

N-Methyl-D-Aspartate Receptor Subunit Changes after Traumatic Injury to the Developing Brain

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ABSTRACT

Traumatic brain injury (TBI) is a major cause of disability in the pediatric population and can result in abnormal development. Experimental studies conducted in animals have revealed impaired plasticity following developmental TBI, even in the absence of significant anatomical damage. The *N*-methyl-D-aspartate receptor (NMDAR) is clearly involved in both normal development and in the pathophysiology of TBI. Following lateral fluid percussion injury in postnatal day (PND) 19 rats, we tested the hypothesis that TBI sustained at an early age would result in impaired NMDAR expression. Using immunoblotting and reverse transcriptase–polymerase chain reaction (RT-PCR), protein and RNA levels of NMDAR subunits were measured in the cerebral cortex and hippocampus on post-injury days (PID) 1, 2, 4, and 7 (though the PID7 analysis was only for protein) and compared with age-matched shams. Significant effects of hemisphere (analysis of variance [ANOVA], $p < 0.01$), and interactions between hemisphere and injury (ANOVA, $p < 0.05$) and hemisphere and PID (ANOVA, $p < 0.05$) were found for synaptic protein levels of the NR2A subunit in hippocampus. Specifically, within the ipsilateral hippocampus, NR2A was reduced by 9.9%, 47.9%, 40.8%, and 6.3% on PID1, PID2, PID4, and PID7, respectively. Within the cortex, there was a significant effect of injury (ANOVA, $p < 0.05$) without any hemispheric differences. These bilateral cortical reductions measured 30.5%, 3.2%, 5.7%, and 13.4% at the same timepoints after injury. Injury had no significant main effect on NR1 or NR2B protein levels. RT-PCR analysis showed no significant changes in NR1, NR2A, or NR2B gene expression; however, as a positive control, hsp70 was induced more than twofold in ipsilateral cortex and hippocampus on PID1. It is known that NR2A expression levels increase during normal development, and in response to environmental stimuli. Our data suggest that injury-induced reduction in the expression of NR2A is one likely mechanism for the impaired experience-dependent neuroplasticity seen following traumatic injury to the immature brain.

Key words: glutamate; immature; pediatric; plasticity; synapse; synaptoneuroosomes; traumatic brain injury

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INTRODUCTION

PEDIATRIC TRAUMATIC BRAIN INJURY (TBI) results in 3,000–7,000 deaths and an estimated 100,000 hospitalizations in the United States annually, making it the major cause of mortality and morbidity in this age group (Weiner and Weinberg, 2000). Traumatic injury to the immature brain can result in abnormal development and lead to persistent cognitive and behavioral problems (Bloom et al., 2001; Ewing-Cobbs et al., 1998; Fineman et al., 2000; Giza et al., 2005; Levin, 2003; Max et al., 1997; Taylor and Alden, 1997).

N-Methyl-D-aspartate receptor (NMDAR) activation has an important role in traumatic brain injury (Faden et al., 1989; Yoshino et al., 1992) and is a necessary component of experience-dependent plasticity and normal development (Carroll and Zukin, 2002; Kind and Neumann, 2001; Perez-Otano and Ehlers, 2005). *N*-methyl-D-aspartate receptor-mediated neurotransmission is regulated by the number of receptors expressed at the synapse and the subunit composition of these receptors (Chen et al., 1999; Scheetz and Constantine-Paton, 1994). Changes in NMDAR-mediated neurotransmission lead to alterations in synaptic plasticity (Jablonska et al., 1995; Kleinschmidt et al., 1987; Maletic-Savatic et al., 1999; Quinlan et al., 1999b). Structurally, the NMDAR is composed of two major subunits, NR1 and NR2. The NR1 subunit is required for the function of the ion channel, but does not confer substantial glutamate sensitivity. Rather, differential glutamate sensitivity is conferred by the NR2 subunit, with four subtypes designated A–D. NR2A subunit-predominant receptors are less sensitive to glutamate, generate lower peak currents, and remain open for a shorter time than those with predominantly NR2B subunits (Scheetz and Constantine-Paton, 1994).

While both NR2A and NR2B are found extensively in neocortex and hippocampus and both increase during development, they are differentially expressed as the brain matures. In the neonatal period, NR2B predominates and there is little NR2A expression, but with cerebral maturation, NR2A increases much more rapidly than NR2B (Monyer et al., 1994; Sheng et al., 1994). This preferential increase in NR2A results in a developmental increase in the relative ratio of NR2A/NR2B expression (as measured by mRNA or protein). In visual cortex, this change in the subunit ratio of NR2A/NR2B occurs at the time of ocular dominance column formation (in ferret; Roberts and Ramoa, 1999) and is altered by visual experience (in rat; Quinlan et al., 1999a,b). Similar changes in NR2A/NR2B ratio can be seen as somatosensory cortex matures, that is, an increased NR2A/NR2B ratio is associated with a change in NMDAR function as measured by duration of *N*-methyl-D-aspartate (NMDA)-mediated

currents (Flint et al., 1997). The remaining NR2 subtypes—NR2C and NR2D—are not predominantly expressed in our regions of interest (cortex and hippocampus; Scheetz and Constantine-Paton, 1994), nor have they been as closely tied to experience-dependent developmental plasticity.

The consequences of NMDAR manipulation in the developing brain are distinct from similar responses in the mature brain. For example, while overstimulation of NMDARs in both the developing and adult brain results in excitotoxicity, NMDAR antagonists are neuroprotective preferentially in the adult. In fact, administration of NMDAR antagonists early in development (before postnatal day 7) has been shown to result in significant cell death via apoptotic mechanisms (Bittigau et al., 1999; Ikonomidou et al., 1999; Ikonomidou and Turski, 1996; Pohl et al., 1999).

One of the neurochemical hallmarks of TBI is the indiscriminate release of glutamate (Faden et al., 1989; Katayama et al., 1990), which results in ionic fluxes and is associated with altered cerebral glucose metabolism (Yoshino et al., 1991). Preventing NMDAR activation using pharmacological antagonists or by lesioning glutamatergic pathways mitigates this post-traumatic ionic flux (Katayama et al., 1990) and elevated glucose uptake (Kawamata et al., 1992; Yoshino et al., 1992). Other studies have shown evidence for NMDAR dysfunction following experimental TBI in adult animals, including decreased NMDAR binding within 24 h of injury (Miller et al., 1990) and impaired induction of long-term potentiation up to 15 days post-injury (D'Ambrosio et al., 1998; Reeves et al., 1995; Sick et al., 1998).

To induce cerebral trauma without significant cell death, we utilized a model of concussive brain injury in the preweanling rat. This fluid percussion (FP) model induces a biomechanical injury to the brain without extensive histological damage (Fineman et al., 2000; Gurkoff et al., 2006; Prins et al., 1996). Within a day after injury, rat pups subjected to a moderate FP injury demonstrated no deficits in open field behavior or Morris Water Maze acquisition when compared with age-matched shams (Fineman et al., 2000; Prins and Hovda, 1998). However, when postnatal day (PND) 19 pups were reared in an enriched environment (EE) after FP, the FP/EE rats failed to demonstrate increases in cortical thickness and improvements in Morris Water Maze performance seen in sham-injured/EE-reared controls (Fineman et al., 2000; Giza et al., 2005). Therefore, while developmental FP injury alone does not lead to gross histological or behavioral deficits, it does impair experience-dependent neuroplasticity (and concomitant anatomical changes) induced by EE rearing. This, combined with the above-mentioned evidence of altered glutamatergic neurotrans-

mission following adult TBI, led to our hypothesis that TBI during development would induce molecular changes in the NMDAR, either by a reduction in number or by alteration of subunit composition.

METHODS

Subjects

Animals studied ($n = 80$) were all male Sprague-Dawley rat pups that underwent sham or FP injury on PND19. For protein studies, at each time point, animals were randomly divided into two groups: age-matched sham ($n = 7$) and FP-injured ($n = 7$) animals. For gene expression studies, at each time point, animals were also randomly divided into the same two groups: age-matched sham ($n = 4$) and FP injured ($n = 4$). Tissue harvesting time points for protein were post-injury day (PID) 1, 2, 4, and 7. Tissue for RNA was harvested on PID1, PID2, and PID4. A total of 94 animals underwent surgery, with 12 excluded for mild injury and two that died after injury. All pups for all experiments were housed as litters with access to mothers until weaning on PND20, 1 day following surgery. An additional six naive animals were used to characterize the synaptoneurosome preparation. All procedures were approved by the UCLA Chancellor's Animal Research Committee.

Fluid Percussion Injury

PND19 Sprague-Dawley male pups were anesthetized with 1–2 mL/min isoflurane in 100% O₂ using a mask. After reaching a surgical level of anesthesia (complete loss of response to painful stimuli), the animals were secured in a stereotaxic frame, where the body temperature was kept constant (37–38°C) by a thermostatic heating pad. The skin was sterilely prepped with betadine and ethanol, and a sagittal incision was made in the scalp, exposing the pericranium. A 3.0-mm-diameter craniotomy was made 3.0-mm posterior to bregma and 6.0-mm lateral (left) to the midline, using a high-speed drill (Dremel). A plastic injury cap was secured over the craniotomy using silicone adhesive, cyanoacrylate, and dental cement. When the dental cement completely hardened, the injury cap was filled with saline. Anesthesia was then removed, and the animal was attached to the FP device (Dixon et al., 1987). At the first sign of hindpaw withdrawal reflex, a moderate fluid pulse was administered (2.65–2.7 atm). Apnea and loss of consciousness (LOC) times were recorded, and were determined by the resumption of spontaneous respiration and by responsiveness to toe pinch, respectively. If no spontaneous respirations were evident by 40 sec post-injury, resuscitation

with 100% O₂ was administered until spontaneous respirations returned. For the level of injury severity used in this study, all animals required some resuscitation, as more mildly injured animals (LOC < 120 sec) were excluded. After recovery of the hindpaw withdrawal reflex, the animal was re-anesthetized for injury cap removal and closure of the surgical incision. Local infiltration of marcaine (0.25%) and topical application of antibiotic ointment completed the procedure. Sham animals underwent the similar preparation as above, up to and including craniotomy, but without attachment of the injury cap or administration of the fluid pulse.

Tissue Harvesting and Synaptoneurosome Preparation

For protein analysis, brains of the FP-injured pups and their age-matched shams were harvested on PID1, PID2, PID4, and PID7. To enrich the samples for synaptic proteins and reduce variability in total protein content, synaptoneurosomes were prepared following standard protocols (Johnson et al., 1997; Quinlan et al., 1999a,b; Scheetz et al., 2000). The brains were immediately placed in ice-cold homogenization buffer (HB; 0.137 M NaCl, 2.7 mM KCl, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 10 mM HEPES, 10 mM glucose, pH 7.4). The brains were divided into 3-mm sections using a brain slicer. Ipsilateral and contralateral parietal cortex and hippocampus were dissected and then homogenized in 1.0 mL of HB. Following this, each sample was diluted with an additional 1.8 mL of ice-cold HB. The mixture was loaded into a 3.0-mL syringe attached to a 13-mm-diameter Millipore syringe filter holder, forced through a 100- μ m nylon mesh filter, and collected in a polystyrene round bottom tube. The filtered homogenate was then loaded into another 3.0-mL syringe and sequentially filtered through two, pre-wetted 5- μ m nitrocellulose membranes. The homogenate was kept on ice at all times. The filtrate was then centrifuged at 1000 \times *g* for 20 min. The supernatant was removed, and the remaining pellet was resuspended in 1.0 mL of homogenization buffer and spun at 1000 \times *g* for 10 min. After the second supernatant was removed, the remaining pellet (synaptoneurosome fraction) was resuspended in 150 μ L of homogenization buffer and stored at –70°C for immunoblot analysis.

Protein Quantitation and Immunoblotting

Total protein of each brain synaptoneurosome fraction was determined using the BioRad DC assay. Synaptoneurosome samples (10 μ g protein each) were resolved on a 7.5% Tris-HCl ready-made minigel (Bio-Rad, 2006) at 160 V for 40 min, then transferred onto nitrocellulose (Bio-Rad Criterion Transfer Apparatus) at 100 V for 60

min. The nitrocellulose membrane was stained for protein using the Bio-Rad Sypro Ruby Protein Stain solution and imaged using the Bio-Rad Fluor-S MultiImager.

After imaging for total protein, the nitrocellulose was blocked in 7% milk in Tris-buffered saline with 1% Tween-20 (TTBS) for 60 min and then incubated overnight with various primary antibodies diluted in TTBS. Primary antibodies (Chemicon, Temecula, CA) were used at the following dilutions: NR1, 1:100; and NR2A and NR2B, 1:750. This was followed by peroxidase-conjugated secondary antibody incubation, diluted 1:10,000 in 1% milk in TTBS. Bands on the Western blots were then visualized using Supersignal Chemiluminescent substrate (Pierce product no. 34080) and detected with the Bio-Rad Fluor-S MultiImager.

Tissue Harvesting and mRNA Preparation

At each time point—i.e., PID1, PID2, or PID4—rats were quickly decapitated and their brain regions were isolated and dissected at 4°C in a manner similar to that of the protein samples above, without the synaptosome preparation. The dissected brain regions were stored at -70°C until used for RNA isolation. Total RNA was isolated from each sample using the RNA-STAT-60 solution (Tel-Test Inc.) and purified by the RNeasy Mini Kit spin columns (Qiagen).

Real-Time Reverse Transcription–Polymerase Chain Reaction and Analysis

Relative expression of mRNA for the genes encoding the NMDA subunits NR1, NR2A, and NR2B was performed by the Comparative C_T (ddC_T) Method (Perkin-Elmer) using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as control. A validation experiment was performed for each gene of interest and its control in order to determine the conditions for optimal concentration of primers and probes. The threshold cycle CT of each gene was recorded by a real-time polymerase chain reaction (PCR) machine (iCycler iQ Detector, Bio-Rad). The normalized C_T (dC_T) was obtained by subtraction of the C_T for GAPDH from the C_T for the gene of interest. The difference between the dC_T for experimental and control samples gave rise to the ddC_T value that was used for the calculation of the relative mRNA expression using a formula 2^{-ddC_T} as modified by Pfaffl (2001). The relative mRNA level is expressed as fold change in injured samples over sham.

The following primer pairs were used for each gene of interest: **NR 1**: Forward primer: 5'-GTT CTT CCG CTC AGG CTT TG-3'; Reverse primer: 5'-AGG GAA ACG TTC TGC TTC CA-3'; Probe: 5'-CGG CAT GCG CAA GGA CAG CC-3'.

NR2A: Forward primer: 5'-AGC CCC CTT CGT CAT CGT A-3'; Reverse primer: 5'-GAC AGG GCA CCG TGT TCC T-3'; Probe: 5'-AGG ACA TAG ACC CCC TGA CTG AGA CCT GTG-3'. **NR2B**: Forward primer: 5'-AGC TGG TAG CCA TGA AC-3'; Reverse primer: 5'-GAT CTT CCG GTC AGA CAT-3'; Probe: 5'-CTG ACC CAA AGA GCA TCA TCA CCC G-3'. **GAPDH**: Forward primer: 5'-TGC ACC ACC AAC TGC TTA-3'; Reverse primer: 5'-GGA TGC AGG GAT GAT GTT-3'; Probe: 5'-CAG AAG ACT GTG GAT GGC CCC TC-3'. **HSP70**: Forward primer: 5'-AGG TTG CAT GTT CTT TGC G-3'; Reverse primer: 5'-AAA CTG TAC ACA GGG TGG C-3'; Probe: 5'-CTT CCT GCG AAC ACC TCA GCA CT-3'.

Immunoblot Analysis

Once the Sypro Ruby stain protein image was acquired, lane length and width were defined for signal quantification using the Bio-Rad Quantity One software. The software identifies and subtracts background within each lane using the mathematical rolling disc method. Trace quantity values of intensity count per unit length were generated and used as a measure of total protein detected on the nitrocellulose membrane post-transfer (Bio-Rad, Sypro Ruby Instruction Manual) (Berggren et al., 2002).

After antibody reaction, volumetric analyses were performed on the immunoblot bands using Quantity One software. Volume values of intensity count per unit area are generated for each band and used as a measure of the specific subunit being detected (Bio-Rad, Quantity One Software Manual). The immunoblot signal values were then normalized to the total protein signal (Sypro Ruby stain) from the same lane.

Statistical Analysis

At each postnatal age/PID, the average of protein-normalized sham values was then set at 100%. This was necessary to control for age-specific changes in NMDAR protein levels, and also to allow comparisons of sham versus injured differences between blots. Each immunoblot thus contained sham and injured samples for a given postnatal age/PID. Individual percent values for each sample were calculated for both sham and injured specimens. These individual percent values of the normalized immunoblot signals were then subjected to analysis of variance (ANOVA) by brain region using group (sham or injured) and time point (PID1, PID2, PID4, PID7) as between-subject variables and hemisphere (ipsilateral or contralateral) as a within-subject variable. If a main effect or interaction was detected by ANOVA in the overall model and since separate animals were analyzed for each individual PID, descriptive sta-

tistics using *t*-tests were performed between sham and injured samples on individual PIDs to illustrate the time-course differences. All analyses were performed using SPSS v11.0 software. All values are presented as mean \pm standard error of the mean.

RESULTS

Subject and Injury Characteristics

Characteristics for all groups are summarized in Table 1. The average apnea time for all injured animals was 213 ± 17 sec. There were no significant differences in apnea time across all post-injury time points (PID1 220 ± 38 sec, PID2 266 ± 44 sec, PID4 165 ± 15 sec, PID7 195 ± 20 sec; ANOVA $F_{3,27} = 1.8$, NS). Duration of loss of consciousness (LOC) averaged 265 ± 18 sec for all injured animals. Unconsciousness times for each post-injury time point were as follows: PID1 280 ± 36 sec, PID2 307 ± 45 sec, PID4 202 ± 16 sec; PID7 273 ± 40 sec. No significant differences were found at any time point (ANOVA $F_{3,27} = 2.1$, NS). There was a trend toward shorter apnea and unconsciousness in the PID4 group; however, no animal in any group experienced unconsciousness of <120 sec. At the time of surgery, no significant weight differences were detected between injury groups (i.e., sham vs. FP; ANOVA $F_{1,56} = 0.12$, NS) or between post-injury time point groups (PID1, PID2, PID4, PID7; ANOVA $F_{3,56} = 2.4$, NS). The animals randomized into the PID4 and PID7 groups tended to weigh slightly more (Table 1).

Furthermore, no significant differences in apnea or LOC were detected when animals harvested for measurement of protein expression or gene expression were analyzed separately (apnea: ANOVA $F_{1,40} = 0.37$, NS; LOC: ANOVA $F_{1,40} = 2.4$, NS). There was a small but significant starting weight difference (4.4 g) between all animals harvested for protein studies (49.4 ± 0.7 g) compared with those used for gene expression analysis (45.0 ± 0.7 g; ANOVA $F_{1,80} = 14.1$, $p < 0.001$).

Synaptoneurosome Preparation

We utilized a well-described synaptoneurosome preparation to enhance the immunoblot signal of synaptic NMDA receptor subunit proteins and reduce sample variability (Johnson et al., 1997; Quinlan et al., 1999b; Scheetz et al., 2000). To demonstrate the benefits of the synaptoneurosome fraction in our hands, a representative set of naive control preparations ($n = 6$) was prepared for both crude protein homogenates and synaptoneurosome. After normalizing for total protein content, crude homogenates and synaptoneurosome samples from identical cortical brain regions were immunoblotted for NR2A and NR2B in triplicate. On average, the synaptoneurosome fractions demonstrated a $181 \pm 31\%$ enrichment in NR2A antibody signal and a $280 \pm 51\%$ increase in NR2B signal when compared to crude homogenates. The NMDAR subunit signals were significantly higher in the synaptoneurosome fraction than in the crude homogenate for both subunits (one-tailed *t*-test; NR2A, $p < 0.02$; NR2B, $p < 0.002$). Furthermore, the coefficient of variance (CV) of the synaptoneurosome

TABLE 1. SUBJECT CHARACTERISTICS

Post-injury day (PID)/ group	Weight, start (g)	Drop angle	Apnea (sec)	Duration of unconsciousness (sec)	N (protein)	N (RNA)
PID1 Sham	46.7 ± 1.4	NA	NA	NA	7	4
FP	48.0 ± 2.0	14.9 ± 0.4	220 ± 38	280 ± 36	7	4
PID2 Sham	45.6 ± 1.1	NA	NA	NA	7	4
FP	46.6 ± 1.5	15.5 ± 0.1	266 ± 44	307 ± 45	7	4
PID4 Sham	50.1 ± 1.4	NA	NA	NA	7	4
FP	48.1 ± 1.9	15.6 ± 0.1	165 ± 15	202 ± 16	7	4
PID7 Sham	51.7 ± 2.0	NA	NA	NA	7	—
FP	50.4 ± 1.8	15.4 ± 0.1	195 ± 20	273 ± 40	7	—

Mean (\pm SEM) starting weight (in g) for all age-matched sham and FP-injured groups. Mean (\pm SEM) drop angle, apnea (in sec), and unconsciousness (in sec) following moderate-severe lateral FP injury. Groups were studied for both *N*-methyl-D-aspartate receptor subunit protein ($n = 7$) and mRNA ($n = 4$) characterization. Post-injury time points were 1, 2, 4, and 7 days, and all groups had the appropriate age-matched shams. There were no significant differences in starting weight, drop angle, apnea, or unconsciousness times between sham and injured groups, or between injured groups sacrificed at different time points (analysis of variance), although there was a trend towards shorter apnea and unconsciousness times in the animals randomized to the PID 4 group. FP, fluid percussion.

immunoblot signals was 3–6% lower than the crude homogenate, suggesting that analysis of the synaptoneurosome preparation would provide enhanced sensitivity to detect subunit changes.

Immunoblotting

NMDAR1. Immunoblotting for the NR1 subunit showed no significant overall effect of injury in any brain region (cortex: $F_{1,47} = 0.79$, NS; hippocampus $F_{1,47} = 1.04$, NS), nor was there any significant effect of hemisphere, PID, or interaction between these variables (Fig. 1A,B). In cortex, ipsilateral sham values were 100 ± 3.6 , and ipsilateral FP were 98.0 ± 5.9 ; cortical contralateral sham signals averaged 100 ± 5.6 , and contralateral FP averaged 91.0 ± 7.3 .

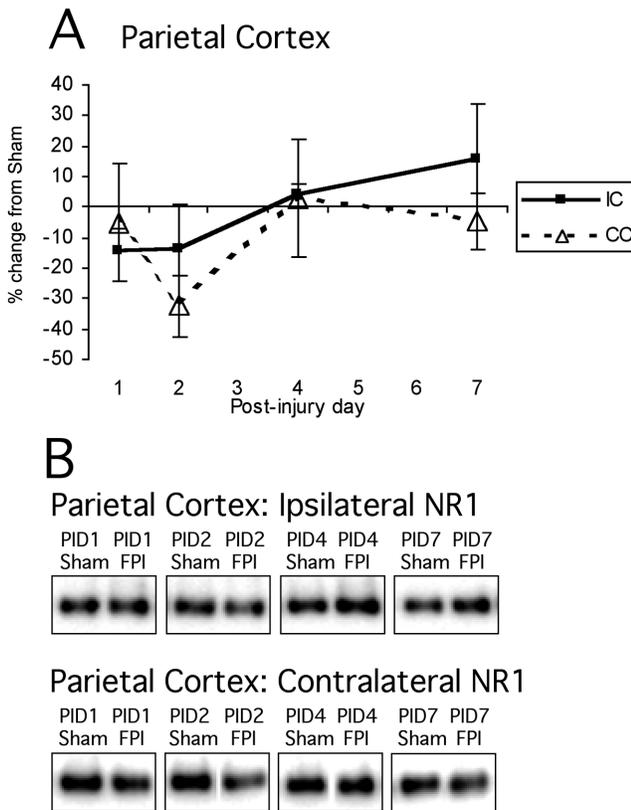


FIG. 1. Mean percent change (\pm SEM) of NR1 in lateral fluid percussion-injured (FPI) rats in parietal cortex (A) at post-injury day (PID) 1, 2, 4, and 7 days post-injury, as compared to age-matched shams (defined as 0%), with representative immunoblots of pooled sham and lateral FPI ipsilateral hippocampal synaptoneurosome at PID1, PID2, PID4, and PID7 (B). No overall main effects of injury were detected for any region. IH, ipsilateral hippocampus; CH, contralateral hippocampus; IC, ipsilateral cortex; CC, contralateral cortex.

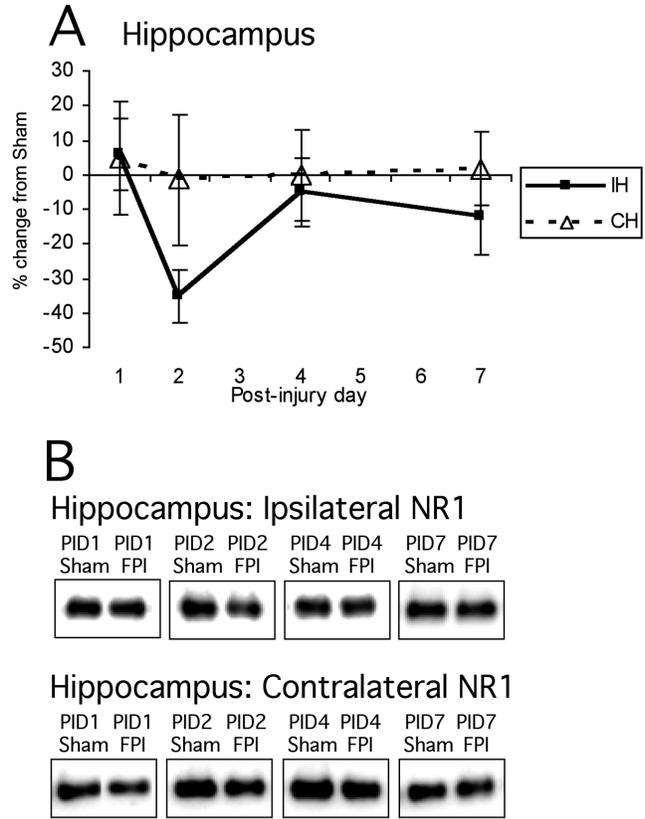


FIG. 2. Mean percent change (\pm SEM) of NR1 in lateral FPI rats in hippocampus (A) on post-injury day (PID) 1, 2, 4, and 7 as compared to age-matched shams (defined as 0%), with representative immunoblots of pooled sham and lateral FPI ipsilateral hippocampal synaptoneurosome at PID1, PID2, PID4, and PID7 (B). No overall main effects of injury were detected for any region. For abbreviations, see legend to Figure 1.

Normalized NR1 signals for hippocampus were as follows: ipsilateral sham 100 ± 5.7 , ipsilateral FP 87.7 ± 5.2 , contralateral sham 100 ± 6.4 , and contralateral FP 100.5 ± 8.4 . As in cortex, there was no overall effect of hemisphere, PID, or interaction between these variables (Fig. 2A,B).

NMDAR2A. Normalized NR2A signal in cortex showed a significant main effect of injury ($F_{1,48} = 4.74$, $p < 0.05$; Fig. 3A,B). There was no significant main effect of hemisphere ($F_{1,48} = 0.14$, NS) or interaction between hemisphere and injury ($F_{1,48} = 0.14$, NS) or hemisphere and PID ($F_{1,48} = 0.09$, NS). Thus, there was clearly a post-injury reduction in cortical NR2A that was essentially similar bilaterally (Fig. 3A,B).

Descriptive analysis of each individual post-injury time point showed a 27.7% reduction of NR2A levels in

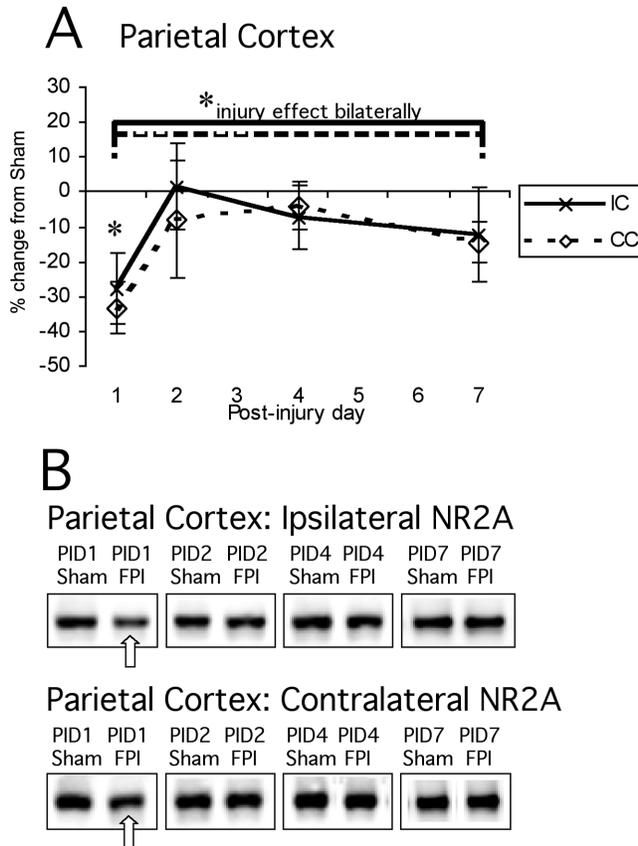


FIG. 3. Mean percent change (\pm SEM) of NR2A in lateral FPI rats in the parietal cortex (A) at post-injury day (PID) 1, 2, 4, and 7 as compared to age-matched shams (defined as 0%). Representative immunoblots of pooled synaptoneurosomes from sham and FPI ipsilateral and contralateral cortices (B) are shown at the same time points. There were significant main effects of injury on NR2A levels (analysis of variance, $p < 0.05$), but no difference between hemispheres across all time points. When individual time points were tested, on PID1 there was a reduced level of NR2A in contralateral cortex (-33% , $*p < 0.05$) and a trend in ipsilateral cortex (-27% , $p < 0.13$). For abbreviations, see legend to Figure 1.

ipsilateral cortex on PID1 that did not achieve statistical significance ($p < 0.13$); on PID2, PID4, and PID7, cortical NR2A in FP animals was indistinguishable from shams. Contralateral cortex also showed a reduction on PID1, and this decrease of 33.3% was significant at this time point ($p < 0.01$), before returning to age-matched sham levels on PID2, PID4, and PID7 (Fig. 3A,B).

In hippocampus, NR2A protein signals showed a main effect of hemisphere ($F_{1,46} = 8.5$, $p < 0.01$) and significant interactions between hemisphere and injury ($F_{1,46} = 5.2$, $p < 0.05$) and hemisphere and PID ($F_{1,46} = 3.0$, $p < 0.05$). This indicates that NR2A levels change differen-

tially in ipsilateral and contralateral hippocampus after FP, and that there is a significant effect of time post-injury on hippocampal NR2A levels. Even with both hemispheres included, there was a strong trend towards an overall effect of injury ($F_{1,46} = 3.6$, $p = 0.06$; Fig. 4A,B).

Descriptive analysis of each individual post-injury time point showed reductions in ipsilateral hippocampal NR2A at all times (-9.9% at PID1, -47.9% at PID2, -40.8% at PID4, -6.3% at PID7). The decreases were greatest at PID2 (sham 100 ± 17.6 , FP 52.1 ± 11.9 , $p = 0.05$) and PID4 (sham 100 ± 14.3 , FP 59.2 ± 9.9 , $p < 0.05$; Fig. 4A,B). Contralateral hippocampal NR2A did not differ between injured animals and age-matched shams at any post-injury time-point.

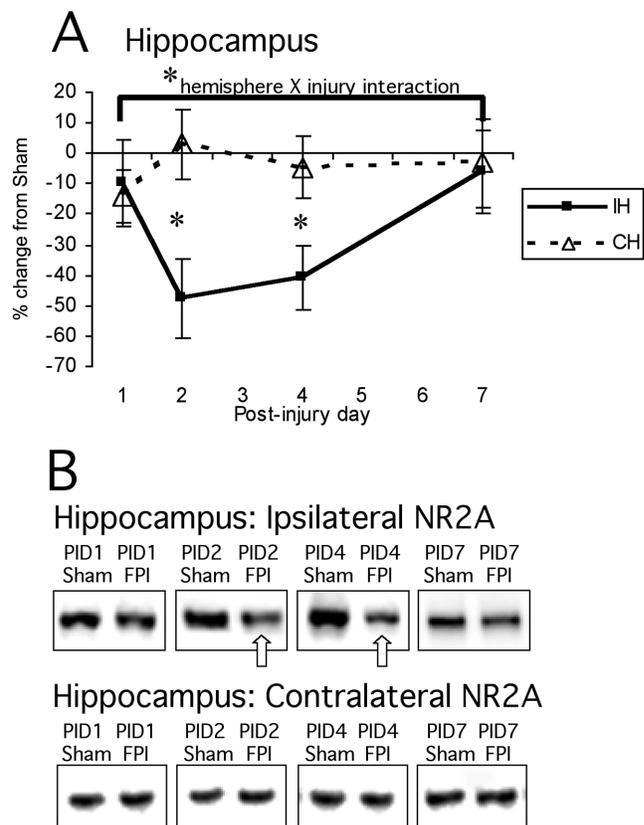


FIG. 4. Mean percent change (\pm SEM) of NR2A in lateral FPI rats in the hippocampus (A) at post-injury day (PID) 1, 2, 4, and 7 as compared to age-matched shams (defined as 0%). Representative immunoblots of pooled synaptoneurosomes from sham and FPI ipsilateral and contralateral hippocampi (B) are shown at the same time points. There was a significant main effect of hemisphere (analysis of variance, $p < 0.01$), as well as significant interactions between hemisphere and injury ($p < 0.05$) and hemisphere and PID ($p < 0.05$). When individual time points were tested, NR2A showed a 47.9% reduction on PID2 ($*p < 0.05$) and a 40.8% reduction on PID4 ($*p < 0.05$). For abbreviations, see legend to Figure 1.

NMDAR2B. NR2B protein levels showed a distinctly different post-injury profile from NR2A (Figs. 5A,B and 6A,B). Normalized NR2B signal did not show any significant effect of injury in cortex ($F_{1,48} = 1.23$, NS) or hippocampus ($F_{1,48} = 0.62$, NS). There were no effects of hemisphere or interactions between hemisphere and injury, or hemisphere and PID in either cortex or hippocampus, so no descriptive statistics were performed. NR2B levels did not change significantly after FP injury in any brain region analyzed (cortex: sham 100 ± 4.7 , FP 93.8 ± 4.8 ; hippocampus: sham 100 ± 5.7 , FP 94.9 ± 5.4).

Real-Time RT-PCR

To determine whether alterations in gene expression level could be responsible for the reductions in NMDAR

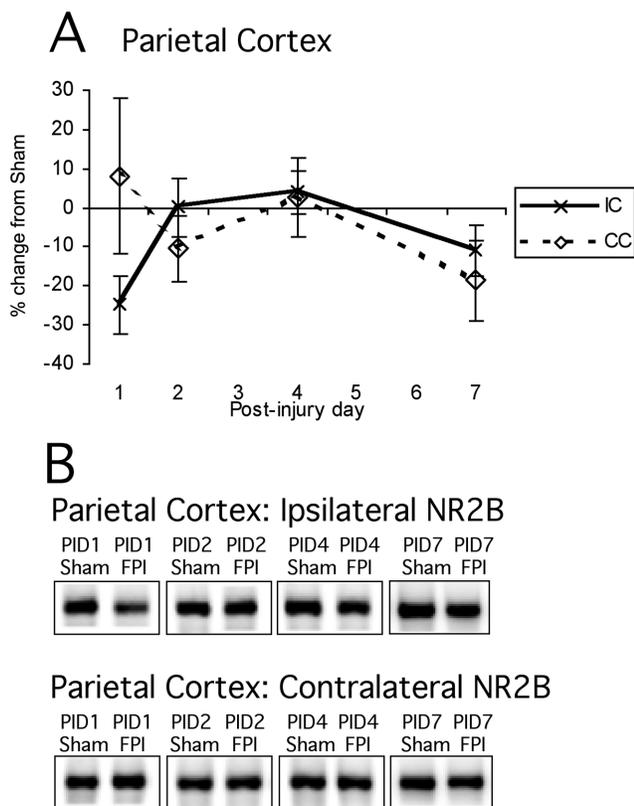


FIG. 5. Mean percent change (\pm SEM) of NR2B in lateral FPI rats in parietal cortex (A) at post-injury day (PID) 1, 2, 4, and 7 as compared to age-matched shams (defined as 0%). Again, representative immunoblots of pooled synaptically enriched sham and FPI ipsilateral and contralateral cortices (B) are shown for PID1, PID2, PID4, and PID7. Time course of post-injury NR2B cortical protein expression shows no significant main effects. For abbreviations, see legend to Figure 1.

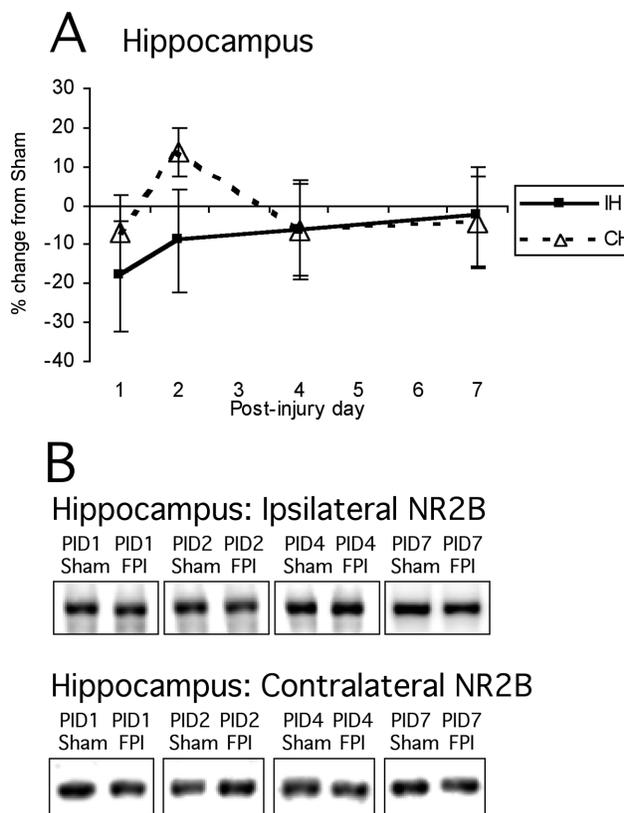


FIG. 6. Mean percent change (\pm SEM) of NR2B in lateral FPI rats in hippocampus (A) at post-injury day (PID) 1, 2, 4, and 7 as compared to age-matched shams (defined as 0%). Again, representative immunoblots of pooled synaptically enriched sham and FPI ipsilateral and contralateral hippocampi (B) are shown for PID1, PID2, PID4, and PID7. Time course of post-injury NR2B hippocampal protein expression shows no significant changes. For abbreviations, see legend to Figure 1.

subunit protein expression detected after developmental TBI, real-time RT-PCR was performed on RNA harvested from a parallel group of animals. Analysis of relative gene expression using primers for each of the NMDAR subunits did not show any changes greater than twofold after developmental lateral FP injury (Table 2). However, as a positive control, RT-PCR for a gene known to be altered by traumatic brain injury, heat shock protein 70, did show a significant upregulation in both ipsilateral cortex and hippocampus at PID1 (2.56- and 2.05-fold induction, respectively).

DISCUSSION

The primary finding of this study is that developmental lateral FPI results in a preferential downregulation of

TABLE 2. RELATIVE GENE EXPRESSION (FOLD-CHANGE OF INJURED VERSUS SHAM) FOR CORTEX AND HIPPOCAMPUS ON POST-INJURY DAYS 1, 2, AND 4

	<i>PID1</i>	<i>PID2</i>	<i>PID4</i>
Hsp70			
IC	2.56		
IH	2.05		
NR1			
IC	-1.00	-1.09	1.03
CC	-1.30	1.11	1.00
IH	-1.11	-1.03	-1.01
CH	-1.30	1.22	-1.03
NR2A			
IC	-1.27	-1.24	-1.08
CC	-1.30	-1.07	-1.06
IH	-1.42	-1.02	-1.08
CH	-1.28	1.06	-1.04
NR2B			
IC	1.43	-1.10	1.03
CC	1.08	-1.21	-1.18
IH	-1.62	1.10	1.06
CH	-1.26	-1.17	-1.14

Hsp 70 showed greater than twofold change in ipsilateral cortex and hippocampus, as a positive control. None of the *N*-methyl-D-aspartate receptor subunits (NR1, NR2A, NR2B) showed a twofold change (up or down).

the NMDAR subunit, NR2A, in a synaptic preparation. This effect was seen bilaterally in cortex, particularly early after injury (*PID1*); however, it was most profound in the ipsilateral hippocampus, where it was significantly reduced as late as *PID4*. NR1 and NR2B levels did not change significantly over the course of the first post-injury week. While these injury-induced changes were evident at the protein level, the relative levels of transcription of the NMDAR subunit mRNAs did not show consistent and significant reductions.

Functionally, increased NR2A levels allow for a more precise glutamatergic signal by altering kinetics of the NMDAR (Flint et al., 1997; Roberts and Ramoa, 1999). Enhanced NR2A gene expression during development occurs in response to environmental differences such as more nurturing maternal nursing and grooming behavior (Liu et al., 2000) and the change from dark rearing to light (Quinlan et al., 1999a,b). Increased NR2A also correlates with functional changes in the NMDAR complex in developing visual cortex (Roberts and Ramoa, 1999), parietal cortex (Flint et al., 1997; Kew et al., 1998), hippocampus (Gottmann et al., 1997), and cerebellum (Takahashi et al., 1996). It appears, then, that increases in NR2A may be a fundamental response to differential experience, and a crucial part of normal maturation of

neuronal circuitry. Furthermore, it appears that NR2A expression in adults may also be responsive to environmental stimulation, as it is capable of being suppressed into adulthood by dark rearing (Quinlan et al., 1999a) or induced in adults by voluntary exercise (Molteni et al., 2002).

Previous studies of traumatic brain injury in developing animals have demonstrated a reduction in experience-dependent plasticity induced by rearing in an EE. PND19 rats subjected to lateral FP injury prior to rearing in EE do not show the cognitive enhancements manifest in sham-injured pups reared in EE (Fineman et al., 2000). Furthermore, increases in cortical depth and expansion of dendritic arbors seen in sham-injured/EE reared animals are mitigated when EE rearing follows early FP injury (Fineman et al., 2000; Ip et al., 2002).

Given the role played by NMDAR in normal and experience-dependent plasticity, and the fact that excessive NMDAR activation is a proven component of the pathophysiological cascade following TBI, it is reasonable to hypothesize a mechanism of inhibited plasticity after early TBI that involves alterations in the NMDAR. This could occur simply through a reduction in NMDAR numbers (and be manifest by diminished NR1 levels) or by a change in proportion of NR2 subunits.

Our results show no lasting change in synaptic NMDAR numbers by demonstrating no decline in protein levels of the NR1 subunit in cortex and hippocampus following PND19 lateral FP injury. This is corroborated by qualitative RT-PCR for NR1 expression, which also shows no significant change over *PID1*–4.

Besides reduction in numbers of NMDARs, NMDAR function may also be affected by changes in subunit composition. As discussed above, increased NR2A naturally occurs in response to various forms of environmental stimulation. The results presented here reveal substantial reductions of the NR2A subunit during the 1st week post-injury, particularly in ipsilateral hippocampus. It follows, then, that environment-induced increases in NR2A may be inhibited in this post-injury period. Further studies are ongoing to investigate the effects of EE rearing on NR2A levels in sham-injured and FP injured animals.

The selective loss of synaptic NR2A protein could occur in several ways. First, NR2A expression may be inhibited at the level of transcription. However, our results with RT-PCR suggest that this is not the case. Alternatively, synaptic NR2A protein turnover might increase following TBI. This could be mediated by an increase in NR2A subunit-specific turnover, with NR2A receptors either being selectively endocytosed for proteolysis or by specific translocation of NR2A-containing receptors away from the synapse (Carroll and Zukin, 2002). Since NR2A is rapidly being integrated into maturing synapses

at this developmental stage, it follows that this process would be vulnerable to a diffuse pathophysiological process such as TBI. The apparent 1–2-day delay in the reduction of synaptic NR2A suggests that the release of NR2A-containing NMDAR from synapses may be a slower, multi-step process. Such a contention is supported by literature showing the relative stability of NR2A-containing NMDARs when localized in the post-synaptic density (Lavezzari et al., 2004).

An additional factor in interpreting NMDAR subunit changes after brain injury is the likelihood of significant cell death, perhaps with selective loss of NR2A-rich neurons. Adult FP injury leads to significant levels of neuronal loss (Conti et al., 1998). However, FP injury in immature animals is distinct from adult FP injury, in that concussed preweanling rat pups demonstrate minimal gross anatomic change or loss of neurons (Fineman et al., 2000; Gurkoff et al., 2006; Prins et al., 1996). Long-standing neurocognitive deficits in a setting of substantial cell death may be due both to the loss of cells as well as impaired function of the surviving cells. However, in this model of developmental FP injury, we report that significant dysfunction of an excitatory neurotransmitter system can occur with little or no cell death, and propose this as a primary mechanism for previously described deficits in experience-dependent plasticity after FP (Fineman et al., 2000; Giza et al., 2005; Ip et al., 2002).

The concept that a brain insult during development leads to chronic dysfunctional neurotransmission is also supported by studies of neonatal NMDA blockade. Chronic administration of the noncompetitive NMDA antagonist, MK-801 to rat pups results in deficits in glutamatergic and monoaminergic neurotransmission later in development (Bellinger et al., 2002; Gorter et al., 1992). Similarly, prenatal exposure to ethanol leads to decreased NR2A and NR2B expression in rat forebrain into the 3rd postnatal week (Hughes et al., 1998). Developmental exposure to lead is also associated with altered NMDAR subunit composition in hippocampus up to PND28 (Nihei and Guilarte, 1999). Interestingly, the lead-exposed rats demonstrated reduced NR2A protein levels in a setting of NR2B levels indistinguishable from age-matched controls (and thus a *decreased* 2A:2B ratio), and other studies clearly show cognitive deficits as a result of developmental lead exposure (Jett et al., 1997; Kuhlmann et al., 1997). This result is consistent with our findings following developmental brain injury, where disruption of neurotransmission during hippocampal maturation leads to reductions in NR2A subunit composition without significant alterations in NMDAR number (as determined by NR1 immunoreactivity) or NR2B expression.

This suggests that developmental deficits and impaired plasticity may occur when the NR2A/NR2B ratio devi-

ates substantially from normal proportions. Alternative mechanisms of impairment include the possibility that NMDAR subunit types not normally seen in specific brain regions may be expressed after insults, such as the induction of NR2C following ischemia (Small et al., 1997), and that the effect of a change in NMDAR subunit composition may depend upon the relative developmental stage of the brain region at the time of injury. In this case, the structure undergoing more marked maturational change (hippocampus) may be more susceptible to derangement in neural activity and thus more likely to demonstrate persistent dysfunction.

In summary, we report that following developmental TBI there is a subunit-specific reduction in synaptic NR2A. These alterations of NR2 composition occur transiently in neocortex and persist at least for 4 days after injury in ipsilateral hippocampus. The changes in protein described do not appear to be mediated at the transcriptional level. We suggest that significant dysfunction of excitatory neurotransmission can occur after moderate traumatic injury to the immature brain, probably via decreased synaptic stability of existing NR2A-containing NMDARs. This is an appealing mechanism for post-traumatic deficits in plasticity and resulting neurocognitive impairment.

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