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Neurotrophin-mediated neuroprotection of hippocampal neurons following traumatic brain injury is not associated with acute recovery of hippocampal function

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Abstract

Traumatic brain injury (TBI) causes selective hippocampal cell death which is believed to be associated with the cognitive impairment observed in both clinical and experimental settings. The endogenous neurotrophin NT-4/5, a TrkB ligand, has been shown to be neuroprotective for vulnerable CA3 pyramidal neurons after experimental brain injury. In this study, infusion of recombinant NT-4/5 increased survival of CA2/3 pyramidal neurons to 71% after lateral fluid percussion injury in rats, compared to 55% in vehicle-treated controls. The functional outcome of this NT-4/5-mediated neuroprotection was examined using three hippocampal-dependent behavioral tests. Injury-induced impairment was evident in all three tests, but interestingly, there was no treatment-related improvement in any of these measures. Similarly, injury-induced decreased excitability in the Schaffer collaterals was not affected by NT-4/5 treatment. We propose that a deeper understanding of the factors that link neuronal survival to recovery of function will be important for future studies of potentially therapeutic agents.

Keywords

Neurotrophins; Rats; Hippocampal neurons; Neuroprotection; Neuropharmacology; NT-4/5; behavior; cognition; brain injury; Neurotrophin

INTRODUCTION

Chronic cognitive impairments such as deficits in attention, learning and memory, or slow processing speed are frequently reported as a consequence of traumatic brain injury (TBI) in

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humans (Salmond and Sahakian, 2005). These long-lasting cognitive impairments are among the most debilitating outcomes in patients, yet, to date, no treatment can prevent or counteract these effects in TBI patients (Maas, 2001, Narayan et al., 2002, Tolias and Bullock, 2004).

Hippocampal pyramidal neurons are particularly vulnerable to brain injury in humans (Tate and Bigler, 2000, Maxwell et al., 2003). Bilateral loss of hippocampal neurons has been observed in 85% of fatal human head injury cases (Kotapka et al., 1992). Cell death in hippocampal area CA3 observed in TBI patients has been replicated by experimental TBI in nonhuman primates (Kotapka et al., 1991), rodents (Cortez et al., 1989, Lowenstein et al., 1992, Hicks et al., 1993, Hicks et al., 1994, Hicks et al., 1995, Colicos and Dash, 1996, Colicos et al., 1996, Hicks et al., 1996, Baldwin et al., 1997, Pierce et al., 1998, Carbonell and Grady, 1999, Golarai et al., 2001, Sato et al., 2001, Grady et al., 2003) and pigs (Smith et al., 1997b). In the lateral fluid percussion (LFP) model of TBI in the rat, significant neuronal cell death occurs in hippocampal area CA2/3 and the hilus of the dentate gyrus, and cognitive deficits have been documented in tests such as the Morris water maze (MWM; for a review see (Fujimoto et al., 2004)). Interestingly, spatial navigation deficits have also been documented in TBI survivors using a computer simulation of the MWM task (Skelton et al., 2006).

We recently demonstrated that the neurotrophin NT-4/5, a TrkB ligand, is highly neuroprotective for hippocampal CA3 pyramidal neurons after experimental TBI (Royo et al., 2006). Administration of recombinant NT-4/5 following LFP TBI in rats increased survival of pyramidal neurons in area CA2/3. In addition, we showed that endogenous levels of NT-4/5 protein are transiently elevated after injury and that genetic deletion of NT-4/5 increases hippocampal vulnerability to TBI in knockout mice (Royo et al., 2006). These results demonstrate that both endogenous and exogenous NT-4/5 are protective. Here, we tested the hypothesis that NT-4/5-mediated sparing of hippocampal neurons would improve hippocampal function after experimental TBI. This study was designed to test whether NT-4/5-induced neuroprotection would improve injury-induced cognitive impairment and altered hippocampal excitability.

EXPERIMENTAL PROCEDURES

All procedures involving animals were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and the Children's Hospital of Philadelphia and conform to federal guidelines (National Research Council, 1996).

Lateral fluid percussion (LFP) traumatic brain injury in rats

Young adult male Sprague-Dawley rats weighing 350-400g (Harlan) were housed at 24°C in a 12 hour light/dark cycle, with access to food and water *ad libitum*. Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and placed in a stereotaxic frame, on a 37°C heating pad. Following the reported protocol for LFP TBI in rats (McIntosh et al., 1989), a midline scalp incision was made and a 5 mm diameter craniotomy was created over the left cortex midway between lambda and bregma and midway between the sagittal suture and the temporal ridge. The *dura mater* was left intact and a plastic female Luer-Lok connector (3 mm inner diameter) was secured in the craniectomy with cyanoacrylate adhesive and dental cement. Ninety minutes after receiving anesthesia, the animals to be injured (TBI; n=30) were attached to the fluid-percussion device and injured at a moderate level (~2.9 atm) by percussion of a fluid (saline) pulse applied to the dural surface for 20 ms, causing mechanical deformation of the brain. Animals were removed from the device, the dental cement and Luer-Lok connector were removed, and the scalp sutured. Sham-injured animals (SHAM; n=18) were used as controls and underwent all anesthetic and surgical procedures but did not receive LFP brain injury.

Brain cannulation for NT-4/5 administration

One day before surgery, osmotic mini-pumps (1007D, Alzet, CA) were filled with vehicle (100mM PBS 0.1% BSA, pH 7.4) or recombinant human NT-4/5 (166.7µg/ml in vehicle; R&D Systems), connected to a 28 gauge brain infusion cannula via a 3.5 cm catheter (Alzet brain infusion kit II) under sterile conditions and primed for 18h at 37°C. The recombinant NT-4/5 used in this study was generated by expressing human preproNT4 in Sf21 insect cells. The product consisted of mature 14kDa NT-4/5 as determined by SDS-PAGE reducing gel (R&D Systems, personal communication). Fifteen minutes following surgery/injury, the pump was inserted subcutaneously in the back of the animal and the connected infusion cannula was inserted stereotactically in the injured primary somatosensory cortex at 3mm posterior to bregma, 3mm lateral and the tip was positioned –3mm from skull surface. The site of cannula implantation is immediately anterior to the site of maximal cortical injury as previously described (Sinson et al., 1995, Sinson et al., 1997, Royo et al., 2006). Animals were randomly assigned to the following groups: sham-injured and vehicle-infused (SHAM/VEH, n=15); LFP-injured and vehicle-infused (TBI/VEH, n=14); or LFP-injured and NT-4/5-infused (TBI/NT4, n=16). The implantation of the osmotic pump and the cannula took an average of 15min. Animals were sutured and allowed to recover from surgery on a 37°C heating pad. The nominal pumping rate was 0.5µl/h for 7 days delivering a dose of 2 µg/day (approximately 5µg/kg/day). At 1 mg/ml NT-4/5 retains more than 70% of its activity after 14 days at 37°C in mini-osmotic pumps (Altar et al., 1994). Pumps were checked after removal for any remaining volume. All animals were subjected to behavioral testing; subsets of animals from each experimental group were used for histological analysis and electrophysiology as indicated below.

Neurological motor function

The composite neuroscore test was previously shown to be sensitive for evaluation of the motor component of LFP injury severity and recovery (McIntosh et al., 1989, Pierce et al., 1998). Composite neuroscore was assessed at 48h, 1 and 2 weeks post-injury using seven 4-point tests (maximal composite neuroscore = 28). Animals were scored on a 4 (normal) to 0 (severely impaired) integral scale for left and right forelimb flexion, left and right lateral pulsion, left and right hindlimb flexion and the ability to stand on an inclined angle board in the vertical and horizontal positions. Animals were scored on the angle board with respect to their change in angle from pre-injury testing, where 0 degrees change = 4; 2.5 degrees = 3; 5 degrees = 2, 7.5 degrees = 1; 10+ degrees = 0, based on the average score for all positions. These are ordinal data and not normally distributed. Data were analyzed with the nonparametric Kruskal-Wallis one-way analysis of variance test followed by Dunn's multiple comparison test (Pierce et al., 1998).

Morris water maze (MWM) test for visuo-spatial learning

Behavioral tests were conducted over several days to minimize stress on the animals, with the most aversive tests (MWM and CFC) separated by five days. The MWM was used to evaluate learning and anterograde memory for 8 trials/day over 3 days beginning at 1 week after the injury. The maze is a black circular pool (1.80 m diameter, 0.6 m depth) filled with water (22–24°C). A 10-cm diameter, clear Plexiglas escape platform was placed 1 cm below the surface of the water in the learning phases. The task requires that the animals learn to locate the submerged platform using external visual cues after being placed at 1 of 4 sites in the pool. Latencies to find the platform were recorded for each trial, with a maximal allowed time of 60 seconds, and averaged for each group of 4 trials. The investigator was blinded to the injury/treatment status of the animals. Twenty-four hours following the last learning trial, animals were placed back in the maze for two probe trials (60s each) without the platform. The time spent in each zone was recorded using video monitoring and tracking software (Accutrak, CA) and the zones were mathematically weighted based on the proximity to the platform (see inset

in Figure 4B). The score for the probe trials were determined by summing the weighted time spent in each zone: $(0 \times \text{time in zone 1}) + (1 \times \text{time in zone 2}) + (5 \times \text{time in zone 3}) + (10 \times \text{time in zone 4})$ and using the better of the two scores for each animal (see Thompson et al., 2006). Data were analyzed with a one-way ANOVA followed by the Newman-Keuls multiple comparison test.

Contextual Fear Conditioning (CFC) test for associative learning

Retrograde amnesia was tested by conditioning the animals one day before the injury and testing on day 14 post-injury. For the CFC test, animals are exposed to an aversive stimulus (foot shock) in a particular context (a cage). The association of these two events is indicated by freezing behavior (becoming immobile) when rats are placed back in the same context without receiving the shocks. Naïve rats were placed in a cage with an electrified grid floor and received foot shocks (5×1.5 mA, 2s duration), one minute apart, delivered by a computer-controlled system (Colbourn Instruments, PA) and then randomly assigned to experimental groups for surgery/injury and infusion on the following day. At 2 weeks post-injury, animals were individually placed in the electrified cage without foot shocks for 5 min. Freezing behavior was measured using an infrared motion sensor detecting the onset and offset of any movements. Freezing behavior was defined as any period of inactivity greater than 10 seconds during the 5 min testing period and the total amount of freezing time was expressed as a percentage. In pilot experiments, a cutoff time of 10 sec was sufficient to distinguish freezing from pauses in movement (data not shown). Contextual fear was distinguished from general fear by placing each animal in a novel context and measuring freezing behavior for 5 min. Data were analyzed with a one-way ANOVA followed by the Newman-Keuls multiple comparison test.

Open Field test for exploratory behavior

A modified version of the open field test was conducted in a brightly lit circular field (2 meter diameter) on day 13 post-injury. Three virtual concentric circular zones (zone 1:radius=1; zone 2:radius=2/3 and zone 3:radius=1/3, see inset on figure 4F) were defined using video monitoring coupled to tracking software (Accutrak). An exploratory score was calculated as the sum of all entries into any zone over a 5 minute observation period, similar to the standard protocol which sums entries into square zones (see O'Connor et al., 2003). Data were analyzed with a one-way ANOVA followed by the Newman-Keuls multiple comparison test.

Stereological estimation of neuronal cell loss in the hippocampus following TBI

At 15 days post-injury a randomly selected subset of animals (6 per group) were over-anesthetized with sodium pentobarbital (200 mg/kg, ip), transcardially perfused with heparinized saline (1 unit/ml) for 1 min followed by 4% paraformaldehyde in phosphate buffered saline (PBS, 100 mM, pH 7.4) for 5 min. Brains were removed, post-fixed for 4h at 4°C, infiltrated with 30% sucrose for 72 hours and snap frozen in isopentane at -40°C . Frozen sections of 50 μm thickness were cut using a sliding microtome (Microm, Germany) and stained with cresyl violet.

Estimation of pyramidal neuron number in the hippocampal subfields was based on the optical fractionator sampling scheme as described by West and colleagues (West et al., 1991) and was carried out exactly as previously described (Royo et al., 2006). The optical microscope used for this study (Nikon Microphot, NY) was equipped with a 3D motorized stage (Prior Scientific), a depth gauge (Heidenhain, Germany), and a digital camera (Toshiba, Japan). Stereological quantification was performed using the computer assisted stereological toolbox (CAST-Grid Olympus, Denmark). Following a systematic random sampling pattern, every 6th section (section sampling fraction: $ssf=1/6$) was selected between -2.5 mm and -5.5 mm relative to bregma. The region of interest was defined and delineated under a 2X objective on

each section as the pyramidal neurons in subfield CA2 and subfield CA3a/b, excluding subfield CA3c. Pyramidal cell counting was conducted using an oil immersion 63X objective lens with a 1.4 numerical aperture. The thickness (t) of each section was defined as the average of 3 measurements randomly selected within the sampling area using the depth gauge (microcator). Estimation of neuronal cell number N was then calculated as $N = \Sigma Q^- \cdot t/h \cdot 1/asf \cdot 1/ssf$ with Q^- being the number of cells counted per area, h , the disector height and asf , the area sampling fraction. A 5% area sampling fraction and a 12 μ m disector height (h) were computer-guided for randomization. The coefficient of error (CE) and coefficient of variation (CV) were calculated as described (Royo et al., 2006). The mean CE in this study was 0.066. The CV was 0.13 for the SHAM/VEH group, 0.11 for the TBI/VEH group and 0.23 for the TBI/NT4 group. Estimation of cell death in the hilus was performed in a similar fashion except the area sampling fraction was 10%.

Fluorochrome staining for neuronal cell death

Alternate 50 μ m sections from the same brains (TBI/VEH and TBI/NT4 groups, $n=6$ per group) were used to estimate the amount of Fluorochrome staining in the hippocampus CA1, CA2/3, dentate gyrus and hilus subfields. For each animal, 5 sections selected between -2.30 and -3.80 mm relative to bregma were mounted onto slides and dried overnight. Sections were gradually dehydrated in ethanol, rehydrated and placed in 0.06% potassium permanganate for 20 min, then rinsed with distilled water and immersed in a staining solution of 0.001% Fluoro-Jade-B (Chemicon International, CA) in 0.1% acetic acid for 30 min. After rinsing in distilled water, the slides were dried at 40°C, dehydrated in xylene, and coverslipped in mounting media. Cellular staining was semi-quantified by an observer blinded to injury/treatment status using an ordinal scale: 0: no cells; 1: 1-5 cells; 2: 6-20 cells; 3: >20 cells.

Immunostaining for NT-4/5 diffusion

To evaluate drug diffusion into the brain parenchyma following NT-4/5 infusion, brain sections were prepared as described above, from a group of animals ($n=3$) at 30 hours post-TBI. Sections were washed in 0.1 M TBS; pH 7.4 and blocked in 0.1% Triton-X; 2% bovine serum albumin (BSA); 2% normal horse serum for 1 h at room temperature. Sections were then incubated overnight at 4°C with the primary antibody (NT-4/5 rabbit polyclonal IgG diluted 1:100, Chemicon, CA). Control sections were processed in parallel by omitting the primary antibody. Following washing, sections were incubated with a biotinylated conjugated secondary antibody (goat anti-rabbit IgG; Jackson Immunoresearch, PA). Following avidin-biotin amplification (Vector ABC Elite kit, Vector Laboratories, CA), sections were developed with 3,3'-diaminobenzidine tetrahydrochloride.

Field potential recording in acutely isolated hippocampal slices

Extracellular recordings for CA3 output were conducted on transverse hippocampal slices at 14 days post-injury in a subset of animals randomly selected from the experimental groups: SHAM/VEH ($n=4$); TBI/VEH ($n=5$); TBI/NT4 ($n=4$). Animals were anesthetized with halothane, decapitated and the brains quickly removed and chilled for 2 min in a modified sucrose-based artificial cerebrospinal fluid (aCSF) solution (201mM sucrose; 3.2 mM KCl; 1.25mM NaHPO₄; 2mM MgCl₂; 2mM CaCl₂; 26mM NaHCO₃; 10mM glucose, equilibrated with 95% O₂/5% CO₂ at 4°C.) The hippocampus from the injured hemisphere was dissected *en bloc*, placed back in the bubbling aCSF at 4°C for 2 min and subsequently, 400 μ m slices from the dorsal hippocampus (first 12 sections) were cut perpendicular to the long axis using a mechanical tissue chopper (Brinkman Instruments). Slices were quickly transferred to a holding chamber at room temperature and equilibrated for at least 1 hr before being placed in the recording chamber. A stimulating bipolar tungsten electrode was placed in the Schaffer collaterals and a monopolar recording electrode in stratum radiatum of CA1 (Figure 4-G).

Stimulation intensities ranging from 50-500 μ A (200 μ s duration) were applied to the slices and the slopes of the recorded field excitatory postsynaptic potentials (fEPSPs) were used to construct input/output curves. Four to six slices per animal were evaluated (23-28 slices per experimental group).

Statistical Analysis

Statistical analyses were performed using Prism 3.02 (GraphPad Software Inc., CA). The Pearson product-moment correlations and their significance levels were calculated using STATA. The specific statistical tests that were used are listed with the methods for each behavioral or histological test above. The data are presented as mean $\pm$ standard deviation (SD) or mean $\pm$ standard error of the mean (SEM) as detailed in each figure legend. Statistical significance was set at $P < 0.05$.

RESULTS

NT-4/5 administration reduces pyramidal cell loss in area CA3 following TBI

We examined the ability of a TrkB ligand, the mammalian neurotrophin NT-4/5, to protect hippocampal pyramidal neurons after experimental TBI. LFP-injured rats were infused with 2 μ g/day of recombinant NT-4/5 (TBI/NT4, $n=6$) or the same volume of vehicle (TBI/VEH, $n=6$), beginning 30 minutes after the injury and continuing for 1 week. A control group of sham-injured animals was infused with vehicle only (SHAM/VEH, $n=6$). Two weeks after TBI, the number of CA2/3 pyramidal neurons was estimated on cresyl violet-stained coronal brain sections using the optical fractionator method.

Brain injury induced substantial pyramidal neuronal death in the CA2/CA3 subfield, which was significantly ameliorated by NT-4/5 administration (Figure 1-A). Animals in the TBI/VEH group lost approximately 45% of pyramidal neurons in the CA2/3 region studied, compared to the SHAM/VEH group (48512 ± 5142 cells in TBI/VEH vs 88957 ± 11620 cells in SHAM/VEH, mean $\pm$ SD) (Figure 1-B). NT-4/5 administration reduced pyramidal cell death at 2 weeks, resulting in survival of 71% of CA2/3 pyramidal neurons compared to 55% in TBI/VEH (63088 ± 14445 cells in TBI/NT4). A one-way ANOVA test revealed that the mean values of the three groups (SHAM/VEH, TBI/VEH, TBI/NT4) were significantly different from each other [$F(2, 15)=20.5$, $p < 0.001$]. Subsequent testing of the difference between TBI/VEH and TBI/NT4 showed a statistically significant difference using an unpaired t-test ($P < 0.05$) and a Tukey test ($P < 0.05$). Because of the small group sizes ($n=6$ per group), we conducted a further nonparametric test of the difference in CA3 neuron number in the TBI/VEH and TBI/NT4 groups: a permutation test was performed to create a reference distribution for the test statistic (Phillip, 2005). Using this approach we obtained a p value of less than 0.03. These results demonstrate that early and prolonged administration of NT-4/5 significantly improves pyramidal cell survival in the CA2/3 subfield of the hippocampus following experimental TBI.

Brain injury also caused significant cell loss in the hilus of the dentate gyrus when compared to sham-injured animals (7475 ± 1642 cells in TBI/VEH vs 11323 ± 3284 cells in SHAM/VEH, $P < 0.05$). Hilar cell death appeared to be slightly improved by NT-4/5 administration but the difference did not reach significance (9118 ± 1854 cells in TBI/NT4) (Figure 1-C). We performed immunostaining for NT-4/5 to determine the targeted regions of the infused recombinant protein. At 30hr post-infusion, NT-4/5 protein had diffused ventrally from the cannula tip throughout the primary target, the hippocampal CA region, but had spread only sparsely into the hilar region (Figure 2).

Rescued CA3 neurons do not undergo delayed degeneration

To address the concern that NT-4/5 administration had merely delayed cell death, we evaluated Fluor Jade-B (FJB) histofluorescence on brain sections at 2 weeks post-TBI. After experimental TBI in rats, FJB staining peaks on the first day post-injury and declines thereafter to nearly undetectable levels in the hippocampus by 14 days (Sato et al., 2001, Hallam et al., 2004, Anderson et al., 2005). If NT-4/5 treatment had delayed cell death, we would expect more staining in the TBI/NT4 group than the TBI/VEH group at the 2 week time point. However, FJB staining was not increased in any area of the hippocampus in the NT-4/5-treated animals. Very low levels of staining (typically 1-5 FJB-positive cells per section) were present in the CA1 and CA2/3 subfields of the hippocampus and slightly more staining in the dentate gyrus and hilus of TBI/VEH animals (Figure 3), indicating that the cells rescued by NT-4/5 infusion did not undergo delayed degeneration.

NT-4/5 treatment does not improve performance on hippocampal-dependent behavioral tasks

1. Visuo-spatial learning, anterograde paradigm—The Morris water maze is an accepted test of visuo-spatial learning in rodents. Anterograde learning was assessed by testing for the ability to learn to find a hidden platform over 6 trials, beginning at 1 week post-injury. All animals, regardless of injury and treatment status, exhibited a pattern of decreasing latencies to find the hidden platform in the maze over the six testing trials (Figure 4-A). Brain-injured animals showed a significant deficit in spatial learning ability compared to sham-injured animals at each trial, consistent with many previous reports of learning deficits in this model (reviewed in Thompson et al., 2005). Interestingly, NT-4/5 administration following LFP injury did not reduce the latency to platform for any of the 6 trials. At the end of the 6 trials, the SHAM/VEH animals located the platform after an average of 8 ± 1 s compared to 36 ± 4 s for TBI/VEH ($P < 0.001$ vs SHAM/VEH) and 33 ± 3 s for TBI/NT4 ($P < 0.001$ vs SHAM/VEH). It has been shown that LFP-injured and sham-injured rats do not differ in their swim speeds or paths in this model of TBI (Passineau et al., 2001, Lenzlinger et al., 2005).

A probe trial was performed on day 10 at 24h following the last learning trial. Consistent with previous reports, there was a significant effect of brain injury ($P < 0.001$ for TBI/VEH vs SHAM/VEH) but NT-4/5 treatment did not improve performance (Figure 4-B).

2. Associative learning, retrograde paradigm—Retrograde amnesia is a feature of human TBI. We hypothesized that brain-injured animals would show impairments in a model of retrograde amnesia, the contextual fear conditioning test. Foot shocks were administered to rats 24 hr before the injury/pump implantation. When returned to the context cage (in which the shocks were originally administered) at 14 days post-injury, SHAM/VEH rats responded by freezing on average $83\% \pm 5\%$ of the 5 min observation period (Figure 4-C). In contrast, TBI/VEH rats froze only $51\% \pm 9\%$ of the time ($P < 0.01$ vs SHAM/VEH), suggesting that they had impaired recall of the association of the context with the painful stimulus. NT-4/5 administration did not improve the injury-induced retrograde memory impairment: TBI/NT4 rats froze $48\% \pm 8\%$ of the time ($P < 0.01$ vs SHAM/VEH, NS vs TBI/VEH). As a control for freezing behavior due to generalized fear, animals were then placed in a novel context and freezing behavior was measured. All groups demonstrated similar low levels of freezing ($< 10\%$ of the observation period) when placed in a novel context (Figure 4-D).

3. Exploratory behavior and anxiety—Exploratory behavior was assessed using a modified version of the open field test at 13 days post-injury. The exploratory score of SHAM/VEH animals of 32 ± 6 entries across border zones was significantly higher than the score of TBI/VEH animals (14 ± 3 , $P < 0.05$). The TBI/NT4 group scored between the SHAM/VEH and

TBI/VEH groups (19 ± 4) but this value was not significantly different from either group (Figure 4-F). Total distance path was comparable in all groups (data not shown).

Neurological motor function

Brain injury induced a significant deficit in gross motor function as assessed using a composite neuroscore from day 2 to day 14 post-injury when compared to sham injury, regardless of treatment status (Figure 4-E). NT-4/5 administration did not improve the neurological function of treated animals at any time point.

Behavioral scores of injured animals are uncorrelated with CA3 pyramidal neuron number

To further address the possibility that neuronal number is not correlated with function in injured animals, we analyzed those animals in each group for which both neuronal counts and behavioral scores were available ($n=6/\text{group}$). Regression analysis showed that, within the injured groups, neuron number was *not* correlated with the final MWM score, the probe trial score or the percent freezing time (Fig. 5A-F). This held true for TBI/VEH and TBI/NT4 analyzed separately and for all the TBI animals grouped together (TBI/ALL).

NT-4/5 administration does not reduce hippocampal hypoexcitability following TBI

We next determined the effects of LFP brain injury and NT-4/5 administration on the function of CA3 pyramidal neurons. Input/output curves were generated from hippocampal slices acutely isolated at 2 weeks post-injury. A stimulating electrode was placed in the Shaffer collateral pathway (CA3 output) and excitability was recorded in the target CA1 dendritic region (Figure 4-G). In SHAM/VEH animals ($n=23$ slices), the mean fEPSP slope increased with stimulation intensity. The entire input/output curve from TBI/VEH animals ($n=28$ slices) was decreased by 16% on average when compared to SHAM/VEH animals, suggesting that this region is hypoexcitable following TBI (Figure 4-H). Treatment with NT-4/5 ($n=25$ slices) did not offset the TBI-induced decrease in excitability (17% decrease on average for TBI/NT4 vs SHAM/VEH).

DISCUSSION

We have confirmed that infusion of recombinant NT-4/5 is neuroprotective for CA3 pyramidal neurons after experimental TBI (Royo et al., 2006). In addition, this study shows that the cellular protection is not reflected by an improvement in behavioral or electrophysiological measures, suggesting that post-injury protection of pyramidal neurons does not ensure a corresponding recovery of hippocampal function.

Hippocampal damage has been documented in human TBI cases, and the resulting impairments in learning and memory are common, long-lasting and debilitating. These issues have led our group and many others to use hippocampal-dependent behavioral tests in rodent models to examine cognitive recovery from experimental TBI. The lateral fluid percussion model of TBI in the rat causes extensive cell loss in the hippocampus (Cortez et al., 1989, Lowenstein et al., 1992, Hicks et al., 1993, Smith et al., 1994, Hicks et al., 1996, Smith et al., 1997a, Pierce et al., 1998, Grady et al., 2003, Royo et al., 2006), an area which has been shown to play a central role in both the standard MWM learning paradigm and the contextual fear conditioning test (Hamm et al., 1993, Gewirtz et al., 2000, LeDoux, 2000, Anagnostaras et al., 2001, Sanders et al., 2003, Bannerman et al., 2004). Indeed, we confirmed a significant injury effect in both the MWM and CFC tests, consistent with many previous studies (for example Smith et al., 1991, Witgen et al., 2005). Although injury-induced cell death disrupts the circuit initially, our data call into question a causal relationship between protection of the CA3 neurons and post-injury performance in the MWM. Several groups have reported a treatment-related improvement in learning and/or memory after experimental TBI in conjunction with CA3

neuronal survival (Sanderson et al., 1999, Leoni et al., 2000, Kline et al., 2001, Philips et al., 2001, Floyd et al., 2002, Kline et al., 2002, Lee et al., 2004, Ozdemir et al., 2005, Statler et al., 2006). However, other reports have clearly documented learning deficits in brain-injured animals in the absence of discernable hippocampal damage (Lyeth et al., 1990) or the converse, an improvement in MWM performance after injury without CA3 neuroprotection (Dixon et al., 2003, Hoover et al., 2004).

We speculate that circuit function in the hippocampus ipsilateral to the injury may not be responsible for improvements in behavioral scores seen in the studies cited above. TBI-induced cell loss in CA3 causes a sharp (60%) loss of synaptic contacts in CA1 dendritic fields, but even a dramatic recovery of normal-appearing CA1 synapses after TBI (up to nearly 75% of preinjury levels by 60 days post-injury) was not reflected by the MWM acquisition scores (Scheff et al., 2005). Although cognitive tests such as the Morris water maze depend on an intact hippocampus, damage to other regions may also impair performance (reviewed in D'Hooge and De Deyn, 2001). Therefore, these intriguing results provide further evidence that a post-injury improvement on some behavioral tests could be due to plasticity in other brain regions rather than functional recovery of the injured hippocampal circuit.

Analysis of our own data showed that CA3 pyramidal cell number and behavioral outcomes were uncorrelated in injured animals. We addressed the concern that the MWM and CFC behavioral tests might not be sensitive enough to distinguish cell rescue by examining hippocampal circuit function directly, using electrophysiological recording in acutely isolated slices. Our data show that CA3 output is significantly impaired by the injury, as shown previously (Reeves et al., 1997, D'Ambrosio et al., 1999, Witgen et al., 2005), but is not improved by NT-4/5 treatment. It has previously been demonstrated that area CA1 decreased excitability caused by lateral fluid percussion injury does not appear to be mediated by presynaptic mechanisms but rather is due to augmented inhibition and decreased postsynaptic glutamatergic receptor function (Schwarzbach et al., 2006). In addition to decreased CA3 output and decreased CA1 excitability, other injury-induced changes to hippocampal excitability include loss of LTP induction, lack of post-tetanic potentiation in area CA1 ipsilateral to the injury and decreased paired-pulse inhibition. Fiber volley amplitude and LTD remain unaffected and the effect on paired-pulse facilitation may depend on the model used (Reeves et al., 1995, Reeves et al., 1997, Reeves et al., 2000, Schwarzbach et al., 2006). These parameters may be useful to examine in future studies of neuroprotective agents.

Our work therefore adds to a growing literature suggesting that cell survival, as measured by histological techniques (Nissl stain, FJB), is not sufficient to predict functional improvement as measured by standard techniques used by many groups in the field. More detailed electrophysiological analysis would be useful, with the caveat that single cell recordings could be difficult to interpret because "rescued" CA3 neurons cannot be distinguished from the rest of the CA3 neurons in an acutely isolated hippocampal slice at present. In addition, behavioral tests that more precisely reflect the function of CA3 neurons may be warranted (Nakazawa et al., 2002, Nakazawa et al., 2003).

It is unknown how long the rescued CA3 neurons persist in an inactive state. Fluoro-Jade staining showed that pyramidal cell death was not merely delayed by NT-4/5 treatment, suggesting that the rescued cells are likely to persist and may regain function over time as axonal transport is reestablished. Besides promoting cell survival, another possible role for NT-4/5 is suggested by the finding that continuous NT-4/5 administration promotes plasticity in hippocampal slice cultures by increasing spontaneous excitatory post-synaptic currents and the growth-associated protein GAP-43 (Schwyzer et al., 2002). Dumas and Sapolsky have pointed out that "little is accomplished if spared neurons do not function normally afterward" (Dumas and Sapolsky, 2001). We believe it is important to determine whether these

rescued neurons eventually undergo functional recovery at later times post-injury or whether their function is permanently impaired and the neuroprotection is to no avail.

The mammalian neurotrophins include NGF, BDNF, NT-4/5 and NT3. The NT-4/5 gene codes for a 26-kDa homodimeric protein of 123 amino acids, sharing 54% homology with BDNF (Ip et al., 1992, Ibanez, 1996, Salin et al., 1997). Although NT-4/5 and BDNF are both high-affinity ligands for the TrkB receptor, the two neurotrophins have distinct biological activities in the nervous system (Klein et al., 1992, Curtis et al., 1995, McAllister et al., 1995, Ryden et al., 1995, Strohmaier et al., 1996, Minichiello et al., 1998), and some populations of neurons require NT-4/5 but not BDNF for survival (Ardelt et al., 1994, Conover et al., 1995, Liu et al., 1995, Riddle et al., 1995). Furthermore, replacing the spatio-temporal expression pattern of BDNF with NT4/5 by 'knocking-in' NT4/5 to the BDNF locus does not rescue all of the deficits, providing direct evidence that the two neurotrophins have some non-overlapping actions (Fan et al., 2000). Interestingly, infusion of NT-4/5 but not BDNF is neuroprotective after LFP-TBI (Blaha et al., 2000, Royo et al., 2006). The mechanisms for this difference may include partially different binding sites on the trkB receptor (Klein et al., 1992), differential activation of a particular shc domain on the cytoplasmic portion of the trkB receptor (Minichiello et al., 1998), and/or differential downregulation of trkB (Frank et al., 1996, Knusel et al., 1997).

The specific mechanism by which NT-4/5 protects CA3 neurons after LFP-TBI has not yet been determined, but NT-4/5 signaling is known to interact with neuronal survival pathways. CA3 pyramidal neurons and hilar interneurons express the TrkB receptor (Yan et al., 1997) and the levels of TrkB in area CA3 are unaffected by LFP-TBI (Hicks et al., 1998, Royo et al., 2006). Signaling through the TrkB receptor activates the PLC γ /IP3/DAG, PI3K/Akt and MAPK/RSK pathways, promoting both cytoplasmic and nuclear events that contribute to cell survival (reviewed in Chao, 2003). We are currently characterizing BDNF- and NT-4/5-regulated genes in the injured brain and investigating the possibility of differential gene regulation by the two neurotrophins.

Numerous compounds have been tested for histological and functional neuroprotection in animal models of TBI, and a few of the successful studies showing neuroprotection and/or improvement in the Morris water maze have provided preclinical data for human trials. Pharmacologic strategies have included blocking calcium channels and the NMDA receptor and inhibiting lipid peroxidation, free radical generation and inflammation, among many others (see Maas, 2001, Maas et al., 2005). Unfortunately, to date, no neuroprotective agent has shown efficacy in a clinical trial in TBI patients (reviewed in Maas et al., 1999, Narayan et al., 2002, Tolia and Bullock, 2004). We propose that a more mechanistic understanding of cognitive recovery after TBI will prove to be very important in evaluating new compounds intended for future clinical trials.

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Comprehensive List of Abbreviations

aCSF, Artificial cerebrospinal fluid
 Akt, Akt or protein kinase B
 BDNF, Brain derived neurotrophic factor

BSA, Bovine serum albumin
 CE, Coefficient of error
 CFC, Contextual fear conditioning
 CV, Coefficient of variance
 DAG, Diacylglycerol
 fEPSP, Field excitatory postsynaptic potential
 FJB, Fluoro-Jade B
 IP3, Inositol 1,4,5-triphosphate
 LFP, Lateral fluid percussion
 MAPK, Mitogen-activated protein kinase
 MWM, Morris water maze
 NT-4/5, Neurotrophin-4/5
 PBS, Phosphate-buffered saline
 PI3K, Phosphoinositide-3 kinase
 PLC γ , Phospholipase C gamma
 RSK, Ribosomal S6 kinase
 TBI, Traumatic brain injury

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Figure 1. NT-4/5 administration reduces hippocampal pyramidal cell loss following traumatic brain injury (TBI) in rats

(A) Photomicrographs of area CA3 in cresyl violet-stained brain sections from (left panel) SHAM/VEH; (center panel) TBI/VEH; (right panel) TBI/NT4. Bar, 100 microns. (B and C) Hippocampus pyramidal cell number was estimated 2 weeks following LFP TBI in sham animals treated with vehicle (black column; n=6), TBI animals treated with vehicle (white column; n=6) and TBI animals treated with NT-4/5 (grey column; n=6). Percentages are normalized to vehicle-treated sham animals. (B) In the CA2/3, 45% cell death was observed in TBI animals treated with vehicle. This cell death was reduced to 29% in animals treated with NT-4/5. (C) In the hilus of the dentate gyrus, 34% cell loss occurred in TBI animals treated

with vehicle and 19% in animals treated with NT-4/5. Data are presented as mean $\pm$ SD ($\dagger$: $P < 0.05$ vs TBI/VEH; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ vs SHAM/VEH).

Figure 2. Distribution of infused NT-4/5

Immunohistochemistry for NT-4/5 was performed on coronal brain sections harvested 30h after brain cannula and osmotic pump implantation. NT-4/5 diffused several millimeters away from the tip of the cannula symbolized by the bar, into part of the parietal cortex and corpus callosum and throughout most of the ipsilateral hippocampus. Scale bar represents 1 mm. Cx: cortex; Th: Thalamus; Hi: hippocampus. Inset: Area CA of the hippocampus of an animal infused with vehicle. This section was immunostained for NT-4/5 in parallel as a control for specificity of NT-4/5 staining.

Figure 3. Hippocampal neuronal cell death 2 weeks following traumatic brain injury (TBI) was not delayed by NT-4/5 infusion

Semi-quantitative analysis of cell death was evaluated using Fluor Jade-B staining. Scarce cell death was observed in the ipsilateral hippocampus at 2 weeks following TBI, especially in the CA subfields. NT-4/5 administration was not associated with an increase in delayed cell death in CA, the dentate gyrus (DG) or the hilus. Data presented as mean $\pm$ SD (0= no cells; 1= 1-5 cells; 2= 6-20 cells; 3= more than 20 cells).

Figure 4. NT-4/5 treatment does not improve behavioral and physiological deficit following traumatic brain injury (TBI) in rats

(A) Anterograde visuo-spatial learning deficit was assessed in the Morris water maze (MWM) one week following TBI. The graph represents average latencies to find the platform over 6 testing blocks (2 blocks/day for 3 days; 4 trials/block). The learning curve of brain-injured animals show a significant deficit regardless of treatment. Data presented as mean $\pm$ SEM in seconds. (B) Probe trial scores of animals tested in the Morris water maze at 10 days after TBI. The deficit observed in brain-injured animals was unaffected by the NT-4/5 treatment. Data presented as mean $\pm$ SEM in arbitrary units. (C) Retrograde amnesia resulting from TBI was assessed using contextual fear conditioning (CFC) in animals trained 24 h prior to injury and tested 2 weeks after injury. The injury caused significantly less freezing behavior when animals were placed back in the test cage, while NT-4/5 treatment had no effect. (D) No injury or treatment effect was observed when animals were placed in a new context, as expected. Data presented as mean $\pm$ SEM in percentage of time over the 5 min observation period. (E) Neuromotor deficit in brain-injured animals was not altered by NT-4/5 administration. (F)

Exploratory behavior was evaluated 2 weeks following TBI in a circular open field. Brain-injured animals showed a deficit in exploration regardless of treatment when compared to SHAM/VEH. **(G)** Hippocampus excitability was assessed in acutely isolated hippocampal slices in the Shaffer collateral to CA1 pathway (schematic adapted from <http://www.bris.ac.uk/Depts/Synaptic/info/pathway/hippocampal.htm>). Positions of the stimulating (STIM) and recording (REC) electrodes are shown. **(H)** Brain-injured animals showed an overall decrease in hippocampal excitability that was not improved by NT-4/5 administration. Data are presented as mean $\pm$ SEM. For all panels: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for TBI/VEH vs. SHAM/VEH; † $P < 0.05$; †† $P < 0.01$; ††† $P < 0.001$ for TBI/NT4 vs. SHAM/VEH.

Figure 5. Regression analysis of behavioral scores and neuron number

Upper panels (**A, C, E**): For each behavioral test, the mean and one standard deviation of the full groups are shown. Individual scores are shown in circles (TBI/VEH, $n=13-14$) or triangles (TBI/NT4, $n=16$). Open circles or triangles indicate the six animals per group for which neuron counts were obtained; the neuronal counts are listed with the corresponding open symbol.

Lower panels (**B, D, F**): Scatterplot graphs showing the data from only those animals in which both neuron counts and behavioral scores were obtained ($n=6$ per group). Behavioral scores for each test are plotted as a function of neuronal number for the TBI/VEH group (circles) and the TBI/NT4 group (triangles). There was no significant correlation between neuron number and any behavioral score ($P>0.2$ in all cases). This was true for the injured groups analyzed separately or together (TBI/ALL).