Hippocampal immediate early gene transcription in the rat fluid percussion traumatic brain injury model

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Traumatic brain injury (TBI) is one of the leading causes of neurological disability and death in the USA across all age groups, ethnicities, and incomes. In addition to the short-term morbidity and mortality, TBI leads to epilepsy and severe neurocognitive symptoms, both of which are referenced to post-traumatic hippocampal dysfunction, although the mechanisms of such hippocampal dysfunction are incompletely understood. Here, we study the temporal profile of the transcription of three select immediate early gene (IEG) markers of neuronal hyperactivation, plasticity, and injury, *c-fos*, *brain-derived neurotrophic factor (BDNF)*, and *Bax*, in the acute period following the epileptogenic lateral fluid percussion injury in a rodent TBI model. We found that lateral fluid percussion injury leads to enhanced expression of the selected IEGs within 24 h of TBI. Specifically, *BDNF* and *c-fos* increase maximally 1–6 h after TBI in the ipsilesional hippocampus, whereas *Bax* increases in the hippocampus bilaterally in this time window. Antagonism of the *N*-methyl-D-aspartate-type glutamate receptor by MK801 attenuates the increase in *BDNF* and *Bax*, which underscores a therapeutic role for *N*-methyl-D-aspartate-type glutamate receptor antagonism in the acute post-traumatic time period and suggests a value to a hippocampal IEG readout as an outcome after injury or acute therapeutic intervention. *NeuroReport* 25:954–959
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NeuroReport 2014, **25**:954–959

Keywords: glutamate, hippocampus, immediate early gene (IEG), MK801, *N*-methyl-D-aspartate, traumatic brain injury

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Received 28 April 2014 **accepted** 27 May 2014

Introduction

Traumatic brain injury (TBI) is a common cause of severe neurological disability and is one of the most common causes of death and disability in the USA across all age groups [1]. Immediately following TBI, and for hours to days afterwards, excessive glutamate release from dead and injured neurons [2], coupled with decreased expression of glial glutamate transporters at the injury site [3], leads to the hyperexcitation of neurons and excessive activation of Ca^{2+}-permeable glutamate receptors, leading to excitotoxicity and cell death [2,4,5]. Relevant to the present report, prevalent post-traumatic sequelae include post-traumatic epilepsy [6] and long-term cognitive and memory deficits [7], both of which are attributable to hippocampal dysfunction, independent of initial mechanical hippocampal damage [8,9]. Although the pathophysiology of post-traumatic hippocampal dysfunction is incompletely understood, two of the more common factors that contribute to both epilepsy and mnemonic deficits are delayed hippocampal neuronal death and exaggerated long-term potentiation of excitatory synaptic connections among the surviving neurons [10].

Extensive experimental data suggest that glutamate-mediated transcription of immediate early genes (IEGs) related to neuronal activity and apoptosis is a critical step toward hippocampal degeneration and synaptic plasticity abnormalities that follow TBI by days to weeks [10–14]. Yet, the temporal pattern of hippocampal post-traumatic IEG expression has not been studied extensively, particularly in the acute post-traumatic period.

In the rat lateral fluid percussion injury (LFPI) TBI model (one of the most-studied rat TBI models), excitotoxic neuronal death and continuing secondary damage – particularly to the hippocampus – after the initial focal neocortical insult lead to long-term neurologic symptoms such as post-traumatic epilepsy and memory deficits [15]. In moderate LFPI, differential hippocampal gene expression patterns, assayed using semi-quantitative microarray techniques, show a delayed and progressive response to injury even in the acute phase (0–24 h). However, the individual IEG data from these experiments have not been validated by quantitative real-time PCR (RT-PCR) [13].

As understanding the temporal progression of acute post-traumatic IEG expression may provide insights into early molecular mechanisms of post-traumatic neurologic symptoms [6,7] and may identify the window of opportunity for therapeutic intervention, we focused on IEG

0959-4965 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/WNR.0000000000000219

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assays in this period. Accordingly, we used the rat LFPI TBI model to investigate the transcriptional pattern of hippocampal IEG markers related to neuronal hyperactivation, initiation of long-term potentiation, and neuronal injury in the acute post-TBI period, and tested whether such markers are dependent on post-TBI glutamate-mediated activation of the *N*-methyl-D-aspartate (NMDA)-type receptor (NMDAR) – a potential therapeutic target [4,5] – that has been studied extensively in the acute post-traumatic time window [16,17], but not in the context of IEG expression.

Specifically, we measured the expression of *brain-derived neurotrophic factor (BDNF)*, which controls synaptic protein translation and potentiates synaptic strength [18]; *c-fos*, which codes for transcription factors controlling the expression of other target genes and reflects excessive neuronal activation [19]; and *Bax*, which is a reliable biomarker for the initiation of apoptotic cell death following brain injury [20].

Our results show that these select IEGs are upregulated in the ipsilesional hippocampus, whereas *Bax* increases bilaterally, within hours of focal cortical LFPI. Further, to underscore the critical role of glutamate in this process, we show that treatment with the NMDAR antagonist MK801 leads to attenuation of the changes in *BDNF* and *Bax* – but not *c-fos* – expression. Taken together, these data suggest that in the hours after injury, TBI leads to a glutamate-mediated increase in the expression of *BDNF*, *c-fos*, and *Bax*, and these changes may be mediated through NMDAR activation by excessive post-traumatic glutamate release and, therefore, are potentially preventable.

Materials and methods

Animals

Young adult male Long–Evans rats (305.7 ± 10.2 g) were used for the present experiments ($N = 4$ for all experimental groups). All animals were housed in a temperature-controlled animal care facility with a 12 h light–dark cycle. All procedures were approved by and in accordance with the guidelines of the Animal Care and Use Committee at Boston Children's Hospital (Boston, Massachusetts, USA) and the NIH Guide for the Care and Use of Laboratory Animals.

Lateral fluid percussion injury

Rats were anesthetized and surgically prepared for moderate LFPI as described previously [21]. Briefly, anesthetized rats were placed in a stereotaxic frame, and anesthesia was maintained with 1–3% isoflurane vapor throughout the surgery, which lasted 30 min. An incision was made into the scalp and a craniotomy was performed anterior to the lambda over the left parietal cortex, leaving the dura intact. A tube with a pipette tip cannula attached at one end was connected to the LFPI device (AmScien Instruments, Richmond, Virginia, USA) and filled with sterile saline so that there were no air bubbles in the tubing or the LFPI device fluid chamber. The cannula was positioned over the craniotomy using a micromanipulator, and a percussion wave of 2.15 (± 0.08) atm was delivered rapidly to induce injury. Sham rats were anesthetized and received a craniotomy but were not subjected to brain injury. For NMDAR antagonist experiments, rats underwent pretreatment with (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo [*a*,*d*]-cyclohepten-5,10-imine maleate (MK801, 3 mg/kg intraperitoneally) 15 min before LFPI, and an equal number of rats received intraperitoneal saline.

Rat body temperature was maintained with a thermostatically controlled heating pad set at 37°C until the animals were able to ambulate. Rats were administered opioid analgesics every 12 h after rLFPI.

mRNA isolation from hippocampal tissue

Animals were decapitated, using a sharp guillotine, 1, 3, 6, 12, and 24 h after injury. The hippocampus from both hemispheres was rapidly dissected out onto a chilled plate and immersed in RNA later (Life Technologies, Grand Island, New York, USA). Total RNA was extracted using the RNeasy Mini kit (Qiagen Inc., Valencia, California, USA) and stored at −80°C.

Quantitative real-time polymerase chain reaction

Total RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA). RT-PCR was carried out using the TaqMan Gene Expression Kit and *BDNF* (Rn02531967_s1), *c-fos* (Rn00487426_g1), *Bax* (Rn01480160_g1), and *GAPDH* (Rn01775763_g1) gene expression assays (Applied Biosystems) on an ABI PRISM 7300 system (Applied Biosystems). All reactions were carried out in triplicate. IEG expression was calculated using the $^{\Delta\Delta}C_t$ method, as described previously [22], and IEG expression after injury was normalized to expression in sham controls.

Data analysis

Data were expressed as mean ± SEM. Statistical significance was assessed by repeated-measures analysis of variance (ANOVA). Verum or Sham TBI and time after injury were used as between-subject factors and side (ipsilesional or contralesional hippocampus) as the within-subjects factor for IEG and *Bax* expression after TBI. Following MK801 administration, antagonist treatment was the between-subject factor for IEG expression, whereas treatment and time after injury were the between-subject factors for *Bax* expression, and side was the within-subject factor. Post-hoc analyses were carried out using Fisher's least significant difference tests.

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Results

Expression of activity and plasticity-dependent immediate early genes after lateral fluid percussion injury

Previous reports describe increases in hippocampal IEG mRNA in the acute phase following TBI in rodents [13, 14], but these semi-quantitative microarray data on temporal expression patterns of select IEGs in the acute post-traumatic time window after LFPI have not been validated by quantitative RT-PCR. We studied the temporal profile of the expression of *BDNF* and *c-fos* in the ipsilesional and contralesional hippocampus 1, 3, 6, 12, and 24 h after LFPI, compared with sham controls.

Repeated-measures ANOVA revealed significant main effects of injury [*c-fos*: $F_{(1,30)} = 26.97$, $P < 0.001$; *BDNF*: $F_{(1,30)} = 19.57$, $P < 0.001$], time after injury [*c-fos:* $F_{(4,30)} = 4.13$, $P < 0.01$; *BDNF*: $F_{(4,30)} = 3.73$, $P < 0.05$], and side [*c-fos*: $F_{(1,30)} = 23.29$, $P < 0.001$; *BDNF*: $F_{(1,30)} = 30.81$, $P < 0.001$], as well as significant interaction between injury and time after injury [*c-fos*: $F_{(4,30)} = 4.20$, $P < 0.01$; *BDNF*: $F_{(4,30)} = 3.81$, $P < 0.05$], injury and side [*c-fos*: $F_{(1,30)} = 23.70$, $P < 0.001$; *BDNF*: $F_{(1,30)} = 30.96$, $P < 0.001$], side and time after injury [*c-fos*: $F_{(4,30)} = 3.76$, $P < 0.05$; *BDNF*: $F_{(4,30)} = 6.51$, $P < 0.001$], and all three factors [*c-fos*: $F_{(4,30)} = 3.77$, $P < 0.05$; *BDNF*: $F_{(4,30)} = 6.80$, $P < 0.001$].

Post-hoc analysis revealed significantly elevated *c-fos* expression compared to sham 1 h (21.28 ± 7.24-fold, $P < 0.001$), 3 h (15.21 ± 9.29-fold, $P < 0.001$), and 6 h (7.40 ± 1.16-fold, $P < 0.05$) after TBI in the ipsilesional hippocampus (Fig. 1a). Expression of *BDNF* was elevated 3 h (9.33 ± 1.53-fold; $P < 0.001$) and 6 h (7.40 ± 2.19-fold, $P < 0.001$) after injury in the ipsilesional hippocampus (Fig. 1b) compared to sham. The contralesional hippocampi remained unaffected.

Bax expression after lateral fluid percussion injury

Bax is a member of the *Bcl2* family of genes and is a marker of cellular injury and apoptosis [20]. We thus evaluated *Bax* expression as a marker of cellular trauma and as a positive control for the molecular changes following the induction of LFPI. Analysis of *Bax* expression levels showed significant main effects of injury [$F_{(1,30)} = 7.26$, $P < 0.05$] and time after injury [$F_{(4,30)} = 2.95$, $P < 0.05$], as well as interaction between the two [$F_{(4,30)} = 3.15$, $P < 0.05$]. Post-hoc tests showed a significant, bilateral increase in *Bax* expression 6 h after LFPI (ipsilesional: 2.86 ± 0.49-fold, $P < 0.001$; contralesional: 2.33 ± 0.70-fold, $P < 0.01$; Fig. 1c).

Effect of *N*-methyl-D-aspartate-type glutamate receptor antagonism on acute immediate early gene and *Bax* expression following lateral fluid percussion injury

Given the critical role played by the NMDAR in neuronal dysfunction and death following injury and epileptic seizures [4,5], we hypothesized that blocking the receptor may selectively reduce pathological IEG expression following LFPI. We measured IEG expression levels 3 h after injury and MK801 (a competitive NMDAR antagonist) treatment, as expression was seen to peak at this time point. Repeated-measures ANOVA of *BDNF* expression levels showed significant main effects of treatment [$F_{(1,6)} = 9.57$, $P < 0.05$], side [$F_{(1,6)} = 34.14$, $P < 0.01$], and interaction between the two [$F_{(1,6)} = 16.93$, $P < 0.01$]. Post-hoc analysis revealed significantly attenuated upregulation of *BDNF* expression in the ipsilesional hippocampus of injured rats after administration of MK801, as compared with TBI controls (1.49 ± 0.45 vs. 9.33 ± 1.53-fold, $P < 0.01$), whereas no significant difference between vehicle and treatment was observed in the contralesional hemisphere (Fig. 2a). In contrast, *c-fos* expression was not significantly altered by MK801 therapy.

We measured *Bax* levels both 3 and 6 h after injury and MK801 administration, as *Bax* peaked at 6 h after LFPI. Analysis of *Bax* expression showed significant main effects of MK801 treatment [$F_{(1,12)} = 8.57$, $P < 0.05$], time after injury [$F_{(1,12)} = 10.25$, $P < 0.01$], and side [$F_{(1,12)} = 9.59$, $P < 0.01$], as well as significant interaction between treatment and side [$F_{(1,12)} = 5.51$, $P < 0.05$]. Post-hoc tests showed significantly attenuated upregulation of *Bax* expression bilaterally in rats treated with MK801, as compared with vehicle-treated controls, 6 h after LFPI (ipsilesional: 1.56 ± 0.07 vs. 2.86 ± 0.49-fold; contralesional: 1.35 ± 0.08 vs. 2.33 ± 0.70-fold, $P < 0.05$; Fig. 2b).

Discussion

Pathological TBI-associated alterations in hippocampal circuitry likely play an important role in the pathogenesis of post-traumatic epilepsy, memory deficits, and other post-traumatic symptoms [10]. Here, we describe the temporal pattern of the expression profile of IEG markers of neuronal activity and synaptic plasticity, and a marker of cellular injury following LFPI in the rat and demonstrate that the selected IEGs are activated within hours of TBI. We also show that treatment with the competitive NMDAR antagonist MK801 attenuates the expression of some of these genes in the hippocampus. These results may provide important clues with regard to the timing of the biological processes underlying the dysplastic response of the brain following TBI.

Post-traumatic immediate early gene expression suggests early pathologic potentiation of excitatory hippocampal circuits

Hippocampal expression of *c-fos* and *BDNF* is enhanced maximally 1 and 3 h after TBI, respectively. These IEGs are especially interesting given the contribution of aberrant potentiation of excitatory synaptic strength to epileptogenesis [23]. The upregulation of hippocampal *c-fos* reflects excess post-traumatic neuronal activity

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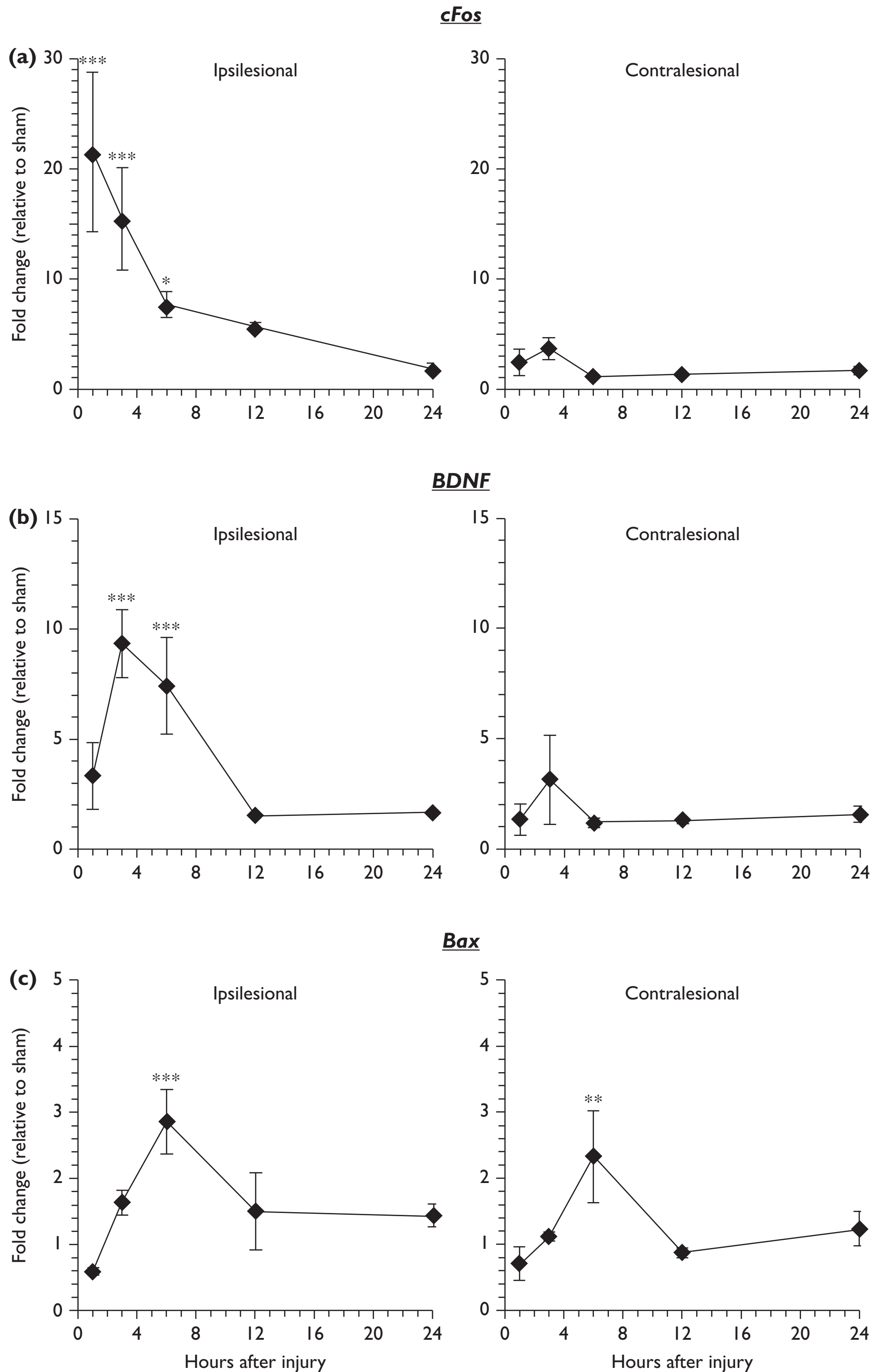
Fig. 1



Gene expression after LFPI. (a, b) There is significantly enhanced expression of the LTP-related gene *BDNF* (3, 6 h), and the neural activity marker *c-fos* (1, 3, 6 h) after injury in the ipsilesional (left) hippocampus compared with sham controls. (c) *Bax* expression is significantly increased in ipsilesional and contralesional hippocampus 6 h after LFPI, compared with sham controls. Data are expressed as average fold change compared with sham animals at each time point ($n = 4$/group). Error bars show SE of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *BDNF*, *brain-derived neurotrophic factor*; LTP, long-term potentiation; LFPI, lateral fluid percussion injury.

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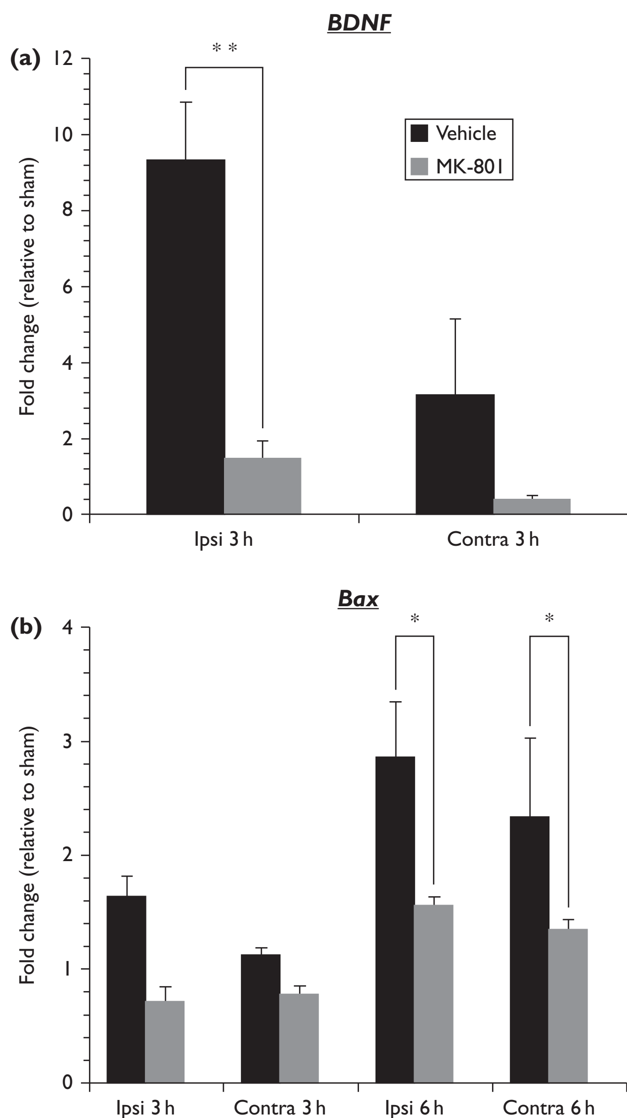
Fig. 2



NMDA receptor blockade changes post-LFPI gene expression in the hippocampus. (a) MK801 administration results in a significantly lower expression of *BDNF* in the ipsilesional hippocampus of treated animals 3 h after TBI as compared with vehicle-treated controls. (b) MK801 administration also significantly attenuates *Bax* upregulation bilaterally, 6 h after TBI in treated animals as compared with controls. Data are expressed as average fold change compared with sham animals ($n=4$/group). Error bars show SE of the mean. *$P<0.05$, **$P<0.01$.BDNF, brain-derived neurotrophic factor; NMDA, *N*-methyl-D-aspartate; TBI, traumatic brain injury.

previously demonstrated by gene expression studies [19] and corresponds to the timing of post-traumatic glutamate release and neuronal overactivation that accompanies brain injury [4,5]. The increase in *BDNF* expression corroborates the contribution of the acute processes to lasting hippocampal dysfunction, as *BDNF* is a critical growth factor that mediates neuronal survival as well as synaptic potentiation [18]. Our results show for the first time that hippocampal *BDNF* expression increases in the acute phase after LFPI. We note that whereas hippocampal *BDNF* upregulation has been documented in closed head injury [24] and controlled cortical impact [14] animal models, Li *et al.* [13] did not report a similar increase in an LFPI model. This discrepancy may be due to differences in the LFPI technique – we use a rapid LFPI procedure that allows us to shorten surgery time and minimize exposure to neuroprotective anesthetic agents [21]. In addition, the authors of the prior study noted that their microarray technique underestimated expression levels of half the genes in a quality control panel as compared with PCR, and they did not validate *BDNF* microarray data by RT-PCR [13].

We hypothesize that the increased expression of these IEGs initiates a series of events that culminate in remodeling and potentiation of specific hippocampal circuits. If these changes are early initiators of the pathologic cascades that lead to spontaneous recurrent seizures and memory deficits, then our data support their value as either targets or biomarkers for post-traumatic antiepileptogenic therapies.

Hippocampal injury following lateral fluid percussion injury

We studied the expression of the injury marker *Bax* and observed a significant increase 6 h following LFPI bilaterally. The *Bax* expression pattern suggests that the cellular injury following LFPI extends to both hippocampi very early after injury, even though the mechanical insult is limited to the cortex directly underlying the craniotomy. This delayed spread of the injury to the hippocampus and the fact that it is preceded by the early appearance of the *c-fos* marker of excess neuronal activity suggest that excess neuronal activity and glutamate-mediated excitotoxicity may be involved in the initiation of hippocampal injury. Previous studies have reported a lack of cell death in the hippocampus immediately after LFPI [25]. Yet, there is strong evidence of neuronal death and hippocampal atrophy developing in the late post-traumatic period [11,21], and our data suggest that despite the lack of early cell death, the hippocampi still experience cellular injury, which may contribute to the emergence of epileptogenic cascades and the development of epilepsy.

N-methyl-D-aspartate-type glutamate receptor blockade alters post-traumatic gene expression in the hippocampus

NMDA receptors are critical in modulating neuronal plasticity by virtue of being permeable to calcium. MK801 is a competitive NMDAR antagonist that reduces excitotoxic neuronal damage mediated by activation of NMDAR-dependent signaling cascades [5]. Our data show that MK801 treatment attenuates the enhanced expression of a marker of plasticity (*BDNF*) in the hippocampus, whereas it has no observable effect on a

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marker of neuronal hyperactivation (*c-fos*). Further, MK801 suppresses *Bax* expression in both the ipsilesional and the contralesional hippocampus. These data suggest that uncoupling and attenuation of early pathological plasticity from glutamate-mediated excitotoxicity, through NMDAR blockade in the immediate post-traumatic period, may be a viable therapeutic strategy after TBI. Ours are not the only data to support the therapeutic value of NMDAR blockade – it has been explored as a potential therapy in several excitotoxicity-mediated neurological disorders [4,16,17]. However, our results are the first to demonstrate the response of pathologic hippocampal IEG expression to NMDAR antagonism in the acute post-traumatic period.

Although limited only to three IEGs, our results support the overall hypothesis that NMDAR blockade after TBI reduces the hippocampal expression of early pathological plasticity and injury markers. More conclusive evidence that this intervention mitigates pathological synaptic potentiation and post-traumatic neurologic symptoms will need to be obtained in additional experiments beyond the scope of this work. We also cautiously conclude that our data indicate the potential value of the selected IEG expression as readouts for both injury and therapeutic intervention.

Acknowledgements

The authors thank Dr Roman Gersner for his advice related to statistical analysis. This work was supported by the Boston Children's Hospital Translational Research Program (A.R.) and the Department of Defense PT073873 (A.R.).

Conflicts of interest

Dr Sanjay N. Rakhade is currently an employee of Genzyme, a Sanofi Company.

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