that some factors such as variable conditions of brain tissue extraction and homogenization, and the specificity, selectivity, and reproducibility of the antibodies can lead to double bands on immunoblotting instead of a single band. In the present study, we optimized standardization of the procedure and minimized those factors that could influence the results of the Western blot analysis.

Data analysis and quantification of immunoblots

Exposed films from the immunoblots were analyzed and quantified by computer-assisted densitometry using the LabWorks 4.0 (UVP) system, which calculates the intensity of a band using both the darkness and the size of the band. The autoradiographs were calibrated using standard amounts of protein obtained from a nucleus-free homogenate from the brain of a control animal. Four standards (15, 30, 45, and 60 μg of total protein) were applied to each gel. For these standards, the optical densities of the immunostained bands ($GABA_AR$ α1, α4, γ2, and δ subunit protein, NR2B, GluR1, HSP70, and HSP90) were plotted against the amounts of protein; in all these standards, least squares regression showed a significant fit to a straight line (e.g., see Fig. 2B and D). To obtain the data presented in the Western blot figures and Table 1, the optical density of each band from each experimental sample was divided by the optical density which, from the calibration of the same autoradiograph, corresponded to 30 μg of total protein in the standard (Solomonia et al., 2005). The data expressed in this manner are referred to as ''standardized relative amount'' of $GABA_AR$ α1, α4, γ2, δ, NR2B, and GluR1 subunit proteins, HSP70 and HSP90. The investigated $GABA_A$ and glutamate receptor subunit proteins and HSPs were not normalized to the amount of total actin. TBI can alter the expression of β-actin (Aldridge et al., 2008), which can be an unreliable loading control in Western blot analysis (Dittmer and Dittmer, 2006). Due to the different states of hippocampal neurons assessed in this study (control, CCI, PTE), it is likely that cytoskeletal actin was affected, resulting in alteration of total amounts of actin in the three animal groups.

Statistical analysis

The mean relative amount of protein obtained from Western blot experiments was used for statistical analysis. Data were analyzed by one-way ANOVA. If a significant effect was found by ANOVA, further statistical comparison between different experimental groups was made using Student's t-tests. All statistical tests were two-tailed unless otherwise stated and a probability (p) of 0.05 was used as statistically significant unless otherwise indicated. Data are presented as mean ± standard error of the mean (SEM).

Results

A total of 15 animals was used in the study. Five naive young adult animals were assigned to the control group and 10 animals were injured by CCI; no injured animal demonstrated convulsions during the first week after injury. Four CCI animals did not demonstrate convulsive seizure activity during extended monitoring and were considered to be ''seizure-free'' (CCI group, non-epileptic). For these animals, the average survival time (the time elapsed between CCI and animal sacrifice) was 278.25 ± 1.31 days. Six CCI-injured animals demonstrated spontaneous class 3—5 seizure activity during extended monitoring and were considered ''epileptic'' (PTE group). The average survival time of these animals after CCI was 156.33 ± 24.61 days. Five of six animals demonstrated only one class 3—5 seizure, which occurred at different times (86—276 days range) following CCI; one animal had five class 5 seizures. No animal had a recurrent seizure during the 7 days prior to sacrifice, ensuring that the acute effects of seizures did not affect the analysis of protein levels following animal sacrifice. Because of the inherent variability of the latent period between TBI and the onset of spontaneous seizure activity, animals in the CCI group were monitored longer to determine whether they would develop PTE during an extended period of observation. Therefore, CCI animals had longer post-injury survival times compared to PTE group animals. The expression of various receptors, including $GABA_ARs$, NMDARs, and AMPARs, can be altered with aging (Gutiérrez et al., 1996; Clayton et al., 2002); however, the total number of hippocampal $GABA_A$/benzodiazepine receptors does not change with age (Ruano et al., 1991). In our study, we compared animals within an age range of 5—11 months, well within the period of young adulthood during which there are minimal age-related receptor changes.

Due to the extensive injury to the ipsilateral cerebral cortex and hippocampus, the contralateral hippocampus was harvested and investigated. Fig. 1 shows a representative CCI-injured rat brain (Fig. 1A) and a single coronal section (Fig. 1B) demonstrating long-term morphological changes in the cortex (Fig. 1B) and in the ipsilateral and contralateral hippocampi (Fig. 1B—D). The hippocampus is particularly vulnerable to TBI, which can result in chronic cellular alterations and dysfunction that may predispose or contribute to posttraumatic epileptogenesis (Lowenstein et al., 1992; Toth et al., 1997; Dixon et al., 1999). Neuronal loss was assessed with neuron-specific nuclear antigen (NeuN, Fig. 1B—D). Immunostaining was performed according to a previously described protocol (Kharlamov et al., 2007).

Protein changes

Expression of $GABA_AR$ α1, α4, γ2, and δ subunit proteins Phasic inhibition: expression of $GABA_AR$ α1 and γ2 subunit proteins: Polyclonal anti-$GABA_AR$ α1-specific antibody detected immunoreactive bands at ~51 kDa, corresponding to the size of the α1 subunit protein (De Blas, 1996; Miranda and Barnes, 1997; Fig. 2A). No significant effect of CCI was found on the amount of this protein by ANOVA and Student's *t*-test (Fig. 2B, Table 1).

Polyclonal anti-$GABA_AR$ γ2 antibodies detected immunoreactive bands at ~46 kDa, corresponding to the size of the γ2 subunit (Araki et al., 1993; Tretter et al., 1997; Fig. 2C). The effect of injury on $GABA_AR$ γ2 protein expression across the groups was significant with ANOVA (Table 1). A significant trend toward a reduction of the γ2

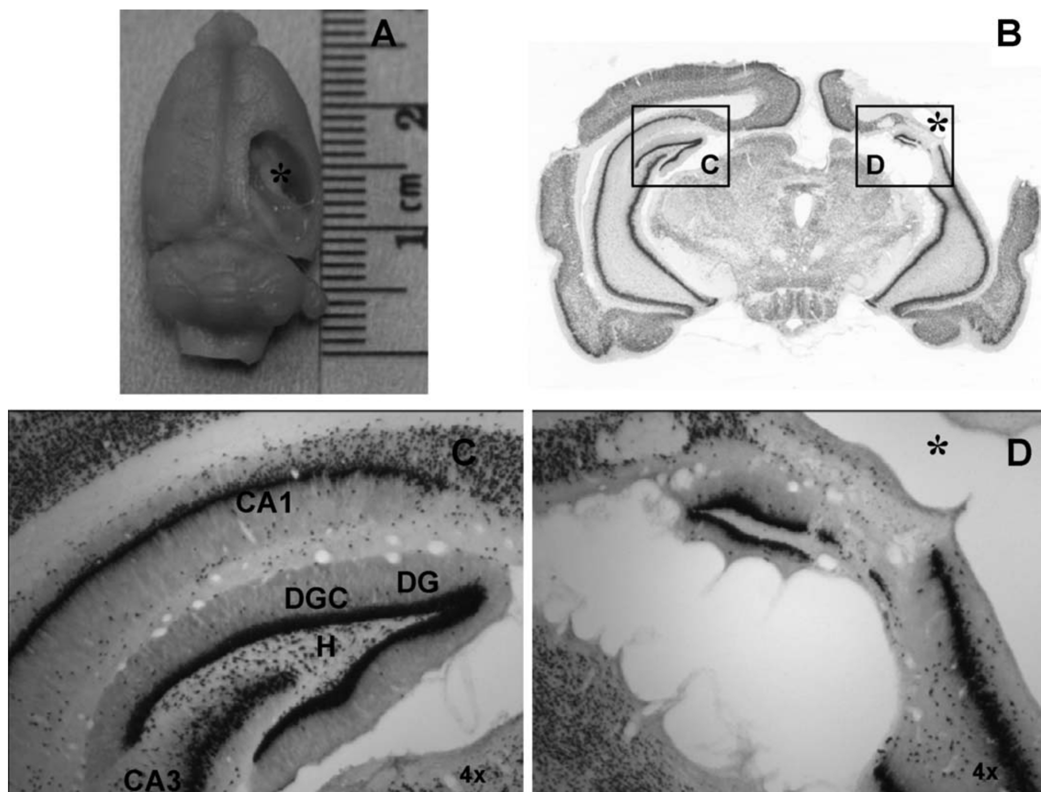

Figure 1 A representative photomicrograph of an adult rat brain demonstrates a loss of tissue and necrotic cavity in the right ipsilateral hemisphere following a single episode of controlled cortical impact (CCI; A). Neuronal loss was assessed with neuron-specific nuclear antigen (NeuN, 1:1000; Chemicon; B—D). A representative NeuN-stained coronal section (B) shows the evidence of morphological changes in the ipsilateral hippocampus (D) compared to the contralateral side (C). Abbreviations: H, hilus; DG, dentate gyrus; DGC, dentate granule cells. An asterisk (*) marks the necrotic cavity at the cortical impact site.

Table 1 Summary of protein analysis by Western blot.

Protein	Mean relative amount of protein ± SEM			*F*, variance ratio from one-way ANOVA, and *p*, probability
	Control group	CCI group	PTE group	
$GABA_AR\ \alpha1$	0.78 ± 0.10	0.88 ± 0.05	0.88 ± 0.04	$F_{2.14} = 1.36$, $p = 0.29$
$GABA_AR\ \gamma2$	0.75 ± 0.07	0.59 ± 0.04	0.57 ± 0.03*↓	$F_{2.14} = 4.60$, $p = 0.033$
$GABA_AR\ \alpha4$	0.95 ± 0.13	1.06 ± 0.09	0.77 ± 0.08	$F_{2.14} = 2.14$, $p = 0.16$
$GABA_AR\ \delta$	1.21 ± 0.15	3.56 ± 0.17**↑	1.57 ± 0.24	$F_{2.14} = 32.91$, $p < 0.0001$
NR2B	1.02 ± 0.15	2.41 ± 0.09**↑	2.28 ± 0.32**↑	$F_{2.14} = 10.00$, $p = 0.003$
GluR1	1.29 ± 0.07	0.85 ± 0.06**↓	0.63 ± 0.01**(*)↓	$F_{2.14} = 22.01$, $p = 0.00001$
HSP70	0.58 ± 0.04	0.76 ± 0.01**↑	0.80 ± 0.04**↑	$F_{2.14} = 10.39$, $p = 0.002$
HSP90	0.85 ± 0.38	0.85 ± 0.06	0.85 ± 0.04	$F_{2.14} = 0.001$, $p = 0.998$

Data represent the mean ± standard error of the mean (SEM) of the relative amounts of protein (see Methods) from Western blot runs from control ($n = 5$), CCI ($n = 4$), and PTE ($n = 6$) animal groups. ** represents a significant difference between the experimental groups in Student's two-tailed t-test, $p < 0.05$. * represents a significant difference between the experimental groups in Student's one-tailed t-test, $p < 0.05$. ↑ represents an increase, ↓ represents a decrease. $GABA_AR\ \gamma2$ subunit — * protein expression is significantly decreased in the PTE group compared to the control group; $GABA_AR\ \delta$ subunit — ** protein expression is significantly increased in the CCI group compared to the control and the PTE groups; NR2B subunit — ** protein expression is significantly increased in the CCI and PTE groups compared to the control group; GluR1 subunit — ** protein expression is significantly decreased in the CCI and PTE groups compared to the control group, and (*) protein expression is significantly decreased in the PTE group compared to the CCI group; HSP70 — ** protein expression is significantly increased in the CCI and PTE groups compared to the control group.

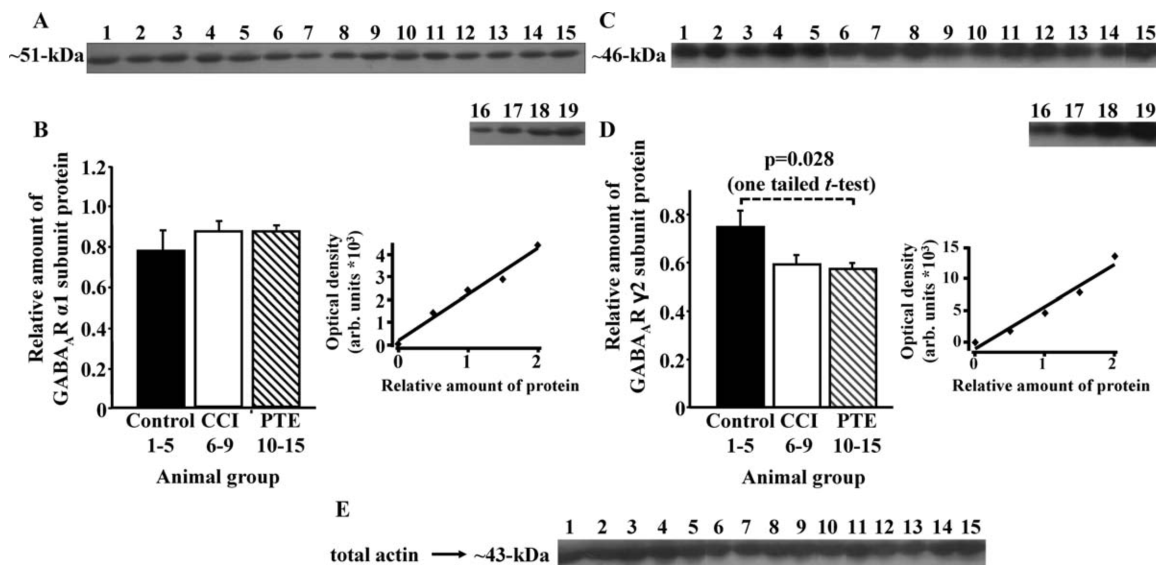

Figure 2 Western blot analysis of GABA$_A$R α1 (A and B) and GABA$_A$R γ2 (C and D) subunits, and total actin (E; to verify equal protein loading). Each lane represents a single sample containing in an equal volume 30 μg of protein. The samples represent the nuclear free homogenates prepared from the contralateral hippocampus of control (n = 5; lanes 1—5) and CCI-injured animals (n = 10) some of which did not demonstrate convulsive seizures and were considered to be ''seizure-free'' or CCI group (n = 4, non-epileptic, lanes 6—9), whereas the animals which had at least one convulsive seizure were considered epileptic, or PTE group (n = 6; epileptic, lanes 10—15), and an internal standard (15, 30, 45, and 60 μg/ml/per lane; 16—19, respectively). The blots were stained with the corresponding primary anti-GABA$_A$R α1 (A) and anti-GABA$_A$R γ2 (C) antibodies and subunit-specific bands were detected (A and C). Mean relative amounts of GABA$_A$R α1 (B) and GABA$_A$R γ2 (D) subunit proteins in the contralateral hippocampus of control, TBI and PTE animals and the calibration plots (D; see Data analysis and quantification of immunoblots for the details). Error bar represents a standard error of the means (SEM). The data were analyzed with the one-way analysis of variance (one-way ANOVA). The comparisons between different experimental groups were made with Student's t-test; all statistical tests were two-tailed unless otherwise stated and unless indicated on the figures. Statistical significance is set at $p < 0.05$.

protein level was observed in the PTE group compared to the control group in a one-tailed test ($p = 0.028$, $t = 2.48$; Fig. 2D, Table 1).

Tonic inhibition: expression of GABA$_A$R α4 and δ subunit proteins: Polyclonal anti-GABA$_A$R α4 antibody detected specific immunoreactive bands at ~67 kDa, corresponding to the size of the α4 subunit protein (Bencsits et al., 1999; Fig. 3A); nearby additional bands were also detected (Fig. 3A). The optical densities of both bands were measured and used for further analysis. The same results were obtained when the measurements were made for either the upper or lower band (data not shown). No significant differences were found across the groups using ANOVA (Table 1).

Specific immunoreactive bands at ~55 kDa, corresponding to the size of the δ subunit protein (Joshi and Kapur, 2009; Fig. 3C), were detected with monoclonal anti-GABA$_A$R δ antibody. Additional bands were also detected on the film (Fig. 3C). The optical densities of both bands were measured and used for further analysis; however, the same results were obtained when the measurements were made for either the upper or lower band (data not shown). The effect of the injury on GABA$_A$R δ protein expression across the groups was significant (Table 1). There was a 194% increase of δ protein expression in the CCI group compared to the control group ($p < 0.0001$, $t = 10.28$; Fig. 3D, Table 1), and a 127% increase of δ protein compared to the PTE group ($p < 0.0001$, $t = 6.74$; Fig. 3D, Table 1).

Ionotropic glutamate receptor subunit expression

Expression of NMDAR 2B (NR2B) subunit protein: Polyclonal anti-NR2B antibody detected specific immunoreactive bands at ~180 kDa, corresponding to the size of the NR2B subunit protein (Portera-Cailliau et al., 1996; Fig. 4A). The mean relative amount of NR2B protein was significantly higher in the CCI and PTE groups when compared to the control group: CCI vs. control $p = 0.0001$, $t = 8.90$; and PTE vs. control $p = 0.011$, $t = 3.66$ (Fig. 4B, Table 1). Thus, CCI altered the expression of NR2B subunit protein during long-term survival and following the development of PTE.

Expression of AMPAR GluR1 subunit protein: Polyclonal anti-GluR1 antibody detected specific immunoreactive bands at ~106 kDa, corresponding to the size of the GluR1 subunit protein (Blackstone et al., 1992; Fig. 4C). The effect of the injury on GluR1 protein expression across the groups was significant with ANOVA (Table 1). GluR1 protein expression was decreased in the CCI and PTE groups compared to the control group: $p = 0.003$, $t = 4.92$; and $p = 0.0001$, $t = 6.31$, respectively (Table 1; Fig. 4D). The mean amount of GluR1 protein in the PTE group compared to the CCI group demonstrated a trend toward a decrease in a one-tailed test ($p = 0.036$, $t = 2.13$; Fig. 4D, Table 1).

Expression of stress proteins

HSPs are among established injury-induced genes and/or proteins that constitute an endogenous cellular defense

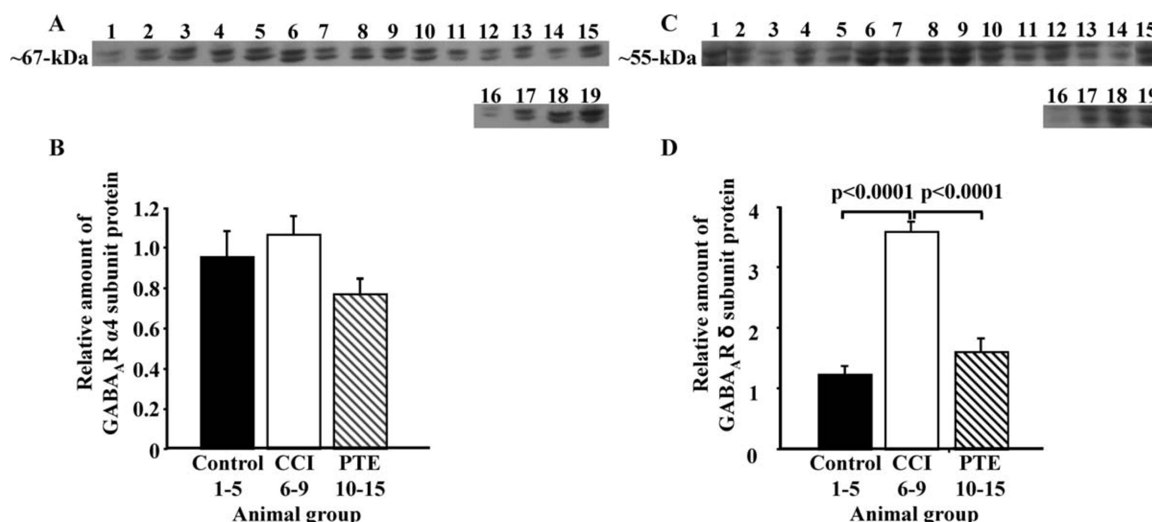

Figure 3 Western blot analysis of GABA$_A$R a4 (A and B) and GABA$_A$R δ (C and D) subunits. The experimental groups, Western plot procedure, data analysis, and statistical tests are the same as in Figure 2. The blots were stained with the corresponding primary anti-GABA$_A$R a4 and anti-GABA$_A$R δ antibodies. Computerized scan of a representative immunoblot illustrates a specific a4 (A) and δ (C) subunit protein band and an additional nearby protein band (A and C). Mean relative amounts of GABA$_A$R a4 and GABA$_A$R δ (D) in the contralateral hippocampus of control, CCI and PTE group of animals. The optical densities of both bands were measured and used for the analysis. The same results were obtained if the measurements were done only for the upper or lower band (data not shown). Error bar represents SEM.

mechanism against cerebral ischemia, trauma, seizures, and stress (Gass et al., 1995; Truettner et al., 2007, 2009; Ekimova et al., 2010). To assess long-term effects of CCI on cellular stress responses, the expression of HSP70 and HSP90 was investigated.

Expression of HSP70

A monoclonal anti-HSP70 antibody was used for the detection of specific immunoreactive bands at ∼70 kDa, corresponding to the size of HSP70 (Dutcher et al., 1998; Fig. 5A). Significant differences for a relative amount of HSP70 were found across the animal groups (Table 1). There was a significant increase of HSP70 expression in the CCI and PTE groups compared to the control group: $p = 0.014$, $t = 4.17$; and $p = 0.005$, $t = 3.68$, respectively (Fig. 5B, Table 1).

Expression of HSP90

Monoclonal anti-HSP90 antibody detected immunoreactive bands at ∼90 kDa, corresponding to the size of HSP90 (Gass et al., 1994; Solomonia et al., 2010; Fig. 5C). Comparison of the expression of HSP90 among control, CCI, and PTE

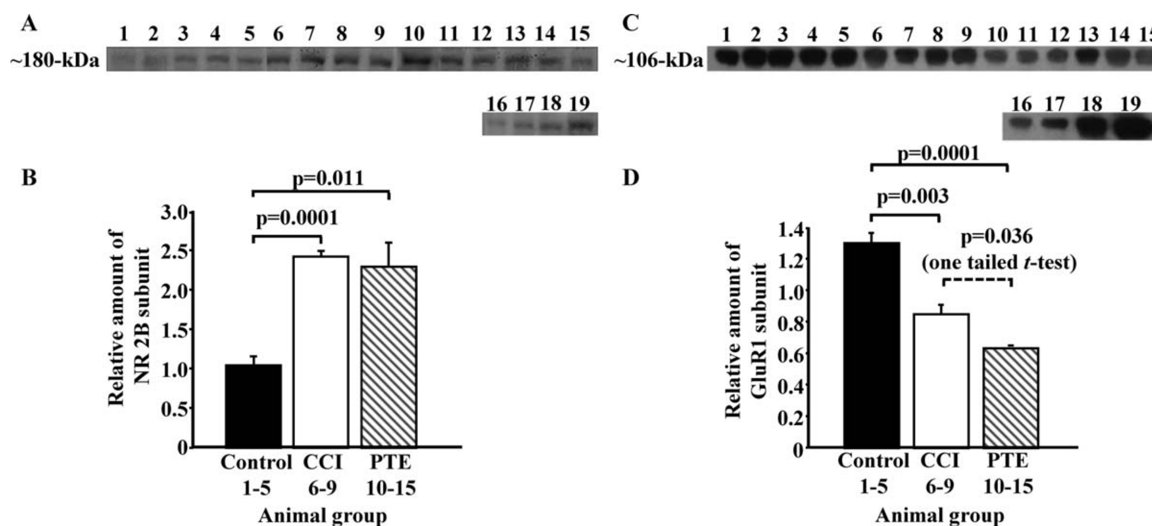

Figure 4 Western blot analysis of NMDA NR2B (A and B) and AMPA GluR1 (C and D) subunits. The experimental groups, Western plot procedure, data analysis, and statistical tests are the same as in Figure 2. The blots were stained with the corresponding primary anti-NR2B (A) and anti-GluR1 (C) antibodies and subunit-specific bands were detected (A and C). Mean relative amounts of NR2B (B) and GluR1 subunit proteins (D) in the contralateral hippocampus of control, CCI, and PTE animals. Error bar represents SEM.

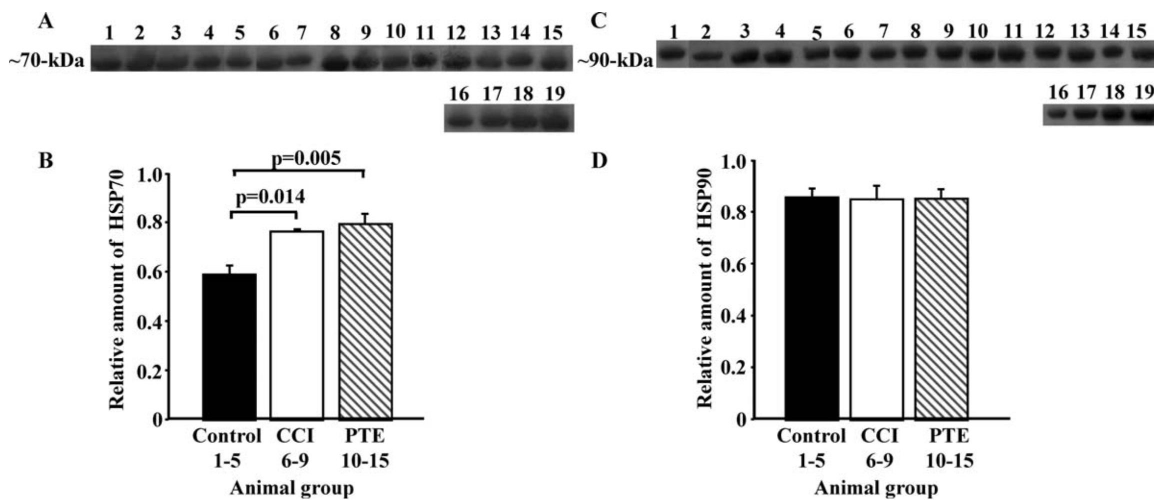

Figure 5 Western blot analysis of HSP70 (A and B) and HSP90 (C and D). The experimental groups, Western plot procedure, data analysis, and statistical tests are the same as in the Figure 2. The blots were stained with the corresponding primary anti-HSP70 (A) and anti-HSP90 (C) antibodies and subunit-specific bands were detected (A and C). Mean relative amounts of HSP70 (B) and HSP90 (D) in the contralateral hippocampus of control, CCI, and PTE animals. Error bar represents SEM.

groups did not reveal any significant alterations (Fig. 5D; Table 1).

Discussion

The purpose of this study was to determine whether TBI and/or the spontaneous seizures in the CCI model of PTE altered the expression of selected $GABA_A$ and glutamate receptor subunits and HSPs. The main findings were: (1) differential alterations of $GABA_AR$ subunit protein expression; (2) increased expression of the NR2B subunit protein and decreased expression of the GluR1 subunit protein; and (3) increased expression of inducible HSP70 and no change in the expression of HSP90. This study identified for the first time specific proteins involved in the complex pathophysiological mechanisms of brain trauma and PTE following long-term survival, provided new insights into potential molecular mechanisms of posttraumatic epileptogenesis and PTE, and corroborated earlier published findings that some CCI-injured animals develop epilepsy.

Differential alterations of $GABA_ARs$ after CCI

This study demonstrated plasticity of specific $GABA_AR$ subunits after CCI and in the subsequent development of PTE. $GABA_AR$ subunits maintain two forms of inhibition: phasic (synaptic) and tonic (extrasynaptic). Phasic inhibition is mediated by activation of postsynaptic receptors by saturating concentrations of vesicular GABA, and tonic inhibition results from activation of extrasynaptic $GABA_ARs$ by low concentrations of ambient GABA. In the adult brain, both forms of GABA signaling are mediated by distinct $GABA_AR$ subtypes that are predominantly composed of α, β, and $\gamma 2$ subunits (Mody and Pearce, 2004; Scimemi et al., 2005; Prenosil et al., 2006; Glykys et al., 2008). In hippocampal and cerebellar granule cells, tonic inhibition is associated with the expression of $\alpha 4$, $\alpha 6$, and δ subunits (Belelli et al., 2009), whereas phasic inhibition is mediated by $\alpha 1$ and $\gamma 2$ subunit-containing receptors (Nusser and Mody, 2002; Lüscher and Keller, 2004). Changes in $GABA_AR$ subunits following CCI and long-term animal survival are not known. CCI triggers widespread molecular and cellular alterations in neocortex, white matter, hippocampus, and thalamus, results in hyperexcitability, and can lead to the development of PTE (Santhakumar et al., 2000, 2001; Ratzliff et al., 2004; Hall et al., 2008; Hunt et al., 2009; Yang et al., 2010). Our recent finding of increased tonic current and concomitant benzodiazepine-insensitive pharmacology of synaptic current in DGCs 90 days after CCI (Mtchedlishvili et al., 2010) suggested the likelihood of altered $GABA_AR$ subunit expression in these neurons. Epileptic seizures were not observed during this 90-day survival period; however, in the present study, animals had significantly longer survival periods, resulting in the detection of convulsive seizures in a subset of animals. For these reasons, $GABA_AR$ $\alpha 1$, $\alpha 4$, $\gamma 2$, and δ subunits were assessed in CCI and PTE animals in this study.

We found that δ subunit protein expression was significantly increased in CCI-injured animals compared to controls. Up-regulation of δ subunit protein in the hippocampus likely contributed to increased tonic current in DGCs of CCI-injured animals 90 days after impact (Mtchedlishvili et al., 2010) and may be associated with an unrecognized latent period of epileptogenesis in the present study. However, in the PTE group, there was a significant decrease of the δ subunit protein compared to the CCI group. Down-regulation of δ subunit protein levels in PTE animals might be due to altered trafficking of receptor subunits, which can be caused by and further reinforce hyperexcitability and seizure susceptibility in established epileptic circuits. Decreased levels of δ subunit in epileptic hippocampus (Peng et al., 2004; Cendes, 2005) might be a consequence, rather than a cause, of seizures. Because δ subunits preferentially co-assemble with $\alpha 4$ subunits (Sun et al., 2004) — the latter is a key subunit linked to enhanced neuronal excitability, seizure susceptibility, and benzodiazepine resistance (Schwarzer et al., 1997; Lagrange et al., 2007) — loss

of the δ subunit may lead to a concomitant reduction in hippocampal α4 subunit levels (Spigelman et al., 2003). In the present study, there was no significant difference in α4 subunit protein expression in the three animal groups. This result stands in contrast to the finding of α4 subunit expression that was increased during the chronic period of temporal lobe epilepsy (Peng et al., 2004) and decreased in the forebrain of δ subunit-deficient mice (Peng et al., 2002). These observed alterations following loss of the δ subunit may reflect local changes in $GABA_AR$ subunit composition and function.

The γ2 subunit is important for assembly of $GABA_ARs$, accumulation at synapses, surface stability, and synaptic clustering (Essrich et al., 1998; Schweizer et al., 2003; Alldred et al., 2005). Diffuse reduction in γ2 subunit expression or mutations and/or deletions in the γ2 subunit gene are associated with some forms of epilepsy (Lagrange, 2005; Eugène et al., 2007; Frugier et al., 2007; Macdonald et al., 2010). $GABA_AR$ γ2 protein expression demonstrated a trend toward reduction in the PTE group compared to controls and may play a significant role during epileptogenesis after CCI. The down-regulation of δ subunits and the trend toward decreased α4 and γ2 subunit expression in PTE animals compared to CCI animals are consistent with the positive correlation of recurrent seizure activity and a use-dependent decline in $GABA_AR$-mediated inhibition (Kapur and Lothman, 1989).

In our study, $GABA_AR$ α1 subunit protein expression was unchanged in control, CCI, and PTE groups. This finding is in contrast to those of several other studies of TBI or epilepsy. For example, increased α1 subunit protein expression was detected at 3 and 6 h after TBI, but decreased at 24 h and 7 days compared to shams (Gibson et al., 2010). In epilepsy studies, up- or down-regulation of α1 subunit gene/protein expression was found depending on the epilepsy model, investigated brain region, or cell type (Brooks-Kayal et al., 1998; Fritschy et al., 1999; Raol et al., 2006; Loup et al., 2009). In addition, mutations of the α1 subunit can alter receptor function, impair subunit messenger RNA stability, subunit protein folding and stability, and $GABA_AR$ assembly and trafficking, and be associated with seizures and epilepsy (Cossette et al., 2002; Gallagher et al., 2005, 2007). It is important to note that our results cannot be compared directly to the above mentioned studies given that none of them investigated long-term changes in CCI and PTE animal groups.

Collectively, our results demonstrated differential alterations of hippocampal $GABA_AR$ subunit proteins involved in phasic and tonic inhibition in both CCI and PTE animals, indicating pathological and/or compensatory changes following brain injury and the establishment of an epileptic state. These alterations may reflect impairments of $GABA_AR$ protein trafficking and cell-surface localization, and protein—protein interactions following brain trauma.

Differential alterations of ionotropic glutamate receptor proteins in CCI

Expression of NMDAR NR2B subunit

The NMDAR-type of glutamate receptor mediates excitotoxicity, which has an important role in the pathophysiologic mechanisms of TBI, seizures, and epilepsy. NMDARs are located on neurons and glial cells and composed of seven subunits: NR1, NR2A—D, and NR3A—B (Monyer et al., 1992). In the adult brain, NR2B subunit-containing receptors have an important role in protein—protein interactions, synaptic plasticity, and regulation of calcium (Ca^{2+}) influx (Carroll and Zukin, 2002; Barria and Malinow, 2005; Groc et al., 2006; Foster et al., 2010; Rebola et al., 2010). Compared to other NMDAR subtypes, NR2B-containing receptors appear to contribute preferentially to pathological processes linked to over-excitation of glutamatergic pathways (Mony et al., 2009). Impairments of NMDA-mediated transmission have been associated with increased neuronal Ca^{2+} influx (Faden et al., 1989; Miller et al., 1990). Following brain injury, prolonged elevations of intracellular Ca^{2+} in neurons may initiate molecular mechanisms that cause cellular injury and death and result in epileptogenesis within surviving neurons (DeLorenzo et al., 2005). Alterations in Ca^{2+} homeostasis have been observed following brain injury and the generation of epileptiform discharges (Pal et al., 2000; Raza et al., 2004; Deshpande et al., 2008) and have been shown to be dependent on an NMDAR pathway (Blair et al., 2008).

In the present study, increased levels of NR2B subunit protein were found in CCI and PTE animals compared to controls. Multiple studies have demonstrated that the effect of CCI on the expression of NMDAR subunits likely depends on several factors, including injury severity, brain region, and post-injury survival time. For example, the expression of hippocampal NR1, NR2A, and NR2B subunits as well as the GluR1 subunit of the AMPAR was increased 15 min following closed head injury, whereas in the ipsilateral cortex, the expression of these subunits was decreased (Schumann et al., 2008). In the rat hippocampus, NR1, NR2A, and NR2B protein expression was significantly decreased at 6 and 12 h following CCI, but was restored to baseline levels 24 h post-injury (Kumar et al., 2002). TBI had no significant effect on NR1 or NR2B protein expression at 1, 2, 4, and 7 days following FPI, whereas within the ipsilateral hippocampus, NR2A expression was reduced (Giza et al., 2006).

NR2B-containing NMDARs can mediate signaling for neuronal survival and synaptic potentiation, and as well as neuronal death (Hetman and Kharebava, 2006; Liu et al., 2007; Martel et al., 2009). Recent studies have demonstrated that in normal cortex, a portion of the NR2B receptor, the postsynaptic density protein-95 (PSD-95; regulated by phosphorylation), and the autophagic protein Beclin-1 can localize in membrane rafts forming a novel multi-protein complex (Lavezzari et al., 2003; Diskin et al., 2005; Park et al., 2008; Zhang et al., 2008). Brain injury may induce a dissociation of this multi-protein complex and cause a rapid increase in the levels of NR2B and the translocations of pCaMKII (autophosphorylated calcium-calmodulin-dependent protein kinase II) and Beclin-1 to or out of membrane rafts, suggesting that the membrane rafts may mediate trafficking and signaling of NR2B following TBI (Meng et al., 2003; Bigford et al., 2009; Raveendran et al., 2009). The redistribution of NMDARs in membrane rafts is one possible mechanism for regulating the efficiency of NMDAR signaling following brain trauma and epilepsy.

Brain injury also may result in increased tyrosine phosphorylation of NR2A and NR2B (Besshoh et al., 2005; Goebel et al., 2005). The inhibition of NR2B expression by blocking

its phosphorylation (Schumann et al., 2008) or by a selective antagonist (Yurkewicz et al., 2005) may improve functional recovery after TBI. In addition, long-term depotentiation of synapses involved in epileptic activity can be induced by partial block of NMDARs using NR2B- but not NR2A-selective antagonists (Hellier et al., 2009). In the kindling and pilocarpine models of epilepsy, activation of NR2A-containing NMDARs were required for limbic epileptogenesis and the development of mossy fiber sprouting, but not the NR2B subunit, whereas NMDARs comprised of either subunit may be involved in seizure-induced neuronal cell death (Chen et al., 2007). In human studies, differential expression of the NR2B subunit contributed to epileptogenesis in human cortical dysplasia (Möddel et al., 2005) and NR2B mRNA was upregulated in brain tissue from patients with TLE (Liu et al., 2007a). In our study, increased expression of the NR2B subunit protein clearly appeared to be a consequence of CCI and may have contributed to posttraumatic epileptogenesis.

Expression of AMPAR GluR1 subunit

In the brain, activity-dependent changes in excitatory synaptic transmission have been shown to depend on the regulation of AMPARs (Dingledine et al., 1999; Emond et al., 2010; Lee et al., 2010). AMPARs are composed of combinations of four subunits: GluR1-, GluR3-, and GluR4-containing receptors form Ca^{2+}-permeable, inwardly rectifying channels, whereas GluR2-containing receptors form Ca^{2+}-impermeable channels with linear or outward rectification (Petralia and Wenthold, 1992; Jonas and Burnashev, 1995). Up- or down-regulation of functional AMPARs located at the synaptic membrane is regulated by changes in their phosphorylation state or by their trafficking (Barria et al., 1997; Malinow and Malenka, 2002). GluR1 subunits have several sites for phosphorylation: serine (S818, S831, and S845) and threonine (T840; Lee et al., 2007, 2010); GluR1 phosphorylation at T840 is regulated by NMDAR activation (Delgado et al., 2007). The expression of GluR1 was increased in the hippocampus and cortex 1 h after TBI (Atkins et al., 2006) and by neonatal seizures (Rakhade et al., 2008). However, GluR1 was decreased in the ipsilateral cortex 15 min following closed head injury (Schumann et al., 2008).

Somewhat surprisingly, our study revealed decreased levels of GluR1 subunits in CCI and PTE groups compared to controls, as well as a decreased level in the PTE group compared to the CCI group; the latter finding is in contrast to the lack of changed expression of NR2B protein between PTE and CCI groups. However, a marked decrease of GluR1 expression was found in the hippocampus on the 28th day following kainate-induced status epilepticus (Solomonia et al., 2010). Our results suggest differential alterations in hippocampal NMDA- and non-NMDA-type glutamate receptors associated with TBI and the development of posttraumatic epilepsy, possibly related to AMPAR-mediated plasticity and NMDAR-dependent long-term potentiation in the hippocampus. Chronic epileptic activity may induce molecular and functional alterations of glutamatergic transmission in the CCI-injured brain. Down-regulation of the GluR1 subunit may be a result of altered phosphorylation and/or reflect brain compensatory responses following TBI and seizures.

Differential expression of HSPs in CCI

HSPs are sensitive and reliable biomarkers of cell stress and injury and have multiple functions. HSP70 and HSP90 are expressed in different brain regions and are abundant in limbic structures (Izumoto and Herbert, 1993; D'Souza and Brown, 1998). HSP70 is the major stress-induced cytoplasmic chaperone and is an important part of the cell's machinery for protein folding (Mallouk et al., 1999; Pratt and Toft, 2003). HSP90 is involved in cellular growth, signaling, survival, and trafficking, chaperoning misfolded proteins into mitochondria, targeting and transport of AMPARs into synapses, and in control of neurotransmitter release at the presynaptic terminal (Gerges et al., 2004; Pratt et al., 2006; Zara et al., 2009; Hota et al., 2010; Solomonia et al., 2010).

In the present study, HSP70 expression was increased in CCI and PTE groups, whereas HSP90 expression levels were similar across all animal groups. Up-regulation of HSP70 may indicate an adaptive brain response that is induced after brain trauma and persists during seizures. In PTE, the increased expression of HSP70 may reflect the CCI-injured brain response to decrease seizures and may be related to its ability to modulate GABA neurotransmission (Ekimova et al., 2010). However, the role of increased HSP70 expression in both animal groups was beyond the scope of the present study and was not investigated in detail. HSP70 may have a protective role due to its chaperone function in preventing abnormal protein folding and/or aggregation by multiple mechanisms, which are associated with necrotic and apoptotic cell death and anti-inflammatory activity (Giffard and Yenari, 2004; Didelot et al., 2006; Zheng et al., 2008). Conversely, HSP70 expression in epilepsy may represent a response to stress rather than a protective role (Yang et al., 2008). Altered expression of HSP70 in the CCI and PTE groups was consistent with previous reports of altered HSP signals following excitotoxicity, ischemia, TBI, seizures, and epilepsy, which are dependent on brain injury severity, seizure severity, and regional and cellular vulnerability (Vass et al., 1988; Sharp et al., 1993; Dutcher et al., 1998; Hellmich et al., 2005). In previous studies, HSP90 expression was not altered following limbic seizures (Gass et al., 1995) and was unchanged in the hippocampus following pilocarpine-induced status epilepticus (Lively and Brown, 2008).

In summary, this study demonstrates for the first time long-term changes in $GABA_A$ and glutamate receptor subunit proteins, and a HSP following CCI and the establishment of PTE. These novel findings reflect alterations in inhibitory and excitatory neurotransmission and likely adaptive stress responses following brain trauma and epileptogenesis. These findings identify potential biological mechanisms underlying the pathophysiology of brain trauma and posttraumatic seizures and suggest molecular targets for the prevention or treatment of TBI and/or PTE.

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