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Institution: Rutgers-Robert Wood Johnson Medical School

3.Grant title: The Role of Brain-Derived Neurotrophic Factor Signaling in Traumatic Brain Injury

4.Grant number: 08.3205-BIR-E-1

5.Grant Period covered by Report: 7/1/08-5/31/11. In No Cost Extension

6.Date of Submission of the Report: August 12, 2014

The Role of Brain-Derived Neurotrophic Factor Signaling in Traumatic Brain Injury
Final Report, Smita Thakker-Varia, Ph.D.

1. Original aims of the project

Aim 1 will test the hypothesis that proBDNF and/or the p75 receptor are upregulated following TBI. The injury paradigm used in all our studies will be the lateral fluid percussion injury (LFP) model. We will perform a careful analysis to decipher the specific BDNF isoforms upregulated at different time points following LFP by Western blot analysis in the cortex, hippocampus and corpus callosum. We will also examine trkB and p75 receptor expression. The identity of the cells expressing the specific BDNF receptors will be determined by immunocytochemistry co-localizing trkB or p75 to neurons, oligodendrocyte (OLGs) and astrocytes. Our preliminary data indicates that the proBDNF/p75 pathway is the predominant one upregulated following TBI, suggesting that the toxic pathway is associated with the deleterious effect following brain injury. If our studies indicate that mBDNF/trkB is also induced following TBI then it that would suggest that the trophic pathway is not sufficient to override the damage induced by the injury.

Aim 2 will test the hypothesis that lack of p75 signaling confers protection specifically against neuronal damage following TBI. To study the response to TBI in the absence of p75 signaling, we will use p75-/- mice. TBI is reported to result in death of neurons and OLGs and proliferation of astrocytes. We will examine the cortex, hippocampus and corpus callosum of p75 (knockout) KO compared to wildtype (WT) mice following LFP and sham injury. The differences in neuronal and OLG lineage cell proliferation and survival will be analyzed using quantitative immunocytochemistry. To this end, we will co-localize markers associated with neurons and OLG lineage cells with bromodeoxyuridine (BrdU) or TUNEL positivity. Axonal damage will be assessed by quantitating neurofilament markers. If the p75 KO mice have less damage compared to WT mice then this will suggest that p75 signaling is inhibiting recovery following TBI. On the other hand, if the p75 KO mice have more damage compared to WT mice, we will conclude that the p75 signaling pathway is necessary for recovery from injury.

Aim 3 will test the hypothesis that the impairment of cognitive and motor function following TBI is reduced in the absence of p75 signaling. To study behavior following LFP in the absence of p75 signaling, p75 KO mice will be subjected to TBI. Cognitive function will be examined using Morris Water Maze (MWM) and motor function will be assessed by generation a composite neuroscore and the rotarod test. Improved cognitive and motor function obtained in the p75 KO animals after TBI when compared to WT animals will implicate p75 signaling pathways in causing damage. On the other hand, exacerbated behavioral response in the mutant mice compared to WT mice will demonstrate that p75 signaling is required for recovery after injury.

2. Project successes

The project has been successfully completed and all the aims have been accomplished. The one small component of one of the sub aims that was not assessed was the axonal damage after TBI. However, instead the PI included additional studies involving use of pharmacological compounds to block p75 signaling and assessing the cellular and behavioral outcomes following TBI, this study was not proposed in this grant.

3. Project challenges

There were some challenges in the early phase of the grant which have bee indicated in the earlier reports as has been stated earlier and listed below:

In 2009 Progress report: the main problem we faced in the beginning of the grant period was the time delay in obtaining the LFP injury equipment. Since it is specific for

mouse and custom-made it took the vendor over 3 months from time of contact to deliver us the complete equipment, longer than predicted time.

In 2010 Progress Report: we faced 2 major problems. We are experiencing problems with the double-immuno-staining procedures to localize p75 with the cellular markers, NeuN and GFAP in the tissue sections. We are trying to trouble shoot these problems. The other problem we encountered was in reproducing the genotyping protocol sent by Jackson labs for the p75 mice we are breeding. We lost some time trying to make the PCR to work. We are now using a modified protocol and have started genotyping our mouse colony and should have mutant animals for the behavioral studies for the summer. We are also focusing in maintaining a reproducible injury on animals and are trying to increase the number of animals in the study for statistical analysis.

4. Implications for future research and/or clinical treatment

As has been shown in the submitted manuscript, after identifying the activation of the toxic pathway following LFP injury in mice the author's decided to study the effects of two pharmacological compounds that interact p75 and TrkB receptor signaling. Cellular and behavioral outcomes were assessed following TBI and drug treatments. The PI's lab is now beginning to explore other compounds on this TBI model as well as other traumatic brain injury models. We are also starting preliminary studies to use stem cells as therapeutic tools and use small animal imaging to assay for injury assessment as well as a tool for biomarker analysis.

5. Plans to continue the research, including applications submitted to other sources for ongoing support. Explain how you have leveraged NJCBIR funding to obtain additional federal or other support for brain injury research and list the appropriate funding organizations.

Four grants listed below were funded following our initial NJCBIR award; one brain injury core grant is being planned for submission and one or two grants with my role as collaborator will be submitted.

1. The PI and the co-investigator have also received another NJCBIR grant; Ephrin Signaling in Axon Regeneration for the Treatment of Traumatic Brain Injury; PI Smita Thakker-Varia; CBIR13IRG003

2. The PI of this grant, Smita Thakker-Varia and the co-investigator, Janet Alder are co-investigators on NJCBIR grant, "Role of TRPM7 in Traumatic Brain Injury; PI Loren Runnels.

3. The PI and the co-investigator are co-principal investigators on a grant from NFL Charities "Protein Therapeutics for the Treatment of Traumatic Brain Injury", in collaboration with Noah Weisleder.

4. A medical student in the PI's lab was awarded an Alpha Omega Alpha 2012 Carolyn L. Kuckein Student Research Fellowship to study the effects of the pharmaceutical compounds following LFP injury; Effects of p75 and TrkB Signaling on Cognitive and Motor Function Following Traumatic Brain Injury.

Submission planned: Brain injury Core grant to NJCBIR, October 2014.

Submission planned: NJCBIR individual grant in collaboration with Frederico Sesti: Oxidation of K+ channels in Traumatic Brain Injury, October 2014.

6. List and include a copy of all publications emerging from this research, including those used in preparation.

1. Alder, J., Fujioka, W., Lifshitz, J., Crockett, D. P., **Thakker-Varia**, S., *Lateral Fluid Percussion: Model of Traumatic Brain Injury in Mice.* http://www.jove.com/details.php?id=3063 doi: 10.3791/3063. J Vis Exp. 54 (2011)
2. Alder, J., Fujioka, W., Giarratana, A., Wissocki, J., Thakkar, K., Vuong, P., Patel, B., Chakraborty, T., Elsabeh, R., Parikh, A., Girn, H.S., Crockett D. and **Thakker-Varia, S.,** *"Genetic and Pharmacological Intervention of the p75NTR Pathway Promotes Morphological and Behavioral Recovery Following Traumatic Brain Injury in Mice"* J. Neurotrauma, (Submitted, 2014).

PUBLICAITONS AND PRESENSTATIONS

All papers, presentations, chapter, and abstracts should mention that the research was supported by a grant from the New Jersey Commission on Brain Injury Research. Copies must be sent to the NJCBIR office.

The following posters were presented and the support from New Jersey Commission on Brain Injury Research was acknowledged in each of them. Many undergraduate and medical students were trained in research specifically about traumatic brain injury and contributed towards the projects.

1. Alder, J., Fujioka, W., Giarratana, A., Crockett, D., **Thakker-Varia, S.,** *Genetic and Pharmacological Intervention of the p75NTR Pathway Promotes Morphological and Behavioral Recovery Following Traumatic Brain Injury in Mice.* Neuroscience Day at Rutgers University, June 2014
2. Alder, J., Giarratana, A., Fujioka, W., Elsabeth, R., **Thakker-Varia, S.,** *Targeting the p75NTR signaling pathway by genetic and pharmacological approaches following moderate traumatic brain injury* (2014) TBI conference Washington DC.
3. Alder, J., Patel, B., Giarratana, A., Elsabeh, R., Fujioka, W., Thakker-Varia, S., *Pharmacological intervention of the BDNF signaling pathway improves cellular and behavioral outcomes following traumatic brain injury* (2013) Society for Neuroscience, 628.04
4. Alder, J., Patel, B., Giarratana, A., Elsabeh, R., Fujioka, W., Wissocki, J., Thakkar, K., Vuong, P., Chakraborty, T., Parikh, A., Girn, H.S., Crockett, D., Thakker-Varia, S., *Genetic and pharmacological intervention of the p75NTR signaling pathway improves cellular and behavioral outcomes following traumatic brain injury* (2013) Gordon Research Conference
5. Alder, J., Patel, B., Giarratana, A., Elsabeh, R., Fujioka, W., Thakker Varia, S. *Pharmacological intervention of the BDNF signaling pathway improves cellular and behavioral outcomes following traumatic brain injury.* (2013) Society for Neuroscience.
6. Patel, B., Fujioka, W., Giarratana, A., Alder, J. and Thakker-Varia, S., *The Effect of Tat-Pep5 and 7, 8-Dihydroxyflavone on Cellular and Behavioral Outcomes following Traumatic Brain Injury* (2013) 6th Annual Nutrition, Endocrinology & Animal Biosciences (NEAB) Graduate Student Conference
7. Miller, S., Crockett, D., Scharf, B., Hershey, J., Alder, J., and Thakker-Varia S. *Mouse traumatic brain injury model: clinical care, humane endpoints and*

neurological assessment. (2012) American Association for Laboratory Animals Science.

8. Alder, J., Fujioka, W., Giarratana, A., Patel, B., Chakraborty, T., Parikh, A., Girn, H.S., Crockett, D., and Thakker-Varia. S. *Inhibition of p75 pathway improves outcome following traumatic brain injury.* (2012) Society for Neuroscience. 864.22.
9. Alder, J., Fujioka, W., Giarratana, A., Patel, B., Chakraborty, T., Crockett, D., and Thakker-Varia, S. *Enhanced signaling of p75 pathway following traumatic brain injury may inhibit recovery.* (2012) Brain Health Institute Poster Session.
10. Alder, J., Fujioka, W., Parikh, A., Girn, H.S., Crockett, D.P., and Thakker-Varia, S. *Enhanced signaling of p75 pathway following traumatic brain injury may inhibit recovery.* (2011). Gordon Research Conference.

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

Janet Alder, Wendy Fujioka, Anna Giarratana, David Crockett, and Smita Thakker-Varia

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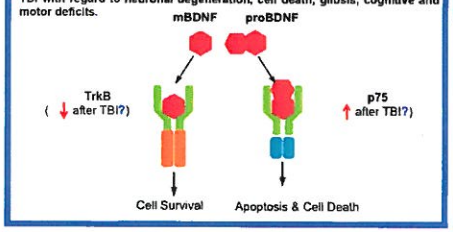
RUTGERS
Robert Wood Johnson Medical School

Introduction

Following traumatic brain injury (TBI), changes in metabolic, cytotoxic and apoptotic processes are observed. These processes lead to pathophysiological alterations in the cortex and hippocampus. Along with mature neurotrophins' well-established role in neural development, maintenance, and adult synaptic plasticity, the precursor form of neurotrophins, pro-neurotrophins, are known to control neuronal apoptosis and long-term depression. Studies indicate that Brain-Derived Neurotrophic Factor (BDNF) is transiently elevated after TBI. However, the increase in expression of the trophic factor in the brain following TBI is not sufficient to support complete regeneration, suggesting that the role of BDNF in pathological conditions is not entirely understood. As it is not yet known whether the mature BDNF and its receptor TrkB which supports cell survival or the proBDNF isoform and its receptor p75 which causes cell death are upregulated following TBI, the consequences of the increased BDNF levels for recovery processes remain to be elucidated. We hypothesize that the toxic proBDNF/p75 pathway is the predominant one upregulated after injury, suggesting that it may be partly responsible for the deleterious consequences of brain injury. We are using lateral fluid percussion injury (LFP) to model moderate TBI and examine whether there is increased signaling through p75 receptor either through upregulated proNeurotrophins and/or p75 expression. We are also analyzing the outcome for p75 -/- mice relative to WT mice after TBI. Finally, we are examining pharmacological antagonism of p75 or activation of TrkB after TBI with regard to neuronal degeneration, cell death, gliosis, cognitive and motor deficits.



Methods

Lateral Fluid Percussion Injury: Animals were anesthetized and placed in a stereotaxic frame. Using a trephine (3mm outer diameter), a craniectomy was performed over the right hemisphere, halfway between lambda and bregma, between the sagittal suture and the lateral ridge. Leaving the dura intact, a rigid Luerloc needle hub (3mm inside diameter) was secured to the skull. After recovery, animals were re-anesthetized and connected to the fluid percussion injury device. A 1.2-1.5 ATM fluid pressure pulse (20-25msec) was generated to strike the intact dura of the brain. Upon return of righting reflex (approximately 4-10 min), the hub and dental acrylic were removed and the scalp incision was closed. Mice that underwent the surgical procedure but not the injury were used as sham controls.

Western Blot Analysis: Hippocampal tissue, ipsilateral to the injury side at 1 day, 7 days, and 21 days post injury (dpi) was flash frozen. 25µg of protein was loaded on a 4-12%Tris-Glycine gel and probed with antibodies to p75 (1:200; sc-8317), TrkB (1:400; sc-12), proNGF (1:200, Alomone Labs; AN-005) and GAPDH (1:1000, Biodesign). BDNF and proBDNF were identified (1:200, Santa Cruz; sc-546) on 12% MOPS NuPage gel. Secondary anti-mouse or anti-rabbit HRP-conjugated IgG (1:5000) antibodies were used. The immunopositive bands were visualized by chemiluminescence and quantitated on a BioRad GelDoc 2000.

Mice: p75 mutant mice (B6.129S4-$Ngfr^{tm1Jae}$/J) and WT C57Bl/6 mice were obtained from Jackson Labs. Adult male mice (~10 weeks) were used for all experiments.

Drug Administration: Vehicle was 6% DMSO/6.1% Tween-20/PBS. TrkB agonist 7,8 Dihydroxyflavone (7,8 DHF from Sigma) was given at 5 mg/kg. p75 antagonist (TAT-Pep5 from Calbiochem) was given at 1.7 mg/kg in 1% DMSO. Each mice was subjected to a dose of either the vehicle or 7,8 DHF (22ul/g), or TAT-Pep5 (10ul/g) via an intraperitoneal injection two hours following the LFP procedure. For Morris Water Maze testing, 7,8 DHF or vehicle was also administered 2 hours prior to testing every trial day. For the Rotarod test, 7, 8-DHF or vehicle was administered daily and 2 hours prior to trials on test days; TAT-Pep5 was administered only on 1, 7, 14, and 21 dpi and 2 hours prior to trials on test days.

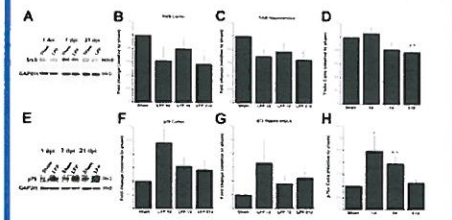
Caspase and Fluorojade C staining: Mice were perfused with PBS followed by 4% paraformaldehyde. The tissue was impregnated with sucrose before sectioning (20µm frozen). A 1:20 series of frozen sections throughout the site of injury on the cortex and the hippocampus was prepared 1, 7 and 21 days post injury. To measure cell death, sections were pretreated with 0.01M citrate buffer, then anti-cleaved caspase3 (1:1000, Cell Signaling 9661) was applied overnight. Secondary goat anti-rabbit (1:200, Vector Labs BA-1000) was applied for 1hr followed by DAB reaction. To measure neuronal degeneration, sections were pretreated with 1% NaOH and 0.06% KMnO4, then 0.0005% Fluoro-Jade C (Millipore AG325)/0.0001% DAPI (Sigma D9564) was applied for 10min. Quantitation of 6 fields in the cortex or the entire hippocampus was performed at 40x. Staining was visualized on a Zeiss Axiophot microscope.

Double Immunohistochemistry: Sections were pretreated with 0.01M citrate buffer and 5% NGS/0.3% TX-100. Caspase3 (1:1000, Cell Signaling 9661) or p75 (1:1500, Cell Signaling 8232) antibody was applied overnight followed by secondary goat anti-rabbit 594 (1:250). Then NeuN (1:200, Millipore MAB377) or GFAP (1:500, Millipore MAB3402) antibody was applied overnight followed by secondary goat anti-mouse 488 (1:250). DAPI (1:1000) was used to label nuclei and staining was visualized on a LSM 510 Meta Zeiss Confocal.

Morris Water Maze: 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 at various time points (1, 7 and 21dpi). 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. Mice were subjected to 8 trials/day for 3 consecutive days or 4 trials/day for 6 days.

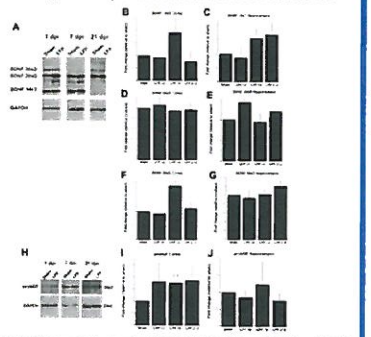
Vestibulomotor Rotarod Test: Mice were trained on the rotarod device 3 times per day with 1-hour inter-trial intervals for the two days prior to the injury. The latency to balance was measured on a 36-mm outer diameter, rotating rod whose velocity increases from 4 to 40 rpm over a maximum 180 sec interval. Each trial ended when the animal fell off the rotarod. At 1, 7, and 21dpi, each subject underwent 3 trials a day on the rotarod device. The average latency to fall of injured mice was compared to that of sham mice.

1 TrkB expression decreases and p75 protein levels increase following Lateral Fluid Percussion (LFP)



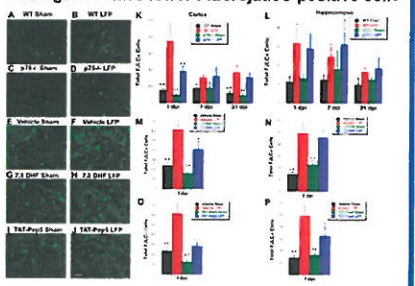
(A, E) Representative Western blots showing TrkB and p75 expression in the ipsilateral cortex and hippocampus, respectively at 1, 7, 21 days post injury. (B-C, F-G) Quantitation of protein levels in the cortex and hippocampus (n = 5-7). (D, H) The average number of TrkB+ and p75+ cells after injury in the hippocampus at 1, 7 and 21 dpi (n = 5,6). * p < 0.05, ** p < 0.01, t-test relative to the sham control from that time point.

2 proNeurotrophin expression increases after LFP



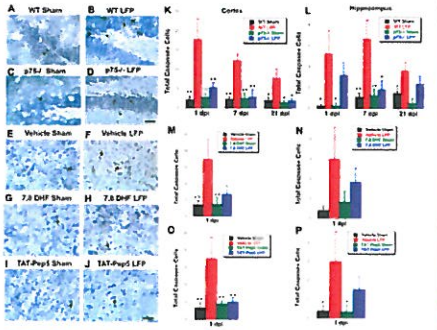
(A, H) Representative Western blots showing BDNF (14kD, 28kD and 36kD) and NGF (26kD) expression in the ipsilateral cortex and hippocampus, respectively. (B-G, I-J) Quantitation of protein levels in the cortex and hippocampus (n = 4,5). * p < 0.05, ** p < 0.01, t-test relative to the sham control from that time point.

3 p75 -/- mice and mice treated with p75 antagonist or TrkB agonist have fewer FluorojadeC positive cells



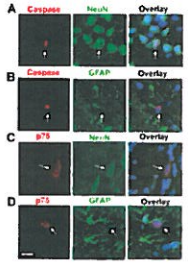
(A-J) Representative images of cortical sections at 1 dpi (E-J) stained with FluorojadeC. Scale bars = 100µm. (K-P) Quantitation of the total number of FLJC+ cells per cortex or hippocampus ± SEM for WT or p75-/- mice (K,L) and mice treated with Vehicle, 7,8 DHF or TAT-Pep5 (M-P) subjected to Sham or LFP procedures (n = 6-8). * p < 0.05, ** p < 0.01 relative to WT LFP or Vehicle LFP, ANOVA Fisher's LSD posthoc test.

4 p75 -/- mice and mice treated with p75 antagonist or TrkB agonist have fewer Caspase3 positive cells



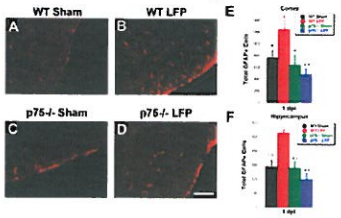
Representative images of hippocampal sections at 7 dpi (A-D) and cortical sections at 1 dpi (E-J) immunostained for activated caspase-3 (indicated by arrow). Scale bars = 50µm. (K-P) Quantitation of the total number of caspase-3+ cells per cortex or hippocampus ± SEM for WT or p75-/- mice (K,L) and mice treated with Vehicle, 7,8 DHF or TAT-Pep5 (M-P) subjected to Sham or LFP procedures (n = 8). * p < 0.05, ** p < 0.01 relative to WT LFP or Vehicle LFP, ANOVA Fisher's LSD posthoc test.

5 Activated Caspase-3 and p75 co-localize with neuronal but not glial markers after injury



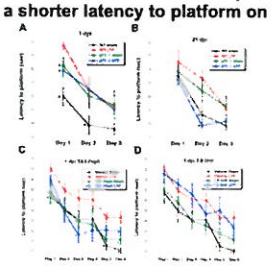
Representative images of cells from the cortex of mice subjected to LFP mice stained with antibodies against Caspase3 (red) or p75 (red) and NeuN or GFAP (green). Scale bar = 30µm.

6 p75-/- mice have fewer GFAP+ astrocytes than WT mice following LFP



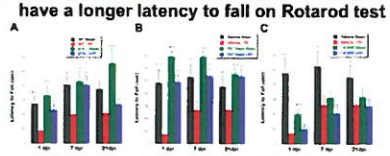
(A-D) Representative images of cortical sections from WT (A,B) and p75-/- (C,D) subjected to sham and LFP injuries and immunostained for GFAP at 1dpi. (E,F) Quantitation of the average number of GFAP+ cells per cortical and hippocampal section ± SEM (n = 3-6). * p < 0.05, , ** p < 0.01 relative to WT LFP mice, ANOVA Fisher's LSD posthoc test.

7 p75 -/- mice and mice treated with p75 antagonist have a shorter latency to platform on MWM test



(A,B) Average latency to platform per day ± SEM for p75 -/- or WT mice subjected to sham or LFP injuries at 1dpi and 21dpi (n = 6-10). (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 at 1dpi (n = 8,9). * p < 0.05 relative to WT LFP or vehicle LFP, ANOVA Fisher's LSD posthoc test.

8 p75 -/- mice and mice treated with p75 antagonist have a longer latency to fall on Rotarod test



(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). (B, C) Average latency to fall per day ± SEM at 1, 7, 21 dpi for mice treated with vehicle, TAT-Pep5 (B) or 7,8 DHF (C) following sham or LFP injuries (n = 8,9) * p < 0.05, ** p < 0.01 relative to WT LFP or vehicle LFP, ANOVA Fisher's posthoc test.

Summary and Conclusions

1. p75 receptor expression is enhanced and TrkB receptor expression is decreased in the cortex and hippocampus following lateral fluid percussion injury (LFP).
2. ProNeurotrophins are moderately increased following LFP.
3. Cell death and degeneration are reduced in p75 -/- LFP mice relative to WT LFP mice. In addition, there is less cell death and degeneration in mice that have been treated with a p75 antagonist (TAT-Pep5) or a TrkB agonist (7,8 DHF).
4. The cells that are dying appear to be neurons rather than glia.
5. There are fewer astrocytes in p75-/- mice suggesting that improved outcome may result from less gliosis.
6. p75-/- LFP mice and mice treated with p75 antagonist show improved spatial learning relative to WT or vehicle treated mice.
7. p75 mutant mice and mice treated with p75 antagonist exhibit improved motor function compared to WT or vehicle treated mice.
8. In conclusion, proNeurotrophin/p75 may be the predominant pathway activated following LFP. Our data suggest that the toxic pathway prevents recovery after injury, possibly by promoting neuronal death and degeneration and increasing gliosis. The effectiveness of eliminating activation of the p75 pathway genetically and the potential use of small molecules to treat TBI is suggested by improved cellular and behavioral outcomes of mice in which the p75 pathway is inhibited.

Supported by New Jersey Commission on Brain Injury Research and by an Alpha Omega Alpha Carolyn L. Kuckein Student Research Fellowship

Future Studies and Collaborations

1. We are examining the effects of a p75 antagonist on repeated mild TBI which mimics sports concussions.
2. We are beginning to correlate MRI imaging of mice with cellular and behavioral outcomes following various treatments to identify biomarkers for injury and regeneration. We plan to do the same with proteomic analysis of the CSF.
3. In collaboration with Renping Zhou we are currently studying the role of the Eph/Ephrin pathway in TBI using Eph-Fc fusion proteins to block signaling.
4. In collaboration with Loren Runnels we are determining the role of TRPM7 in damage caused by TBI using transgenic mice.
5. In collaboration with Huaye Zhang and Victoria DiBona we are analyzing the effect of Par1 polarity protein and microglia after TBI.
6. In collaboration with Jim Millonig and Myka Ababon we are exploring the role of GPR161 on neurogenesis after TBI.
7. In collaboration with Federico Sesti we are exploring how KCNB1 is involved in TBI using pharmacological and genetic approaches.
8. In collaboration with the BME department, we are starting to implant encapsulated human mesenchymal stem cells post TBI to determine if they help regeneration.

Pharmacological Intervention of the BDNF Signaling Pathway Improves Cellular and Behavioral Outcomes following Traumatic Brain Injury

Janet Alder, Wendy Fujioka, Bijal Patel, Anna Giarratana, Rami Elsabeh, Jenna Wissocki, Keya Thakkar, Phung Vuong, Trisha Chakraborty, Ankit Parikh, Hartaj S. Girn, David Crockett and Smita Thakker-Varia

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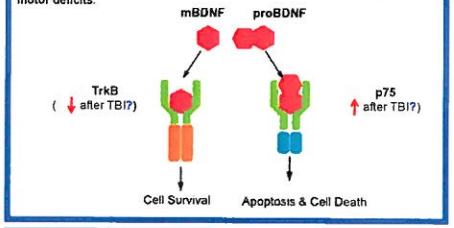
Robert Wood Johnson Medical School

628.04

Introduction

Following traumatic brain injury (TBI), changes in metabolic, cytotoxic and apoptotic processes are observed. These processes lead to pathophysiological alterations in the cortex and hippocampus. Along with mature neurotrophins' well-established role in neural development, maintenance, and adult synaptic plasticity, the precursor form of neurotrophins, pro-neurotrophins, are known to control neuronal apoptosis and long-term depression. Studies indicate that Brain-Derived Neurotrophic Factor (BDNF) is transiently elevated after TBI. However, the increase in expression of the trophic factor in the brain following TBI is not sufficient to support complete regeneration, suggesting that the role of BDNF in pathological conditions is not entirely understood. As it is not yet known whether the mature BDNF and its receptor TrkB which supports cell survival or the proBDNF isoform and its receptor p75 which causes cell death are upregulated following TBI, the consequences of the increased BDNF levels for recovery processes remain to be elucidated. We hypothesize that the toxic proBDNF/p75 pathway is the predominant one upregulated after injury, suggesting that it may be partly responsible for the deleterious consequences of brain injury. We are using lateral fluid percussion injury (LFP) to model moderate TBI and examine whether there is increased signaling through p75 receptor either through upregulated proNeurotrophins and/or p75 expression. We are also analyzing the outcome for p75 -/- mice relative to WT mice after TBI. Finally, we are examining pharmacological antagonism of p75 or activation of TrkB after TBI with regard to neuronal degeneration, cell death, gliosis, cognitive and motor deficits.



Methods

Lateral Fluid Percussion Injury: Animals were anesthetized and placed in a stereotaxic frame. Using a trephine (3mm outer diameter), a craniectomy was performed over the right hemisphere, halfway between lambda and bregma, between the sagittal suture and the lateral ridge. Leaving the dura intact, a rigid Luerloc needle hub (3mm inside diameter) was secured to the skull. After recovery, animals were re-anesthetized and connected to the fluid percussion injury device. A 1.2-1.5 ATM fluid pressure pulse (20-25msec) was generated to strike the intact dura of the brain. Upon return of righting reflex (approximately 4-10 min), the hub and dental acrylic were removed and the scalp incision was closed. Mice that underwent the surgical procedure but not the injury were used as sham controls.

Western Blot Analysis: Hippocampal tissue, ipsilateral to the injury side at 1 day, 7 days, and 21 days post injury (dpi) was flash frozen. 25pg of protein was loaded on a 4-12%Tris-Glycine gel and probed with antibodies to p75 (1:200; sc-8317), TrkB (1:400; sc-12), proNGF (1:200, Alomone Labs; ANR-005) and GAPDH (1:1000, Biodesign). BDNF and proBDNF were identified (1:200, Santa Cruz; sc-546) on 12% MOPS NuPage gel. Secondary anti-mouse or anti-rabbit HRP-conjugated IgG (1:5000) antibodies were used. The immunopositive bands were visualized by chemiluminescence and quantitated on a BioRad GelDoc 2000.

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Cassase and Fluorojade C staining: Mice were perfused with PBS followed by 4% paraformaldehyde. The tissue was impregnated with sucrose before sectioning (25µm frozen). A 1:20 series of frozen sections throughout the site of injury on the cortex and the hippocampus was prepared 1, 7 and 21 days post injury. To measure cell death, sections were pretreated with 0.01M citrate buffer, then anti-cleaved caspase3 (1:1000, Cell Signaling 9661) was applied overnight. Secondary goat anti-rabbit (1:200, Vector Labs BA-1000) was applied for 1hr followed by DAB reaction. To measure neuronal degeneration, sections were pretreated with 1% NaOH and 0.06% KMnO4, then 0.0005% Fluoro-Jade C (Millipore AG325)/0.0001% DAPI (Sigma D9564) was applied for 10min. Quantitation of 5 fields in the cortex or the entire hippocampus was performed at 40x. Staining was visualized on a Zeiss Axiophot microscope.

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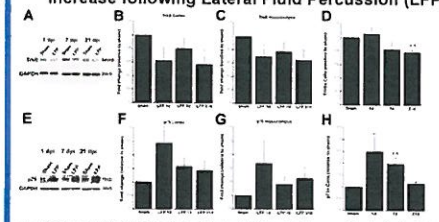
1 TrkB expression decreases and p75 protein levels increase following Lateral Fluid Percussion (LFP)



(A, E) Representative Western blots showing TrkB and p75 expression in the ipsilateral cortex and hippocampus, respectively at 1, 7, 21 days post injury. (B-C, F-G) Quantitation of protein levels in the cortex and hippocampus (n = 5-7). (D, H) The average number of TrkB+ and p75+ cells after injury in the hippocampus at 1, 7 and 21 dpi (n = 5,6). * p < 0.05, ** p < 0.01, t-test relative to the sham control from that time point.

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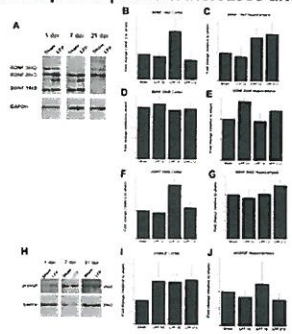
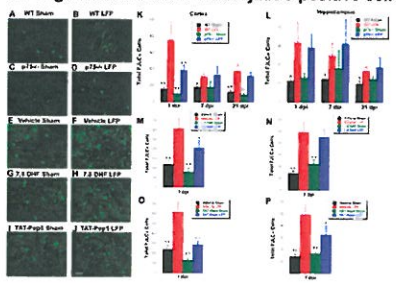


(A, H) Representative Western blots showing BDNF (14kD, 28kD and 35kD) and NGF (26kD) expression in the ipsilateral cortex and hippocampus, respectively. (B-G, I-J) Quantitation of protein levels in the cortex and hippocampus (n = 4,5) * p < 0.05, ** p < 0.01, t-test relative to the sham control from that time point.

3 p75 -/- mice and mice treated with p75 antagonist or TrkB agonist have fewer FluorojadeC positive cells



(A-J) Representative images of cortical sections at 1 dpi (E-J) stained with FluorojadeC. Scale bars = 100µm. (K-P) Quantitation of the total number of FJJC+ cells per cortex or hippocampus ± SEM for WT or p75-/- mice (K,L) and mice treated with Vehicle, 7,8 DHF or TAT-Pep5 (M-P) subjected to Sham or LFP procedures (n = 6-8). * p < 0.05, ** p < 0.01 relative to WT LFP or Vehicle LFP, ANOVA Fisher's LSD posthoc test.

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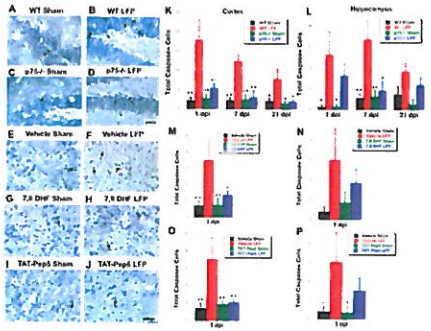
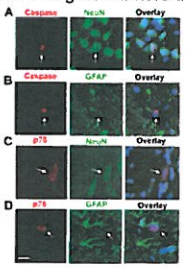


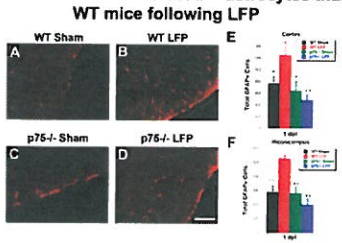
Representative images of hippocampal sections at 7 dpi (A-D) and cortical sections at 1 dpi (E-J) immunostained for activated caspase-3 (indicated by arrow). Scale bars = 50µm. (K-P) Quantitation of the total number of caspase-3+ cells per cortex or hippocampus ± SEM for WT or p75-/- mice (K,L) and mice treated with Vehicle, 7,8 DHF or TAT-Pep5 (M-P) subjected to Sham or LFP procedures (n = 8). * p < 0.05, ** p < 0.01 relative to WT LFP or Vehicle LFP, ANOVA Fisher's LSD posthoc test.

5 Activated Caspase-3 and p75 co-localize with neuronal but not glial markers after injury



Representative images of cells from the cortex of mice subjected to LFP mice stained with antibodies against Caspase3 (red) or p75 (red) and NeuN or GFAP (green). Scale bar = 30µm.

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 = 3-5). * p < 0.05, , ** p < 0.01 relative to WT LFP mice, ANOVA Fisher's LSD posthoc test.

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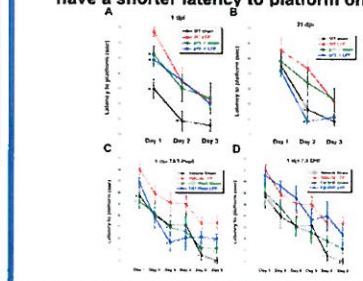

(A,B) Average latency to platform per day ± SEM for p75 -/- or WT mice subjected to sham or LFP injuries at 1dpi and 21dpi (n = 6-10). (C,D) Average latency to platform per day ± SEM for WT mice treated with vehicle, TAT-Pep5 (C) or 7,8 DHF (D) following sham and LFP injuries at 1dpi (n = 8,9). * p < 0.05 relative to WT LFP or vehicle LFP, ANOVA Fisher's LSD posthoc test.

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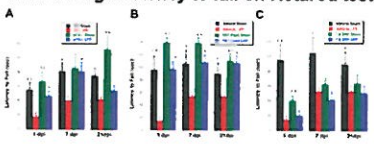

(A) Bars represent average latency to fall per day ± SEM at 1, 7, and 21 dpi for WT and p75 -/- mice subjected to sham or LFP injuries (n = 8-10). (B, C) Average latency to fall per day ± SEM at 1, 7, 21 dpi for mice treated with vehicle, TAT-Pep5 (B) or 7,8 DHF (C) following sham or LFP injuries (n = 8,9) * p < 0.05, ** p < 0.01 relative to WT LFP or vehicle LFP, ANOVA Fisher's posthoc test.

Summary and Conclusions

1. p75 receptor expression is enhanced and TrkB receptor expression is decreased in the cortex and hippocampus following lateral fluid percussion injury (LFP).
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5. There are fewer astrocytes in p75-/- mice suggesting that improved outcome may result from less gliosis.
6. p75-/- LFP mice and mice treated with p75 antagonist show improved spatial learning relative to WT or vehicle treated mice.
7. p75 mutant mice and mice treated with p75 antagonist exhibit improved motor function compared to WT or vehicle treated mice.

In conclusion, proNeurotrophin/p75 may be the predominant pathway activated following LFP. Our data suggest that the toxic pathway prevents recovery after injury, possibly by promoting neuronal death and degeneration and increasing gliosis. The effectiveness of eliminating activation of the p75 pathway genetically and the potential use of small molecules to treat TBI is suggested by improved cellular and behavioral outcomes of mice in which the p75 pathway is inhibited.

Supported in part by an Alpha Omega Alpha Carolyn L. Kuckein Student Research Fellowship. Also supported by New Jersey Commission on Brain Injury Research.

Genetic and Pharmacological Intervention of the p75NTR Signaling Pathway Improves Cellular and Behavioral Outcomes following Traumatic Brain Injury

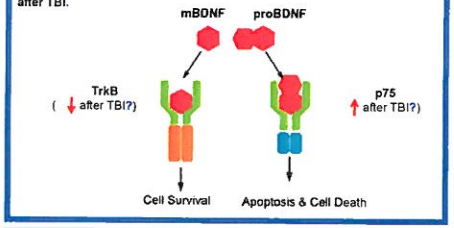
Janet Alder, Bijal Patel, Anna Giarratana, Rami Elsabeh, Wendy Fujioka, Jenna Wissocki, Keya Thakkar, Phung Vuong, Trisha Chakraborty, Ankit Parikh, Hartaj S. Girn, David Crockett and Smita Thakker-Varia

ROBERT WOOD JOHNSON MEDICAL SCHOOL

Department of Neuroscience and Cell Biology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, USA

Introduction

Following traumatic brain injury (TBI), changes in metabolic, cytotoxic and apoptotic processes are observed. These processes lead to pathophysiological alterations in the cortex and hippocampus. Along with mature neurotrophins' well-established role of regulation in neural development and maintenance, and adult synaptic plasticity, the precursor form of neurotrophins, pro-neurotrophins, are known to control neuronal apoptosis and long-term depression. Studies indicate that Brain-Derived Neurotrophic Factor (BDNF) is transiently elevated after TBI. However, the increase in expression of the trophic factor in the brain following TBI is not sufficient to support complete regeneration, suggesting that the role of BDNF in pathological conditions is not entirely understood. As it is not yet known whether the mature BDNF and its receptor TrkB which supports cell survival or the proBDNF isoform and its receptor p75 which causes cell death are upregulated following TBI, the consequences of the increased BDNF levels for recovery processes remain to be elucidated. We hypothesize that the toxic proBDNF/p75 pathway is the predominant one upregulated after injury, suggesting that it may be partly responsible for the deleterious consequences of brain injury. We are using lateral fluid percussion injury (LFP) to model moderate TBI and examine whether there is increased signaling through p75 receptor either through upregulated proNeurotrophins and/or p75 expression. We are also analyzing the outcome for p75 -/- mice and antagonism of p75 or activation of TrkB after TBI.



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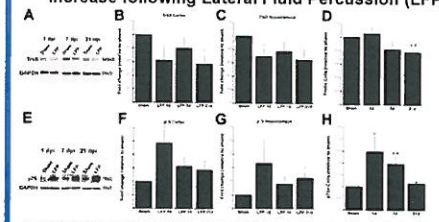

(A, E) Representative Western blots showing TrkB and p75 expression in the ipsilateral cortex and hippocampus, respectively at 1, 7, 21 days post injury. (B-C, F-G) Quantitation of protein levels in the cortex and hippocampus (n = 5, 6, 7). (D, H) The average number of TrkB+ and p75+ cells after injury in the hippocampus at 1, 7 and 21 dpi (n = 5,6). * p < 0.05, ** p < 0.01, t-test relative to the sham control from that time point.

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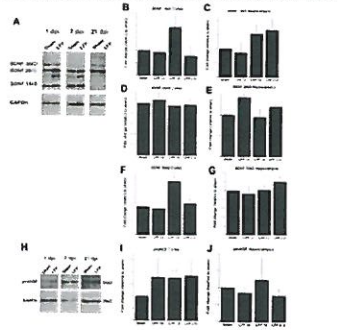

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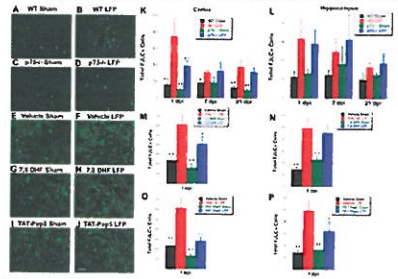

(A-J) Representative images of cortical sections at 1 dpi (E-J) stained with FluorojadeC. Scale bars = 100µm. (K-P) Quantitation of the total number of FJJC+ cells per cortex or hippocampus ± SEM for WT or p75-/- mice (K,L) and mice treated with Vehicle, 7,8 DHF or TAT-Pep5 (M-P) subjected to Sham or LFP procedures (n = 8). * p < 0.05, ** p < 0.01 relative to WT LFP or Vehicle LFP, ANOVA Fisher's LSD posthoc test.

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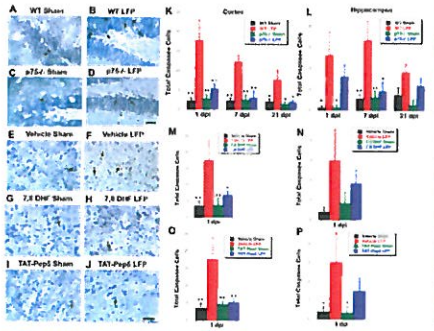

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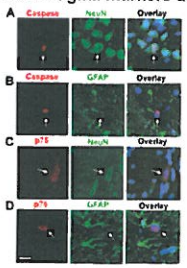

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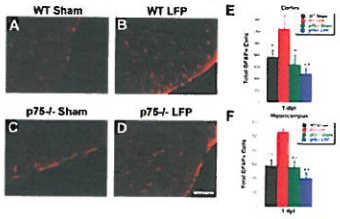

(A-D) Representative images of cortical sections from WT (A,B) and p75-/- (C,D) subjected to sham and LFP injuries and immunostained for GFAP at 1dpi. (E,F) Quantitation of the average number of GFAP+ cells per cortical and hippocampal section ± SEM (n = 4,5). * p < 0.05, ; ** p < 0.01 relative to WT LFP mice, ANOVA Fisher's LSD posthoc test.

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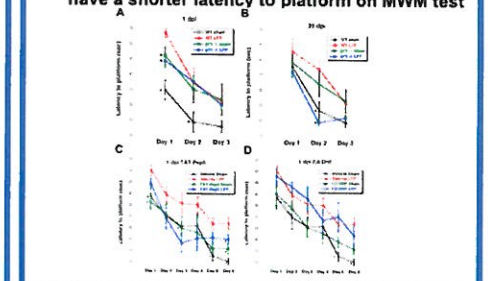

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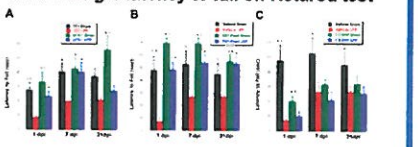

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6. p75-/- LFP mice and mice treated with p75 antagonist show improved spatial learning relative to WT or vehicle treated mice.
7. p75 mutant mice and mice treated with TAT-Pep5 exhibit improved motor function compared to WT or vehicle treated mice subjected to LFP.

In conclusion, proNeurotrophin/p75 may be the predominant pathway activated following LFP. Our data suggest that the toxic pathway prevents recovery after injury, possibly by promoting neuronal death and degeneration and increasing gliosis. The effectiveness of eliminating activation of the p75 pathway genetically and the potential use of small molecules to treat TBI is suggested by improved cellular and behavioral outcomes of mice in which the p75 pathway is inhibited.

Supported by New Jersey Commission on Brain Injury Research Grant

THE EFFECT OF TAT-PEP5 AND 7, 8-DIHYDROXYFLAVONE ON CELLULAR AND BEHAVIORAL OUTCOMES FOLLOWING TRAUMATIC BRAIN INJURY

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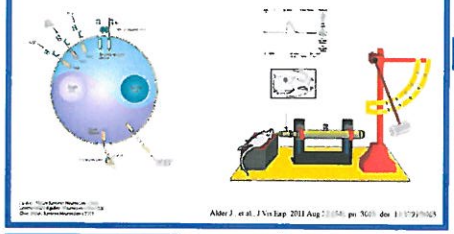
Introduction

Traumatic Brain Injury (TBI) may lead to severe and permanent impairment of nervous tissue, and can cause deficits in cognition and motor-sensory skills. Among the factors known to be regulated following TBI are the neurotrophin family of growth factors. Along with mature neurotrophins' role of regulation in neural development, the precursor form, the pro-neurotrophins, control neuronal apoptosis. Our previous studies indicate that it is the toxic proBDNF/p75NTR pathway, rather than the growth promoting-mature BDNF/TrkB pathway, that is predominantly upregulated after injury. It has been previously demonstrated that genetic inhibition of this p75 toxic pathway promotes recovery from TBI. We tested the role of these pathways post-TBI using pharmaceutical agents. TAT-Pep5 (p75 antagonist), 7, 8-dihydroxyflavone (7,8-DHF: TrkB agonist), or vehicle were administered to sham and lateral fluid percussion (LFP) injured mice. We hypothesized that inhibiting the p75 receptor or activating the TrkB receptor will reduce cell death and improve the cognitive, behavioral, and motor outcome following traumatic brain injury. Morris Water Maze training indicates cognitive function. Motor skills were tested using the Rotarod. We also observed the effect of these drugs by applying immunohistochemistry methods to detect cell death (activated caspase-3) or cell degeneration (Fluoro Jade-C). By targeting different components of the neurotrophic pathway we have shown a possible clinical paradigm to counteract the damaging effects of TBI.



Alder J. et al. J Neurosci 2011 Aug

Methods

Lateral Fluid Percussion Injury: Animals were anesthetized and placed in a stereotaxic frame. Using a trephine (3mm outer diameter), a craniectomy was performed over the right hemisphere, halfway between lambda and bregma, between the sagittal suture and the lateral ridge. Leaving the dura intact, a rigid Luerloc needle hub (3mm inside diameter) was secured to the skull. After recovery, animals were re-anesthetized and the hub is connected to the fluid percussion injury device. A 1.2-1.5 ATM fluid pressure pulse (20-25msec) was generated to strike the intact dura of the brain. Upon return of righting reflex (approximately 4-10 min), the hub and dental acrylic were removed and the scalp incision was closed. Mice that underwent the surgical procedure but not the injury were used as sham controls.

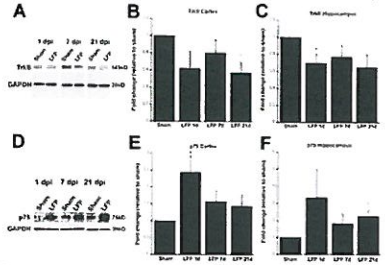
Drug Administration (Vehicle/7, 8-DHF/TAT-Pep5): The vehicle was prepared by a 1:10 dilution of the stock solution—6% dimethyl sulfate (DMSO)/0.1% Tween-20/PBS—in PBS (phosphate buffered saline). The 7, 8-DHF solution was prepared by 1:10 dilution of a stock solution—50 mg of stock 7,8-DHF/ 60% DMSO, 1% Tween-20/PBS—in PBS. The stock preparation of TAT-Pep5 (500ug TAT-Pep5/1% DMSO/PBS) was directly administered to the animals, without dilution. Each mice was subjected to a dose of either the vehicle (22ul/g), 1:10 diluted 7, 8 DHF (22ul/g), or stock TAT-Pep5 (10ul/g) via an intraperitoneal injection two hours following the LFP procedure. For Morris Water Maze testing, 7, 8-DHF or vehicle was administered, additionally, 2 hours prior to testing every trial day. For the Rotarod test, 7, 8-DHF or vehicle was administered daily and 2 hours prior to trials on test days; TAT-Pep5 was administered only on 1, 7, 14, and 21 dpi and 2 hours prior to trials on test days.

Caspase and Fluorolade –C staining: A 1:20 series of frozen sections throughout the site of injury on the cortex and the hippocampus was prepared 1 day post injury. To measure cell death, sections were pretreated with 0.01M citrate buffer, then anti-cleaved caspase3 (1:1000, Cell Signaling, 9661) was applied overnight. Secondary goat anti-rabbit (1:200, Vector Labs BA-1000) was applied for 1hr followed by DAB reaction. To measure neuronal degeneration, sections were pretreated with 1% NaOH and 0.06% KMnO4, then 0.0001% Fluoro-Jade C (Millipore AG325)/0.0001% DAPI (Sigma D9564 was applied for 10min. Quantitation of 6 fields in the cortex or the entire hippocampus was performed at 40x.

Morris Water Maze: 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 one day post injury and continuing for 6 days. 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 15 min between trials. Mice were subjected to 4 trials/day for 6 consecutive days. A probe trial was completed on the 7th day, in which the platform was removed and time spent in target quadrant was recorded.

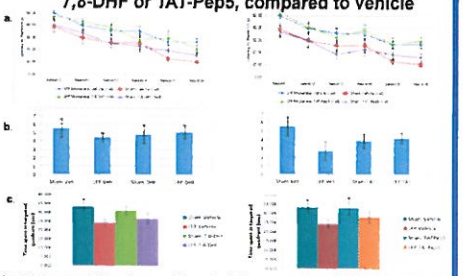
Vestibulomotor Rotarod Test: Mice were trained on the rotarod device 3 times per day with 1-hour inter-trial intervals for the two days prior to the injury. The latency to balance was measured on a 36-mm outer diameter, rotating rod whose velocity increases from 4 to 40 rpm over a maximum 180 sec interval. Each trial ended when the animal fell off the rotarod. At 1, 7, and 21dpi, each subject underwent 3 trials a day on the rotarod device. The average latency to fall of injured mice was compared to that of sham mice.

1 TrkB expression decreases and p75 protein levels increase following Lateral Fluid Percussion (LFP)



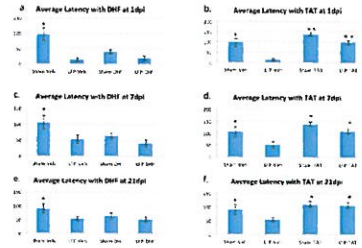
A. Representative Western blots showing TrkB expression in the ipsilateral cortex after injury (each lane represents one animal). B-C, Quantitation of protein levels in the cortex (B) and hippocampus (C). D, Representative Western blots showing p76 expression in the ipsilateral hippocampus after injury. E-F, Quantitation of protein levels in the cortex (E) and hippocampus (F). All data is normalized to GAPDH to control for protein loading and expressed as an average fold change relative to the time matched sham controls ± SEM (n = 5, 6, 7). * p < 0.05, t-test relative to the sham control.

2 Positive Trends in learning in mice administered 7,8-DHF or TAT-Pep5, compared to vehicle



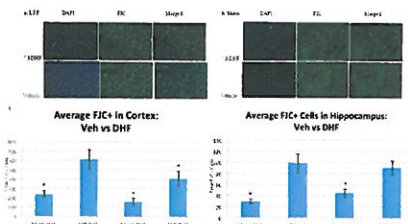
Graphical representation of average latency to platform per session ± SEM for Sham-Veh, LFP-Veh, Sham-DHF, LFP-DHF, Sham-TAT, and LFP-TAT (a). Latency times to platform over 6 days (session 1 to session 6) were re-quantified into slopes for each mouse, in which a steeper slope indicated greater learning. The slope for the average latency of the LFP injured mice administered 7,8-DHF and TAT-Pep5 is shown to be steeper in the downward direction than the LFP mice administered the vehicle (b). Representative bar graphs for probe trials showed longer latency in target platform by LFP-drug mice, compared to LFP-vehicle (c). * p<0.05 relative to LFP-Veh ** p< 0.0001 relative to LFP-Veh. ANOVA Fisher's LSD posthoc test.

3 Motor Function Recovery on Rotarod in mice administered TAT-Pep5 1, 7, 21dpi



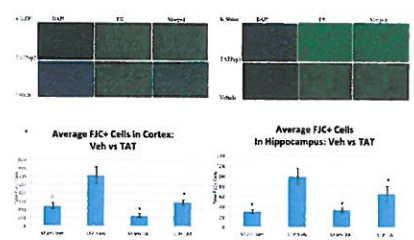
Graphical representation of average latency to fall per day ± SEM at 1, 7, and 21 dpi for Sham-Veh, LFP-Veh, Sham-DHF, LFP-DHF, Sham-TAT, and LFP-TAT. (a, c, and e) LFP-injured mice administered 7, 8-DHF do not show significantly better motor function compared to LFP-vehicle mice. (b, d, and f) Longer latency to fall by LFP-TAT group relative to the LFP-Veh mice group, indicated TAT-Pep5 injection leads to significantly repaired motor function 1, 7, and 21 days post-TBI. n=8 for all experimental groups. * p<0.05 relative to LFP-Veh ** p< 0.0001 relative to LFP-Veh. ANOVA Fisher's LSD posthoc test.

4 Decrease in FJC+ cells in cortex with 7, 8-DHF administration, compared to vehicle



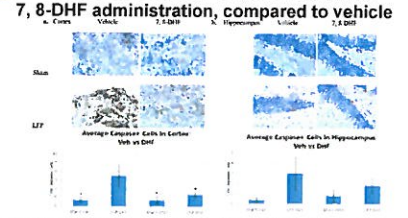
Representative Fluro-JadeC histofluorescence of brain sections following administration of 7, 8-DHF or vehicle 2 hours post-LFP injury or sham procedures. DAPI was used as a counterstain to view hippocampal cells. Significant decrease in neuronal degeneration is seen in in LFP-DHF mice, compared to LFP-Veh mice. Sham groups showed consistent cellular degeneration amongst all experimental groups (b). Quantification of histological findings, indicating total FLJC+ neurons per brain ± SEM. Neurons in cortical regions were significantly rescued from damage post-TBI, due to the 7,8-DHF injection (c). * indicates statistically significant difference from LFP-Veh (p<0.05). n=6 for every experimental group. ANOVA Fisher's LSD posthoc test.

5 Decrease in FJC+ cells in cortex and hippocampus with TAT-Pep5 administration, compared to vehicle



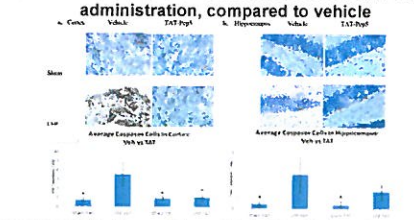
Representative Fluro-JadeC histofluorescence of brain sections following administration of TAT-Pep5 2 hours post-LFP injury or sham procedures. DAPI was used as a counterstain to view hippocampal cells. Significant decrease in neuronal degeneration is seen in LFP-TAT mice, compared to LFP-Veh mice. Sham groups showed consistent cellular degeneration amongst all experimental groups (b). Quantification of histological findings, indicating total FLJC+ neurons per brain ± SEM (c). * indicates statistically significant difference from LFP-Veh (p<0.05). n=6 for every experimental group. ANOVA Fisher's LSD posthoc test.

6 Decrease in Caspase+ cells in cortex with 7, 8-DHF administration, compared to vehicle



Representative anti-activated caspase3 immunohistochemistry of brain sections following administration of 7, 8-DHF 2 hours post-LFP injury and sham procedures. Significant decrease in neuronal apoptosis is seen in the cortex of LFP-DHF mice, compared to LFP-Veh mice (a). Apparent decrease in cell death in the hippocampus following administration of 7,8-DHF (b). Bars represent quantification of histological findings, total Caspase+ neurons per brain ± SEM.. * indicates statistically significant difference from LFP-Veh (p<0.05). n=6 for every experimental group. ANOVA Fisher's LSD posthoc test.

7 Decrease in Caspase+ cells in cortex with TAT-Pep5 administration, compared to vehicle



Representative anti-activated caspase3 immunohistochemistry of brain sections following administration of TAT-Pep5 2 hours post-LFP injury and sham procedures. Significant decrease in neuronal apoptosis is seen in the cortex of LFP-TATPep5 mice, compared to LFP-Veh mice (a). Apparent decrease in cell death in the hippocampus following administration of TAT-Pep5 (b). Bars represent quantification of histological findings, indicating total Caspase+ neurons per brain ± SEM. * indicates statistically significant difference from LFP-Veh (p<0.05). n=6 for every experimental group. ANOVA Fisher's LSD posthoc test.

Summary and Conclusions

Tested a clinically relevant paradigm of blocking the p75 pathway using TAT-Pep5 or activating the TrkB pathway using 7,8-DHF post TBI and found:

1. Preliminary evidence of the downregulation of TrkB expression (generative pathway) and upregulation of p75 expression (toxic pathway) post-TBI.

2. Trends to improved cognitive function (learning and spatial memory) in mice administered either TAT-Pep5 or 7, 8-DHF.

3. Pronounced improvement in motor function of mice injected with TAT-Pep5 at all time points: 1,7, and 21 days post injury.

4,6. Attenuated degeneration and cell death in the cortex but not hippocampus by treatment with 7,8-DHF.

5,7. Reduced degeneration and cell death in the cortex and hippocampus with administration of TAT-Pep5.

Therefore although both compounds show promise of rescuing neurons from cell death and motor outcome following TBI, TAT-Pep5 is more effective than 7,8-DHF. This is consistent with preliminary evidence that p75 is dramatically upregulated after injury and that mice lacking p75 receptors perform better after TBI than wildtype mice. The lack of significant effect on cognitive function may reflect the more apparent histological improvement in the cortex relative to the hippocampus following drug treatments.

Supported by Arosty Research Center and New Jersey Commission on Brain Injury Research Grant

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Enhanced signaling of p75 pathway following traumatic brain injury may inhibit recovery

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INTRODUCTION

Following traumatic brain injury (TBI), changes in metabolic, cytotoxic and apoptotic processes are observed. These processes lead to pathophysiological alterations in the cortex, hippocampus and the limbic regions of the brain. Along with neurotrophins' well-established role of regulation in neural development and maintenance, cytoskeleton restructuring and adult synaptic plasticity, neurotrophins are known to control cell apoptosis of neural cells. Studies indicate that BDNF is transiently elevated after traumatic brain injury (TBI). Induction of BDNF and activation of its intracellular receptors should theoretically produce neural regeneration and dendritic sprouting and improve synaptic efficacy. However, the increase in expression of the trophic factor in the brain following TBI is not sufficient to support complete regeneration, suggesting that the role of BDNF in pathological conditions is not entirely understood. We hypothesize that the toxic proBDNF/p75 pathway is the predominant one upregulated after injury, suggesting that it may be partly responsible for the deleterious consequences of brain injury. We are using the lateral fluid percussion injury (LFP) model to examine whether there is increased signaling through the p75 receptor following TBI either through upregulated proBDNF and/or p75 expression. Our preliminary results indicate that the expression of BDNF receptors TrkB and p75 are altered in specific brain regions following mild TBI suggesting that upregulation of the p75 toxic pathway and downregulation of TrkB may be associated with the harmful effects following TBI. To reveal the identity of the cells expressing the specific BDNF receptors, immunohistochemistry was performed to co-localize TrkB or p75 to neurons, oligodendrocytes and astrocytes and p75 receptors were found to be associated predominantly with astrocytes after TBI and TrkB with neurons. To determine whether lack of p75 signaling confers protection against neuronal damage following TBI, we are using activated caspase-3 and Flouro-Jade C to quantitate neural cells undergoing cell death and neurodegeneration in wild type and p75 null mice. Our ultimate goal is to study whether the impairment of cognitive and motor function following TBI is reduced in the absence of p75 signaling. Preliminary behavioral experiments using p75 mutant mice demonstrate that there is improved response in cognitive and motor function tests in mice with reduced p75 following mild LFP when compared to wild type mice indicating that p75 signaling pathway may be responsible for the damage following TBI.

Methods

Lateral Fluid Percussion Injury: Animals were anesthetized and placed in a stereotaxic frame. Using a trephine (3mm outer diameter), a craniectomy was performed over the right hemisphere, halfway between lambda and bregma, between the sagittal suture and the lateral ridge. Leaving the dura intact, a rigid Luerloc needle hub (3mm inside diameter) was secured to the skull. After recovery, animals were re-anesthetized and connected to the fluid percussion injury device. A 1.2-1.5 ATM fluid pressure pulse was generated to strike the intact dura, creating an elastic decompression (20-25msec) of the brain. Upon return of righting reflex (approximately 4-10 min), the hub and dental acrylic were removed and the scalp incision was closed. Mice that underwent the surgical procedure but not the injury were used as sham controls.

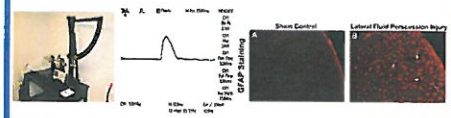
Western Blot Analysis: Hippocampal tissue, ipsilateral to the injury side at 1 day, 7 days, and 21 days post injury (dpi) was flash frozen. p75 and TrkB receptor expression were determined by loading 25µg of protein on a 4-12%Tris-Glycine gel and probing with p75 (1:200: sc-8317), TrkB (1:400: sc-12) and GAPDH (1:1000, Biodesign). BDNF and proBDNF were identified by (1:200, Santa Cruz: sc-646) on 12% MOPS NuPage gel. Secondary anti-mouse or anti-rabbit HRP-conjugated IgG (1:5000) antibodies were used. The immunopositive bands were visualized by chemiluminescence using an ECL detection kit and quantitated on a BioRad GelDoc 2000.

Immunohistochemistry: Mice were perfused with PBS followed by 4% paraformaldehyde. The tissue was impregnated with 10% and 30% sucrose before sectioning (20µm frozen). Sections were preincubated with 0.01M citrate buffer and 6% NGS, then TrkB (1:250: sc-12) or p75 (1:100, Bioscience, MLR 2) antibody was applied overnight. Secondary goat anti-rabbit 488 (1:1000) was used for TrkB and goat anti-mouse (1:1000) was used for p75. NeuN (1:200, Chemicon MAB377) was used to co-label TrkB positive neuronal cells, and GFAP co-labeled p75 positive astrocytes. Staining was visualized on a Zeiss Axiophot microscope at 63X and a LSM 510 Meta Zeiss Confocal.

Morris Water Maze: Cognitive function following LFP was tested using the Morris Water Maze (MWM), a sensitive measure of posttraumatic spatial learning and working memory in rodents. Mice are 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 are trained using a hidden platform fixed in one of 4 quadrants at various time points (1, 7 and 21dpi). Black and white distal cues are placed on the walls. The quadrant in which the mouse is placed is pseudorandomly varied throughout training and the time to locate the platform is recorded. Maximum trial time is 60 sec and the mouse remains or is placed on the platform for 15 sec and warmed for 10 min between trials. Mice are subjected to 8 trials/day for 3 consecutive days. To assess memory retention, the animals are subjected to a 60 sec probe trial the day following the last training. During the probe trial, the platform is removed to determine the time spent in the quadrant where the platform used to be. Finally, a visible platform test is done to rule out possible motor and visual deficits that developed post-injury.

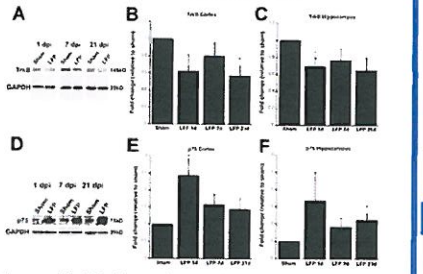
Vestibulomotor Rotarod Test: Mice are trained on the rotarod device 3 times per day with 1-hour inter-trial intervals for the two days prior to the injury. The latency to balance is measured on a 36-mm outer diameter, rotating rod whose velocity increases from 4 to 40 rpm over a maximum 180 sec interval. Each trial ends when the animal falls off the rotarod. At 1, 7, and 21dpi, each subject underwent 3 trials a day on the rotarod device. The average latency to fall of injured mice is compared to that of sham mice.

1 Lateral Fluid Percussion Injury



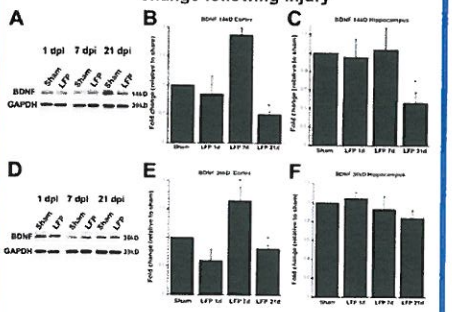
The fluid percussion injury device and an example of the oscilloscope trace obtained during injury. Histopathology following LFP injury. Increased number of GFAP-positive cells (red) are found in the cortex ipsilateral to the injury (A) compared to the sham control mouse, indicating gliosis and validating the injury model.

2 p75 protein levels increase and TrkB expression decreases following LFP



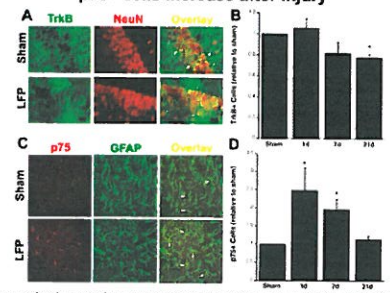
Adult mice were subjected to injury or sham procedures and sacrificed either 1, 7 or 21 d later. *A*, Representative Western blots showing TrkB expression in the ipsilateral cortex after injury (each lane represents one animal). *B-C*, Quantitation of protein levels in the cortex (*B*) and hippocampus (*C*). *D*, Representative Western blots showing p75 expression in the ipsilateral hippocampus after injury (each lane represents one animal). *E-F*, Quantitation of protein levels in the cortex (*E*) and hippocampus (*F*). All data is normalized to GAPDH to control for protein loading and expressed as an average fold change relative to the time matched sham controls ± SEM (n = 5, 6, 7). * p < 0.05, t-test relative to the sham control from that time point.

3 Mature and pro BDNF levels do not consistently change following injury



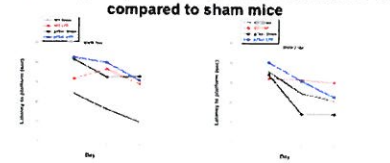
Adult mice were subjected to injury or sham procedures and sacrificed either 1, 7 or 21 d later. *A*, Representative Western blots showing mBDNF expression in the ipsilateral cortex after injury (each lane represents one animal). *B-C*, Quantitation of protein levels in the cortex (*B*) and hippocampus (*C*). *D*, Representative Western blots showing proBDNF expression in the ipsilateral cortex after injury (each lane represents one animal). *E-F*, Quantitation of protein levels in the cortex (*E*) and hippocampus (*F*). All data is normalized to GAPDH to control for protein loading and expressed as an average fold change relative to the time matched sham controls ± SEM (n = 5, 6, 7). * p < 0.05, t-test relative to the sham control from that time point.

4 TrkB+ cells are primarily neurons and p75+ cells are primarily astrocytes; TrkB+ cells decrease and p75+ cells increase after injury



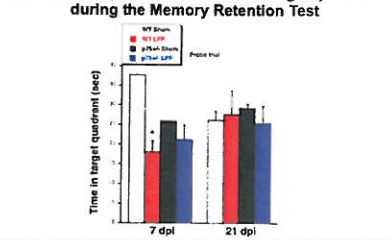
A, Representative images of the hippocampus from sham and LFP mice stained with antibodies against TrkB (green) and NeuN (red). Co-localization is observed as yellow regions around the darker red center of cells and indicated by arrows in the overlayed image. B, Quantitation of the average number of TrkB positive cells in the hippocampus at 1, 7 and 21 days post injury relative to sham controls ± SEM. C, Representative images of the hippocampus from sham and LFP mice stained with antibodies against p75 (red) and GFAP (green). Colocalization is indicated by arrows in the overlayed image. D, The average number of TrkB+ cells after injury (relative to sham values) in the hippocampus at 1, 7 and 21 dpi ± SEM. Six or five fields at 63X were counted per time point. * p < 0.05, t-test relative to the sham control from that time point.

5 Mice subjected to moderate LFP take longer to learn the task of finding the platform in the Morris Water Maze test compared to sham mice



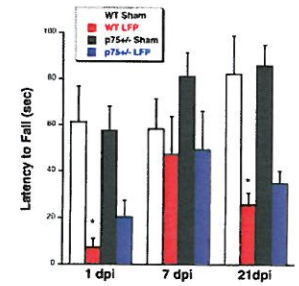
Mice are acclimated to the paradigm and tested for baseline response using a visible platform test 4 days prior to injury and are trained using a hidden platform fixed in one of 4 quadrants at 7 and 21dpi, n = 1 – 10. 8 trials/day for 3 consecutive days are performed. Average latency to platform per session ± SEM is plotted. Sham surgery mice demonstrated improved learning compared to LFP mice. At 21dpi, LFP mutant mice show a trend towards improved learning compared to LFP wildtype mice.

6 Injured mice spend less time in the target quadrant during the Memory Retention Test



To assess memory retention, the animals are subjected to a 60 sec probe trial the day following the last training. During the probe trial, the platform is removed to determine the time spent in the quadrant where the platform used to be. Finally, a visible platform test is done to rule out possible motor and visual deficits that developed post-injury. At 7dpi mice subjected to moderate LFP spend less time in the target quadrant during the probe trial relative to sham mice and there is a trend toward increased time in the target quadrant in p75+/- LFP mice compared to WT LFP mice (7 and 21 dpi, n = 1 - 10). * p < 0.05 relative to WT sham, ANOVA Fisher' s posthoc test.

7 Mice subjected to LFP perform worse than sham mice and p75+/- mice have a trend to increased latency to fall relative to WT mice on the rotarod test



Mice are trained on the rotarod device 3 times per day with 1-hour inter-trial intervals for the two days prior to the injury. The latency to balance is 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 ends when the animal falls off the rotarod. At 1, 7, and 21dpi, each subject underwent 3 trials a day on the rotarod device. The average latency to fall of injured mice is compared to that of sham mice. Bars represent average latency to fall (1, 7, and 21 dpi, n = 3-7). Error bars represent SEM. * p < 0.05 relative to WT sham, ANOVA Fisher' s posthoc test.

Summary and Conclusions

1. p75 receptor expression is enhanced in the cortex and hippocampus following injury.
2. TrkB receptor expression decreases in the cortex and hippocampus following TBI.
3. Pro and mature BDNF levels do not consistently change following TBI.
4. There is an increase in the number of cells that express p75 after injury and the p75+ cells are primarily astrocytes.
5. There is no obvious change in the number of neurons that express TrkB except a significant decrease at 21 days after injury is observed.
6. LFP injured mice perform poorly in the Morris Water Maze test compared to sham injured mice and demonstrate poor memory retention.
7. Overall the p75+/- mice following LFP injury show a trend toward improved learning compared to WT mice subjected to sham injury.
8. LFP injury results in decreased performance on vestibulomotor rotarod tests and p75 mutant mice show a trend towards improved functioning relative to WT mice.

In conclusion, changes in p75 and TrkB levels following TBI and the concurrent lack of consistent change in BDNF suggest that BDNF signaling is regulated at the level of receptor expression. Our results indicate that proBDNF/p75 may be the predominant pathway activated following LFP and suggest that the toxic pathway prevents recovery after injury. In the behavior experiments p75 mutant mice appear to show an overall improvement in their functioning. Future studies will further examine the behavioral effects of TBI in p75 KO mice.

Supported by New Jersey Commission on Brain Injury Research Grant