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INJURY-INDUCED ALTERATIONS IN *N*-METHYL-D-ASPARTATE RECEPTOR SUBUNIT COMPOSITION CONTRIBUTE TO PROLONGED ^{45}CALCIUM ACCUMULATION FOLLOWING LATERAL FLUID PERCUSSION

C. L. OSTEEN,[a,b] C. C. GIZA[a,c,d] AND D. A. HOVDA[a,c,e]*

[a]*Brain Injury Research Center, Division of Neurosurgery/Department of Surgery, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA*

[b]*Interdepartmental Program in Molecular, Cellular, and Integrative Physiology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA*

[c]*Brain Research Institute, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA*

[d]*Division of Neurology/Department of Pediatrics, David Geffen School of Medicine at UCLA, Mattel Children's Hospital at UCLA, Los Angeles, CA 90095-7039, USA*

[e]*Department of Medical and Molecular Pharmacology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA*

Abstract—Cells that survive traumatic brain injury are exposed to changes in their neurochemical environment. One of these changes is a prolonged (48 h) uptake of calcium which, by itself, is not lethal. The *N*-methyl-D-aspartate receptor (NMDAR) is responsible for the acute membrane flux of calcium following trauma; however, it is unclear if it is involved in a flux lasting 2 days. We proposed that traumatic brain injury induced a molecular change in the NMDAR by modifying the concentrations of its corresponding subunits (NR1 and NR2). Changing these subunits could result in a receptor being more sensitive to glutamate and prolong its opening, thereby exposing cells to a sustained flux of calcium. To test this hypothesis, adult rats were subjected to a lateral fluid percussion brain injury and the NR1, NR2A and NR2B subunits measured within different regions. Although little change was seen in NR1, both NR2 subunits decreased nearly 50% compared with controls, particularly within the ipsilateral cerebral cortex. This decrease was sustained for 4 days with levels retuning to control values by 2 weeks. However, this decrease was not the same for both subunits, resulting in a decrease (over 30%) in the NR2A:NR2B ratio indicating that the NMDAR had temporarily become more sensitive to glutamate and would remain open longer once activated. Combining these regional and temporal findings with ^{45}calcium autoradiographic studies revealed that the degree of change in the subunit ratio corresponded to the extent of calcium accumulation. Finally, utilizing a combination of NMDAR and NR2B-specific antagonists it was determined that as much at 85% of the long term NMDAR-mediated calcium flux occurs through receptors whose subunits favor the NR2B subunit. These data indicate that TBI induces molecular changes within the NMDAR, contributing to the cells' post-injury vulnerability to glutamatergic stimulation.

Key words: calcium, glutamate, ifenprodil, MK-801, *N*-methyl-D-aspartate receptor, traumatic brain injury.

Calcium accumulation is an important feature in many types of brain injury (Araki et al., 1990; Cortez et al., 1989; Dienel, 1984; Dubinsky and Rothman, 1991; Katayama et al., 1991; Rappaport et al., 1987; Shirotani et al., 1994) including trauma (Fineman et al., 1993; Osteen et al., 2001; Thomas et al., 1990). Although voltage-gated Ca^{++} channels and release of intracellular stores contribute, the *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) is the major conduit of the post-traumatic calcium load (Bullock and Fujisawa, 1992; Nadler et al., 1995) that endures for at least 2 days following mild-moderate lateral fluid percussion injury in the adult rat (Fineman et al., 1993; Osteen et al., 2001). The NMDAR is also known to be involved in the post-traumatic neurochemical and metabolic cascade, being excessively activated by the indiscriminant release of excitatory amino acids that occurs immediately following traumatic brain injury (Faden et al., 1989; Katayama et al., 1990). Over-activation of the NMDAR is directly related not only to post-injury ionic fluxes (like calcium influx), but also to increases in the metabolic demand for glucose utilization (to restore ionic homeostasis) and excitotoxicity (due to calcium overload and/or energy failure; Hovda, 1996).

Recent descriptions of the NMDAR subunits have provided a molecular understanding of how this receptor functions (Flint et al., 1997; Ishii et al., 1993; Scheetz and Constantine-Paton, 1994; Yamakura and Shimoji, 1999). The NMDAR is a heteromeric assembly, thought to be comprised of two NMDAR subunits (NR1; Behe et al., 1995) and two or three NR2 subunits (Dingledine et al., 1999; Ferrer-Montiel et al., 1995). The fraction of lone NR1 or NR2 subunits is small (Luo et al., 1997), indicating the subunits' preferential incorporation into functional channels. NR1 is the principal constituent of the NMDA channel, homogeneously distributed in the brain, that alone can generate a functional pore *in vitro* (Moriyoshi et al., 1991). The NR2 subunit is the regulatory component, with each of

*Correspondence to: D. A. Hovda, Division of Neurosurgery, David Geffen School of Medicine of UCLA, Room 18–228 NPI, Box 957039, Los Angeles, CA 90095–7039, USA. Tel: +1-310-206-3650; fax: +1-310-794-2147.
E-mail address: dhovda@mednet.ucla.edu (D. A. Hovda).

Abbreviations: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; ANOVA, analysis of variance; L, left (ipsilateral to injury); MK-801, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo(*a*,*d*)cyclohepten-5,10-imine hydrogen maleate; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; NR, *N*-methyl-D-aspartate receptor subunit; PSD, postsynaptic density protein; R, right (contralateral to injury); ROI, regions of interest.

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the four subtypes (NR2A, 2B, 2C and 2D) being differentially distributed in the brain and imparting specific electrophysiological properties to the NMDA channel (Healy and Meador-Woodruff, 2000; Ishii et al., 1993; Scheetz and Constantine-Paton, 1994; Seeburg et al., 1994; Wang et al., 1995; Yamakura and Shimoji, 1999). For example, cortical neurons with NMDARs containing more NR2B than NR2A (a low NR2A:NR2B ratio) are more sensitive to glutamate, open for a longer amount of time, and conduct larger currents than a population with a high NR2A:NR2B ratio. Furthermore, NR1/NR2B receptors lack calcium-dependent inactivation that is prominent in NR1/NR2A receptors (Yamakura and Shimoji, 1999) and thus may make an important contribution to the prolonged calcium influx.

The notion that NMDAR subunit composition (i.e. NR2A:NR2B ratio) dictates the channel's function is supported in the neuroplasticity, aging, and transgenic literature. Specifically, neonatal NMDARs are rich in NR2B, but as the immature brain develops, the NR2A:NR2B ratio increases by a disproportionate increase in expression of NR2A. These changes make NMDARs less active both in terms of the sensitivity to glutamate and the NMDA excitatory post synaptic current (Flint et al., 1997; Quinlan et al., 1999; Scheetz and Constantine-Paton, 1994). Moreover, aged rats have significantly lower levels of NR2B mRNA and protein than younger animals (Clayton and Browning, 2001; Magnusson et al., 2002) and this high NR2A:NR2B ratio correlates to aged rats' deficits in long-term potentiation and learning/memory. Transgenic studies have likewise shown that knocking down NR2B in young rats replicates the impairment of NMDAR function (Clayton et al., 2002) and that overexpressing NR2B leads to enhanced learning and memory (Tang et al., 1999). Clearly, the subunit composition of the NMDAR is important in normal development, aging, and plasticity.

Previous studies using receptor binding techniques have suggested that the composition and function of the NMDAR might be altered after traumatic brain injury in regions near the primary injury site (Gorman et al., 1995; Miller et al., 1990; Sihver et al., 2001). However, these binding studies did not use the subunit-specific functional attributes of post-injury NMDARs to differentiate between the NR2 subtypes. Subunit-specific changes in NMDAR gene expression and protein expression have been better addressed following ischemia. Ninety minutes after ischemic injury, hippocampal gene expression displayed an increased NR2A:NR2B ratio (Small et al., 1997), perhaps indicating an acute down-regulation of NR2B in response to high glutamate levels. At 1 day post-ischemia, gene expression and protein levels of both NR2A and NR2B were decreased (Hsu et al., 1998; Zhang et al., 1997). These studies not only suggest that brain injury can induce dynamic changes in NMDAR subunit expression, but also reiterate the functional consequences of NMDAR subunit alterations, as injured NMDARs displayed differential electrophysiological properties as compared with shams (Hsu et al., 1998; Zhang et al., 1997).

Since traumatic brain injury activates NMDARs and results in a prolonged calcium influx, and since the NR2 subunit composition of the NMDAR directly reflects the function of the receptor, a change in NR2 subunit composition is a reasonable mechanism to explain calcium-related pathophysiology following traumatic brain injury. As suggested by calcium's prolonged influx into the ipsilateral cortex following traumatic brain injury, the injury may induce a shift toward a more physiologically active composition of the NMDAR. For example, an injury-induced reduction of the NR2A:NR2B ratio could result in increased sensitivity to glutamate, lowering the activation threshold for the neuron, as well as numerous downstream consequences from excessive activation of the NMDAR, such as increased calcium influx. In fact, following *in vitro* injury of cortical neurons, significantly larger ionic currents and calcium influx occur through the NMDAR in response to NMDA application (Zhang et al., 1996). At this time, it is unknown if the NMDAR subunits are altered following lateral fluid percussion brain injury. The primary purpose of the present study was to determine the regional and temporal profile of the NR2 subunit composition of the NMDAR following mild-moderate lateral fluid percussion brain injury in the adult rat and to investigate if injury-induced alterations in NMDAR subunit composition contribute to post-traumatic calcium flux.

EXPERIMENTAL PROCEDURES

Subjects

Ninety-two male Sprague–Dawley adult rats (200–250 g) were obtained from Charles River Laboratory (Rolley 10 facility; Raleigh, NC, USA) for this study. To determine the temporal and regional profile of brain injury-induced changes in NR2A and NR2B subunits of the NMDAR, rats were studied at four time points after sham surgery or lateral fluid percussion injury: 1 day (sham $n=5$, injured $n=6$), 2 days ($n=5$, 6), 4 days ($n=5$, 6), and 14 days ($n=5$, 6). To confirm that acute changes of NMDAR subunits were indeed affecting the synapse, NR1, NR2A, and NR2B subunits were studied in synaptically enriched fractions at 18–24 h post-injury in 11 additional rats (sham $n=5$, injured $n=6$). To evaluate the functional significance of NMDAR subunit changes on the regional extent of $^{45}Ca^{++}$ accumulation, rats were randomly assigned to two time points after injury (1 or 2 days following lateral fluid percussion injury) and administered MK-801 (0.3 mg/kg, i.p.: 1 day $n=5$, 2 days $n=5$), ifenprodil (30 mg/kg, i.p.; $n=5$, 5), or saline ($n=5$, 5). All experimental animal protocols were approved by the UCLA Chancellor's Committee for Animal Research. All experiments described herein conform to all national and international guidelines as outlined in the United States Department of Agriculture Animal Welfare Act, the Public Health Service Policy on Human Care and Use of Laboratory Animals, and by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Consequently, procedures were employed to reduce pain and suffering as well as to minimize the number of animals used.

Fluid percussion injury

Animals were anesthetized with 1.5–2.0 ml/min of enflurane in 100% oxygen, placed in a stereotaxic frame, and prepped in the usual sterile fashion. Body temperature was kept constant at 36.6–38.0 °C with a thermostatically controlled heating pad. A

midline incision was made in the scalp, the temporalis muscle retracted, and the cranium exposed. A craniotomy 3.0 mm in diameter was made 3.0 mm posterior to bregma and 6.0 mm lateral (left) to midline. The injury cap was secured with silicone adhesive and dental cement and, after hardening, filled with 0.9% saline. Anesthesia was removed and the animal was attached to the fluid percussion device (McIntosh et al., 1989). Once the animal responded to a toe pinch, a 2.65–2.75 atm fluid pulse was administered. This type of injury has been described in detail elsewhere (Dixon et al., 1987; Katayama et al., 1990; McIntosh et al., 1989; Prins et al., 1996) and physiological responses to lateral fluid percussion injury have been characterized in adult rats (Dixon et al., 1988). In brief, the fluid percussion device delivers a transient (21–23 ms) fluid pulse into the epidural space causing diffuse biomechanical deformation. Apnea and unconsciousness (as measured by withdrawal response to toe pinch) times were recorded and the injured animal was returned to anesthesia for removal of the injury cap and suturing of the scalp. The sham animals received the same surgical procedures (including craniotomy), but were not administered the fluid pulse. All animals were monitored for up to 3 h after surgery and returned to their home cages when stable.

Homogenate preparation

At 1, 2, 4, and 14 day time points post-injury animals for the NMDAR subunit characterization were killed and their brains removed. These fresh brains were identically sectioned into anterior (+4.0 mm to 0.0 mm from bregma), middle (0.0 mm to −4.0 from bregma), and posterior (−4.0 mm to −8.0 mm from bregma) portions with a brain slicer and bisected into hemispheres ipsilateral and contralateral to the site of fluid pulse administration. Regions of interest (ROI) were isolated on a chilled surface: frontal cortices (from the anterior brain section), parietal cortices (from the middle section), thalami (from the middle section), hippocampi (from both the middle and posterior sections), and occipital cortices (from the posterior section). All ROIs were individually homogenized in PBS buffer containing Complete protease inhibitors (Roche, Foster, CA, USA).

Synaptically enriched fraction

Brains from a separate group of animals studied at 18–24 h following lateral fluid percussion injury were processed to determine the NMDAR subunit characterization at the synapse. After the cerebellum was discarded, these fresh brains were sectioned using a brain slicer, isolating the parietal cortices (0.0 mm to −4.0 from bregma) and hippocampi (−2.0 mm to −6.0 mm from bregma) on a chilled surface. ROI were individually homogenized in PBS buffer containing protease inhibitors. Subcellular fractionation was performed to purify the synaptosomal plasma membrane/mitochondrial fraction in the following manner (Hens, 1997). ROI homogenates were centrifuged at 1000 g for 10 min. After carefully collecting the supernatant, the P1 pellet (containing glia, myelin, nuclei, cell debris, and blood cells) was discarded. The S1 fraction was then centrifuged at 10,000×*g* for 20 min. The supernatant was discarded, and the P2 pellet (the crude synaptosomal/mitochondrial pellet) was reconstituted in PBS buffer with protease inhibitors.

Western blotting

Protein determination analysis was conducted on all samples using the DC Protein Assay (BioRad, Hercules, CA, USA). Ten milligrams of protein from each sample was loaded into individual wells, in randomized order, of a 7.5% polyacrylamide denaturing Ready Gel (BioRad). Each gel was specific for time point and ROI, containing 11 lanes of samples (five sham and six injured), and was duplicated for each subunit investigated. After electrophoresis, protein was transferred to nitrocellulose blotting paper (1 h at 100 V) using the Mini-Trans-Blot apparatus (BioRad). Western blots were incubated in blocking buffer (1 h), washed, and incubated in primary antibody (NR1, NR2A, or NR2B) overnight at room temperature. After incubating in secondary antibody (1 h) and washed, blots were developed using Super West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA). Bands were compared with molecular weight markers, confirming the NR1 subunit at approximately 100 kDa and NR2A and NR2B at approximately 180 kDa. Chemiluminescent images were captured with the Fluor-S Max Multimager (BioRad) and protein bands were volumetrically analyzed by a blinded investigator (C.O.) using Quantity One software (BioRad).

Pharmaceuticals

In order to investigate the role that NMDAR subunit composition plays in the post-traumatic accumulation of calcium, two distinct pharmaceuticals were used to differentially block the NMDAR during the uptake of $^{45}Ca^{++}$ at 1 or 2 days post-injury. MK-801 (0.3 mg/kg) is a noncompetitive NMDAR antagonist that is non-subunit-selective, while ifenprodil [α-(4-hydroxyphenyl)-β-methyl-4-benzyl-1-piperidineethanol] (30 mg/kg) is a noncompetitive NMDAR antagonist that is selective for NR2B subunit-containing NMDARs (Coughenour and Barr, 2001; Kew et al., 1996; Williams, 1993). MK-801, ifenprodil, and saline (all 10 ml/kg i.p.) were administered 10 min prior to radioisotope administration and again 2 h later, to ensure continuous antagonist action of the compounds during the 5 h $^{45}Ca^{++}$ uptake period (Dienel, 1984). Gross behavioral assessment was conducted during that time frame to confirm activity of the drugs. The doses used in the current study were selected by binding, biochemical, and behavioral data from other studies (Chizh et al., 2001; Dawson et al., 2001; Dempsey et al., 2000; Dravolina et al., 2000; Guzikowski et al., 2000; Healy and Meador-Woodruff, 2000; McIntosh et al., 1990; Migaud et al., 1998; Murray et al., 2000; Nash et al., 1999; Vezzani et al., 1989) with the purpose of maximizing specific antagonism at the NMDAR while minimizing nonspecific effects on other systems. Since during this 5 h uptake of $^{45}Ca^{++}$ (Dienel, 1984) the blood and brain levels of pharmaceuticals are changing (due to metabolism as well as booster shots) and since the rate of Ca^{++} exchange between blood and brain is unknown (Betz et al., 1989; Ghosh et al., 1997), the degree of Ca^{++} flux per time cannot be determined. Thus, appropriate dose response experiments could not be conducted. However, our goal was not to determine absolute values of calcium flux inhibited by these pharmaceuticals but rather to investigate region-specific patterns of a NR2B subunit-specific antagonist as compared with a non-subunit-specific antagonist, so the current experiment performed with the stated doses are valid to this end.

Histology

Brains of animals in the pharmaceutical/$^{45}Ca^{++}$ experiment were processed for histological analysis. The 20 μm fresh frozen brain sections (at 400 μm intervals) were stained with Cresyl Violet and examined under the light microscope. Since we were interested in calcium accumulation in viable cells, areas of contusion and/or gross reductions in neuronal abundance were noted and excluded from optical densitometry of ROIs.

Fluoro-Jade

Brains of animals in the pharmaceutical/$^{45}Ca^{++}$ experiment were processed for Fluoro-Jade (Schmued et al., 1997). This marker is positive within cells which experience a drop in intracellular pH and therefore is related to cellular stress. The regional extent of injured neurons, which may be destined for degeneration, was determined by gross characterization of Fluoro-Jade positivity

using a fluorescent microscope and FITC filter. Fluoro-Jade positive neurons were mapped in template-defined ROI, for a subset of saline, MK-801, and ifenprodil treated rats and qualitatively compared between groups.

$^{45}Ca^{++}$ autoradiography

In order to determine the regional accumulation of $^{45}Ca^{++}$ following mild lateral fluid percussion injury during differential antagonism of the NMDAR, $^{45}Ca^{++}$ autoradiography was performed similarly to the procedures previously reported (Fineman et al., 1993; Osteen et al., 2001). At either 1 or 2 days post-injury, the animal was anesthetized and prepared for surgery (as above). The tibial vein was exposed and 10 min after an i.p. injection of the appropriate pharmaceutical treatment (MK-801, ifenprodil, or saline), $^{45}Ca^{++}$ (1 μCi/g) in 0.9% saline was injected into the vessel. After suturing, the animal was allowed to recover and received a second i.p. injection of drug 2 h after the first. Five hours (Dienel, 1984) after isotope injection, the animal was killed by overdose of Nembutal (100 mg/kg). The brain was then quickly removed and frozen. Coronal 20 μm sections were cut using a cryostat. Five samples were taken every 400 μm: two were mounted on separate slides for histological (Cresyl Violet) and fluorescence analysis (Fluoro-Jade), while three consecutive sections were mounted on coverslips for autoradiography. Brain slices were exposed to Biomax film (Kodak, Rochester, NY, USA) for 4 days.

Autoradiographic ROI analysis

The autoradiographic images were analyzed using Scion Image at five ROI: frontal, parietal, and occipital cortices, hippocampus, and thalamus as defined by a template generated from an atlas (Paxinos and Watson, 1986). As described in our previous publications (Fineman et al., 1993; Osteen et al., 2001), this template acted only as a guide (see Fig. 6) so as to standardize measurements between sides across sections and between animals. Optical densities were measured bilaterally over the three consecutive sections at three to five structure-specific depths and relative ipsilateral optical density values were calculated by subtracting the contralateral (right) value from the ipsilateral (left) value, dividing by the sum (left+right) and multiplying by 100, as previously reported (Fineman et al., 1993; Osteen et al., 2001; Samii et al., 1999). This formula $[(L-R)/(L+R)\times 100]$ was used to reflect the ROI ipsilateral to the injury relative to the contralateral homotypic region, while controlling for the amount of radioactivity that entered the brain. Means and standard deviations were calculated for each structure at each time point and drug treatment.

To calculate the amount of calcium influx associated with the NMDAR ion channel and with the NR2B subunit-containing receptors, individual difference scores were calculated. Relative ipsilateral optical densities within ROI and group were ranked and individual differences between saline and MK-801 treated animals were calculated to represent the amount of calcium accumulation occurring through the NMDAR ion channel. Similarly, individual differences between saline- and ifenprodil-treated animals were calculated to represent the amount of calcium accumulation occurring through NR2B subunit-containing receptors. Individual percentages of NMDAR-associated calcium flux that occurs through NR2B-subunit containing receptors were then calculated from the difference scores.

Statistical analysis

Given that traumatic brain injury models can vary in terms of their severity, it is important to determine if the physiological variables that reflect severity were different between the different injury groups (assigned to different times post-insult). Consequently, the length of time in seconds for the duration of apnea and unconsciousness (as measured by determining if the animal would withdrawal its hindlimb when the corresponding paw was pinched) was compared across all injury groups using a one-way analysis of variance (ANOVA).

For the semi-quantitative Western blotting studies, all optical densities were normalized to sham levels. First, the mean volumetric optical density and variance was calculated for the sham group on each blot. Thereafter, the optical density of every experimental animal was normalized to sham controls on the same blot. This allowed for comparisons of subunit, time, and region between sham and experimental groups. Mean optical densities of each group (and S.E.M.) were then calculated for each blot. In addition, a relative ratio of NR2A:NR2B was calculated for each ROI and time point and normalized to sham levels in the same manner.

From this normalization procedure, group (injured or sham) and time post injury (1, 2, 4 or 14 days) were treated as between subject factors. Within each subject, 10 ROIs were selected. These ROIs consisted of the frontal, parietal, and occipital cortex along with the underling hippocampus and thalamus, with each structure being measured bilaterally. Then, within each ROI, the level of protein for each subunit was determined (NR2A, NR2B, or relative NR2A:NR2B ratio). Consequently, a univariate ANOVA was used with group (injury and sham) having two levels, time (four levels), regions having 10 levels and each subunit treated as the dependent variable of interest. For simple main effects, appropriate contrasts were assigned.

Upon completing these analyses, it was determined that most of the contribution to the significant main effects were due to changes of the subunits within the cerebral cortex ipsilateral to the side of the fluid pulse induction and to the corresponding contralateral parietal cortex. From this discovery, an ANOVA was conducted for each subunit within the ipsilateral parietal and occipital cortex as well as the contralateral parietal cortex. These post hoc comparisons were subjected to a Bonferroni adjustment so that the sum of significance levels for each overall comparison did not exceed $P<0.05$.

To confirm that subunit changes found in brain homogenates reflect changes at the synapse, normalized NR2A, NR2B, and NR2A:NR2B relative ratios were compared between 1 day homogenates and 18–24 h synaptically enriched fractions for each ROI. A *t*-test was used to compare between-preparation values in bilateral parietal cortices and hippocampi. In addition, intra-blot NR1 levels were compared between sham and injured synaptically enriched fractions by use of a *t*-test, for both the L and R parietal cortices.

To test if injury-induced regional alterations in NR2A:NR2B relative ratios were related to the extent of post-traumatic calcium accumulation in the days that follow lateral fluid percussion, a correlation was calculated. By use of Pearson's correlation coefficient, mean percentages of NR2A:NR2B reduction (as compared with shams) was compared with calcium's relative ipsilateral optical densities $[(L-R)/(L+R)\times 100]$ from our previous work (Osteen et al., 2001) for each region with complete data sets for each variable at 1 and 2 days post-injury.

For the pharmaceutical/$^{45}Ca^{++}$ experiment a multivariate ANOVA was performed to see if an overall effect existed between the one within-subject variable (ROI relative ipsilateral optical density) and the two between-subject variables [group (saline, MK-801, or ifenprodil) and time point (1 or 2 days after injury)], followed by Bonferroni's post hoc test.

For the ROI that exhibited a significant effect of group, relative ipsilateral optical densities were ranked in each group and difference scores were calculated to represent the amount of calcium flux occurring through NMDARs (saline–MK-801) and NR2B subunit-containing receptors (saline–ifenprodil) in these ROI. The difference scores were used to calculate individual percentages of NMDAR-associated calcium influx that occurred through NR2B subunit-containing receptors [(saline–ifenprodil/saline–MK-801)×100] for each region and time point

Table 1. Mean (±S.E.M.) apnea and unconsciousness times in seconds of each group following mild-moderate lateral fluid percussion injury[a]

Timepoint	Study	Treatment	Apnea	Unconsciousness
1 Day post-LFP	NMDAR	H	60±23	183±19
		S	14±3	153±33
	$^{45}Ca^{++}$	Saline	24±2	155±11
		MK-801	18±3	142±23
		Ifenprodil	33±7	140±17
2 Days post-LFP	NMDAR	H	19±9	120±18
	$^{45}Ca^{++}$	Saline	24±7	149±23
		MK-801	21±5	156±37
		Ifenprodil	29±7	167±33
4 Days post-LFP	NMDAR	H	39±24	168±30
14 Days post-LFP	NMDAR	H	44±16	145±18

[a] NMDAR stands for NMDAR subunit characterization study and $^{45}Ca^{++}$ stands for the pharmaceutical/$^{45}Ca^{++}$ autoradiography study. Two groups were studied for NMDAR characterization at 1 day post-injury, one processed for synaptically enriched fractions (S), the other for homogenates (H).

demonstrating a significant effect of group. These percentages were subjected to a univariate ANOVA with one within-subject variable (percentage of NMDAR-associated calcium occurring through NR2B subunit-containing receptors) and two between-subject variables (ROI and time), followed by Bonferroni's post hoc test.

All data analysis was conducted using SPSS 9.0 software and a level of significance of 0.05 was maintained throughout statistical analysis.

RESULTS

Injury severity

Seven lateral fluid percussion brain injured animals were excluded from the study due to complications from surgery/injury, resulting in five sham and six injured rats in each of the five groups for the NMDAR subunit characterization ($n=55$) and five rats in each of the six groups for the pharmaceutical/$^{45}Ca^{++}$ experiment ($n=30$) for a total of 85 rats completing the study. All animals were injured with a mild-moderate level of injury over the left parietal cortex (2.65–2.75 atm) and all demonstrated a brief period of apnea followed by a longer period of unconsciousness (Table 1). Statistical analysis revealed no difference in severity of injury between groups, as measured by apnea ($F_{10,49}=1.160$, $P=0.340$) and unconsciousness times ($F_{10,49}=0.516$, $P=0.871$). After recovering from anesthesia, all animals displayed normal exploratory, grooming, and feeding behavior in their home cages.

Subunit protein levels from brain homogenates

The 82 Western blots obtained were of sufficient quality to analyze for optical densities. Each blot was region and time specific, with 11 randomized experimental lanes (five sham and six lateral fluid percussion brain injured rats). Although the figures express the results as a percent of sham controls, all statistical analyses were conducted on the original data incorporating the variance within the sham group. Ten ROIs were measured bilaterally [ipsilateral (L) and contralateral (R)]. These included the frontal, parietal and occipital cortices, the hippocampus, and the thalamus. In cases where an artifact occurred over the location of desired data, the blinded investigator (C.O.) excluded that band from analysis. This occurred in less than 10% of the bands, with no systematic pattern evident among groups.

In general, changes in NR2A and NR2B levels were ROI and time-after-injury dependent. Animals that were subjected to a mild-moderate left-sided fluid percussion injury had a reduction in their levels of NR2A and NR2B as compared with shams, primarily in regions closest to the injury site (the ipsilateral cortex) within the first few days following injury, returning to control values by 14 days (Fig. 1). However, the magnitudes of reduction of NR2A and NR2B were not equal. In the affected regions, NR2A was reduced to a greater degree than was NR2B resulting in a decrease of the NR2A:NR2B relative ratio at 1 and 2 days post-injury. At 4 days post-injury, alterations in NR2A and NR2B were similar, so that the primary time frame when NR2A:NR2B was reduced was the first 2 days following injury (Fig. 1).

Using the full model, the between group variables consisted of group (injury or sham), subunit (NR2A, NR2B, NR2A:NR2B relative ratio) and time (1, 2, 4 and 14 days). ROI (frontal, parietal and occipital cortices, hippocampus and thalamus) were designated as within subject factors. Using a univariate ANOVA, the analysis revealed a significant main effect for group (injury versus sham; $F_{1,38}=4.238$, $P<0.001$) and time (1–14 days after injury; $F_{3,76}=1.996$, $P<0.017$). In addition, there was a main effect of subunit (NR2A, NR2B or the NR2A:NR2B relative ratio; $F_{2,76}=1.822$, $P<0.033$). Using appropriate contrasts for post hoc analysis, the main effect of group (injury vs sham) was primarily due to the changes in subunits following injury within the left (ipsilateral) parietal cortex ($F_{7,23}=20.809$, $P<0.001$), the left occipital cortex ($F_{7,23}=20.637$, $P<0.001$) and the right (contralateral) parietal cortex ($F_{7,23}=12.927$, $P=0.001$). There was no significant difference of the volumetric density of protein bands or relative NR2A:NR2B ratio measurements between lateral fluid percussion brain injured and sham-injured animals in the L frontal cortex (Fig. 1, Panel A), L hippocampus, L thalamus, R frontal

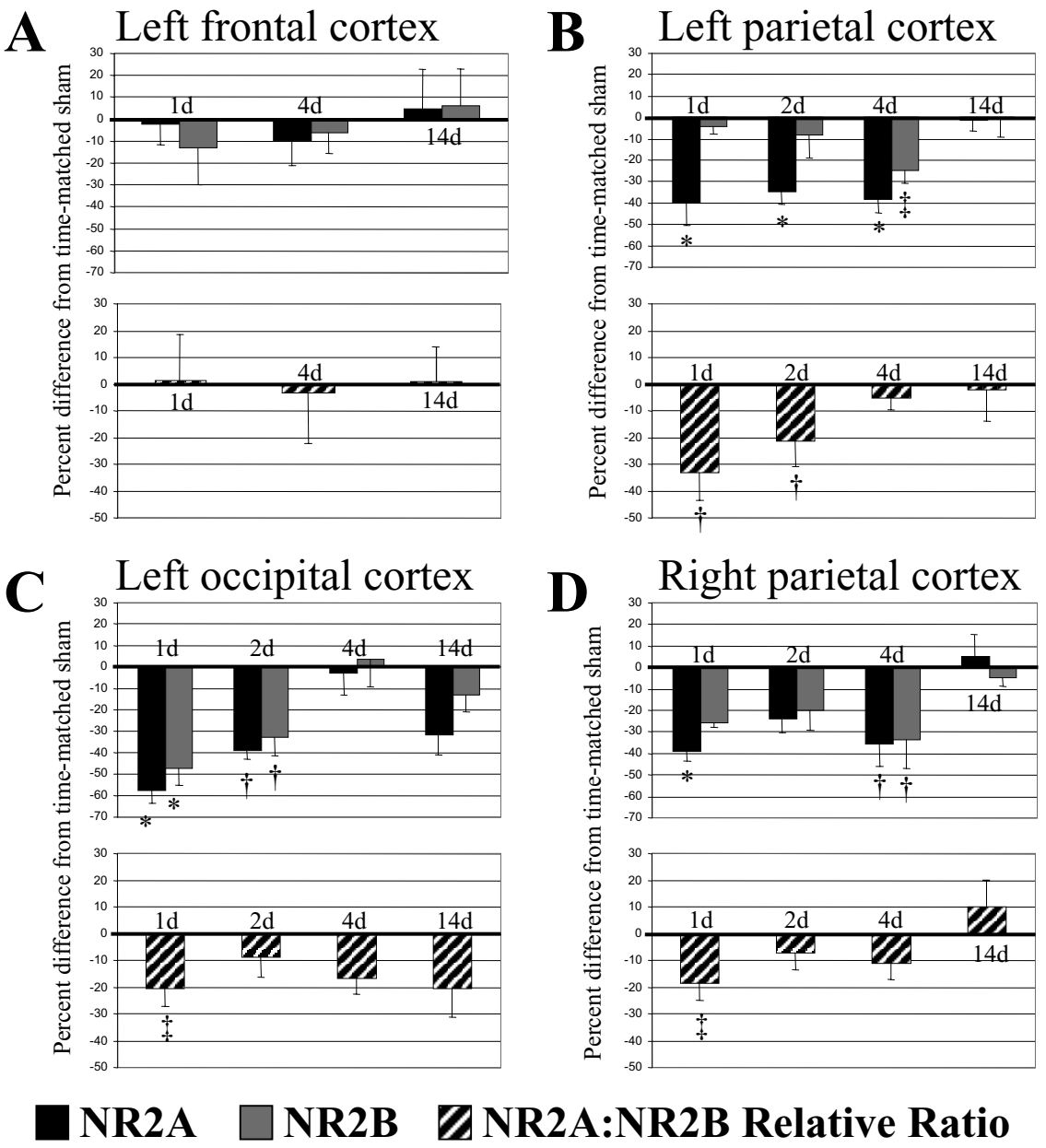

Fig. 1. Normalized group means (±S.E.M.) of NR2A, NR2B, and the NR2A:NR2B relative ratio in selected regions of interest, presented as percentage difference from sham levels with *x* axis being time after left-sided mild-moderate lateral fluid percussion injury (LFP). The left frontal cortex (A) is included as an example of a region whose NR2 subunits are unaffected by LFP, while the left parietal cortex (B) has a significant depression of the NR2A:NR2B relative ratio at 1 and 2 days post-injury. There is also a trend for the left occipital (C) and the right parietal (D) cortices' NR2A:NR2B relative ratio to be diminished 1 day post-injury. * $P<0.01$, † $P<0.05$ and ‡ $P\leq0.10$ (trend), as compared with time-matched shams.

cortex, R hippocampus, R occipital cortex and R thalamus.

Ipsilateral parietal cortex

Within the L parietal cortex, there was a significant reduction (35–40%) of the volumetric density of NR2A protein bands in the injured group at 1 day ($P<0.01$), 2 days ($P<0.01$), and 4 days ($P<0.01$) as compared with shams. Studies of the NR2B subunit revealed that within the same region there was also a trend toward reduction (25%) restricted to the fourth day after injury ($P<0.06$; Fig. 1).

When normalized relative ratios (NR2A:NR2B) were subjected to statistical analysis, only the L parietal cortex exhibited a significant difference between lateral fluid percussion brain injured and sham-injured animals. This difference was due to a significant reduction (21–33%) of the NR2A:NR2B ratio in the L parietal cortex of the injured group at 1 and 2 days ($P<0.05$) as compared with shams. See Fig. 1, panel B and Fig. 2.

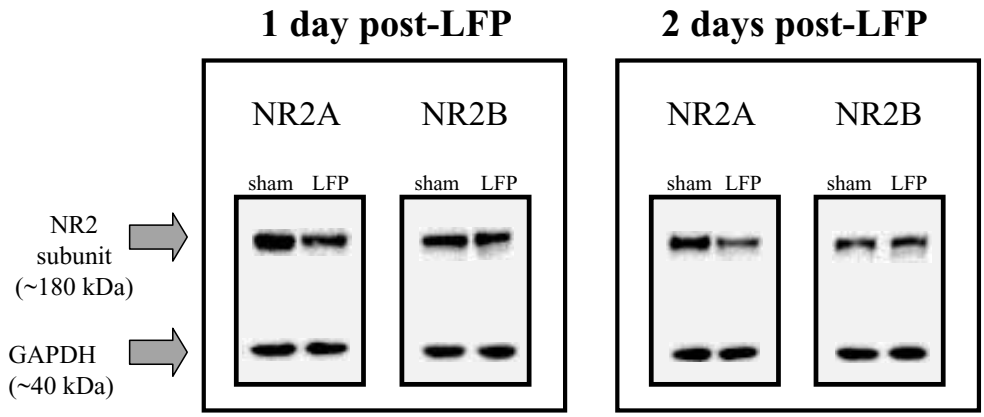

Fig. 2. Western blots from the ipsilateral parietal cortex of sham and lateral fluid percussion (LFP)-injured rats at 1 and 2 days post-injury. Although raw data were obtained by loading each lane with 10 mg of protein from individual animals for each time and region specific blot (total of 82), the above illustration was obtained by pooling the animals into four group samples (1d sham, 1d LFP, 2d sham, 2d LFP). Blots were incubated with primary antibodies against NR2A, NR2B, and GAPDH (a housekeeping protein, used as a control for amount protein loaded/transferred).

Ipsilateral occipital cortex

Within the L occipital cortex there was a significant reduction (39–58%) of the NR2A subunit in the injured group at 1 day ($P<0.01$) and 2 days ($P<0.02$) as compared with shams. There was also a significant reduction (33–48%) of NR2B in the injured group at 1 day ($P<0.01$) and 2 days ($P<0.05$) as compared with shams.

Addressing the NR2A:NR2B ratio, analysis reveals a trend toward a reduction. However, these values did not achieve statistical significance (20%; Injured mean=.668; S.E.M.=0.11; Sham normalized mean=1.00; S.E.M.=0.15, $P<0.10$). See Fig. 1, panel C.

Contralateral parietal cortex

Within the R parietal cortex, there was a significant reduction (36–39%) of NR2A in the lateral fluid percussion brain injured group at 1 day ($P<0.01$) and 4 days ($P<0.02$) as compared with shams. There was also a significant reduction (34%) of NR2B in the injured group at 4 days ($P<0.03$) as compared with shams.

As with the ipsilateral occipital cortex, there was only a trend for the NR2A:NR2B relative ratio to be reduced (19%; Injured mean=0.81, S.E.M.=0.07; Sham normalized mean=1.00, S.E.M.=0.11) when measured on the first day after injury ($P<0.10$). See Fig. 1, panel D.

Homogenates and synaptically enriched preparations

Although NMDARs are known to preferentially localize in synaptic structures (Monaghan and Cotman, 1986), to ensure that the alterations demonstrated by the brain ROI homogenates reflect changes at the synapse in our post-injury model, a separate group of animals was investigated at 18–24 h post-injury and their tissue was prepared to the level of the P2 fraction (containing synaptosomes and mitochondria). A *t*-test was used to compare the normalized NR2A, NR2B, and NR2A:NR2B relative ratios between these synaptically enriched fractions and the 1 day post-injury homogenates in the bilateral parietal cortices and hippocampi. There were no differences between the synaptically enriched fractions and homogenates in any ROI: L parietal cortex ($P=0.240-0.878$), R parietal cortex ($P=0.100-0.816$), L hippocampus ($P=0.269-0.558$), and R hippocampus ($P=0.107-0.558$). See Fig. 3.

There are consistently two NR1 subunits per NMDAR complex (Behe et al., 1995) and thus NR1 can be used as an estimate of the number of functional NMDARs in a ROI. To investigate if there is a difference in the number of NMDARs after injury, the amount of NR1 was compared between sham and lateral fluid percussion brain injured rats in the 18–24 h synaptically enriched preparations for the L and R parietal cortices. Although there was a trend for injured group to be reduced (14–21%; L parietal cortex mean=0.86; S.E.M.=0.12; Sham normalized mean=1.00, S.E.M.=0.12; R parietal cortex mean=0.80, S.E.M.=0.10; Sham normalized mean=1.00; S.E.M.=0.14) as compared with shams, there were no significant differences in NR1 protein levels between sham and injured animals in

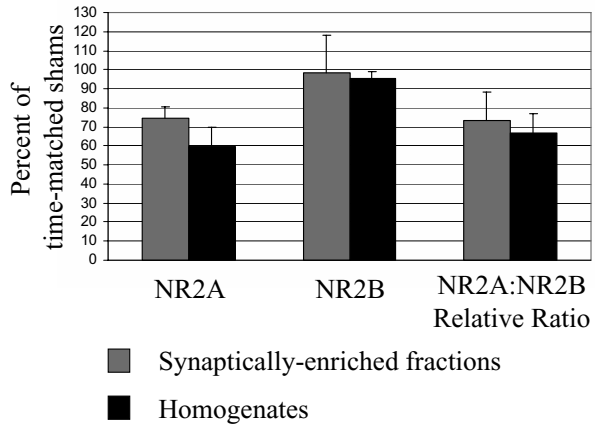

Fig. 3. Comparison of NR2A, NR2B, and the NR2A:NR2B relative ratio (normalized to intra-blot shams) in the ipsilateral parietal cortex at 1 day post-injury in synaptically enriched preparations as compared with homogenates.

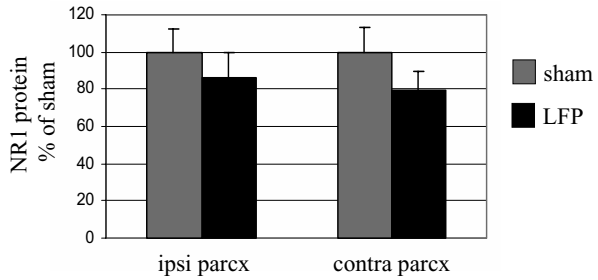

Fig. 4. Mean amount (±S.E.M.) of NR1 in lateral fluid percussion-injured rats in ipsilateral (ipsi) and contralateral (contra) parietal cortices at 18–24 h post-injury (synaptically enriched preparation) as compared with shams (defined as 100%). Although there are no significant differences between sham and LFP groups ($P>0.20$), there is a trend for NR1 to be slightly reduced in the LFP rats, both ipsilateral and contralateral to the fluid pulse administration.

the L parietal cortex ($P=0.387$) or R parietal cortex ($P=0.215$). Interestingly, following injury, both the ipsilateral and contralateral parietal cortices showed similar levels indicating that lateral fluid percussion does not cause a unilateral effect on the number of NMDARs (Fig. 4). This nonsignificant reduction in NR1 protein levels within the L and R parietal cortices opens the possibility that some of the trauma-induced changes in subunits could be due to selective loss of receptors having more or less of either the NR2A or NR2B subunit.

^{45}Calcium accumulation

Pearson's correlation coefficient revealed that there was a significant correlation between the injury-induced reduction of NR2A:NR2B relative ratio and the accumulation of calcium in ROI (Osteen et al., 2001) at 1 and 2 days post-injury ($\rho=0.89$, $P=0.003$; Fig. 5). This association suggests a relationship between NR2A:NR2B ratio and regional post-lateral fluid percussion brain injury calcium influx.

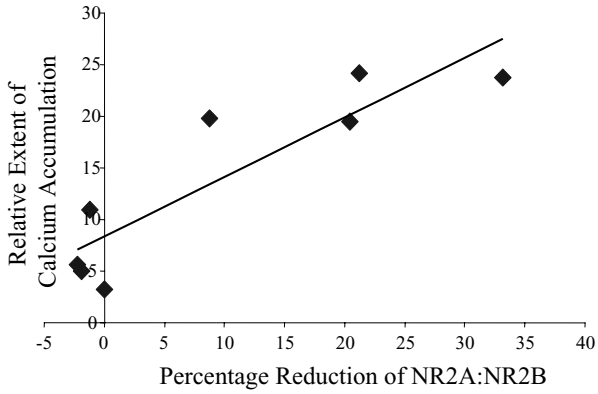

Fig. 5. Mean values of relative ipsilateral $^{45}Ca^{++}$ accumulation [(L−R)/(L+R)×100] from lateral fluid percussion injured adult rats as function of percentage of injury-induced reduction of NR2A:NR2B relative ratios. The eight data points represent ipsilateral parietal and occipital cortices and hippocampus at 1 and 2 days post-injury, and ipsilateral frontal cortex and thalamus at 1 day post-injury (all of the regions that have both sets of data at the given time points). Pearson's correlation coefficient revealed significant correlation between the two variables ($\rho=0.89$, $P=0.003$).

Pharmaceuticals

Animals in the pharmaceutical portion of the study were studied at 1 or 2 days following lateral fluid percussion, referring to the time point at which $^{45}Ca^{++}$ was injected. During the 5-h period of $^{45}Ca^{++}$ uptake, animals received MK-801 (0.3 mg/kg), ifenprodil (30 mg/kg), or saline (control). Gross behavioral observation confirmed the activity of the drugs: saline-treated rats behaved normally, MK-801 treated rats appeared intoxicated (body rolling, head weaving, ataxia, loss of balance when rearing), while ifenprodil-treated rats displayed a reduction in spontaneous activity, behaviors that have been previously reported at these doses (Boyce et al., 1999).

Histology

Histological analysis was performed on fresh frozen 20 μm brain sections at 400 μm intervals that were stained with Cresyl Violet. Light microscope analysis revealed abnormalities typical for this level of injury, with no differences noted between groups. In general, there was slight damage evident at the site of fluid pulse administration, with the vast majority of the underlying parietal cortex appearing completely normal (Fig. 6). Almost all rats did, however, exhibit a focal area of cell loss at an intracortical location lateral to the site of fluid pulse administration, as previously reported in this model (Cortez et al., 1989; Hicks et al., 1996; Osteen et al., 2001) and 50% also showed evidence of a smaller area of cell loss at the superior-posterior occipital cortex. Since dying cells are known to accumulate calcium, and as we were interested in calcium accumulation occurring in viable cells of normal histology, contused areas were excluded from optical densitometry analysis. The majority of rats demonstrated disruption of the gray-white interface as well as intracerebral hemorrhage at both the gray-white interface and superior border of the thalamus. In most rats, the CA3 region of the hippocampus was somewhat affected, displaying some cell loss. The ipsilateral thalamus appeared mostly normal at 1 day post-injury, but by 2 days thalamic cell loss was a consistent finding (Nagasawa and Kogure, 1990; Pierce et al., 1998; Smith et al., 1997; Watanabe et al., 2000).

Fluoro-Jade

Six 1 day post-injury brains (saline $n=2$, MK-801 $n=2$, ifenprodil $n=2$) were analyzed under the fluorescent microscope and cells staining positive for Fluoro-Jade were mapped. Despite distinct, potent pharmaceutical interventions, the three groups displayed a pattern of Fluoro-Jade positivity that was remarkably similar. Starting at bregma, Fluoro-Jade positive cells were evident in the cortex lateral to the site of fluid pulse administration, near the gray-white interface. Moving posteriorly through the entire cerebrum, the region of Fluoro-Jade positivity expanded to a maximum of 20% the area of the ipsilateral hemisphere, expanding 75% of the way through the cortical mantle from the gray-white interface. In addition, all brains exhibited a small area of Fluoro-Jade positivity in the ipsilateral superior parietal–occipital region, just above the crest of the

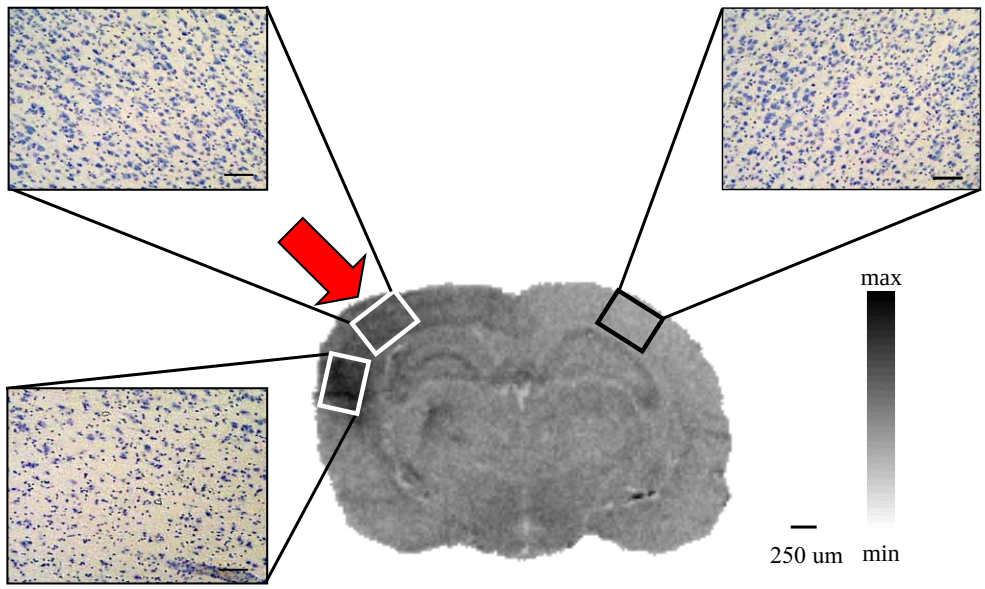

Fig. 6. A representative autoradiograph from a control rat (saline treatment) injected with $^{45}Ca^{++}$ at 1 day post lateral fluid percussion, with corresponding representative histology from ipsilateral and contralateral parietal cortex (illustrating the ROI for densitometry measurements) and the intra-cortical contusion lateral to the site of fluid pulse administration (indicated with the arrow).Histological inserts are Cresyl Violet-stained sections revealing normal morphology in both the ipsilateral and contralateral parietal cortex as well as a loss of cells within the above described contusion. In general, the frontal, parietal, and occipital cortices displayed diffuse calcium accumulation, with no gross histological damage. In addition, almost all animals exhibited an intracortical contusion lateral to the site of fluid pulse administration, evident by a "hotspot" of calcium signal and marked cell loss; these contused areas were excluded from optical densitometry analysis.

corpus callosum, from bregma −1.5 mm to bregma −8.5 mm. The dorsal hippocampus exhibited small clusters of Fluoro-Jade positive neurons in CA2, CA3, and dentate gyrus with a more pronounced pattern at CA3 from bregma −4.5 mm to bregma −6.0 mm. The ipsilateral thalamus exhibited a pattern of Fluoro-Jade positivity scattered throughout its boundaries. See Fig. 7. The Fluoro-Jade results support the Cresyl Violet histological findings, confirming that the areas of ROI analyzed by optical densitometry consisted of viable cells.

$^{45}Ca^{++}$ autoradiography ROI analysis

Autoradiographs were of sufficient quality to ensure accurate measurements of the ROI at their predetermined locations. The findings of the current $^{45}Ca^{++}$ studies conducted in the injured saline-treated animals were virtually identical to what we have previously reported (Fineman et al., 1993; Osteen et al., 2001). In all groups and time points, $^{45}Ca^{++}$ accumulation was diffuse in nature, extending out in all directions from the site of fluid pulse administration. It encompassed approximately 30% of the brain's surface area, stopping at the midline. When a contusion was present at an intracortical site (as verified by histology) it presented as an intense, focal signal and that portion of the ROI was excluded from optical densitometric analysis. This exclusion was necessary due to the potential of a calcium sink (Young and Koreh, 1986), so that the massive calcium load occurring in dying cells would not artificially elevate the value representing sublethal calcium influx into viable cells.

In general, MK-801 and ifenprodil decreased the extent of $^{45}Ca^{++}$ accumulation in a ROI-specific manner, which was consistent across time. For the regions in which the pharmaceutical treatment was effective, the greatest magnitude of calcium load was in the saline-treated groups, the least in MK-801-treated groups, with the ifenprodil-treated groups falling somewhere in between (Figs. 8, 9).

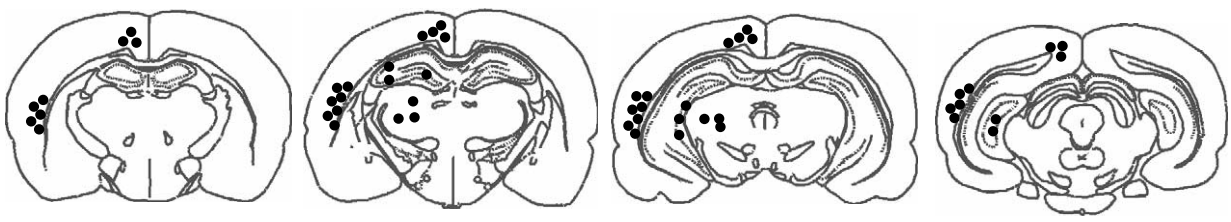

Fig. 7. Representative mapping of Fluoro-Jade positive cells for all groups at 1 day following lateral fluid percussion injury. Coronal sections are indicative of Bregma 1.5 mm, Bregma 3.0 mm, Bregma 4.5 mm, and Bregma 6.0 mm respectively and black circles represent small clusters (five to 10) of Fluoro-Jade positive neurons.

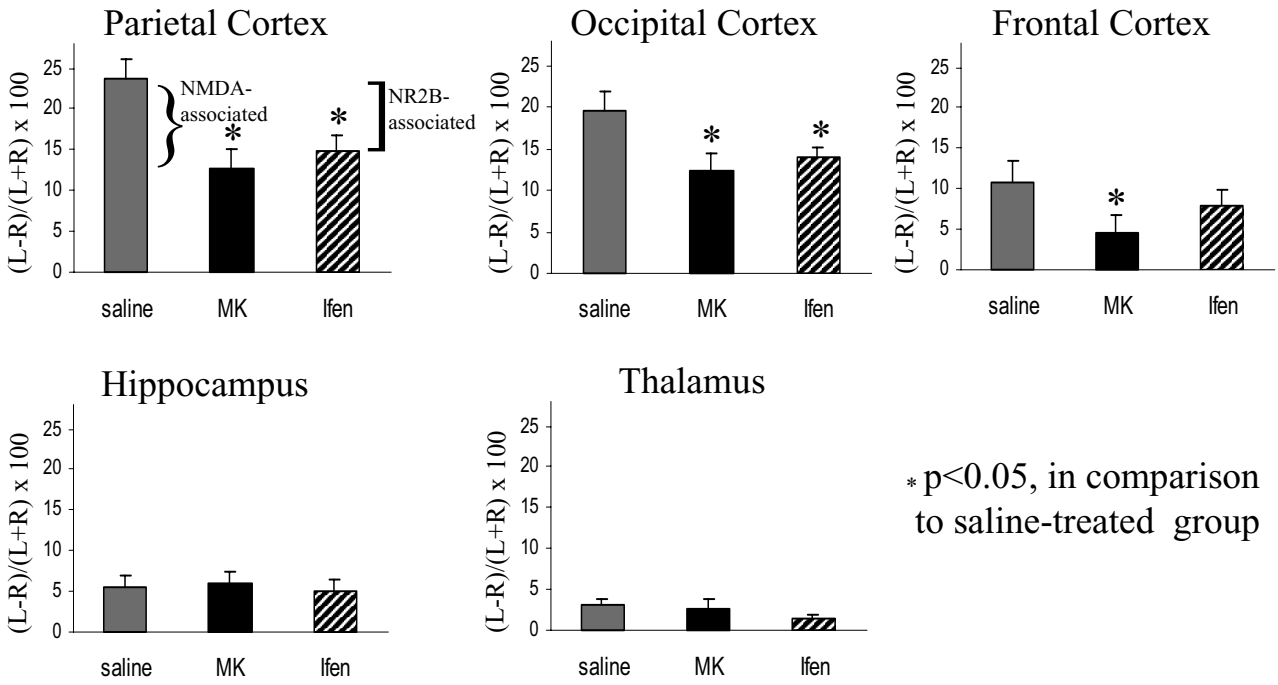

Fig. 8. Amount of post-traumatic calcium accumulation [relative ipsilateral optical density=(L−R)/(L+R)×100] in animals treated with saline, MK-801, and ifenprodil (prior to $^{45}Ca^{++}$ injection), at 1 day following mild-moderate lateral fluid percussion injury. The difference between saline- and MK-801-treated rats represents the amount of calcium influx associated with the NMDA receptor (which can contain both NR2A and NR2B subunits), while the difference between saline and ifenprodil represents the calcium specifically associated with NR2B-containing NMDA receptors.

Statistical analysis revealed an overall effect of group (saline, MK-801, or ifenprodil-treated; $F_{5,20}=6.562$, $P=0.001$) with a main effect of group present in the frontal cortex ($F_{2,27}=4.651$, $P=0.02$), parietal cortex ($F_{2,27}=15.616$, $P<0.001$), and occipital cortex ($F_{2,27}=9.214$, $P=0.001$), but not in the hippocampus ($F_{2,27}=0.404$, $P=0.673$) or thalamus ($F_{2,27}=1.806$, $P=0.187$). There was also an overall effect of time ($F_{5,19}=11.117$, $P<0.001$), but this was solely due to the thalamus ($F_{1,28}=20.460$, $P<0.001$) having substantially more $^{45}Ca^{++}$ accumulation at 2 days than 1 day (Figs. 8 and 9). The delayed thalamic ipsilateral accumulation of $^{45}Ca^{++}$ has previously been described (beginning at 2 days post-injury) and shown to be associated with cell death (Nagasawa and Kogure, 1990; Osteen et al., 2001; Pierce et al., 1998; Smith et al., 1997; Watanabe et al., 2000). There was no main effect of time in the frontal cortex, hippocampus, parietal cortex, or occipital cortex ($F_{1,28}=0.023–0.883$, $P=0.357–0.881$).

Bonferroni post hoc analysis revealed that the MK-801 group accumulated significantly less $^{45}Ca^{++}$ than the saline-treated group in the frontal cortex ($P=0.019$), parietal cortex ($P<0.001$), and occipital cortex ($P=0.002$) across time. In addition, the ifenprodil-treated group accumulated significantly less $^{45}Ca^{++}$ than the saline-treated group in the parietal cortex ($P=0.001$) and occipital cortex ($P=0.009$) across time.

Since there were significant differences between groups (saline, MK-801, or ifenprodil-treated) in the frontal cortex, parietal cortex and occipital cortex at 1 and 2 days post-injury, difference scores were calculated to represent the amount of calcium flux occurring through NMDARs (saline minus MK-801) and those NMDARs containing a NR2B subunit (saline minus ifenprodil) in these ROIs. See Fig. 10.

Individual percentages of NMDAR-associated calcium flux that occurred through NR2B subunit-containing receptors (which can also contain NR2A subunits) were calculated and subjected to statistical analysis, revealing an overall effect of ROI ($F_{5,24}=8.319$, $P=0.002$), but not of time ($P=0.684$). Bonferroni's post hoc test revealed that the frontal cortex had a significantly lower percentage of calcium flux that occurred through the NR2B subunit-containing receptors than did both the parietal and occipital cortex ($P=0.005$). See Fig. 11.

DISCUSSION

The data reported in this study characterize lateral fluid percussion brain injury-induced molecular changes in the NMDAR and determine a functional significance (enhanced calcium influx) of the injury-induced alteration of NMDAR subunit composition. This suggests that NMDA receptor changes following traumatic brain injury contribute mechanistically to the pathophysiological cascade and are capable of inducing dysfunction, even in regions without overt cell death.

Summary of findings

The first experiment was conducted to determine the regional and temporal profile of the NR2 subunit composition of the NMDAR following mild-moderate lateral fluid percus-

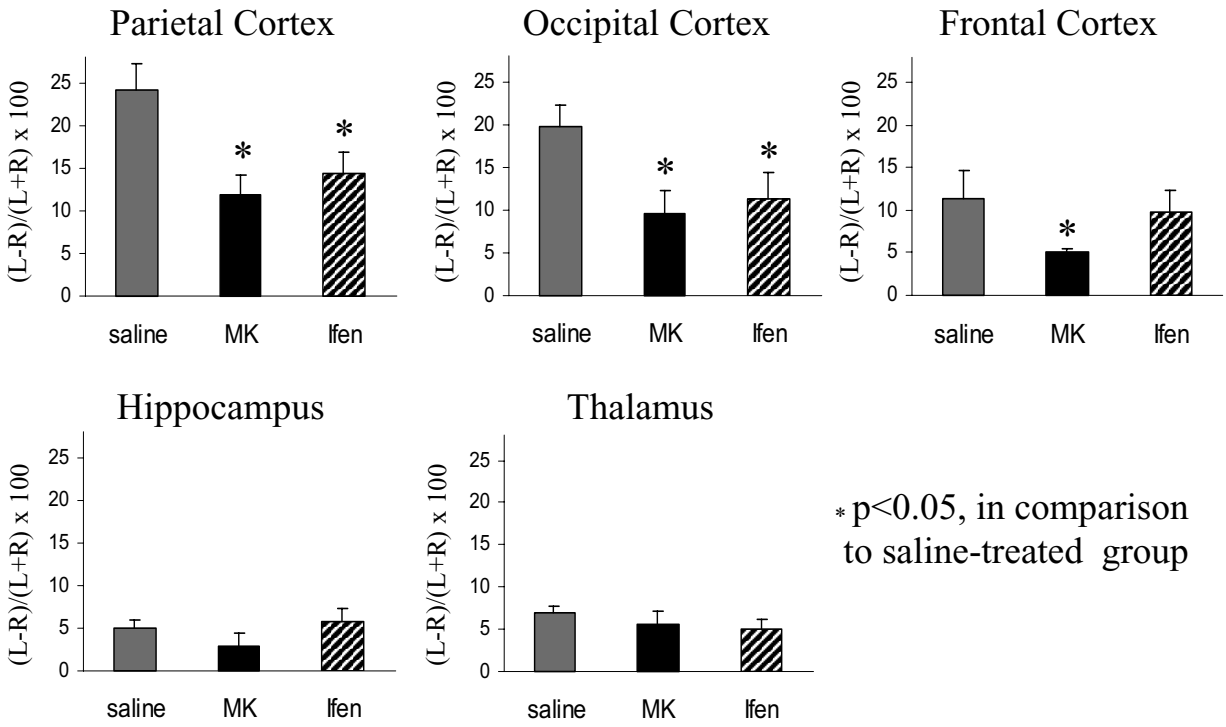

Fig. 9. Amount of post-traumatic calcium accumulation [relative ipsilateral optical density=[(L−R)/(L+R)×100] in animals treated with saline, MK-801, and ifenprodil (prior to $^{45}Ca^{++}$ injection), at 2 days following mild-moderate lateral fluid percussion injury.

sion injury in the adult rat. The NR2 subunit composition dictates the functional properties of the NMDAR; therefore, an alteration of such may make an important contribution to the post-traumatic milieu. It was found that lateral fluid percussion alters the levels of NR2A and NR2B primarily in regions proximal to the site of impact (ipsilateral parietal and occipital cortices), and in regions remote from this location (contralateral parietal cortex). The injury-induced NMDAR subunit composition alteration occurs early after injury (1–4 days) and normalizes over time (by 14 days). Importantly, the magnitude of alteration is not uniform across subunits, as NR2A is affected to a greater degree than is NR2B at 1 and 2 days post-injury. The result of this unequal injury-induced reduction is a decreased NR2A:NR2B ratio, meaning that more NR2B is available for incorporation into the NMDAR heteromer than NR2A. Since receptors with more NR2B than NR2A are more sensitive to glutamate, conduct more current, and are open for a longer amount of time (Flint et al., 1997; Ishii et al., 1993; Quinlan et al., 1999; Scheetz and Constantine-Paton, 1994; Seeburg et al., 1994; Yamakura and Shimoji, 1999), this reduced NR2A:NR2B ratio seems to indicate an injury-induced shift toward a sensitized NMDAR, regardless of the absolute receptor numbers. Although this composition may indicate a period of neuroplastic potential, it is being expressed acutely after brain injury, a time when many other post-traumatic processes are active (such as enhanced Ca^{++} influx). Thus, the injury-induced alteration in NMDAR subunit composition may actually be contributing to the post-traumatic pathophysiology.

Post-traumatic calcium overload is an important feature following traumatic brain injury, and is known to occur predominantly, but not exclusively, through the NMDAR (Bullock and Fujisawa, 1992; Nadler et al., 1995). Thus, it is likely the injury-induced alteration in the NMDAR subunit composition contributes to an increased influx of calcium into viable cells following injury, especially since NR1/NR2B receptors lack calcium-dependent inactivation. The comparison of the relative ipsilateral extent of post-traumatic calcium accumulation from our previous work (Osteen et al., 2001) with post-traumatic alterations in NMDAR subunit composition supported this idea, as the *temporal* and *regional* profiles are identical. Specifically, at 1 and 2 days post-injury, ROIs in injured animals demonstrating the lowest NR2A:NR2B relative ratios (ipsilateral parietal and occipital cortices) accumulate the greatest amount of calcium. The strong correlation between these two factors (Fig. 5) led us to investigate whether traumatic brain injury-induced alterations in NMDAR subunit composition contribute to the prolonged post-traumatic accumulation of calcium.

To address this question, the $^{45}Ca^{++}$ autoradiographic experiment utilized distinct NMDAR antagonists to differentiate between the calcium flux occurring through all NMDARs (MK-801-sensitive) and the flux occurring through NMDARs specifically containing the NR2B subunit (ifenprodil-sensitive). First, it was found that calcium influx occurs through the NMDAR in a ROI-specific manner. Neither MK-801 nor ifenprodil significantly reduced the calcium load in the hippocampus and thalamus at either 1

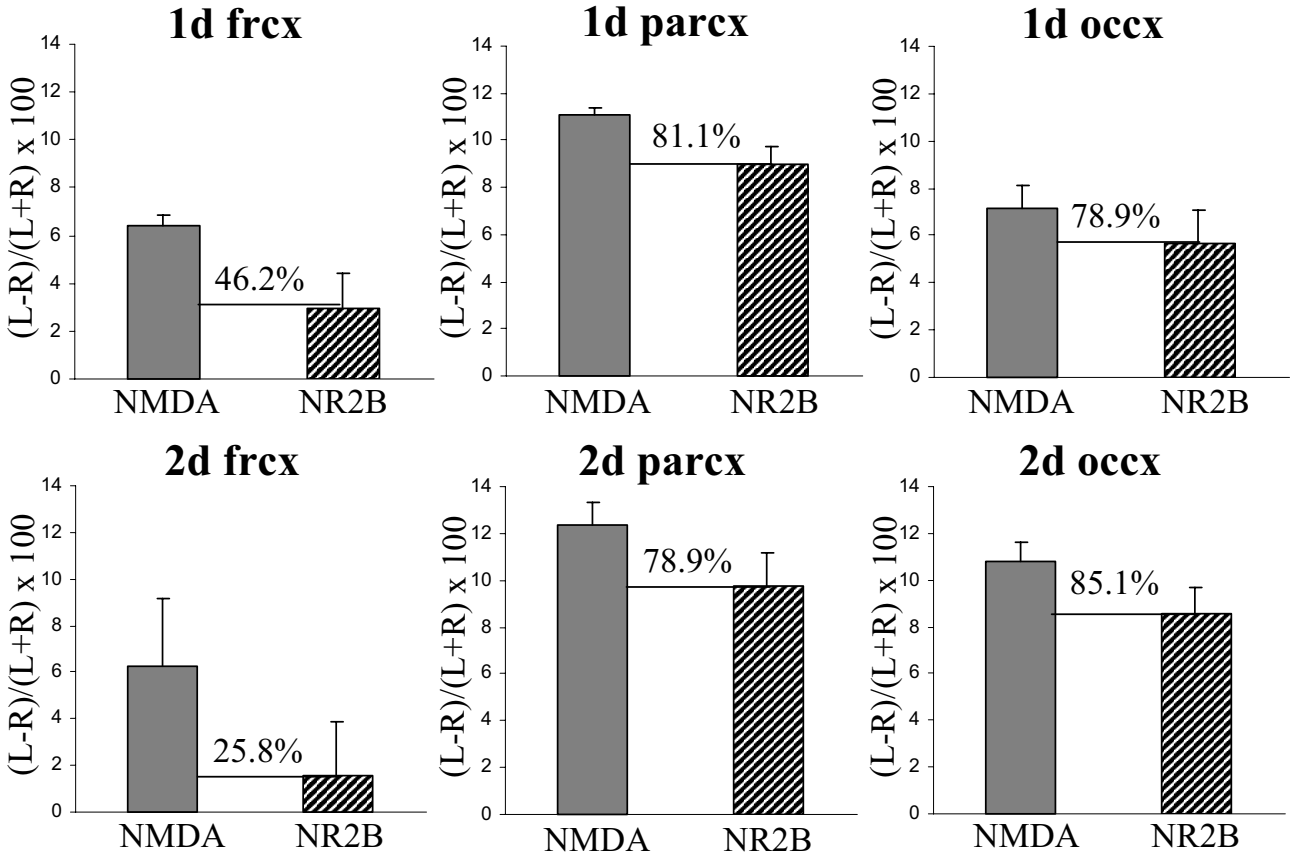

Fig. 10. Amount of post-traumatic calcium accumulation [relative ipsilateral optical density=[(L−R)/(L+R)×100] associated with all NMDARs (saline-MK801) vs the NR2B-containing NMDARs (saline-ifenprodil) in regions in which MK-801 significantly diminished calcium accumulation at 1 and 2 days following mild-moderate lateral fluid percussion injury.

or 2 days following injury. However, in this model, roughly 50% of the post-traumatic calcium influx occurring in the in the ipsilateral frontal, parietal, and occipital cortices at 1 and 2 days post-injury is associated with the NMDAR. The remaining proportion of $^{45}Ca^{++}$ accumulation is most likely due to flux through voltage-gated Ca^{++} channels (Samii et al., 1999), Ca^{++}-permeable α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) receptors, breakdown of the blood–brain barrier (Osteen et al., 2001), the Ca^{++} permeable nonselective cation conductance via TRPM7 channel (Aarts et al., 2003) and, possibly, extracellular signals.

Second, it was found that the amount of post-traumatic calcium flux blocked by these pharmaceutical agents was greater for MK-801 than it was for ifenprodil. These were the expected results, since not all NMDARs contain the NR2B subunit. However, the proportion of calcium flux that was sensitive to ifenprodil differed between regions. The ipsilateral frontal cortex had only 25.8–46.2% of the NMDAR-associated calcium flux occurring through NR2B subunit-containing receptors, while the ipsilateral parietal and occipital cortices had 78.9–81.1% and 78.9–85.1%, respectively, of the NMDAR-associated calcium flux occurring through NR2B-containing receptors.

The mutual consideration of these two experiments suggests a biological consequence, or functional significance, to the injury-induced alterations in the NMDAR. Regions lacking an injury-induced shift in NMDAR subunit composition (i.e. the ipsilateral frontal cortex) have a much smaller proportion of calcium flux occurring through NR2B-containing receptors than regions demonstrating an injury-induced reduction of the NR2A:NR2B relative ratio (i.e. the ipsilateral parietal and occipital cortices). Therefore, it seems that lateral fluid percussion brain injury induces proximal regions to shift toward NMDARs containing NR2B subunits, and this sensitized form of the receptor (more sensitive to glutamate, conducts larger currents, stays open longer, lacks calcium-dependent inactivation) contributes to the post-traumatic accumulation of calcium.

Interpretations of data

The current data describe an alteration in the NMDAR subunit composition following trauma and a corresponding functional consequence of this change. The demonstration that NR2B subunit-containing NMDARs are preferentially expressed in specific regions following TBI in adult rats taken together with the fact NR2B-contain-

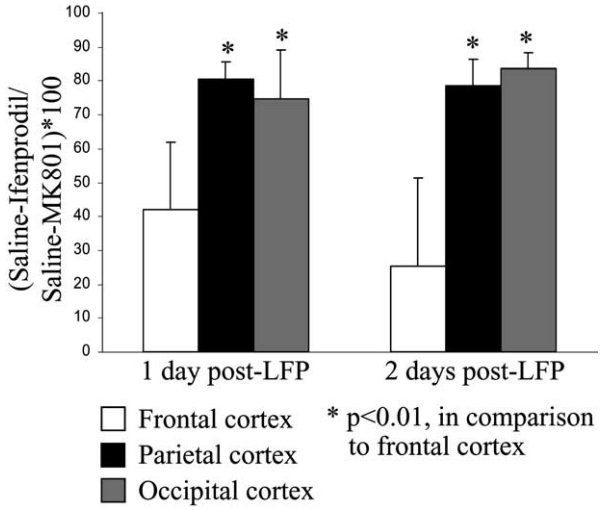

Fig. 11. Percentage of NMDAR-associated (saline–MK801) calcium accumulation that is specifically associated with the NR2B subunit (saline–ifenprodil) in the ipsilateral frontal, parietal, and occipital cortices in the 2 days following LFP injury. Notice the difference between regions that have an injury-induced reduction of the NR2A:B ratio (parietal and occipital cortices) and the region that does not (frontal cortex).

ing receptors inherently allow enhanced calcium influx suggests that this glutamate receptor alteration contributes to the post-traumatic accumulation of calcium in adult rats' intact cortical neurons.

Since calcium plays a key role in the extensive signaling pathways of the NMDAR, there are a large number of potential downstream effects resulting from post-traumatic calcium accumulation. It is likely that in addition to contributing to traumatic brain injury pathophysiology, the post-traumatic subunit alteration has other important functional roles, perhaps even relating to neuroplasticity and recovery of function. The potential contribution of the NMDAR subunit composition alteration to vulnerability and neuroplasticity is discussed below.

Jenkins and colleagues (1988) have addressed the idea that post-traumatic neuronal vulnerability may be receptor-mediated. The combined pharmacological blockade of muscarinic and NMDA receptors was found to attenuate the secondary insult-induced neuronal degeneration of the hippocampus. Furthermore, since lateral fluid percussion injury reduces the threshold to kainic acid-induced seizures (Zanier et al., 2003), stimulation of sensitized NMDARs may also have a role in post-traumatic epilepsy. However, the role NMDAR subunit composition plays in the vulnerability to secondary injury is unknown. The mere fact that NR2B subunit-containing NMDARs make a significant contribution to the post-traumatic accumulation of calcium links the post-traumatic alteration of NMDAR subunit composition to the concept of vulnerability. For example, if a secondary injury occurs when neurons are already burdened with a sublethal load of intracellular calcium, an additional surge of calcium influx from the secondary insult (such as ischemia or hypotension) may trigger excitotoxic cell death. Alternatively, the ionic flux caused by a secondary insult may place additional energy demands on a neuron in energy crisis, forcing it into energy failure and the fate of secondary cell death. In either case, the injury-induced sensitized composition of the NMDAR may render neurons vulnerable to a secondary insult-induced glutamate surge that otherwise would have been well tolerated if the NMDAR were in its native subunit composition.

Changes in NMDAR subunit composition, as seen in the current study, may instead render the brain primed for neuroplasticity. In development, a change in the NR2A:NR2B ratio is associated with a critical period for visual plasticity and occurs in response to experience-dependent plasticity (Quinlan et al., 1999). Since NMDAR activation is required for plasticity, it is possible that a receptor primed for activation may foster recovery of a dysfunctional neuron (or, in other words, a viable neuron in an injured brain). If the post-traumatic reduction of NR2A:NR2B indeed reflects a critical window for neuroplastic responses, the amount and timing of appropriate stimuli could be key to optimizing recovery.

Although the relative change of the NR2 subunit composition has not previously been addressed following fluid percussion injury in the adult rat, binding studies have suggested that the NMDAR composition and function may be altered after traumatic brain injury (Gorman et al., 1995; Miller et al., 1990; Sihver et al., 2001). After a moderate central fluid percussion brain injury in adult rats, Miller et al. (1990) reported that binding of [^{3}H]glutamate in the cortex was reduced by up to 66% from control values at 5 min, 3 h, and 24 h after injury. Moreover, Gorman et al. (1995) found a 52% and 71% reduction of NMDAR binding in the frontal and entorhinal cortex, respectively, at 1 h post-injury, but at 1 day post-injury NMDAR binding had returned to sham levels in the frontal cortex while the entorhinal cortex was reduced by 66%. More recent work has investigated NMDAR binding following lateral fluid percussion brain injury at 12 h post-injury and found that the ipsilateral cortex exhibited an increase in MK-801's equilibrium dissociation constant (K_D) and a decrease in maximum specific binding (B_{max}), resulting in a significant reduction in binding potential (B_{max}/K_D) as compared with shams (Sihver et al., 2001). Since the binding site for glutamate is on the NR2 subunit, all of these studies support the reported trends for reduction of the NR2 subunit following trauma. This acute post-traumatic reduction of NMDARs may be the cells' immediate effort to protect themselves from excitotoxicity, as they are faced with a massive, traumatically induced glutamate surge.

Changes in NMDAR structure and function have been more extensively characterized using a model of ischemia, in which the hippocampus is the most vulnerable structure. When Small et al. (1997) investigated NMDAR gene expression at 90 min following *in vitro* ischemic injury, the proportion of NMDARs containing the NR2B subunit dropped by approximately 50% while the proportion of NR2A subunit-containing NMDARs was unchanged, indi-

cating a down-regulation of the sensitized form of the NMDAR early after injury. At 1 day post-injury, gene expression of both NR2A and NR2B were found to be reduced (Hsu et al., 1998; Zhang et al., 1997), whereas NR1 was not (Gass et al., 1993). Unfortunately, when investigating post-injury NMDAR protein levels, experimenters used an antibody that did not differentiate between NR2A and NR2B, but instead reported that the NR2A/NR2B combination was significantly reduced at this time point (Zhang et al., 1997). Importantly, post-ischemic changes in the NMDAR gene expression and protein levels were found to have functional implications as electrophysiological properties were significantly different than those of shams (Hsu et al., 1998; Zhang et al., 1997). Although helpful in terms of linking post-injury changes in NMDAR structure with post-injury NMDAR function, ischemic injury is substantially different than traumatic injury; in particular, it lacks the hallmark diffuse, biomechanical load of trauma.

Enhancement of current through glutamate's ionotropic receptors has been reported following traumatic brain injury, including both NMDA (Zhang et al., 1996) and AMPA (Goforth et al., 1999) receptors. Using a model of stretch injury on cultured cortical neurons that produces strain comparable to *in vivo* traumatic brain injury (Schreiber et al., 1995), Zhang et al. (1996) found that injury induced significantly larger ionic currents and calcium influx through the NMDAR in response to NMDA application, which was related to a reduction of the Mg^{++} block. Post-traumatic enhancement of AMPA-mediated current has also been demonstrated and attributed to the reduction of desensitization (Goforth et al., 1999). Interestingly, both the strength of NMDAR's Mg^{++} block and the rate of AMPA receptor desensitization are determined, at least in part, by subunit composition of their respective receptors (Burnashev et al., 1992; Dingledine et al., 1999). This suggests that different subtypes of ionotropic glutamate receptors may be modulated post-injury by similar mechanisms.

The mechanism by which the NMDAR subunits are selectively altered following traumatic brain injury remains unknown. Since lateral fluid percussion brain injury recognizably causes a small degree of cell death (depending on the level of injury severity), the overall pattern of NR2 subunit reduction in injured animals may simply be due to the selective loss of cells. However, this is not likely due to the fact that the same amount of protein was analyzed for each Western sample, subunit changes were seen even in contralateral ROIs, and the ROIs selected for protein analysis and $^{45}Ca^{2+}$ imaging showed little histological damage with both Fluoro-Jade and Cresyl Violet staining.

The biomechanical load of the fluid percussion injury itself leads to many downstream changes, each of which may be involved in triggering the observed changes in the NMDAR subunits. The NMDAR itself interacts with numerous associated signaling pathways (Husi and Grant, 2001b) and thus it is tempting to hypothesize that changes in the levels of NMDAR subunits are NMDAR-mediated. A similar hypothesis was made regarding changes in NMDARs following ischemia, and although post-injury alterations were found to be associated with glutamate release, selective antagonists demonstrated that it proceeded via an AMPA/kainate pathway (Heurteaux et al., 1994). Alterations in NMDAR subunits reported in this study may be due to changes in gene expression, protein synthesis, and/or receptor degradation, and further study will be necessary to precisely elucidate these mechanisms.

The most direct explanation for the reduction of NMDAR subunit levels, as well as the decrease in the NR2A:NR2B relative ratio, is an injury-induced down-regulation in gene expression. However, it has been reported that subunit-specific binding of the NMDAR does not necessarily correspond with subunit mRNA levels (Healy and Meador-Woodruff, 2000), and that acutely following cortical contusion injury, the hippocampus exhibits a reduction of NR1, NR2A, and NR2B protein levels but no difference in the amount of the NMDAR subunit mRNA as compared with shams (Kumar et al., 2002). In contrast, the reduction of NMDAR subunit levels at 24 h post-ischemic injury had a corresponding reduction in mRNA expression at 12 and 24 h post-injury (Zhang et al., 1997). The discrepancy between these data may be due to the difference of the injury models, and it is unknown how these findings may extrapolate to lateral fluid percussion injury.

The general reduction of NR1, NR2A, and NR2B subunits in viable brain ROIs following lateral fluid percussion brain injury may be due to post-traumatic internalization and degradation of the NMDAR complex, which is recognized as an important regulatory mechanism of glutamate receptors at post-synaptic sites. The expression of both AMPA and NMDA receptors is now known to be a dynamic process, with receptors cycling in and out of the post-synaptic membrane (Carroll and Zukin, 2002; Luscher et al., 1999). It is unknown, however, how NMDAR stability changes in pathological conditions, such as the aftermath of traumatic brain injury. Calpain-mediated degradation of glutamate receptors has been demonstrated in a seizure model (Bi et al., 2000). Since calpain (a calcium-dependent cysteine protease) activity has also been found to increase significantly after traumatic brain injury (Kampfl et al., 1997), similar mechanisms may contribute to reduction of NR1, NR2A, and/or NR2B following lateral fluid percussion brain injury.

NMDAR degradation may occur via a more indirect means as the loss of NR1 and NR2 subunits may be due to calpain's degradation of a protein that interacts with the glutamate receptor (Lu et al., 2001) versus the subunits themselves. The NMDAR complex (approximately 2000 kDa) is now recognized to include 75 or more proteins (Husi et al., 2000; Husi and Grant, 2001a,b) and it is likely that traumatic brain injury affects a subset of these proteins, possibly ones involved in anchoring the receptor in the plasma membrane. For example, postsynaptic density protein 95 (PSD-95) binds the NMDAR (Kornau et al., 1995; Sheng and Pak, 2000) and is implicated in both the stability of the receptor (Roche et al., 2001) and the coupling of the NMDAR to signal transduction pathways that

regulate synaptic plasticity and learning (Migaud et al., 1998). It is possible, albeit speculative, that the injury-induced increase in calpain activity affects a protein such as PSD-95, having secondary effects on the NMDAR, such as acute internalization and degradation. Perhaps as the neuron recovers, other mechanisms such as gene expression, post-transcriptional modifications, or post-translational modifications become responsible for the prevalence of NR2B over NR2A and the resultant sensitized NMDAR.

Determining the precise mechanisms of post-traumatic NMDAR subunit composition alteration will require ongoing investigation. However, from the patterns of injury-induced NR2A and NR2B expression demonstrated in this study, it appears that very soon after traumatic brain injury there is a drastic reduction in all NMDAR subunits at the synapse, with the expression of NR2B recovering prior to that of NR2A.

Alternative hypotheses

As described above, traumatic brain injury induces a shift in the NMDAR subunit composition to a state that renders the receptor more easily activated by glutamate and that allows enhanced ionic flux. The post-traumatic, sensitized NMDARs seem to be repetitively stimulated by post-injury levels of extracellular glutamate, resulting in chronic channel opening and continuous ionic flux. Whereas the model of cortical contusion has been reported to elevate extracellular levels of glutamate for an extended period of time following injury (van Landeghem et al., 2001), fluid percussion injury induces a massive release of glutamate immediately after injury that lasts only on the order of minutes (Faden et al., 1989; Katayama et al., 1990).

Therefore, intermittent glutamate release (rather than an elevated basal level of glutamate) remains the source of stimulation for the sensitized receptors. For one, a mild-moderate lateral fluid percussion brain injury does not decrease exploratory behavior in the rats at 1 and 2 days post-injury. So, the synaptic activity accompanying typical rat behavior leads to the continual release of glutamate and this normal physiological activity may relate to enhanced activation of sensitized NMDARs. In addition to the normal synaptic release of glutamate activating the receptors, the post-traumatic brain is susceptible to secondary insults (like ischemia or hypotension) that can cause large glutamate surges (Benveniste et al., 1984). In the face of deficient glutamate uptake mechanisms, the typical glutamate spike of such insults may be prolonged, resembling a plateau. Indeed, the secondary injury-induced elevation of extracellular glutamate may cause overactivation of NMDARs, but secondary injury or not, there is adequate glutamate available to activate NMDARs. Glutamatergic activation of NMDARs with sensitized electrophysiological properties seems to contribute to chronic functional hyperactivity of these receptors, which in turn contributes to the pathophysiology of traumatic brain injury.

The current studies focused on relative changes of NR2A and NR2B (the major subtypes of the NR2 subunit in the cerebral cortex and hippocampus) following traumatic brain injury, and specific pharmacological blockade of receptors containing the NR2B subunit confirm the importance of these subtypes of NR2 subunit. However, it is also possible that other NMDAR subunits besides NR2A an NR2B play an important role after trauma, such as NR2C. In fact, following an *in vitro* model of ischemia, NR2C was found to increase in the hippocampus relative to NR2A and NR2B (Small et al., 1997). Although it is unknown if NR2C is altered at 1 and 2 days post-lateral fluid percussion brain injury, the possibility that cortical levels may be up-regulated does not contradict the interpretation of our findings. In fact, properties of NMDARs containing the NR2C are remarkably similar to those of NR2B (including sensitivity to glutamate, length of time channel is open, and absence of Ca-dependent inactivation) (Flint et al., 1997; Ishii et al., 1993; Quinlan et al., 1999; Scheetz and Constantine-Paton, 1994; Seeburg et al., 1994; Yamakura and Shimoji, 1999) and, in addition, NR2C-containing receptors lack a significant Mg^{++} block. Therefore, the potential post-injury induction of NR2C may be an additional mechanism that results in a sensitized form of the NMDAR.

NR1 has eight different splice variants (Hollmann et al., 1993; Yamakura and Shimoji, 1999), and although not as critical to function as the NR2 subunit, channels containing different splice variants can have different electrophysiological properties (Durand et al., 1993; Hollmann et al., 1993; Sugihara et al., 1992). The antibody we used did not differentiate between variants, but rather recognized a sequence common to all. Thus, the possibility exists that traumatic brain injury induces different splice variants of NR1, which can alter function of the NMDAR.

Besides structural changes in the NMDAR subunits, changes in regulatory mechanisms of the NMDAR may also contribute to altered function post-injury. For example, the properties of NMDAR seem to be regulated by tyrosine phosphorylation, and injury may change patterns of phosphorylation. Following ischemic injury, the phosphorylation of NR2A and NR2B is enhanced (Takagi et al., 1997) which may contribute to alterations in NMDAR function by altering signaling pathways and receptor mobility.

In conclusion, lateral fluid percussion brain injury induces dynamic molecular alterations in the NMDA receptor resulting in a sensitized form of the receptor. Importantly, this structural change is accompanied by a functional consequence, the enhanced influx of calcium. Injury-induced alteration in receptor structure and function is a new way to think about traumatic brain injury. As molecular consideration of this unique pathology continues, it is our hope that appropriate targets will be defined, resulting in effective treatment options for traumatic brain injured patients.

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