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Developmental traumatic brain injury decreased brain derived neurotrophic factor expression late after injury

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Abstract

Pediatric traumatic brain injury (TBI) is a major cause of acquired cognitive dysfunction in children. Hippocampal Brain Derived Neurotrophic Factor (BDNF) is important for normal cognition. Little is known about the effects of TBI on BDNF levels in the developing hippocampus. We used controlled cortical impact (CCI) in the 17 day old rat pup to test the hypothesis that CCI would first increase rat hippocampal BDNF mRNA/protein levels relative to SHAM and Naïve rats by post injury day (PID) 2 and then decrease BDNF mRNA/protein by PID14. Relative to SHAM, CCI did not change BDNF mRNA/protein levels in the injured hippocampus in the first 2 days after injury but did decrease BDNF protein at PID14. Surprisingly, BDNF mRNA decreased at PID 1, 3, 7 and 14, and BDNF protein decreased at PID 2, in SHAM and CCI hippocampi relative to Naïve. In conclusion, TBI decreased BDNF protein in the injured rat pup hippocampus 14 days after injury. BDNF mRNA levels decreased in both CCI and SHAM hippocampi relative to Naïve, suggesting that certain aspects of the experimental paradigm (such as craniotomy, anesthesia, and/or maternal separation) may decrease the expression of BDNF in the developing hippocampus. While BDNF is important for normal cognition, no inferences can be made regarding the cognitive impact of any of these factors. Such findings, however, suggest that meticulous attention to the experimental paradigm, and possible inclusion of a Naïve group, is warranted in studies of BDNF expression in the developing brain after TBI.

Keywords

Anesthesia; Controlled cortical impact; Brain derived neurotrophic factor; Developmental; Traumatic brain injury

Introduction

Pediatric traumatic brain injury (TBI) is the leading cause of traumatic death and disability in children 1–14 years of age (Langlois et al. 2006) in the United States. The most common neurologic disabilities suffered by survivors of pediatric TBI involve impaired learning and memory (Yeates et al. 2002, 2005).

Important learning and memory functions are performed by the hippocampus, a brain region that is particularly vulnerable to TBI (Gao et al. 2008). The hippocampus is rich in binding sites for Brain Derived Neurotrophic Factor (BDNF) (Lessmann 1998). BDNF is an endogenously produced neurotrophin necessary for normal cognitive function and brain

development (Binder and Scharfman 2004). Hippocampal BDNF expression is highly sensitive to numerous factors, including age at injury (Shah et al. 2006), severity of injury (Hicks et al. 1999), neuronal activation (Binder and Scharfman 2004) and glutamatergic activity (Lindholm et al. 1994; Zafra et al. 1991). Little is known about the expression of BDNF in the developing hippocampus after TBI.

We used an established model of pediatric TBI, controlled cortical impact (CCI) in the 17 day old rat pup, to generate three groups: CCI (exposed to impact), SHAM (sham surgery) and Naïve (no intervention) groups. As outlined in the Methods, we included Naïve animals to control for expected changes in BDNF expression over time in the developing rat (Kim et al. 2007; Liu et al. 2001). Hippocampi were collected on postinjury days (PID) 1, 2, 3, 7 and 14. Based on the expected rise in glutamatergic activity early after TBI (Palmer et al. 1993) and on data from experimental TBI (Griesbach et al. 2002; Hicks et al. 1999; Oyesiku et al. 1999), we hypothesized that hippocampal BDNF mRNA/protein levels would increase in the CCI group, relative to SHAM and Naïve, during the first 24–48 h after injury and subsequently decrease over the next 14 days.

Experimental procedures

Animals

All experimental protocols were approved by the Animal Care and Use Committees at the University of Utah, in accordance with US NIH guidelines and carried out at the University of Utah. All surgical procedures were performed using aseptic technique.

Male Sprague—Dawley rats were obtained from Charles Rivers Laboratories (Raleigh, NC) on post-natal day (P) 7–10. We studied only males to eliminate any confounding effects of gender. Rats were housed in litters of 10 with the lactating dam until weaning on P 21–23. After weaning, rats were housed 3–5 per cage and allowed free access to food and water. All cages were kept in a temperature- and light-controlled (12 h on/12 h off) environment. The three experimental groups were CCI, SHAM, and Naïve. We included Naïve animals to control for possible changes in BDNF expression over time in the developing animal. Rats were randomized to experimental group on the day of surgery, P17. In order to control for maternal rearing characteristics, randomization was distributed evenly within litters. At least two litters of 10 rats each were used to generate the numbers needed per group (6–8 rats per group) for each time point in our study.

CCI procedure

We used an established model of pediatric TBI as previously described (Schober et al. 2010). Rats underwent CCI (CCI), SHAM craniotomy (SHAM), or were left with the dam (Naïve).

On P17, rats undergoing CCI ($n=3-4$ /litter) were anesthetized with 3 % isoflurane for induction followed by 2–2.5 % isoflurane for the duration of surgical preparation, using a VetEquip Bench Top Isoflurane Anesthesia System (Pleasanton, CA). Core temperature was monitored via a rectal probe and continuously controlled at 37 ± 0.5 °C using a servo-controlled heating pad. Oxygenation, heart rate and respiratory rate were monitored using femoral probe pulse oximetry (Mousox[®], Starr Life Sciences, Oakmont, PA).

The rat was placed into a stereotaxic frame (David Kopf, Tujunga, CA). After shaving, prepping with povidone-iodine and incising the scalp, a craniotomy (6-mm×6-mm) was performed over the left parietal cortex (centered at the point 4-mm posterior and 4-mm lateral to bregma) using a high-speed dental drill. Care was taken not to perforate the dura. Once the craniotomy was complete, anesthesia was reduced to 1 % isoflurane for a 5-min

equilibration period. CCI was then delivered (Pittsburgh Precision Instruments, Pittsburgh, PA) to the left parietal cortex using a 5-mm rounded tip to deliver a 2-mm deformation at 4 meters/second velocity and 100 millisecond duration. Immediately after CCI, isoflurane was increased to 2–2.5 %, and the bone flap was replaced and secured with dental cement (Patterson Dental, Salt Lake City, UT). The scalp incision was sutured, and triple antibiotic ointment and bupivacaine 0.25 % were applied topically. Isoflurane was stopped, and rats were allowed to recover in a temperature-controlled chamber. Once fully awake, rats were returned to their dams and littermates. SHAM rats ($n=3-4$ /litter) underwent the same surgical craniotomy, equilibration, anesthesia exposure and closure procedures save for the actual impact. Naïve rats (3/litter) underwent randomization with no further intervention.

Tissues

Hippocampal tissue was collected at PID 1, 2, 3, 7 and 14. Rats were anesthetized with intraperitoneal (IP) xylazine (8 mg/kg) and ketamine (40 mg/kg), and killed by swift decapitation ($n=6$ per group). After brain removal, the hippocampus was quickly dissected on ice. The hippocampus on the left (ipsilateral to injury) from CCI and SHAM animals was labeled CCI_{ipsi} and SHAM_{ipsi}, respectively. The hippocampus on the right (contralateral to injury) from CCI and SHAM animals was labeled CCI_{con} or SHAM_{con}, respectively. The two hippocampi from each Naïve rat were not analyzed separately, as genomic microarray studies have not disclosed differences between left and right hippocampi (Stansberg et al. 2007). Tissue was snap frozen in liquid nitrogen and stored at -80°C . Ground frozen tissue was used to make cDNA or protein, as detailed below.

RNA isolation and real-time RT-PCR

Hippocampal mRNA levels of BDNF were measured by real-time RT-PCR. In brief, total RNA was extracted with Nucleospin II (Macherey-Nagel Inc. Bethlehem, PA), treated with DNase I (Ambion, Austin, TX), and quantified by spectrophotometry (Nano-Drop ND-1000, NanoDrop Technologies, DE). Sample integrity was confirmed by gel electrophoresis. cDNA was synthesized from 1 microgram of DNase-treated total RNA. cDNA- and gene-specific probe and primers were added to Taqman universal PCR master mix (PE Applied Biosystems), and samples were run on the 7900HT Sequence Detection System (Applied Biosystems Foster City, CA). Real-time RT-PCR quantification was then performed with Taqman GAPDH as an internal control. The primers and probes used for BDNF were Rn 02531967_s1 (Applied Biosystems). For each set of reactions, samples were run in quadruplicate. Relative quantification of PCR products was based on value differences between the target and GAPDH control by the comparative threshold cycle method (Taqman Gold RT-PCR manual, PE Applied Biosystems).

Protein isolation and determination of proteins of interest

Proteins of interest were extracted by homogenizing hippocampi in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris pH7.4, 1 mM EDTA, 0.5 % Na-deoxycholate, 1 % Igepal CA-630) with protein inhibitors (Roche Applied Science, Indianapolis, IN). After centrifugation at 13,000 rpm at 4°C for 10 min, the supernatants were stored at -80°C until use. Protein concentration was determined by a colorimetric assay, the bicinchoninic acid or BCA, method (Pierce Protein Research Products, Rockford, IL) and used to calculate volume for equal protein loading.

Proteins were separated by SDS PAGE using Criterion XT Precast 4–12 % Bis-Tris Gels (Bio-Rad, Hercules, CA), followed by transfer to PVDF membranes in CAPS buffer (10 mM 3-cyclohexylamino-1-propane sulfonic acid, 20 v/v% methanol, PH 11), at room temperature. After blocking at room temperature for 1 h, bound proteins were exposed to

specific antibodies against BDNF (rabbit polyclonal 1:1000 ab46176 Abcam, Cambridge, MA) for 1 h at RT.

After extensive washing in TBST, a 1:5,000 dilution of goat anti-rabbit HRP secondary antibody (Cell Signaling Technology MA) was applied, and incubated for 1 h at room temperature. After extensive washing, signals were detected with Western Lightning ECL (PerkinElmer Life Sciences) and quantified relative to GAPDH (rabbit monoclonal 1:5000 14 C10 Cell Signaling Technology, Danvers, MA) by densitometry on a Kodak Image Station 2000R (Eastman Kodak/SIS, Rochester, NY).

Statistics

All data are expressed as mean $\pm$ SE and/or as percentage of control. Data was analyzed using ANOVA for all five groups, with $p<0.05$ defined as the cutoff for statistical significance, using Statview software.

Results

mRNA results

Statistical significance was calculated using all five groups, as outlined in the methods. Because we found no difference in gene expression between SHAMipsi or SHAMcon hippocampi, only SHAMipsi (denoted SHAM) values are shown in the figures. All results are presented relative to Naïve. GAPDH mRNA expression did not vary between groups at any time.

CClpsi BDNF mRNA values did not differ from SHAM at any time point. In contrast, CCIcon BDNF mRNA increased relative to SHAM (114 ± 6 % Naïve vs 48 ± 7 % Naïve, $p<0.0001$) and to Naïve at PID3 (114 ± 6 % Naïve, $p<0.0001$). Differences between CCI hemispheres were only significant at PID3, in which CCIcon was greater than CClpsi, as shown in Fig. 1.

BDNF mRNA decreased in CClpsi relative to Naïve at PID1 (66 ± 5 % Naïve, $p<0.0001$), PID7 (63 ± 1 % Naïve, $p<0.0001$), and PID14 (75 ± 5 % Naïve, $p<0.01$). BDNF mRNA also decreased in SHAM relative to Naïve at PID1 (60 ± 2 % Naïve, $p<0.0001$), PID3 (48 ± 7 % Naïve, $p<0.0001$), PID 7 (62 ± 6 % Naïve, $p<0.0001$) and PID 14 (58 ± 9 % Naïve, $p<0.0001$) as shown in Fig. 1.

Protein results

Hippocampal BDNF protein levels decreased in CClpsi relative to SHAMipsi at PID14 (76 ± 14 % Naïve vs 136 ± 9 % Naïve, $p<0.01$). Relative to Naïve, hippocampal BDNF protein levels decreased in CClpsi and SHAMipsi at PID2 (44.6 ± 11 % Naïve and 40 ± 6 % Naïve, respectively, $p<0.05$ for each) as shown in Fig. 2. GAPDH protein levels did not differ between groups.

Discussion

To our knowledge, the report by Griesbach et al. is the only other study of BDNF expression in the developing hippocampus after traumatic brain injury (Griesbach et al. 2002). Our results differ from theirs. While they showed that hippocampal BDNF mRNA increased at 24 h after mild TBI in the 19 day old rat pup using a fluid percussion model (Griesbach et al. 2002), we found that BDNF mRNA did not increase at 24 or 48h after CCI. As suggested by other studies on BDNF expression, the greater severity of injury in our model may account for this difference. Severe injury both in the adult and neonatal rat is associated with

upregulation of BDNF mRNA during the first few hours after TBI, followed by rapid normalization. Studies of experimental moderate to severe adult TBI show that hippocampal BDNF peaked at 2 h and normalized by 24 h after TBI (Skoglosa et al. 1999), peaked at 12 h after injury and normalized by 36 h (Oyesiku et al. 1999), and that BDNF was unchanged at 24 h after TBI (Shah et al. 2006). In a neonatal rat model of TBI, BDNF mRNA peaked in the injured thalamus of the 7 day old rat at 8 h after injury, and subsequently normalized at the 48 h time point (Felderhoff-Mueser et al. 2002). In conclusion, it is possible that BDNF mRNA in our model had peaked prior to the 24 h measurement. In the setting of severe TBI, a rapid increase in BDNF expression in response to glutamate release would be expected early after injury, followed by a decline due to the loss of BDNF-producing neuronal and glial cells. Indeed, injured hippocampal neurons produce less BDNF than adjacent, uninjured neurons (Hellmich et al. 2005). Further, the age difference between the two models may not be trivial. Hippocampal BDNF expression rises from birth (Kim et al. 2007), peaks at 21 days of life and then declines to adult levels (Liu et al. 2001); thus, small differences in developmental age could also influence the capacity to up-regulate BDNF production after injury.

Griesbach et al. additionally reported that BDNF mRNA and protein increased at PID7 (bilaterally) and PID14 (limited to the ipsilateral hippocampus) in the 19 day old rat pup after mild TBI (Griesbach et al. 2002). In contrast, we found that CCI increased BDNF mRNA in the contralateral hippocampus only on PID 3 and that CCI decreased BDNF protein at PID14 in the ipsilateral hippocampus. While different from those of Griesbach et al., our findings are more consistent with what has been reported in adult rats. Moderate to severe TBI decreased hippocampal BDNF mRNA and protein at PID 7 (Aiguo et al. 2010; Wu et al. 2003, 2006) and at PID21 (Griesbach et al. 2009) in adult rats. Decreased BDNF over time may result from the progressive cortical and hippocampal tissue loss that occurs after moderate to severe TBI over the first year after injury (Dixon et al. 1999) (Smith et al. 1997).

Interestingly, we found significant differences between CCI/SHAM and Naïve hippocampi; specifically, in BDNF mRNA levels during the first 14 days after injury, and BDNF protein at PID2. We have previously shown that expression levels of various genes, including insulin-like growth factor 1 (IGF-1) and IGF-1 receptor, erythropoietin (EPO) and EPO receptor, did not differ between Naïve and SHAM hippocampus. Further, because BDNF levels vary with the light/dark cycle (Hamatake et al. 2011) and age of the rat (Kim et al. 2007; Liu et al. 2001) we collected hippocampi at the same time of day from age-matched littermates as described in the Methods. Thus, differences between Naïve and SHAM/CCI rats are unlikely to result from developmental factors or diurnal variation. Instead, these results suggest that hippocampal BDNF expression in the rat pup is exquisitely sensitive to the experimental paradigm. As discussed below, elements of the experimental paradigm that may affect BDNF expression include anesthetic exposure and maternal separation.

Isoflurane, an anesthetic commonly used in experimental and clinical pediatric TBI, increases gamma aminobutyric acid (GABA) activity and decreases activation of the glutamate receptor, n-methyl-d-aspartate receptor (NMDAR) (Ming et al. 2001). Hippocampal BDNF expression decreases in response to pharmacologic agents that enhance GABA activity or antagonize NMDAR activity (Lindholm et al. 1994; Zafra et al. 1991). It is thus plausible that isoflurane decreased BDNF expression in CCI and SHAM rats early after exposure. Indeed, isoflurane alone decreased BDNF protein levels in the thalami of neonatal rats (Lu et al. 2006), supporting the contention that isoflurane can modulate BDNF expression in the developing brain. Further, enhancement of GABA activity or antagonism of NMDAR activity decreased BDNF mRNA expression in cultured hippocampal neurons (Lindholm et al. 1994) and in adult rats (Zafra et al. 1991). It is not known if isoflurane

exposure could affect BDNF expression for as long as 14 days. Thus, it is reasonable to consider other factors that may influence long-term BDNF expression.

Rodent studies suggest that maternal separation in the pre-weanling period decrease BDNF expression at adulthood (Kikusui et al. 2009) (Aisa et al. 2009). There are few studies that have measured BDNF expression in rat pups early after maternal separation. One study found that maternal separation at day 9 of life did not change hippocampal BDNF mRNA at day 10 (Roceri et al. 2002), while another found that hippocampal BDNF mRNA at day 8 of life increased in rat pups exposed to high maternal care (Liu et al. 2000). It is possible then, that the maternal separation incurred by both CCI and SHAM rats affected hippocampal BDNF mRNA levels during the first 14 days after separation.

An additional explanation for the differences in BDNF expression between Naïve and SHAM/CCI rats may be found in the fact that even meticulous, careful craniotomy induces a small injury. As shown by Cole et al., craniotomy by drill (as used in this study) generates a visible MRI lesion, behavioral changes, and cytokine differences relative to Naïve rats (Cole et al. 2011).

Conclusions from this study are limited to male rats. Our conclusions are also limited to the time points we chose, as we did not measure daily BDNF mRNA and protein levels. While not exhaustive, however, our study covers a relatively high number of time points early after injury relative to similar studies of gene expression in experimental TBI in immature (Cook et al. 1998) and adult rodents (Matzilevich et al. 2002; Shah et al. 2006). We also cannot extricate the effects of maternal separation from those of anesthesia or the craniotomy itself, as SHAM and CCI rats were exposed to all three elements of the paradigm.

The discrepancies between mRNA and protein levels in our study are not entirely unexpected. The rat BDNF gene encodes at least 11 mRNA variants (Aid et al. 2007), but only one mature BDNF protein. We used primers and probes common to all BDNF mRNA variants. Given that mRNA variants often differ in terms of translational efficiency, it is possible that overall translation was unaffected despite the decrease in total BDNF mRNA. Yet another explanation lies in the fact that BDNF is subject to anterograde transport from the neuronal nuclei to the nerve terminal. Indeed, BDNF protein is widely distributed in nerve terminals even in brain regions that lack BDNF mRNA (Altar et al. 1997). Finally, since previous studies have shown associations between brain BDNF mRNA levels and cognitive outcome, it is reasonable to consider that mRNA changes alone may have functional implications. For example, in adult rats after TBI, decreased brain BDNF mRNA levels correlated with decreased performance on a test of cognitive function (Griesbach et al. 2009); in uninjured adult rats, increased brain BDNF mRNA was associated with improved performance on tests of learning and memory.

In summary, we found that, relative to SHAM, CCI in the 17 day old rat was associated with decreased BDNF protein at PID 14. We have previously shown that CCI rats perform poorly relative to SHAM and Naïve rats on Morris Water Maze testing at PID14 (Schober et al. 2010). We speculate that decreased hippocampal BDNF late after injury plays a role in decreased cognitive function after TBI. We also found that expression of BDNF mRNA in the hippocampus of the developing rat appears to be exquisitely sensitive to the experimental paradigm, even in the absence of impact injury. We speculate that anesthesia, maternal separation and/or mild brain injury from the craniotomy itself can decrease BDNF after experimental SHAM surgery. BDNF is important for normal cognition. Results from this study, however, are not sufficient to make any inferences as to the cognitive impact of any of these factors, alone or in combination. Our findings suggest that carefully controlling for the effects of anesthesia, maternal separation and/or mild brain injury from the

craniotomy, as well as consideration of inclusion of a naïve group, is important in studies of BDNF expression in the developing brain after traumatic injury.

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Fig. 1.

BDNF mRNA Levels in CCI, SHAM and Naïve Hippocampus. CCI increased BDNF mRNA relative to SHAM only in the hippocampus contralateral to injury (CCIcon, shown in *gray*) on day 3 after injury, but did not change BDNF mRNA levels in hippocampus ipsilateral to injury (CCIipsi, shown in *black*) at any time. BDNF mRNA decreased in both CCI and SHAM (shown in *dotted white*) over the first fourteen days after injury. The *dashed line* represents 100 % of Naive mRNA. Results are presented as percent of Naive animals from the same day post injury, $\pm$ SEM, $n=6$ /group. * $p<0.05$ relative to Naive, # $p<0.05$ relative to SHAM and & $p<0.05$ relative to CCIipsi

Fig. 2.

BDNF protein Levels in CCI, SHAM and Naïve Hippocampus. Results from hippocampi ipsilateral to injury or SHAM craniotomy are represented as CCI or SHAM, respectively. CCI decreased BDNF protein at postinjury (PID) 14 relative to SHAM rats. BDNF protein decreased in both CCI (shown in *black*) and SHAM (shown in *dotted white*) hippocampi relative to Naïve at PID2. Protein results are expressed as densitometry units relative to GAPDH and normalized to Naïve at the various days after injury. The *dashed line* represents 100 % of Naïve BDNF protein levels. Representative blots from CCI, SHAM and Naïve are shown as follows: the top band is BDNF and the bottom band is GAPDH. Results are presented as BDNF/GAPDH ratio $\pm$ SEM, $n=4-8$ * $p<0.05$ relative to Naïve, # $p<0.05$ relative to SHAM