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Acute Neuroprotection to Pilocarpine-Induced Seizures is Not Sustained After Traumatic Brain Injury in the Developing Rat: “Neuroprotection Following Traumatic Brain Injury”

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Abstract

Following central nervous system injury there is a period of vulnerability when cells will not easily tolerate a secondary insult. However recent studies have shown that following traumatic brain injury (TBI), as well as hypoxic-ischemic injuries, the central nervous system may experience a period of protection termed “preconditioning.” While there is literature characterizing the properties of vulnerability and preconditioning in the adult rodent, there is an absence of comparable literature in the developing rat. To determine if there is a window of vulnerability in the developing rat, post-

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natal day 19 animals were subjected to a severe lateral fluid percussion injury followed by pilocarpine-induced status epilepticus at 1, 6 or 24-hours post TBI. During the first 24 hours after TBI, the dorsal hippocampus exhibited less status epilepticus-induced cell death than that normally seen following pilocarpine administration alone. Instead of producing a state of hippocampal vulnerability to activation, TBI produced a state of neuroprotection. However, in a second group of animals evaluated 20-weeks post-injury, double-injured animals were statistically indistinguishable in terms of seizure threshold, mossy fiber sprouting and cell survival when compared to those treated with pilocarpine alone. TBI, therefore, produced a temporary state of neuroprotection from seizure-induced cell death in the developing rat; however, this ultimately conferred no long-term protection from altered hippocampal circuit rearrangements, enhanced excitability or later convulsive seizures.

Keywords

long-term outcome; preconditioning; secondary insult; vulnerability; hippocampus

Introduction

There is considerable evidence that following injuries such as traumatic brain injury (TBI) (Jenkins et al., 1989, Dixon et al., 1994, Longhi et al., 2005) and hypoxia-ischemia (Kirino and Sano, 1984) the brain is vulnerable to secondary insults. However, in both adult (Kato et al., 1992b, Perez-Pinzon et al., 1997) and developing (Vannucci et al., 1998, Gustavsson et al., 2005) models of hypoxia-ischemia and adult models of epilepsy (Sasahira et al., 1995, Najm et al., 1998) and TBI (Otori et al., 2004), there is a window of time after the primary insult when affected regions of the brain exhibit relative protection (preconditioning) against a subsequent insult. Therefore, a dynamic relationship between vulnerability and preconditioning has been observed, with vulnerability acutely following injury (Jenkins et al., 1989, Zanier et al., 2003) followed by a period of protection induced by preconditioning that appears to develop over a number of days (Kato et al., 1992a, Geeraerts et al., 2007). Consequently some aspects of the post-injury neurobiology may actually reflect the evolution of protective mechanism(s). It is not clear, however, what the long-term consequences of vulnerability or preconditioning might be since previous studies rarely extended beyond 4 weeks post-insult.

TBI is the leading cause of death and disability in the pediatric population (Weiner and Weinburg, 2000, Langlois and Sattin, 2005) yet is not well characterized in terms of injury-induced vulnerability and/or preconditioning. Studies of the relationship between brain lesions and plasticity in primates found that the developing brain exhibits greater cell survival and sparing of function when compared to a similarly injured adult brain (Kennard, 1936, Kennard and Fulton, 1942). Similar conclusions about age and recovery of function were observed in the rat (Kolb and Nonneman, 1978). This improved outcome is ascribed to inherent developmental neuroplasticity (Huttenlocher, 1979, Huttenlocher and Dabholkar, 1997). It remains to be seen, however, whether young, plastic cells are better able to survive and developing animals better able to recover from secondary insults following TBI. Furthermore, it is known that while plasticity can lead to a favorable functional outcome after injury, it can also lead to adverse affects such as epileptogenesis (dos Santos et al., 2000, Giza and Prins, 2006).

Based on previous literature from our lab demonstrating vulnerability to secondary insults in adult rats (Zanier et al., 2003, Maeda et al., 2005), we hypothesized that lateral fluid percussion injury (LFP), an extensively characterized model of TBI in the developing rat (Prins et al., 1996, Fineman et al., 2000, Ip et al., 2002, Giza et al., 2005, Gurkoff et al., 2006), would make the hippocampus vulnerable to secondary status epilepticus (SE) in the first hours post-TBI

with vulnerability decreasing at longer intervals between the two injuries. More specifically, we hypothesized that there would be an interval-dependent increase in both acute and long-term neuronal cell death in the hippocampus as well as an increase in the incidence of mossy fiber sprouting and epileptogenesis in response to pilocarpine (Pc)-induced SE within the first 24 hours post-LFP. To test our hypothesis, post-natal day 19 (P19) rat pups were subjected to a LFP, followed by Pc-induced SE (Turski et al., 1983, Hirsch et al., 1992, Sankar et al., 1998) at either 1, 6, or 24-hours post-TBI (LFP+Pc(1), LFP+Pc(6) and LFP+Pc(24) respectively). What we observed, contrary to our hypothesis, was that compared to animals receiving SE alone, animals in the LFP+Pc(1) and LFP+Pc(6) groups had a significant decrease in acute hippocampal cell death. However, LFP+Pc(1) animals observed 20 weeks post-injury developed hilar cell death, mossy fiber sprouting and seizures similar to animals receiving Pc alone.

Experimental Procedures

Subjects

A total of 241 male, P19, Sprague Dawley (Charles River) rats (34–58 grams) were used in the following experiments. In order to quantify acute cell death 6 groups were used: sham, Pc and LFP as well as LFP+Pc(1), LFP+Pc(6) and LFP+Pc(24). Animals were sacrificed 24 hours following their final insult. The second experiment utilized 5 groups: naïve, sham, Pc and LFP as well as LFP+Pc(1). LFP+Pc(1) was chosen specifically based on preliminary data pertaining to acute hippocampal cell death which suggested this time point as having maximal neuroprotection. These animals were used to observe long terms outcomes (20 weeks post-injury). All group assignments were done in a random fashion. The UCLA Chancellor's Committee for Animal Research approved all procedures detailed within this report.

Surgical Preparation

As previously described (Gurkoff et al., 2006), animals were anesthetized with isoflurane (1.5 – 2.5%) in 100% oxygen while body temperature was maintained at 37 – 38°C using a thermostatically controlled heating pad. Surgical areas were shaved and antiseptically cleaned prior to positioning in a stereotaxic frame. The temporalis muscle was resected on the left side and a 3-mm diameter craniotomy was centered 3 mm posterior to bregma and 6 mm lateral to the midline. A plastic injury cap was then affixed over the craniotomy and was filled with saline. Randomly assigned sham controls were maintained on isoflurane to ensure that duration of anesthesia was comparable to those animals receiving fluid percussion.

Injury Induction

The following injury induction has been described previously in detail (Prins et al., 1996, Gurkoff et al., 2006). Animals were removed from isoflurane anesthesia, connected to the injury device and monitored for the return of a hind paw withdrawal reflex to toe pinch to determine the threshold of surgical anesthesia. Immediately following the return of this withdrawal reflex, fluid percussion was initiated. After the insult, hind-paw withdrawal reflex was lost for a period of seconds-to-minutes which operationally defined the time of loss-of-consciousness (LOC). The duration of LOC was assessed by pinching the hind-paw at 15s intervals and recorded when the response returned.

In addition to LOC, animals were monitored for apnea. If animals exhibited apnea for more than 45s post-injury, they were resuscitated using 100% oxygen until they exhibited spontaneous breathing. Once both spontaneous respiration and the hind paw withdrawal reflex recovered, general anesthesia was reinstated for removal of the injury cap and the suturing of the wound. Anesthesia was removed and animals were placed in a cage maintained at room temperature until recovery.

LOC was used to evaluate severity of injury (Giza et al., 2002, Gurkoff et al., 2006). The current set of experiments was restricted to animals that endured a severe injury (LOC > 120s). The average atmospheric pressure (ATM) for these severe injuries was 2.20 ± 0.02 (mean $\pm$ standard error of the mean) ATM. A total of 19 animals did not exhibit a severe LFP as defined by LOC and were excluded. One animal in the long-term outcome study died within the first 30 minutes post-LFP.

Induction of Secondary Insult: Lithium Pilocarpine Status Epilepticus

As described previously (Jope, 1979), all animals were given a single s.c. injection of 3 mEq/kg of lithium chloride, 9 – 16 hours prior to injection of Pc or Saline. Following LFP animals were given either 1, 6 or 24 hours to recover before the injection of Pc (30 – 60 mg/kg or equivalent volume of saline). For the Pc-alone group, sham animals received Pc 1-hour following recovery from anesthesia. Animals were not exposed to warmth after Pc injection as motor seizures generate hyperthermia (Meldrum and Brierley, 1973) and any further heating could lead to increased mortality. Animals were observed periodically over the course of the day to determine the extent of SE. For the animals receiving Pc, animals were included only if they exhibited Racine level 5 (rearing and loss of posture) SE (Racine, 1972, Hirsch et al., 1992). Within the first 60 minutes all animals receiving Pc experienced Racine 5 or 6 seizures. Pc did lead to a significant amount of mortality, all within the first 48 hours following administration. Total mortality due to Pc was 23% for acute neuronal counts (Pc = 6, LFP+Pc(1) = 3, LFP+Pc(6) = 3) and 45% (Pc = 15, LFP+Pc(1) = 10) in the 20-week outcome study.

Tissue Processing for Acute Cell Death Analysis Using Hematoxylin & Eosin (H&E) Fluorescence

Animals were placed under deep pentobarbital anesthesia (100 μ l/kg) 24-hours post-injection of Pc and transcardially perfused using 4% paraformaldehyde. This time point was selected as studies of LFP (Conti et al., 1998), and Pc-induced seizures (Sankar et al., 1998) suggested that the period of maximal hippocampal cell death is 24-hours. Following perfusion, brains were post-fixed for 4 hours in 4% paraformaldehyde. Fluorescent H&E staining was performed as previously described (Sankar et al., 1997). Briefly, brains were dehydrated in graded ethanol solutions, cleared with xylene and embedded in paraffin. Coronal, 8 μ m sections were cut and sections saved throughout the entire span of the hippocampus. These sections were then deparaffinized, hydrated, and stained with modified H&E (0.03% eosin).

Quantifying Acute Cell Death Within the Dorsal Hippocampus Using H&E Fluorescence

Cell death was analyzed in three sections spanning from the beginning of the dorsal hippocampus to the most caudal aspects. The first section sampled corresponded to approximately –2.3 mm bregma in the adult rat atlas (Paxinos and Watson, 1986) based on specific anatomical landmarks including the size and shape of the hippocampus and the lateral ventricles. Subsequent sections were sampled at 160 μ m intervals. All fluorescent cells were counted bilaterally in the hippocampus (a sum of the hilus, CA3 and CA1) to assess the total number of acidophilic (eosinophilic) cells of each animal. As both LFP and Pc-induced SE generate specific patterns of cell death in the hilus, CA3 and CA1, these sub regions were analyzed individually to determine whether changes in cell viability were regionally specific. The criterion for counting a profile as a dying cell included an easily identifiable, bright, fluorescent soma with a dark pyknotic nucleus (Sankar et al., 1998).

Seizure Threshold

Following Pc-induced SE at ages P14–28 epileptogenesis occurs between post-SE weeks 8 and 16 (Sankar et al., 1998). In the current study, animals were individually observed over a four-day period at post-injury weeks 12 and 20 to determine if they had developed convulsive

seizures. Briefly animals were removed from their cages and handled by the investigator for roughly 2 minutes. As it is difficult to assess mouth and facial movement as well as heading nodding, only animals exhibiting bilateral forelimb clonus or greater seizures were considered to have convulsive seizures. Following observation at post-week 20, rats were infused with the GABA antagonist pentylenetetrazol (PTZ) (Davidoff and Hackman, 1978) in order to measure the threshold for both bilateral forelimb myoclonus and for generalized seizure (Pollack and Shen, 1985) providing a quantitative measurement of seizure threshold. A tail-vein catheter was secured, flushed with 0.5 mL of 0.9% saline and 10 mg/mL PTZ (in sterile saline) was infused at a rate of 3 mL/hour. The time from PTZ infusion to the first bilateral, forelimb myoclonus and generalized seizure was recorded. For analysis of seizure threshold data, time-to-seizure was converted to mg/kg measurements based on the infusion rate and the weight of each animal. After the onset of generalized seizure, animals were euthanized (100 mg/kg pentobarbital i.p.) and processed for histology.

Tissue Processing for Timm and NeuN

After animals were transcardially perfused using 0.9% saline (200 mL), 0.1% sodium sulfide (200 mL) and 4% paraformaldehyde (300 mL), brains were removed and post-fixed for 4 hours in 4% paraformaldehyde, placed in a 30% sucrose solution and stored at 4°C for at least 72 hours. The brain was then quick frozen in 2-methylbutane at -55°C, embedded in media (Cytoseal 60) and sectioned into 40 µm coronal sections. Every section was saved between the genu and the splenium of the corpus callosum and stored in sets of sections at 400 µm intervals for unbiased, systematic analysis. One series of these sections was randomly selected and stored in 0.1 M Tris Buffered Saline (TBS) at 4°C for Timm staining. The remaining sections were preserved in 0.1 M Tris Buffered Saline (TBS) with 0.05% azide and stored at 4°C for immunohistochemical staining with NeuN.

Timm Histochemistry

From the serial, 40 µm, coronal sections, only those containing the dorsal hippocampus (approximately -2.3 mm bregma, to approximately 4.48 mm bregma) were stained. Sections were developed in the dark for 1 to 2 hours (based on staining intensity) in a 6:3:1 mixture of gum arabic (20%, w/v), hydroquinone (5.6%, w/v), citric acid-sodium citrate buffer with 1.5 ml of a silver nitrate (17%) solution (Sankar et al., 1998). Sections were then rinsed for 10 minutes in running tap water, dehydrated and cover-slipped. The last three sections (400 µm intervals) containing the dorsal hippocampus were blindly evaluated at high magnification (400X) and scored on a scale of one-to-five (Huang et al., 1999) based on the degree of supragranular mossy fiber sprouting (Table 1). This scale has been used to characterize supragranular sprouting in rats whose initial epileptic insult was between P0 and P20 (Huang et al., 1999, Rutten et al., 2002, Cilio et al., 2003). We experienced some difficulty with the Timm stain. Tissue from 24 animals was excluded from this study because there was either a failure to stain or staining artifact. Failure to stain was defined as a complete failure to stain any part of the tissue while an excess of staining throughout the entirety of the tissue characterized staining artifact. In both cases it was impossible to objectively assess whether there was supragranular sprouting. All sections with successful Timm staining were included in the final analysis. (Insert Table 1 about here)

NeuN Immunohistochemistry

Sections were stained at 200 µm intervals with the neuron specific marker NeuN (Chemicon MAB377, Temecula CA) starting at the genu of the corpus callosum and spanning the hippocampus (22 – 26 sections). Sections were washed three times in TBS and then placed in a blocking solution (10% normal horse serum, Vector Labs, in 0.1 M TBS with 0.5% triton) for one hour. Sections were then incubated in the primary antibody (diluted 1:10,000 in 0.1 M

TBS with 0.5% triton) at room temperature overnight. The following day, sections were washed with 0.1 M TBS and then placed in the secondary antibody (biotinylated anti-mouse IgG, rat-absorbed, Vector Labs; diluted 1:400 in 10% horse normal serum in 0.1 M TBS). Sections were then washed in Tris buffer to remove saline. To visualize the stain, sections were incubated in 3,3'-diaminobenzidine (DAB; Vector Labs, Burlingame, CA) for 60s followed by immediate washes in Tris buffer. Sections were then mounted serially onto subbed slides and allowed to dry overnight.

Stereological Estimation of NeuN-Positive Neurons

All counts were made using an optical fractionator probe as previously described (Gurkoff et al., 2006). Briefly, the optical fractionator probe utilizes systematic, random, multilevel sampling to estimate both the volume and the number of cells per a designated region of interest (ROI) (Gundersen et al., 1988). Stereological software (Stereo Investigator, Microbrightfield, Williston VT) was used to create the probe, determine the number of neurons in each ROI and the coefficient of error. Contouring was performed at 400x and counting was done under 1,000x oil immersion. Three ROIs of the hippocampus were counted bilaterally: the hilus, CA3 and CA1. Each ROI was quantified starting from the first section containing dorsal hippocampus (approximately -2.3 mm bregma) and spanning until the section before the dorsal and ventral hippocampus fuses (approximately 4.48 mm bregma). The hilus was operationally defined as the area between the blades of the dentate gyrus including CA3/CA4 interface (Figure 1). The border between CA2 and CA3 was operationally defined using anatomical markers to ensure that each region had similar boundaries (Figure 1). For the CA1, the contour was started at the border of the CA2/CA1 region as determined by the reduction in thickness of the pyramidal layer. The CA1 was then contoured to the border of the subiculum (Figure 1). Neurons were selected for counting only if they were evenly stained and with a sharp contour of the soma. Furthermore, cells were only counted if part of the soma resided within the counting frame following standard stereological rules (Gundersen et al., 1988).

Statistical Analysis

Statistical analysis was performed using SPSS software (version 17.0, SPSS, Chicago, IL). Measures including apnea, LOC, and weight (before and after injury) were analyzed with a one-way ANOVA. Post-traumatic SE was analyzed with a two-way ANOVA [group $\times$ time] followed by Dunnett's post-hoc to compare all LFP+Pc animals with those receiving Pc alone. For the long-term study, including physiological measures, NeuN stereology and seizure threshold analysis, separate ANOVAs were conducted comparing the five groups (naïve, sham, LFP, Pc, LFP+Pc). For the stereological counts, we used a two-way ANOVA [group $\times$ time] to evaluate significance. For seizure threshold, animals were not only compared by group but also based on the presence and absence of seizures. As Timm analysis used a non-linear scale, the non-parametric Mann-Whitney Test was used to compare both the ipsilateral and contralateral hippocampus between all groups. Comparisons that reached $P < 0.05$ were considered statistically significant.

Results

Acute Cell Death: Physiological Measures

There was no significant difference in weight among any of the groups at the time of injury. There was a difference in the duration of anesthesia among groups ($F_{(5,49)}=6.202$, $p < 0.001$) with a simple main effect detected between sham-Pc animals and LFP ($p < 0.04$), LFP+Pc(1) ($p < 0.001$) and LFP+Pc(6) ($p < 0.001$). There was no significant difference between apnea and LOC among LFP groups (Table 2).

Acute Hippocampal Cell Death

Sham injuries were conducted to ensure that surgery alone did not lead to significant cell death. There was minimal cell death in both the ipsilateral (0.14 ± 0.34 cells per section) and contralateral (0.26 ± 0.57 cells per section) hippocampi 24 hours following injury. As there is virtually no cell death in sham animals, any cell death seen in experimental groups must be due to the specified insults (LFP, Pc or LFP+Pc). For these reasons, shams were not included in the statistical analysis of acute cell death.

Previous stereological studies in our laboratory have indicated that LFP in P19 rats does not cause significant hippocampal neuron loss (Gurkoff et al., 2006). Conversely, studies in P21 animals receiving Pc have indicated that prolonged seizures do generate significant hippocampal cell death (Sankar et al., 1998). Therefore, to determine the potential for LFP to increase vulnerability to seizure-induced cell death all statistical comparisons were made against animals receiving Pc alone.

We hypothesized that LFP would increase vulnerability to Pc-induced cell death. In order to assess the timing of vulnerability animals were given Pc 1, 6 or 24 hours after LFP. Total hippocampal cell death was assessed (Figure 2A,B) 24-hours following administration of Pc using H&E fluorescence (Figure 3). There was a significant effect of injury on group in both the ipsilateral ($F_{(4,50)} = 5.108$, $p < 0.002$; Figure 2A) and contralateral hippocampus ($F_{(4,50)} = 4.801$, $p < 0.002$; Figure 2B). Post-hoc analysis revealed significantly less cell death bilaterally in animals subjected to LFP alone ($p < 0.02$ ipsilateral, $p < 0.02$ contralateral), as well as when Pc followed LFP by 1 or 6 hours (LFP+Pc(1) $p < 0.03$ ipsilateral, $p < 0.05$ contralateral; LFP+Pc(6) $p < 0.02$ ipsilateral, $p < 0.02$ contralateral) as compared to animals receiving Pc alone. In contrast, when LFP preceded Pc by 24 hours (LFP+Pc(24)), there was no significant difference in hippocampal cell death compared to Pc alone.

In order to determine whether these potential neuroprotective effects showed regional distinctions within the hippocampus, we broke down the total hippocampal cell counts to examine differences in the hilus (Figures 4A,B), CA3 (Figures 4C,D) and CA1 (Figures 4E,F). In the hilus there was a statistically significant main effect of injury in both the ipsilateral ($F_{(4,50)} = 9.880$, $p < 0.001$; Figure 4A) and contralateral hilus ($F_{(4,50)} = 7.498$, $p < 0.001$; Figure 4B). Post-hoc revealed significantly less cell death bilaterally in the LFP only group ($p < 0.001$ ipsilateral, $p < 0.001$ contralateral) and a trend toward a reduction in the LFP+Pc(6) group ($p < 0.09$ ipsilateral, $p < 0.06$ contralateral) as compared to animals receiving Pc alone.

In the CA3 there was a significant effect of group ipsilaterally ($F_{(4,50)} = 3.425$, $p < 0.02$; Figure 4C) but did not reach statistical significance contralaterally ($F_{(4,50)} = 2.406$, $p < 0.06$; Figure 4D). Post-hoc analysis of the ipsilateral CA3 revealed significantly less cell death in the LFP group ($p < 0.007$) and trends toward a reduction in cell death in the LFP+Pc(1) and LFP+Pc(6) groups ($p < 0.08$ and $p < 0.06$ respectively) as compared to animals receiving Pc alone.

Finally, in the CA1, there was a significant effect of group in both the ipsilateral ($F_{(4,50)} = 2.852$, $p < 0.03$; Figure 4E) and contralateral ($F_{(4,50)} = 3.023$, $p < 0.03$; Figure 4F) hemispheres. Post-hoc analysis revealed significantly less cell death ipsilaterally in animals receiving LFP alone ($p < 0.05$) as compared to animals receiving Pc alone. There were trends towards reductions in the LFP+Pc(1) and LFP+Pc(6) groups ($p < 0.1$ and $p < 0.07$ respectively). In the contralateral CA1 cell death was significantly less in animals receiving LFP alone ($p < 0.03$) and LFP+Pc(6) ($p < 0.05$). Additionally there was a trend towards a reduction in cell death in the contralateral CA1 of animals receiving LFP+Pc(1) ($p < 0.08$).

In sum, these data indicate that Pc generates a significant amount of cell death compared to animals receiving LFP alone. In the LFP+Pc paradigm, LFP does not increase the vulnerability

of the hippocampus to Pc-induced cell death across the intervals of 1h, 6h and 24h between injuries. In fact, analysis of the entire hippocampus revealed a significant bilateral reduction in cell death when Pc was administered either 1 or 6 hours post LFP. This apparent neuroprotective effect was evident to varying degrees across each of the sub-regions of the hippocampus within this 6-hour temporal window.

Long-Term Outcome: Naïve vs. Sham Animals

For the long-term study both age-matched sham and naïve animals were utilized as controls. Although sham-injured animals were subjected to both isoflurane anesthesia and a craniotomy, there were no significant differences between the sham and naïve groups on any measure other than anesthesia time. For this reason these two groups were combined into one “control” group (N = 33) for all remaining aspects of this study.

Long-Term Outcome: Physiological Measures

Analysis of pre-injury weight found no difference between groups (Table 3). There was no difference in mortality between animals receiving Pc (47%) and LFP+Pc(1) (46%) injuries. Over the first four days post-injury, animals receiving LFP, Pc and LFP+Pc(1) experienced a significant ($p < 0.001$) drop in weight compared to control animals (16%, 13% and 14% respectively). Two-weeks post-injury LFP and Pc-injured animals still had significantly lower weights ($p < 0.05$) than controls (13% and 6% respectively). By 12-weeks post-injury the weights between groups were not different. After post-injury day 4 there was no significant difference in the rate of weight gain between any of the groups.

All surgical groups received similar durations of anesthesia (Table 3); (sham = 59.1 ± 3.6 , LFP = 64.7 ± 2.9 , Sham+Pc = 56.2 ± 3.6 and LFP+Pc(1) = 62 ± 3.5 minutes $\pm$ SEM). There was no significant difference in anesthesia time ($F_{(3,60)} = 1.271$, $p = 0.292$).

In addition, there was no significant difference in duration of apnea after injury (LFP = 316.2 ± 4.9 , LFP+Pc(1) = 299.1 ± 42.8 seconds $\pm$ SEM; $p = 0.79$) or LOC (LFP = 386.1 ± 49.6 , LFP+Pc = 329 ± 47.9 seconds $\pm$ SEM; $p = 0.41$) between groups (Table 3). Analysis of the fluid pulse found that there was no significant difference in the output from the fluid percussion device between groups (LFP = 2.28 ± 0.02 , LFP+Pc(1) = 2.29 ± 0.01 ; Mean ATM $\pm$ SEM).

Long-Term Outcome: Seizures and Pentylentetrazol Threshold

None of the controls or animals receiving LFP alone displayed any convulsive seizure activity over the 20-week period. Animals receiving Pc alone (8 of 15; 53%) and LFP+Pc(1) (11 of 15; 73%) displayed convulsive seizures. While seizures were sometimes spontaneous, they were frequently observed in response to external stimulation including the opening of the door to the vivarium, opening of the cages, or the actual handling of the animals.

As described in the methods, a subset of animals (Control = 24, LFP = 12, Pc = 13 and LFP +Pc(1) = 10) was infused with the GABA antagonist PTZ in order to determine whether injured animals had a reduction in seizure threshold (Figure 5A,B). There was a significant main effect of group indicating a reduction in the seizure threshold for bilateral myoclonus ($F_{(3,55)} = 4.511$, $p < 0.007$; Figure 5A). Post-hoc analysis revealed a significant difference between the control and LFP+Pc(1) group ($p < 0.005$; Figure 5A) only but no differences between LFP+Pc(1) and animals receiving Pc alone. There was no main effect of group on the threshold for generalized seizure ($F_{(3,55)} = 1.634$, $P < 0.192$).

However, due to the fact that some of the animals treated with Pc develop seizures and others do not, there is an a priori reason to divide injured groups (controls excluded) based on the presence (epileptic, N=15) or absence (non-epileptic, N=20) of convulsive seizures in the

observation period. When PTZ data was analyzed based on the presence of convulsive seizures during this observation period, there was a significant reduction in threshold to both bilateral forelimb myoclonus ($F_{(1,34)}=41.9$, $p < 0.001$) as well as to generalized seizure ($F_{(1,34)}=13.8$, $p < 0.001$) in epileptic animals compared to non-epileptic animals (Figure 5B). These data indicate that animals in the LFP+Pc(1) group have seizures and a reduction in seizure threshold similar to those receiving Pc alone.

Long-Term Outcome: Timm Scoring

A subset of the subjects in each group was analyzed for mossy fiber sprouting (Table 4). Of the 24 animals not quantified, there was either no Timm staining or significant staining artifact as described in the methods section. Analysis of Timm staining revealed that many animals had mossy fiber sprouting (Figure 6A-D) ranging from 58% (7 of 12) in shams to 100% in the LFP+Pc(1) group (7 of 7; Table 4). Sprouting occurred both ipsilaterally and contralaterally and all scores ranged from 0 (no sprouting 6D) to 3 (near continuous sprouting; Figure 6C). There was no significant mossy fiber sprouting in LFP-injured animals as compared to controls (Figure 6E). There was a statistically significant increase in sprouting in the ipsilateral supragranular layer between controls and Pc-treated animals ($p < 0.045$) as well as between controls and LFP+Pc(1)-treated animals ($p < 0.028$; Figure 6E). In the contralateral supragranular layer there was a trend toward an increase in sprouting in both the Pc ($p < 0.142$) and LFP+Pc(1) animals ($p < 0.083$; Table 4). There was no difference between Pc and LFP+Pc(1) groups in either the ipsilateral ($p < 0.383$) or contralateral ($p < 0.71$) dentate gyrus. Therefore while animals receiving LFP+Pc had significant mossy fiber sprouting, these data indicate that this sprouting was not significantly influenced by LFP.

Long-Term Outcome: Stereological Counts of the Hippocampus

From the 59 animals tested for seizure threshold using PTZ, a total of 29 representative animals were randomly selected to estimate the total neuronal number in the hilus, CA3 and CA1 bilaterally (control = 7, LFP = 6, Pc = 7, LFP+Pc(1) = 8). The average coefficient of error for stereological counts was 0.08 with a range of 0.06 to 0.12. Within each region, there was no significant difference in the average number of counting frames analyzed or total neurons counted.

Stereological estimations of the total number of neurons per region of interest indicated no significant reduction in the number of neurons in the ipsilateral hilus, CA3 or CA1 between groups (Table 5). There was no difference in the estimated volume of each ROI between groups (Table 6). It should be noted that one animal was excluded from this analysis as it exhibited morphological pathology consistent with cerebral ischemia (Pulsinelli et al., 1982).

When the same stereological data was analyzed based on the presence ($N=9$) or absence ($N=12$) of convulsive seizures in the observation period in injured subjects, animals with seizures had significant reductions in neuronal number in both the ipsilateral and contralateral hilus ($F_{(1,19)}=10.0$, $p < 0.005$; $F_{(1,19)}=13.4$, $p < 0.002$ respectively; Figure 7). There was no difference in neuronal number in either the CA3 or CA1 based on the presence or absence of convulsive seizures. Therefore, while there was no relationship between LFP+Pc(1) injury and long-term cell death, there was significant hilar cell loss in animals that developed convulsive seizures. These data indicate, similar to seizure threshold data, that acute neuroprotection in the LFP+Pc(1) group did not prevent loss of hilar neurons 20-weeks post-injury in similarly injured animals.

Discussion

Our original hypothesis was that during the first hours post-TBI, the developing brain would be vulnerable to a secondary injury. However, contrary to what was anticipated, there was no evidence of acute enhancement of hippocampal cell death in animals receiving both LFP and Pc regardless of the interval between the two insults. In fact, our data indicate that LFP produced a state of transient neuroprotection, as there was a significant reduction in cell death bilaterally in the hippocampus when the interval between insults was either 1 or 6 hours.

In order to determine whether this acute neuroprotection led to an improved long-term outcome, a second group of animals was studied 20-weeks post-injury. Previous long-term data following Pc-induced SE in both the P14 (Sankar et al., 1998) and P21 rat (Sankar et al., 1998, Sankar et al., 2000, Sankar et al., 2002) indicated that a subset of these animals (30% and 70% respectively) developed seizures between 16–20 weeks post-injury. Additionally, Pc-induced SE in the P21 rat leads to supragranular mossy fiber sprouting (Liu et al., 1994, Sankar et al., 1998, Cilio et al., 2003). Based on our acute data showing similar degrees of neuroprotection at both the 1h and 6h interval, for the chronic study we selected the 1-hour interval between LFP and Pc to compare to animals receiving LFP, or Pc alone. Our data found, similar to the literature, that Pc-alone led to the development of convulsive seizures, a reduction in seizure threshold, and an increase in mossy fiber sprouting when analyzed 20-weeks post-injury. Animals receiving Pc 1 hour post-LFP also developed seizures, a decrease in seizure threshold and similar mossy fiber sprouting to animals receiving Pc alone. These long-term data suggest that early apparent TBI-induced neuroprotection did not ultimately prevent the development of chronic enhanced excitability and hippocampal sprouting. However, these data indicate that LFP also did not increase their vulnerability to subsequent Pc-induced seizures.

Experimental design considerations

To determine whether developing animals were vulnerable to secondary insults following LFP, we chemically induced SE using Pc in P19 rat pups. This age was chosen in part based on the extensive literature characterizing LFP (Prins et al., 1996, Prins and Hovda, 1998, Fineman et al., 2000, Giza et al., 2002, Giza et al., 2005, Giza et al., 2006, Gurkoff et al., 2006) and Pc-induced status epilepticus (Hirsch et al., 1992, Sankar et al., 1998, Sankar et al., 2000, Dubé et al., 2001, Rutten et al., 2002, Cilio et al., 2003), specifically in relation to patterns of acute and long-term anatomical pathologies. Additionally, this approach for double insult was selected as early post-traumatic seizures are a relevant clinical issue (Asikainen et al., 1999, Barlow et al., 2000, Vespa et al., 2007) in both the pediatric and adult populations.

Our principal early finding was that in the developing brain a prior TBI (within 6 hours) reduced the subsequent vulnerability to Pc-induced hippocampal cell death. The decrease in acute cell death might be explained by a difference in the magnitude of post-LFP seizures between the LFP+Pc and Pc only groups. However, after Pc administration, all animals were monitored visually and 100% experienced Racine 5 seizures, indicating generalized seizures (Racine, 1972, Hirsch et al., 1992). While this ensured a certain minimal level of seizure activity, we did not specifically quantify seizures using EEG. Additionally we did not interrupt seizure activity with diazepam to control the duration of SE as is frequently done in Pc studies (Walton and Treiman, 1988, Suchomelova et al., 2006). As we originally predicted that TBI would increase the vulnerability to SE, and we wanted to observe large increases in cell death, we chose not to stop the seizure activity or apply a benzodiazepine that is known to affect both behavioral and anatomical outcomes associated with brain injury (Turski et al., 1983, Statler et al., 2006). Our long-term data indicate that animals receiving both LFP and Pc have mossy fiber sprouting, hilar cell death and convulsive seizures similar to animals injured with Pc-alone. These long-term data, along with visual observation of Racine 5 seizures immediately

after injury, suggest that animals have a similar magnitude of seizures whether or not animals receive a LFP prior to Pc administration.

An additional concern in the experimental design is that lithium chloride (LiCl) is a proven neuroprotectant in models of ischemia (Nonaka and Chuang, 1998, Nonaka et al., 1998, Bian et al., 2007) and nerve crush (Schuetauf et al., 2006). LiCl is routinely pre-administered in studies utilizing Pc to reduce the peripheral effects of Pc (Honchar et al., 1983, Jope et al., 1986). To control for the potential neuroprotective effects, all animals were administered LiCl, including sham animals and animals receiving LFP alone. However it is unlikely that LiCl provided neuroprotection in these studies since previous stereological experiments have demonstrated that there is no significant cell death in the CA3 following the 6 mm lateral injury in P19 animals (Gurkoff et al., 2006). Furthermore animals receiving LiCl prior to Pc or Pc 24-hours post-LFP had significant hippocampal cell death.

One other consideration when using Pc as a model of secondary insult is the high mortality rate of animals primarily due to the peripheral effects of the drug. It was determined that the combination of LiCl with a sub-threshold dose of Pc generates a pattern of seizures and cell death similar to animals receiving a higher dose of Pc (Honchar et al., 1983, Jope et al., 1986) but with a diminished side effects profile (Clifford et al., 1987, Persinger et al., 1988). For initial experiments animals received a 60 mg/kg dose of Pc. As 100% of animals reached Racine 5 seizures Pc administration was reduced to 30 mg/kg. Regardless of the concentration of Pc 100% of animals reached Racine 5 or greater seizures and mortality was equivalent. While the dose of Pc ranges from 30–60 mg/kg per animal, once seizures are initiated in the first 15 minutes, seizure progression occurs independent of cholinergic input (Goodkin et al., 2003). These data suggest that seizure severity is likely not dependent on the initial dose of Pc.

Preconditioning vs neuroprotection

Preconditioning and neuroprotection have been well documented following serial lesions (Kennard, 1936, Kennard and Fulton, 1942, Finger et al., 1973), repeated cerebral ischemia (Kato et al., 1992b, Perez-Pinzon et al., 1999), epilepsy (Kelly and McIntyre, 1994, Najm et al., 1998) and in experimental traumatic brain injury (Otori et al., 2004) in mature animals. While there are limited studies addressing preconditioning and neuroprotection in young animals (Kennard, 1936, Kennard and Fulton, 1942, Vannucci et al., 1998, Gustavsson et al., 2005), our data demonstrate injury-induced neuroprotection following developmental experimental TBI. Additionally there are published experimental (Jenkins et al., 1989, Dixon et al., 1995, Zanier et al., 2003, Longhi et al., 2005) and clinical (Bouma and Muizelaar, 1992, Vespa et al., 1998) data indicating that TBI increases the vulnerability to a second insult. Our data suggest that in the developing rat, LFP reduces the acute effects of subsequent insults within the first six hours in terms of acute hippocampal cell death. Considering the previously cited literature on both neuroprotection and vulnerability, the transient neuroprotection observed in this study are likely related to the age at injury, injury type, injury severity, and the interval between insults.

Potential mechanisms of neuroprotection

One important finding of the current reported LFP-induced neuroprotection is that protection was observed in the first few hours post-injury. The majority of preconditioning literature finds neuroprotection not in the first hours post-injury but the first days (Kato et al., 1992b, Perez-Pinzon et al., 1999). This time window of the observed acute neuroprotective effect, when Pc is administered 1 or 6 hours following LFP, must be taken into account when considering potential mechanisms. As first proposed by Walker and colleagues (Walker et al., 1944), one of the hallmarks of experimental TBI is an elevation of extracellular potassium. Following LFP in the adult rat elevated extracellular potassium (Katayama et al., 1990) and resulting negative

shifts in DC voltage (Kubota et al., 1989) were observed in the hippocampus and described as “spreading depression-like” by Katayama and colleagues. Additional studies have described how direct application of potassium to the hippocampus results in waves of hippocampal spreading depression (Somjen, 1984, Herreras and Somjen, 1993b, a). Following LFP in rat, the use of a multi electrode probe demonstrated that TBI generated conditions resembling cortical spreading depression (Rogatsky et al., 1996) with cycles of spreading depression lasting at least 6-hours in animals receiving a severe LFP. Additionally, TBI has been shown to stimulate cortical spreading depression in the cat (Ishijima and Walker, 1969) and in humans (Oka et al., 1977, Strong et al., 2002, Strong and Dardis, 2005). It has been demonstrated that spreading depression can propagate from the cortex into the hippocampus leading to hippocampal spreading depression (Wernsmann et al., 2006). Therefore in the hours following LFP in the developing rat, increases in extracellular potassium in the hippocampus, and waves of cortical spreading depression may lead to a state of hippocampal spreading depression.

During spreading depression, neither sensory nor direct stimulation can produce an evoked potential in the cortex (Leão, 1944). There is evidence that cortical spreading depression alone can precondition the rodent hippocampus to secondary insult as cortical spreading depression reduced the cortical lesion volume in response to a secondary ischemic insult (Matsushima et al., 1996) in the first days post-injury. The peak period of Pc-induced seizures in the 1-hour group for this study corresponds to the period in which the cortex was observed to undergo regular cycles of spreading depression (Rogatsky et al., 2003). Rogatsky and colleagues found that severely injured animals were still experiencing significant spreading depression at 6-hours (the last published data point) which corresponds with the start of Pc-induced seizures in the 6-hour interval group. Furthermore D’Ambrosio and colleagues reported an elevation of extracellular potassium in the hippocampus of the injured rat pup lasting out to 48-hours post-injury (D’Ambrosio et al., 1999). Perhaps TBI-induced spreading depression in the first hours post-injury could reduce neuronal activation in response to a secondary insult leading to transient neuroprotection.

An additional feature of the LFP is that it leads to an acute reduction in glucose metabolism bilaterally in the first hours following injury in the developing rat brain (Thomas et al., 2000) as well as an reduction in mean arterial blood pressure (Prins et al., 1996). It is possible that cells undergoing metabolic dysfunction may be depressed and unable to respond to a second insult that may account for the observed bilateral acute neuroprotection following a lateral TBI. In fact it has been demonstrated that following injury and during the prolonged metabolic depression that hippocampal neuronal excitability and long-term potentiation are significantly reduced (Reeves et al., 1995, Reeves et al., 1997). Furthermore as early as 1-hour post-injury paired-pulse facilitation was impaired in the CA1 of the hippocampus suggesting a transient impairment of presynaptic terminal functioning (Reeves et al., 2000). It was also demonstrated that extracellular glucose concentrations during stimulation 1 day following LFP decreased compared to sham controls (Ip et al., 2003). These data suggest that LFP alters the underlying hippocampal circuitry in the hours-to-days following injury. If, for example, the CA3 of the hippocampus is lesioned prior to induction of LFP, eliminating the primary excitatory input into the CA1, the characteristic injury-induced CA1 metabolic dysfunction was prevented (Yoshino et al., 1992). Therefore overall changes in excitability and reduction in the function of the hippocampal circuit may be responsible for the reduced excitotoxic effect of Pc in the first hours post-injury.

An alternative and controversial hypothesis is that activation of cells after brain injury may improve cell survival and recovery of function. For example in both rodents and feline studies of acute post-injury activation (Feeney and Hovda, 1983, Hovda and Fenney, 1984, Hovda et al., 1989, Sutton et al., 1989) and electroconvulsive seizures in rats (Feeney et al., 1987), stimulated animals demonstrated behavioral improvement compared to animals receiving a

contusion only. In other rodent studies PTZ-induced seizures actually led to improved outcome following cortical lesions (Hernandez and Schallert, 1988) or fluid percussion injury (Hamm et al., 1995). Additional studies have described that following TBI, the activity of the brain in response to a secondary stimulation is significantly reduced, not eliminated (Dietrich et al., 1994, D'Ambrosio et al., 1999, Ip et al., 2003). It was hypothesized that moderate seizure activity may stimulate an adaptive response while severe SE generates a maladaptive response following TBI (Schallert et al., 1986). This idea is supported by our data where the injured developing brain has the capacity for acute neuroprotection but the interaction between developmental plasticity and post-injury activation ultimately led to epileptic pathology.

In summary, this research demonstrates that severe LFP injury in the P19 rat produces a temporary state of neuroprotection of the neurons within the hippocampus in terms of reduced SE-induced cell death 24-hours post-injury. However, in the weeks after, seizure threshold was reduced, supragranular mossy fiber sprouting occurred, and over 70% of double-injured rats developed convulsive seizures. These data highlight the complexity of central nervous system injury in the developing mammal. While the central nervous system is still undergoing maturation it retains a higher capacity for adaptation however, there may be no innate mechanism guiding plasticity to a strictly beneficial outcome. While LFP protected cells acutely, reducing Pc-induced cell death as compared to animals receiving Pc alone, the chronic network response to seizure activation was unaffected, leading to aberrant plasticity and the eventual development of convulsive seizures. Based on these data, combined with the more extensive literature of preconditioning and vulnerability, one might predict that vulnerability and preconditioning will not only vary depending on the age at injury, but also on injury severity, type of injury, interval between injuries and time post-injury that the subject is evaluated. More importantly, neuroprotective strategies that preserve cells acutely may not by themselves alter the long-term consequences, such as seizures and/or neurocognitive disorders that accompany TBI.

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Abbreviations

TBI	Traumatic Brain Injury
LFP	Lateral Fluid Percussion Injury
Pc	Pilocarpine
SE	Status Epilepticus
LFP+Pc	Lateral Fluid Percussion combined with Pilocarpine
P19	Post-Natal Day 19
LOC	

Loss Of Consciousness

PTZ

Pentylentetrazol

ROI

Region of Interest

SEM

Standard Errors of the Mean

ANOVA

Analysis of Variance

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Figure 1.

Contours for Stereological Counts. The contour of the hilus encompasses all of the area in-between the dorsal and ventral blades of the dentate gyrus (gray hatched area; "H"). The CA3 region (gray line) includes the entire CA4 as well as the CA3 up to the border defined by drawing a line from the tip of each blade of the dentate gyrus extending to the CA3. The CA1 (black line) extends from the border of the CA2/CA1 region to the subiculum.

Figure 2.

Hippocampal Cell Death. Compared to animals receiving Pc alone, there was statistically less cell death in the ipsilateral (A) and contralateral (B) hippocampus in animals receiving only LFP as well as in animals receiving Pc either 1 or 6 hours after TBI. By 24 hours post-LFP, Pc-induced cell death was comparable to animals receiving Pc alone. * = $p < 0.05$

Figure 3.

H&E Staining. H&E fluorescence was accomplished by using a diluted concentration of eosin compared to normal staining. Dying neurons are clearly indicated by a strong eosin fluorescence signal as well as a pyknotic nucleus. Following LFP there is no indication of cell death in either the hilus (A), CA3 (C) or CA1 (E) of the hippocampus. Following Pc-induced status there are a number of dying cells in the hilus (B), CA3 (D) and CA1 (F). Scale bar A,B= 150 μ m, D-F = 50 μ m

Figure 4.

Analysis of Acute Cell Death by Region. Compared to animals receiving Pc alone, animals subjected to LFP alone demonstrated significantly less cell death in the ipsilateral (A) and contralateral (B) hilus. There were trends towards a bilateral decrease in cell death in animals receiving Pc 6-hours post-LFP. Additionally there were statistically fewer dying cells counted in the ipsilateral (C) CA3 in animals receiving only LFP when compared to those receiving Pc. There was no main effect of group in the contralateral CA3 (D). There were trends towards a decrease in cell death in the ipsilateral CA3 in animals receiving Pc either 1 or 6-hours post-LFP. Analysis of the CA1 found that animals that experienced LFP alone showed significantly less cell death in the ipsilateral (E) and contralateral (F) than those receiving only Pc. Animals

receiving Pc-6 hours post-LFP showed significantly less cell death in the contralateral CA1 than Pc only animals. Additionally there were trends towards a decrease in cell death in the ipsilateral CA1 of animals receiving Pc either 1 or 6-hours post-LFP and the contralateral CA1 of animals receiving Pc 1-hour post-LFP. * = $p < 0.05$

Figure 5.

PTZ seizure thresholds. (A) PTZ infusion at 20-weeks post-injury demonstrated a significant reduction in bilateral forelimb myoclonus (* $P=0.007$) with a simple main effect of group between controls and LFP+Pc (** $P=0.005$). (B) When compared based on the presence ($N=15$) or absence ($N=20$) of convulsive seizures there were significant differences between groups for both bilateral forelimb myoclonus and generalized seizure. Values are the mean PTZ concentration to evoke seizure behavior $\pm$ SEM. * $p < 0.001$.

Figure 6.

Timm staining in the hippocampus. Animals were scored on a 0 – 5 scale based on sprouting of mossy fibers into the supragranular layer. In a sham-injured animal, the presence of a single fiber, sprouting from the dentate gyrus into the supragranular layer, while difficult to detect at low power (A) was quite obvious at higher magnification (B). While several sections exhibited a score of 2, only one LFP+Pc animal had near continuous sprouting, a score of 3, in the supragranular layer (C). Several animals from sham (4), LFP (2) and Pc (1) groups had no supragranular sprouting (D). Based on mean scores, there was no difference in mossy fiber sprouting between controls and animals receiving LFP alone (E). However animals receiving Pc, regardless of prior LFP, developed significant mossy fiber sprouting into the supragranular layer of the dentate gyrus. Scale bars A, C, D 500 μ m, and B, 25 μ m. Individual data is represented in the figure (●)

Figure 7.

Long-term stereological counts. Stereological counting revealed a significant reduction in the estimated number of hilar neurons in injured animals with convulsive seizures as compared to injured animals with no observed seizures. Individual data is represented in the figure (●)

Scoring mossy fiber sprouting. Supragranular mossy fiber sprouting was scored on a scale of 1 – 5. Adapted from Rutten et al., 2002).

Table 1

Score:	Description:
0	No granules observed.
1	Only occasional granules in the supragranular layer.
2	Numerous granules in the supragranular layer occurring in a patchy distribution.
3	Numerous granules in the supragranular layer occurring in a near continuous distribution.
4	Highly concentrated band of granules appearing either in a near-continuous or continuous distribution
5	Continuous dense layer of granules.

Table 2

Acute H&E Physiological Data.

	N	wt(g)	Apnea (s)	LOC (s)	Anesth (min)
Sham	12	45.5±2.1	0	0	52.3±9.0
LFP	9	46.0±2.5	260.3±50.7	272.6±44.6	57.6±2.7
Pc	16	43.2±1.7	0	0	30.0±5.8
LFP+Pc(1)	10	43.0±1.8	178.1±29.8	221.0±30.1	60.0±2.8
LFP+Pc(6)	9	45.3±1.5	283.4±56.7	373.6±90.5	65.0±4.6
LFP+Pc(24)	12	48.8±0.7	225.9±23.5	274.6±27.2	48.3±5.9

N = Sample Size; wt = weight at time of LFP; Apnea = the duration of loss of spontaneous respiration in seconds; LOC = duration of loss-of-consciousness in seconds; Anesth = Anesthesia in minutes.
All values represent mean ± SEM.

Table 3

Long-Term Physiological Data.

	N	Wt(0) (g)	Wt(20) (g)	Apnea (s)	LOC (s)	Anesth (min)
Control	33	45.4±0.9	638.4±10.4	0	0	59.1±3.6
LFP	16	44.9±1.3	606.8±11.2	316.2±49	386.1±49.6	64.6±2.9
Pc	17	43.9±1.0	623.2±17.9	0	0	56.2±3.6
LFP+Pc(1)	15	45.9±1.1	656.7±37.9	299.1±42.8	329.7±47.9	62.0±3.5

N = Sample Size; wt(0) = weight at the time of LFP; wt(20) = weight 20 weeks post-insult; Apnea = the duration of loss of spontaneous respiration in seconds; LOC = duration of loss-of-consciousness in seconds; Anesth = Anesthesia in minutes. All values represent mean ± SEM.

Table 4
Timm scoring in the supragranular layer of the dentate gyrus.

Group	N	Sprouting	Ipsi Score	Contra Score
Control	12	7	0.47±0.14	0.47±0.17
LFP	8	6	0.58±0.17	0.67±0.27
Pc	6	7	1.00±0.41 *	0.95±0.39
LFP+Pc(1)	7	7	1.38±.36 *	1.19±0.37

N = Sample size for each group; Sprouting = The number of animals exhibiting any mossy fiber sprouting in that group; Ipsi/Contra Score = Average score within the ipsilateral and contralateral side relative to the side of the fluid pulse ± SEM;

* p < 0.05 as compared to controls.

Hippocampal Cell Counts. Stereological estimation of the total number of cells in the hilus, CA3 and CA1 of the dorsal hippocampus 20-weeks post-insult.

Group	N	Hilus		CA3		CA1	
		Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Control	7	13.0±0.8	13.4±1.1	63.8±3.9	68.9±3.9	80.2±5.4	87.3±3.5
LFP	6	13.5±0.8	14.5±0.9	61.7±4.0	60.6±1.9	68.1±6.2	78.8±5.1
Pc	7	12.0±1.5	11.2±1.3	62.0±3.2	63.6±3.9	83.1±7.2	81.4±7.8
LFP+Pc(1)	8	12.2±0.8	11.7±0.5	66.0±3.1	70.8±3.4	86.3±3.5	94.7±3.4

N = sample size. Values represent means in thousands ± SEM.

Table 6
Volumes of hippocampal regions 20-weeks post-injury. Stereologically measured volume estimations (million μm^3) for each ROI.

Group	Hilus		CA3		CA1	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Control	909 $\pm$ 78	990 $\pm$ 65	529 $\pm$ 55	548 $\pm$ 49	419 $\pm$ 33	437 $\pm$ 31
LFP	879 $\pm$ 42	986 $\pm$ 64	506 $\pm$ 15	540 $\pm$ 22	375 $\pm$ 22	423 $\pm$ 17
Pc	864 $\pm$ 67	847 $\pm$ 63	514 $\pm$ 35	525 $\pm$ 31	402 $\pm$ 25	387 $\pm$ 28
LFP+Pc(1)	843 $\pm$ 50	873 $\pm$ 43	508 $\pm$ 32	540 $\pm$ 25	395 $\pm$ 41	414 $\pm$ 31

Values represent means $\pm$ SEM.