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Cortical Brain Injury Causes Retrograde Degeneration of Afferent Basal Forebrain Cholinergic Neurons via the p75NTR

Abbreviated title: p75NTR-mediated axonal degeneration following TBI

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Abstract

Traumatic brain injury elicits neuronal loss at the site of injury and progressive neuronal loss in the penumbra. However, the consequences of TBI on afferent neurons projecting to the injured tissue from distal locations is unknown. Basal forebrain cholinergic neurons (BFCNs) extend long projections to multiple brain regions including the cortex, regulate many cognitive functions and are compromised in numerous neurodegenerative disorders. To determine the consequence of cortical injury on these afferent neurons, we used the Fluid Percussion Injury (FPI) model of traumatic brain injury and assessed the effects on BFCN survival and axon integrity in male and female mice. Survival or death of BF neurons can be regulated by neurotrophins or proneurotrophins, respectively. The injury elicited an induction of proNGF and proBDNF in the cortex, and a loss of BFCNs ipsilateral to the injury compared to sham uninjured mice. p75NTR knockout mice did not show loss of BFCN neurons, indicating a retrograde degenerative effect of the cortical injury on the afferent BFCNs mediated through p75NTR. In contrast, locus coeruleus (LC) neurons which also project throughout the cortex were unaffected by the injury, suggesting specificity in retrograde degeneration after cortical TBI. Proneurotrophins (proNTs) provided directly to basal forebrain axons in microfluidic cultures triggered retrograde axonal degeneration and cell death, which did not occur in the absence of p75NTR. This study shows that after traumatic brain injury, proNTs induced in the injured cortex promote BFCN axonal degeneration and retrograde neuron loss through p75NTR.

Significance Statement

TBI is well-known to elicit direct neuronal loss at the site of injury and secondary loss in the penumbra, however the effect on afferent neuronal populations that project axons from distal locations such as the basal forebrain, has not been elucidated. Basal forebrain cholinergic neurons (BFCNs) project to a myriad

of brain regions and regulate cognitive processes such as learning, attention, and memory, and are compromised in neurodegenerative diseases such as Alzheimer's disease. These neurons constitutively express p75NTR, a receptor that can promote neuronal degeneration following injury. We demonstrate here that cortical injury promotes degeneration of afferent BFCNs, mediated by p75NTR, indicating that TBI causes neuronal loss in brain regions distal to the site of injury via retrograde axonal degeneration.

Introduction

Traumatic brain injuries have immediate as well as long term neurological consequences and thus progressively affect behavior and quality of life over time. Primary and secondary degeneration as a consequence of TBI has been studied in detail with respect to effects on the injured region and the surrounding penumbra (Loane and Faden, 2010; Montroull et al., 2020; Raghupathi et al., 2000). Secondary injury after cortical TBI includes neuronal, glial, and white matter loss (Raghupathi et al., 2000). Studies using the Fluid Percussion Injury (FPI) model of TBI show a massive change in the injury microenvironment, with an acute inflammatory response, excitotoxicity, increase in reactive oxygen species, as well as changes in neurotrophin mRNA and protein expression (Schimmel et al., 2017; Thompson et al., 2005) which may affect not only the cells in the penumbra but also neuronal populations that extend their axons to cortical targets from distal locations in the brain. Basal forebrain cholinergic neurons (BFCNs), through long and extensive axonal projections, release acetylcholine in the cortex to regulate cognitive functions such as emotion, attention, and memory (Boskovic et al., 2019). They are composed of several nuclei among which the nucleus basalis of Meynert (NBM) and substantia innominate (SI), comprising the Ch4 cluster, innervate the cortex (Mesulam et al., 1983; Rye et al., 1984). BFCNs require neurotrophic factors for their survival, differentiation, maintenance, and function (Nonomura et al., 1995) which are produced by their neuronal target regions, and signal via cognate

receptor complexes through the projecting axon terminals. BFCNs are unique in their expression of all the neurotrophin receptors, including the pan-neurotrophin receptor p75NTR as well as the receptor tyrosine kinases TrkA, TrkB and TkC throughout life. Mature neurotrophins (NTs) promote a pro-survival response through Trk signaling (Alderson et al., 1990; Hefti et al., 1985 Friedman et al., 1993; Kromer, 1987), while proneurotrophins (proNTs) bind p75NTR and sortilin to promote apoptosis in BFCNs (Volosin et al., 2006). The degenerative role of p75NTR in BFCNs has been established in mass cultures and after seizure

conditions in vivo (Volosin et al., 2006). Proneurotrophins, which are high affinity ligands for p75NTR, are upregulated in the region of injury (Alder et al., 2015; Sebastiani et al., 2015), and they promote apoptotic signaling in the injury penumbra in the cortex via p75NTR (Montroull et al., 2020), which is a known contributor to secondary neurodegeneration after TBI (Delbary-Gossart et al., 2016; Alder et al., 2015; Montroull et al., 2020). However, whether the changes in the neurotrophic environment of BFCN terminals after TBI has a retrograde effect on survival of afferent projections has not been investigated. The constitutive expression of p75NTR in BFCN throughout life, coupled with the complexity of maintaining an elaborate axonal arbor may make BFCNs vulnerable to degeneration. Recent studies suggest that TBI increases the risk of neurological disorders such as Alzheimer's disease (Tajiri et al., 2013), and BFCN loss is a hallmark of this disease (Whitehouse et al., 1982, 1983).

In our study, moderate cortical FPI was used to investigate the retrograde effect of cortical injury on the projecting BFCNs. Retrograde degeneration through p75NTR has been studied in peripheral neurons (Sørensen et al., 2003; Yano et al., 2009), but not in the context of brain injury and its effect on afferent neurons that project to the injury site from distal locations. We investigated the effect of cortical injury on the afferent BFCN neurons and compared p75NTR KO mice with WT mice to understand the role of p75NTR in retrograde BFCN degeneration after injury. To specifically determine the effects of proNTs on retrograde axonal degeneration of basal forebrain neurons, neurons were cultured in

microfluidic chambers to investigate whether direct stimulation of axon terminals with proneurotrophins can signal through p75NTR to affect BFCN axon integrity and cell death. These findings indicate that the constitutive expression of p75NTR in BFCNs promotes cell type specific retrograde degeneration of BFCNs following cortical TBI.

Materials and Methods

Reagents

Recombinant human proNGF (cleavage resistant) protein (Cat# N-285) and recombinant mouse proBDNF (cleavage resistant) protein (Cat# B-243) were purchased from Alomone Labs (Tel Aviv, Israel). Poly-d-lysine, glucose, transferrin, insulin, putrescine, selenium, progesterone, penicillin, and streptomycin were purchased from Sigma Aldrich. Minimum Essential Medium (MEM), Ham's F-12 Media and B-27™ Plus Supplement (50X) (Cat# A3582801) was purchased from Gibco. Microfluidic chambers were prepared using Microfluidic chamber master molds which were a generous gift from Dr. Eran Perlson, Tel Aviv University, using the protocol described by (Harris et al., 2007). Cholera Toxin Subunit B (recombinant), Alexa Fluor™ 488 Conjugate (CTB) (Cat# C34775) was purchased from Invitrogen. Propidium Iodide (PI) (Cat# P1304MP) was obtained from Molecular Probes. Cytoplasmic dynein inhibitor, Ciliobrevin D (Cat# 250410) was purchased from Calbiochem. Antibody to BDNF (Cat# 327-100, RRID:AB_2927780) was obtained from Icosagen. Anti-NGF (Cat# N6655, RRID:AB_477660) and mouse- anti- β- actin (Cat# A5441, RRID:AB_476744) antibodies was purchased from Sigma Aldrich. Goat anti-choline acetyltransferase (ChAT) (Cat# AB 144-P, RRID:AB_2079751), and rabbit-anti-p75NTR (Cat# 07-476, RRID:AB_310649) were purchased from Millipore. Goat-anti-p75NTR antibody (Cat# AF1157, RRID:AB_2298561) was purchased from R & D Systems. Mouse-anti-tyrosine hydroxylase (TH) (Cat# 58844S, RRID:AB_2744555) was

obtained from Cell Signaling Technology. Mouse-anti- β-Tubulin III (Tuj1) (Cat# G712A, RRID:AB_430874) antibody was purchased from Promega. Alexa Fluor-488 (Cat # A-11055) and Alexa Fluor-555 (Cat # A-31572) anti-goat and anti-rabbit secondary antibodies respectively, Alexa Fluor- 647 anti-mouse secondary antibody (Cat # A32787) were purchased from Invitrogen. Donkey-anti-goat Alexa Fluor 647 (Cat# 705-607-003, RRID:AB_2340439) was obtained from Jackson ImmunoResearch. LICOR mouse 800 (P/N: 926-32210, RRID AB_621842), rabbit 800 (P/N: 926-32213, RRID AB_621848), mouse 680 (P/N: 926-68020, RRID AB_10706161) secondary antibodies for Western blots were purchased from LICOR. Fast blue (5%) was purchased from Polysciences, (Cas no: 73819-41-7). DRAQ5 (Code# DR05500) was obtained from Biostatus. Fluoromount-G (Cat# 0100-01), DAPI Fluoromount-G (Cat# 0100-20) was obtained from Southern Biotech.

Mice

All experiments were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) policies and approved by Rutgers University, Newark. Adult mice between the ages of 2 and 3 months were maintained on a 12h light/dark cycle with free access to food and water. Wild type (WT) mice were purchased from The Jackson Laboratory. p75NTR global KO mice with an exon III deletion (Lee et al., 1992) are bred in house. Both males and females were used in all experiments.

Neuronal cultures

WT and p75NTR KO pregnant mice were euthanized by exposure to CO_2 and soaked in 70% ethanol for 5 min for sterilization. Embryonic day 15 (E15) mouse fetuses were removed under sterile conditions and kept in PBS on ice. Basal forebrains were dissected and dissociated in serum-free medium (SFM) (Friedman et al., 1993) comprised of a 1:1 mixture of Eagle's MEM and Ham's F-12 supplemented with glucose (6 mg/ml), putrescine (60 µm), progesterone (20 nm), transferrin (100 µg/ml), selenium (30 nm), penicillin (0.5 U/ml), and streptomycin (0.5 µg/ml). The cells were then plated in microfluidic chambers

(Taylor et al., 2005) attached to glass coverslips in tissue culture dishes that were precoated overnight with poly-d-lysine (0.2 mg/ml). 200µl of media was added to the soma compartment while 100µl media was maintained in the axon compartments to maintain a media volume difference that facilitated the growth of axons through the microgrooves towards the distal compartment. The cells were maintained with the volume difference between the soma and axon compartments in SFM supplemented with 1% B-27 for 5 days at 37°C to obtain compartmentalized BFCN cultures which could be treated separately at the axons or somas.

Live Imaging of BFCNs in Microfluidic cultures

BFCN microfluidic cultures were prepared for live imaging after 5 days *in vitro* (DIV). The axon compartment was treated with Alexa488 labeled choleratoxin B (CTB, 1µg/ml), a retrograde tracer, for 20min and washed twice with SFM+1%B27 to retrogradely label the BFCNs which extended axons to the distal compartment through the microgrooves. After 5h, the CTB from the axons was found to be transported into the cytoplasm of the BFCNs that projected axons to the distal compartment. The soma compartment was treated with propidium iodide (PI, 1µg/ml) to label dying neurons. BFCNs were then treated with proNGF (20ng/ml) or proBDNF (40ng/ml) in the axon compartment and compared with control untreated compartmentalized BFCNs to assess the effect of axonal stimulation with proNTs on neuronal degeneration. Growth media volume difference was maintained as described in the neuronal culture method to restrict stimulation with proNTs exclusively to the axons. To assess surviving versus dying neurons, live imaging of neurons was performed using a Zeiss LSM 510 confocal microscope maintaining constant temperature (37°) and CO_2 (5%) for the duration of the experiment. Incorporation of PI in the nucleus of CTB positive neurons after 24h axonal treatment was assessed.

Lateral Fluid Percussion Injury (FPI)

Traumatic brain injury (TBI) was induced in mice using the FPI model following a protocol adapted from Alder et al. (2011) Adult mice (3- 5 months of age) were anesthetized with ketamine (80mg/kg) and xylazine (10mg/kg). Craniotomy was performed on the right cortical hemisphere midway between Bregma and Lambda, 2 mm lateral to the midline, 3mm in diameter. 1 day post craniotomy we performed a moderate FPI using 30PSI or 2ATM injury pressure. Sham mice underwent craniotomy but were not subjected to FPI. Sham and injured mice were injected with buprenorphine (0.05mg/kg weight) after the craniotomy and the injury. Injured and sham mice were perfused 1 to 14 days post injury (DPI) to obtain brain sections for immunohistochemistry or euthanized by CO_2 exposure to obtain brain lysates for analysis by western blot.

Western blot analysis of TBI mouse brains

Sham and TBI mice were sacrificed 1 day post injury (DPI), 3 DPI or 7DPI by exposure to CO_2. Brains were dissected on ice to obtain the area of craniotomy in cortex, as well as the basal forebrain tissue from the injured and uninjured hemisphere and lysed in 300µl of RIPA lysis buffer comprised of NP40 (10%), deoxycholic acid (10%), SDS (10%), EDTA (0.5M), NaCl (5M), Tris (1M) and protease and phosphatase inhibitors. After protein quantification, equal amounts of protein were run on a 15% polyacrylamide gel and transferred to nitrocellulose membrane. Equal protein loading was assessed by Ponceau staining, which was washed out with TBS with 0.05% Tween 20 (TBST). The membranes were then blocked with 5% nonfat milk prepared in TBST for 1 hour and incubated with primary antibodies to BDNF (Cat# 327-100, RRID:AB_2927780) or NGF (Cat# N6655, RRID:AB_477660) overnight. After washing 3 X 10 min with TBST, the blots were incubated with appropriate secondary antibodies for 1 h at room temperature. The membrane was washed 3 X 10 min with TBST and then scanned with the Odyssey infrared imaging system (LI-COR Bioscience). The same procedure was repeated with antibodies to β- actin (Cat# A5441, RRID:AB_476744). LC tissue was harvested from Naïve WT and p75KO mice and processed for

quantifying the expression of p75NTR using rabbit-anti-p75NTR (1:1000, Cat# 07-476, RRID:AB_310649, TH using mouse-anti-tyrosine hydroxylase (TH) (1:1000, Cat# 58844S, RRID:AB_2744555), and β- actin. All blots shown are representative of at least three independent experiments.

Immunocytochemistry

Basal forebrain microfluidic cultures were fixed with 4% paraformaldehyde for 20 min, washed with PBS and permeabilized with 0.5% Triton X-100 in PBS for 10min. The cells were then blocked for 1h with 5% normal goat serum and 1% bovine serum albumin (BSA) in PBS and incubated overnight at 4°C with primary antibody prepared in 1%BSA in PBS. Primary antisera were directed against β-Tubulin III (Tuj1) (mouse 1:1000, Cat# G712A, RRID:AB_430874), rabbit-anti-p75NTR (1:1000, Cat# 07-476, RRID:AB_310649), goat-anti-choline acetyltransferase (CHAT) (1:1000, Cat# AB 144-P, RRID:AB_2079751). Cells were then washed with PBS, exposed to the appropriate secondary antibodies coupled to different fluorophores, and highly cross-adsorbed against different species (Alexa 488, Alexa 594 and Alexa 647; Invitrogen). Coverslips were mounted on slides using DAPI Fluoromount-G to label the nuclei. Images were obtained using Zeiss LSM 510 META confocal microscope and analyzed to measure axon fragmentation using Image J software. Degeneration index was calculated as the ratio of the area of fragmented axons over the total area of axons (intact axons + fragmented axons) by using Tuj1-stained fluorescence images. A total of 8 images were analyzed per chamber. All images were processed using ImageJ software. To analyze size fragment of particles, binary masks were created of each image. Particles with a size area equal or lower than 60 μm^2 and with a circularity index higher than 0.03 were classified as degenerated neurites fragments.

Immunohistochemistry

TBI and sham animals were anesthetized with ketamine/xylazine 7DPI and 14DPI and perfused with PBS followed by 4% paraformaldehyde. After perfusion, the brains were removed and postfixed in 4%

paraformaldehyde overnight and cryoprotected in 30% sucrose for 2 days. 20µm sections were obtained using a cryostat (Leica, Nussloch, Germany) and mounted onto charged slides and stored at -20ºC. The basal forebrain was analyzed by staining coronal sections starting from Bregma 0.50mm to Bregma 0.50mm, with an interval of 200µm between each section spanning the diagonal band of Broca (DBB), NBM and SI which comprise the Ch4 nuclei of the basal forebrain (Mesulam et al., 1983). To analyze the locus coeruleus, coronal brain sections (20 µm) starting from Bregma -5.00mm to Bregma -6.0mm, with an interval of 200µm between each section were processed. Sections were processed as above and then exposed overnight at 4°C to the following primary antibodies: anti-rabbit-p75NTR (1:1000, Cat# 07-476, RRID:AB_310649), anti-goat-choline acetyltransferase (CHAT) (1:1000, Cat# AB 144-P, RRID:AB_2079751), anti-mouse-tyrosine hydroxylase (TH) (1:1000, Cat# 58844S, RRID:AB_2744555) diluted in 1%BSA in PBS. The next day slides were washed three times in PBS for 10min each and exposed for 1 h at room temperature to secondary antibodies coupled to the Alexa 488 or 555 fluorophores (1:1000) prepared in 1%BSA in PBS. Sections were then washed again in PBS three times for 10min each. Sections were coverslipped with DAPI Fluoromount-G and analyzed by fluorescence microscopy using Nikon Eclipse microscope. ChAT and p75NTR labeled neurons in the basal forebrain and TH labeled neurons in the locus coeruleus were counted using Image J software.

Fast Blue Injection and retrograde tracing in vivo

Following craniotomy on the right cortical hemisphere midway between Bregma and Lambda, 2 mm lateral to the midline, 3mm in diameter, adult WT mice were injected with Fast blue (0.25%) (Polysciences, Cas no: 73819-41-7) in 5 injection sites, at 2 layers in the cortex: in the center of the craniotomy, and 4 injections 1.5mm from the center diametrically opposite to each other, 90 degrees apart. Injections were performed at a rate of 35 nanolitres/min, 30 nanolitres per injection site, at 300 µm and 450 µm depth, targeting the cortical layers 4 and 5, which receive projections from the nucleus

basalis and substantia innominate of the basal forebrain, with a 5 min wait period between each injection. Injected brains were harvested 14 days after injection. Sections of the basal forebrain and LC were obtained as described for the immunohistochemistry, and processed by immunostaining for rabbit-anti-p75NTR (1:1000, Cat# 07-476, RRID:AB_310649), goat-anti-choline acetyltransferase (CHAT) (1:1000, Cat# AB 144-P, RRID:AB_2079751), mouse-anti-tyrosine hydroxylase (TH) (1:1000, Cat# 58844S, RRID:AB_2744555) and DRAQ5 (1:1000, Biostatus, Code# DR05500). Sections were coverslipped with Fluoromount-G and Fast blue + cells were analyzed by fluorescence microscopy using Nikon Eclipse microscope and processed using Image J software.

Whole mount Imaging

Fixed adult mouse brains with unilateral FPI treatment to induce TBI were delipidated with a modified Adipo-Clear protocol (Hou et al., 2021). Briefly, perfusion fixed brain samples were washed with B1n buffer (H2O/0.1% Triton X-100/0.3 M glycine, pH 7), then transferred to a methanol gradient series (20%, 40%, 60%, 80%) in B1n buffer, 4 mL for each brain, 1 h for each step; then 100% methanol for 1 h; then overnight incubation in 2:1 mixture of DCM: methanol and a 1.5 h incubation in 100% DCM the following day; then 100% methanol for 1 h three times, and reverse methanol gradient series (80%, 60%, 40%, 20%) in B1n buffer, 30 min for each step. Samples were then washed in B1n buffer for 1 h and overnight. The above procedures were done at room temperature with rocking to complete delipidation. The delipidated samples were then blocked in PTxwH buffer (PBS/0.1% Triton X100/0.05% Tween 20) with 5% DMSO and 0.3M glycine for 3 h and overnight at 37°C, then washed with PTxwH for 1 h, 2 h, and overnight at room temperature. For staining, brain samples were incubated in primary antibody (R&D goat-anti-p75NTR, 1:500, Cat# AF1157, RRID:AB_2298561) diluted in PTxwH for 14 days at 37°C. After primary antibody incubation, samples were washed in PTxwH for 1 h, 2 h, 4 h, overnight, then 1 d three times, and then incubated in secondary antibody (Jackson ImmunoResearch 705-607-003, Alexa Fluor

647 donkey-antigoat, 1:100) diluted in PTxwH for 10 days. Samples were then washed in PTxwH for 1 h, 2 h, 4 h, overnight, then 1 d three times. Samples were finally washed in PBS for one day, then proceeded for clearing with iDISCO+ (Hou et al., 2021). Samples were dehydrated with methanol gradient with water, then 100% methanol, DCM/methanol mixture overnight, and 100% DCM for 1h twice the next day. Brains were finally cleared for 4 h in dibenzyl ether and then stored in a fresh tube of dibenzyl ether before imaging with a LifeCanvas SmartSPIM lightsheet microscope. 647 nm laser was used for whole mount IHC imaging with the 3.6×/0.2 detection lens. Lightsheet illumination is focused with NA 0.2 lens, and axially scanned with electrically tunable lens coupled to the camera (Hamamatsu Orca Back-Thin Fusion) in slit mode. Camera exposure was set at fast mode (2 ms) with 16b imaging. The *X*/*Y* sampling rate was 1.866 µm and *Z* step at 2 µm. 3D imaging datasets were processed using ImageJ to generate the selected volume flatten views in coronal directions.

Experimental Design and Statistical Analyses

Statistical analysis was performed using Prism 5.0 software (GraphPad). Image analysis was performed using ImageJ software. All measurements are shown as mean ± SEM. For samples defined by one factor, data was analyzed by one-way ANOVA with Tukey's post hoc multiple-comparisons test when three or more independent group of samples were compared. For samples defined by two factors, data were compared by two-way ANOVA with Sidak's post hoc multiple-comparisons test. For *in vivo* experiments, sample size (n) was defined as the number of mice that were quantified. For the *in vitro* experiments, sample size (n) was defined as the number of independent cultures of embryos obtained from separate pregnant rats. The null hypothesis was rejected at the 0.05 level. p values <0.05 are considered significant. The statistical test, sample size (n), and the p values are reported in the figure legends specific to each experiment. Epifluorescent images were assembled using Adobe Photoshop.

Results

Cortical FPI causes an induction of proneurotrophins in the cortex ipsilateral to the injury.

To investigate the effect of moderate cortical injury on afferent basal forebrain neurons, we first examined proNT induction at the region of injury in the cortex as well as in the basal forebrain. Cortical FPI was performed on wild-type (WT) adult mice, and tissue lysates were collected 1DPI, 3DPI and 7DPI from both hemispheres of the naïve, sham and TBI mouse brains from the cortex and basal forebrain for biochemical analysis of proNTs. A dramatic increase in proBDNF was observed at the injury site of the cortex ipsilateral to the injury, in comparison to the contralateral side at 1DPI and 3DPI, which was reduced by 7DPI in injured mice (Fig. 1 a). Naïve mice without a craniotomy or injury had comparable levels of proBDNF (Fig.1 a) and proNGF (Fig.1 c) in the right and left hemisphere of the cortex and basal forebrain. A trend towards proBDNF induction was observed in sham mice due to the craniotomy, but no difference was observed in comparison to the contralateral side of the cortex (Fig.1 a). No changes in proBDNF levels were observed in the basal forebrain at 1DPI, 3DPI or 7DPI (Fig.1 b). In addition to proBDNF, a clear induction of proNGF was observed at the injury site in the cortex compared to the contralateral side at 3DPI (Fig.1 c), but no changes in proNGF levels were observed in the basal forebrain (Fig.1 c). These results indicate that after moderate cortical FPI proneurotrophins are induced in the injured cortex in target brain regions of the basal forebrain neurons, but not locally near the basal forebrain soma.

Cortical FPI promotes a retrograde loss of afferent basal forebrain neurons ipsilateral to the injury

Studies reporting neuronal loss in the acute as well as chronic phases after TBI have been limited to the cortical penumbra of the injury, attributed in part to the concurrent induction of proNTs and increased expression of their cognate receptor p75NTR in the injured cortex (Alder et al., 2016; Montroull et al., 2020). However, the effect of cortical FPI on afferent neurons that project to the injured area from distal locations has not been explored. WT naïve, sham, and injured mice were analyzed for the number of surviving neurons expressing p75NTR and Choline Acetyl Transferase (ChAT), well-established markers for the basal forebrain cholinergic population, throughout the Diagonal band of Broca, Nucleus basalis, and Substantia Innominate (Fig.2a). Cortical FPI induced a significant reduction in p75NTR+ neurons after 7 days in comparison to the contralateral side, and in comparison to sham uninjured animals (Fig.2 a, b). A greater effect on p75NTR+ neuron loss was observed after 14DPI (Fig.2 a, d). A significant loss of ChAT+ neurons (Fig.2 a) was also observed in the basal forebrain 7DPI (Fig 2 c) and 14DPI (Fig.2 e). Although a trend towards an increase in p75NTR and ChAT+ BFCNs in the contralateral side was observed after 7 days post injury, the trend was not found to be significant (p75NTR+ Sham contra vs injured contra: n.s. $p = 0.1121$; ChAT+ Sham contra vs injured contra: n.s. $p = 0.1521$). Co-immunolabeling with Ki67, a proliferation marker and p75NTR did not show any proliferating cells in the injured versus uninjured basal forebrain, (data not shown). A significant loss of ipsilateral p75NTR and ChAT double positive neurons was observed compared to the contralateral side in injured mice, which was absent in sham mice (Fig. 2 f, g). Although a trend towards reduction in p75NTR and Chat positive BFCNs was observed in the contralateral side 14DPI, the effect was not significant compared to the contralateral side in sham mice (Sham:Contra vs. Injured:Contra: $p= 0.8846$). Retrograde tracing using Fast blue injections in the cortex showed Fast Blue+ cells in the cortex, and in the basal forebrain that co-express p75NTR and ChAT on the ipsilateral side of the injection (Fig 2. h, i). However no Fast blue positive cells were observed on the contralateral basal forebrain, suggesting the absence of contralateral connections from the basal

forebrain to the injection site in the cortex (Fig 2. i). These results suggest that BFCNs undergo retrograde cell death after cortical FPI, which leads to a progressive loss of p75NTR+ and ChAT + BFCNs 7 and 14 days after injury.

Cortical FPI does not promote neuronal loss of afferent locus coeruleus neurons ipsilateral to the injury

To compare the effects of FPI on different afferent neuronal populations, we investigated whether cortical FPI promotes a similar degenerative effect on locus coeruleus (LC) afferent neurons as on the BFCNs. The LC noradrenergic neurons send long axonal projections throughout the cortex (Jones and Moore, 1977; Nomura et al., 2014; Pickel et al., 1974). Brain sections through the LC were examined to quantify the number of tyrosine hydroxylase (TH)+ neurons in sham and injured WT mouse brains (Fig 3). No loss of TH+ neurons was observed in the LC ipsilateral or contralateral to the injury at 7DPI (Fig 3 a, c), 14DPI (Fig. 3 a, d) or even after 21DPI (Fig. 3 a, e), in contrast to the loss of basal forebrain neurons ipsilateral to the injury (Fig.2), suggesting that cortical FPI does not promote neuronal loss in all afferent neurons. Interestingly, the TH positive neurons in the LC were found to co- express p75NTR even in adulthood (Fig. 3 b). This was also observed by Western blot analysis of LC tissue from WT naïve mice, where p75NTR and TH were detected (Fig. 3 f). However, fast blue injections in the craniotomy site at the cortex (Fig. 2h), did not result in any fast blue positive cells in the ipsilateral or contralateral LC in TH+p75NTR+ neurons even 14 days after fast blue injection (Fig. 3 f), which is in contrast to results observed in the basal forebrain (Fig. 2 i), suggesting that the LC neurons may not be projecting to the specific cortical injury site. Overall, these results indicate the specificity of retrograde basal forebrain neuronal loss after cortical injury.

p75NTR is necessary for retrograde loss of afferent basal forebrain neurons after cortical FPI

To investigate the role of p75NTR in BFCN loss after cortical injury, moderate cortical FPI was performed on adult p75NTR knock-out mice (p75NTR KO) (Fig. 4a). No significant changes in the number of ChAT+ BFCNs were observed in the absence of p75NTR (Fig. 4a) in the ipsilateral versus contralateral basal forebrain of the p75NTR KO mice after cortical FPI 7DPI (Fig. 4 b) or 14DPI (Fig. 4 c), in contrast to WT mice (Fig 2). To assess whether proNTs were still induced after cortical FPI in the p75KO mice as observed in WT mice, brain lysates were obtained from p75NTR KO naïve, injured, and sham mice 3DPI and analyzed by western blot for levels of proNTs. A dramatic increase in levels of proBDNF and proNGF were detected in the injured cortex in comparison to the uninjured side in the p75NTR KO mice at 3DPI (Fig. 4 d, e), similar to the results observed in WT mice (Fig. 1). No changes in proNT levels were seen in the BF, as observed in the WT mice (Fig. 4 d, e). These results show that although FPI induced elevated proNT levels in the p75KO mice as in WT mice, no loss of BFCNs occurred in the absence of p75NTR, indicating that retrograde degeneration of BFCNs after FPI requires p75NTR.

Cortical FPI promotes axonal degeneration of afferent basal forebrain neurons ipsilateral to the injury 7DPI

WT mouse brains with moderate FPI were fixed 7DPI and processed for iDISCO whole mount immunolabeling with anti-p75NTR to investigate the consequences of cortical FPI on the projecting BFCN axon integrity (Fig 5.a). Induction of p75NTR expression at the cortical injury site was observed in accordance with previous studies (Montroull et al., 2020) (Fig 5. a,b). Additionally, the ipsilateral (IPSI) side of the brain showed p75NTR+ axon projections with varicosities, tortuosity and retraction bulbs extended towards the injured cortex (Fig 5.b, yellow arrowheads) indicative of axon degeneration, suggesting that axonal integrity of projecting BFCNs was compromised. In contrast, the uninjured or

374 contralateral (CONTRA) side of the brain was devoid of degenerating p75NTR+ axons (Fig 5.c). These
375 results suggest that retrograde degeneration of basal forebrain afferent axons occurs after injury to the
376 cortex, leading to loss of basal forebrain neurons. Blood vessels in the mouse brain express abundant
377 levels of p75NTR, although the function of this receptor in blood vessels is unknown (5 c, yellow arrows).
378 Interestingly, p75NTR expression in the blood vessels was lost in the region of injury (Fig 5b).

379 *Proneurotrophins signal through p75NTR to promote retrograde degeneration of basal forebrain*
380 *cholinergic neurons in vitro*

381 Studies in mass cultures have demonstrated that proneurotrophins signal through the p75NTR-sortilin
382 receptor complex to promote BFCN death (Volosin et al., 2006). To investigate whether direct stimulation
383 of axon terminals with proneurotrophins can induce retrograde cell death of BFCNs via p75NTR, an *in*
384 *vitro* microfluidic culture model was used (Fig. 6 b). BFCNs grown in the microfluidic system extended
385 their axons to the distal compartment over 5DIV and express the BFCN markers ChAT and p75NTR (Fig. 6
386 b). Basal forebrain neurons from WT and p75NTR KO mouse embryos were cultured in microfluidic
387 chambers for localized stimulation of the axons (Fig.6a). Axons were treated with Alexa 488-labeled
388 choleratoxin B (CTB) to identify the neurons that projected their axons to the distal chamber. ProNGF or
389 proBDNF was added to the axon compartment, followed by live imaging from 0h to 24h. Propidium
390 iodide (PI) was added to the soma compartment to monitor dying neurons, and the number of CTB Alexa
391 488 positive neurons that incorporated PI was quantified in comparison to control untreated BFCNs (Fig.
392 6 b, c, d). Axonal stimulation with proNGF or proBDNF resulted in a significant increase in CTB+/PI+ dying
393 neurons (Fig. 6 c) quantified as percentage of total CTB+ neurons after 24h, suggesting that proNGF and
394 proBDNF can promote retrograde cell death initiated from the axons in WT, but not p75NTR KO neurons
395 (Fig. 6 d). To investigate the effect of proNT-p75NTR signaling on axonal integrity, cells were
396 immunostained for β-Tubulin III (Tuj1) after 24h axonal treatment with proNGF or proBDNF (Fig. 6 e).
397 Both proNGF and proBDNF treatment of WT BFCN axons for 24h promoted a significant increase in axon

fragmentation in comparison to control untreated WT BFCNs (Fig. 6 e, f). In contrast, proNGF or proBDNF axonal stimulation of p75NTR KO BFCN cultures did not result in BFCN cell death (Fig. 6 d) nor promote axon degeneration (Fig.6 e, f). To determine whether retrograde transport was necessary for axonal proNT induced BFCN degeneration, we inhibited the function of the retrograde motor dynein by pretreating the axon compartment for 20 mins with ciliobrevin-D, a dynein functional inhibitor, prior to axonal stimulation with proNGF or proBDNF. Blocking retrograde motor function significantly rescued the BFCNs from retrograde axon degeneration (Fig 6 h,i) as well as cell death (Fig 6 g) even after 24h of axonal proNGF or proBDNF stimulation compared to BFCNs that did not receive ciliobrevin-D pretreatment, suggesting that the proNT-p75NTR degenerative signal requires retrograde transport to the soma to promote BFCN axon degeneration as well as cell death. These results demonstrate that proNTs, which are induced in the injured cortex following TBI, can promote retrograde degeneration of afferent basal forebrain neurons via p75NTR, which may contribute to the progressive retrograde loss of these neurons after cortical injury *in vivo*.

Discussion

Recent studies have shown that brain injury induces increased expression of proneurotrophins and p75NTR at the site of injury and in the penumbra, with a prominent role in mediating the secondary neuronal degeneration that occurs in the penumbra after TBI (Alder et al., 2015; Montroull et al., 2020; Sebastiani et al., 2015). The loss of cortical neurons after injury is reduced when p75NTR is deleted or the proNT ligands that bind to this receptor are inhibited (Montroull et al., 2020). However, in addition to the induction of p75NTR on injured neurons in the cortex, this receptor is constitutively expressed on basal forebrain neurons that project their axons throughout the cortex. Therefore, we investigated whether the constitutive expression of p75NTR might render the basal forebrain neurons vulnerable to

degeneration due to the induction of proNTs in their cortical target region after TBI, eliciting retrograde cell death initiated at the axon terminal.

Cortical FPI promotes a retrograde loss of projecting basal forebrain neurons

Proneurotrophin induction is a consequence of TBI in the area of impact (Alder et al., 2015; Montroull et al., 2020), and we confirmed the increase in proBDNF and proNGF by 3 days after the injury. To assess whether there were any consequences for the afferent BFCNs, we examined the number of neurons in the Diagonal Band of Broca (DBB), substantia innominate (SI) and Nucleus Basalis of Meynert (NBM) that project to the cortex and express p75NTR and ChAT, both well-established markers of basal forebrain cholinergic neurons. Cortical FPI elicited a significant loss of BFCNs that express both p75NTR and ChAT in the basal forebrain ipsilateral to the injury compared to the contralateral side of WT mice, indicating that, in addition to the local damage at the site of injury, spatially distant neuronal populations such as the BFCNs that send afferent projections to the region of injury may be adversely affected by cortical TBI. The trend towards an increase in contralateral BFCNs observed in injured mice 7DPI was not statistically significant. Retrograde tracing of BFCN afferents which project to the injury site confirmed that BFCNs do not project contralaterally. The trend towards contralateral BFCN loss at 14DPI may be attributed to indirect effects of the injury, such as inflammation. Our previous study had shown that seizure-induced injury in the brain elicited increased proNGF levels in basal forebrain astrocytes with a consequent loss of basal forebrain neurons (Volosin et al., 2006), suggesting that BFCNs may be exposed to altered proNT levels in their local environment after certain types of injury. To assess whether the loss of BFCN was due to increased proneurotrophin expression within the basal forebrain, or alterations in the trophic environment in their injured target regions elicited by TBI, we investigated levels of proNTs in the basal forebrain after injury. Following moderate FPI we found no differences in the basal forebrain between the ipsilateral and contralateral sides of the brain, and no differences compared to sham animals,

445 suggesting that the cortical injury did not induce alterations in proNT levels within the basal forebrain,
446 and that the neuronal loss observed in the ipsilateral basal forebrain after FPI may be attributed to the
447 proNT exposure of the BFCN axon terminals at their injured targets.

448 Traumatic axonal injury in the region of injury has been a long-standing focus of study in relation to
449 secondary degeneration after TBI (Johnson et al., 2012). In addition to progressive neuronal death in the
450 injured cortex as a consequence of TBI (Alder et al., 2016; Montroull et al., 2020), injury-induced axon
451 degeneration in cortical neurons has also been established after frontal TBI (Chen et al., 2009). Using
452 whole mount immunostaining for p75NTR of injured brains cleared with iDisco, we identified p75NTR+
453 axon projections with varicosities, tortuosity and retraction bulbs extended towards the injured cortex on
454 the ipsilateral side of the brain. These hallmarks of degenerating axons, and their subcortical location,
455 suggest that afferent neurons projecting to the injured cortex undergo retrograde axonal degeneration.

456

457 *Specificity of afferent neuronal loss after injury*

458 To assess the specificity of retrograde neuronal loss after TBI, we examined another afferent population
459 of neurons that projects to the cortex, the noradrenergic neurons of the locus coeruleus (LC).

460 Interestingly, LC neurons showed no change in the number of TH+ neurons even at 21DPI, suggesting
461 that specific afferent neuronal populations are adversely affected by an injury to their target brain
462 regions, while others are spared. Interestingly, the LC neurons were also found to express p75NTR,
463 similar to BFCNs even in adulthood. However retrograde tracing from the craniotomy site in the cortex
464 indicated that the LC neurons may not specifically project to the cortical region targeted for injury in this
465 TBI model, and therefore may play a role in the contrasting response noted in the LC compared to the
466 basal forebrain after injury. These observations also suggest that spatial differences in the injury location
467 versus distribution of axonal terminals of projecting neurons determine the degenerative effect on distal

468 neuronal populations. The specificity of the degenerative effect of cortical TBI on BFCNs might also be
469 due differences in other cell type specific protein expression, subcellular localization of components for
470 the required cell signaling cascades, and more, which need further investigation to be elucidated.

471 To establish whether the loss of BFCN was due to the expression of p75NTR, we compared p75NTR KO
472 mice with WT mice. Although proneurotrophins were similarly induced in p75NTR KO mice as in WT
473 mice, no loss of BFCNs was seen in the p75NTR KO mice after cortical FPI, in contrast to our observations
474 with WT mice, indicating that retrograde neurodegeneration of BFCNs after TBI was mediated by
475 p75NTR.

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477 *Proneurotrophins signal through p75NTR to promote retrograde degeneration of basal forebrain*
478 *cholinergic neurons in vitro*

479 To investigate whether proneurotrophins could directly elicit retrograde degeneration of basal forebrain
480 neurons initiated at the axon terminal, we used *in vitro* microfluidic chambers to separate the axons from
481 the somas. ProNGF or proBDNF treatment of axons elicited axonal fragmentation during 24 hr of
482 treatment, leading to neuronal cell death. The mechanisms governing p75NTR induced apoptosis in cells
483 have been studied in detail in the CNS (Lee et al., 2001; Nykjaer et al., 2004; Troy et al., 2002; Volosin et
484 al., 2006). Previously described downstream signaling mechanisms governing p75NTR induced cell death,
485 such as the intrinsic caspase pathway (Troy et al., 2002), may be a potential pathway involved in p75NTR
486 mediated retrograde cell death as well as axon degeneration. However, other established axon
487 degeneration mechanisms (Coleman & Höke, 2020) may also be involved in conjunction with cell death
488 signaling to specifically affect the axonal integrity. Whether the same mechanisms govern p75NTR
489 mediated axon degeneration and cell death, or whether axonal degeneration involves an independent
490 signaling mechanism remains to be investigated.

A major consequence of traumatic brain injury is the progressive neuronal loss that occurs over days and weeks following the initial insult. Previous studies have shown that the induction of p75NTR on injured cortical neurons plays a significant role in mediating neuronal loss in the penumbra of the injury. However, in addition to the local effects of injury eliciting loss of cortical neurons, projecting BFCN afferent neurons that constitutively express p75NTR can respond to proneurotrophins induced by injury to their target and promote retrograde degeneration. Interestingly, an increase in proneurotrophin expression in the cortex has been observed in several conditions of brain insults such as seizures (Friedman, 2010.; Volosin et al., 2006) as well as in degenerative diseases such as Alzheimer's Disease (Cuello and Bruno, 2007; Pedraza et al., 2005) which also show BFCN loss. The progressive worsening of cognitive functions such as memory and learning that occurs over time following cortical injuries (Thompson et al., 2005) may be due in part to loss of BFCNs as well as cortical neurons. Rescue of medial septal cholinergic neurons by NGF infusion has been shown to improve cognitive behavior after FPI (Sinson et al., 1997), suggesting that loss of cholinergic basal forebrain neurons contributes to progressive cognitive decline following FPI. The contribution of NBM or SI BFCN loss after TBI remains uninvestigated. Therefore, determining key regulators of the retrograde BFCN degeneration after TBI, as well as parsing out the mechanistic differences between axonal degeneration and cell death signaling in BFCNs after TBI is essential to our understanding of the spatial impact and temporal aspect of BFCN loss under injury conditions.

Figure Legends

Figure 1. Proneurotrophins are induced in the ipsilateral cortex but not the basal forebrain after cortical FPI. Brain tissue lysates from naïve, sham and injured (2ATM) wild-type adult mice were obtained 1DPI, 3DPI and 7DPI to determine levels of proBDNF (a- b) and proNGF (c) in the injured versus uninjured side. (a) Cortical tissue lysate harvested for western blot was probed for proBDNF (32 kDa) in the ipsilateral and contralateral cortex 1DPI, 3DPI and 7DPI in naïve, sham and injured mice. (b) Basal forebrain tissue lysate harvested for western blot was probed for proBDNF (32 kDa) in the ipsilateral vs contralateral basal forebrain 1DPI, 3DPIand 7DPI in naïve, sham and injured mice. (c) Cortex and basal forebrain tissue lysates harvested for western blot were probed for proNGF (37kDa) at 3DPI after FPI in the ipsilateral versus contralateral side of the cortex and the basal forebrain. n= 4 (naïve), n= 4 (sham 1DPI), n= 4 (injured 1DPI), n= 4 (sham 3DPI), n=4 (injured 3DPI), n= 3 (sham 7DPI), n = 3 (injured 7DPI) (a-b); n= 3 (naïve), n= 4(sham 3DPI), n= 4 (injured 3DPI) (c). The established size of proBDNF is 32 kDa, however a prominent band of 25 kDa was also recognized by the BDNF antibody that appeared to be regulated by injury, however the identity of that band is unclear.

Figure 2. Cortical FPI leads to a retrograde loss of afferent basal forebrain neurons ipsilateral to the injury 7DPI and 14DPI. (a) Coronal brain sections of the basal forebrain show immunostaining for p75NTR (red) and ChAT (green) 7 and 14 days after injury in sham and injured mice. Scale bar = 50µm. (b) Quantification of p75NTR+ basal forebrain neurons ipsilateral to the injury in comparison to the contralateral side in sham and injured mice at 7DPI. n = 5 sham and 5 injured mice, 7DPI. The asterisk indicates p = 0.0257 comparing contra versus ipsi in injured mice. Sham contra vs injured contra: n.s. p = 0.1121. (c) Quantification of ChAT+ basal forebrain neurons ipsilateral to the injury in comparison to the contralateral side in sham and injured mice at 7DPI. n = 6 sham and 7 injured mice. The asterisk indicates

p = 0.0487 comparing contra versus ipsi in injured mice. Sham contra vs injured contra: n.s. p = 0.1521. (d) Quantification of p75NTR+ basal forebrain neurons ipsilateral to the injury in comparison to the sham mice at 14DPI. n = 6 sham and 6 injured mice, 14DPI. The asterisk indicates p = 0.0109 comparing sham ipsi versus injured ipsi. Injured contra vs injured ipsi: n.s. p = 0.1937. (e) Quantification of ChAT+ basal forebrain neurons ipsilateral to the injury in comparison to sham mice at 14DPI. n = 6 sham and 6 injured mice, 14DPI. The asterisk indicates p = 0.0010 comparing sham ipsi versus injured ipsi. Injured contra vs injured ipsi: n.s. p = 0.0883. (f) Quantification of p75NTR and ChAT double-positive basal forebrain neurons ipsilateral to the injury in comparison to the contralateral side in sham and injured mice at 7DPI. n = 5 sham and 5 injured mice, 7DPI. The asterisk indicates p = 0.0391 comparing contra versus ipsi in injured mice. Sham contra vs injured contra: n.s. p = 0.3753. (g) Quantification of p75NTR and ChAT double-positive basal forebrain neurons ipsilateral to the injury in comparison to the contralateral side in sham and injured mice at 14DPI. n = 6 sham and 6 injured mice, 14DPI. The asterisk indicates p = 0.0112 comparing contra versus ipsi in injured mice, p = 0.0016 comparing sham ipsi versus injured ipsi. Statistical analysis was performed by 2-way ANOVA, Sidak's multiple comparison tests. (h) Coronal brain sections of the naïve cortex showing immunostaining for Fast Blue (blue) and DRAQ5 (grey) 14 days after cortical Fast Blue injection in the uninjected versus injected side. n = 4; Scale bar = 50µm. (i) Coronal brain sections of the naïve basal forebrain showing immunostaining for Fast Blue (blue), p75NTR (red), ChAT(green) 14 days after cortical Fast Blue injection in the uninjected versus injected side. Scale bar = 50µm.

Figure 3. TH positive cells in the Locus Coeruleus after cortical FPI. 7DPI,14DPI and 21 DPI sham and TBI brains were obtained from wild-type adult mice after FPI. (a) Coronal brain sections of the LC immunostained for tyrosine hydroxylase (TH) (green) 7DPI, 14DPI and 21DPI (b) Coronal brain sections of

559 the naïve LC showing absence of staining for Fast Blue (blue) 14 days after cortical Fast Blue injection in
560 the uninjected versus injected side, co-immunolabeled with TH (green) and p75NTR (red). n = 4. (c)
561 Quantification of TH+ neurons in the ipsilateral versus contralateral side of the LC 7DPI in sham and
562 injured mice. (d) Quantification of TH+ neurons in the ipsilateral versus contralateral side of the LC 14DPI
563 in sham and injured mice. (e) Quantification of TH+ neurons in the ipsilateral versus contralateral side of
564 the LC 21DPI in sham and injured mice. Statistical analysis was performed using one-way ANOVA, Tukey's
565 multiple comparisons test. (g) Western blot showing expression of p75NTR, and TH in the LC in naive WT
566 mice, along with a negative control using LC from p75NTR KO mice. Each Western blot lane represents an
567 n. n= 5 WT LC, n = 4 p75NTR KO LC. All scale bars = 50 µm.

568

569 Figure 4. The absence of p75NTR abrogates the retrograde loss of projecting basal forebrain neurons
570 after cortical FPI. (a) Coronal brain sections of the basal forebrain from injured p75NTR KO mice show
571 immunostaining for ChAT (green) at 7DPI and 14DPI. Scale bar= 50µm. (b) Quantification of ChAT+ BFCNs
572 in the ipsilateral versus contralateral side of the basal forebrain 7DPI in sham and injured p75NTR KO
573 mice. Statistical analysis was performed using 2-way ANOVA, Sidak's multiple comparisons tests. (c)
574 Quantification of ChAT+ BFCNs in the ipsilateral versus contralateral side of the basal forebrain 14DPI in
575 sham and injured p75NTR KO mice. Statistical analysis was performed using 2-way ANOVA, Sidak's
576 multiple comparisons tests. (d) Cortex and basal forebrain tissue lysates obtained from 3DPI sham and
577 TBI p75NTR KO mice were probed for proBDNF (32 kDa) by western blot. n=3 sham and 3 injured brains.
578 (e) Cortex and basal forebrain tissue lysates harvested from 3DPI sham and TBI p75NTR KO mice were
579 probed for proNGF (37 kDa) by western blot. n=3 sham and 3 injured brains.

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Figure 5. Cortical FPI leads to a retrograde axonal degeneration of afferent basal forebrain neurons ipsilateral to the injury 7DPI. (a) WT mouse brains were fixed 7 DPI after cortical FPI were cleared by iDISCO prior to immunostaining for p75NTR. Whole brains were analyzed by light-sheet microscopy. Areas highlighted in rectangles (yellow) are magnified in (b) and (c) to show the ipsilateral (IPSI) and contralateral (CONTRA) regions in further detail. (b) p75NTR staining ipsilateral to the injury shows p75NTR+ basal forebrain afferents with varicosities, tortuosity, and retraction bulbs (yellow arrowheads). (c) p75NTR staining in the cortex contralateral to the injury. Yellow arrows denote p75NTR+ blood vessels in the uninjured cortex. n = 3 (7DPI). Scale bar = 50 µM.

Figure 6. proNGF and proBDNF promote retrograde degeneration of basal forebrain cholinergic neurons (BFCNs) in microfluidic cultures via p75NTR. (a) Basal forebrain neurons were cultured from E15 mouse embryos in microfluidic chambers for 5 DIV. (b) BFCNs grown in microfluidic chambers co-immunolabeled for Tuj1 (grey), p75NTR (green), and ChAT (red). Scale bar: 50 µM. (c) The axon compartment was treated with CTB Alexa- 488 (green) to retrogradely trace neurons that extended their axons to the distal compartment. Propidium iodide (PI) (red) was added to the soma compartment before axonal treatment to study dying (PI+ CTB+) neurons after 24h of axonal treatment with proneurotrophins. Arrows indicate CTB+ neurons that incorporate PI in their nucleus after 24h of treatment. Scale bar: 20 µM. (d) Quantification of dying neurons in WT and p75NTR KO cultured BFCNs after axonal treatment with proNGF or proBDNF. n = 5 (Control, WT), n = 4 (Control, KO), n= 5 (proNGF(A), WT), n=4 (proNGF(A), KO), n=3 (proBDNF(A), WT), n= 4 (proBDNF(A), KO) where (A) indicates axonal treatment. All asterisks indicate $p < 0.0001$ by 2-way ANOVA, Sidak's multiple comparisons tests. (e) Axon fragmentation in WT or KO BFCNs after proNGF or proBDNF treatment assessed using β-Tubulin III (Tuj1) staining represented as binary images. Scale bar (red): 20 µM. (f) Quantification of axonal degeneration in WT and p75NTR KO

cultured BFCNs after axonal treatment with proNGF or proBDNF. n = 4 (Control, WT), n = 4 (Control, KO), n= 4 (proNGF(A), WT), n=4 (proNGF(A), KO), n=3 (proBDNF(A), WT), n= 4 (proBDNF(A), KO). The asterisk indicates $p<0.0001$ comparing WT:control versus proNGF(A), and WT:control versus proBDNF(A), $p = 0.0007$ comparing WT:proNGF(A) versus KO:proNGF(A), and WT:proBDNF(A) versus KO:proBDNF(A) by 2-way ANOVA, Sidak's multiple comparisons tests. (g) Quantification of dying neurons in WT cultured BFCNs with or without pretreatment with ciliobrevinD (50µM) before proNGF or proBDNF treatment in the axons. n = 5 (Control), n= 5 (proNGF(A)), n=5 (proNGF(A)+ CilioD(A)), KO), n=3 (proBDNF(A)), n= 3 (proBDNF(A)+ CIlioD(A)). All asterisks indicate $p<0.0001$ by One-way ANOVA, Tukey's multiple comparison tests. (h) Axon fragmentation in WT cultured BFCNs with or without pretreatment with ciliobrevinD (50µM) before proNGF or proBDNF treatment in the axons assessed using β-Tubulin III (Tuj1) staining represented as binary images, and (i) quantification of axonal degeneration. n = 4 (Control), n= 4 (proNGF(A)), n=3 (proNGF(A)+ CilioD(A)), n=3 (proBDNF(A)), n= 3 (proBDNF(A)+ CilioD(A)). Scale bar (red) : 20 µM. The asterisk indicates $p = 0.0001$ comparing control versus proNGF(A), $p = 0.0002$ comparing control versus proBDNF(A), $p = 0.0102$ comparing proNGF(A) versus proNGF(A)+ CilioD(A), $p = 0.0011$ comparing proBDNF(A) versus proBDNF(A)+ CilioD(A) by One-way ANOVA, Tukey's multiple comparison tests.

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632 References

633 Acosta, S. A., Tajiri, N., de la Pena, I., Bastawrous, M., Sanberg, P. R., Kaneko, Y., & Borlongan, C. v. (2015).
634 Alpha-Synuclein as a Pathological Link Between Chronic Traumatic Brain Injury and Parkinson's
635 Disease. *Journal of Cellular Physiology*, *230*(5), 1024. https://doi.org/10.1002/JCP.24830

636 Alder, J., Fujioka, W., Giarratana, A., Wissocki, J., Thakkar, K., Vuong, P., Patel, B., Chakraborty, T., Elsabeh,
637 R., Parikh, A., Girn, H. S., Crockett, D., & Thakker-Varia, S. (2016). Genetic and pharmacological
638 intervention of the p75NTR pathway alters morphological and behavioural recovery following
639 traumatic brain injury in mice Genetic and pharmacological intervention of the p75NTR pathway
640 alters morphological and behavioural recovery following traumatic brain injury in mice. *Brain Inj*,
641 *30*(1), 48–65. https://doi.org/10.3109/02699052.2015.1088963

642 Alderson, R. F., Alterman, A. L., Barde, Y. A., & Lindsay, R. M. (1990). Brain-derived neurotrophic factor
643 increases survival and differentiated functions of rat septal cholinergic neurons in culture. *Neuron*,
644 *5*(3), 297–306. https://doi.org/10.1016/0896-6273(90)90166-D

645 Boskovic, Z., Meier, S., Wang, Y., Milne, M. R., Onraet, T., Tedoldi, A., & Coulson, E. J. (2019). Regulation of
646 cholinergic basal forebrain development, connectivity, and function by neurotrophin receptors.
647 *Neuronal Signaling*, *3*(1). https://doi.org/10.1042/ns20180066

648 Chen, X. H., Johnson, V. E., Uryu, K., Trojanowski, J. Q., & Smith, D. H. (2009). A lack of amyloid beta
649 plaques despite persistent accumulation of amyloid beta in axons of long-term survivors of
650 traumatic brain injury. *Brain Pathology (Zurich, Switzerland)*, *19*(2), 214–223.
651 https://doi.org/10.1111/J.17503639.2008.00176.X

652 Coleman, M. P., & Höke, A. (2020). Programmed axon degeneration: from mouse to mechanism to
653 medicine. *Nature Reviews Neuroscience 2020 21:4*, *21*(4), 183–196.
654 https://doi.org/10.1038/s41583-020-0269-3

655 Delbary-Gossart, S., Lee, S., Baroni, M., Lamarche, I., Arnone, M., Canolle, B., Lin, A., Sacramento, J.,
656 Salegio, E. A., Castel, M. N., Delesque-Touchard, N., Alam, A., Laboudie, P., Ferzaz, B., Savi, P.,
657 Herbert, J. M., Manley, G. T., Ferguson, A. R., Bresnahan, J. C., … Beattie, M. S. (2016). A novel
658 inhibitor of p75-neurotrophin receptor improves functional outcomes in two models of traumatic
659 brain injury. *Brain*, *139*(6), 1762–1782. https://doi.org/10.1093/BRAIN/AWW074

660 Friedman, W. J., Ibáñez, C. F., Hallböök, F., Persson, H., Cain, L. D., Dreyfus, C. F., & Black, I. B. (1993a).
661 Differential Actions of Neurotrophins in the Locus Coeruleus and Basal Forebrain. *Experimental*
662 *Neurology*, *119*(1), 72–78. https://doi.org/10.1006/EXNR.1993.1007

663 Friedman, W. J., Ibáñez, C. F., Hallböök, F., Persson, H., Cain, L. D., Dreyfus, C. F., & Black, I. B. (1993b).
664 Differential actions of neurotrophins in the locus coeruleus and basal forebrain. *Experimental*
665 *Neurology*, *119*(1), 72–78. https://doi.org/10.1006/EXNR.1993.1007

666 Harris, J., Lee, H., Vahidi, B., Tu, C., Cribbs, D., Jeon, N. L., & Cotman, C. (2007). Fabrication of a
667 Microfluidic
668 Device for the Compartmentalization of Neuron Soma and Axons. *JoVE (Journal of Visualized*
669 *Experiments)*, *7*, e261. https://doi.org/10.3791/261

670 Hefti, F., Hartikka, J., Eckenstein, F., Gnahn, H., Heumann, R., & Schwab, M. (1985). Nerve growth factor
671 increases choline acetyl-transferase but not survival or fiber outgrowth of cultured fetal septal
672 cholinergic neurons. *Neuroscience*, *14*(1), 55–68. https://doi.org/10.1016/0306-4522(85)90163-0

673 Hou, Y., Zhang, Q., Liu, H., Wu, J., Shi, Y., Qi, Y., Shao, M., Yang, Z., Lu, J., Wu, Z., Gong, L., & He, M. (2021).
674 Topographical organization of mammillary neurogenesis and efferent projections in the mouse
675 brain. *Cell Reports*, *34*(6), 108712. https://doi.org/10.1016/J.CELREP.2021.108712

676 Johnson, V. E., Stewart, W., & Smith, D. H. (2012). *Axonal pathology in traumatic brain injury*.
677 https://doi.org/10.1016/j.expneurol.2012.01.013

678 Jones, B. E., & Moore, R. Y. (1977). Ascending projections of the locus coeruleus in the rat. II.
679 Autoradiographic study. *Brain Research*, *127*(1), 23–53.
680 https://doi.org/10.1016/00068993(77)90378-X

681 Kromer, L. F. (1987). Nerve Growth Factor Treatment After Brain Injury Prevents Neuronal Death. *Science*,
682 *235*(4785), 214–216. https://doi.org/10.1126/SCIENCE.3798108

683 Lee, K. F., Li, E., Huber, L. J., Landis, S. C., Sharpe, A. H., Chao, M. v., & Jaenisch, R. (1992). Targeted
684 mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral
685 sensory nervous system. *Cell*, *69*(5), 737–749. https://doi.org/10.1016/0092-8674(92)90286-L

686 Lee, R., Kermani, P., & Teng, K. K. (2001). *Regulation of Cell Survival by Secreted Proneurotrophins*.
687 *294*(November), 1945–1949.

688 Loane, D. J., & Faden, A. I. (2010). Neuroprotection for traumatic brain injury: translational challenges
689 and emerging therapeutic strategies. *Trends in Pharmacological Sciences*, *31*(12), 596–604.
690 https://doi.org/10.1016/J.TIPS.2010.09.005

691 Mesulam, M.-M., Mufson, E. J., Wainer~, B. H., & Levey, A. I. (1983). *CENTRAL CHOLINERGIC PATHWAYS IN*
692 *THE RAT: AN OVERVIEW BASED ON AN ALTERNATIVE NOMENCLATURE (Ch1-Ch6)*. *10*(4), 76.

693 Montroull, L. E., Rothbard, D. E., Kanal, H. D., D'Mello, V., Dodson, V., Troy, C. M., Zanin, J. P., Levison, S.
694 W., & Friedman, W. J. (2020). Proneurotrophins Induce Apoptotic Neuronal Death After Controlled
695 Cortical Impact Injury in Adult Mice. *ASN Neuro*, *12*. https://doi.org/10.1177/1759091420930865

696 Nomura, S., Bouhadana, M., Morel, C., Faure, P., Cauli, B., Lambolez, B., & Hepp, R. (2014). Noradrenalin
697 and dopamine receptors both control cAMP-PKA signaling throughout the cerebral cortex. *Frontiers*
698 *in Cellular Neuroscience*, *8*(AUG), 247. https://doi.org/10.3389/FNCEL.2014.00247/BIBTEX

699 Nonomura, T., Nishio, C., Lindsay, R. M., & Hatanaka, H. (1995). Cultured basal forebrain cholinergic
700 neurons from postnatal rats show both overlapping and non-overlapping responses to the
701 neurotrophins. *Brain Research*, *683*(1), 129–139. https://doi.org/10.1016/0006-8993(95)00357-V

702 Nykjaer, A., Lee, R., Teng, K. K., & Jansen, P. (2004). Sortilin is essential for proNGF- induced neuronal cell
703 death. *Nature*, *427*(February), 15–20. https://doi.org/10.1038/nature02289.

704 Pickel, V. M., Segal, M., & Bloom, F. E. (1974). A radioautographic study of the efferent pathways of the
705 nucleus locus coeruleus. *Journal of Comparative Neurology*, *155*(1), 15–41.
706 https://doi.org/10.1002/CNE.901550103

707 Raghupathi, R., Graham, D. I., & McIntosh, T. K. (2000). Apoptosis after traumatic brain injury. *Journal of*
708 *Neurotrauma*, *17*(10), 927–938. https://doi.org/10.1089/NEU.2000.17.927

709 Rye, D. B., Wainer, B. H., Mesulam, M. M., Mufson, E. J., & Saper, C. B. (1984). Cortical projections arising
710 from the basal forebrain: A study of cholinergic and noncholinergic components employing
711 combined retrograde tracing and immunohistochemical localization of choline acetyltransferase.
712 *Neuroscience*, *13*(3), 627–643. https://doi.org/10.1016/0306-4522(84)90083-6

713 Schimmel, S. J., Acosta, S., & Lozano, D. (2017). Neuroinflammation in traumatic brain injury: A chronic
714 response to an acute injury. *Brain Circulation*, *3*(3), 135. https://doi.org/10.4103/BC.BC_18_17

715 Sebastiani, A., Gölz, C., Werner, C., Schäfer, M. K. E., Engelhard, K., & Thal, S. C. (2015). Proneurotrophin
716 Binding to P75 Neurotrophin Receptor (P75ntr) Is Essential for Brain Lesion Formation and
717 Functional Impairment after Experimental Traumatic Brain Injury.
718 *Https://Home.Liebertpub.Com/Neu*, *32*(20), 1599–1607. https://doi.org/10.1089/NEU.2014.3751

719 Sinson, G., Perri, B. R., Trojanowski, J. Q., Flamm, E. S., & McIntosh, T. K. (1997). Improvement of
720 cognitive deficits and decreased cholinergic neuronal cell loss and apoptotic cell death following
721 neurotrophin infusion after experimental traumatic brain injury. *Journal of Neurosurgery*, *86*(3),
722 511–518. https://doi.org/10.3171/JNS.1997.86.3.0511

723 Sørensen, B., Tandrup, T., Koltzenburg, M., & Jakobsen, J. (2003). No further loss of dorsal root ganglion
724 cells after axotomy in p75 neurotrophin receptor knockout mice. *Journal of Comparative Neurology*,
725 *459*(3), 242–250. https://doi.org/10.1002/CNE.10625

726 Tajiri, N., Kellogg, S. L., Shimizu, T., Arendash, G. W., & Borlongan, C. v. (2013). Traumatic Brain Injury
727 Precipitates Cognitive Impairment and Extracellular Aβ Aggregation in Alzheimer's Disease
728 Transgenic Mice. *PLoS ONE*, *8*(11), 78851. https://doi.org/10.1371/JOURNAL.PONE.0078851

729 Taylor Anne M, Blurton-Jones Mathew, Rhee Seog Woo, Cribbs David H, Cotman Carl W, and J. N. L.
730 (2005). *A microfluidic culture platform for CNS axonal injury, regeneration and transport*. *2*(8), 599–
731 605.
732 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1558906/pdf/nihms-11752.pdf

733 Thompson, H. J., Lifshitz, J., Marklund, N., Grady, M. S., Graham, D. I., Hovda, D. A., & Mcintosh, T. K.
734 (2005). Lateral Fluid Percussion Brain Injury: A 15-Year Review and Evaluation.
735 *Https://Home.Liebertpub.Com/Neu*, *22*(1), 42–75. https://doi.org/10.1089/NEU.2005.22.42

736 Troy, C. M., Friedman, J. E., & Friedman, W. J. (2002). *Mechanisms of p75-mediated Death of*
737 *Hippocampal Neurons*. *277*(37), 34295–34302. https://doi.org/10.1074/jbc.M205167200

738 Volosin, M., Song, W., Almeida, R. D., Kaplan, D. R., Hempstead, B. L., & Friedman, W. J. (2006).
739 Interaction of Survival and Death Signaling in Basal Forebrain Neurons: Roles of Neurotrophins and
740 Proneurotrophins. *Journal of Neuroscience*, *26*(29), 7756–7766.
741 https://doi.org/10.1523/JNEUROSCI.1560-06.2006

742 Whitehouse, P. J., Hedreen, J. C., White, C. L., & Price, D. L. (1983). Basal forebrain neurons in the
743 dementia of Parkinson disease. *Annals of Neurology*, *13*(3), 243–248.
744 https://doi.org/10.1002/ANA.410130304 Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W.,
745 Coyle, J. T., & DeLong, M. R. (1982). Alzheimer's disease and senile dementia: Loss of neurons in the
746 basal forebrain. *Science*, *215*(4537), 1237–1239. https://doi.org/10.1126/SCIENCE.7058341

747 Yano, H., Torkin, R., Martin, L. A., Chao, M. v., & Teng, K. K. (2009). Proneurotrophin-3 Is a Neuronal
748 Apoptotic Ligand: Evidence for Retrograde-Directed Cell Killing. *Journal of Neuroscience*, *29*(47),
749 14790–14802. https://doi.org/10.1523/JNEUROSCI.2059-09.2009

750