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The long term objective of this study was to improve functional outcomes following traumatic brain injury (TBI) by investigating potential mechanisms of neuroprotection. Brain-derived neurotrophic factor (BDNF) has a well-established role in promoting cell survival during development. Using a well characterized animal model of TBI, the lateral fluid percussion (FP) brain injury model, we first characterized acute alterations in gene expression of BDNF and its receptor, trkB in response to injury. This study demonstrated that regions of the brain that are resistant to cell damage have increased gene expression for BDNF and its high affinity receptor, tyrosine kinase B (trkB) during the acute periods after injury. Study 2 examined whether the alterations in mRNA levels following FP injury resulted in subsequent alterations in protein levels of BDNF and trkB and activation of the ERK/MAP kinase signal transduction pathway. In contrast to the robust changes in BDNF mRNA levels, smaller and more transient alterations were observed in BDNF protein levels, and these protein alterations did not alter levels of activated ERK. Study 3 investigated whether administration of BDNF attenuated the neuropatholoical and behavioral deficits that are associated with FP injury. BDNF attenuated the size of the cortical lesion by approximately 30% after a FP injury of moderate severity, but did not attenuate cognitive deficits. These studies support a role for BDNF in providing partial neuroprotection following FP injury.

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Ramona Stiehm

PI - Signature Date

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INTRODUCTION

Traumatic brain injury (TBI) is the leading cause of death in the United States for people under age 45. Despite marked improvements in the medical management of TBI, only minor reductions (10-15%) have been observed in the incidence of fatal and severe injuries (Bullock and Fusijawa, 1992). The morbidity and neurological deficits associated with TBI are hypothesized to be attributable to both primary and secondary neuropathological events. Although the damage associated with primary events may be difficult to reverse, it is believed that secondary damage may be largely preventable with appropriate pharmacological intervention. Brain-derived neurotrophic factor (BDNF) has a well-established role in promoting cell survival during development, and may also provide neuroprotection following central nervous system insults in mature animals. The purpose of this study was to investigate the role of BDNF in attenuating neurological deficits in a well-characterized rodent model of traumatic brain injury, the lateral fluid percussion (FP) brain injury model.

BODY

The purpose of the first year of the grant was to **determine if alterations in BDNF and its receptor, trkB, are associated with neuronal survival following lateral FP brain injury.** Using in-situ hybridization and histological analysis following FP injuries of moderate severity we found that BDNF and trkB mRNA levels increased significantly in regions of the hippocampus that are resistant to cell death (Hicks et al., 1997, Appendix I; Hicks et al., 1998a, Appendix II). In cortical regions with the most profound loss of cells, we saw a significant decrease in BDNF and trkB mRNA (Hicks et al., 1999a, Appendix III). Following a FP injury of mild severity, we saw similar temporal and spatial patterns of gene expression in the hippocampus, except for one notable difference (Hicks et al., 1999b, Appendix IV). Alterations in gene expression in the hippocampus following a moderate injury were bilateral, whereas following a mild injury there were restricted to the hippocampus ipsilateral to the injury (Hicks et al., 1999b). In addition, because recent studies suggest that BDNF and NT-3 may have opposing roles, we also examined alterations in this neurotrophin and its receptor trkC mRNA levels. As expected based on previous studies in other injury models, NT-3 mRNA levels were significantly decreased in regions of the hippocampus where BDNF mRNA levels were increased, (Hicks et al., 1997). No significant alterations were observed in trkC (Hicks et al., 1998a).

The purpose of the second year of the grant was to **determine if alterations in BDNF/trkB signal transduction are associated with neuronal survival following lateral FP brain injury.** Specifically, we examined protein levels of BDNF, trkB, phosphorylated trkB, extracellular signal-regulated kinase (ERK, a member of the MAP kinase family), and phosphorylated ERK (MAPk) in the injured cortex and bilateral hippocampus between 6 and 96 h after FPI.

Male Sprague-Dawley rats (300-350g; n=32) received an experimental brain injury of moderate severity (2.0-2.1 atm) using the lateral fluid percussion brain injury

model. This model is well-characterized and has been previously described in detail (McIntosh et al., 1989; Hicks et al., 1996). Briefly, animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) 10 min after receiving 0.15 ml of atropine (0.4 mg/ml, i.m.), and given a 5 mm diameter craniotomy which was centered 3 mm lateral to the sagittal suture and 4.5 mm posterior to bregma. A Luer-loc hub was rigidly fixed with dental cement to the craniotomy. The hub was then attached to the FPI device, and a pressure fluid pulse was rapidly transmitted to the epidural space, generating mechanical stress forces to the brain. Following FPI injury, rats were euthanatized at 6, 24, 48, or 96 h in order to assess alterations in protein levels in response to the injury. Additional control animals (n=32) underwent anesthesia and surgery but were not injured (sham injury).

After the appropriate survival times, the rats were deeply anesthetized with an overdose of sodium pentobarbital, decapitated, and the brains were rapidly removed and dissected. Injured cortical tissue from the left neocortex and the bilateral hippocampi were collected in eppendorf tubes, weighed, frozen in liquid nitrogen, and then stored in at –80° C until used. Samples from half of the animals were used to measure BNDF protein levels with a two-site ELISA, and the other half were used to measure trkB and ERK protein levels with Western blot procedures (4/group).

For the ELISA procedure, samples were homogenized in 350 µl of sample buffer (0.1 M PBS, 0.4 M NaCl, 0.1% Triton X-100, 2 mM EDTA, 1 mM benzethonium chloride, w mM benzamidine, 0.1 mM PMSF, 20 trypsan inhibitor unit/ml aprotinin, and 0.5% BSA, pH 7.4) and spun at 14,000 rpm for 30 min at 4° C. The supernatants were assayed in triplicate using the BDNF E_{max} Immunoassay system (Promega). Dynatech immunolon plates were coated with an anti-BDNF monoclonal antibody to capture the soluble BDNF from the supernatant solution. Bound BDNF complexes were exposed to an anti-human BDNF polyclonal antibody, and the amount of antibody was detected using an anti-IgY antibody conjugated to horseradish peroxidase. Unbound conjugate was removed by washing, and the chromogenic substrate 3,3',5,5'-tetramethyl benzidine (TMB) was added. BDNF protein levels in the samples were determined by colorimetric analysis with a microplate reader. Unknown sample values were normalized to a BDNF standard curve with values ranging between 0.005 ng/ml and 5 ng/ml.

The primary antibodies used for Western blot analysis included anti-trkB and anti-ERK (Transduction Laboratories, Lexington, KY), and anti-ACTIVE MAPk (Promega, Madison, WI). The anti-trkB antibody is reported to be able to recognize the 145 kD full length catalytic form of trkB, as well as the 95 kD truncated, non-catalytic version of this protein (Barbacid et al., 1991). The anti-ERK antibody recognizes the 42 kD MAP Kinase protein (Boulton and Cobb, 1991). The anti-ACTIVE MAPK antibody is specific for the phosphorylated and activated form of MAP Kinase. Tissue sections were homogenized in Tris-saline containing leupeptin (10 µM), EDTA-Na^{2+} (1 µM), Pepstatin A (1 µM), and AEBSF (0.25 µM). The homogenates were centrifuged twice at 14,000 g for 15 at 4° C. The protein content in the supernatants was determined by the micro-BCA method (Pierce). Equivalent amounts of protein from 4 injured and 4 control animals from the same post-injury time points were separated by SDS-polyacrylamide

gel electrophoresis for each antibody. For trkB, 60 μg of protein was loaded per lane on a 6.5% gel, and for ERK and MAPk, 20 μg of protein on a 10% gel. After electrophoretic separation, the proteins were transferred to nitrocellulose membranes (Bio-Rad) and immunoreacted with the proteins of interest (trkB, 1:200; ERK, 1:5000; MAPk, 1:20,000). Immunoreactive bands were visualized using a chemiluminescence method (Pierce) and quantitated by densitometric analysis (NIH Image, version 1.60). Results were expressed as percent of control. Levels of protein immunoreactivity were linear over a threefold range of tissue concentration for each of the proteins analyzed.

The immunoprecipitations were conducted as described previously (Stein-Behrens et al.,1994; Keller et al.,1997), with some modifications. Briefly, homogenates (300 μg) from fluid percussion and sham injured animals were heated for 5 min at 60oC and brought to a volume of 1 ml in ice cold RIPA buffer (50 mM Tris-HCl [pH 7.5], 10% glycerol, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, 5mM EDTA, 2 mM PMSF, and 1 μg/ml leupeptin. Protein A was then added, and the solution incubated for 30 min at 4°C, then centrifuged at 500*g* for 3 min to preclear the sample. The first antibody (anti-TRKB or anti-PhosphoTyrosine) was added, and allowed to incubate at 4°C for 2 h on shaker. Protein A was then added and allowed to incubate on shaker for 2 h. The solution was then pelleted by centrifugation at 3000*g* and washed 3 times in PBS. The pellet was then resuspended in 25 μl of RIPA buffer and electrophoresed by 7.5% SDS-polyacrylamide gel electrophoresis. Following transfer to nitrocellulose sheet, the protein was incubated with primary antibody and then processed further with alkaline phosphatase-conjugated secondary antibody and resolved using an enhanced chemiluminescent system.

A student's T-test was used to determine if there were significant differences in protein levels ($p < 0.05$) between the injured or control groups. All analyses were performed with SYSTAT 9 (Chicago, IL). All values are presented as percent control ± SEM.

The BDNF ELISA revealed significant increases in BDNF protein in the injured cortex at 6 h post-FP injury and in the bilateral hippocampi at 48 h (Fig. 1) compared to sham-injured controls. There was also a trend toward a decrease in the injured cortex at 48 h ($p < 0.1$). Western blots for analysis of nonphosphorylated trkB protein levels yielded consistent blots only for the noncatalytic 95 kD protein ($trkB_{95}$) and not for the full-length, catalytic 145 kD isoform. Therefore, only $trkB_{95}$ protein levels were evaluated. No significant differences were observed in $trkB_{95}$ in either the injured cortex or in the hippocampus, although there was a trend toward an increase at 96 h in the hippocampus (Table 1). However, phosphorylated trkB protein levels increased in the injured cortex at 6 h in the injured animals (Fig. 2), although not in the hippocampus at 48 h postinjury. Despite the increases in phosphorylated trkB protein, no significant changes were seen in ERK or phosphorylated ERK (MAPk) protein levels between FPI and sham-injured animals, except for a decrease in MAPk in the injured cortex at 24 h after FPI (Table 1).

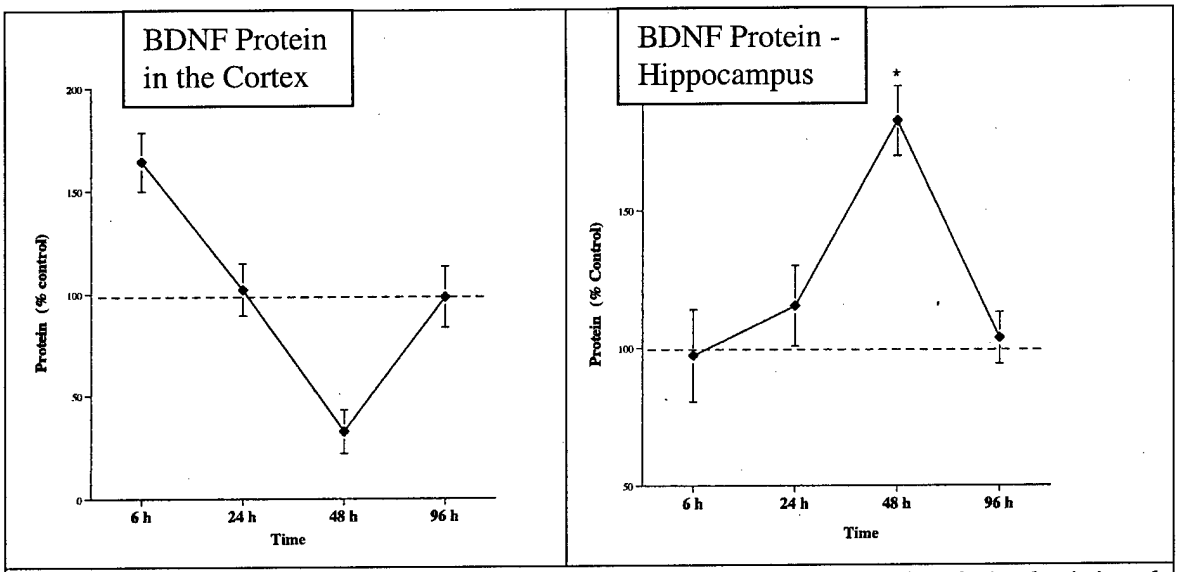


Fig. 1. Temporal pattern of alterations in endogenous BDNF protein levels in the injured left parieto-occipital cortex and in the bilateral hippocampus of rats following FP injury. BDNF ELISA kits were used to measure protein levels. Note the significant increase in BDNF protein levels in the hippocampus at 48 h. In the injured cortex there was an increase at 6 h and a trend toward a decrease at 48 h. Values represent percent control levels (sham injury) ± SEM..

Table 1. Percent change for injured rats compared to control (sham-injured) rats in the injured cortex and hippocampus at various postinjury time points for BDNF, trkB, ERK, and MAPk protein levels.

Region & Time	BDNF (%)	*p* =	$TrkB_{95}$	*p* =	ERK	*p* =	MAPk	*p* =
Cor-6h	+51.4	0.028	-13.3	0.427	+4.2	0.692	-3.1	0.877
-24h	+2.2	0.912	-14.2	0.485	+1.6	0.788	-42.9	0.039
-48h	-58.1	0.076	+ 9.4	0.468	+4.2	0.602	-5.5	0.615
-96h	-1.5	0.965	-34.6	0.374	-5.6	0.929	-34.7	0.203
Hip–6h	+2.7	0.805	+8.0	0.704	+6.2	0.671	+28.6	0.800
-24h	+15.5	0.501	+10.0	0.504	-5.8	0.719	+14.9	0.122
-48h	+82.3	0.001	+14.6	0.265	+13.2	0.082	+20.6	0.766
-96h	-2.7	0.927	+31.5	0.069	+11.0	0.582	+37.7	0.269

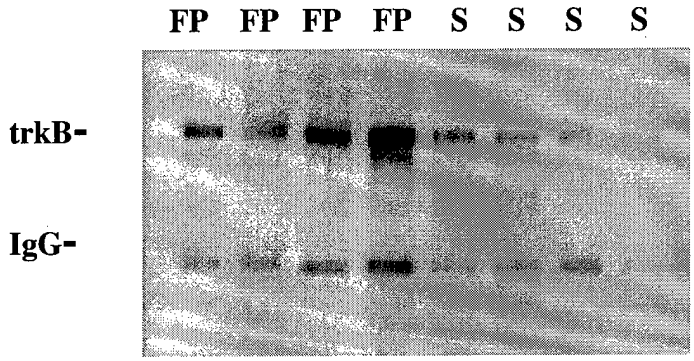


Fig. 2. Fluid percussion injury increases trkB phosphorylation. Brain homogenates, collected from rats 6 h following either fluid percussion (FP) or sham (S) surgery, were immunoprecipitated with anti-TRKB antibody, subject to electrophoresis, and immunoreacted using anti-Phosphotyrosine antibody. Data presented are typical of results from 2 separate experiments. Similar results were obtained when immunoprecipitation using anti-Phosphotyrosine were immunoreacted with anti-trkB (data not shown). The high molecular weight trkB and IgG artifact are indicated on the margin of Western blot.

Numerous investigations are currently underway to elaborate the specific actions of BDNF and how it may promote neuronal survival during development or after brain injury. BDNF is believed to initiate its effects by binding to either a common low-affinity neurotrophin receptor, p75 (Rabizadeh et al., 1993; Carter et al., 1996) and/or to a high-affinity tyrosine kinase receptor (trkB) (Meakin and Shooter, 1992; Barbacid, 1994). It is believed to act primarily in an autocrine or paracrine fashion (Acheson et al., 1995), although there is also evidence of retrograde (Watson et al., 1999) and anterograde transport (Altar et al., 1994, Tonra, 1999) of BDNF. BDNF/trkB interactions result in trkB dimerization and phosphorylation, which can lead to the subsequent activation of two known intracellular signaling cascades, the extracellular-regulated kinase/mitogen-activated protein kinase (ERK/MAP kinase) pathway or the phosphotidylinositol-3 kinase (PI3 kinase) pathway (Gottshalk et al., 1999). In vitro studies have begun to differentiate the roles of these two pathways. Apoptosis secondary to serum deprivation can be blocked by BDNF, and activation of the PI3, but not the MAP kinase pathway is reported to be critical for this process (Hetman et al., 1999, Bhave et al., 1999, Dolcet et al., 1999; Encinas et al., 1999). Although studies have primarily linked MAP kinase signal transduction with neuronal plasticity (Derkinderen et al., 1999; Impey et al., 1999), a recent study reported that apoptosis secondary to DNA damage was blocked by BDNF activation of the MAP kinase pathway (Hetman et al., 1999). These studies suggest that both of these BDNF/trkB downstream pathways can mediate neuroprotection, but that they may be specific for various types of cellular insults. However, we did not see

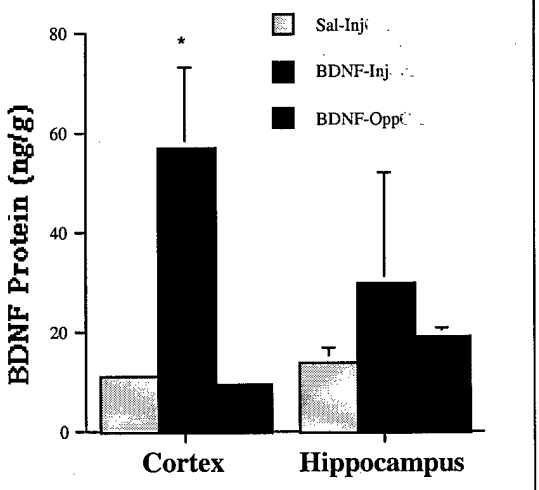
evidence of activation of the ERK/MAP kinase pathway following modest injury-induced increases in BDNF protein levels.

The purpose of the third year of the study was to **determine if BDNF improves outcome following FP brain injury.** Specifically we examined the effects of intravenous administration of BDNF following FP injuries of mild and moderate severity on BDNF protein levels, weight loss, neuropathology in the cortex and hippocampus, and cognitive deficits.

Animals (n=49) were anesthetized and received a FPI of either moderate severity or mild severity (1.0 atm), or a sham injury as described above. Fifteen minutes after the FP injury, animals received an injection of either BDNF (10 mg/kg in 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0, and 0.004% Tween -20, Regeneron, N.J., i.v.) or an equivalent amount of sterile saline (approximately 0.3 ml) into the femoral vein. Animals were euthanized with an overdose of sodium pentobarbitol at either 2 h, 7 or 14 days after the FP injury.

Experiment 1: Determine if the BDNF is reaching the injured cortex and hippocampus. Animals were euthanized 2 h after FPI (2.0 atm) and injection of either BDNF or saline (n=3/group) and the brains rapidly removed and dissected into the following regions: injured cortex (InjCor), contralateral cortex (OppCor), injured hippocampus (IH), and contralateral hippocampus (OH). The tissue was rapidly frozen in liquid nitrogen and stored at -80 °C until ready for use. The ELISA procedure as described above was used to measure BDNF protein levels. Protein levels were significantly increased in the injured cortex ($p < 0.02$) compared to the contralateral side and to animals that received saline (Fig. 3). In the hippocampus, there were no significant differences between the groups (Fig. 3).

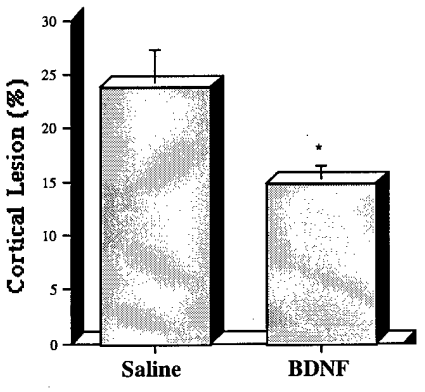
Fig. 3. Graph shows BDNF protein levels in the cortex and hippocampus 2 hours after animals were given a FPI followed by administration of either BDNF (10 mg/kg, i.v.) or saline (n=3/group) 15 min after the injury. BDNF ELISA kits (Promega) were used to measure protein levels in the injured and contralateral (homotypic) cortex and hippocampus in animals that received either BDNF or saline after FPI. BDNF was significantly increased ($p < 0.02$) in the injured cortex after BDNF injection compared to the contralateral cortex or to animals that received saline. There was a trend toward an increase in the hippocampus, but this did not reach significant levels.



Experiment 2: Determine if BDNF attenuates neuropathology after FP. Animals received a FP injury of moderate severity, followed by injection of either BDNF or saline (n= 5/group). Seven days post-FP injury, animals were anesthetized, and then perfused with saline followed by 4% paraformaldehyde. The brains were removed, post-fixed with paraformaldehyde overnight, and then transferred into a solution of 25% sucrose in PBS for cryoprotection. Brain sections throughout the extent of the injury (bregma 0 to -6.5 mm) were cut into 40 μm thick sections on a microtome. Sections were mounted onto slides and stained with cresyl violet to evaluate the extent of neuronal injury. The cortical damage was quantified by digitizing 9 brain sections/animal with image processing software (Image 1.60, NIH) and measuring the area of the neocortex on the injured and uninjured sides of the brain. Neocortical volumes were then determined using the Cavalieri method (Michel and Cruz-Orive, 1988). Neuropathology was assessed by calculating the percent difference in the volume of the injured and uninjured cortices [uninjured cortex (mm^3) - injured cortex (mm^3)/ uninjured cortex (mm^3)]. This method takes into consideration differences in animal brain size, tissue shrinkage secondary to processing, and the collapse of cortical tissue into the damaged region. In the hippocampus, a semi-quantitative scoring method was used to evaluate cell loss. Scores were assigned as follows: 0 = no visible cell loss or damage; 1 = cell loss restricted to the hilar region of the dentate gyrus; 2 = cell loss in the hilar region and slight cell loss in the CA3 pyramidal cell layer; and 3 = cell loss in the hilar region and a marked cell loss in the CA3 region.

In animals that received BDNF there was a significant reduction in the lesion volume in the cortex 7 days post-FP injury ($p < 0.05$) compared to animals that received saline (Fig. 4 and 5). In the hippocampus, there were no significant differences in neuropathology between groups (data not shown).

Fig. 4. The percentage of the cortex that was damaged after FPI was calculated by measuring the area of the injured and contralateral neocortices from 9 brain sections taken throughout the level of the injury site. The mean difference in areas was expressed as a percentage of the uninjured neocortical area and compared between animals that received BDNF or saline after FPI [volume of contralateral cortex – volume of ipsilateral cortex/volume of contralateral cortex]. BDNF significantly attenuated the cortical damage ($p < 0.05$).



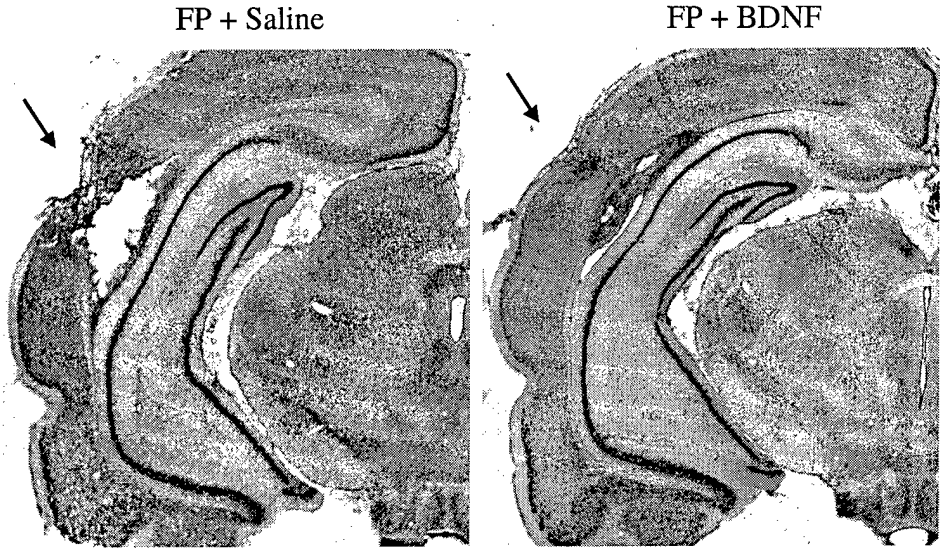


Fig. 5. Photomicrographs of coronal sections of the side of the brain ipsilateral to the FP injury in representative animals that received either saline or BDNF after the injury. Note the attenuation of damage to the cortex (arrows) and subcortical white matter with BDNF treatment after FP injury.

Experiment 3: Determine if BDNF attenuates cognitive deficits after FP injury of moderate severity. Animals received either a FP injury of moderate severity or a sham injury, followed by either administration of BDNF or saline as described above. Twelve days post-FP injury, animals were tested for spatial learning and memory deficits using a modified 2 day Morris Water Maze (MWM) procedure as described previously (Kraemer and Randall, 1995; Kraemer et al., 1996). All testing was performed by an observed blinded to each animal's treatment. Animals were trained to find a hidden platform in the pool using external visual cues. Animals were trained beginning on day 12, performing two blocks of four acquisition trials per day for 2 days, for a total of 16 trials. The time required for each animal to find the platform (goal latency) was recorded for each trial. After completing the last acquisition trial, animals underwent a single probe test to assess their spatial memory. The platform was removed and the swim pattern of each animal was videotaped for 90 seconds. The distance, time, and number of visits to the previous platform location, as well as visits to similar regions in the other quadrants was computed by a video motion analyzer (Videomex V, Columbus Instruments). Mean zone differences were calculated by subtracting the visits to the wrong target from visits to the former platform location.

FPI animals were significantly impaired in learning to find the hidden platform after the 4^{th} trial block ($p < 0.01$) compared to sham injured animals (Fig. 6). Administration of BDNF following FP injury or sham injury was without significant

effects on goal latencies (Fig. 6). Mean zone differences also revealed no significant effects of BDNF on spatial memory following FP injury (data not shown).

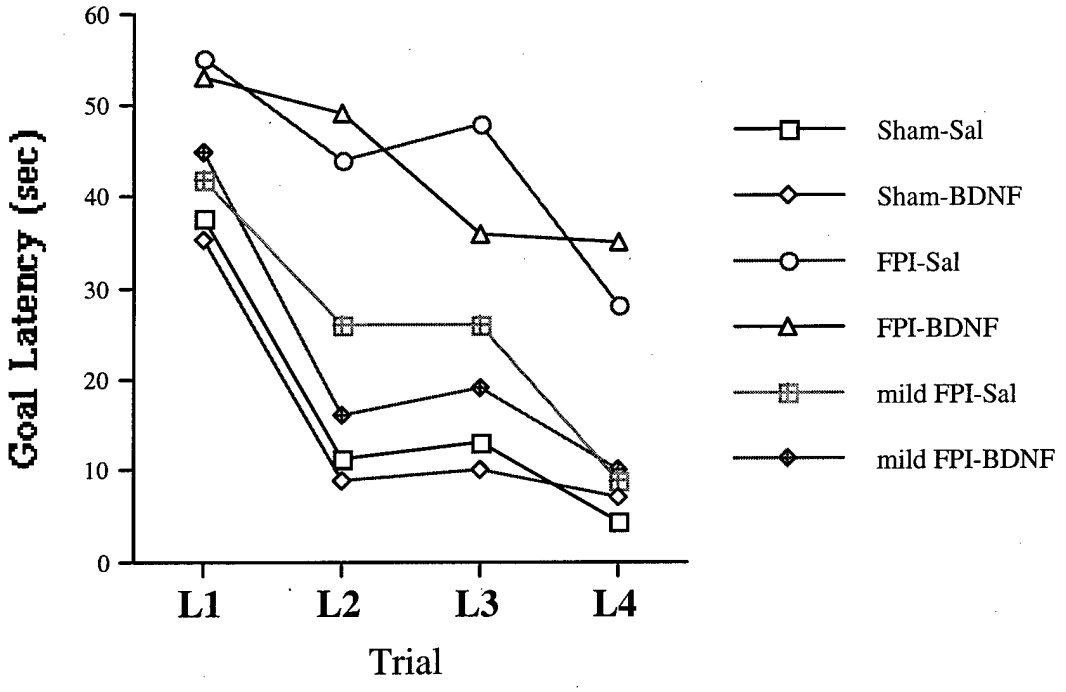


Fig. 6. Spatial learning and memory was evaluated in a Morris Water Maze two weeks after animals were subjected to either a mild or moderate FPI or a sham injury, followed by administration of BDNF or saline 15 min later. The time it took the animals to swim to the platform (goal latency) was significantly longer after moderate FPI compared to sham injury ($p < 0.01$). Adminstration of BDNF did not attenuate these impairments in spatial learning. Although there was a trend toward a delay in learning after mild FPI, all the animals learned the procedure by the fourth trial block (L4).

After the behavior testing was completed, animals were weighed, euthanized and their brains removed and analyzed for neuropathological damage as described above. There was a trend toward attenuation of the cortical lesion volume 14 days post FP injury in the group that received BDNF, but this did not reach stastitical significance (Table 2). This is in contrast to the animals that were analyzed 7 days post-FP where there was a modest but significant improvement. The data demonstrate that the differences between 7 and 14 days are between the animals that received saline, not BDNF (Table 2).

Table 2. Cortical lesion volume (%) in animals with 7 and 14 day survival periods following FP injury.

	FP + Saline	FP + BDNF	*p*
7 day survival	23.8%	14.8%	0.042
14 day survival	20.3%	14.6%	0.126

Weight loss was also investigated in this experiment because of a previous study which demonstrated an association between BDNF and weight loss in rats (Pellymounter, 1995). Animals that received BDNF lost significantly more weight (16.6 g) compared to animals that received saline (1.6 g) ($p < 0.001$) (Table 3). This finding is surprising given that animals received just one acute injection of BDNF after FP injury. However, it suggests that BDNF is reaching the brain and exerting physiological effects.

Table 3. Change in weight after FP injury in animals that received either saline or BDNF treatment.

	FP + Saline	FP + BDNF	*p*
14 day survival	-1.6 g	-16.6 g	0.001

Experiment 4. Determine if BDNF attenuates cognitive deficits after FP injury of mild severity. This study was identical to that described above except that the animals received a FP injury of mild severity. Previous studies have demonstrated that mild FP injury is also associated with acute deficits in retrograde spatial memory and learning (Hicks et al., 1993). However, the MWM testing procedure employed in this study examines anterograde memory deficits, and we did not observe any significant differences in goal latencies or mean zone differences between mild FP and sham injured animals (Fig. 6). Thus, this test was not sensitive enough to detect behavioral deficits after mild FP injury, and thus cannot evaluate the effects of BDNF. We also investigated effects of BDNF on cortical lesion volume following FP injury and found no differences between those that received BDNF (cortical damage = 8.0 ± 2.0 %) or saline (7.0 ± 2.0 %).

Experiment 5. Determine if exercise-induced increases in endogenous BDNF are associated with a reduction in cognitive deficits after FP injury. Using in-situ hybridization and histological analysis following FP injuries of moderate severity we found that treadmill exercise increased BDNF mRNA levels significantly in the hippocampus (Hicks et al., 1998b, Appendix V). However, the exercise-induced increases in endogenous BDNF mRNA were not associated with attenuation of impairments in spatial learning and memory (Hicks et al., 1998b).

Numerous studies have demonstrated that BDNF affords neuroprotection in a variety of in vitro and in vivo animal models of brain injury, including ischemia (Kindy et al., 1993, Beck et al., 1994, Larsson et al., 1999, Yamashita et al., 1997, Schabitz et al., 1997), hypoglycemia (Nakao et al., 1995), excitotoxicity (Dekker et al., 1994, Cheng and Mattson, 1994, Hayes et al. 1995, Mattson et al., 1995) oxidative damage (Skaper et al., 1998, Yamagata et al.,1999), glucocorticoid toxicity (Nitta et al., 1999), and nerve transection (Mansour-Robaey et al., 1994, Klocker et al, 1998, Hagg et al., 1998). Our

findings are in agreement with these previous studies undertaken in different injury models. In contrast to studies demonstrating neuroprotective effects of BDNF, there have also been a few studies that reported an actual increase in cell death associated with BDNF/trkB signal transduction following injury, both in vitro (Koh et al., 1995, Kim, 1999) and in vivo (Rudge et al., 1997, Alessandrini, 1999). These conflicting results underscore the need to understand molecular events associated with various injury processes and how BDNF interacts with these events prior to clinical applications.

Enhancing cell survival after injury is important, but the ultimate goal is to enhance recovery of function. Much less is known about the role of BDNF in restoring function after brain injury. However, following spinal cord injury, BDNF promoted regeneration as well as functional improvements (Liu et al., 1999; Jakeman et al., 1998). Interestingly, in animals with basal ganglia lesions, BDNF improved locomotor activity, but did not alter neuronal survival (Klein et al., 1999). We did not find evidence of enhanced cognitive performance with BDNF following FP injury. However, the tests we administered are believed to be dependent upon the hippocampus, and we did not see significant alterations in BDNF protein levels or attenuation of neuropathology in this region with our intervention. Thus, whether BDNF can attenuate deficits associated with the cortex following FP injury is unknown, and should be investigated in future studies. Furthermore, whether the modest neuroprotection afforded by BDNF following moderate FP injury can be enhanced by using it at other doses or in combination with other drugs is unknown and should be investigated in the future.

KEY RESEARCH ACCOMPLISHMENTS

- Characterized effects of mild and moderate experimental brain trauma on gene expression of BDNF, trkB, NT-3, and trkC in the cortex and hippocampus.
- Demonstrated that BDNF and trkB gene expression increases in brain regions that are resistant to cell damage and decreases in regions that are vulnerable following experimental brain trauma.
- Demonstrated that alterations in endogenous BDNF protein levels following FP injury do not activate the ERK/MAP kinase signal transduction pathway.
- Demonstrated that administration of BDNF following moderate FP injury attenuates neuropathological damage in the cortex, but does not attenuate cognitive deficits.
- Demonstrated that exercise following FP injury induces BDNF gene expression in the hippocampus, but is not associated with improvements in cognitive performance.

REPORTABLE OUTCOMES

Manuscripts

1. Hicks RR, Numan S, Dhillon HS, Prasad MR, Seroogy KB. 1997. Alterations in BDNF and NT-3 induction in the hippocampus following lateral fluid percussion brain injury in the rat. **Mol. Brain Res.** 48:401-406.
2. Hicks RR, Zhang L., Dhillon H.S., Prasad M.R., Seroogy K.B. 1998a. Expression of *trk*B is altered in rat hippocampus after experimental brain trauma. **Mol. Brain. Res.** 59: 264-268.

3. Hicks RR, Boggs A, Leider D, Kraemer P, Brown R, Scheff SW, Seroogy KB. 1998b. Effects of exercise following lateral fluid percussion brain injury in rats. **Res. Neurol. Neurosci.** 12:1-6.
4. Hicks RR, Li C, Zhang L, Prasad MR, and Seroogy KB. 1999a. Alterations in BDNF and trkB mRNA levels in the cerebral cortex following experimental brain trauma in rats. **J. Neurotrauma** 16:501-510.
5. Hicks RR, Martin VB, Zhang L, Seroogy KB. 1999b. Mild experimental brain injury differentially alters the expression of neurotrophin and neurotrophin receptor mRNAs in the hippocampus. **Exp. Neurol.** 160:469-478.

Manuscripts in Preparation

1. Hicks RR, Keller JN., 2000a. BDNF and Phosphorylated trkB Protein Levels are Altered in the Cortex and Hippocampus after Lateral Fluid Percussion Brain Injury.
2. Hicks RR, Mace D, Billings B, Li C, Kraemer P. 2000b. BDNF attenuates cortical damage but not cognitive deficits after experimental brain trauma in rats.

Presentations

1. Hicks RR, Prasad MR, Dhillon HS, Dose JM, Numan S, Seroogy K. 1996. Alterations in BDNF and NT-3 mRNAs in the hippocampus following traumatic brain injury in the rat. **Society for Neuroscience Annual Meeting.** Washington, D.C.
2. Hicks RR, Boggs A, Scheff SW, Kraemer P, Brown R, Zhang L, Seroogy KB. 1997. Exercise alters BDNF mRNA expression, but not behavior, after fluid percussion brain injury in rats. **Society for Neuroscience Annual Meeting**, New Orleans.
3. Hicks RR, Prasad MR, Zhang L, Dhillon HS, Li C, Seroogy KB. 1997. Alterations in BDNF mRNA in the cortex after lateral fluid percussion brain injury. **Neurotrauma Society Annual Meeting**, New Orleans, and **Seventh International Neural Regeneration Symposium**, Pacific Grove, Ca
4. Boggs A, Scheff SW, Kraemer P, Brown R, Zhang L, Seroogy KB, Hicks RR. 1998. Effects of exercise on neural plasticity and behavior after traumatic brain injury in rats. **Combined Sections Meeting, APTA**, Boston.
5. Hicks RR, Prasad MR, Zhang L, Dhillon HS, Seroogy KB. 1998. Lateral fluid percussion brain injury alters trkB and trkC mRNA levels in rat hippocampus. **Society for Neuroscience Annual Meeting**, Los Angeles, and **The Fourth Annual Kentucky Spinal Cord and Head Injury Research Symposium**, Lexington, KY.
6. Hicks RR, Perkins VB, Zhang L, Seroogy KB. 1998. Mild experimental brain injury produces alterations in BDNF, trkB, and NT-3 mRNA in the hippocampus. **National Neurotrauma Society Meeting**, Los Angeles.
7. Meeker P, Lear B, Li C, Hicks RR. 1998. Is exercise neuroprotective for traumatic brain injury? **APTA Section on Research Neuroplasticity Research Retreat**, Newport, RI. and **APTA Combined Sections Meeting,** Seattle.
8. Li, C, Billings B., **Hicks, RR**. 1999. Lateral fluid percussion brain injury alters brain-derived neurotrophic factor protein levels in the cortex and hippocampus. Submitted to **Society of General Physiologists, 53rd Annual Meeting and Symposium**, Woods Hole, MA.

CONCLUSIONS

1. Experimental brain trauma induces acute alterations in BDNF, trkB, NT-3 and trkC mRNA levels in the cortex (ipsilateral to the injury) and the bilateral hippocampus.
2. Endogenous increases in BDNF and trkB mRNA levels following experimental brain trauma result in relatively minor alterations in BDNF protein levels, and these alterations do not activate the ERK/MAP kinase signal transduction pathway.
3. Intravenous administration of BDNF following moderate FP injury reaches the injured cortex. This finding has important clinical relevance because it demonstrates that a simple intravenous injection given shortly after TBI can be effective in targeting injured regions of the brain. Personnel with first aid training, such as emergency medical technicians, could give intravenous injections as soon as they arrive at the scene of the injury.
4. Administration of BDNF after a moderate FPI provides modest neuroprotection to cells in the injured cortex, but does not attenuate cognitive deficits. Future studies should be undertaken to determine if these neuroprotective effects of BDNF after FP injury can be enhanced, either by increasing the dose or by using it in combination with other neuroprotective agents.

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Alterations in BDNF and NT-3 mRNAs in rat hippocampus after experimental brain trauma

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Short communication

Alterations in BDNF and NT-3 mRNAs in rat hippocampus after experimental brain trauma

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Abstract

Previous studies have suggested that the neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are neuroprotective or neurotrophic for certain subpopulations of hippocampal neurons following various brain insults. In the present study, the expression of BDNF and NT-3 mRNAs in rat hippocampus was examined after traumatic brain injury. Following lateral fluid percussion (FP) brain injury of moderate severity (2.0–2.1 atm) or sham injury, the hippocampi from adult rats were processed for the in situ hybridization localization of BDNF and NT-3 mRNAs using ^{35}S-labeled cRNA probes at post-injury survival times of 1, 3, 6, 24 and 72 h. Unilateral FP injury markedly increased hybridization for BDNF mRNA in the dentate gyrus bilaterally which peaked at 3 h and remained above control levels for up to 72 h post-injury. A moderate increase in BDNF mRNA expression was also observed bilaterally in the CA3 region of the hippocampus at 1, 3, and 6 h after FP injury, but expression declined to control levels by 24 h. Conversely, NT-3 mRNA was significantly decreased in the dentate gyrus following FP injury at the 6 and 24 h survival times. These results demonstrate that FP brain injury differentially modulates expression of BDNF and NT-3 mRNAs in the hippocampus, and suggest that neurotrophin plasticity is a functional response of hippocampal neurons to brain trauma. © 1997 Elsevier Science B.V.

Keywords: Traumatic brain injury; Lateral fluid percussion; Brain-derived neurotrophic factor; Neurotrophin-3; Hybridization, in situ; Neuronal plasticity

Secondary or delayed injury processes that begin to develop within minutes and continue to develop for hours after traumatic brain injury can contribute to irreversible tissue damage [3]. Although the sequence and timing of these processes are largely unknown, they are thought to be initiated by the release of neurotransmitters such as excitatory amino acids [8,23,35,37] and acetylcholine [14], and by the subsequent activation of neurotransmitter receptors, including NMDA-receptor subtypes, muscarinic cholinergic receptors, and opioid receptors [6,16,29,38]. An increase in intracellular calcium and the subsequent stimulation of calcium-dependent enzymatic activities are implicated mediators in some of the neurotransmitter-induced generation of secondary injury factors [6,10,16,19,45].

Several studies indicate that activation of excitatory amino-acid receptors in neurons can also result in the induction of neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) [34,47] (for review, see [26]). Accordingly, increased gene expression for BDNF and NGF in the brain has been observed in several models of central nervous system injury, such as ischemia and seizures, where excitatory amino-acid receptors are implicated in the pathogenesis [12,21,27,46]. In contrast to the up-regulation of NGF and BDNF mRNAs, down-regulation of neurotrophin-3 (NT-3) mRNA has also been observed with cerebral ischemia and seizures [12,27]. It has been suggested that these neurotrophins may provide neuroprotection by playing a role in the maintenance and survival of neurons after traumatic brain injury [31,33]. Therefore, it is important to characterize the spatial and temporal patterns and levels of neurotrophic factor expression after experimental brain injury.

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Relatively few studies have examined alterations in the neurotrophin family of trophic factors after traumatic brain injury. Recent reports have, however, demonstrated increases of BDNF and NGF mRNAs and NGF protein in cortical areas after cortical contusion brain injury [5,15,48]. The present study characterized the changes in BDNF and NT-3 mRNAs in the hippocampus after lateral fluid percussion (FP) brain injury, another established model of traumatic brain injury. The hippocampus was of particular interest because of its prominent expression of and responsiveness to neurotrophins [2,13,22,25], its vulnerability to neurodegeneration subsequent to various brain insults [41], and its role in learning and memory dysfunction following FP injury [18,44].

Male Sprague-Dawley rats (325–350 g) were anesthetized with sodium pentobarbital (60 mg/kg i.p.) 10 min after receiving 0.15 ml of atropine (0.4 mg/ml i.m.), and placed in a stereotaxic frame. The scalp and temporal muscles were reflected, and a stainless-steel screw was secured to the skull 1 mm anterior to bregma. A hand-held trephine with a 4.9 mm diameter was used to make a craniotomy, which was centered between bregma and lambda, 3 mm lateral to the sagittal suture. A Luer-loc hub was rigidly fixed with dental cement to the craniotomy. Experimental lateral FP brain injury of moderate severity (2.0–2.1 atm) was induced in the anesthetized animals ($n = 20$) using a well-characterized model that has been previously described in detail [20,32]. Following FP injury, rats were allowed to survive for 1, 3, 6, 24 or 72 h before euthanasia, in order to assess the acute response of the neurotrophins to the injury. A subset of animals ($n = 4$; 3 h survival period) underwent anesthesia and surgery but were not injured (sham treatment).

After the appropriate survival times, the rats were deeply anesthetized with an overdose of sodium pentobarbital and decapitated. Brains were rapidly removed and frozen over dry ice. Tissue sections through the hippocampus were cut in the coronal plane at 10 μm in a cryostat, thaw-mounted onto Superfrost Plus (Curtin Matheson Scientific) glass slides, and stored at -20°C until processing for hybridization. Adjacent sections throughout the hippocampus of animals from the various injury and sham groups were processed for the in situ hybridization localization of mRNAs for BDNF and NT-3 as previously described [11,42,43]. The cRNA probes were prepared by in vitro transcription from linearized cDNA constructs with the appropriate RNA polymerase in the presence of [^{35}S]UTP. The 550-base rat NT-3 cRNA is complementary to 392 bases of the mature rat NT-3 coding region, whereas the 540-base BDNF cRNA includes 384 bases complementary to the rat BDNF mRNA coding region [11,21]. Hybridization was conducted at 60°C for 18–24 h with the ^{35}S-labeled cRNA at a concentration of 1×10^6 cpm/50 μl/slide. Following post-hybridization washes and ribonuclease treatment, the sections were air-dried and exposed to β-Max Hyperfilm (Amersham) for 14–18 days at room temperature for generation of film autoradiograms. After autoradiographic film development, the sections were dipped in NTB2 nuclear track emulsion (Kodak; 1 : 1 in H_2O), air-dried, and exposed in light-tight boxes at 4°C for 4–6 weeks. After autoradiographic development of the emulsion, the sections were counterstained with Cresyl violet, coverslipped in D.P.X. mounting medium (Fluka), and analyzed with a Nikon Optiphot-2 microscope equipped with brightfield and darkfield optics. Cells were considered labeled if the density of reduced silver grains overlying the perikarya was at least 10-fold greater than background. Control sections that had been treated with ribonuclease A (45°C for 30 min) before hybridization or processed for hybridization with appropriate sense-strand riboprobes (see [11]) were devoid of specific labeling.

Film autoradiograms were analyzed with Image 1.50 software (NIH) to compare the density of hybridization for the neurotrophin mRNAs in various hippocampal subfields (dentate gyrus, CA1 and CA3) after sham treatment to that found after the various survival periods following lateral FP injury. Three to seven sections were analyzed per animal. All measurements are expressed as the mean $\pm$ S.E.M values. The data sets were compared using a two-way analysis of variance (ANOVA) for side (ipsilateral and contralateral to the injury) and groups (5 injury survival times and sham treatment). Newman-Keuls post-hoc analyses were used for pairwise comparisons with a significance level set at $P < 0.05$. BDNF and NT-3 mRNA levels did not differ by side for any of the hippocampal regions investigated, nor was there a side by group interaction (data not shown). Therefore, the hybridization data from the ipsilateral and contralateral sides were combined. The mean value of the sham control (3 h survival) was also compared to additional sham treatment animals with survival periods of 1, 6, 24, and 72 h ($n = 3$/group) post-surgery. No significant differences were found in hybridization densities among sham treatment groups with various survival periods for either BDNF or NT-3 mRNAs in any of the hippocampal subfields (data not shown).

Hybridization for BDNF mRNA was present in the granule cell layer of the dentate gyrus (stratum granulosum) and in regions CA1-CA3 of the hippocampus in the control (sham treatment) animals (Fig. 1A), similar to previous reports in normal, uninjured rats [7,21]. Unilateral FP injury resulted in a marked bilateral increase in the expression of BDNF mRNA in the dentate gyrus granule cell layer, which peaked at 3 h and remained above control levels for up to 72 h after injury (Fig. 1B–D). Densitometric measurements of film autoradiograms demonstrated that BDNF mRNA hybridization was significantly increased in the granule cell layer at all post-injury time points ($P < 0.001$; Fig. 2A). Expression of BDNF mRNA was also significantly elevated bilaterally in the hippocampal CA3 region at 1, 3 and 6 h after FP injury ($P < 0.001$), but returned to control levels by 24 h (Figs. 1 and 2B). Again, the most pronounced increase in labeling in the

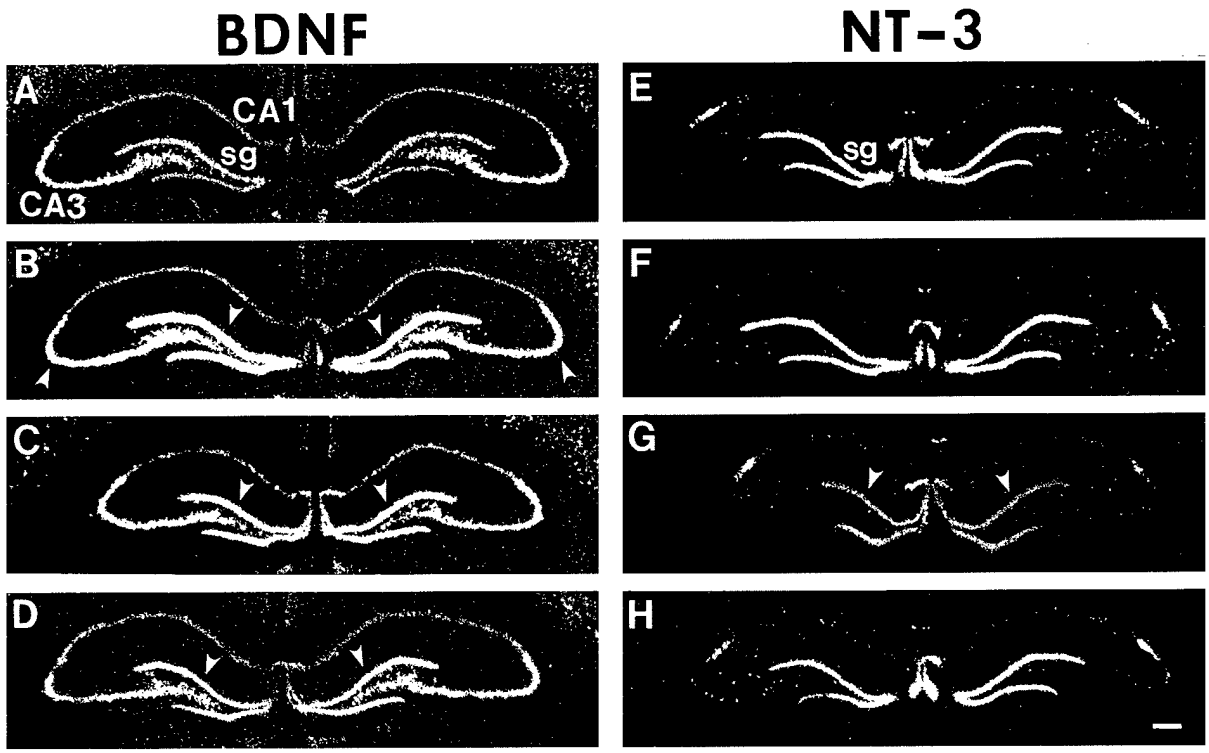

Fig. 1. Prints of film autoradiograms showing expression of BDNF (A–D) and NT-3 (E–H) mRNAs in coronal sections from control (sham) rats (A,E), and from rats subjected to moderate unilateral FP brain injury with 3 (B,F), 24 (C,G), and 72 h (D,H) survival periods. Note the increased hybridization for BDNF mRNA bilaterally in the dentate gyrus granule cell layer (stratum granulosum; sg) at all survival times following injury (B–D), and in the hippocampal CA3 region at the 3 h post-injury time point (B). In contrast, NT-3 mRNA levels are decreased bilaterally in the dentate gyrus at 24 h post-injury (G). Arrowheads in appropriate panels indicate the cell layers and survival times which differ significantly from the sham controls (see quantification in Figs. 2 and 3). Scale bar = 500 μm.

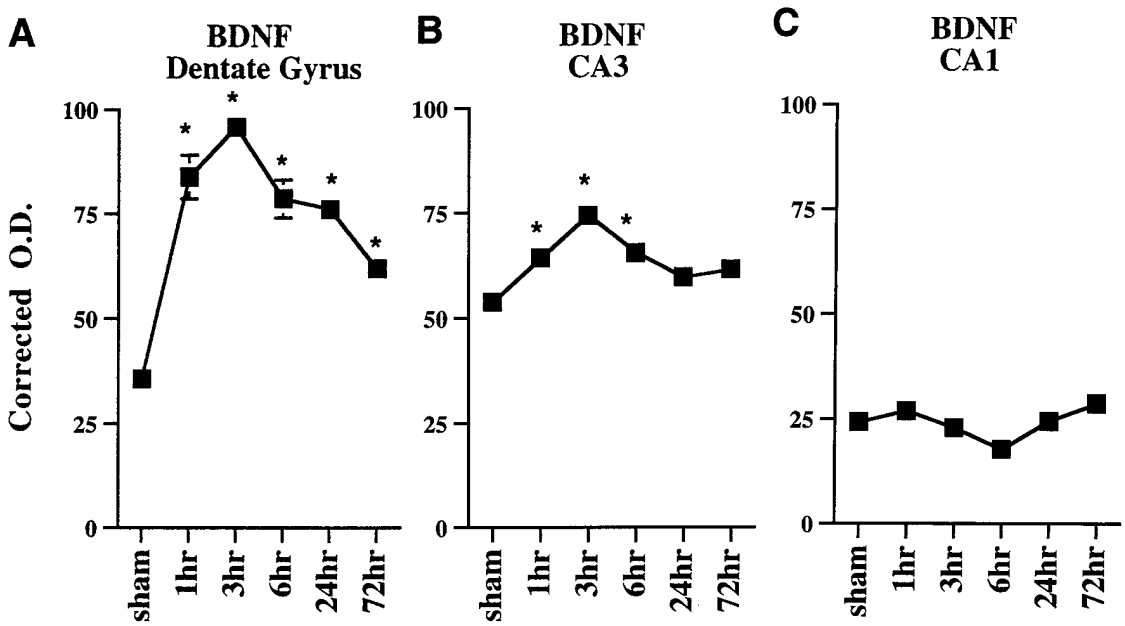

Fig. 2. Graphs showing corrected optical density (O.D.) measurements of hybridization for BDNF mRNA in the dentate gyrus stratum granulosum (A), hippocampal CA3 (B), and hippocampal CA1 (C) regions over time following lateral FP brain injury. Note the significant increase in BDNF mRNA expression in the dentate gyrus granule cell layer at all post-injury times (A), and in the hippocampal CA3 region at 1, 3, and 6 h following injury (B), compared to the sham treatment group ($^{*}P < 0.001$). Lateral FP injury did not alter BDNF mRNA levels in the hippocampal CA1 region at any of the survival times (C). Values represent mean $\pm$ S.E.M.

CA3 region was evident at 3 h after injury (Fig. 1B). No changes in expression of BDNF mRNA occurred in the CA1 region of the hippocampus following FP injury (Figs. 1 and 2C).

In the control, sham-injured animals the most prominent expression of NT-3 mRNA was localized to the dentate gyrus granule cell layer (Fig. 1E). Labeled cells were also present in regions CA2 and extreme medial CA1 of the hippocampal pyramidal cell layer, as well as infrequently scattered throughout the dentate gyrus hilus and hippocampal molecular layers (Fig. 1E). This distribution is in good agreement with previous descriptions in normal rats [7,13]. Following unilateral FP injury, hybridization for NT-3 mRNA was decreased bilaterally in the dentate gyrus granule cell layer at the 6 and 24 h survival times (Fig. 1G). By 72 h post-injury, hybridization levels had returned to near-control (sham injury) levels (Fig. 1H). Quantitative measurements of film autoradiograms confirmed that NT-3 mRNA expression was significantly reduced in the granule cells at both 6 and 24 h after FP injury ($P < 0.001$; Fig. 3), compared to sham controls. Although not analyzed densitometrically, visual examination of NT-3 mRNA hybridization in CA2 and medial CA1 indicated no apparent change in expression at any of the survival times post-injury, compared to sham controls.

The present results demonstrate that FP brain injury induces pronounced alterations in the expression of neurotrophin mRNAs in the hippocampus. Levels of BDNF mRNA were substantially increased post-injury in both the dentate gyrus granule cell and CA3 pyramidal cell layers. In contrast, expression of NT-3 mRNA was transiently decreased in the dentate gyrus, and the response was delayed relative to the early change in BDNF. Thus, traumatic brain injury differentially modulates neurotrophin gene expression in the hippocampus, in patterns and directions similar to findings in other brain injury paradigms including ischemia and seizures [12,13,27,28]. Although the present study focused on the hippocampal formation, it should be noted that obvious alterations in neurotrophin expression following FP injury were also observed in other brain regions, including the cortical lesion site, adjacent neocortical areas, the piriform cortex, and several medial thalamic nuclei (data not shown).

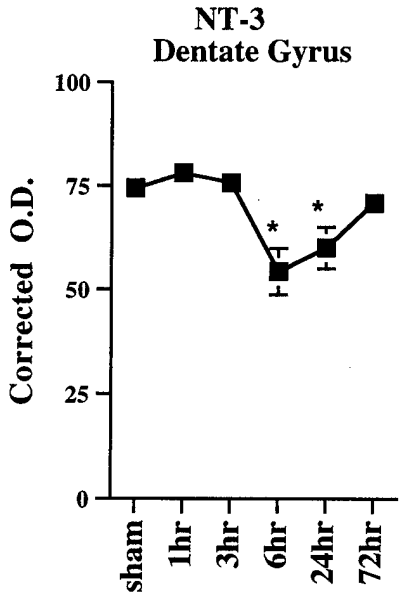

Fig. 3. Graph showing corrected optical density (O.D.) measurements of NT-3 mRNA hybridization in the dentate gyrus granule cell layer over time following lateral FP brain injury. Note the significant decline in NT-3 mRNA expression at 6 and 24 h after injury compared to the sham treatment group ($^{*}P < 0.001$). Values represent mean ± S.E.M.

Our results are consistent with recent data on the acute modulation of neurotrophin gene expression obtained with another model of traumatic brain injury, the cortical contusion impact model [48]. That study reported an increase in BDNF, but no change in NT-3, mRNA levels in the dentate gyrus granule and hippocampal pyramidal cell layers at 1, 3, and 5 h (the longest survival time examined) post-injury. The lack of change in NT-3 mRNA expression may reflect the acute time course of their study, since in the present study the decrease in NT-3 expression was not evident until 6 h after FP injury. In any event, it is now apparent from two different paradigms that a consistent response of hippocampal neurons to traumatic brain injury is dramatic, differential regulation of neurotrophin expression.

The bilateral alterations in BDNF and NT-3 expression are in contrast to the gross morphological and histological damage which has been primarily identified in hippocampal regions ipsilateral to the impact site [4,20]. However, they are consistent with more subtle changes, such as the bilateral loss of hilar neurons [30] and bilateral alterations in the expression of immediate-early genes and tumor necrosis factor-α [9,39], which have been observed following unilateral FP injury. Whereas no evidence of abnormal behavior or overt seizure activity was noted in any of the experimental groups in this study, nor in a previous study with FP injury of this severity [30], it is possible that post-traumatic subclinical seizures contributed to the alterations in expression of BDNF and NT-3. However, neurochemical changes that would be expected to occur bilaterally following seizures have only been observed unilaterally following FP brain injury [6,36,38].

The significance of the alterations in BDNF and NT-3 expression on cell survival following lateral FP injury is unclear. The dentate gyrus showed the greatest increase in BDNF compared to control values and cells in this region are selectively resistant to cell death following lateral FP injury [4,20,30]. However, BDNF expression was also elevated bilaterally in the hippocampal CA3 region, which contains numerous injured neurons on the side ipsilateral to the impact [4,20]. Numerous previous studies have supported the hypothesis that BDNF is neuroprotective following injury [1,2,17,27,28,46], whereas others have found no trophic effect [40] or an actual increase in

neuronal death [24] with BDNF treatment. Although further studies are necessary to clarify the role of BDNF following injury, one hypothesis is that it is the *amount* of BDNF available that is critical for promoting cell survival. The functional consequences of the concurrent decrease in NT-3 expression in the same cells (stratum granulosum) marked by the BDNF increase, also remain unknown. It is possible that whereas optimal neurotrophin levels may promote survival, insufficient or excessive levels may exacerbate neuronal loss. Moreover, injury-induced alterations in levels and functional states of appropriate neurotrophin receptors may also be important determinants of resulting neurotrophic functions.

In conclusion, lateral FP brain injury differentially modulates expression of BDNF and NT-3 in hippocampal neurons. These alterations are consistent with the hypothesis that widespread secondary events, including neurotrophin plasticity, occur following traumatic brain injury. Further investigations are necessary to evaluate the role of these neurotrophic factors on cell survival after experimental brain trauma.

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Short communication

Expression of trkB mRNA is altered in rat hippocampus after experimental brain trauma

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MOLECULAR BRAIN RESEARCH

Short communication

Expression of trkB mRNA is altered in rat hippocampus after experimental brain trauma

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Abstract

Recent investigations have shown that expression of mRNAs for the neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) is differentially altered in the hippocampus following traumatic brain injury. In the present study, modulation of neurotrophin receptor expression was examined in the hippocampus in a rat model of traumatic brain injury using in situ hybridization. Messenger RNA for trkB, the high-affinity receptor for BDNF and neurotrophin-4 (NT-4), was increased between 3 and 6 h bilaterally in the dentate gyrus following a lateral fluid-percussion brain injury of moderate severity (2.0–2.1 atm). No time-dependent alterations were observed for trkB mRNA in hippocampal subfields CA1 and CA3. Levels of mRNA for trkC, the high-affinity receptor for NT-3, did not change in any region of the hippocampus. These data demonstrate that lateral fluid-percussion injury modulates expression of trkB mRNA in the hippocampus and support a role for BDNF/trkB signalling mechanisms in secondary events associated with traumatic brain injury.

Keywords: Traumatic brain injury; Lateral fluid percussion; Neurotrophin receptors; In situ hybridization; Neuronal plasticity

The trophic properties of neurotrophins are mediated through interactions with their high-affinity receptors, which are transmembrane protein–tyrosine kinases (trks) [4,14,20]. The widely expressed trkB protein is the high-affinity receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) [4,20]. The trkC protein is also broadly expressed throughout the nervous system and is the high-affinity receptor for neurotrophin-3 (NT-3) [4,17]. Activation of trk receptors involves ligand binding by the neurotrophin, receptor dimerization, autophosphorylation, and activation of tyrosine residues on various intracellular substrates [14]. Under normal developmental and mature conditions, these intracellular substrates serve as signals for survival, proliferation, differentiation, and synaptogenesis, as well as for other forms of neural plasticity [18,33]. Following various types of neural injury, neurotrophin/trk interactions have been linked to neuroprotection and recovery of function [22,28].

Previous investigations into the role of neurotrophins in experimental brain trauma have demonstrated that BDNF mRNA is elevated in the hippocampus as early as 1 h after lateral fluid percussion (FP) [12] or cortical contusion [35] brain injury, and expression remains elevated up to 72 h post-injury [12]. Conversely, NT-3 mRNA levels decrease in the hippocampus following lateral FP brain injury [12]. However, the injury responsiveness of neurotrophin receptors in such brain trauma paradigms has yet to be determined. Therefore, to further investigate the role of neurotrophin/trk interactions in brain trauma, we examined whether trkB and trkC mRNAs in the hippocampus are altered following lateral FP brain injury in rats.

Male Sprague–Dawley rats (325–350 g) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) 10 min after receiving 0.15 ml of atropine (0.4 mg/ml, i.m.), and placed in a stereotaxic frame. The scalp and temporal muscles were reflected, and a stainless-steel screw was secured to the skull 1 mm anterior to bregma. A craniotomy with a 5-mm diameter was centered between bregma and lambda, 3 mm lateral to the sagittal suture. A Luer-loc hub was rigidly fixed with dental cement to the craniotomy. Experimental brain injury of moderate sever-

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ity (2.0–2.1 atm) was induced in the anesthetized animals ($n = 20$), using the lateral FP brain injury model. This model is well-characterized and has been previously described in detail [6,13,24]. Following FP injury, rats were euthanatized at 1, 3, 6, 24, or 72 h (four per survival period), in order to assess the acute response of trkB and trkC to the injury. Additional animals ($n = 15$; three per survival period) underwent anesthesia and surgery but were not injured (sham treatment).

After the appropriate survival times, the rats were deeply anesthetized with an overdose of sodium pentobarbital, decapitated, and their brains were rapidly removed and frozen over powdered dry ice. Coronal sections (10 μm thick) were cut through the hippocampus in a cryostat, thaw-mounted onto Superfrost Plus (Curtin Matheson Scientific) glass slides, and stored at −20°C until processing for hybridization. Adjacent sections throughout the hippocampus of animals from the various injury and sham groups were processed for the localization of mRNAs for trkB and trkC using in situ hybridization as previously described [30,31]. Sense and antisense cRNA probes were prepared by in vitro transcription using appropriate linearized DNA constructs in the presence of the proper RNA polymerase (T3 or T7) and [^{35}S]UTP (New England Nuclear). The cDNA constructs for trkB and trkC (plasmids kindly provided by D. McKinnon, State University of New York at Stony Brook) resulted in antisense RNA transcripts that were 196 and 374 bases in length, respectively. The trkB cRNA probe detects the kinase-specific, full-length catalytic form of the receptor mRNA [10,27]. The probe used to detect trkC mRNAs recognizes transcripts for both the full-length catalytic and kinase domain insertion/deletion non-catalytic forms of the receptor [1,7,34]. Sections were hybridized at 60°C for 18–24 h with [^{35}S]labelled cRNA probes at a concentration of 1.0×10^6 c.p.m./50 μl/slide. After post-hybridization ribonuclease treatment and washes, film autoradiograms were generated by exposure of the sections to β-Max Hyperfilm (Amersham) for 7 days. Following film development, the sections were dipped in NTB2 nuclear track emulsion (Kodak; 1:1 in H_20), air-dried, and exposed in sealed slide boxes at 4°C for 4–6 weeks. The emulsion was developed in D19 (Kodak) and fixed with Rapidfix (Kodak). The slides were then counterstained with Cresyl violet (Sigma) and coverslipped with DPX mountant (Fluka). Pre-hybridization treatment of tissue with ribonuclease A and hybridization of sections with sense transcript controls resulted in no specific hybridization signal.

Film autoradiograms were analysed with Image 1.60 software (NIH) to measure the density of hybridization for the trk mRNAs in several hippocampal subfields (stratum granulosum of the dentate gyrus, strata pyramidale of CA1 and CA3). Background optical density (O.D.) measurements were taken in adjacent white matter of the corpus callosum and subtracted from the O.D. measurements in the hippocampal subregions in order to obtain corrected O.D. measurements. At least three sections were analysed per animal. All measurements are expressed as the mean values plus or minus the standard error of the mean (S.E.M.). The effects of treatment (injury vs. sham), survival time, and their interaction effects were analysed with a two-way analysis of variance (ANOVA) in each hippocampal subfield for the side of the brain ipsilateral to the injury, contralateral to the injury, and for the bilateral sham data. Bonferonni post-hoc analyses were used for pairwise comparisons with a significance set at $P < 0.05$.

Hybridization for trkB and trkC mRNAs was present in the granule cell layer of the dentate gyrus (stratum granulosum) and in regions CA1–CA3 of the hippocampus in the control (sham treatment) animals (Fig. 1A, Fig. 3A), similar to previous reports in normal, uninjured rats [3,9,25]. Following FP injury, increases in trkB mRNA levels were evident in the dentate gyrus granule cell layer at 3 and 6 h post-injury, but not at 1, 24 or 72 h (Figs. 1 and 2), in comparison to levels in sham animals. Densitometric measurements of film autoradiograms confirmed

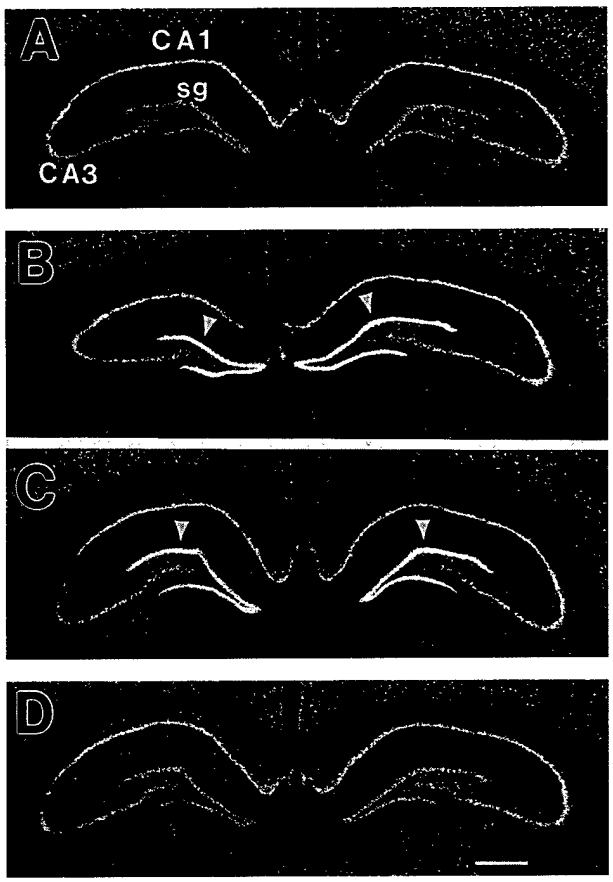

Fig. 1. Film autoradiograms showing hybridization for trkB mRNA in coronal sections through the hippocampus of a control (3 h sham) rat (A) and of rats subjected to lateral FP brain injury of moderate severity with 3 (B), 6 (C), and 72 (D) h survival times. Note the increase in hybridization signal in the dentate gyrus granule cell layer (stratum granulosum; sg) at the 3 and 6 h post-injury timepoints [arrowheads in (B), (C)], and the return to control levels by 72 h (see quantification in Fig. 3). Scale bar = 1 mm.

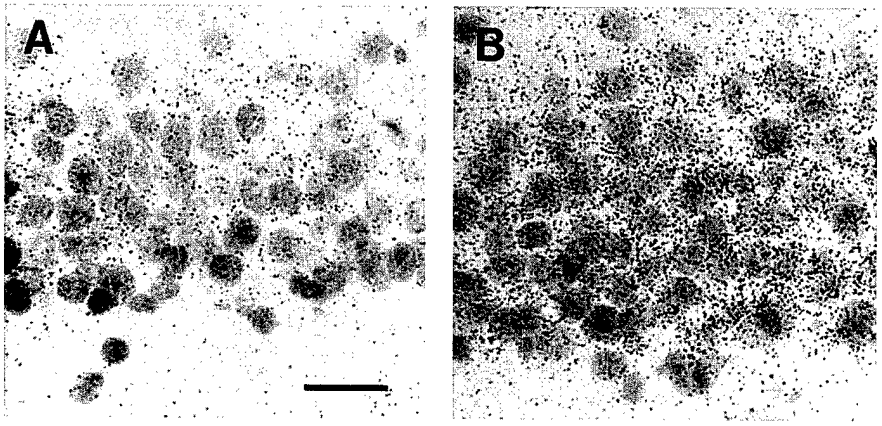

Fig. 2. High-power brightfield photomicrographs showing autoradiographic labeling for trkB mRNA in the dentate gyrus stratum granulosum in a control (6 h sham injury) rat (A) and in a rat subjected to FP brain injury with a 6-h survival time (B). Note the substantial increase in hybridization density over the granule cells at the 6 h post-injury timepoint (B). Scale bar = 20 μm.

that hybridization for trkB mRNA was significantly elevated in both the ipsilateral and contralateral stratum granulosum of FP-injured animals as compared to sham controls at 3 h ($P < 0.005$) and at 6 h ($P < 0.05$) (Fig. 3). In the strata pyramidale subfields, CA1 and CA3 (Fig. 1), O.D. measurements indicated that there were no time-dependent changes in trkB mRNA after FP injury (data not shown). Hybridization for trkC mRNA in the dentate gyrus granule cell layer, CA1 or CA3 hippocampal subfields, was unchanged following FP injury for all timepoints (Figs. 4 and 5).

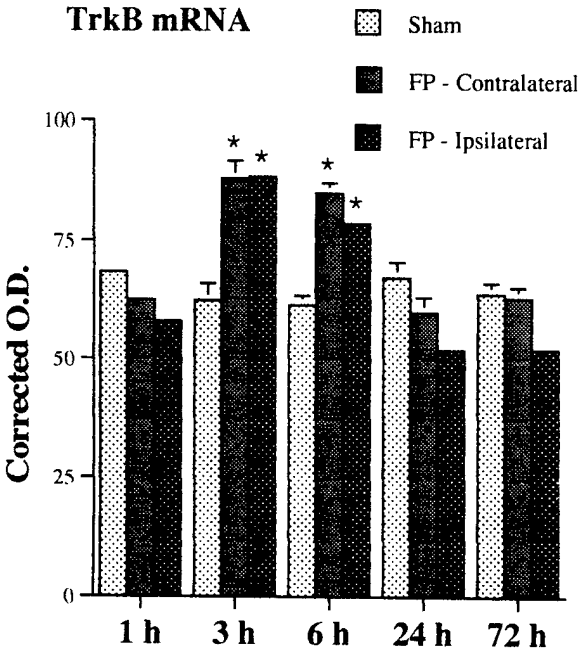

Fig. 3. Changes over time in corrected optical density (O.D.) measurements of trkB mRNA hybridization in the ipsilateral and contralateral dentate gyrus in rats subjected to FP injury and in the bilateral dentate gyrus in control (sham injury) rats. Note the significant increase in trkB mRNA expression at 3 and 6 h after injury as compared to the sham treatment group (* $P < 0.05$). Values represent mean ± S.E.M.

The results of the present study demonstrate that expression of trkB mRNA in the hippocampus is transiently up-regulated following lateral fluid-percussion brain injury. The alterations in trkB mRNA that we observed in the dentate gyrus at 3 and 6 h post-injury are in general agreement with previous investigations utilizing other models of central nervous system (CNS) injury. For example, induction of seizures following kindling caused a rapid and transient elevation in trkB mRNA in the dentate gyrus, which peaked at 30 min and tapered off to near control values by 4 h [26]. Increases in trkB mRNA in the dentate gyrus were also observed at 2 h following an ischemic insult [26], and at 2–4 h following a mechanical injury to one side of the brain [29]. Our finding that trkC expression in the hippocampus was not affected by lateral FP injury is in agreement with observations reported after seizures [26]. However, in contrast to these similar observations, 2–4 h after a focal mechanical injury, an increase in trkC mRNA was found in the dentate gyrus [29]. Thus, despite some differences in trkC responsiveness following injury, the similar alterations in trkB mRNA across models suggest that common mechanisms may be involved in the modulation of neurotrophin expression after CNS injury.

Our previous study demonstrated that levels of BDNF mRNA in the dentate gyrus increased by approximately 200% of sham values between 1 and 72 h after FP injury [12]. The present increase in trkB mRNA (approximately 35%) in the dentate gyrus after FP injury was much less robust and slightly delayed relative to the onset of significant alterations in its ligand, BDNF. Nevertheless, the complementary increase in BDNF and trkB mRNAs suggests that this signal transduction pathway may be further enhanced in the dentate gyrus during the acute periods following FP injury. This is a noteworthy observation because chronic exposure to exogenous BDNF has been shown to down-regulate trkB mRNA and protein in vivo [8,16]. Thus, since acute increases in BDNF mRNA following FP injury are not associated with decreases in trkB

mRNA, acute administration of BDNF may be more effective than chronic administration in activating BDNF/trkB signal transduction pathways and influencing hippocampal cell survival and function.

The functional significance of the increase in BDNF/trkB mRNA in the dentate gyrus following FP injury is presently unknown. Neurotrophin/trk interactions are believed to be important for selective neuronal survival [2,15], and this role may also be important following FP injury. BDNF/trkB signalling mechanisms may have a neuroprotective effect on the granule cells of the dentate gyrus following FP injury, as these cells are selectively resistant to death [6,13,21]. However, CA1 pyramidal cells are also resistant to lateral FP-induced degeneration, and increases in BDNF/trkB were not observed in this region of the hippocampus ([12]; present study). NT-3/trkC signal transduction has also been found to have neuroprotective effects in some models of neuronal injury [11,19], but this appears unlikely following FP injury, as the NT-3 mRNA levels are decreased [12] and the trkC mRNA levels remain unchanged.

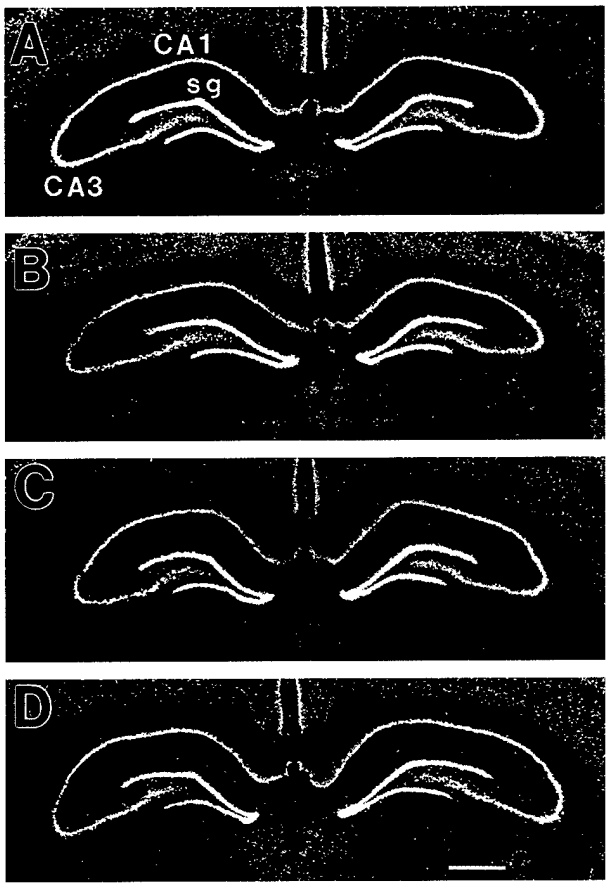

Fig. 4. Film autoradiograms showing hybridization for trkC mRNA in coronal sections through the hippocampus of a control (24 h sham) rat (A) and of rats subjected to lateral FP brain injury of moderate severity with 3 (B), 24 (C), and 72 (D) h survival times. Hybridization signal for trkC mRNA is unchanged for any of the timepoints that were analysed (see quantification in Fig. 5). Scale bar = 1 mm.

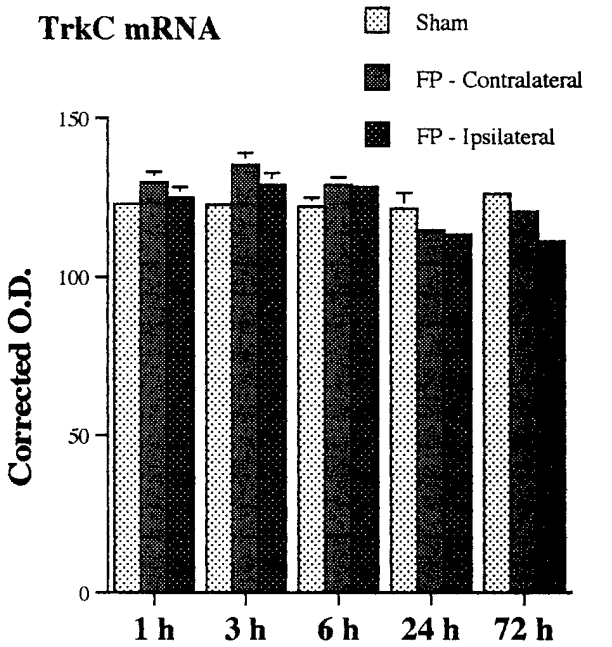

Fig. 5. Corrected O.D. measurements over time of trkC mRNA hybridization in the ipsilateral and contralateral dentate gyrus in rats subjected to FP injury and in the bilateral dentate gyrus in control (sham injury) rats. No statistically significant differences were observed in injured rats as compared to control rats. Values represent mean ± S.E.M.

Both BDNF and trkB have also been associated with synaptogenesis and neural plasticity, and thus, may be important for recovery of function following injury. Blocking trkB receptors interfered with the normal development of ocular dominance columns in the visual cortex [5]. Removal of facial vibrissae in mice during development resulted in a decrease in BDNF mRNA in the corresponding cortical barrel region, but an increase in the contralateral barrel region [32]. Moreover, recent evidence indicates that BDNF and NT-3 may have antagonistic actions on dendritic growth in cortical neurons [23]. If a similar relationship exists in the hippocampus, then the decrease in NT-3 mRNA following FP injury [12] may further amplify the effects of BDNF/trkB signal transduction on neural plasticity.

In summary, our results demonstrate that increases in trkB occur bilaterally in the dentate gyrus following lateral FP injury. These data complement our previous studies that have shown similar alterations in BDNF mRNA in this same region of the hippocampal formation. The coordinated up-regulation of BDNF/trkB expression may be important for neuronal survival or neural plasticity following traumatic brain injury.

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Alterations in BDNF and trkB mRNA Levels in the Cerebral Cortex Following Experimental Brain Trauma in Rats

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ABSTRACT

Recent studies have suggested that brain-derived neurotrophic factor (BNDF) and its receptor, trkB, may provide neuroprotection following injury to the central nervous system. Conversely, other studies have implicated BDNF as a contributing factor to neurodegenerative events that occur following injury. In order to further investigate the role of BDNF in neuroprotection, we subjected adult rats to a lateral fluid percussion (FP) injury of moderate severity (2.0–2.1 atm) or sham injury. After survival periods of 1, 3, 6, 24, or 72 h, the brains were processed for the in situ hybridization localization of BDNF and trkB mRNAs using ^{35}S-labeled cRNA probes. Hybridization levels were compared between injured and sham animals for regions of the cortex that were located within, adjacent to, and remote from the site of the cortical contusion. BDNF mRNA levels were significantly decreased in the injured cortex at 72 h, increased in adjacent cortical areas at 3 h, and increased bilaterally in the piriform cortex from 3 to 24 h post-FP injury. Expression of trkB mRNA was significantly decreased at all postinjury time-points in the injured cortex and at 24 h in the adjacent cortex. These results demonstrate that, following lateral FP injury, BDNF and trkB mRNA levels are decreased in cortical regions that contain degenerating neurons, generally unchanged in adjacent regions, and increased in remote areas. Thus, injury-induced decreases in the expression of BDNF and trkB may confer vulnerability to neurons within the cortical contusion.

Key words: head injury; neurotrophic factors; traumatic brain injury

INTRODUCTION

TRAUMATIC BRAIN INJURY (TBI) is a major health problem, especially for adolescents and young adults. It accounts for 25% of injury-related deaths and is also a major cause of lifelong disability and impairment (Sosin et al., 1989). Although the neuropathology associated with TBI is variable, a cortical contusion is a frequently encountered form of neuronal injury (Adams, 1992). The lateral fluid percussion rodent model of experimental brain trauma effectively reproduces this feature of TBI, as a cortical contusion is the most pronounced form of neuropathology associated with this injury device (McIntosh et al., 1989; Hicks et al., 1996).

Previous studies have suggested that the neuronal damage following experimental TBI may initially be reversible (Cooper, 1985). It is believed that the impact and shear forces generated at the time of the injury cause primary neuronal damage, followed by a cascade of secondary excitotoxic biochemical events (Faden et al.,

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1989; Katayama et al., 1990). Identifying pharmacologic agents to block these secondary neurodegenerative events has been a primary focus of TBI research. To this end, administration of the trophic factor basic fibroblast growth factor has been reported to result in a significant decrease in the cortical contusion volume following fluid percussion (FP) injury in rats (Dietrich et al., 1996).

Another potentially neuroprotective agent for TBI is brain-derived neurotrophic factor (BDNF). Numerous studies have demonstrated that administration of BNDF can protect cells from a variety of central nervous system insults both *in vitro* (Skaper et al., 1993; Cheng and Mattson, 1994; Hayes et al., 1995; Mattson et al., 1995; Kubo et al., 1995; Nakao et al., 1995) and *in vivo* (Kindy, 1993; Beck et al., 1994; Mansour-Robey et al., 1994; Yurek et al., 1996; Cheng et al., 1997; Schabitz et al., 1997; Klocker et al., 1998; Hagg, 1998). However, there is also evidence to suggest that BDNF potentiates neuronal injury *in vitro* (Koh et al., 1995) and *in vivo* (Rudge et al., 1998).

BDNF mediates its effects through interaction with a tyrosine kinase receptor, trkB (Barbacid, 1994; Lindsay et al., 1994). Both BDNF and trkB mRNA levels increase significantly in the hippocampus following lateral FP (Hicks et al., 1997, 1999) and cortical impact injury (Yang et al., 1996). Acute increases in BDNF mRNA levels have also been observed in the ipsilateral neocortex up to 5 h after a cortical impact injury (Yang et al., 1996). In the present study, we were interested in exploring alterations in expression of BDNF and its functional receptor, trkB, in relationship to their potential roles in neuroprotection and/or neural plasticity following the lateral FP model of TBI. Thus, we investigated time-dependent (1–72 h) alterations in expression of both BDNF and trkB mRNAs in specific subregions of the cortex that undergo varying degrees of neuronal degeneration following a lateral FP injury.

METHODS

Surgery and Fluid Percussion Injury

Male Sprague-Dawley rats (325–350 g) were injected with atropine (0.15 ml i.m.) 10 min prior to being anesthetized with sodium pentobarbital (60 mg/kg i.p.) and placed in a stereotaxic frame. After reflecting the scalp and temporal muscles, the animals were given a 5-mm craniotomy with a hand-held Michele trephine over the left parieto-occipital cortex. The craniotomy was centered between bregma and lambda, and was lateral to the sagittal suture. A hollow Luer-Lok fitting was fixed rigidly with dental cement to the craniotomy. Experimental brain injury of moderate severity (2.0–2.1 atm) was induced in the anesthetized animals ($n = 20$), using the lateral FP brain injury model. This model is well-characterized and has been previously described in detail (McIntosh et al., 1989). Following FP injury, rats were euthanatized at 1, 3, 6, 24, or 72 h ($n = 4$ per survival period), in order to assess the acute response of BDNF and trkB mRNAs to the injury. Additional animals ($n = 15$; 3 per survival period) underwent anesthesia and surgery but were not injured (sham injury).

After the appropriate survival times, the rats were deeply anesthetized with an overdose of sodium pentobarbital and decapitated. Brains were rapidly removed and frozen over dry ice. Tissue sections through the level of the hippocampus were cut in the coronal plane at 10 μm in a cryostat, thaw-mounted onto Superfrost (Plus Fisher Scientific) glass slides, and stored at −20°C until processing for hybridization. Adjacent sections throughout the cerebral cortex of animals from the various injury and sham groups were processed for the in situ hybridization localization of mRNAs for BDNF and trkB as previously described (Gall et al., 1992; Seroogy et al., 1994; Numan and Seroogy, 1997; Seroogy and Herman, 1997). The cRNA probes were prepared by in vitro transcription from linearized cDNA constructs with the appropriate RNA polymerase in the presence of ^{35}S-UTP. The 540-base BDNF cRNA (plasmid kindly provided by J. Lauterborn and C. Gall, University of California, Irvine) includes 384 bases complementary to the rat BDNF mRNA coding region (Isackson et al., 1991; Gall et al., 1992). The cDNA construct for trkB (plasmid kindly provided by D. McKinnon, State University of New York at Stony Brook) resulted in an antisense RNA transcript that was 196 bases in length. The trkB cRNA probe detects the kinase-specific, full-length catalytic form of the receptor mRNA (Middlemas et al., 1991; Goodness et al., 1997). Hybridization was conducted at 60°C for 18–24 h with the ^{35}S-labeled cRNAs at a concentration of 1×10^6 cpm/50 μl/slide. Following posthybridization washes and ribonuclease treatment, the sections were air-dried and exposed to β-Max Hyperfilm (Amersham) for 14–18 days at room temperature for generation of film autoradiograms. After autoradiographic film development, the sections were dipped in NTB2 nuclear track emulsion (Kodak; 1:1 in H_2O), air-dried, and exposed in light-tight boxes at 4°C for 4–6 weeks. After autoradiographic development of the emulsion, the sections were counterstained with cresyl violet, coverslipped in DPX mounting medium (Fluka), and analyzed with a Nikon Optiphot-2 microscope equipped with brightfield and darkfield optics. Cells were considered labeled if the density of reduced silver grains overlying the perikarya was at least tenfold greater than background. Control sections that had been treated with ribonuclease A (45°C for

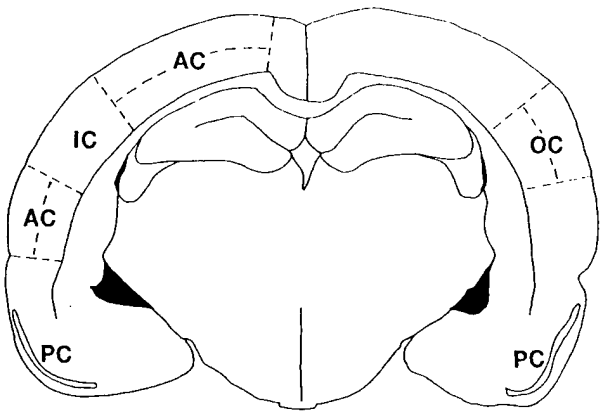

FIG. 1. Schematic representation of cortical regions analyzed for optical density measurements of hybridization for BDNF and trkB mRNAs: IC, the cortical region that comprises the lesion site following lateral FP injury; AC, the adjacent neocortical regions that surround the injury site; OC, the cortical region on the opposite side of the brain that is comparable in size and location to the IC; and PC, the bilateral piriform cortices. The AC and OC were subdivided into superficial and deep layers.

30 min) before hybridization or processed for hybridization with appropriate sense-strand riboprobes were devoid of specific labeling.

Film autoradiograms were analyzed with Image 1.60 software (NIH) to measure the density of hybridization for BDNF and trkB mRNAs in the following cortical regions: injured cortex (IC), superficial and deep layers of the adjacent cortex (AC) and opposite (homotypic) cortex (OC), and the bilateral piriform cortex (PC) (Fig. 1). Background optical density (OD) measurements were taken in adjacent white matter of the corpus callosum and subtracted from the OD measurements in the cortical regions in order to obtain corrected OD measurements. Hybridization levels observed after lateral FP injury were compared to those found after sham injury with equivalent survival periods. At least three and an average of seven sections were analyzed per animal. All measurements are expressed as the mean values plus or minus the standard error of the mean (SEM). The effects of treatment (injury versus sham), survival time, and their interaction effects were analyzed with a two-way analysis

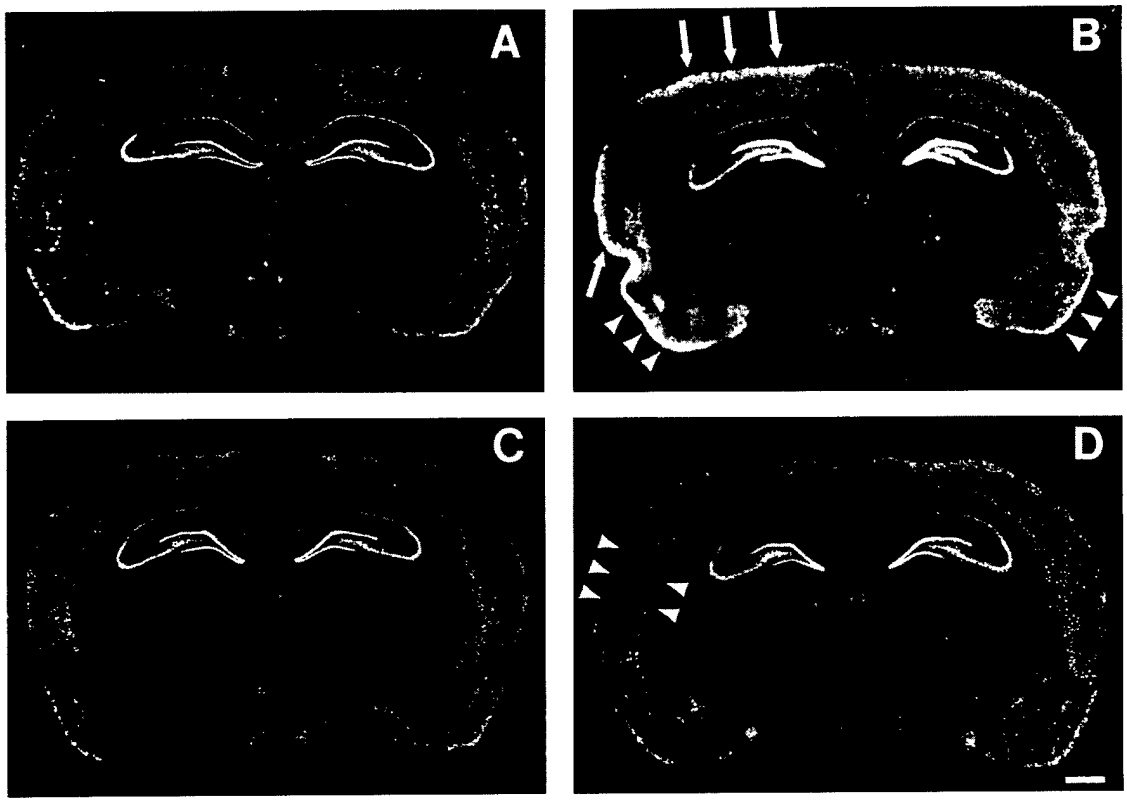

FIG. 2. Prints of autoradiograms showing expression of BDNF mRNA in coronal sections from a rat 3 h after a sham injury (A), 3 h after a lateral FP injury (B), 72 h after a sham treatment (C), and 72 h after a FP injury (D). Note the up-regulation of BDNF 3 h after a FP injury in the bilateral piriform cortices (arrowheads, B) and in the superficial layers of the adjacent cortex (arrows, B), and the decrease in BDNF mRNA in the injured cortex at 72 h post-injury (arrowheads, D). The mild increase in BDNF mRNA hybridization levels in the superficial layers of the OC 3 h after FP injury (B), was not significantly different compared to sham animals. Bar = 1,000 μm.

of variance (ANOVA) for each cortical region. BDNF and trkB mRNA levels did not differ by side for the PC. Therefore, the hybridization data from the right and left sides were combined. Bonferonni post hoc analyses were used for pairwise comparisons with a significance set at $p < 0.05$.

RESULTS

Hybridization for BDNF and trkB mRNAs in the cortex in the control (sham injury) animals (Fig. 2A,C; also see Fig. 4A below), was similar to previous reports described for normal, uninjured rats (Ernfors et al., 1990; Fryer et al., 1996). Following FP injury, BDNF mRNA levels in the IC did not differ from sham levels until 72 h, when they were significantly decreased ($p < 0.01$; Figs. 2D and 3A). This is in contrast to the superficial layers of the AC, where BDNF mRNA was significantly increased at 3 h postinjury ($p < 0.02$; Figs. 2B and 3B), and to the PC, where levels were significantly increased bilaterally ($p < 0.02$) at 3, 6, and 24 h in the FP injured-animals (Figs. 2B and 3C), compared to shams. In the deep layers of the AC and in all layers of the OC (Fig. 2), densitometric measurements revealed no time-dependent changes in BDNF mRNA after FP injury (quantitative data not shown).

The response of cortical trkB mRNA expression to FP injury was generally quite different from that of BDNF mRNA. No cortical regions for any of the survival peri-

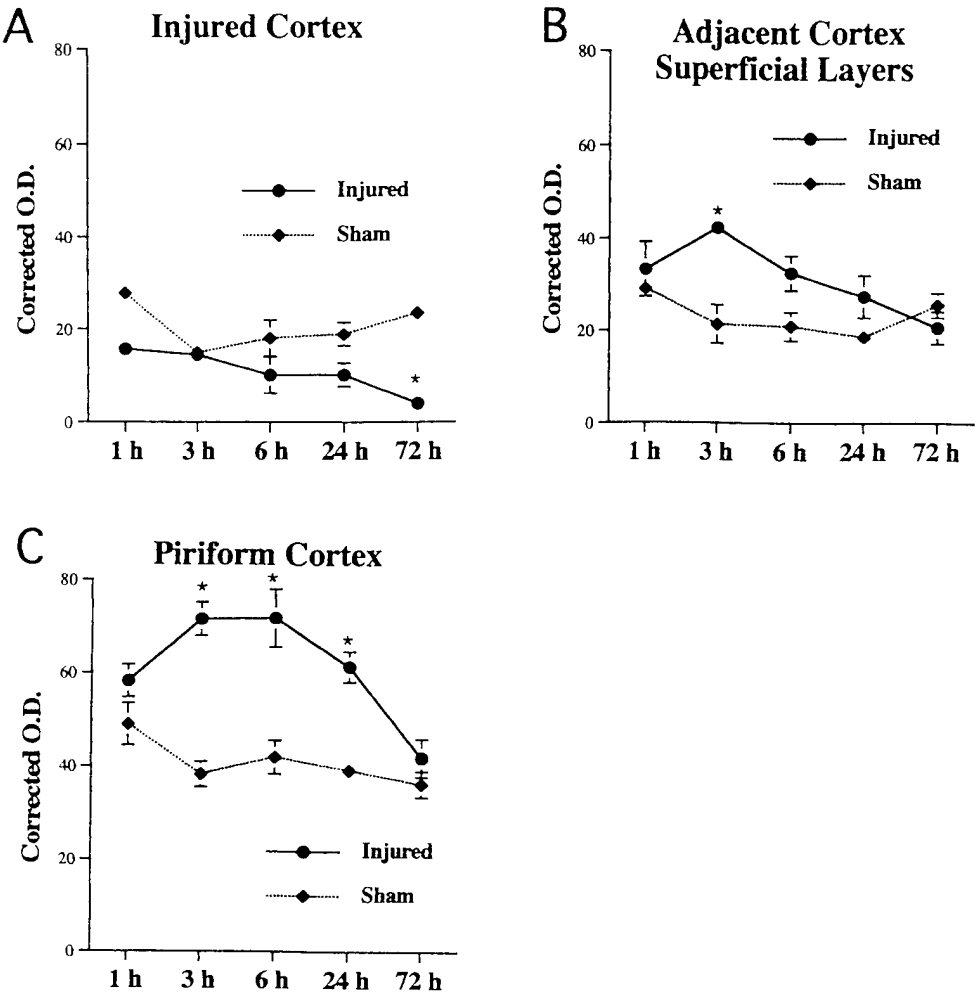

FIG. 3. Graphs showing corrected optical density (OD) measurements of hybridization for BDNF mRNA in the injured cortex (A), superficial layers of the adjacent cortex (B), and bilateral piriform cortex (C) over time following lateral FP brain injury. Note the significant decrease in BDNF mRNA expression in the injured cortex at 72 h following injury (A), and the significant increase in the superficial layers of the adjacent cortex at 3 h post-injury (B), and in the bilateral piriform cortex at 3, 6, and 24 h following injury (C) compared to the sham injury groups (*$p < 0.02$).

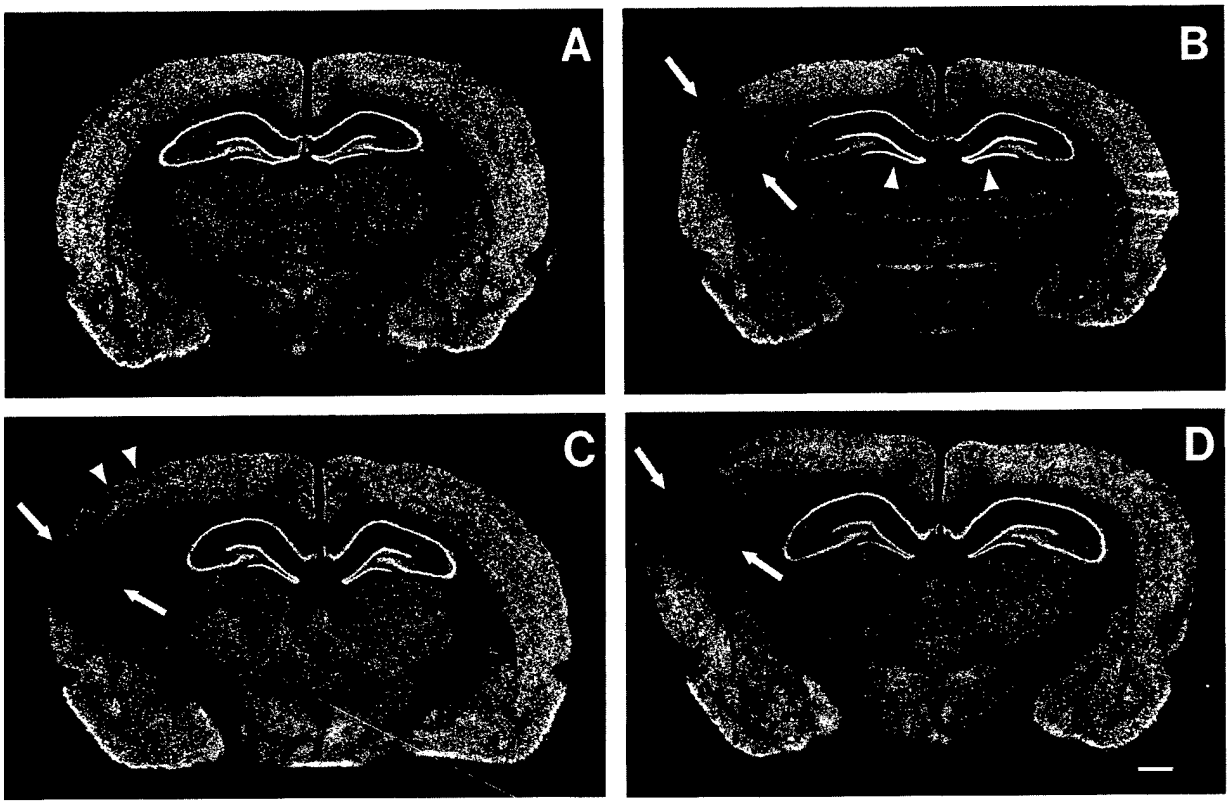

FIG. 4. Prints of autoradiograms showing expression of trkB mRNA in coronal sections from a rat 24 h after a sham injury (A), 3 h after a lateral FP injury (B), 24 h after a lateral FP injury (C), and 72 h after a lateral FP injury (D). Note the decreased hybridization for trkB mRNA in the injured cortex at all time-points (arrows, B,C,D) and in the superficial layers of the adjacent cortex at 24 h postinjury (arrowheads, C). Note also the increased hybridization for trkB mRNA in the granule cell layer of the dentate gyrus of the hippocampus at 3 h post-FP injury (arrowheads, B). Bar = 1,000 μm.

ods following FP injury demonstrated an increase in hybridization for trkB mRNA. Rather than an increase, expression of trkB mRNA in the IC was significantly decreased ($p < 0.001$) at all times following the FP injury compared to sham animals (Figs. 4 and 5A). There was also a significant decrease ($p < 0.03$) of trkB mRNA levels in the superficial layers of the AC at 24 h post-FP injury (Figs. 4C and 5B). In the deep layers of the AC, all layers of the OC, and bilaterally in the PC, no time-dependent alterations in trkB mRNA were observed after FP injury (Fig. 4; quantitative data not shown).

DISCUSSION

The purpose of this study was to characterize the response of BDNF and trkB mRNAs to lateral FP brain injury in cortical regions that undergo varying degrees of neurodegeneration. To this end, we evaluated time-dependent changes in BDNF and trkB mRNA levels in cortical tissue within, adjacent to, and remote from the contusion site. The hybridization levels for BDNF mRNA varied by region, with a persistent increase bilaterally in the PC, a transient increase in the superficial layers of the AC, no change in the deep layers of the AC, no change in the OC, and a delayed decrease in the IC. These cortical alterations in BDNF mRNA are in general agreement with those observed in a different model of experimental brain trauma, the cortical impact injury (Yang et al., 1996). The hybridization levels for trkB mRNA did not change in the cortex following FP injury, except for an immediate and persistent decrease in the IC, and a delayed transient decrease in the superficial layers of the AC.

This study also expands upon our previous investigations into alterations of neurotrophin levels in the hippocampus following FP (Hicks et al., 1997, 1999). The persistent increase in BDNF mRNA observed throughout most of the acute period following FP injury in the PC is similar to the increases we observed in the dentate gyrus granule cell layer and the CA3 pyramidal cell layer in the hippocampus (Hicks et al., 1997). Conversely, the

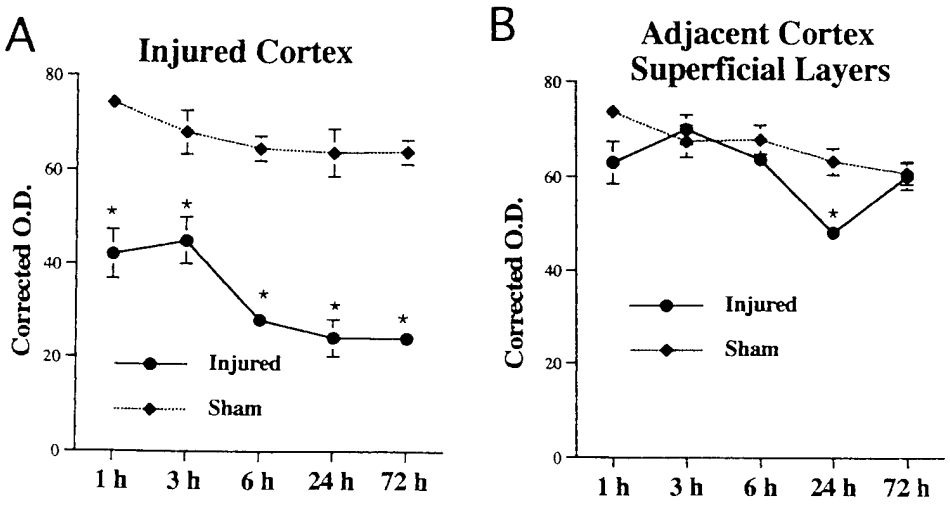

FIG. 5. Graphs showing corrected optical density (OD) measurements of hybridization for trkB mRNA in the injured cortex (A) and superficial layers of the adjacent cortex (B) over time following lateral FP brain injury. Note the significant decrease in trkB mRNA expression in the injured cortex at all time points following injury (A), and at 24 h in the superficial layers of the adjacent cortex (B) compared to the sham injury groups ($*p < 0.03$).

alterations in trkB mRNA following FP differed for cortical and hippocampal regions. No increases were observed in trkB mRNA in any of the cortical areas we investigated, whereas significant increases were observed in the granule cell layer of the dentate gyrus at 3 and 6 h post-FP (Hicks et al., 1998; see also Fig. 4B).

The most pronounced increases in BDNF mRNA hybridization levels were observed in the bilateral PC at 3, 6, and 24 h post-FP injury. Conversely, trkB mRNA levels remained unchanged in these cortical regions. Similar observations have been reported following seizures, with significant increases in BDNF mRNA, and no change in trkB mRNA in the PC between 1 and 24 h postkindling (Ernfors et al., 1991; Kokaia et al., 1996a). However, other studies have not only observed increases in BDNF mRNA in the PC, but also trkB mRNA 3–4 h following seizures (Mudo et al., 1993, 1996).

The PC has been described as a selectively vulnerable region for seizures (Tanaka et al., 1996). The possibility that the increases in BNDF mRNA in the PC following FP injury are attributable to seizures cannot be completely eliminated in the present study. However, evidence against this possibility include the absence of abnormal behavior or overt seizure activity in any of the animals in this study, or in a previous study of moderate FP injury (Lowenstein et al., 1992), and a lack of bilateral neurochemical changes that would be expected with seizures (Padmaperuma et al., 1996; Prasad et al., 1994). Furthermore, increases in BDNF mRNA in the neocortex following FP injury differ from those observed following seizures. FP injury-induced changes are restricted to the superficial layers of the neocortex, whereas seizure-induced changes are observed in all neocortical layers (Gall, 1993).

Another possible explanation for the robust increase of BDNF mRNA in the PC, a region that is remote from the FP injury and associated with a role in memory and learning (Litaudon et al., 1997), may be its relatively high density of glutamate receptors (Sato et al., 1995). Within the hippocampus, another region with high levels of glutamate receptors (Sato et al., 1995), the CA3 pyramidal cell layer and the granule cell layer of the dentate gyrus also undergo marked increases in BDNF and trkB expression following FP injury (Hicks et al., 1997, 1998). Previous studies have demonstrated that basal levels of BDNF are regulated by *N*-methyl-D-aspartate (NMDA) receptor activation, whereas alterations in response to acute injuries are regulated by non-NMDA receptors (Lindholm et al., 1994; Lindvall et al., 1992; Wetmore et al., 1994). Glutamate increases after FP injury (Faden et al., 1989; Katayama et al., 1990) and may thus mediate the increases in BDNF mRNA expression in the PC. Following ischemic injury in the rat, BDNF mRNA also increases acutely in both the PC and hippocampus (Tsukahara et al., 1998). Inhibition of specific glutamate receptors with selective antagonists differentially modulated the expression of BDNF in these two areas following ischemic injury (Tsukahara et al., 1998). These findings suggest that the increased expression of BDNF in the PC and hippocampus following ischemia involves in-

dependent regulatory mechanisms. Whether this is also true following FP injury remains to be determined with future studies.

The results of the present study also suggest that up-regulation of BDNF/trkB mRNA within the IC does not contribute to neuronal degeneration as hybridization remained at or below control levels for all time points. These findings are consistent with those observed following ischemic injury (Kokaia et al., 1996b). However, it does not eliminate the possibility that anterograde transport of BDNF from the AC may contribute to degenerative events. Anterograde transport of BDNF in mossy fibers of the dentate gyrus following kainic acid injections is believed to exacerbate the neuronal degeneration in the CA3 pyramidal cells of the hippocampus (Rudge et al., 1998). This appears unlikely following FP injury, though, because whereas corticocortical connections are prevalent between neurons in adjacent cortical regions in layers II and III (Kolb, 1990), neuronal degeneration is observed fairly uniformly throughout all layers (Hicks et al., 1996).

However, the cortical afferent projections from the IC may be related to the transient increase in BDNF mRNA that is observed at 3 h postinjury in the AC, because the increase was localized to cells in the superficial but not the deep layers of this region. Alternatively, because non-NMDA receptors are more prevalent in the superficial than deep layers of the neocortex (Huntley et al., 1994), the differential response between layers may be attributable to post-FP injury elevations in glutamate. Similar increases in BDNF hybridization were not observed in the OC. Thus, while adjacent and opposite cortical regions have been observed to undergo cortical reorganization following various injuries and are hypothesized to play a role in recovery of function (Barneoud et al., 1991; Castro-Almancos and Borrel, 1995; Dietrich et al., 1987; Dunn-Meynell and Levin, 1995), we did not observe sustained alterations in BDNF or trkB mRNA in either of these cortical regions, at least during the initial (72 h) period following FP injury.

In summary, lateral FP injury produces alterations in BDNF and trkB mRNA levels in several regions of the cerebral cortex. The largest increase in BDNF mRNA following FP injury was observed in the bilateral PC, regions relatively remote from the impact and injury sites. The functional significance of the increase in the bilateral PC remains to be determined. The IC, the site of the contusion, demonstrates normal or below normal levels for both BDNF and trkB mRNA throughout the acute period following injury, suggesting that up-regulation and enhanced activation of BDNF/trkB signal transduction pathways are not contributing to the neuropathology. However, it is possible that the injury-induced loss in expression of BDNF and trkB may confer vulnerability to neurons within the cortical contusion. Other neocortical regions either showed no significant alterations, or changes of very short duration, suggesting that injury-induced regulation of BDNF/trkB pathways is somewhat unlikely to have a significant influence on neuronal survival, at least during the acute periods following FP injury. Future studies are needed to examine the effects of manipulating BDNF/trkB signal transduction activity on neuroprotection following lateral FP brain injury.

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Mild Experimental Brain Injury Differentially Alters the Expression of Neurotrophin and Neurotrophin Receptor mRNAs in the Hippocampus

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The molecular events responsible for impairments in cognition following mild traumatic brain injury are poorly understood. Neurotrophins, such as brain-derived neurotrophic factor (BDNF), have been identified as having a role in learning and memory. We have previously demonstrated that following experimental brain trauma of moderate severity (2.0–2.1 atm), mRNA levels of BDNF and its high-affinity receptor, trkB, are increased bilaterally in the hippocampus for several hours, whereas NT-3 mRNA expression is decreased. In the present study, we used *in situ* hybridization to compare BDNF, trkB, NT-3, and trkC mRNA expression in rat hippocampus at 3 or 6 h after a lateral fluid percussion brain injury (FPI) of mild severity (1.0 atm) to sham-injured controls at equivalent time points. Mild FPI induced significant increases in hybridization levels for BDNF and trkB mRNAs, and a decrease in NT-3 mRNA in the hippocampus. However, in contrast to the bilateral effects of moderate experimental brain injury, the present changes with mild injury were restricted to the injured side. These findings demonstrate that even a mild traumatic brain injury differentially alters neurotrophin and neurotrophin receptor levels in the hippocampus. Such alterations may have important implications for neural plasticity and recovery of function in people who sustain a mild head injury. © 1999 Academic Press

Key Words: **BDNF; NT-3; trkB; trkC; hippocampus; traumatic brain injury.**

INTRODUCTION

Clinically, the vast majority of head injuries (75–90%) are classified as mild (46, 49), because posttraumatic amnesia is present for less than 24 h (37). Despite the rather benign acute symptoms, 50% of the individuals who sustain a mild head injury demonstrate residual impairments 1 year later (86). These impairments include cognitive deficits (66), emotional disturbances (65), and abnormal EEG recordings (86). Relatively little is known about the neuropathological consequences of mild head injury and how they might contribute to acute and chronic impairments in function.

Neurotrophins are a family of structurally related polypeptides that have been shown to play a critical role during neuronal development and appear to mediate a protective response in mature animals (26, 54). Members of the neurotrophin family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin 4/5 (NT-4/5), neurotrophin 6 (NT-6), and neurotrophin-7 (NT-7) (4, 5, 24, 80). Neurotrophins are believed to promote their cell survival, growth, and differentiation effects through interactions primarily with specific high-affinity tyrosine kinase (trk) receptors and subsequent activation of intracellular signal transduction pathways (4, 58, 78).

Numerous investigations are currently underway to elaborate the specific actions of the various neurotrophins and how they may promote neuronal survival or functional recovery after brain injury. In brief, NGF/trkA interactions are believed to be important for cell survival during development and following injury (for review, see 54) and to promote sprouting and synaptogenesis of cholinergic neurons (13, 82). In an animal model of traumatic brain injury (TBI), infusion of NGF into the injured cortex improved cognitive function without reducing the size of the lesion, although cell death in the septum was attenuated (76). BDNF is the most abundantly expressed neurotrophin in the mature central nervous system (32) and supports the survival of many types of neurons (51). BDNF/trkB interactions appear to be neuroprotective following various forms of brain injury (6, 11, 25, 41, 47, 70, 77), although this is controversial as a few studies have implicated BDNF as a contributing factor to neuronal degeneration (42, 69). BDNF also appears to be important for learning and memory, including roles in long-term potentiation (15, 18, 45), dendridogenesis (55), and activity-dependent neuroplasticity (21, 68). NT-3/trkC signal transduction has also been found to have neuroprotective effects in some models of neuronal injury (23, 51) and has recently been found to promote axon regeneration after spinal cord injury (84). Less is

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known about the other members of the neurotrophin family, but they may also have a role in neuroprotection following various CNS disorders (51, 54).

The expression of BDNF, trkB, and NT-3 in the hippocampus is altered following experimental traumatic brain injury of *moderate* severity (27, 30, 87). However, much less is known about modulation of neurotrophin and neurotrophin receptor expression following traumatic brain injury of *mild* severity. The purpose of the present study was to examine mRNA levels of BDNF, NT-3, trkB, and trkC in the hippocampus following mild experimental brain injury.

MATERIAL AND METHODS

Surgical Procedures

Mild lateral fluid percussion injury (FPI) or sham-injury was produced in male Sprague–Dawley rats (n = 14, 300–350g) as previously described (28, 57). The rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.) 10 min after receiving 0.15 ml of atropine (0.4 mg/ml, i.m.). The head was rigidly fixed in a stereotaxic frame while the scalp and temporal muscles were reflected, and a 5.0 mm craniotomy was made with a hand-held trephine over the parieto-occipital cortex. The craniotomy was centered between bregma and lambda, 3 mm lateral to the sagittal suture. A Luer-Loc hub was fixed to the craniotomy with dental acrylic and filled with sterile saline. One hour after the animals were injected with sodium pentobarbital, a fluid percussion device (Department of Biomedical Engineering, Medical College of Virginia) was used to induce a lateral FPI of mild severity (1.0 atm). At the time of injury, the animals were fully anesthetized, as indicated by absence of a corneal reflex and toe-pinch withdrawal response, but their breathing was deeper and more regular than at earlier postanesthesia time points. Control animals received a sham injury, which consisted of anesthesia, a craniotomy, and attachment of the hub with dental acrylic. The sham injury procedure was identical to the surgical preparation for the FPI except that the animals were not subjected to the injury. Following FPI, rats were allowed to survive 3 or 6 h before euthanasia. These time points were selected because following moderate FPI, pronounced alterations in BDNF, trkB, and NT-3 gene expression occurred at 3 and 6 h postinjury (27, 30).

Tissue Processing and in Situ Hybridization

Following deep anesthesia with an overdose of sodium pentobarbital, the animals were decapitated and the brains rapidly removed and frozen over dry ice. Coronal brain sections (10 μm) were cut in a cryostat, thaw-mounted onto Superfrost Plus (Fisher Scientific) glass slides, and stored at −20°C until processing for hybridization. Adjacent sections through the hippocampal formation were processed for the detection of BDNF, NT-3, trkB, and trkC mRNAs by using *in situ* hybridization with ^{35}S-labeled cRNA probes as described in detail previously (27, 63, 64, 71, 72). Briefly, following pretreatment consisting of fixation in 4% paraformaldehyde, rinses in 0.1 M phosphate buffer, acetylation, dehydration, and delipidation, the sections were hybridized for 18–24 h at 60°C in hybridization cocktail. The hybridization solution consisted of 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 0.15 mg/ml yeast tRNA, 0.33 mg/ml denatured salmon sperm DNA, 40 mM dithiothreitol, 1 mM EDTA, 20 mM Tris–HCl, and the ^{35}S-labeled cRNA probe at a concentration of 1.0×10^6 cpm/50 μl/slide. The sense and antisense cRNA probes were prepared by *in vitro* transcription using appropriate linearized DNA constructs in the presence of the proper RNA polymerase (T3 or T7) and ^{35}S-UTP (New England Nuclear). The cDNA constructs for BDNF and NT-3 (kindly provided by J. Lauterborn and C. Gall, University of California-Irvine) resulted in antisense RNA transcripts of 550 and 540 bases, respectively (36, 50). The cDNA constructs for trkB and trkC (plasmids kindly provided by D. McKinnon, State University of New York at Stony Brook) resulted in antisense cRNAs that were 196 and 374 bases in length, respectively. The trkB cRNA probe detects the kinase-specific, full-length catalytic form of the receptor mRNA (22, 60), whereas the trkC cRNA probe recognizes transcripts for both the full-length catalytic and kinase domain insertion/deletion noncatalytic forms of the receptor (2, 14, 83). After posthybridization ribonuclease treatment and washes, film autoradiograms were generated by exposure of the sections to β-Max Hyperfilm (Amersham) for 10 (trkB and trkC), 14 (BDNF), or 21 (NT-3) days. Following film development, the sections were dipped in NTB2 nuclear track emulsion (Kodak; 1:1 in H_2O), air-dried, and exposed in light-tight boxes at 4°C for 6–10 weeks. The emulsion was developed in D19 (Kodak) and fixed with Rapidfix (Kodak). The slides were then counterstained with cresyl violet (Sigma) and coverslipped with DPX mountant (Fluka). The emulsion-dipped slides were analyzed under both brightfield and darkfield conditions using a Nikon Optiphot-2 microscope. In control procedures, pre-hybridization treatment of tissue with ribonuclease A or use of sense-strand riboprobes resulted in no specific hybridization signal.

Quantitative Image Analysis

Film autoradiograms were digitized and analyzed with image processing software (Image 1.60, NIH). An average of 5, and at least 4, sections were analyzed per animal to compare the density of hybridization for BDNF, NT-3, trkB, and trkC mRNAs in the hippocampus between the FPI and sham groups. Sections were

spaced 150 μm apart and analyzed in the dorsal hippocampus (approximately −3.14 to −4.3 mm posterior to bregma). Optical densities (O.D.) of BDNF, trkB, and trkC hybridization signal were measured by manually outlining the stratum granulosum of the dentate gyrus and the strata pyramidale of subfields CA1 and CA3 for the left (ipsilateral to the impact site) and right (contralateral) sides of the brain for the FPI group. Right and left side data for the sham animals were combined. NT-3 cRNA hybridization was only measured in the dentate gyrus, because it is not expressed in the CA1 or CA3 regions (17). Background O.D. measurements were taken in adjacent white matter of the corpus callosum and subtracted from the O.D. measurements in the hippocampus in order to obtain corrected values. All measurements are expressed as the mean values plus or minus the standard error of the mean (SEM). The effects of group (FPI-ipsilateral, FPI-contralateral, and sham-bilateral), survival time (3 and 6 h), and their interaction effects were analyzed for each hippocampal subfield with a two-way analysis of variance (ANOVA). Bonferonni post-hoc analyses were used for pairwise comparisons with a significance set at $P < 0.05$.

RESULTS

Hybridization patterns and levels for BDNF, NT-3, trkB, and trkC mRNAs in the sham animals were similar to previous reports in normal, uninjured rats (3, 17, 20, 36, 59). There were also no differences in the mRNA levels in the corpus callosum (background measurements) between the FPI and sham-injured animals (data not shown).

BDNF mRNA

Increases in hybridization for BDNF mRNA were evident in the dentate gyrus and CA3 regions of the hippocampus on the ipsilateral side of the brain following mild FPI compared to the contralateral side or the sham animals (Figs. 1A, 1B, and 2). Statistical analysis revealed no time-dependent changes, but there were significant differences across groups. At both 3 and 6 h postinjury, O.D. measurements of BDNF mRNA in the ipsilateral dentate gyrus were significantly greater than on the contralateral side or in sham animals ($P < 0.001$) (Fig. 3). Increases were also observed in the CA1 and CA3 regions on the ipsilateral side of the brain compared to sham animals (Figs. 1A and 1B), but these increases did not reach statistical significance unless the 3- and 6 h data was pooled. When the 3- and 6-h data were combined, hybridization in the CA3 region was greater on the ipsilateral than on the contralateral side after FPI and greater than sham animals ($P < 0.01$) (Fig. 4). Combining the data also revealed small but significant differences between mRNA levels in the CA1 region on the ipsilateral side following FPI compared to sham injured animals ($P < 0.05$) (Fig. 4).

trkB mRNA

The mild FPI induced marked increases in trkB hybridization levels in the dentate gyrus on the ipsilateral side of the brain compared to the contralateral side or sham animals (Figs. 1C and 1D). Alterations were not observed in the CA1 and CA3 regions (Figs. 1C and 1D). Statistical analysis confirmed that there was a significant difference in trkB mRNA levels in the dentate gyrus on the side ipsilateral to the injury compared to the contralateral side or to levels in sham animals at 3 h ($P < 0.001$) and at 6 h ($P < 0.005$) (Fig. 5).

NT-3 mRNA

Hybridization for NT-3 mRNA was visibly decreased following mild FPI in the ipsilateral dentate gyrus compared to the contralateral side or sham animals (Figs. 1E and 1F). Statistical analysis revealed that there were time and group differences and a significant interaction between them. At 6 h postinjury, NT-3 mRNA levels were significantly reduced in the dentate gyrus on the ipsilateral side compared to both the contralateral side ($P < 0.001$) and to sham levels ($P < 0.001$) (Fig. 6). In addition, NT-3 hybridization levels in the ipsilateral dentate gyrus were significantly lower at 6 than at 3 h after FPI ($P < 0.05$) (Fig. 6).

trkC mRNA

No differences were revealed either in the film autoradiograms (Figs. 1G and 1H) or by statistical analysis of densitometric measurements (data not shown) in trkC mRNA levels between FPI and sham animals at either 3 or 6 h postinjury.

DISCUSSION

The major finding of this study is that even mild head injury induces alterations in neurotrophin gene expression in the hippocampus. BDNF mRNA expression was markedly increased in the granule cells of the dentate gyrus and moderately increased in the CA1 and CA3 pyramidal cell layers of the hippocampus. Conversely, NT-3 mRNA was significantly decreased in the dentate gyrus. Mild FPI also increased trkB mRNA levels in the dentate gyrus, but did not alter trkC mRNA levels. The overall timing, subregional localization, and direction of the acute alterations in neurotrophin and trk mRNA levels are in general agreement with those observed after a moderate FPI (27, 30), except for one major difference. Unlike the bilateral changes in neurotrophin gene expression observed in the hippocampus

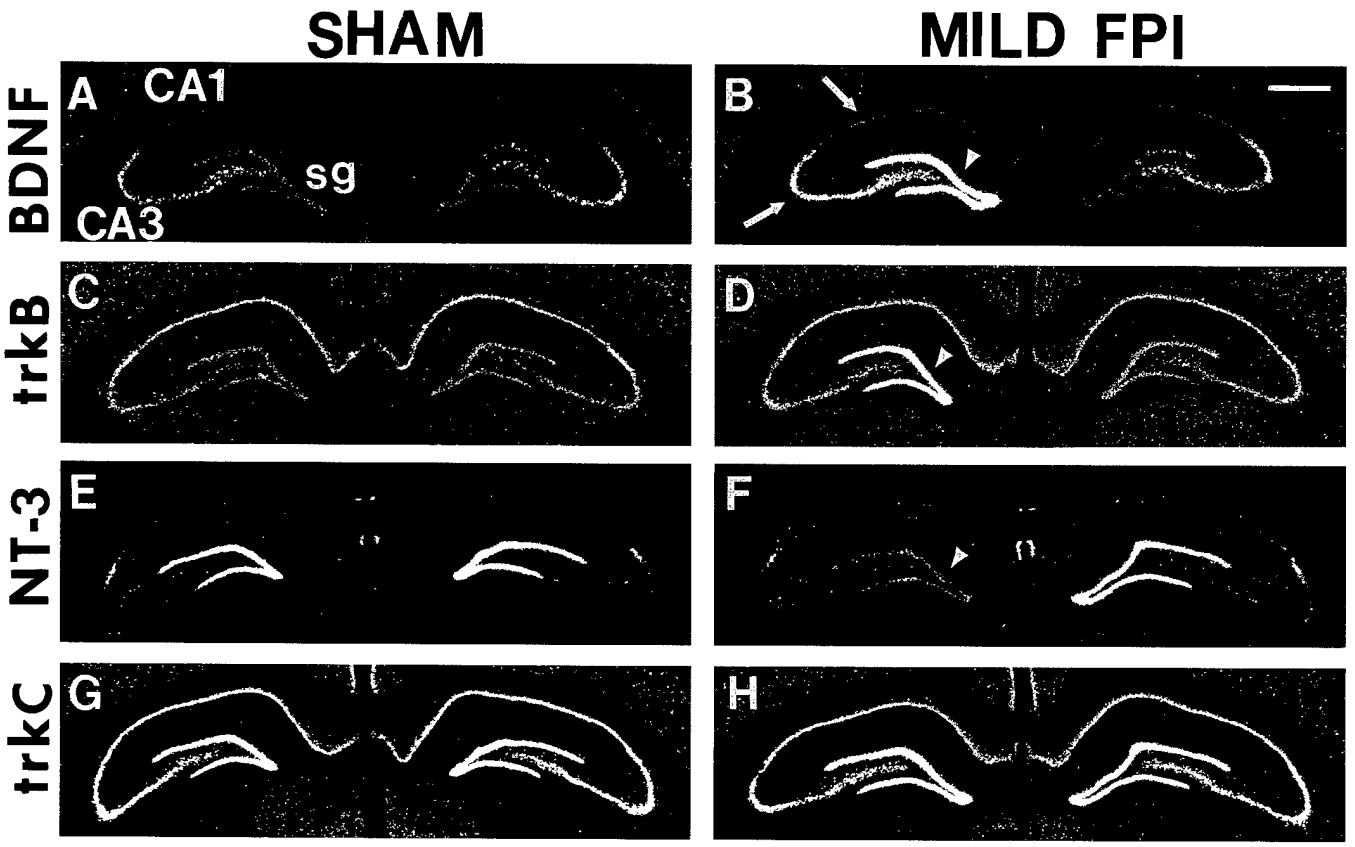

FIG. 1. Film autoradiograms showing expression of BDNF (A, B), trkB (C, D), NT-3 (E, F), and trkC (G, H) mRNAs in coronal sections through the hippocampus in control (sham injury) rats (A, C, E, G) and in rats subjected to mild FPI (B, D, F, H). Survival periods shown were either 3 h (A–D) or 6 h (E–H) following FPI or sham injury. Following the mild injury, hybridization for BDNF mRNA is dramatically increased in the dentate gyrus stratum granulosum (sg) (arrowhead, B) and less so in hippocampal CA3 and CA1 regions (arrows, B) ipsilateral to the injury. Similarly, expression of trkB mRNA is also elevated in the sg ipsilateral to the mild FP injury (arrowhead, D). In contrast, NT-3 mRNA levels are unilaterally decreased in the dentate gyrus sg ipsilateral to the mild injury (arrowhead, F). Hybridization for trkC mRNA is unaltered in the hippocampal formation after mild FP brain injury (H). Note that all changes in expression are restricted to the side ipsilateral to the unilateral FPI. Scale bar, 1000 μm.

after an injury of moderate severity (27, 30), the alterations following a mild injury were restricted to the side of the brain ipsilateral to the impact site. These findings demonstrate that there is a clearly graded response to sham, mild, and moderate FPI and suggest that some sort of threshold for regulating neurotrophin gene expression may exist.

This pattern, unilateral response following mild FPI and bilateral response following moderate FPI, was also characteristic of alterations in the immediate early genes c-fos, c-jun, and junB (67). The present changes, however, are probably not directly related to the activation of the above transcription factors because, to our knowledge, the BDNF, NT-3, and trkB genes do not contain AP-1 binding sites. Since BDNF promoters are regulated by CREB (74), it would be interesting to examine the levels of CREB phosphorylation in the hippocampus in the present head injury paradigm. It has also been suggested that up-regulation of BDNF mRNA following various brain injuries, including trauma, may be caused by spreading depression (40, 44, 79). Although this is possible, it appears unlikely because we did not see evidence of seizure activity after FPI, and sodium pentobarbital anesthesia is associated with decreased cortical activity (85). In addition, spreading depression was not observed following a mild injury with the cortical contusion model of experimental brain injury (62).

The coincident increases in mRNA for BDNF and its high-affinity receptor, trkB, in the dentate gyrus suggest that mild FPI may lead to activation of the BDNF/trkB signal transduction pathways. Further studies examining alterations in protein levels after mild FPI are required to confirm this, but in our laboratory, increases of a similar magnitude in BDNF mRNA in the hippocampus following a moderate FPI were associated with significant increases in BDNF protein levels (unpublished data). The functional consequences of these alterations in neurotrophin gene expression following injury are unknown, but intriguing

because of their putative roles in neuroprotection (6, 11, 25, 41, 47, 70, 77) and learning and memory (1, 35, 38, 39). In the CA1 and CA3 regions of the hippocampus, alterations in BDNF and trkB mRNA levels did not covary. BDNF, but not trkB, mRNA levels were significantly increased in the CA1 and CA3 regions, although these alterations were not as robust as in the dentate gyrus. Similarly, the increases in mRNA levels in the dentate gyrus were more pronounced for BDNF than for trkB. Thus, it is possible that the regulatory mechanisms that resulted in these smaller increases in BDNF mRNA were not of a sufficient magnitude to induce alterations in trkB mRNA levels in the CA1 and CA3 regions. Alternatively, the differential response may be related to differences in basal levels of the full-length isoform of trkB, which are lower in the CA3 region than the dentate gyrus in normal adult rats (81).

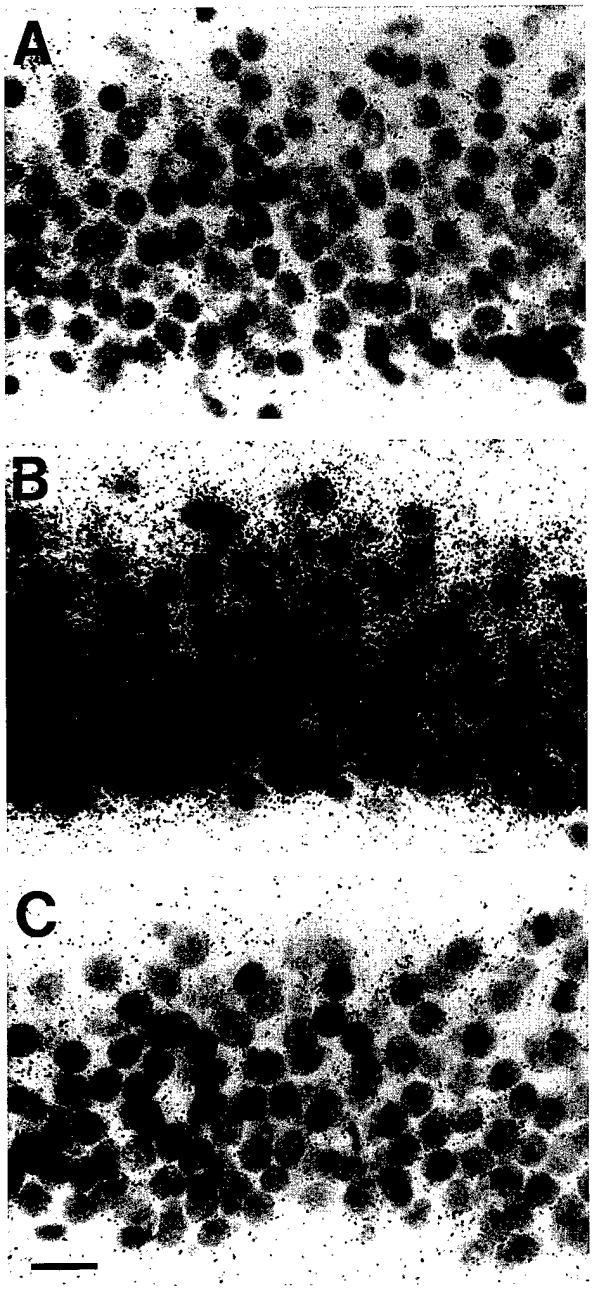

FIG. 2. High-power brightfield photomicrographs showing autoradiographic labeling for BDNF mRNA in the dentate gyrus stratum granulosum in a control (3-h sham injury) rat ipsilateral to sham surgery (A) and in a rat subjected to mild unilateral FPI with a 3-h survival time on the side ipsilateral to injury (B) and on the side contralateral to injury (C). Note that the substantial increase in hybridization density over the granule cells following mild FPI is limited to the side ipsilateral to the injury (B). Scale bar, 20 μm.

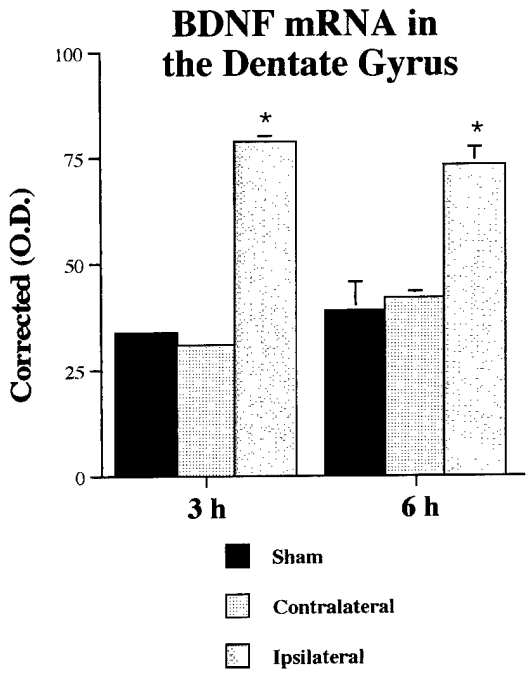

FIG. 3. Alterations in BDNF mRNA levels in the dentate gyrus following mild lateral FPI. Corrected O.D. measurements of hybridization for BDNF mRNA demonstrated a marked increase in the ipsilateral dentate gyrus granule cell layer ($^*P < 0.001$) compared to the contralateral side or to sham injury. These differences were observed at both 3 and 6 h after mild FPI. Values represent mean ± SEM.

Although numerous studies have demonstrated neuroprotective effects of BDNF in rescuing injured neurons (6, 11, 25, 41, 47, 70, 77), other studies suggest that it may potentiate neurodegeneration (7, 42). The reasons for these different findings are unclear, but the type of cell death (apoptotic or necrotic), the location where BDNF is mediating its effects (autocrine/paracrine or anterograde transport), and the timing of the alterations in gene expression (immediate or delayed) may be important factors (7). After FPI, granule cells in the dentate gyrus are the most resistant to cell death, and CA3 pyramidal cells are the most vulnerable (12, 29). The most pronounced increases in BDNF mRNA are in the dentate gyrus, thus these alterations

may be promoting cell survival in this region. However, increases were also observed in the CA3 region, the most vulnerable region. If BDNF is transported in an anterograde manner in the mossy fibers after FPI, it may lead to further increases of this neurotrophin in the CA3 region and exacerbate the neuronal injury. For example, anterograde transport of BDNF in the mossy fibers is associated with neurodegeneration in the CA3 region following excitotoxic insults (69). Future studies utilizing exogenous administration of neurotrophins or substances that inhibit signal transduction are necessary to clarify the role of BDNF/trkB after FPI.

In contrast to BDNF and trkB, NT-3 mRNA decreased and trkC was unchanged in the dentate gyrus after mild FPI, suggesting that the NT-3/trkC signal transduction pathway may be inactivated. These opposing patterns of BDNF and NT-3 expression have also been observed after a moderate lateral FPI (27), ischemia (52), and seizures (21), as well as during development (55, 56). It is possible that inactivation of NT-3/trkC signal transduction pathways after mild FPI confers vulnerability on neurons, as increases in NT-3 protein levels were associated with neuroprotection and regeneration in other injury models (23, 51, 61, 84). Alternatively, there is also evidence from a study utilizing NT-3 knock-out mice to suggest that inactivation of this pathway may inhibit seizures (16) and attenuate secondary pathological events.

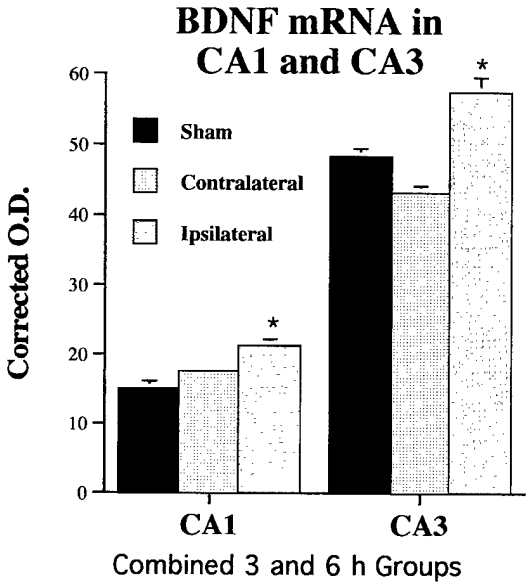

FIG. 4. Regional alterations in BDNF mRNA levels following mild lateral FPI. Slight, but significant increases in the ipsilateral CA1 region of the hippocampus were present compared to levels in sham injured animals ($^*P < 0.05$) when the 3- and 6-h data were combined. There were also group, but not time differences in the CA3 region with a significant increase on the ipsilateral side compared to contralateral or sham levels ($^*P < 0.01$). Values represent mean ± SEM.

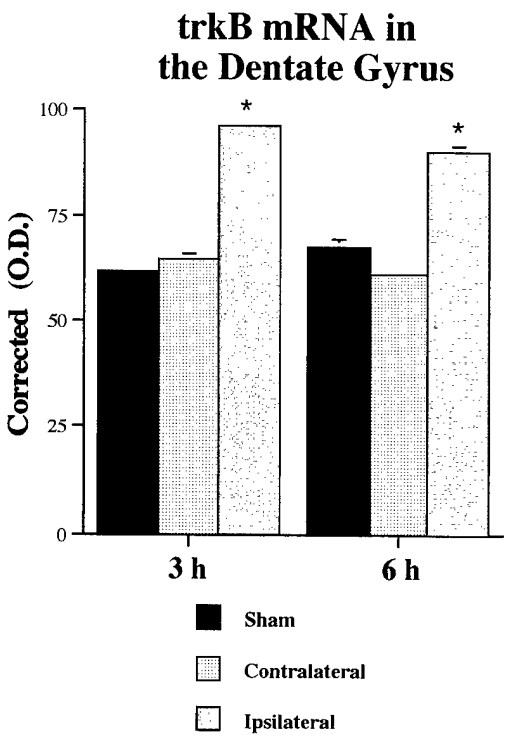

FIG. 5. Alterations in trkB mRNA levels in the dentate gyrus following mild lateral FPI. Corrected O.D. measurements of hybridization for trkB mRNA demonstrated a significant increase in the ipsilateral dentate gyrus granule cell layer compared to the contralateral side or to sham injury at both 3 ($^*P < 0.001$) and 6 h ($^*P < 0.005$) postinjury. There were no significant differences in the CA1 and CA3 regions of the hippocampus. Values represent mean ± SEM.

Posttraumatic amnesia is a common feature during the acute stages following a mild head injury and is associated with impairments in learning and memory (48, 86). These impairments after mild injury have been observed both in clinical cases (48, 86), as well as in rodent models of TBI (28, 33, 53, 73). The underlying cause of the cognitive dysfunction is unclear, but physiological consequences of a mild FPI include an impairment in long-term potentiation (LTP) in the CA1 region of the hippocampus (75). BDNF and NT-3 have both been linked with development of LTP in the hippocampus and visual cortex (1, 18, 35, 38, 39). Since mild FPI alters neurotrophin mRNA levels in the hippocampus, it is possible that these alterations influence the excitability of neurons. The up-regulation of BDNF in the hippocampus following a mild injury would appear to support the generation of LTP, rather than to attenuate it (1, 18, 35). However, there is evidence to suggest that the down-regulation of NT-3 mRNA in the dentate gyrus may influence neural plasticity of hippocampal circuits. A study undertaken in NT-3 knock-out mice demonstrated that short-term facilitation, but not LTP, was reduced in the hippocampus compared to controls (43). Whether the decrease in NT-3 mRNA in the

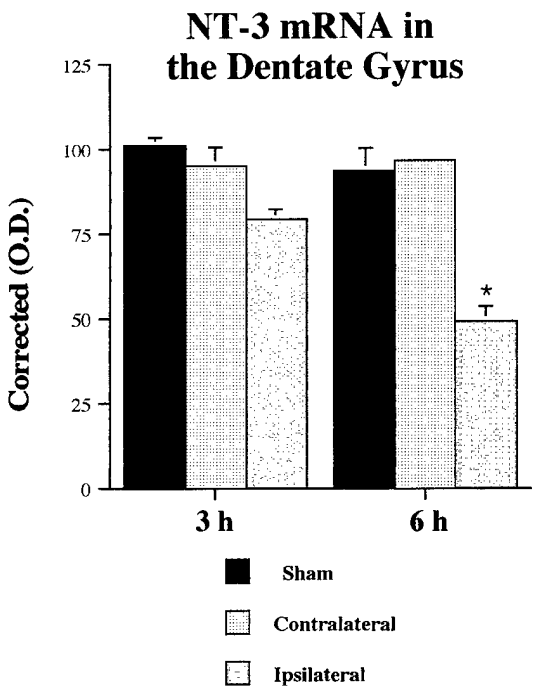

FIG. 6. Alterations in NT-3 mRNA levels in the hippocampal dentate gyrus following a mild lateral FPI. Corrected O.D. measurements of hybridization for NT-3 mRNA demonstrated a significant decrease in the ipsilateral dentate gyrus granule cell layer 6 h after FPI compared to both the contralateral side and to sham injury (*$P < 0.001$). In addition, the decrease in the ipsilateral dentate gyrus was greater at 6 h than at 3 h postinjury ($P < 0.05$). Values represent mean ± SEM.

granule cells of the dentate gyrus following mild FPI is associated with impairments in short-term facilitation or LTP in the hippocampus remains to be determined.

One of the perplexing characteristics of mild head injury is that cognitive deficits often persist despite a lack of pronounced neuropathological alterations (8, 9, 10, 31). It has been suggested that less overt changes in the brain following a mild head injury may be responsible for the cognitive and neurophysiological impairments. In the present study, we only examined acute changes in neurotrophin and trk receptor gene expression after mild FPI. However, a recent paper demonstrated that administration of BDNF enhanced responses for reward-related stimuli following cocaine injection and that these enhancements persisted for 1 month after treatment (34). Therefore, it appears that even temporary changes in BDNF levels may exert long-lasting changes in behavior. Furthermore, BDNF up-regulates tissue-type plasminogen activator, which converts plasminogen to plasmin, a protease capable of degrading most extracellular proteins (19). These alterations could underlie structural changes at the synapse and mediate chronic changes in behavior.

In summary, a FPI of mild severity produces unilateral alterations in expression of BDNF, trkB, and NT-3 in the hippocampus. These alterations may underlie some of the cognitive deficits associated with mild head injury. In addition, because the alterations induced by a mild FPI are distinctly unilateral, this level of trauma may be advantageous for illuminating regulatory mechanisms and functional consequences of neurotrophin/trk signal transduction pathways, as well as other intracellular signaling pathways.

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Effects of Exercise Following Lateral Fluid Percussion Brain Injury in Rats

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Abstract

Previous studies have suggested that brain-derived neurotrophic factor (BDNF) is involved in memory and learning, and may be neuroprotective following various brain insults. Exercise has been found to increase BDNF mRNA levels in various brain regions, including specific subpopulations of hippocampal neurons. In the present study, we were interested in whether following traumatic brain injury, exercise could increase BDNF mRNA expression, attenuate neuropathology, and improve cognitive and neuromotor performance. We subjected adult male Sprague-Dawley rats to a fluid percussion brain injury, followed by either 18 days of treadmill exercise or handling. Spatial memory was evaluated in a Morris Water Maze (MWM) and motor function was evaluated with a battery of neuromotor tests. Neuropathology was evaluated by measuring the cortical lesion volume and the extent of neuronal loss in the hippocampus. Expression of BDNF mRNA in the hippocampus was assessed with *in situ* hybridization and densitometry. Hybridization signal for BDNF mRNA was significantly increased bilaterally in the exercise group in hippocampal regions CA1 and CA3 ($p < 0.05$), but not in the granule cell layer of the dentate gyrus. No significant differences were observed between the groups in neuropathology, spatial memory, or motor performance. This study suggests that after traumatic brain injury, exercise elevates BDNF mRNA in specific regions of the hippocampus.

Keywords: brain-derived neurotrophic factor, cognition, traumatic brain injury

1. Introduction

An important question facing rehabilitation specialists is how to maximize functional recovery after traumatic brain injury (TBI). A variety of functional deficits may be present after TBI, but impairments in information processing, perceptual function, and memory are the most common [27]. Numerous studies have demonstrated that exercise may be important for maintaining cognitive and memory function in humans (for review see [10]). Although the exact physiological mechanisms underlying these improvements are unknown, several molecular events in the brain that are associated with physical activity may be important.

In humans, moderate to high intensity exercise increases regional cerebral blood flow [22,47], as well as plasma levels of noradrenaline and serotonin, neurotransmitters that are thought to be associated with memory storage and retrieval [9]. In animal studies, exercise has been linked to an increase in neuronal activity in the hippocampus, as demonstrated by elevations in extracellular lactate [5]. In aged rats, exercise increases antioxidant enzymes in brain tissue [45]. Exercise prior to an ischemic injury in gerbils, lowered mortality and attenuated damage in the cortex, striatum, and hippocampus [46]. Alterations in neurotrophic factor levels have also been

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associated with exercise. Following 2–7 days of exercise on free-running wheels, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and basic fibroblast growth factor (bFGF) were increased in various regions of the hippocampus and cortex in rats [15,34,35].

BDNF is the most prevalent neurotrophin in the brain, with especially high levels in the neocortex, cerebellum and hippocampus [19,28]. Although the function of BDNF is incompletely understood, it appears to play a role in long-term potentiation (LTP) and memory formation [8,12,24], and activity-dependent neuroplasticity [13,30,39]. Housing rats in an enriched environment not only improves spatial memory [16,32] and increases synaptic density and branching in the hippocampus [23], it also up-regulates BDNF mRNA [11]. The present study tested whether exercise following a lateral fluid percussion (FP) brain injury could increase BDNF mRNA expression in the hippocampus and attenuate the neuropathology and behavioral deficits that are associated with this model of experimental brain injury in rats [17,43].

2. Materials and methods

2.1. Surgical procedures

Lateral FP brain injury was performed as previously described [31]. Briefly, male Sprague-Dawley rats (360–410 g, n = 20) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) ten minutes after receiving 0.15 ml of atropine (0.4 mg/ml), and placed in a stereotaxic frame. The scalp and temporal muscles were reflected, and a stainless-steel screw was secured to the skull 1 mm anterior to bregma. A Luer-Lok connector was rigidly fixed with dental cement to a 5 mm diameter craniotomy centered over the left parieto-occipital cortex, midway between bregma and lambda. Sixty minutes after receiving anesthesia, rats were removed from the stereotaxic frame, attached to the FP device via the connector, and given a unilateral brain injury of moderate severity (2.0–2.1 atm). Following the injury, the Luer-Lok connector was removed from the scalp and the skin was sutured. Normothermia was maintained throughout the procedure by placing the animal on a heating pad.

2.2. Treadmill training

Beginning the day after FP injury, the experimental group of rats (n = 10) was given daily incremental exercise on a treadmill during the diurnal part of the light-dark cycle. On the first day, rats were given 5 minutes of exercise, which was increased by 5 minutes per day until they were exercising for 60 minutes. The rate of the treadmill was set at 11.3 meters per minute with a belt inclination of 6°. On days 5–10 (20–45 min of exercise) rats were given one 2 min rest, and on days 11–14 (50–60 min of exercise) they were given two 2 min rests. Animals were not exercised on days that they were undergoing cognitive and neuromotor testing (days 15–18). After the completion of neuromotor testing, animals resumed exercise for 60 min without a rest period (days 18–21). Rats ran a total of 7.8 km over the course of 18 days of exercise. On the last six days of exercise, the animals reached the maximum period of 60 minutes and were running 0.68 km/day. The control group of rats (n = 9) did not exercise on the treadmill, but were handled 30–60 s daily. All of the animals were housed 2–3/cage with a 12 hr light-dark cycle. Treadmill exercise and handling procedures were given during the light part of the cycle.

2.3. Cognitive and neuromotor function

Spatial learning and memory were evaluated by using a Morris Water Maze (MWM) procedure as described previously [26]. All testing was performed by an observer blinded to each animal's treatment. The MWM is a 1.15 m diameter circular pool filled with water. All animals were trained to find a hidden platform using external visual cues. Animals were trained on post-surgery days 15–17, performing one block of four acquisition trials per day, for a total of twelve trials. The time required for each animal to find the platform (goal latency) was recorded for each trial. After completing the last acquisition trial, each rat underwent a single probe test to assess their spatial memory. The platform was removed and the swim pattern of each animal was videotaped for 90 seconds. The distance, time, and number of visits to the previous platform location as well as visits to similar regions in the other quadrants were computed by a video motion analyzer (Videomex V, Columbus Instruments). Relative target visits were calculated by dividing visits to the platform location by the sum of the visits to all zones.

The day after the MWM test (day 18), animals underwent a battery of tests for neurologic motor function that were adapted from previously published reports. All testing was performed by two independent observers that were blinded to each animal's treatment. The motor tests used were the inclined plane test for the right and left side [38], visual limb placing [6], vertical righting response [29], and the grip test [1]. The inclined plane test measured the animal's ability to maintain its body position on an inclined board for 5 seconds. Animals were placed sideways on a board, which is covered with a rubber mat and inclined to a 45° angle. Animals received a score of 2 (able to maintain body position for ≥ 5 sec), 1 (able to maintain position for 1–4 sec), or 0 (unable to maintain position). Rats were tested for both sides of the body. For visual limb placing, a rat was held 10 cm above a table top and slowly lowered toward it with free hanging forelimbs. Normal rats reach, stretch and place both forepaws on the table top. Animals received a score of 0 (no placing, limb flexion), 1 (incomplete and/or delayed), or 2 (immediate and complete placing). The righting response records the amount of time it takes within a 60 second interval for rats that are placed face down on a vertically oriented wire grid, to assume a head up position. For the grip test, rats were suspended from a wooden dowel (1 cm diameter) that is positioned 40 cm above a foam mat. The length of time the animals held on to the wire within a 30 second interval was measured. Mean scores or latencies for each test were calculated by averaging the values assigned to each rat by the two testers.

2.4. Weight loss

Weight loss or gain was calculated by subtracting the animals weight on day 1 (prior to the FP injury) from the weight on day 18.

2.5. In situ hybridization

Animals were deeply anesthetized with an overdose of sodium pentobarbital and decapitated 3 weeks after the FP injury. Brains from 6 randomly selected rats from each group were rapidly removed and frozen over dry ice. Tissue sections through the hippocampus were cut in the coronal plane at 14 μm in a cryostat, thaw-mounted onto Superfrost Plus (Fisher Scientific) glass slides, and stored at –20 °C until processing for the *in situ* hybridization localization of BDNF mRNA as previously described [41,42]. The BDNF cRNA probe was prepared by in vitro transcription from a linearized cDNA construct with T3 polymerase in the presence of ^{35}S-UTP. The 540-base rat BDNF probe includes 384 base pairs complementary to the rat BDNF mRNA coding region [14,20]. Hybridization was conducted at 60 °C for 18–24 h with the ^{35}S-labeled cRNA at a concentration of $1x10^6$ cpm/50 μl/slide. Following post-hybridization washes and ribonuclease treatment, the sections were air-dried and exposed to β-Max Hyperfilm (Amersham) for 15 days at room temperature for generation of film autoradiograms. After autoradiographic film development, the sections were dipped in NTB2 nuclear track emulsion (Kodak; 1:1 in H_2O), air-dried and exposed in light-tight slide boxes at 4 °C for 4–6 weeks. After autoradiographic development of the emulsion, the sections were coverslipped in D.P.X. (Fluka) and analyzed with a Nikon Optiphot-2 microscope equipped with brightfield and darkfield optics. Control sections that had been treated with ribonuclease A before hybridization or processed with an appropriate sense-strand riboprobe (see [14]) were devoid of specific labeling. Film autoradiograms were analyzed with Image 1.57 software (NIH) to compare the density of hybridization for BDNF mRNA in various hippocampal subfields (dentate gyrus, CA1 and CA3) between the exercise and control groups after FP injury. Background hybridization in the corpus callosum was subtracted from the hybridization in the hippocampal subfields to obtain corrected optical density measurements (OD). At least three sections taken from the dorsal hippocampus between bregma –2.80 to –4.30 [37] were analyzed per animal.

2.6. Histological Evaluation

Alternate sections were fixed in formalin for 10 min, taken through xylenes and graded ethanols, stained with hematoxylin and eosin, dehydrated, cleared, and coverslipped with Permount. The areas of the right and left neocortices were measured every 500 μm between bregma –2.56 and –6.04 [37] using an image processor (NIH Image, 1.57). Damaged or necrotic tissue in the neocortex was omitted from the area measurements. Neocortical volumes were calculated by summation of neocortical areas for each animal. The percentage of the tissue that was damaged by the FP injury (% lesion volume) was calculated by dividing the tissue volume on the side ipsilateral to the impact by the volume on the contralateral side and subtracting this number from 100. Previous studies have demonstrated that a moderate lateral FP injury typically damages portions of the posterior parietal, temporal, and occipital cortices [17].

Hippocampal neuropathology was assessed by scoring neuronal cell loss and injury in the CA3 region by two independent, blinded investigators. The CA3 region was selected because previous studies have demonstrated that after FP injury, hippocampal damage is most visible in this region [4,17]. Neuronal loss in the CA3 region was scored as 0 (normal), 1 (barely visible thinning of the cell layer or a few abnormal appearing cells), 2 (cell loss estimated to be less than 25 %), 3 (cell loss estimated between 25–50 %), and 4 (cell loss estimated to be greater than 50 %).

2.7. Statistical analysis

All measurements were analyzed with a statistical software package (SYSTAT, version 5.2) and expressed as means ± SEM. Percent lesion volume, weight loss, grip test, vertical righting latencies, and MWM goal latencies and relative target visits were compared with a *t*-test. Hippocampal damage and the inclined plane and limb placing scores were compared with the Mann-Whitney *U*-test. A one-way ANOVA followed by the Newman-Keuls post-hoc test was used to compare right and left neocortical volumes between groups. A two-way ANOVA was used to compare the BDNF mRNA optical density measurements by group and by side. Statistical significance was obtained with p values < 0.05.

3. Results

One animal died shortly after the FP injury, therefore 9 animals were placed in the control (handling) group. In addition, one animal from each group was euthanized 12 days after the FP injury because of severe weight loss and debilitation. In the surviving animals, mean weight loss was not significantly different between the exercise (–2.8 ± 3.6 g) and control animals (–6.9 ± 4.8 g).

Treadmill exercise following FP injury increased the hybridization density of BDNF mRNA in the hippocampus compared to injured animals that were not exercised (Fig. 1). *In situ* hybridization for BDNF mRNA showed a clear pattern of hybridization in the hippocampal pyramidal cell layers and the granule cell layer of the dentate gyrus, with emulsion grains closely associated with the cell bodies in these layers. No differences were observed between O.D. values for the right and left sides of the hippocampus, so the data were combined. Comparison of group mean corrected O.D. values revealed that significant increases in BDNF mRNA were present in the CA1 ($F_1 = 7.78$, $p < 0.02$) and CA3 ($F_1 = 5.11$, $p < 0.05$) pyramidal cell layers in the exercise group (Fig. 2). An small increase was also observed in the granule cell layer of the dentate gyrus, but this did not reach statistical significance.

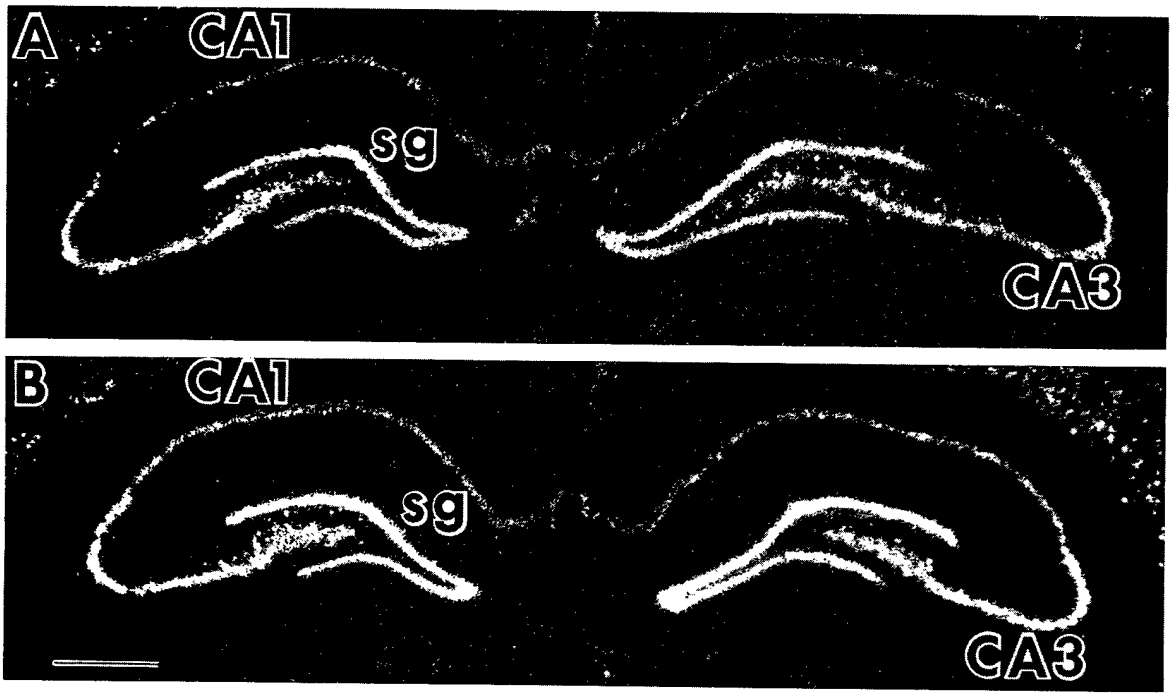

Fig. 1. Prints of autoradiograms showing the localization of hybridization for BDNF mRNA in hippocampi from (A) an FPI-control (no exercise after lateral fluid percussion injury) and (B) an FPI-exercise rat (18 days of exercise after lateral fluid percussion injury). Note the bilateral increase in BDNF mRNA hybridization in the hippocampal CA1 and CA3 regions in B; the apparent increase in expression seen in the dentate gyrus granule cell layer (stratum granulosum; sg) did not reach statistical significance (see quantification in Fig. 2). Scale bar = 1000 μm.

Histological evaluation revealed that there were no significant differences in the scores for hippocampal damage between the exercise and control groups (Table 1). Nor was the cortical lesion volume significantly different between the exercise and control groups (Table 1). However, there was a trend toward attenuation of damage in the exercise group, in that significant asymmetries between the left (injured) and right neocortical volumes were observed in the control group, but not in the exercise group ($F_3 = 5.43$, $p < 0.01$, Table 1).

Analysis of MWM performance (Table 1) revealed that there were no significant differences in goal latencies or relative target visits between the exercise and control injured animals, although both were significantly impaired compared to uninjured (sham) animals (unpublished data). Neuromotor scores and timed tests after FP injury were also unaffected by exercise (Table 1).

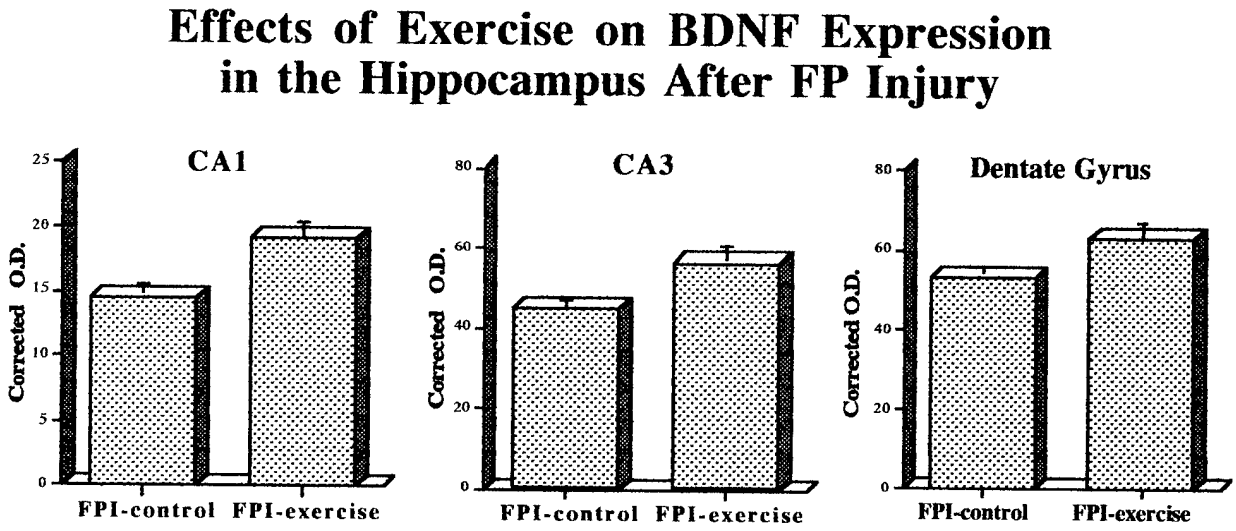

Fig. 2: Corrected optical density (O.D.) ± SEM measurements of BDNF hybridization in various regions of the hippocampus 21 days following a FP injury. Statistically significant increases were observed in the CA1 and CA3 regions in animals that received exercise after injury (FPI-exercise) compared to those that were not exercised (FPI-control).

TABLE I. Summary of histological and behavioral data

	Control	*Treadmill*
	Mean ± SEM	*Mean ± SEM*
Hippcampal cell loss (score)	1.4 ± 0.5	1.3 ± 0.3
Cortical lesion volume (%)	29.6 ± 7.8	17.4 ± 4.5
Left neocortical volume (mm^3)	45.7 ± 5.8*	54.0 ± 2.2
Right neocortical volume (mm^3)	64.1 ± 2.7	63.4 ± 3.2
Goal latency (s)	22.2 ± 5.5	21.6 ± 7.3
Relatively target visits (%)	30.2 ± 3.0	34.8 ± 8.1
Inclined plane – left (score)	1.3 ± 0.2	1.3 ± 0.2
Inclined plane – right (score)	1.9 ± 0.1	1.8 ± 0.2
Limb placing (score)	1.3 ± 0.3	1.7 ± 0.2
Righting response (s)	13.4 ± 3.1	11.6 ± 4.4
Grip test (s)	7.0 ± 2.4	7.0 ± 2.3

* The volume of the left neocortex (side ipsilateral to the injury) was significantly less ($p < 0.01$) than the volume of the right neocortex in the control group, but not in the treadmill group.

1. Discussion

To our knowledge, this paper is the first investigation of the effects of an exercise program on neural plasticity, neuropathology, and behavior following experimental brain trauma. Our results demonstrate that exercise on a treadmill following FP injury significantly increases hippocampal BDNF mRNA levels compared to injured, unexercised animals, but does not attenuate histological, cognitive, or neuromotor deficits. The increase in BDNF mRNA following exercise is in agreement with a previous study conducted on normal rats [34,35]. Traumatic brain injury by itself can lead to up-regulation of BDNF mRNA in the hippocampus [18,51]. However, we believe that the increases observed in the present study are separate from those associated with FP injury because post-injury elevations in BDNF mRNA are acute, not chronic, and are found in different subregions of the hippocampus [18]. These spatial and temporal differences suggest that FP injury and exercise may involve separate pathways for the up-regulation of BDNF.

Under normal conditions, BDNF appears to be an important factor for neural plasticity and LTP [8,12,24,30,39]. BDNF has also been linked to neuroprotection following injury [2,40,48]. In the present study, the increases in BDNF mRNA were not associated with a significant attenuation of the neuropathology, however there was a trend toward improvement in the injured cortex (Table 1). This trend appears to be related to events in the damaged tissue, rather than to non-specific increases in cortical volume, because the contralateral cortex was not affected by the exercise. Also, it is important to note that the exercise did not worsen the cortical lesion, as has been reported after a forced-use paradigm following cortical ablation [25].

There are several possible explanations for why the treadmill exercise did not enhance recovery of cognitive or neuromotor function after FP injury. The role of exercise on cognitive performance is controversial, with some studies reporting positive correlations, and others negative (see [10] for review). It has been suggested that the duration and intensity of the exercise are important factors, and that low levels of physical activity are ineffective in improving cognitive performance. The graduated exercise program in the present study may not have been intense or long enough to produce effects.

Another possible explanation is that forced, diurnal treadmill training may have induced a stress response [50]. Exposure to stress is associated with impaired spatial memory and neuronal damage in the hippocampus [49]. Stress also significantly decreases BDNF mRNA levels in the hippocampus [44,49]. Thus, while exercise is generally associated with attenuation of the stress response [7], the conditions in which we exercised our rats may have actually contributed to it and interfered with beneficial effects of exercise on memory and learning. A stress response may also have attenuated the increase in BDNF mRNA that we observed in the hippocampus.

It has also been suggested that activities that require motor learning are better than repetitive exercise at enhancing cognitive and neuromotor performance [3]. Exposure to an enriched environment provides animals with an opportunity for motor learning as they explore novel objects placed in their cages. Animals placed in an enriched environment after a midline FP injury had improved spatial memory [16]. After an ischemic injury, rats placed in an enriched social environment or just a social environment (no access to exercise equipment) outperformed isolated rats with access to a free-running wheel on neuromotor tasks [21].

In conclusion, this study demonstrates that even animals that have undergone experimental brain trauma are able to increase neurotrophin levels in the brain in response to exercise. Despite these molecular events, the treadmill exercise program used to rehabilitate the rats after FP injury was not associated with an improvement in cognitive or neuromotor function. Whether the lack of behavioral effects are attributable to specific features of the exercise paradigm employed in this study or to exercise in general should be investigated in future studies.

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