Faculty of Medicine | University of Manitoba

B.Sc.(Med)Program
Office of the Associate Dean, Research

Student Name: Alysa Almojuela **Date:** 8/8/14

Project Title: Investigation of Potential Therapeutic Application of Thioredoxin Therapy for Enhancement of NPCs Proliferation After Stroke

Primary Supervisor Name: Dr. Eftekhar Eftekharpour

Department: Physiology and Pathophysiology

SUMMARY: (no more than 250 words single spaced)

Stroke is a leading cause of death and disability in Canada. The acute management of stroke involves the use of thrombolytics and/or mechanical recanalization. Despite successful implementation of these techniques, limited therapeutic benefits emphasize a current need for novel treatments.

The presence of endogenous neural precursor cells (NPCs) in the subventricular zone of the adult human central nervous system represents a potential therapeutic cell source in the setting of stroke. These NPCs have a limited capacity to replace neurons, astrocytes and oligodendrocytes in response to stroke, but tissue demand ultimately exceeds this proliferative capacity.

In this project, we show that thioredoxin (Trx) treatment enhances NPC proliferation and modifies their differentiation. Application of Trx in an in-vitro model of NPC culture induced cell proliferation. The effect of Trx on NPC differentiation was investigated using cell specific immunocytochemistry, which displayed significantly higher numbers of oligodendrocyte (Olig2+ cells) and lower numbers of GFAP+ cells in Trx pre-treated cultures. To investigate the effects of Trx in-vivo, a focal permanent devascularisation lesion (stroke) was implemented in experimental rat models, and Trx was delivered after intraventricular infusion. Immunohistochemistry revealed enhanced NPC proliferation in animals that received Trx treatment versus animals that received the vehicle, as indicated by the presence of significantly higher levels of proliferation marker (BrdU) and stem cells (Sox2) expressing cells in both the subventricular zone and cortex.

Ultimately, these results highlight Trx as a potential neuroprotective therapy in the setting of stroke that can enhance the endogenous NPC proliferation.

Alysa Almojuela
Student Signature

E. Eftekharpour
Digitally signed by Eftekhar Eftekharpour
DN: cn=Eftekhar Eftekharpour, o=University of Manitoba, ou=Deapratment of Physiology, email=eftekhar@cc.umanitoba, c=CA
Date: 2014.08.11 04:10:19 -05'00'
Supervisor Signature

ACKNOWLEDGEMENTS:
I gratefully acknowledge the support by one or more of the following sponsors;
H.T. Thorlakson Foundation
Dean, Faculty of Medicine
Manitoba Health Research Council
Manitoba Institute of Child Health
Kidney Foundation of Manitoba
Leukemia and Lymphoma Society of Canada
Other:
CancerCare Manitoba
Manitoba Medical Service Foundation
Associate Dean (Research), Faculty of Medicine
Heart and Stroke Foundation
Health Sciences Centre Research Foundation

Introduction and Background:

Stroke is the third leading cause of death in Canada and a major source of physical and social disability. The pathophysiology of stroke includes a blockage of blood supply to the brain, resulting in brain ischemia or hemorrhage. The majority of stroke cases (80%) are caused by obstruction of an artery leading to ischemia, which decreases the arterial blood supply carrying oxygen and glucose vital for proper brain function. The remaining 20% of stroke cases are caused as a result of rupture of weakened blood vessels such as after an aneurism. Overall, limited supply of nutrients leads to an interruption of energy production in mitochondria which leads to membrane depolarization and spontaneous neuronal activity. Synaptic glutamate receptors are then activated leading to a massive influx of calcium ions, which leads to a cascade of molecular and cellular events, known as secondary injury that result in cell death. Oxidative stress is a major player in the secondary injury process, as excess mitochondrial damage results in upregulation of reactive oxygen species (ROS). This will lead to activation of inflammation and cell death. The nerve cells near the ischemic core are lost within few minutes after stroke; however these molecular events are significantly slower in the tissues surrounding the ischemic core, known as penumbra. In this region, although the cells are still below the functional threshold, they are temporarily above the threshold of cell death but can die over days to weeks after stroke. Optimistically, this delayed form of cell death provides a potential therapeutic window to limit the extent of cell death in penumbra that can potentially have significant impact in patients' quality of life.

Currently, the acute management of stroke involves the use of thrombolytics (recombinant tissue plasminogen activator) and/or mechanical recanalization. A major drawback in these approaches is the inevitable second wave of oxidative stress that is caused by restoration of blood supply to the infarcted tissue and increased levels of ROS (Kahles and Brandes, 2012; Molina, 2011; Nagakannan et al., 2012) which contribute toward increased cell death that hinders the full potential of these treatments. Over the last few decades, targeting the damaging ROS using antioxidant therapy has been an attractive goal in experimental medicine and despite positive results in animal studies, human trials have failed to generate any significant recovery (Kahles and Brandes, 2012; Kelly et al., 2008; Legos et al., 2002; Slemmer et al., 2008). This emphasizes the complexity of antioxidant therapies in the setting of stroke. Alternative novel approaches include the use of neural stem and progenitor cells, here referred to as neural precursor cells (NPCs). These cells are located in the subventricular zone throughout the CNS as well as dentate gyrus in hippocampus. Although quiescent in the adult brain, these cells have the capacity to self-renew and differentiate to all three main neural cell types after injury (Kolb et al., 2007). Increased proliferation of the endogenous or transplantation of exogenous NPCs can have significant therapeutic application.

An optimal treatment for stroke must include antioxidant therapy to prevent the oxidative stress-mediated damage and to enhance the regenerative capacity of the stem cells. Increasing evidence indicate the potential role of Thioredoxin-1 (Trx1) as a multifaceted protein that can deliver such extensive range of protective effects. This small protein (12kDa) contains two thiol (-SH) groups at the active site that can undergo oxidation upon interaction with oxidants such as ROS or oxidized proteins. Trx1 is oxidized but the target is reduced. The oxidized Trx1 is quickly reduced to engage in its protective effects.

The regulatory role of Trx1 has also been implicated in regulation of gene expression that can affect many aspects of the cell's life. Trx1 is widely distributed in neural cells(Patenaude et al., 2005) in all rat brain regions, especially in cortex and hippocampus.

Upon mechanical injury or ischemia Trx1 expression is highly upregulated in the traumatic tissue(Hattori et al., 2002) suggesting a neuroprotective role for Trx1. Over-expression of Trx1 or its intravenous administration results in increased life span and resistance to oxidative damage in ischemia(Tian et al., 2014; Zhou et al., 2009), glutamate toxicity(Takagi et al., 1999) and brain injury in mouse(Hattori et al., 2002). Alzheimer brains contain lower level of Trx1 than the age-matched controls suggesting the neuroprotective role of Trx in human brain(Akterin et al., 2006). In cultures, astrocytes also respond to H_2O_2-induced injury by upregulation of Trx1 release into the culture medium that might be a reflection of their neuroprotective role in nervous system(Hori et al., 1994).

Although the intravenous use of Trx1 in previous stroke models has shown promise, a major drawback is that the molecule has a short half-life (Nakamura et al., 2009)and is unable to cross the blood brain barrier (BBB) to enter the central nervous system. A very recent report showed that a single intraperitoneal injection of 10mg/kg Trx1 before a middle cerebral artery occlusion model of stroke in mice resulted in an enhanced proliferation of NPCs. Therefore, in this study we aimed to investigate the effect of intraventricular continuous delivery of Trx on NPCs population. Additionally we hypothesized that intracellular delivery of Trx1 using a cell penetrating peptide known as TAT can enhance its effects. We observed an enhanced cell proliferation after Trx1 therapy in cultures of adult-brain derived NPCs as well as after intraventricular delivery in a permanent focal cortical devascularization model. These results are the first report on intraventricular Trx1 delivery in CNS. This is the first use of intracellular delivery of Trx1. Furthermore, our observations confirm the previous reports on the capacity of Trx1 as a potential therapeutic treatment where induction of NPCs is intended.

Materials and Methods:

Recombinant Protein generation and purification: A plasmid (pET-28a(+)-TAT-EGFP) (Provided by Dr. Cathy Tsilfidis, (Ottawa Hospital Research Institute) was used in these studies. Full length cDNA containing TAT, human Trx1, and red fluorescent protein (RFP) and appropriate restriction sites was synthesized by the IDTDNA, and then cloned into pET-28a(+)-TAT-EGFP. One Shot Top10© Chemically Competent E. coli (Invitrogen) were used for cloning experiments. For the expression of the fusion protein an *E. coli* BL21 (DE3) host strain was used. To distinguish the effect of extracellular Trx1 (E-Trx) and intracellular Trx1 (I-Trx1) proteins, we used Trx-RFP and TAT-Trx1-RFP constructs respectively.

Single colony of recombinant bacteria producing E-Trx and I-Trx were picked and grown in small culture for overnight in kanamycin containing LB media at 37˚C. Then the culture was mass produced by transferring into larger volume of LB media and were induced with IPTG to produce recombinant proteins for six hours at 37˚C. The bacteria were pelleted down and lysed using a French Press (Thermo Fisher, Canada). Qiagen Ni-NTA columns were used for protein purification according to the manufacturer's protocol. Then the proteins were eluted in 250mM imidazole containing binding buffer that was removed using the GE-10 desalting column. Finally the proteins were sterile filtered (0.2um syringe filter) and stored at -80˚C.

Generation of mouse SVZ neurospheres and Protein transduction: Neural stem cells were isolated brain subventricular zone of 6-8 weeks old mice as we have done previously. Briefly the subventricular zone was dissected in sterile condition and transferred to artificial cerebrospinal fluid (aCSF). The tissue is enzymatically dissociated in Trypsin and single cells suspension was seeded in 6 well plate at $5X10^5$ cells in growth media DMEM:F12 with 20ng/ml EGF(Epidermal Growth Factor) and FGF2(Fibroblast Growth Factor). Neurospheres are generated and are weekly passaged. Only passages 5-10 were used for these experiments.

Proliferation of NPCs: Neurospheres were dissociated and seeded at 2,000 cells per well (200µl) in matrigel coated 8well chamber slide. The cells were grown overnight and treated with E-Trx and I-Trx at 1µM concentration or vehicle control for 3days. The cells were grown with or without proteins for 5 days and further processed for immunocytochemistry. At the termination of experiment (day 4) 10µM Bromodeoxyuridine (BrdU) was added in the cultures for an hour. Medium was removed and cells were washed with PBS and then fixed with 4% PFA for immunocytochemistry.

Differentiation of NPCs: NPCs were grown at 20,000 cells per well for 2 days in growth media in Matrigel coated 8well chamber slides. Then the cells were transferred to 2%FBS containing DMEM:F12 with or without our recombinant proteins. The cells were allowed to grow for 6days in differentiating conditions then processed for immunocytochemistry.

Immunocytochemistry: Undifferentiated and differentiated cells in chamber slides were fixed in 4% paraformaldehyde (PFA). Immunocytochemistry were performed as we have shown previously; for BrdU incorporation slides, antigen retrieval was done using an acid treatment (2N HCl+0.5% TritonX at 37˚C for 30mins), followed by a wash in 0.1 M Sodium Borate (pH 8.5) for 10 min. The cells were blocked in 5% BSA+0.3%TritonX for an hour. Fluorescence immunocytochemistry was performed for the following antibodies: anti-BrdU (1:150; BDBiosciences), Ki67 (1:150; BDBiosciences), markers for Oligodendrocytes- Olig2 (1:400, Chemicon) and astrocytes-GFAP (1:1000, Sigma) were used. All antibodies were incubated overnight at 4˚C in a humidified chamber. After brief washing cells were probed for respective secondary antibodies conjugated with Alexa flour 488, Alexa flour 568 and Alexa flour 647 (1:600, Life technologies). The slides were gently washed and then mounted using Vectashield with 4,6-diamidino-2-phenylindole (DAPI) (Vector Labs). For cell quantification, total cells were counted using DAPI nuclear marker in five random microscopic fields in each well (n=2/chamber, giving a total of n=6 for each experimental condition). The percentage of DAPI-positive cells that express the marker of interest was used for statistical analysis. Images were taken using Axiovision imaging station (Zeiss).

Permanent focal cortical devascularization stroke model (PFCD): Twenty male Sprague-Dawley rats (250-300g) used in this study according to the guidelines set forth by the Canadian Council on Animal Care and the University of Manitoba Animal care Committee. The procedure was performed as described by Kolb et al (Journal of Cerebral Blood Flow & Metabolism (2007) 27, 983–997). Animals were anaesthetized using Isoflurane inhalation and the surgery area was shaved closely and prepared for surgery. Animals were placed in a rat stereotaxic frame and a midline incision was done to expose the skull. A rectangular hole was cut using a dental drill in the left frontal and parietal bones from +3 to -4mm (anterior/posterior) to Bregma and +1.5 to +4.5mm laterally. The bone and underlying dura matter was removed and a sterilized saline-cotton swap was used to remove the pia and the attached blood vessels from the cortical surface.

Drug delivery: We used the Alzet osmotic mini pumps for intraventricular delivering our recombinant proteins. The pumps used in this study allowed for continuous delivery of our recombinant proteins or saline control for one week at a rate of 0.5µl/hr. The pump is attached to a size 26 needle via a catheter. The needle is inserted in the contralateral hemisphere at a depth of 3-4mm at 1.5mm Bregma (medial and posterior).

Post-surgical observations: After surgery, rats recovered and survived for one week and were observed closely to monitor any functional deficits. Asymmetrical use of right limb was detected at day 4/5 after the surgery. We did not quantify any functional deficits in this study.

Immunohistology: At the end of one week, animals were euthanized after transcardial perfusion using ice-cold phosphate buffered saline (PBS) followed by 3% paraformaldehyde in PBS. After cryoprotection in 20% sucrose in PBS for 24–48 h at 4°C, the whole brain was embedded in tissue embedding media (HistoPrep; Fisher Scientific, Houston, TX) on dry ice. Cryostat sections (35µm) were cut and mounted onto gelatin-subbed slides and stored at -20°C. For immunostaining, the frozen slides were air dried at room temperature for 10 min and then were washed with PBS for 10 min. Sections were blocked with 1% BSA, 5% nonfat milk, and 0.3% Triton X-100 in PBS for 1 h at room temperature and then were incubated with primary antibody in the same blocking solution for overnight at 4°C in humidified chambers. Sections were then washed with PBS and incubated with the appropriate secondary fluorescent antibody for 1 h at room temperature. After washing again, sections were mounted with DAPI. Images of the sections were taken using Zeiss(Axio Imaging Station??). The following primary antibodies were used in this study: mouse anti-nestin (1:200; Chemicon) for neural precursor cells, rabbit anti-GFAP (1:1000; Dako) for astrocytes/GFAP-positive NPCs, mouse anti-BrdU (1:400; BD), mouse anti-sox-2 for NPCs (1:500; Sigma). Appropriate secondary fluorescent antibodies were used (1:400, Invitrogen).

Cresyl violet staining: Cresyl violet staining was performed on 35 µm sections of brain according to routine laboratory protocols (Emery et al., 1998) to evaluate the extent of injury after stroke. Breifly, frozen sections were allowed to air-dry and then dehydrated using ascending-alcohol series (70-100%) followed by xylene to delipidize the tissue. Sections were then rehydrated in a descending-alcohol series. After a brief (4mins) staining in 0.1% cresyl violet acetate and washing the excess stain in water, a serial dehydration was performed. Sections were mounted and imaged using a brightfield Zeiss Axio Imager (M2), Zeiss.

Results:

Generation and purification of E-Trx and I-Trx

Extracellular Trx1 (E-Trx-1) and Intracellular Trx1 or TAT-Trx1 (I-Trx) were cloned and purified as described in Materials and Methods (Figure 1). In vitro and in vivo staining show that I-Trx can be successfully incorporated in the cytoplasm.

Effect of I-Trx and E-Trx on cell proliferation

To investigate the effect of Trx on NPCs proliferation, an in vitro study was performed using adult mouse-brain derived NPCs. Neurospheres passages 5-10 were dissociated and single cells were plated at clonal density (10cells/1µm proliferation medium) on Matrigel-coated multi-chamber slides. E-Trx and I-Trx at 0.1um and 1um were added to the cultures after the initial plating (12hours). We used two different markers to label the proliferating cells: BrdU pulse labeling was performed for one hour and then cells were washed and fixed for immunocytochemistry. Sister cultures were also stained with anti-Ki-67 antibody. Ten randomly chosen field were imaged and the total number of Dapi-positive nuclei was determined using Image J software from NIH that allows for unbiased cell quantification. Although we observed a general (20%) increase in E-Trx treated, overall we did not observe any significant changes in proliferating cells (Ki-67/BrdU-positive cells). (Data not shown)

Effect of I-Trx and E-Trx on cell differentiation

To investigate the Trx regulatory role on NPCs fate, Trx proteins were added to differentiation medium as described above. Adult brain-derived NPCs are mostly gliogenic and only less than %1 of these cells may differentiate to neurons ((Eftekharpour et al., 2007). We therefore focused on oligodendroglia and astrocytes. Using cell specific markers for astrocytes and oligodendrocyte we observed that cells receiving E-Trx expressed a higher percentage of Olig2

positive cells over DAPI *(P < 0.01*) than the control, and cells receiving I-Trx showed an even higher percentage of Olig2 expression (*P < 0.01)* than the control. However, oneway ANOVA showed that I-Trx is the most effective treatment to increase oligodendroglial cells. Conversely, when incubated with E-Trx, cells expressed a lower percentage of GFAP positive cells *(P < 0.01)* than the control, and cells incubated with I-Trx expressed even less GFAP *(n=6, P < 0.001).(*Figure 2)

Permanent focal cortical devascularization results in cortical neuronal loss.
Permanent devascularization of the cortex was performed as described. This model results in changes in cortical structure. The superficial layer (molecular layer is lost at day 7 post-injury). In some cases, signs of hemorrhage was observed in cortex, ventricles and basal ganglia. The contralateral cortex was used to deliver the intraventricular drug delivery (Figure 3). No changes in cortex structure were observed. The ipsilateral cortical site (damaged) did not show any significant difference between the treated and non-treated animals. Although Cresyl violet is usually used to observe the ischemic tissue, we did not observe any changes in staining that will indicate the induction of ischemic insult in this model. This correlates with the lack of functional deficits we observed in this model.

Intraventricular Trx delivery results in enhancement of cell proliferation:
BrdU labeling was performed to investigate the effect of Trx1 treatment on cell proliferation as described above. 4-7 tissue sections 210 um apart (between two adjacent sections) containing the ventricle region were chosen from each animal. For cell quantification, 20x ApoTome-generated images of the sections were taken using Zeiss Axio Imaging System. Imaging was always performed with a similar setting across the different samples and experimental groups. Adobe Photoshop (CS.6) was used uniformly across all the samples to enhance fluorescent signals for better specificity while counting. Using ImageJ, a grid (3 in^2) was placed on the image of the tissue section and a total of 25 squares per animal were manually quantified (10 in the subventricular zone and 15 squares in the surrounding cortical area representing proliferating parenchymal stem cells). Total DAPI-positive nuclei were counted in each square. We then counted the number of BrdU-positive nuclei that were also DAPI-positive in the same square. The ratio of BrdU-positive nuclei to total DAPI-nuclei was used for statistical analysis.
In the subventricular zone, animals that received Trx treatment post-stroke had higher levels of BrdU positive cells compared to those that did not (*P < 0.01*). In the cortex, similar results were found in that animals that received Trx treatment after injury showed an increase in BrdU cells (*P < 0.05*) that was more than twice that seen in untreated animals. The significance of all values was evaluated via unpaired T-tests with Welch's correction. (Figure 4)

Intraventricular Trx delivery enhances cell proliferation in sox2-positive NPCs.
Sox2 labeling was performed to investigate the effect of Trx1 treatment on cell proliferation of NPCs. 3-9 tissue sections 210 um apart (between two adjacent sections) containing the ventricle were chosen from each animal. For cell quantification, 20x ApoTome-generated images of the sections were taken using Zeiss Axio Imaging System. Imaging was always performed with a similar setting across the different samples and experimental groups. Adobe Photoshop (CS.6) was used uniformly across all the samples to enhance fluorescent signals for better specificity while counting. Using ImageJ, a grid (3 in^2) was placed on the image of the tissue section and a total of 25 squares per animal were manually quantified (10 in the subventricular zone and 15 in the surrounding cortical area representing proliferating parenchymal stem cells). Total DAPI-positive nuclei were counted in each square. We then counted the number of sox2-positive nuclei that were also DAPI-positive in the same square. The ratio of sox2-positive nuclei to total DAPI-nuclei was used for statistical analysis.

In the subventricular zone, sox2 expression was significantly higher (*P < 0.0001*) in animals that received treatment, the percent Sox2-positive cells/DAPI being roughly double the percent seen in animals that did not receive treatment. A similar increase in Sox2 expression (*P < 0.0001*) was also seen in the cortical area; percent Sox2/DAPI in treated animals was more than twice than that seen in untreated animals. The significance of all values was again evaluated via unpaired T-tests with Welch's correction. (Figure 5)

Discussion:

In this study, we aimed to investigate the effect of Trx-transduction for enhancement of NPCs proliferation. The importance of Trx regulatory role in maintaining a reduced environment in the cell has been increasingly identified in different systems. Trx represent a potent anti-apoptotic and anti-inflammatory protein that is upregulated under the oxidative stress conditions such as stroke. The intravenous and intraperitoneal administration of high doses of Trx (10mg/Kg) has been shown to be protective in CNS trauma, although its permeability through the blood brain barrier remains questionable. There is currently no receptors identified for Trx and its protective effects have been linked to its immunosuppressive properties through modulation of immune cell infiltration. Trx can also enhance stem cell proliferation in adipose stem cells (Song et al., 2011) and very recently for NPCs (Tian et al., 2014). The current literature generally ignores the mechanism of action for Trx and often report similar effects for endogenous Trx and externally administered Trx. The protective effects reported for Trx is mediated through its active site and these effects are often disappear (Oblong et al., 1994a; Oblong et al., 1994b) if one or both cysteine residues in the active site are mutated to a serine. Since extracellular Trx is quickly oxidized and therefore is no longer active it is important to understand the mechanism and mode of action for Trx-transduction. We therefore have aimed to distinguish the intracellular and extracellular effects using a Tat-mediated Trx delivery system. Using this method, we showed that Trx transduction did not increase cell proliferation in adult mouse brain-derived NPCs. This is in contrast with the recent report (Tian et al., 2014) by Tian et. al. These authors showed a significant increase in BrdU and Ki-67 labeling of mouse embryonic NPCs which contradicts our data. It is possible that NPCs response to Trx mutagenic effects is more enhanced in embryonic NPCs.

We also investigated the effect of Trx transduction of gliogenic fate. We show that Trx therapy can promote oligodendroglial differentiation, while decreasing GFAP-positive astrocyte. This effect was more potent when I-trx was used. This effect, if confirmed in vivo, can have substantial importance in therapeutic application of trx for diseases or conditions that affect oligodendrocyte populations.

For our in vivo experiments, we used E-Trx and I-Trx, however only E-Trx results were presented in this manuscript. The effect of permanent devascularization on NPCs was previously shown (Kolb et al., 2007) and therefore we only focused on the effect of our treatments in injured animals. Our data shows that E-Trx treatment enhances NPCs proliferation. While this is the first application of intraventricular application of Trx for promotion of NPCs proliferation, our results are in agreement with previous report(Tian et al., 2014).

Our data clearly indicate the potential application of Trx for regenerative purposes involving NPCs. We plan to investigate the effect of Trx-therapy on neuronal population as suggested by previous reports. I-Trx represents a clinically suitable candidate for delivering Trx across the BBB, and therefore we will continue to investigate its effects. I-Trx can be injected intravenously, or intramuscularly to be quickly delivered to the patient without a need for

invasive intrathecal surgery. This can potentially have significant impact in the patients recovery and quality of life.

References:

Akterin, S., R.F. Cowburn, A. Miranda-Vizuete, A. Jimenez, N. Bogdanovic, B. Winblad, and A. Cedazo-Minguez. 2006. Involvement of glutaredoxin-1 and thioredoxin-1 in beta-amyloid toxicity and Alzheimer's disease. *Cell Death Differ* 13:1454-1465.

Eftekharpour, E., S. Karimi-Abdolrezaee, J. Wang, H. El Beheiry, C. Morshead, and M.G. Fehlings. 2007. Myelination of congenitally dysmyelinated spinal cord axons by adult neural precursor cells results in formation of nodes of Ranvier and improved axonal conduction. *J Neurosci* 27:3416-3428.

Emery, E., P. Aldana, M.B. Bunge, W. Puckett, A. Srinivasan, R.W. Keane, J. Bethea, and A.D. Levi. 1998. Apoptosis after traumatic human spinal cord injury. *J Neurosurg* 89:911-920.

Hattori, I., Y. Takagi, K. Nozaki, N. Kondo, J. Bai, H. Nakamura, N. Hashimoto, and J. Yodoi. 2002. Hypoxia-ischemia induces thioredoxin expression and nitrotyrosine formation in new-born rat brain. *Redox Rep* 7:256-259.

Hori, K., M. Katayama, N. Sato, K. Ishii, S. Waga, and J. Yodoi. 1994. Neuroprotection by glial cells through adult T cell leukemia-derived factor/human thioredoxin (ADF/TRX). *Brain Res* 652:304-310.

Kahles, T., and R.P. Brandes. 2012. NADPH oxidases as therapeutic targets in ischemic stroke. *Cell Mol Life Sci* 69:2345-2363.

Kelly, P.J., J.D. Morrow, M. Ning, W. Koroshetz, E.H. Lo, E. Terry, G.L. Milne, J. Hubbard, H. Lee, E. Stevenson, M. Lederer, and K.L. Furie. 2008. Oxidative stress and matrix metalloproteinase-9 in acute ischemic stroke: the Biomarker Evaluation for Antioxidant Therapies in Stroke (BEAT-Stroke) study. *Stroke; a journal of cerebral circulation* 39:100-104.

Kolb, B., C. Morshead, C. Gonzalez, M. Kim, C. Gregg, T. Shingo, and S. Weiss. 2007. Growth factor-stimulated generation of new cortical tissue and functional recovery after stroke damage to the motor cortex of rats. *J Cereb Blood Flow Metab* 27:983-997.

Legos, J.J., R.F. Tuma, and F.C. Barone. 2002. Pharmacological interventions for stroke: failures and future. *Expert Opin Investig Drugs* 11:603-614.

Molina, C.A. 2011. Reperfusion therapies for acute ischemic stroke: current pharmacological and mechanical approaches. *Stroke; a journal of cerebral circulation* 42:S16-19.

Nagakannan, P., B.D. Shivasharan, B.S. Thippeswamy, V.P. Veerapur, and P. Bansal. 2012. Protective effect of hydroalcoholic extract of Mimusops elengi Linn. flowers against middle cerebral artery occlusion induced brain injury in rats. *Journal of ethnopharmacology* 140:247-254.

Nakamura, H., Y. Hoshino, H. Okuyama, Y. Matsuo, and J. Yodoi. 2009. Thioredoxin 1 delivery as new therapeutics. *Adv Drug Deliv Rev* 61:303-309.

Oblong, J.E., M. Berggren, P.Y. Gasdaska, and G. Powis. 1994a. Site-directed mutagenesis of active site cysteines in human thioredoxin produces competitive inhibitors of human thioredoxin reductase and elimination of mitogenic properties of thioredoxin. *J Biol Chem* 269:11714-11720.

Oblong, J.E., M. Berggren, and G. Powis. 1994b. Biochemical, structural, and biological properties of human thioredoxin active site peptides. *FEBS Lett* 343:81-84.

Patenaude, A., M.R. Murthy, and M.E. Mirault. 2005. Emerging roles of thioredoxin cycle enzymes in the central nervous system. *Cell Mol Life Sci* 62:1063-1080.

Slemmer, J.E., J.J. Shacka, M.I. Sweeney, and J.T. Weber. 2008. Antioxidants and free radical scavengers for the treatment of stroke, traumatic brain injury and aging. *Curr Med Chem* 15:404-414.

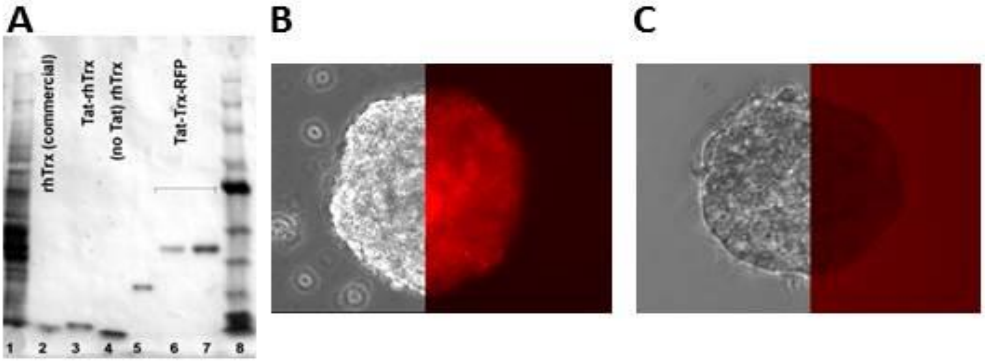
Song, J.S., H.H. Cho, B.J. Lee, Y.C. Bae, and J.S. Jung. 2011. Role of thioredoxin 1 and thioredoxin 2 on proliferation of human adipose tissue-derived mesenchymal stem cells. *Stem cells and development* 20:1529-1537.

Takagi, Y., A. Mitsui, A. Nishiyama, K. Nozaki, H. Sono, Y. Gon, N. Hashimoto, and J. Yodoi. 1999. Overexpression of thioredoxin in transgenic mice attenuates focal ischemic brain damage. *Proc Natl Acad Sci U S A* 96:4131-4136.

Tian, L., H. Nie, Y. Zhang, Y. Chen, Z. Peng, M. Cai, H. Wei, P. Qin, H. Dong, and L. Xiong. 2014. Recombinant human thioredoxin-1 promotes neurogenesis and facilitates cognitive recovery following cerebral ischemia in mice. *Neuropharmacology* 77:453-464.

Zhou, F., M. Gomi, M. Fujimoto, M. Hayase, T. Marumo, H. Masutani, J. Yodoi, N. Hashimoto, K. Nozaki, and Y. Takagi. 2009. Attenuation of neuronal degeneration in thioredoxin-1 overexpressing mice after mild focal ischemia. *Brain Res* 1272:62-70.

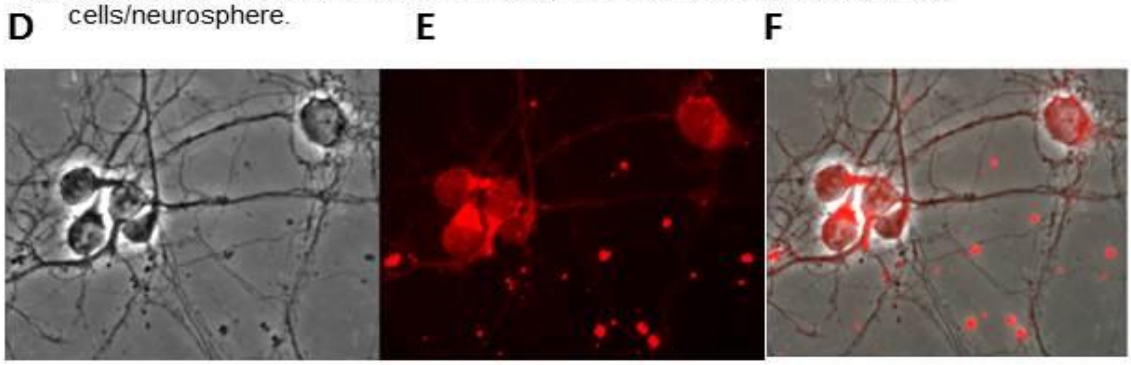
Figure 1) Purification of human Trx proteins and in vitro and in vivo protein delivery

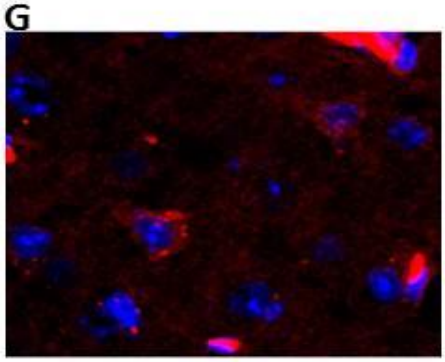


A) Coomassie blue stained gel depicting Trx recombinant human proteins. Lanes 1) bacterial homogenate, 2) commercial rhTrx, 3) Tat-rhTrx, 4) rhTrx (no Tat), 5) Red fluorescent protein (RFP), 6 &7) 25 and 50ng Tat-rhTrx-RFP (l-Trx), 8) protein marker.

B) Adult brain-derived neurosphere treated with (Tat-Trx-RFP) l-Trx. High intensity RFP signal in the sphere indicate the intracellular delivery of l-Trx.

C) Extracellular Trx (Trx-RFP) that contains no Tat peptide can not enter the cells/neurosphere.

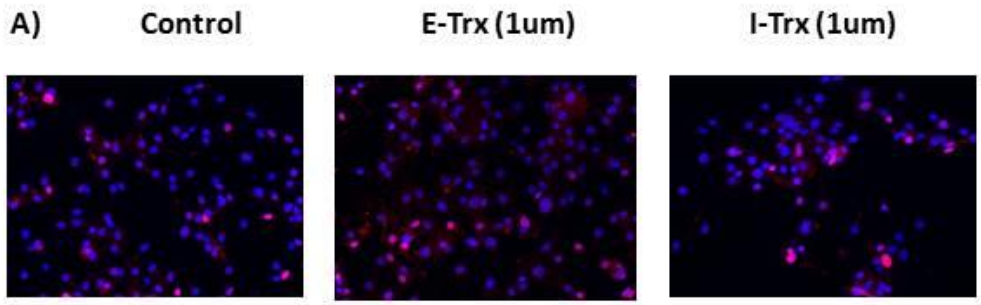


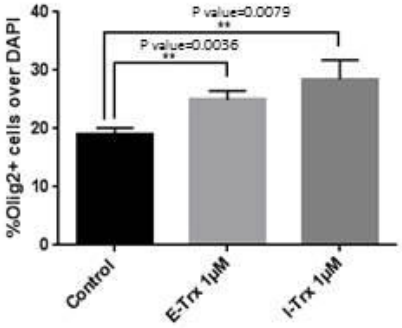


D-F) cultures of cortical neurons treated with 1µm l-Trx. Intracellular delivery is shown as RFP signal in the cytoplasm in the cell body and cell processes.

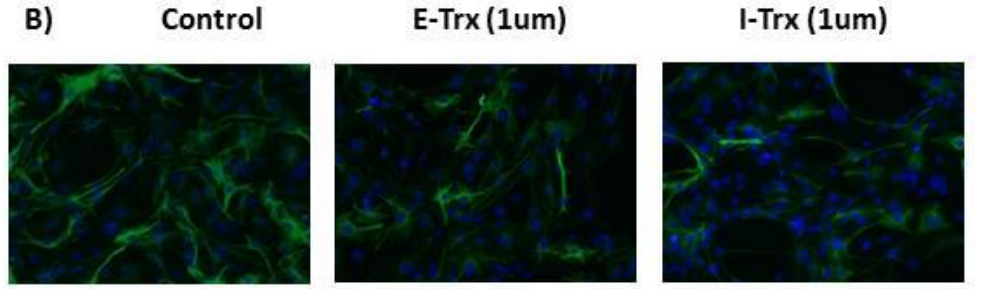
G) Intracellular entry of l-Trx after intraperitoneal injection in the brain cortex. RFP signal indicating the presence of l-Trx in neuronal bodies is shown.

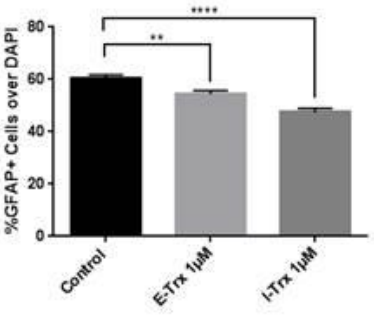
Figure 2) Effect of Trx-transduction on NPCs differentiation





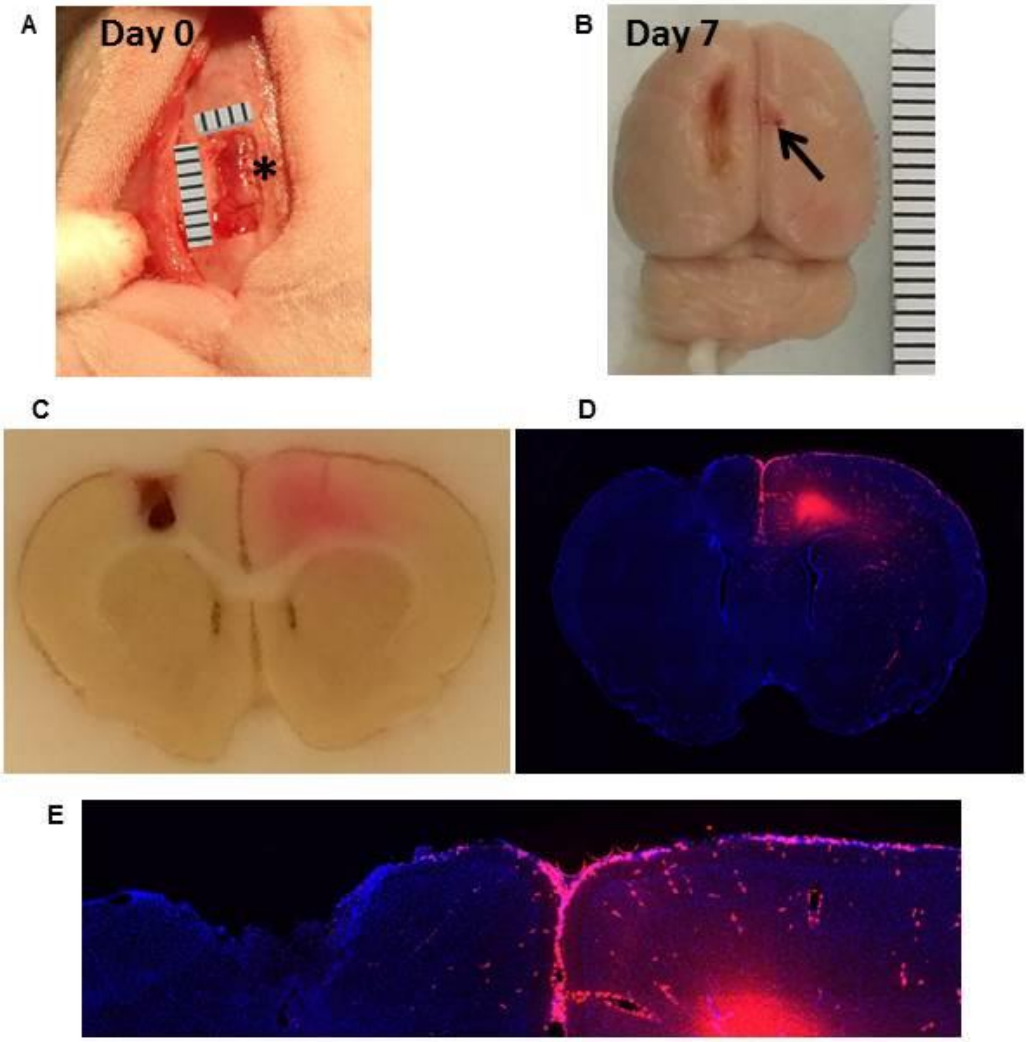
A) Representative fluorescent images depicting oligodendroglial lineage using anti-olig2 antibody. The ratio of Olig2-positive cells to total DAPI-positive cells were used for statistical analysis. Unpaired t-Test was used to compare any changes by Trx-treatment. While both E-Trx and I-Trx enhanced the number of oligodendrocyte significantly (p values are shown), One-way ANOVA NOVA showed that only I-Trx can significantly increase the Olig2-positive cells.





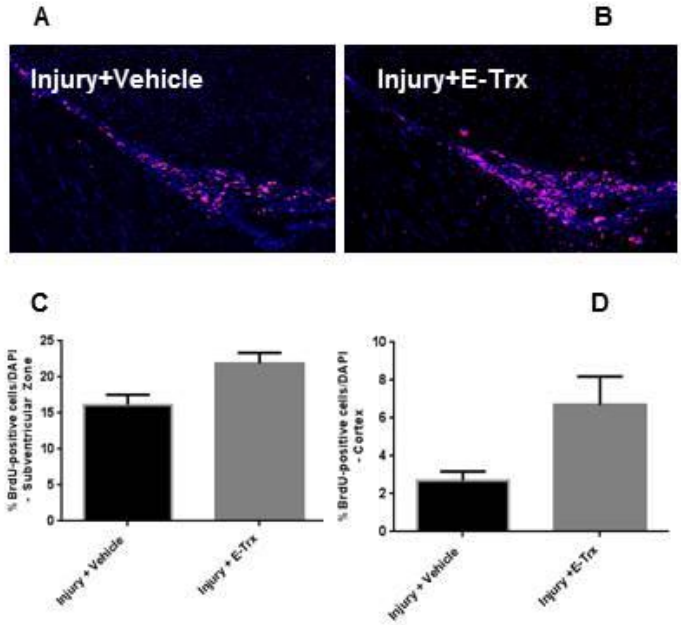
B) Representative fluorescent images depicting GFAP-positive cells. The ratio of GFAP-positive cells to total DAPI-positive cells were used for statistical analysis. One way ANOVA was used to compare any changes by Trx-treatment. Our results show that E-Trx and I-Trx significantly decreased the number of GFAP-positive astrocytes.

Figure 3) Permanent focal cortical devascularization, and drug delivery system.



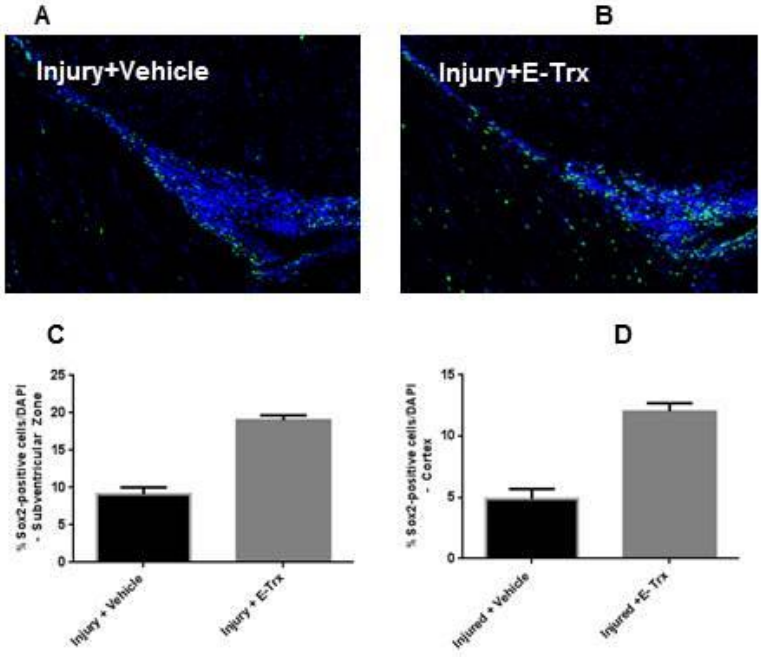
A) Permanent focal cortical devascularization induction as described in the Materials and Methods (A). In B, Rat brain is shown at day 7 after the injury. Arrow shows the drug delivery site in the contralateral hemisphere. C) A cross section of the brain tissue that received E-Trx (Trx-RFP). The pink color of our protein is seen in the tissue block. D) Fluorescent image from the same animal after staining for DAPI. E) shows a higher magnification of the cortex showing the loss of cortical organization in the injured site.

Figure 4) Effect of Trx-transduction on proliferation of endogenous NPCs



A and B) Proliferating BrdU-positive cells were identified in the subventricular zone in injured animals that received controlled vehicle or E-Trx. We manually counted the percentage of nuclei that were double labeled for BrdU and DAPI. These data were used for statistical analysis as shown in C. Un-paired two-tailed t-Test indicated a significant upregulation of BrdU-positive cells in the subventricular zone. D) similar approach was used for the cortical regions. Although BrdU-positive cells were less frequent in these regions, their number increased significantly in E-trx treated animals.

Figure 5) Effect of Trx-transduction on endogenous NPCs



A and B) To further investigate the effect of Trx on NPCs, we use an anti-Sox2 antibody. Sox2 is a reliable marker for multipotent ial neural precursor cells. Sox2 cells were identified in the subventricular zone in injured animals that received controlled vehicle or E-Trx. We manually counted the percentage of nuclei that were double labeled for Sox2 and DAPI. These data were used for statistical analysis as shown in C. Un-paired two-tailed t-Test indicated a significant upregulation of Sox2-positive cells in the subventricular zone. D) similar approach was used for the cortical regions. Although Sox2-positive cells were less frequent in the cortical regions, their number increased significantly in E-trx treated animals.