Genetic and Pharmacological Intervention of the p75NTR Pathway Alters Morphological and Behavioural Recovery Following Traumatic Brain Injury in Mice

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Abstract

Primary objective: Neurotrophin levels are elevated after TBI yet there is minimal regeneration. It was hypothesized that the pro-neurotrophin/p75NTR pathway is induced more than the mature neurotrophin/Trk pathway and that interfering with p75 signaling improves recovery following TBI.

Research design: Lateral Fluid Percussion (LFP) injury was performed on wildtype and p75 mutant mice. In addition, TrkB agonist 7,8 Dihydroxyflavone or p75 antagonist TAT-Pep5 were tested. Western blot and immunohistochemistry revealed biochemical and cellular changes. Morris Water Maze and Rotarod tests demonstrated cognitive and vestibulomotor function.

Main outcomes and results: p75 was upregulated and TrkB is downregulated 1 day post LFP. p75 mutant mice as well as mice treated with the p75 antagonist or the TrkB agonist exhibited reduced neuronal death and degeneration and less astrocytosis. The cells undergoing apoptosis appear to be neurons rather than glia. There was improved motor function and spatial learning in p75 mutant mice and mice treated with the p75 antagonist.

Conclusions: Many of the pathological and behavioural consequences of TBI might be due to activation of the pro-neurotrophin/p75 toxic pathway overriding the protective mechanisms of the mature neurotrophin/Trk pathway. Targeting p75 can be a novel strategy to counteract the damaging effects of TBI.
Introduction

Traumatic brain injury (TBI) is an alternation in brain function, or other evidence of brain pathology, caused by an external force [1]. In recent years there is increasing literature deciphering the neuropathology associated with TBI, however the molecular mechanisms that account for the lack of regeneration following TBI are not well understood. TBI is divided into two stages: acute and chronic. The acute phase of injury can be initiated by a direct contusion to the brain or from shearing and stretching forces causing displacement of brain tissue [2, 3]. The acute and subacute stage lasts for hours after initial trauma and immediately results in changes in cell structure that lead to apoptosis and cell death. The chronic phase of injury persists for days after the initial event and is a continuation of cell death and neuronal degeneration in regions both focal and distal to the original site of injury [4, 5]. In addition, astrogliosis leads to glial scar formation that may impede recovery [6]. These injuries affect many processes including motor function, memory and spatial learning [7-11]. For example, in humans, hippocampal cell death can lead to verbal memory deficits, retrograde and anterograde amnesia, and learning deficits [12, 13]. Motor deficits are also prevalent after TBI, although they are more likely to persist after severe TBI [14].

Among the numerous physiological outcomes following both spinal cord injury and TBI is the induction of mRNA and protein expression of the neurotrophin family of growth factors. In both humans and animal models, brain-derived neurotrophic factor (BDNF) has been found to be upregulated following TBI in neurons and glia [15-20]. For example, transient increases in expression of
BDNF have been detected in the CSF of children with severe TBI [21, 22]. However, because past studies of BDNF levels after TBI have mainly focused on the message levels or the protein levels as determined by immunocytochemistry or ELISA, these previous studies could not discriminate between the neurotrophin protein isoforms which are known to have important functional consequences. Mature neurotrophins bind with low affinity to the p75 receptor however, pro-neurotrophins including proBDNF, proNGF, proNT3 and proNT4/5 bind with high affinity to the p75 receptor to induce cell death [23]. Although proNT3 and proNT4 binding to p75 have been associated with apoptosis [24] most studies have focused on the role of proBDNF and proNGF binding to p75 to induce cell death [25]; [26] as well as long-term depression [27]. In contrast, mature neurotrophins bind tightly to the tropomyosin-receptor kinase (Trk) receptors to mediate cell survival [28]; NGF binds to TrkA, BDNF and NT4/5 bind to TrkB, and NT3 binds primarily to TrkC [29]. In addition to cell survival, the interaction of mature BDNF with TrkB promotes differentiation and long-term potentiation in the hippocampus [30]. Based on the approaches previously used to analyze neurotrophin expression, it is unclear whether neurotrophins are predominantly present in their precursor or mature form and therefore which receptor-signaling pathway is activated following injury. Although it is known that p75 is upregulated after various forms of CNS injury [31, 32], the consequence of reducing p75 signaling in lateral fluid percussion injury has not been explored. In addition, due to the high expression of BDNF and TrkB in the hippocampus and cortex [33] and the well-described roles of BDNF in survival and plasticity, much
of the focus for prior studies has been on BDNF. Although BDNF has been demonstrated to potentiate recovery after acute injury [34, 35] and a very recent study demonstrates that a TrkB agonist increases activation of plasticity markers and enhances memory [36] however, conflicting studies show that mature neurotrophins have minimal beneficial effects [37, 38], indicating that the role of neurotrophins as well as the p75 and Trk receptor signaling following injury has not been definitely elucidated.

In this report we have systematically studied expression of different isoforms of neurotrophins and their receptors after moderate lateral fluid percussion injury in mice. Among the TBI models studied, LFP is the most reproducible and commonly used system to evaluate mixed focal and diffuse brain injury [39]. LFP recapitulates acute and chronic injuries observed in humans, rendering it clinically relevant, and allows for exploration of novel therapeutics for clinical translation [40]. We therefore examined the effects of genetic and pharmacologic manipulation of neurotrophin signaling on the cellular, biochemical and behavioural changes following LFP. To examine potential effects of both proBDNF and proNGF, we targeted the p75 receptor and to explore the role of mature BDNF, we activated the TrkB receptor. Our findings indicate that intervention with p75 signaling reduces apoptosis, neuronal degeneration and astrocytosis and improves outcome on two major objective measures of neurological function following injury: the Morris Water Maze (MWM) and Rotarod test to examine spatial learning and sensorimotor function, respectively.
Materials and Methods

Animals: All studies employed male mice ages 10-12 weeks. Wild type C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used for studies exploring receptor and ligand expression as well as pharmacological studies. B6.129-Ngfr\textsuperscript{tm1Jae/J} mice (Jackson Laboratories, Bar Harbor, ME) that had been backcrossed to C57BL/6J inbred mice were used for studies involving p75 mutant mice and their WT controls. p75 mutant mice have been reported to display abnormal sensory neuron innervation resulting in reduced sensitivity to heat and ulcers on their toes by 4 months [41], however since we used the mice prior to this age, we did not observe any phenotype. The procedures described were conducted in accordance with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee.

Lateral fluid percussion injury: Lateral fluid percussion brain (LFP) injury involves the displacement of neural tissue by a rapid fluid pulse to the brain and has been described in detail [42]. For surgery, animals were anesthetized with 4-5% isoflurane in 100% O\textsubscript{2} and placed in a mouse stereotaxic frame. Mice were maintained at 2% isoflurane and respiration was monitored throughout the procedure. A 3-mm thin plastic disc was fixed with Loctite glue (444 Tak Pak, Henkel Corporation, Rocky Hill, CT) onto the skull halfway between lambda and bregma, and between the sagittal suture and the lateral ridge over the right
hemisphere. Using a trephine (3mm outer diameter), a craniectomy was performed, keeping the dura intact. A rigid Luer lock needle hub (3 mm inside diameter) was secured to the skull over the opening with cyanoacrylate adhesive and dental acrylic (Butler Schein, Dublin, OH). The skull sutures were sealed with the cyanoacrylate to ensure that the fluid bolus from the injury remains within cranial cavity and the hub was filled with saline. After a 60 min period of recovery, the animals were re-anesthetized and connected to the fluid percussion injury device (Custom Design and Fabrication, Virginia Commonwealth University) through the Luer-loc fitting of the hub. Once a normal breathing pattern resumed, before sensitivity to stimulation, a ~1.5 ATM pulse (~15 msec) was generated through the LFP device. Upon return of righting reflex (4-10 minutes for moderate injury) the hub and dental acrylic were removed. The scalp incision was sealed with 3M Vetbond (Fisher Scientific, Waltham, MA) and the animals were returned to normal housing conditions. At this moderate level of injury, ~10% of animals died as a result of the injury within the acute post-traumatic period (15 min), generally from respiratory failure and pulmonary edema [39]. This is a normal and anticipated feature of the TBI model because it mimics human TBI [43]. Mice that undergo the surgical procedures but that were uninjured served as the sham controls. Assignment of mice to LFP or sham group was done in a random manner.

**Drug administration:** TrkB agonist, 7,8 Dihydroxyflavone (7,8 DHF) (Sigma, St. Louis, MO) was given intraperitoneally (IP) at 5 mg/kg [44-46]. p75 antagonist
TAT-Pep5 (Calbiochem, Billerica, MA) was given IP at 1.7 mg/kg [47, 48]; [49, 50]. Vehicle for both compounds was 6% dimethyl sulfate (DMSO)/0.1% Tween-20/phosphate buffered saline (PBS) and the vehicle controls were shared between groups to reduce the number of animals. Experiments using the compounds and the vehicles were done concurrently and WT mice were randomly assigned a treatment or vehicle group. Each mice was subjected to a dose of either vehicle, 7,8 DHF (22ul/g), or TAT-Pep5 (10ul/g) via an IP injection two hours following the LFP procedure. For Morris Water Maze testing, a daily dose of 7, 8-DHF or vehicle was administered 2 hours prior to testing. For the Rotarod test, 7, 8-DHF or vehicle was administered daily which on test days was also 2 hours prior to trials; TAT-Pep5 was administered on 1, 7, 14, and 21 dpi; 2 hours prior to trials on test days due to the prohibitive cost of more frequent treatments.

**Western blot analysis:** Tissue from the site of injury at the cortex as well as the ipsilateral hippocampus was flash frozen for biochemical analysis at 1, 7, and 21 days post injury. Five to seven total mice per time point for each sham and injured categories were analyzed. These tissues were solubilized either with RIPA lysis buffer (50ml 2X PBS, 1ml NP-40, 0.5 g Sodium Deoxycholate, 1ml 10% SDS, 47.5 ml deionized water and protease inhibitors) or T-PER with protease inhibitors and EDTA (Pierce, Rockford, IL). The protein lysates were then sonicated for two minutes and centrifuged for 10 min. The protein content of the supernatant was determined using the BCA Protein Assay Reagent Kit.
(Pierce, Rockford, IL). Equal amounts of protein were loaded onto NuPAGE or Tris Glycine gels (Invitrogen, Grand Island, NY). The proteins were transferred onto polyvinylidene difluoride (PVDF)-filter Immobilon-P transfer membranes (Millipore, Billerica, MA). Following blocking in 5% milk + 5% normal horse serum, primary antibody was applied overnight at 4°C. Specifically, 25µg of protein was run on a 12% MOPS NuPage gel and probed with BDNF antibody (1:200 sc-546, Santa Cruz Biotechnology, Inc, Santa Cruz, CA). p75, TrkB and proNGF expression were determined by loading 40µg of protein on a 4-12%Tris-Glycine gel and probing with p75 (1:200 sc-8317, Santa Cruz, Biotechnology, Inc., Santa Cruz, CA), TrkB (1:400 sc-12, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or proNGF (1:200 AN-005, Alomone Labs, Jerusalem, Israel) antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000, Biodesign, Saco, ME) was used as a loading control. Secondary anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated IgG antibodies were used (1:5000, GE Healthcare, South Plainfield, NJ). Proteins were visualized by chemiluminescence using the Enhanced Chemiluminescence (ECL) detection kit (Perkin Elmer, Waltham, MA). Levels of the immunopositive bands were quantified densitometrically using Quantity One version 4.2.1 software on a GelDoc 2000 (Bio-Rad, Hercules, CA). All data is normalized to the sample’s own GAPDH and expressed as a fold change relative to the average of the time matched sham controls.
**Immunohistochemistry:** Mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde at either 1, 7 and 21 dpi. The brains were cryoprotected in 30% sucrose and 20µm frozen sections were prepared throughout the site of injury on the cortex and the hippocampus in a 1:20 series so that the same set of tissue samples could be used for expression of different makers.

For activated Caspase-3 immunohistochemistry, sections were pretreated with 0.01M citrate buffer, then anti-cleaved Caspase-3 antibody (1:1000 9661, Cell Signaling, Danvers, ME) was applied overnight. Secondary goat anti-rabbit horseradish peroxidase (HRP) conjugated antibody (1:200 BA-1000, Vector Labs, Burlingame, CA) was applied for 1hr followed by diaminobenzidine (DAB) reaction and counterstaining with toluidine blue.

For Fluorojade C (FJC) staining, sections were pretreated with 1% NaOH and 0.06% KMnO4, then 0.0005% FluoroJade C (AG325 Millipore, Billerica, MA)/0.0001% 4',6-diamidino-2-phenylindole (DAPI) (D9564 Sigma, St. Louis, MO) was applied for 10min.

For double immunohistochemistry, anti-cleaved Caspase-3 (1:1000 9661, Cell Signaling, Danvers, ME) or p75 (1:1500 8232, Cell Signaling, Danvers, ME) antibody was applied overnight followed by secondary goat anti-rabbit 594 (1:250). Then NeuN (1:200 MAB377, Millipore, Billerica, MA) or GFAP (1:500 MAB3402, Millipore, Billerica, MA) antibody was applied overnight followed by secondary goat anti-mouse 488 (1:250). DAPI (1:1000, Sigma, St. Louis, MO)
was used to label nuclei and staining was visualized at 63X using a z stack on a LSM 510 Meta Zeiss Confocal.

Quantitation of immunohistochemistry: Staining was visualized on a Zeiss Axiophot microscope at 40x. For caspase and FJC eight animals per time point and each treatment were analyzed and for GFAP 4-5 mice were used. Positive cells on the ipsilateral hemisphere were counted in coronal sections representing a 1:20 series throughout the site of injury at the cortex and inclusive of the entire length of the hippocampus. For the cortex, a total of 6 fields of view at 40x (3 most dorsal along the surface of the cortex starting from midline and moving laterally and 3 just ventral to those fields) were counted on the side ipsilateral to the injury. The ipsilateral hippocampus including CA1 and CA3 as well as the dentate gyrus was used for quantitation of cells in the hippocampus.

Morris water maze (MWM): Mice were acclimated to the paradigm and tested for baseline response using a visible platform test 4 days prior to injury. The animals were placed in a small circular pool (1m diameter) of water containing non-toxic white paint and a clear platform for escape. To assess learning, mice were trained using a hidden platform fixed in one of 4 quadrants starting at various time points (1 and 21 dpi) in separate sets of mice. Black and white distal cues were placed on the walls. The quadrant in which the mouse was placed was pseudo-randomly varied throughout training and the time to locate the platform was recorded. Maximum trial time was 60 sec and the mouse remained or was
placed on the platform for 15 sec and warmed for 10 min between trials. For the
p75-/− studies mice were subjected to 8 trials/day for 3 consecutive days starting
at either 1 or 21 days post injury (a separate set of mice was used for each of the
two time points). Six to ten mice per genotype and condition were used for the
p75-/− studies. For the pharmacological treatments, a different set of 8 to 9 WT
mice per treatment group and condition were subjected to 4 trials/day for 6
consecutive days starting at 1 day post injury. Both training regimens resulted in
learning that had leveled out by the last 2 days of training, however the training
schedule was prolonged for the pharmacological studies to allow more time for
the drugs to be effective. Data was recorded using a video-tracking system
(EthoVision XT; Noldus Information Technology, Leesburg, VA).

Vestibulomotor rotarod test: A separate set of mice was used for motor testing.
Eight to 10 mice per treatment group and condition were used. Mice were
acclimated to the Rotarod device 3 times per day with 1-hour inter-trial intervals
for the two days prior to the injury. Balance and motor function was measured on
a 36-mm outer diameter, rotating rod whose velocity increases from 4 to 40 rpm
over a maximum 180 sec interval. Each trial ended when the animal fell off the
Rotarod. At 1, 7, and 21 dpi, each subject underwent 3 trials a day with 1-hour
inter-trial interval on the Rotarod device. The same mice were used for each time
point. The average latency to fall of injured mice was recorded using the Rod
programme and was compared to that of sham mice.
Statistical analyses: Statview software was used for analysis of all data. For Western blots, data were analyzed using two-tailed Student t-test comparing the injured mice to the average of the time-matched shams. For immunocytochemical cell count analysis, ANOVA followed by Fisher’s PLSD post hoc test for multiple comparisons was performed. p < 0.05 is considered significant.

Results

TrkB and p75 receptor and pro and mature neurotrophin expression patterns following LFP injury. In this study we have used a moderate LFP injury procedure in mice [42]. LFP has been shown to destroy the blood brain barrier at the site of injury as well as cause slow and continuous white matter degeneration and an increase in glial fibrillary acidic protein (GFAP) concentration at the site of injury [51, 52]. These responses demonstrate that LFP mimics both acute and chronic injury in animal models. We performed moderate LFP at the sensory-motor cortex and that also affects the underlying hippocampus and corpus callosum. Righting reflex times within 4-10 minutes were used to represent moderate injury resulting in deficits that hamper normal functioning and continued atrophy after the initial insult [53, 54]. Since studies have found that the craniectomy procedure performed prior to the LFP injury may also cause mild injury, we have included sham mice that have received craniectomies but no LFP injury as controls. We have also only analyzed the cortex and hippocampus ipsilateral to the injury. The
advantages of these approaches are that they will reduce the risk of confounding variables [55].

In order to examine the expression of pro and mature forms of the neurotrophins and their respective receptors, p75 and TrkB, tissue lysates from the cortical area surrounding the injury and the whole hippocampus were examined by Western blot analysis 1, 7 and 21 days post injury (dpi). Quantitation of protein levels in both regions showed an overall trend of reduced TrkB levels at all time points following injury with statistical significance at 21 dpi in cortex and 1 dpi in the hippocampus (Fig. 1A-C) when data are normalized to sham animals from the equivalent time point. The protein levels for p75 revealed an overall increase at all the time points with statistical significance of about 2-fold increase at 1 dpi in cortex and 21 dpi in the hippocampus (Fig. 1E-G). The co-receptor for p75, sortilin, also demonstrated an upregulation in the cortex at 1 dpi (Fig. 1H-I). No effect of injury was seen for sortilin in the hippocampus (data not shown). Therefore, the overall effect of LFP injury on receptor expression is a downregulation of TrkB as early as 1 dpi in the hippocampus and an upregulation of p75 as early as 1 dpi in the cortex.

To assess the expression of pro and mature BDNF protein levels, the same lysates were subjected to Western blot analysis and probed with an antibody that recognizes the mature 14kD, truncated 28kD and pro 36kD isoforms of BDNF. Quantitation of the protein levels of each of the isoforms in cortex (Fig. 2B, D, and F) and hippocampus (Fig. 2C, E and G) showed an increase at specific days following injury when normalized to protein levels from animals subjected to
sham surgeries from the equivalent time point. Specifically, the mature form of BDNF (14kD) showed a trend to an increase at 7 dpi in the cortex and at 7 and 21 dpi in the hippocampus. The 28kD truncated BDNF isoform showed little difference in the cortex after LFP injury, while in the hippocampus both at 1 and 21 dpi there was a statistically significant increase observed. The 36kD proBDNF isoform increased at 7 dpi in the cortex but there was no obvious change in the hippocampus at any time point. Since proNGF also binds to the p75 receptor, the protein lysates were analyzed for this neurotrophin as well. There was a trend to an increase in proNGF at all time points after TBI when compared to sham animals in the cortex with a significant increase at 7 dpi (Fig. 2I) with no apparent trend in the hippocampus (Fig 2J). In sum, we observed an overall trend to an increase in expression of the various isoforms of BDNF and NGF with a significant increase in the levels of pro-neurotrophins as early as 1 dpi in the hippocampus and 7 dpi in the cortex. Increased mRNA and protein expression of BDNF has been described in spinal cord and traumatic brain injury [15-20], however this is the first report of a change in expression of different isoforms of BDNF and their receptors along with proNGF after LFP injury.

**Effect of interventions with p75 signaling on neurodegeneration following LFP.**

To begin to explore the role of p75 signaling in the effects of LFP, we performed experiments on p75-/- mice and compared outcomes relative to WT mice. By using p75 mutant mice, we eliminated signaling from both proNGF and proBDNF ligands. To demonstrate the effect of these manipulations on neurodegeneration, Fluorojade C (FJC), a relatively new but widely used fluorescent dye [56, 57] was
used to identify neuronal degeneration after LFP injury both in the cortex and hippocampus. FJC positive cells appear smaller and slightly contracted when compared to normal neurons which is consistent with somatic degeneration. The number of FJC positive cells significantly increased subsequent to LFP injury in WT mice when compared to sham WT mice. The effect was observed as early as 1 dpi and persisted to 21 dpi although the effect was most apparent at 1dpi reflecting a decrease in the rate of cell death over time following TBI (Fig 3A-B, K-L). In p75/- mice subjected to LFP injury, the number of FJC positive cells showed a significant decrease in the cortex relative WT LFP mice at 1 dpi (Fig. 3B, D, and K,). No effect of the p75 mutation on FJC+ cells after injury compared to WT mice was observed in the hippocampus at any time point (Fig. 3L). There was no effect of the p75 deletion on numbers of FJC+ cells in sham relative to WT mice implying that p75 does not play a role in baseline neurodegeneration.

Since the effect of the LFP injury on FJC+ cells was most evident at 1 dpi, analysis of pharmacological treatments on WT mice was performed at that time point. The effect of pharmacological intervention on neurotrophin signaling was examined using either the short peptide p75 receptor antagonist TAT-Pep5 (1.7 mg/kg) or the small molecule TrkB agonist 7,8 Dihydroxyflavone (7,8 DHF) (5 mg/kg). The p75NTR inhibitor Pep5 (TAT-Pep5) is made cell-permeable by fusing it with the N-terminal protein transduction domain sequence (11 amino acids) from HIV protein TAT. TAT-Pep5 has been shown to act as an effective blocker of MAG- and Nogo-induced inhibition of neurite outgrowth in both dorsal root ganglion and cerebellar neurons [47, 48]. 7,8 DHF has been shown to have
potent neurotrophic activities [45] and improves motor function in a mouse model of Huntington’s disease [58]. Both compounds cross the blood brain barrier. Importantly, after LFP injury and treatment with 7,8 DHF or TAT-Pep5 the neurodegeneration as shown by FJC positive cells was significantly lower after 1 dpi in the cortex when compared to vehicle treated animals (Fig. 3M and O). Although treatment with TAT-Pep5 did show a significant reduction after 1 dpi in the hippocampus (Fig. 3P), the reduction in FJC positive cells was not apparent after treatment with 7,8 DHF (Fig. 3N). Neither compound had an effect on baseline FJC levels in sham mice. In sum, interfering with the p75 pathway either through genetic deletion or pharmacological intervention prevents cell degeneration following LFP at 1 dpi, primarily in the cortex. Furthermore, activating TrkB reduces cell degeneration in the cortex at 1 dpi.

*Analysis of cell death following LFP in mice with reduced p75 or enhanced TrkB signaling.* It is established that subsequent to TBI, there is neuronal loss by cell death and that apoptosis is associated with Caspase-3 activation [59]. We have thus quantitated activated Caspase-3 by immunohistochemistry following LFP injury in both in p75-/- mice as well as after treatment with pharmacological agents that affect neurotrophin receptor function. LFP injury results in a significant increase in the number of Caspase-3 positive cells when compared to sham or drug treated mice in both the cortex and hippocampus especially at 1 dpi (Fig. 4). There was an overall trend to fewer Caspase-3 positive cells in WT mice at 7 and 21 dpi in the injured cortex relative to the number of Caspase-3 positive cells at 1 dpi, again reflecting a decrease in the rate of cell death over
time following injury. p75−/− mice exhibited significantly fewer Caspase-3+ cells relative to WT mice at 1, 7 and 21 dpi in the cortex and at 7 dpi in the hippocampus (Fig. 4K and L). There was no effect of the p75 deletion on baseline numbers of Caspase-3+ cells in sham mice.

The effect of pharmacological intervention was examined at 1 dpi given that the highest number of Caspase-3+ cells was observed at that time point. After treatment with either 7,8 DHF or TAT-Pep5, cell death as measured by Caspase-3 positivity was significantly lower after 1 dpi when compared to vehicle treated animals subjected to LFP injury in the cortex (Fig. 4M and O). However, the reduction in Caspase-3 positive cells was less pronounced in the hippocampus after treatment with 7,8 DHF or TAT-Pep5 (Fig. 4N and P). Neither drug had an effect on baseline Caspase-3+ levels in sham animals. Together these data imply that blocking the p75 pathway genetically or pharmacologically or activating TrkB prevents cell death, primarily in the cortex, following LFP at 1 dpi.

**Identification of cell type affected by LFP.** To determine precisely which cells were undergoing apoptosis and which cells expressed p75, cortical tissue sections were stained with either activated Caspase-3 or p75 antibody and subsequently co-labeled with either NeuN or GFAP antibody to identify neuronal and glial cell types respectively. Both Caspase-3 and p75 positive cells also stained with NeuN (Fig. 5A and C) but not with GFAP (Fig. 5B and D) antibody after LFP injury. This finding suggests that the cells undergoing apoptosis after injury via activation of p75 expression and Caspase-3 signaling are most likely neurons rather than astrocytes.
**Effect of p75 deletion on astrocyte cell number following LFP.** Increased gliosis, as evidenced by cells expressing GFAP have been reported following TBI and these cells may be forming a glial scar, preventing regeneration [6]. GFAP is an intermediate filament protein distinctly localized to the cytoskeleton of mature astrocytes, the most abundant cell type in the central nervous system. Our evaluation of GFAP staining of cortical and hippocampal sections respectively showed a significant increase in GFAP positive cells in WT injured mice when compared to sham WT animals at 1 dpi (Fig. 6E-F). Quantitation of GFAP positive cells in injured p75 mutant mice demonstrated a decrease in the number when compared to WT LFP mice. There was no effect of the p75 deletion on baseline levels of GFAP+ cells in sham mice. These results indicate that the mice lacking p75 exhibit less gliosis in the cortex and hippocampus following injury.

**Analysis of learning in mice lacking p75 signaling.** To assess the neurofunctional outcomes of LFP injury, cognitive and motor tests were performed including the Morris Water Maze (MWM). Animals with hippocampal damage perform significantly worse than intact animals on the MWM and have a longer latency to find the platform indicating that this paradigm is an excellent measure of studying the effects of cell death on spatial learning memory [60]. We used the MWM test to measure impairments in spatial learning in mice subjected to LFP injury.

Latency in the ability to find the platform is increased in WT LFP mice when compared to WT sham mice when training is started at 1 dpi or 21 dpi in separate sets of mice (Fig. 7A-B). There is a progressive decrease in the latency
to locate the platform over a three-day training period in all the groups with a
leveling out in the last two days for the WT sham mice, indicating that learning is
taking place. When training is started at 1 dpi, the p75/- LFP mice have a
shorter latency to platform than the WT LFP mice and are similar to the p75/-
sham mice on day 1 of training (Fig. 7A). At 21 dpi the LFP-injured p75/- mice
show a significant improvement in their ability to find the hidden platform relative
to WT LFP mice on day 2 of training. The p75/- sham mice had longer latency
to platform relative to WT sham mice. These findings were consistent with the
results of pretesting in the MWM prior to injury, where p75/- mice exhibited a
trend to increased latency to platform prior to injury (WT 28.57 ± 2.17, p75/-
32.71 ± 2.08 sec, n = 14-21 mice/group, p > 0.05 ANOVA Fisher’s PLSD post-
hoc test). There was no significant effect of any of the treatment groups on swim
speed following injury (WT sham 23.52 ± 1.63, WT LFP 23.67 ± 1.40, p75/-
sham 22.32 ± 1.11, 21.63 ± 1.46 cm/sec, n = 7-9 mice/group, p > 0.05 ANOVA
Fisher’s PLSD post-hoc test). Together these data indicate that the effect
observed of improved learning in the p75/- mice is not due to a shorter baseline
time to platform because the p75 mutant mice have slightly impaired learning in
the absence of injury.

For pharmacological assessments of neurotrophin receptor signaling, all training
was initiated at 1 dpi. Vehicle controls were shared between the two treatment
groups to reduce the use of animals. Animals subjected to injury and treated
with TAT-Pep5 demonstrated significantly shorter latencies relative to vehicle-
treated LFP mice on multiple days of training (Fig. 7C). Conversely, when the
injured mice were treated with the TrkB agonist 7,8 DHF there was no improvement in cognitive function on any of the days of training (Fig. 7D). These data indicate that genetic or pharmacological intervention of p75 receptor signaling but not activation of the TrkB receptor can result in cognitive functional recovery following LFP injury.

Sensorimotor analysis of mutant or pharmacologically-treated mice following LFP. Deficits in motor functioning are a consequence of TBI that causes damage to the sensorimotor cortices and can be measured using the Rotarod test [61]. The Rotarod test involves placing animals on a rotating cylinder and measuring the amount of time the animals can stay balanced without falling; longer times indicate better motor coordination. In this study, average latency to fall was measured in a set of mice subjected to LFP and assayed at 3 time points: 1, 7 and 21 dpi. As shown in figure 8A, WT LFP mice consistently showed a shorter latency to fall when compared to WT sham mice. The latency to fall in injured mice is the shortest at 1 dpi implying that there is partial recovery of motor function over time. Interestingly, when we tested the LFP p75-/- mice on the Rotarod device, we found that these mice exhibited longer latency to fall times when compared to WT LFP mice at 1 and 7 dpi (Fig. 8A). There is no difference in the latency to fall in sham WT and p75-/- mice. Moreover, there was no difference in the latency to fall between p75-/- and WT mice during pre-testing prior to injury (WT 52.54 ± 5.99, p75-/- 64.64 ± 6.32 sec, n = 16 mice/group, p > 0.05, t-test), suggesting that p75 does not play a role in baseline sensorimotor function. When the effect of the two pharmacological agents modulating
neurotrophin receptor was measured after LFP on Rotarod performance, we saw that the p75 antagonist TAT-Pep 5 effectively increased the latency to fall when compared to vehicle LFP mice (Fig. 8B) at 1, 7 and 21 dpi. However, the TrkB agonist 7,8 DHF did not result in an increased latency to fall relative to vehicle mice at any of the time points. In sum, sensorimotor function can be improved after injury in mice in which p75 signaling is inhibited.

Discussion

In this study, we report that following LFP injury, p75 is upregulated and TrkB is downregulated in the cortex and hippocampus with more variable changes in the levels of the neurotrophin ligands. p75 mutant mice as well as WT mice treated with the p75 antagonist TAT-Pep5 or the TrkB agonist 7,8-DHF exhibited less neurodegeneration and cell death relative to control mice after injury. There was also less astrocytosis in mice lacking p75 expression. Behavioural experiments demonstrate that there is improved motor and cognitive function in p75 mutant mice and mice treated with TAT-Pep5 compared to control mice following LFP.

Increased mRNA and protein expression of BDNF has previously been described in traumatic brain injury and spinal cord injury [15-18], however this is the first report of a change in expression of different isoforms of BDNF and their receptors along with proNGF after lateral fluid percussion injury paradigm. The BDNF and TrkB levels after TBI studied so far have focused mainly on the message [15, 62-64] or protein levels determined either by immunocytochemistry
or ELISA, which do not allow for discrimination of the isoforms of the neurotrophin expression.

In this report we have investigated the total protein changes at three time points, 1, 7 and 21 days post injury to include a wide time frame when different biochemical processes are activated. Our data indicated a change in expression of both neurotrophin receptors starting at 1-day post injury (1 dpi); p75 receptor is increased whereas TrkB expression is reduced. This is consistent with previous findings that have determined as early as one day after initial injury there are distinct changes in cell structure leading to cell death [4, 5]. We have observed a reproducible change in receptor protein levels after lateral fluid percussion both in the cortex and the hippocampus indicating the effect of the external injury extends to distal regions including the hippocampus. It has been widely seen that after TBI, secondary injury can continue for days after the initial event and is associated with continuation of cell death in regions that are both focal and distal to the original site of injury [4, 5]. Although the change in protein levels measured at different time points showed an overall trend of a decrease in TrkB levels and an increase in p75 amounts, there are some variations in the extent of protein alterations at different time points. This can be attributed to either differences between animals or the slight unpredictability of the extent of injury.

Conflicting studies have reported that TrkB mRNA and protein levels either increase or decrease after TBI, however these studies differ with regard to timing and proximity to injury site of TrkB expression [62, 63, 65, 66]. The upregulation of p75 after injury is more consistently observed. In spinal cord injury models, the
expression patterns and timing of mRNA for p75 and Trk receptors is complex but p75 is increased as early as 6 hours after injury at sites proximal to the injury [67]. Moreover, a recent report using reverse phase protein microarray demonstrates that as long as 8 weeks after penetrating TBI, there is increase in p75 expression in the area surrounding the cavity [68]. Thus, p75 expression is induced quickly and is maintained for extended periods after injury. It has been shown that the concentration of the p75 receptor is increased in serum and CSF following TBI [22], suggesting that the upregulation of p75 could be used as a clinical biomarker. Moreover, p75 expression after other types of insults has also been reported including Alzheimer’s disease, corticospinal axotomy, seizures and hyper-osmolar stress [69-73]. Recently, microRNA-592 was found to regulate the expression of p75 and apoptotic-inducing activity following neuronal ischemic injury [26]. Whether MiR-592 also plays a role in the upregulation of p75 following TBI remains to be explored.

p75 binds to its sortilin co-receptor and activates the caspases involved in the intrinsic apoptotic cascade [74, 75]. Our results showed that following LFP, sortilin levels significantly increased in the cortex at 1dpi. Corticospinal neurons are protected from death following a lesion in mice lacking the p75 co-receptor sortilin [76], suggesting that the p75/sortilin cell death pathway could be contributing to the damage following LFP.

We observed variability in protein expression patterns with regard to the different isoforms of neurotrophins following LFP, but the overall trend of increase for mature and pro-neurotrophins in both the cortex and hippocampus was evident.
Some of the variability we observed between animals and isoforms may be caused by mild hypoxia resulting from brief apnea-like episodes that most mice experience immediately after the injury. We detected that the 14kD mature BDNF isoform increased by almost two fold after 7 dpi in the cortex and 7 and 21 dpi in the hippocampus. The 14kDa mature form, mBDNF has high affinity for tropomyosin-receptor kinase (TrkB) receptors [77, 78]. Upon binding to TrkB, mBDNF potentiates neuron survival, differentiation, and plasticity [79]. A recent report indicates that intranasal administration of the protease tissue plasminogen activator results in functional recovery after TBI in rats, suggesting that cleavage of proBDNF to mBDNF by TPA enhances recovery [80]. In vitro, mBDNF protects against glutamate toxicity [81-83] and oxygen deprivation [84] and in vivo, BDNF pretreatment protects against chemical-induced injury [85, 86] and axotomy [87, 88]. In spinal cord injury, mBDNF increases the number of axons, promotes regeneration, increases the amount of myelin and improves motor function [89-92]. Experimental models of cerebral ischemia demonstrated that mBDNF reduced infarct size, protects hippocampal neurons, and improves memory and motor function [93-99]. Thus, although mBDNF is protective in some systems, its upregulation in LFP may be too late or at insufficient levels to promote survival.

Interestingly, in the cortex LFP injury did not affect the 28kD truncated BDNF isoform which results from proBDNF processing by a calcium-dependent serine proteinase known as membrane-bound transcription factor protease site 1 (MBTPS-1) [100], while in the hippocampus both at 1 and 21 dpi there was a statistically significant increase in the 28kD truncated form. The larger 36kD
precursor proBDNF isoform, increased at 7 dpi in the cortex, with not much of a trend in the hippocampus. The p75 receptor also induces apoptosis when bound to proNGF [101, 102]. We show that proNGF levels are increased at all time points in the cortex. In sum, although proNGF is upregulated early, the increases in proBDNF occur slightly later than the upregulation of p75 which is detected as early as 1 dpi. Therefore the apoptotic pathway may be regulated by the enhanced receptor activation rather than the augmented presence of neurotrophin ligands. It would be of interest to examine the levels of the proteases that cleave pro-neurotrophins to see if they are regulated by LFP and control how much of the various isoforms are available.

Although cell death and degeneration were increased by LFP in WT and vehicle treated mice relative to sham controls, p75 mutant mice and mice receiving the p75 antagonist TAT-Pep5 had reduced neuronal damage. The TrkB agonist, 7,8 DHF was less effective, demonstrating that activating the TrkB pathway may not be sufficient to prevent the toxic effects of p75 signaling. Alternatively, the treatment schedule or dose of 7,8 DHF may not have been optimal to produce an effect in our assays. **One limitation of this study is that we cannot distinguish short-term (2 hr) versus longer-term (daily) effects of the compounds.** Although the mice were given treatments on a daily basis, on the day of behavioral testing or perfusion for histological analysis, the daily dose of drugs was administered 2 hours prior to the testing. This acute dose could have been sufficient for the drugs’ effects which can be explored in the future. In support of the effect of TrkB activation improving recovery after TBI, a very recent study demonstrates
that 7,8 DHF activates proteins related to plasticity and improves memory function after LFP which could be reversed by a Trk antagonist [36]. Moreover, another recent report demonstrates that TAT-Pep5 reduces lesion volume after controlled cortical impact to a similar degree as p75 deletion in mice [32]. Mature exogenous BDNF has proven protective during neuronal insult in some systems including spinal cord injury [89-92] and TBI [34]. In contrast, other reports reveal that mature neurotrophins have minimal beneficial effects [37, 38], suggesting mature neurotrophins are not sufficient for full recovery of neuronal function.

Perhaps once the cell death pathway is activated by p75 signaling, it is not possible to reverse the apoptotic signaling by promoting survival mechanisms. Studies have indicated that p75 receptors can be present on neurons after injury before the onset of the apoptotic pathway but TrkB only appears after the caspase-3 pathway has been induced [103] thus lending support to the idea that p75 receptor activation overrides TrkB activation and causes cell death during and after TBI. Moreover, proNGF can elicit apoptosis even when the Trk receptors are activated [72], again demonstrating that p75 can override the effects of Trk receptors.

The effects of the injury relative to sham on markers of apoptosis and axonal degeneration were most apparent at 1 dpi indicating that there is some endogenous recovery from the insult by 21 dpi. In parallel to these findings, the genetic and pharmacological interventions were most effective at 1 dpi for reducing cellular loss. Thus, early treatment can potentially inhibit the cellular and molecular pathways that cause long-term damage to the tissue.
Furthermore, the cortical area demonstrated higher numbers of dying and degenerating cells relative to the hippocampus suggesting that the immediate site of injury is more prone to damage than slightly distal regions. Finally, the number of activated capase-3 and FJC+ cells in the p75-/- mouse and mice treated with the p75 antagonist were more robustly reduced in the cortex relative to the hippocampus indicating that the interventions are most effective at the site of the highest damage.

The cells undergoing apoptosis and the cells expressing p75 after injury appear to be neurons rather than glial as determined by immunohistochemistry. This is in contrast to a previous study which showed that p75 is expressed on astrocytes following seizure-induced injury [104]. These variations in findings may be explained by the different forms of injury. In support of our results, induction of p75 expression after injury mediates an apoptotic response in neurons but not astrocytes [105]. We did observe an increase in astrogliosis following lateral fluid percussion in both the cortex and hippocampus that is reduced in the p75 mutant mice suggesting that there is some interaction between glia and the neurons that express p75. The glial scar is thought to prevent axonal regeneration [6]. Specifically, myelin-associated glycoprotein inhibits axonal outgrowth by interaction with the Nogo66 receptor and activation of the p75 receptor [106]. Therefore in the absence of p75 signaling, there may be less glial inhibition of axonal growth. The glial scar and increase in neurons expressing activated caspase-3 and p75 is likely to result in the cognitive and motor deficits observed following LFP relative to sham.
Cognitive function as assessed by the Morris Water Maze and sensorimotor function as determined on the Rotarod are both reduced following lateral fluid percussion injury although there is some natural recovery from the injury over time. There was a slightly longer latency to platform for the p75-/- mice prior to injury and after surgery the p75-/- sham mice had a longer latency to platform than WT sham mice. These observations are consistent with published findings that p75 mutant mice exhibit subtle behavioural impairments in MWM [107]. However, the p75-/- sham mice had a shorter latency to platform on day 1 of training relative to the WT LFP mice indicating that the injury paradigm is capable of exacerbating the effect of the p75 mutation. Thus our findings support the conclusion that the improved outcome in the p75-/- LFP mice relative to WT injured mice can be attributed to the absence of p75 signaling. Since swim speed was not altered by the injury in either WT or p75-/- mice, we can conclude that the injury induces less impairment in learning and memory and motor function when p75 signaling is silenced through either genetic or pharmacological treatment. However, the TrkB agonist did not have an effect on cognition or motor skills consistent with its lack of effect on histological markers and suggesting that activating the TrkB pathway is not sufficient to prevent damage due to the injury.

Several previous studies have manipulated neurotrophin and receptor expression levels to explore the effects on various forms of injury. BDNF+/- mice have improved motor function after stroke, suggesting that reducing BDNF levels enhances recovery [108] and while the authors attributed this to mature BDNF, it
is possible that the effect could be due to a reduction in proBDNF protein. Moreover, when fibroblasts transfected with BDNF cDNA are implanted into a lesioned brain, they enhanced neuronal survival and increased axonal growth only to a small degree [109, 110] which could be due to the fact that both mature and proBDNF levels are increased following transfection. Other methods to reduce p75 signaling have proven beneficial following injury. For example, transplantation of p75-suppressed bone marrow stromal cells promotes functional behaviour in rat spinal cord injury [111], supporting our results that p75 inhibition is beneficial to motor recovery. In addition, histone deacetylase inhibitors downregulate p75 receptors and apoptotic factors after controlled cortical impact and these treatments are associated with increased stem cell and decreased glial cell marker expression in the injured tissue [112]. Furthermore, cells expressing mutant neurotrophins that have been engineered to have reduced binding affinity to p75 when grafted into mice subjected to spinal cord injury result in enhanced axonal growth [113]. Finally, posttraumatic increases in p75 expression are caused by enhanced GABA\textsubscript{A}-mediated depolarization activating Rho kinase and this cascade can be blocked by the loop diuretic bumetanide to improve outcome [114]. These studies above have attempted to alter p75 or pro-neurotrophin expression, in addition to using genetically mutant p75 mice, we have used a pharmacological approach to specifically inhibit p75 activation.

In support of our findings, a different p75 antagonist, LM11A-31, promotes improved motor function and coordination after spinal cord injury. Treatment with
LM11A-31 following contusion injury results in an increase in the number of surviving oligodendrocytes and myelinated axons through inhibition of apoptosis via JNK3 [115]. The LM11A-31 compound also increases neurogenesis, inhibits neuronal death, and prevents activation of astrocytes and microglia following Controlled Cortical Impact model of TBI [116]. In contrast, LM11A-31 has been found to lack protective effects following pilocarpine-induced seizures [117]. Although our data indicate that p75 affect neuronal death and prevents glial activation, whether TAT-Pep5 affects other cellular processes such as myelination and neurogenesis remain to be explored.

The optimization of pharmacological agents that interfere with the p75 pathway should be performed in various models of TBI and spinal cord injury. Moreover, the effectiveness of blocking p75 signaling could be explored using other behavioural and cognitive tests. Although inhibiting the p75/pro-neurotrophin pathway appears to be more effective than activating the TrkB pathway, future studies will also examine a combinatorial effect. In conclusion, our observations illustrate that targeting p75 can be a novel strategy that is non-invasive to counteract the damaging effects of TBI.
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Figures Legends

FIG. 1. TrkB protein expression decreases and p75 and sortilin levels increase following LFP injury. (A, E, H) Representative Western blots showing TrkB, p75 and sortilin expression in the ipsilateral cortex (A and H) and hippocampus (E) (each lane represents one animal). (B-C, F-G and I) Quantitation of protein levels in the cortex and hippocampus, respectively. All data is first normalized to GAPDH to control for protein loading and then expressed as a fold change relative to the average ± SEM of the time matched sham controls which are represented as a single bar in the graph (n = 5-7 mice/group for TrkB and p75. n = 3-4 mice/group for sortilin). * p < 0.05, t-test relative to the sham control from that time point.

FIG. 2. pro-Neurotrohin expression increases following LFP. (A, H) Representative Western blots showing BDNF (mature 14kD, truncated 28kD and pro 36kD) and proNGF (26kD) expression in the ipsilateral cortex (A) and hippocampus (H). (B-G, I-J) Quantitation of protein levels in the cortex and hippocampus for BDNF (B-G) and proNGF (I-J). All data is first normalized to GAPDH to control for protein loading and then expressed as a fold change relative ± SEM to the average of the time matched sham controls which are represented as a single bar in the graph (n = 4,5 mice/group). * p < 0.05, ** p < 0.01, t-test relative to the sham control from that time point.

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**FIG. 3.** p75-/- mice and mice treated with p75 antagonist, TAT-Pep5 or TrkB agonist, 7,8 DHF have fewer FluorojadeC positive cells than WT or vehicle treated mice following LFP. (A-J) Representative images of cortical sections at 1 dpi stained with FJC. Scale bars = 100µm. (K-P) Quantitation of the total number of FJC+ cells per cortex or hippocampus ± SEM for WT or p75-/- mice (K-L) and mice treated with Vehicle, 7,8 DHF (M-N) or TAT-Pep5 (O-P) subjected to Sham or LFP procedures (n = 8 mice/group). * p < 0.05, ** p < 0.01, ANOVA Fisher’s PLSD post-hoc test relative to WT LFP or Vehicle LFP. # p < 0.05, ## p < 0.01, ANOVA Fisher’s PLSD post-hoc test relative to indicated groups.

**FIG. 4.** p75-/- mice and mice treated with p75 antagonist, TAT-Pep5 or TrkB agonist, 7,8 DHF have fewer Caspase-3 positive cells than WT or vehicle treated mice following LFP. (A-J) Representative images of cortical sections at 1 dpi (E-J) immunostained for activated caspase-3 (indicated by arrow). Scale bars = 100 µm. (K-P) Quantitation of the total number of caspase-3+ cells per cortex or hippocampus ± SEM for WT or p75-/- mice (K-L) and mice treated with Vehicle, 7,8 DHF (M-N) or TAT-Pep5 (O-P) subjected to Sham or LFP procedures (n = 8 mice/group * p < 0.05, ** p < 0.01, ANOVA Fisher’s PLSD post-hoc test relative to WT LFP or Vehicle LFP. There was no significant difference between any of the other groups by ANOVA Fisher’s PLSD post-hoc test.

**FIG. 5.** Activated Caspase-3 and p75 co-localize with neuronal but not glial markers after injury. (A-D) Representative images of cells from the cortex of mice subjected to LFP. Cortical sections stained with antibodies against Caspase-3
(red indicated by arrow) (A-B) or p75 (red indicated by arrow) (C-D) and NeuN (green) (A and C) or GFAP (green) (B and D). The overlay images also include DAPI nuclear stain. There is co-localization of Caspase-3 and NeuN (A) as well as of p75 and NeuN (C) as indicated by the overlap between red and green but not of Caspase-3 and GFAP (B) or p75 and GFAP (D). Scale bar = 30 µm.

FIG. 6. p75-/– mice have fewer GFAP+ astrocytes than WT mice following LFP. (A-D) Representative images of cortical sections from WT (A-B) and p75-/– (C-D) subjected to sham and LFP injuries and immunostained for GFAP at 1dpi. (E-F) Quantitation of the average number of GFAP+ cells per cortical and hippocampal section ± SEM (n = 4,5 mice/group). Scale bar = 500 µm. * p < 0.05, ** p < 0.01, ANOVA Fisher’s PLSD post-hoc test relative to WT LFP mice. There was no significant difference between any of the other groups by ANOVA Fisher’s PLSD post-hoc test.

FIG. 7. p75-/– and TAT-Pep5 treated injured mice have a shorter latency to platform on the Morris Water Maze. (A-B) Average latency to platform per day ± SEM for p75-/– or WT mice subjected to sham or LFP injuries at 1dpi (A) and 21dpi (B) (n = 6-10 mice/group). Each subject underwent 8 trials/day for 3 consecutive days beginning at 1 or 21 dpi. (C-D) Average latency to platform per day ± SEM for WT mice treated with vehicle, TAT-Pep5 (C) or 7,8 DHF (D) following sham and LFP injuries and beginning training at 1 dpi (n = 8,9 mice/group). Each subject in this series underwent 4 trials/day for 6 consecutive
days. All statistics are presented relative to WT LFP or vehicle LFP, * p < 0.05, ANOVA Fisher's PLSD post-hoc test.

**FIG. 8.** p75 mutant and TAT-Pep5 treated LFP mice have a longer latency to fall relative to WT LFP or Vehicle LFP mice at 1 and 7 dpi. Each subject underwent 3 trials per day on the Rotarod device. (A) Bars represent average latency to fall per day ± SEM at 1, 7, and 21 dpi for WT and p75/- mice subjected to sham or LFP injuries (n = 8-10 mice/group). (B-C) Average latency to fall per day ± SEM at 1, 7 and 21 dpi for mice treated with vehicle, TAT-Pep5 (B) or 7,8 DHF (C) following sham or LFP injuries (n = 8,9 mice/group) * p < 0.05, ** p < 0.01 relative to WT LFP or vehicle LFP, ANOVA Fisher's PLSD post-hoc test.
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127x127mm (300 x 300 DPI)
FIG. 2. pro-Neurotrophin expression increases following LFP. (A, H) Representative Western blots showing BDNF (mature 14kD, truncated 28kD and pro 36kD) and proNGF (26kD) expression in the ipsilateral cortex (A) and hippocampus (H). (B-G, I-J) Quantitation of protein levels in the cortex and hippocampus for BDNF (B-G) and proNGF (I-J). All data is first normalized to GAPDH to control for protein loading and then expressed as a fold change relative ± SEM to the average of the time matched sham controls which are represented as a single bar in the graph (n = 4,5 mice/group). * p < 0.05, ** p < 0.01, t-test relative to the sham control from that time point.

190x217mm (300 x 300 DPI)
FIG. 3. p75−/− mice and mice treated with p75 antagonist, TAT-Pep5 or TrkB agonist, 7,8 DHF have fewer FluorojadeC positive cells than WT or vehicle treated mice following LFP. (A-J) Representative images of cortical sections at 1 dpi stained with FJC. Scale bars = 100µm. (K-P) Quantitation of the total number of FJC+ cells per cortex or hippocampus ± SEM for WT or p75−/− mice (K-L) and mice treated with Vehicle, 7,8 DHF (M-N) or TAT-Pep5 (O-P) subjected to Sham or LFP procedures (n = 8 mice/group). * p < 0.05, ** p < 0.01, ANOVA Fisher’s PLSD post-hoc test relative to WT LFP or Vehicle LFP. # p < 0.05, ## p < 0.01, ANOVA Fisher’s PLSD post-hoc test relative to indicated groups.

75x53mm (300 x 300 DPI)
FIG. 4. p75R/R mice and mice treated with p75 antagonist, TAT-Pep5 or TrkB agonist, 7,8 DHF have fewer Caspase-3 positive cells than WT or vehicle treated mice following LFP. (A-J) Representative images of cortical sections at 1 dpi (E-J) immunostained for activated caspase-3 (indicated by arrow). Scale bars = 100 µm. (K-P) Quantitation of the total number of caspase-3+ cells per cortex or hippocampus ± SEM for WT or p75R/R mice (K-L) and mice treated with Vehicle, 7,8 DHF (M-N) or TAT-Pep5 (O-P) subjected to Sham or LFP procedures (n = 8 mice/group * p < 0.05, ** p < 0.01, ANOVA Fisher’s PLSD post-hoc test relative to WT LFP or Vehicle LFP. There was no significant difference between any of the other groups by ANOVA Fisher’s PLSD post-hoc test. 104x80mm (300 x 300 DPI)
FIG. 5. Activated Caspase-3 and p75 co-localize with neuronal but not glial markers after injury. (A-D) 
Representative images of cells from the cortex of mice subjected to LFP. Cortical sections stained with 
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There is co-localization of Caspase-3 and NeuN (A) as well as of p75 and NeuN (C) as indicated by the 
overlap between red and green but not of Caspase-3 and GFAP (B) or p75 and GFAP (D). Scale bar = 30 
µm. 
52x73mm (300 x 300 DPI)
FIG. 6. p75-/- mice have fewer GFAP+ astrocytes than WT mice following LFP. (A-D) Representative images of cortical sections from WT (A-B) and p75-/- (C-D) subjected to sham and LFP injuries and immunostained for GFAP at 1dpi. (E-F) Quantitation of the average number of GFAP+ cells per cortical and hippocampal section ± SEM (n = 4.5 mice/group). Scale bar = 500 µm. * p < 0.05, ** p < 0.01, ANOVA Fisher’s PLSD post-hoc test relative to WT LFP mice. There was no significant difference between any of the other groups by ANOVA Fisher’s PLSD post-hoc test.

139x84mm (300 x 300 DPI)
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