Expression of the Prodynorphin Gene after Experimental Brain Injury and Its Role in Behavioral Dysfunction

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Traumatic brain injury (TBI) causes excess release of neurotransmitters, such as glutamate, and increases intracellular calcium levels. Elevated levels of calcium, and perhaps other intracellular second messengers, as a result of TBI can alter the expression of many genes. The protein products of some of these genes may be signals for TBI-associated memory dysfunction. Therefore, identification of genes whose expression is altered after TBI in the hippocampus, a structure in the medial temporal lobe that plays a critical role in memory formation and storage, and elucidation of the role(s) of their protein products may shed light on the molecular mechanisms underlying TBI-elicited memory dysfunction. The prodynorphin gene is expressed in hippocampal granule cells, and its expression has been reported to be enhanced as a result of elevated intracellular calcium. The prodynorphin protein is proteolytically cleaved to generate multiple dynorphin peptides, which can modulate neurotransmitter release through the activation of presynaptic κ opioid receptors. In this study, we report that 1) TBI transiently increases prodynorphin mRNA in the hippocampus, 2) dynorphin peptide immunoreactivity is enhanced for up to 24 hr after TBI and 3) intracerebroventricular infusion of the κ receptor antagonist nor-binaltorphimine (nor-BNI) impairs subsequent performance in a spatial memory task. These results suggest that dynorphin action may serve a beneficial role after TBI. Exp Biol Med 228:261–269, 2003

Key words: dynorphin; behavior; hippocampus; brain trauma; kappa receptor

One of the persistent outcomes of traumatic brain injury (TBI) is impairment of declarative memory (memories for names and places in humans and spatial memory in animals; Refs. 1, 2). Declarative memory is dependent on the hippocampus as well as other structures, including the entorhinal, perirhinal, and parahippocampal cortices. The hippocampus is highly susceptible to TBI, possibly because of intrinsic connectivity and/or to the presence of high levels of glutamate receptors (3–7). TBI not only causes hippocampal neuron loss but also alters the function, growth, and plasticity of the surviving neurons, all of which are thought to contribute to post-traumatic memory dysfunction (8–10). The molecular events activated by TBI that contribute to this impairment, however, are not well characterized.

TBI causes neuronal depolarization, excessive excifatory neurotransmitter release, and increased intracellular calcium levels (11, 12). These events activate a variety of biochemical cascades, some of which may contribute to neuronal death and dysfunction whereas others participate in neuronal survival, growth, and plasticity pathways, possibly via alterations in gene expression. The prodynorphin gene contains several calcium-responsive enhancer elements in its promotor region, including calcium/cAMP-responsive element, TPA-responsive element, and downstream regulatory element and is highly responsive to calcium levels (13–15). The prodynorphin protein is proteolytically processed to generate multiple bioactive dynorphin peptides, which are released after neuronal depolarization and participate in modulating neurotransmitter release, plasticity, long-term potentiation, and learning and memory (16, 17). For instance, dynorphin peptides acting via presynaptic κ receptors have been shown to inhibit the release of glutamate from mossy fiber terminals in the hippocampus and alter long-term potentiation (17, 18). Based on its ability to alter neurotransmitter release and hippocampal plasticity, the examination of dynorphin expression and determination of its role in the behavioral deficits observed after brain injury may provide insights into TBI-associated hippocampal dysfunction. Although acute administration of

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dynorphin A has been reported to worsen neurological outcome (19) and bolus infusions of κ-opioid antagonists have been suggested to improve cellular bioenergetics (20), the consequences of long-term infusions of kappa receptor antagonists are not known.

In this study, we examined the time course of prodynorphin mRNA expression as well as dynorphin peptides after lateral cortical impact injury in rodents. Intracerebroventricular administration of *nor*-binaltorphimine (nor-BNI) for 1 week after the injury adversely affected subsequent performance in a spatial memory task when compared with vehicle-treated controls. Taken together, these data suggest that endogenously generated dynorphin as a result of TBI may be beneficial.

Materials and Methods

Materials. Male Long–Evans rats (275–300 g) were purchased from Charles River Laboratories (Wilmington, MA). Dynorphin antibody (Dyn A1-8) was obtained from Peninsula Laboratories (Belmont, CA), and biotinylated anti-rabbit secondary antibody was from Promega (Madison, WI). The Anti-NeuN antibody was from Chemicon (Temecula, CA). AmpliTaq DNA polymerase and polymerase chain reaction (PCR) reagents were purchased from Applied Biosystems (Foster City, CA). Sybr green I was purchased from Molecular Probes (Eugene, OR), and Superscript II reverse transcriptase was obtained from Invitrogen Life Technologies (Carlsbad, CA). nor-BNI was obtained from Sigma (St. Louis, MO).

Production of Cortical Impact Injury. All protocols involving the use of animals are in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee. A controlled cortical impact device was used to administer brain injury as previously described (21, 22). Briefly, rats were anesthetized with 4% isoflurane and a 2:1 N_2O:O_2 mixture, intubated, and mechanically ventilated with a 2% isoflurane and a 2:1 N_2O:O_2 mixture. The rat was then mounted in the injury device stereotaxic frame. The head was held in a horizontal plane, a midline incision was made, and bilateral 6-mm craniectomies were performed midway between the bregma and lambda with the medial edge of the craniectomies 1 mm lateral to the midline. Rats received a single impact at 2.3 mm deformation with an impact velocity of 6 m/s at an angle of 30 degrees from the vertical plane. Core body temperature was maintained at 37–38°C by use of a rectal thermometer coupled to a heating pad. Sham rats were anesthetized and received a midline incision but were not injured. After injury or sham operation, the scalp was sutured and animals placed in an oxygenated chamber for monitoring during recovery.

Drug Preparation and Administration. nor-BNI was prepared as a 1 mg/ml solution in sterile saline. Immediately after injury, animals were given a bolus infusion (2 μl) of either 1 mg/ml nor-BNI (n = 9) or saline (n = 9) into the contralateral lateral ventricle (−0.8 mm from bregma, 1.5 mm lateral to midline, and 4.0 mm below the surface of the skull). After the completion of the bolus injection, a brain infusion cannulae (Alzet) was implanted using the same stereotaxic coordinates as for the bolus injection and secured by dental cement. The cannulae were connected via catheter tubing to a 7-day mini-osmotic pump (Alzet) containing either 1 mg/ml nor-BNI or saline. The pump was placed in a subcutaneous pouch between the scapulas as described by the vendor. A flow moderator maintained the drug delivery at a rate of 1 μl/hr over the 7-day infusion period. On day 7 post-injury, animals were anesthetized with 4 ml/kg Equithesin (250 mM chloral hydrate, 10 mg/ml pentobarbital, 90 mM $MgSO_4$, 10% ethanol, 40% propylene glycol), a small (1 cm) incision was made above the pump, the pump removed, and the catheter tubing cauterized. The incision was sutured and the animal was allowed to recover for an additional 7 days before being tested in the Morris water maze. Control animals for nonspecific effects of nor-BNI were infused and implanted as described above, but were not injured.

Isolation and Quantification of mRNA. At the indicated time points, animals were killed and the ipsi- and contralateral hippocampi quickly removed while the brain was submerged under ice-cold artificial cerebrospinal fluid (10 mM HEPES, pH 7.2; 1.3 mM NaH_2PO_4; 3 mM KCl; 124 mM NaCl; 10 mM dextrose; 26 mM $NaHCO_3$; and 2 mM $MgCl_2$), snap frozen and stored at −80°C until required for RNA isolation. The tissue was homogenized in a guanadinium thiocyanate solution, and total RNA was isolated by phenol:chloroform extraction and ethanol precipitation (23). The total RNA concentration in each sample was quantified by ribogreen fluorescence according to the manufacturers' protocol (Molecular Probes, Eugene, OR). Five hundred nanograms of total RNA was reverse transcribed for 2 hr at 42°C in a 20-μl reaction containing 50 mM Tris, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 10 mM DTT; 2.5 μM random hexamer; 1 mM each dNTP; 20 U RNasin; and 200 U Superscript II reverse transcriptase. To assess RNA samples for potential amplification from genomic DNA contamination, negative control RT reactions containing RNase A in place of the RNasin and reverse transcriptase were included. The level of expression of each target gene was quantified by amplification in triplicate of 1.3 μl of the resulting cDNA in a 30-μl reaction containing 18 mM Tris, pH 8.3; 55 mM KCl; 2 mM $MgCl_2$; 2 mM DTT; 200 nM each oligonucleotide; 10 nM fluorescein; 1:75,000 dilution of Sybr green I; and 2 U AmpliTaq DNA polymerase. The following primer pairs were used for target mRNA quantification:

prodynorphin: sense (5′-GGAGAACCCCAATACCTATTCCG-3′)
antisense (5′-GGTCTCCTGGATTCTAGGGTGG-3′)

β-*actin*: sense (5′-CTTCACCACCACGGC-3′)
antisense (5′-CCATCTCTTGCTCGAAG-3′).

The amplification protocol consisted of one cycle at 95°C for 3 min followed by 40 cycles of 94°C for 30 s, 60°C (β-actin) or 65°C (dynorphin) for 1 min, 72°C for 1 min. A melt curve protocol consisting of 70 cycles at 60°C for 10 s, +Δ0.5°C/cycle, was performed at the end of the amplification. Fluorescence data were acquired with a Bio-Rad iCycler and optical module and analyzed using the iCycler iQ Real-Time Detection System software. For quantification, a standard curve for each gene product was generated using increasing concentrations of cDNA derived from naive hippocampal total RNA to determine the linear range and amplification efficiency. The results for prodynorphin mRNA levels were normalized against the mRNA values obtained for β-actin. Real-time PCR results were repeated in at least three independent experiments.

Immunohistochemistry. Animals were killed with an overdose of chloral hydrate (1 g/kg) and transcardially perfused with 150 ml of heparinized phosphate-buffered saline (PBS) followed by 150 ml PBS containing 4% paraformaldehyde and 15% picric acid. Brains were removed and post-fixed overnight in perfusant. Brains were then cryoprotected in a 30% sucrose solution, embedded in OCT (Myers Laboratory), and sectioned into 40-micron thick slices using a cryostat. Free-floating slices were incubated overnight in Dyn A1-8 antibody (0.5 μg/ml) in PBS containing 2% bovine serum albumin, 2% normal goat serum, and 0.2% Triton X-100. Immunoreactivity was detected using a biotinylated anti-rabbit secondary antibody and a streptavidin-HRP conjugate. Immunoreactivity was visualized using a DAB kit (Vector) as described by the vendor. Slices were mounted on microscope slides and cover slipped using 50% glycerol/PBS. Immunohistochemistry results were repeated in at least three independent experiments.

Acute Neurological Assessments. Acute neurological assessments were performed blind as to the animal treatments. Assessments of simple nonpostural somatosensory functions were conducted by recording the duration of suppression of responses to stimulation (1, 21). The corneal reflex was evaluated by lightly touching the cornea with a cotton swab to elicit an eye blink. The pinna reflex was elicited by pressure applied to the outer ear with a wooden applicator. Forward body movements in response to a tail pinch defined escape. More complex postural somatosensory function was assessed by recording the duration of suppression of the righting response. The righting response was defined as the animal's ability to right itself three times consecutively after being placed on its back.

Assessment of Vestibulomotor Performance. All behavioral tests were conducted blind. For beam balance, animals were pre-assessed by placing them on a narrow wooden beam (2.0 cm wide) and measuring the duration they remained on the beam for a maximum of 60 sec. Before injury, animals were given repeated training until capable of balancing on the beam for three consecutive trials of 60 sec in duration. After injury, animals were given three daily trials on the beam balance task to give an average daily score (24). Paw placement was evaluated by placing the animal on a wire grid (opening size of 2 × 2 cm) and counting the number of foot faults out of a total of 50 steps (25). A foot fault was defined as when a front paw misses and appears below the plane of the grid. Paw placement was repeated three times daily to give an average daily score.

Assessment of Spatial Memory Performance. All behavioral tests were conducted blind. Cognitive function was examined using the hidden platform version of the Morris water maze (days 14–18 post-injury) as described previously (1, 26). Testing began 14 days post-injury to ensure recovery from the pump-removal surgery. For each daily block of four trials, rats were placed by hand facing the wall in the pool. Animals started a trial once from each of four randomized start locations (north, south, west, southwest). Rats were given a maximum of 60 sec to find the platform. If a rat failed to find the platform after 60 sec, it was placed there by the experimenter. All rats were allowed to remain on the platform for 30 sec before being placed in a 37°C warming cage between trials. The intertrial interval was 4 min. Movement within the maze was recorded by use of a video camera and analyzed using a tracking device and software (*Chromotrack*, San Diego Instruments). After the completion of training, animals were given a 60-sec probe trial in which the platform was removed to determine any nonspatial influences to the water maze performance.

Histopathology. After the completion of the behavioral testing, animals were overdosed with chloral hydrate (1 g/kg), perfused, and brain slices prepared as outlined in the immunohistochemistry section. To examine neuronal cell layers, sections were immunoreacted with an antibody for the neuron-specific marker NeuN (1 μg/ml) and detected with an anti-mouse-Alexafluor 568 secondary antibody by an experimenter who was kept blind with respect to the treatment. Cannulae placement was verified in representative animals by examination of the cannulae track using cresyl violet stained brain sections.

Statistics. Data obtained from PCR reactions were normalized to β-actin mRNA expression levels and compared using a one-way analysis of variance followed by a Student-Newman-Keul *post-hoc* analysis. Acute neurological reflex scores were compared using a Student's *t* test for unpaired variables. For evaluation of behavioral data, a repeated measures analysis of variance was used to determine group main effects. A Fisher's PLSD *post-hoc* test was used to determine group differences on specific days post-injury. Data were considered significant at $P < 0.05$. Data are presented as the mean ± S.E.M.

Results

TBI Increases Prodynorphin mRNA levels in the Hippocampus. Prodynorphin mRNA and dynorphin peptides are expressed at relatively high levels in the granule neurons of the hippocampus. Hippocampal prodynorphin mRNA levels in sham-operated and injured animals were measured using real-time PCR. Figure 1A shows that the relationship between the starting quantity of total RNA

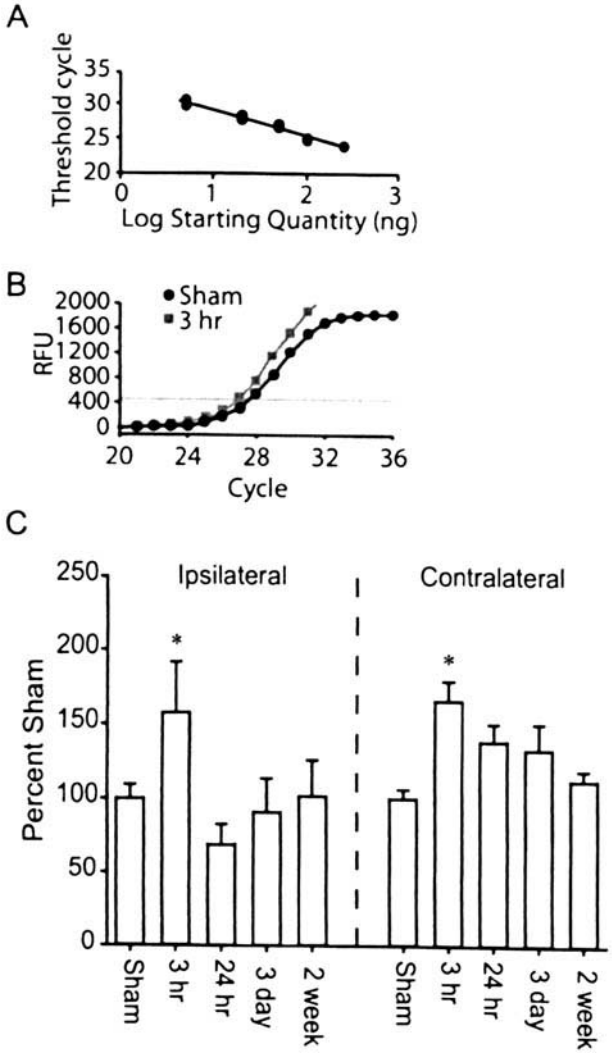

Figure 1. TBI increases the level of prodynorphin mRNA in the hippocampus. (A) Representative standard curve for the detection of prodynorphin mRNA using real-time PCR. A linear relationship exists between the log starting quantity of total mRNA (0.5 ng to 250 ng) and the number of PCR cycles needed to generate a specific amount of product. (B) Real-time PCR reactions for sham and 3 hr post-injury mRNA samples prepared from the ipsilateral hippocampus. As product is formed, the Sybr green dye intercalates with the double-stranded DNA, resulting in a fluorescence enhancement that is detected by the iCycler. The figure shows that the relative fluorescence units increase in the 3-hr sample more rapidly than the sham sample, indicating the presence of more prodynorphin mRNA in the starting sample. (C) Summary figure for the change in prodynorphin mRNA levels at different time points after injury. sham, $n = 6$; 3 hr, $n = 6$; 24 hr, $n = 6$; 3 day, $n = 3$; 2 week, $n = 3$; $^*P < 0.05$ by one-way analysis of variance and Student-Newman-Keul *post-hoc* analysis.

and number of PCR cycles that were required to detect a threshold level of amplified double-stranded specific product using Sybr green fluorescence. The specificity of the measured amplified product was confirmed by melting point analysis and gel electrophoresis. The threshold signal for each of the target genes (dynorphin and β-actin) was linear from at least 10 ng to 300 ng of input total RNA with a correlation coefficient >0.95. Representative profiles for real-time PCR amplification of prodynorphin cDNA using samples obtained from a sham and a 3-hr injured animal are shown in Figure 1B. The figure shows that the 3-hr TBI RNA sample gives rise to a detectable fluorescent signal more rapidly than the sham RNA sample, indicating more abundant prodynorphin mRNA representation in the TBI sample. The gray horizontal line indicates the threshold value at which starting quantity for each sample was calculated by comparison to a simultaneously amplified standard curve. The summary data in Figure 1C show that TBI significantly increases prodynorphin mRNA at the 3-hr time point in both the ipsilateral (control = 99.99 ± 7.8% versus injured = 162.45 ± 37.7%, $P < 0.05$) and the contralateral (control = 99.99 ± 6.4% versus injured = 166.33 ± 13.2%, $P < 0.05$) hippocampi. Although prodynorphin mRNA levels in the ipsilateral hippocampus had returned to control values by the 24-hr time point, contralateral levels remained above control levels for over 3 days post-injury. Although not statistically significant, a transient decrease in the mRNA for the kappa receptor was observed in both hippocampi as early as 3 hr post-injury (data not shown). This is consistent with binding studies which showed that the binding of [^{3}H]bremazocine to kappa receptors was significantly decreased in the CA1 subfield of the hippocampus by 3 hr after fluid percussion injury (27).

TBI Increases Dynorphin Peptide Immunoreactivity in the Hippocampus. We next examined hippocampal dynorphin peptide immunoreactivity at various time points after injury. Figure 2 shows that in both naïve and sham-operated control animals, a low but detectable immunoreactivity was observed. As reported previously, this immunoreactivity was localized to the mossy fiber axons of the dentate gyrus granule cells (28). After injury, dynorphin peptide immunoreactivity was robustly increased by 6 hr and remained elevated for over 24 hr post-injury. By day 3 post-injury, dynorphin peptide immunoreactivity appeared to be similar to that observed in control animals.

Chronic Administration of the Kappa Antagonist nor-BNI Did Not Affect Vestibulomotor Performance. To assess the role of the TBI-associated enhanced expression of dynorphin, a group of animals were injured, given a bolus intracerebroventricular injection of 2 μg nor-BNI, and then chronically supplied with nor-BNI (1 μg/hr) into the contralateral ventricle through implanted 7-day mini-osmotic pumps as outlined in the Materials and Methods section. A control group of animals was treated the same, except vehicle alone was substituted for the nor-BNI. The dose of nor-BNI used in this study has previously been shown to be an effective *in vivo* dose to inhibit κ receptor action (29, 30). The duration of administration is based on the following observations: Because increased levels of prodynorphin mRNA were detected as early as 3 hr post-injury, administration of nor-BNI was initiated as soon as possible after the injury. On average, the time required for the preparation of the burr hole, implantation of the infusion syringe and initiation of the infusion was approximately 8–10 min. The duration of administration was based on our time course of enhanced dynorphin peptide immunoreactivity in

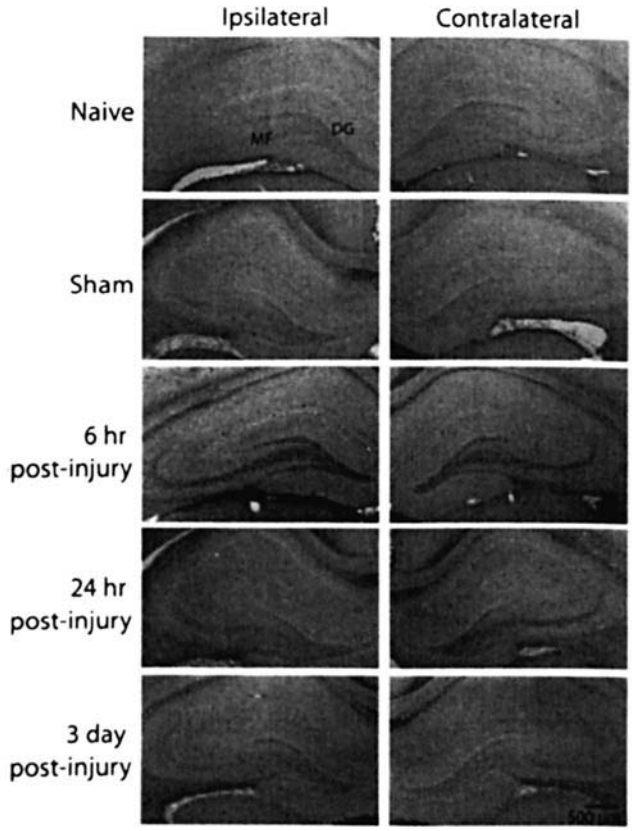

Figure 2. TBI increases the immunoreactivity for dynorphin peptides in the hippocampus. Representative photomicrographs showing dynorphinA1-8 immunoreactivity in the ipsi- and contralateral hippocampi for control and injured animals. Each time point was repeated in three separate animals. DG, dentate gyrus granule cell layer; MF, mossy fibers.

the hippocampus, which appeared to remain elevated for at least 24 hr after the injury. A 1-week infusion paradigm was used to minimize any effect of the pump removal surgery on the motor skills testing that was to be performed on days 1-4 post-injury. Immediately after the surgery, a battery of neurological responses was measured (Fig. 3A). The durations of suppression for both simple and complex somatosensory functions were not significantly different between the vehicle- and nor-BNI-treated groups.

Vestibulomotor skills after injury were assessed using the beam balance and paw placement tasks. Before injury (day 0), all animals were capable of remaining on the balance beam for the entire 60-sec testing period for three consecutive trials (Fig. 3B). As reported previously, cortical impact injury impaired performance on this task (Fig. 3B; Ref. 21). Figure 3C shows that before injury, animals made

A

Reflex	*Vehicle (sec)*	*nor-BNI (sec)*	
Corneal	2.51 ± 0.48	2.47 ± 0.43	n.s.
Pinna	3.29 ± 0.32	3.32 ± 0.36	n.s.
Escape	4.35 ± 0.33	3.99 ± 0.41	n.s.
Righting	4.95 ± 0.82	5.14 ± 0.60	n.s.

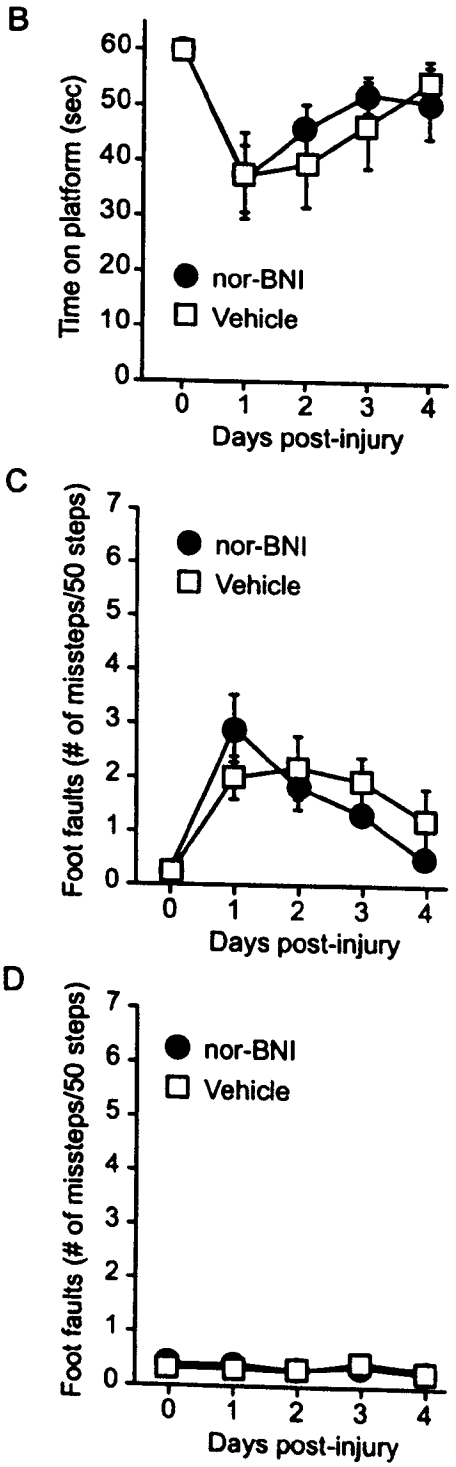

Figure 3. Chronic nor-BNI infusion after injury does not affect acute neurological responses or performance in vestibulomotor tasks. (A) Table showing the duration of suppression of corneal, pinna, righting and escape responses measured immediately after injury. No significant differences (Student's *t*-test for unpaired variables) were seen between the injured group receiving vehicle (n = 9) and the injured group receiving nor-BNI (n = 9). Injured animals infused with either vehicle or nor-BNI were also assessed for their performance on the (B) beam balance, (C) contralateral foot fault, and (D) ipsilateral foot fault tasks.

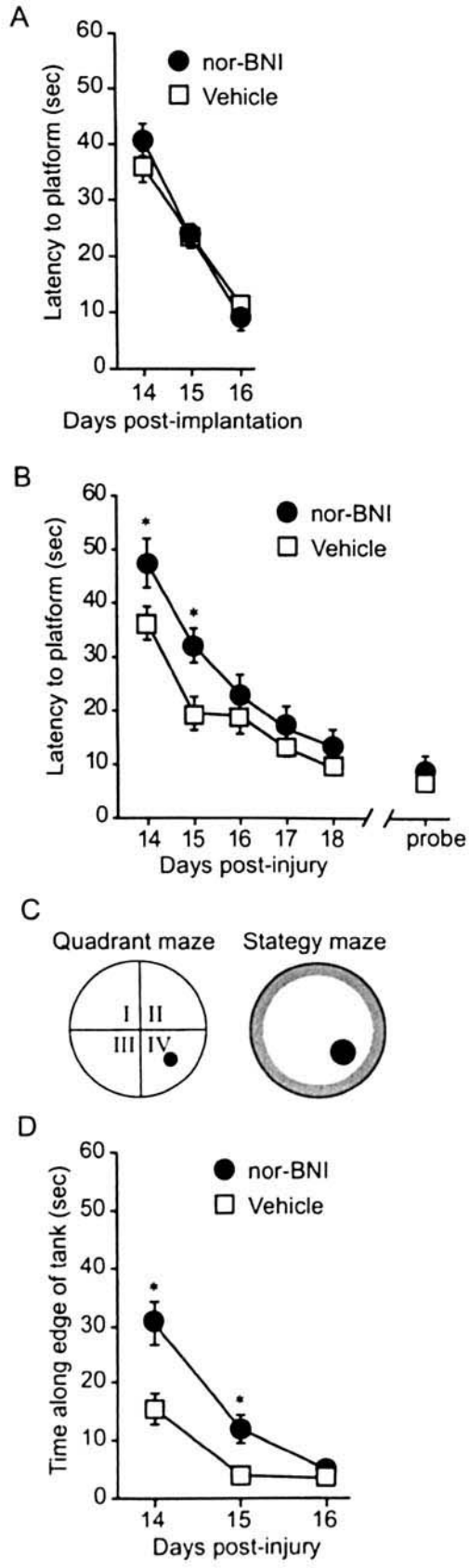

Figure 4. Chronic nor-BNI infusion after injury impairs subsequent performance in a water maze task. (A) When tested in a hidden platform version of the Morris water maze, uninjured animals treated with nor-BNI (n = 9) or vehicle (n = 9) performed equally well. (B) Injured animals treated with nor-BNI (n = 9) had significantly longer latencies (repeated measures analysis of variance followed by a Fisher's PLSD *post-hoc* test) on the first two days of hidden platform training than did their vehicle-treated counterparts (n = 9). No difference in latency was observed on the final days of training, nor during a probe trial. Using a strategy maze diagram (shown in C), the performance of injured animals during the training trials was examined. (D) Nor-BNI-treated injured animals spent significantly more time (repeated measures analysis of variance followed by a Fisher's PLSD *post-hoc* test) searching the perimeter of the tank during the first two days of training than did the vehicle-treated controls. $^*P < 0.05$.

on average less than one contralateral foot fault per 50 steps. After injury, however, the number of contralateral foot faults increased to 2.0 per 50 steps (Fig. 3C). This deficit was not affected by nor-BNI treatment. Ipsilateral to the injury, the number of foot faults did not change after injury compared with the pre-injury level (Fig. 3D). All animals had fully recovered from the motor deficits prior to the initiation of Morris water maze testing.

Chronic Administration of the Kappa Antagonist nor-BNI Worsened Spatial Memory Performance. To assess any effects that nor-BNI may have on Morris maze performance in the absence of injury, groups of uninjured animals were given a bolus, implanted with mini-osmotic pumps and infused for 7 days with either saline or nor-BNI as described above. When tested in the water maze task beginning on day 14 post-implantation, no significant differences in maze performance were detected between the two groups (Fig. 4A). These animals required only 3 days of hidden platform training (12 trials) to be able to consistently locate the platform in under 10 s. By comparison, saline-treated injured animals required 5 days (20 trials) of training to reach the same performance level (Fig. 4B). Figure 4B shows that on the first 2 days of testing, the nor-BNI-treated animals required significantly longer to find the hidden platform compared with the saline-treated controls. By the third day of testing, however, the nor-BNI-treated animals' performance in the maze was not significantly different than the control group. Analysis of swimming speed did not reveal any significant differences between the two groups (vehicle = 25.1 ± 1.8 cm/s vs nor-BNI = 27.9 ± 1.4 cm/s; ns) on the first two days of testing. When the injured animals were given a probe trial after the completion of training, no significant difference in latency was detected between the two groups for animals to cross the previous location of the hidden platform (Fig. 4B), the number of platform crossings or quadrant preference (data not shown).

To further analyze the performance of the animals in the Morris water maze during training, the swimming path for each trial was examined using a strategy maze diagram (Fig. 4C). When first exposed to the Morris water maze, animals typically explore the circumference of the tank for any route of escape. After repeated exposure to the maze, however, animals abandon searching the edge of the tank and use the extra-maze cues to develop successful escape

strategies (31). The time spent within 7.5 cm of the edge of the tank (gray), in open water searching (white), and within one radius (5 cm) of the platform (black), was recorded and averaged for each daily block of four trials. Figure 4D shows that injured animals treated with nor-BNI spent significantly more time along the perimeter of the tank during the first two days of testing than did their saline-treated counterparts. Once animals left the perimeter of the tank, there was no difference in the time spent searching in the open field or in the immediate vicinity of the hidden platform (data not shown).

After the completion of behavioral studies, animals were killed and brain tissues were used for histopathological analysis. Figure 5A shows a representative picture of a cresyl violet stained brain section indicating the position of the injury (filled arrow) and the track of the cannulae (open arrow) used for infusion. Figures 5B and 5C show representative photomicrographs of the hippocampal neuronal cell layers from vehicle and nor-BNI-treated injured animals immunoreacted with the neuron-specific antibody anti-NeuN. Chronic nor-BNI infusion does not appear to cause any overt change in the number of hippocampal neurons or thinning of neuronal layers relative to vehicle-treated controls. In addition, the staining patterns and intensities of the dendritic marker MAP-2a,b,c and the axonal marker synapsin were not grossly altered by the nor-BNI treatment (data not shown). When mRNA samples obtained from the animals used for the behavioral studies were compared for prodynorphin (Fig. 5D) and kappa receptor levels (data not shown), no differences were found as a result of the nor-BNI treatment.

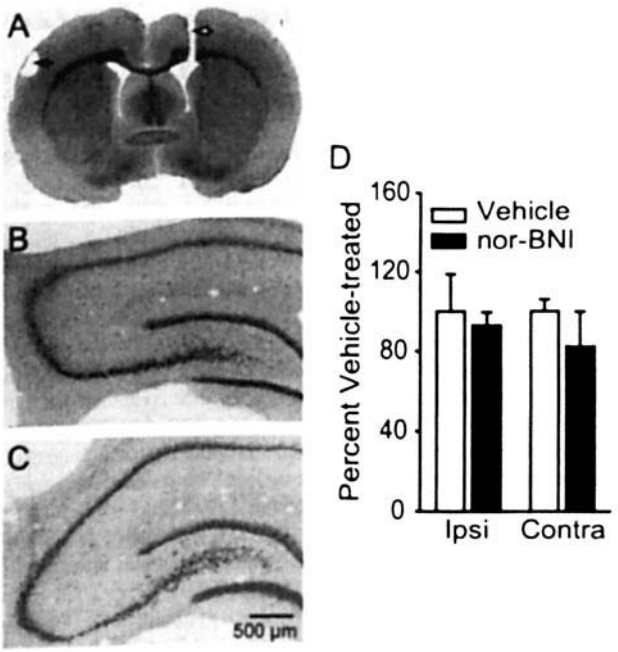

Figure 5. Chronic nor-BNI treatment does not grossly alter hippocampal morphology. (A) Representative picture of a brain slice indicating the position of the injury (filled arrow) and the track of the cannulae used for infusion (open arrow). Representative photomicrographs of hippocampal slices from (B) vehicle- and (C) nor-BNI-treated animals immunoreacted for the neuron-specific marker NeuN. The levels of (D) prodynorphin and (D) kappa receptor mRNAs were not significantly different (Student's *t-test* for unpaired variables) between the animals treated with nor-BNI (n = 3) and those treated with vehicle (n = 5).

Discussion

Traumatic brain injury is known to activate a wide variety of biochemical cascades and biophysical changes, some of which serve beneficial roles, whereas others are deleterious. The findings presented in this report show that experimental brain injury enhances the expression of prodynorphin mRNA and a dynorphin peptide (Dyn A1-8) in the hippocampus. In addition, intracerebroventricular administration of a κ receptor antagonist, nor-BNI, exacerbated the spatial memory defects observed after cortical impact injury. The findings from this study suggest that dynorphin action may be beneficial after TBI, possibly through their previously established role in limiting neuronal excitability by presynaptic inhibition of glutamate receptor-mediated neurotransmission or voltage-dependent Ca^{2+} channels (32).

Although studies have been performed to examine the role of dynorphin and opioid receptors after brain injury, these studies examined the influence of acute modulation of dynorphin peptide levels or κ receptor function. For example, it has been reported that a bolus injection of dynorphin (Dyn A1-17) 15 min before TBI worsened neurological outcome (19, 33). Similarly, Vink *et al.* found that a bolus intravenous injection of the κ opioid antagonist nor-BNI 30 min post-injury resulted in an improved neurological outcome (20). While these studies suggest a detrimental role for dynorphin, other studies have shown that pretreatment with κ receptor agonists (e.g., U50, 488H and GR89696) can protect against ischemic cell death and attenuate neurological deficits (34–36). The results from these and other studies have therefore provided contradictory evidence for a putative role of dynorphin in TBI pathophysiology. The results presented in this study using chronic infusion of a κ receptor antagonist support the previous evidence indicating that dynorphin action after TBI may play a beneficial role.

The increased levels of prodynorphin mRNA detected in the hippocampus after TBI suggest a transcriptional upregulation of the prodynorphin gene. However, post-transcriptional mechanisms such as enhanced stability of mRNA can also result in increased steady-state mRNA levels. We have previously shown that calcium/cAMP response element binding protein phosphorylation in the granule neurons of the hippocampus is increased as early as 5 min after injury (26). In addition, the binding activity of the transcription factor complex activator protein-1 was also increased as a result of TBI (26). Activation of calcium/cAMP response element binding protein, activator protein-1, and/or other transcription factors, such as downstream regulatory element antagonist modulator, also known as calsenilin, could contribute to the elevated prodynorphin mRNA levels we observed (13–15, 37). Although the

mRNA was transiently upregulated, the immunoreactivity of dynorphin A1-8 peptides appeared to remain elevated for a prolonged period after the injury. Although qualitative in nature, dynorphin peptide immunoreactivity did not appear to return to control values until the 3-day time point (Fig. 2). This prolonged increase in immunoreactivity may be due to the sequestration of the dynorphin peptides in synaptic vesicles, resulting in a slower catabolic rate.

Nor-BNI administration did not alter performance in motor and vestibulomotor tasks. However, when tested in the hidden platform version of the Morris water maze, animals treated with nor-BNI for 1 week after injury performed significantly worse than animals treated with vehicle, although no differences in swimming speed were observed. This poor performance was found to be associated with enhanced perimeter swimming (Fig. 4). This deficit was specific to post-injury administration of nor-BNI, as animals that were infused with nor-BNI but not injured showed no significant difference in performance in the water maze task compared with vehicle-treated controls. Consistent with this, it has been previously shown that intrahippocampal administration of nor-BNI does not affect performance in the Morris water maze task (16). Once effective escape strategies were learned and perimeter swimming abandoned, both injured groups performed equally well.

Although performance in the Morris water maze has been reported to be dependent on hippocampal function, other brain structures may have contributed to the behavioral effects we observed after nor-BNI administration. For example, it has been previously shown that animals receiving lesions of the medial caudate putamen have poor performance in the water maze task, and that this performance is associated with enhanced perimeter swimming during the early phases of acquisition (38). Interestingly, similar deficits in the initial phases of acquisition have also been observed after systemic administration of high-doses of NMDA antagonists, suggesting that perturbations of glutamate signaling and/or neuronal degeneration can elicit thigmotaxic swimming behaviors as a result of impaired spatial search strategies (39). Because nor-BNI was infused into the ventricular system, the present study cannot exclude the involvement of other structures on the behavioral effects we observed for nor-BNI administration after injury. Furthermore, our histological and immunofluorescent examination of hippocampal sections did not reveal any gross cellular or morphological changes within the hippocampal subfields that could be associated with nor-BNI administration (Fig. 5, data not shown). However, we cannot rule out any effect that enhanced dynorphin expression may have on the ultrastructural modifications and/or altered neuronal plasticity that have been observed after TBI (40, 41).

In summary, the results presented in this study show that the expression of prodynorphin is increased as a result of trauma, and that the blockade of dynorphin action after injury is detrimental for subsequent spatial performance. These results suggest that the enhanced expression of dynorphin after TBI may be an indicator for molecular events occurring in the hippocampus after TBI. However, the mechanism by which this occurs is not known at present. Based on the well-established role of dynorphin in reducing glutamate receptor-mediated neurotransmission and inhibiting voltage-dependent Ca^{2+} channels, it is possible that transient changes in dynorphin expression reduce these processes and limit the long-term consequences of their action. For example, it has been reported that calcium influx soon after brain trauma can activate calpain and increase the degradation of spectrin leading to delayed axonal failure and disconnection (42). In addition, the use of glutamate antagonists starting as early as 5 min after injury has been reported to decrease neuronal degeneration and reduce water maze deficits testing 11 days post-injury, suggesting that acute alterations in glutamate signaling as a result of trauma may contribute to long-lasting hippocampal dysfunction and memory deficits (43). Future studies aimed at investigating the molecular cascades altered as a result of nor-BNI administration may shed light on the biochemical basis of dynorphin protection.

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