TEL AVIV UNIVERSITY אוניברסיטת תל-אביב
Sagol School of Neuroscience
Felsenstein Medical Research Center,
Sackler School of Medicine

New therapeutic approaches to promote functional outcome and recovery in mouse model of focal ischemic injury

By
Lior Molcho
301097507

The thesis was carried out under the supervision of
Prof. Daniel Offen

September, 2016

אוניברסיטת תל-אביב TEL AVIV UNIVERSITY

בית הספר סגול למדעי המוח
המכון למחקר רפואי ע״ש פלזנשטיין
הפקולטה לרפואה ע״ש סאקלר

שיטות טיפוליות חדשות לשיפור תפקוד והחלמה במודל עכברי של שבץ איסכמי

מאת
ליאור מולכו
301097507

החיבור בוצע בהנחייתו של
פרופ׳ דניאל אופן

ספטמבר, 2016

Table of contents

Abstract

Background: Stroke is a leading cause of death worldwide and inflicts serious long-term damage and disability. The vasoconstrictor Endothelin-1 (ET-1) is increasingly being used to induce focal ischemic injury in rodents as a preclinical model of stroke. Animals in this model, display significant long-term neurological deficits, associated with excitotoxicity, inflammatory response and oxidative stress. Here we show different approaches to minimize neuronal damage and improve functional outcome. First approach used is peptide therapy. A DJ-1 based peptide, called ND-13, has been shown to protect against glutamate toxicity, neurotoxic insults and oxidative stress in various animal models. Moreover, ND-13 provides its neuroprotective effects through regulation of anti-oxidant and anti-inflammatory responses. Second approach used is gene therapy. A novel multifactorial-cocktail treatment of lentiviruses encoding EAAT2, GDH2 and NRF2 genes, has been shown to act synergistically to address the effected excito-oxidative axis, thereby reducing extracellular-glutamate and glutamate availability while improving the metabolic state and the anti-oxidant response. This strategy has yielded impressive results in various animal models of neurodegenerative diseases. The aim of this study is to examine the possible efficacy of peptide and genetic treatments on functional outcome and recovery following focal ischemic injury in mice and reveal possible underlying mechanisms of action.

Methods: focal ischemic injury was induced by injection of ET-1 into the right striatum. Behavioral tests were performed to measure motor function after ischemia. Treatment: in the first approach, ND-13 peptide or vehicle where injected subcutaneously twice a day for five days following surgery, starting three hours after lesion. In the second approach, mice were intrastriatally injected (unilaterally) with lentiviruses carrying Nrf2, GDH2 and EAAT2 genes or with GFP as control 72h prior to ischemia. In search of relevant mechanisms of action, protein analysis and profiling were performed including immunohistochemistry and proteomics analysis.

Results: The evaluation of motor function by elevated bridge test and cylinder test, showed motor deficits following ET-1-induce ischemia. In the first approach, Mice treated with ND-13 improved in the elevated bridge test compared to the control group. ND-13 treatment also attenuated motor asymmetry in the cylinder test compared to control group. The ND-13 treatment improvements were consistent for at least three weeks from surgery. Furthermore, DJ-1 knock-out mice treated with ND-13 showed significant improvement after ET-1 induced ischemia, indicating that ND-13 provides compensation for DJ-1 deficits. Proteomics analysis showed changes in regulatory proteins involved in mitochondria regulation and oxidative stress response, as well as excitotoxicity response. In the second approach, Injection of a viral vector carrying Nrf2, GDH2 and EAAT2

genes, demonstrated marked improvements in motor function following ET-1 induced ischemia. In the elevated bridge test, we observed a decrease in the time spent crossing the bridge compared to the control group. An improvement was also evident after treatment in the cylinder test. Moreover, treatment with mixture of the 3 genes yielded a more significant improvement then each gene separately, suggesting a synergistic effect. Immunohistochemistry showed a decreased inflammation in the treated group compared to the control group, as indicated by GFAP staining.

Conclusion: Our findings suggest that both treatments promote recovery in mouse model of focal ischemic injury. Both treatments seem to activate parallel pathways, reducing the harmful processes that are initiated after stroke, and promoting processes leading to cell survival and functional improvement. These preliminary results demonstrate a possible basis for clinical applications to enhance neuroprotection in stroke patients with various disabilities.

תקציר

הקדמה: שבץ מוחי הוא גורם התמותה שכיח בעולם כולו, ועלול לגרום לנזק ונכות משמעותיים לטווח ארוך. בשנים האחרונות גובר השימוש באנדותלין-1 להשריית נזק איסכמי במכרסמים כמודל פרה-קליני לשבץ. חיות במודל זה מציגות ירידה נוירולוגית משמעותית וארוכת טווח, הקשורה לטוקסיות גלוטמטרגית, תגובה דלקתית מתמשכת וסטרס חמצוני. בעבודה זו מוצגות שתי גישות שונות להפחתת הנזק הנוירולוגי ושיפור תפקודי לאחר שבץ. הגישה הראשונה מתמקדת בטיפול בעזרת פפטיד. מחקרים קודמים במעבדתנו הראו כי פפטיד המבוסס על החלבון DJ-1, בשם ND-13, מגן מפני תהליכים דלקתיים וטוקסיים במודלים למחלות נוירולוגיות שונות. יתרה מכך, ND-13 מספק את האפקט המגן שלו דרך רגולציה של תגובות אנטי-אוקסידנטיות ונוגדות דלקת. הגישה השנייה מתבססת על טיפול גנטי. טיפול זה מתבסס על לנטי-וירוסים המקודדים את הגנים EAAT2, GDH2 and NRF2. מחקרים קודמים במעבדתנו הראו את יעילות הטיפול במודלים למחלות נוירולוגיות שונות. הטיפול מבוסס על עידוד תהליכים כמו הפחתת הזמינות של גלוטמט במרחב החוץ תאי, ושיפור המצב המטבולי והתגובה של נוגדי החמצון. מטרת מחקר זה היא לבחון את יעילותם של טיפולים אלה על התפקוד וההחלמה לאחר נזק איסכמי מוחי בעכברים ולחשוף את מנגנוני הפעולה המאפשרים זאת.

שיטות: שבץ איסכמי מוקדי הושרה ע"י הזרקת אנדותלין-1 לסטריאטום הימני של עכברים. מבחנים התנהגותיים נעשו על מנת למדוד את התפקוד המוטורי לאחר הנזק. טיפול: בגישה הראשונה, ND-13 או סליין הוזרקו תת עורית פעמיים ביום ל-5 ימים, החל מ-3 שעות לאחר השריית הנזק. בגישה השנייה, לנטי-וירוסים הנושאים את הגנים EAAT2, GDH2, Nrf2 או GFP כביקורת הוזרקו סטריאוטקטית לסטריאטום הימני 72 שעות לפני השריית השבץ. הערכת התפקוד המוטורי התבצעה ע"י מבחן הגשר ומבחן הצילינדר. אנליזת חלבונים ואימונוהיסטוכימיה נעשו על מנת לגלות מנגנוני פעולה המגשרים את האפקט הטיפולי.

<u>תוצאות</u>: הערכת התפקוד המוטורי ע״י המבחנים ההתנהגותיים הראתה נזק מוטורי לאחר השריית השבץ בכל המקרים. בגישה הראשונה, חיות שטופלו ב-ND-13 השתפרו במבחן הגשר בהשוואה לקבוצת הביקורת. הטיפול גם עזר לשיפור א-סימטריה מוטורית הנבדקת במבחן הצילינדר בהשוואה לקבוצת הביקורת. השיפור התפקודי היה עקבי למשך 3 שבועות לפחות מהשריית השבץ. בנוסף, חיות DJ1-KO שטופלו עם הפפטיד הראו גם הן שיפור משמעותי לאחר הנזק, דבר המרמז על כך ש-ND 13 מפצה על חוסר בחלבון DJ-1. אנליזת חלבונים (פרוטאומיקה) הראתה שינוי ברמות הביטוי של חלבונים רגולטוריים שמעורבים ברגולציה של המיטוכונדריה, תגובה אנטי-חמצונית ותגובה כנגד רעילות של גלוטמט. בגישה השנייה, הקבוצה לה הוזרקו וירוסים הנושאים את הגנים Nrf2, GDH2, EAAT2 הראתה שיפור מוטורי לאחר איסכמיה בהשוואה לקבוצה שקיבלה GFP. במבחן הגשר, נצפתה ירידה בזמן שלקח לחיות לחצות את הגשר בהשוואה לקבוצת הביקורת. שיפור נרשם גם במבחן הצילינדר בקבוצת הטיפול. יתרה מכך, טיפול ע״י תערובת של שלושת הגנים הניב תוצאות טובות יותר מאשר טיפול עם כל גן בנפרד, מה שמצביע על אפקט סינרגיסטי בפעולת הגנים הללו. בדיקות אימונוהיסטוכימיות גילו ירידה בדלקתיות בקבוצה המטופלת ע״י שלושת הגנים בהשוואה לקבוצת הביקורת.

<u>מסקנות</u>: הממצאים המדווחים בעבודה זו מראים כי שני הטיפולים המוצעים משפרים את התפקוד ומידת ההחלמה של חיות המודל לאחר שבץ איסכמי. נדמה ששני הטיפולים מפעילים מסלולים מקבילים, הגורמים להפחתת התהליכים המזיקים שקורים במוח לאחר שבץ, ולהגברת תהליכים מועילים שמובילים לשרידות הרקמה ושיפור תפקודי. התוצאות הראשוניות הללו יכולות להוות בסיס עבור יישומים קליניים להגברת ההגנה על הרקמה המוחית ושיפור תפקודי בעבור מטופלים עם מוגבלויות שונות לאחר שבץ.

<u>Abbreviations</u>

ET-1, Endothelin 1; KO, knock out; SEM, standard error of the mean; ANOVA, Analysis of variance; EAAT2, Excitatory amino acid transporter 2; GDH2, Glutamate dehydrogenase 2; NRF2, Nuclear factor E2-related factor 2; GFP, green fluorescence protein; PBS, Phosphate buffered saline; TTC, Triphenyl tetrazolium chloride; BSD, blasticidin-S deaminase; GFAP, glial fibrillary acidic protein; DAPI, 4′,6-diamidino-2-phenylindole; PFA, Paraformaldehyde; BSA, Bovine serum albumin; ALS, Amyotrophic Lateral Sclerosis; MSA, Multiple System Atrophy; 3-NP, 3-Nitropropionic acid; ROS, Reactive oxygen species; SOD1, superoxide dismutase 1; Pink1, PTEN Induced Putative Kinase 1; SDH, succinate dehydrogenase; SDHAF4, succinate dehydrogenase assembly factor 4; COMMD1, copper metabolism Murr1 domain containing 1; RHOT2, Rho GTPase 2; USP35, Ubiquitin Specific Peptidase 35; CRBN, Cereblon; Kcnip4, Potassium Voltage-Gated Channel Interacting Protein 4; MCAo middle cerebral artery occlusion; FC, Fold change.

Introduction

Stroke is the second most common cause of death, causing 9% of all deaths around the world, and the most frequent cause of permanent disability in adults worldwide (Donnan et al. 2008). When blood flow to the brain is suddenly stopped, neuronal function is impaired and pathological pathways are triggered, causing irreversible neuronal damage in the ischemic area within minutes of onset. In the hours and days following stroke, the damaged regions undergo a broad scale necrosis, which causes the death of all types of cells (Donnan et al. 2008; Fisher & Garcia 1996; Heiss et al. 1992). Stroke initiates a cascade of pathological processes which lead to permanent neuronal damage including excitotoxicity, oxidative stress, inflammation, ionic imbalance, blood–brain barrier disruption and apoptosis (Guo et al. 2014). Failure of energy production causes a flood of neurotransmitters to be released from neurons, mostly release of the excitatory glutamate, which further amplifies the damage (Choi & Rothman 1990; Chao & Li 2014). Subsequent processes such as oxidative stress, dysfunction of blood brain barrier and inflammatory response all contribute to the outcome of stroke (U Dirnagl, Iadecola & M. a Moskowitz 1999; Lo et al. 2003a).

The high rate of oxidative metabolism in the brain renders it vulnerable to oxidative stress. Reactive oxygen species (ROS) levels increase after ischemic injury, since the endogenous scavenging mechanisms are normally not high enough to match excess radical formation, resulting in massive oxidative stress (Lo et al. 2003b; Chan 2001). When an imbalance between production of free radicals and endogenous scavenging capacity of cellular antioxidants is disturbed, damage to the tissue is unavoidable. The mitochondria is implicated in this process due to excessive superoxide production during the electron transport chain (Lee et al. 2012; Murphy et al. 1999). Free radicals are also generated through multiple injury mechanisms such as mitochondrial inhibition, Ca^{2+} overload, reperfusion injury, and inflammatory response after ischemia (Lakhan et al. 2009a; Coyle & Puttfarcken 1993a; Cuzzocrea et al. 2001). Oxidative stress directly damages proteins, lipids, carbohydrates and nucleic-acids, leading to cell dysfunction and DNA fragmentation, contributing to ischemic cell death (Chan 2001; Lipton et al. 1999; Choi et al. 2009).

Oxidative stress also leads to mitochondrial dysfunction and mitophagy which is one of the major mechanisms of mitochondrial quality control. This process is mediated by pink1 and Parkin (PARK2) proteins. Upon mitochondrial depolarization, likely the result of oxidative damage, pink1 is localized to the outer mitochondrial membrane where it recruits, phosphorylates and activates parkin. This causes the ubiquitination of mitochondrial substrates which are degraded by the proteasome, resulting in fragmentation of mitochondria and removal via mitophagy (Kim et al. 2008; Matsuda et al. 2010; Narendra et al. 2010; Narendra et al. 2008). In addition, pink1 and parkin have a vital role arresting mitochondrial movement. Halting mitochondrial axonal transport

prevents an accumulation of dysfunctional mitochondria and prevents damaged mitochondria from being transported along the axons (Ashrafi et al. 2014; Cai et al. 2012; Sheng & Cai 2012). This is regulated by the expression profile of Miro proteins that are involved in mitochondrial trafficking. Pink1 phosphorylates Miro, resulting in its ubiquitination by parkin and subsequent degradation in the proteasome. The degradation of Miro results in the disassembly of the motor-adaptor complex, thus arresting mitochondrial movement. This quarantines the depolarized mitochondria and subsequently increases the clearance of damaged mitochondria (van der Merwe et al. 2015; Liu et al. 2012).

Nuclear factor E2-related factor 2 (Nrf2), is a transcription factor that binds to the promoter regions of target genes and plays a key role in cellular defense. It enhances expression of protective enzymes and antioxidant response (ARE) elements and up-regulate antioxidant genes, resulting in reduction of the oxidative stress and inflammatory responses (Itoh et al. 1997; Nguyen et al. 2009; Surh et al. 2008; Ishii et al. 2000). Various studies indicate that activation of Nrf2 leads to neuroprotective effects against ischemic injury. Reports include improved survival rate, decreased sensorimotor and behavioral deficits, reduced cortical damage, decreased cerebral infarct volume and brain water content, and improved neurological symptoms (Shih et al. 2005; Takagi et al. 2014; Yamauchi et al. 2016; Yang et al. 2009; Zhao et al. 2006). Thus, increasing levels of NRF2 could serve as a therapeutic target for stroke.

The impairment in energy metabolism and cell death after stroke leads to ionic imbalance. Neurotransmitter spillover occurring after ischemia, especially of the excitatory glutamate, result in neuronal over-excitability and cause excessive excitotoxicity that leads to cell necrosis (Lo et al. 2003a; Lipton et al. 1999; Martin et al. 1998). It is the main contributor to tissue damage and cell death following stroke. Excessive accumulation of the excitatory neurotransmitter glutamate leads to a toxic increase in intracellular calcium, which activates multiple signaling pathways, eventually leading to necrosis and apoptosis (Lai et al. 2014). Blocking the excitotoxicity shows promise in rescue and neuroprotection after stroke (U Dirnagl, Iadecola & M. a Moskowitz 1999; Chamorro et al. 2016).

A potential approach to reduce excitotoxicity is enhancing glutamate reuptake. The excitatory amino acid transporter 2 (EAAT2), is increasingly being investigated as a new target for treatment of neurodegenerative diseases (Lin et al. 2012; Takahashi et al. 2015). The glial glutamate transporter EAAT2 in astrocytes, is associated with excitatory synaptic and is the primary responsible for maintaining low extracellular glutamate concentrations by glutamate reuptake activity (Chaudhry et al. 1995; Rothstein et al. 1996). Knockdown of EAAT2 aggravates the neuronal damage after stroke, whereas overexpression of EAAT2 enhances neuroprotection, reduces infarct volume, decreases cell death and improves behavioral recovery (Rao et al. 2001; Weller et al. 2008; Chu et al. 2007; Harvey et al. 2011). These studies suggest that increased EAAT2 levels can be a therapeutic target for stroke.

An additional way to prevent excitotoxicity is enhancing glutamate turnover. Glutamate dehydrogenase 2 (GDH2), a mitochondrial enzyme that catalyzes the oxidative deamination of glutamate to α-ketoglutarate, is central to glutamate metabolism (Shashidharan et al. 1997; Hudson & Daniel 1993). Studies show that administration of GDH2 reduces glutamate bioavailability in the neuron. Furthermore, GDH2 activity is decreased in multi-systemic neurological disorders (Plaitakis et al. 2000). Specific mutation in the GDH2 gene can encourage an earlier Parkinson's disease onset (Plaitakis et al. 2010). Therefore, increasing levels of GDH2 may also serve as a therapeutic target for stroke.

Another approach to reduce excitotoxicity is through channel regulation. Studies show that modifying channel regulation can promote cell survival by blocking the spreading depolarization and modulating glutamate and calcium levels (Bruno et al. 2001; Horn & Limburg 2001; Pellegrini-Giampietro et al. 1992). Recently, imbalances in potassium have also been implicated in ischemic cell death (Shah & Aizenman 2014). Electrical signaling in the brain is necessary for the most basic biological processes. The largest and most diverse class of ion channels underlying this type of signaling are potassium channels. The opening of K+ channels mediates feedback control of excitability in a variety of conditions. Voltage-gated K+ channels help bring the activated plasma-membrane potential more rapidly back toward its original negative potential (Alberts et al. 2002). Unlike other K+ channels, the large-conductance voltage- and Ca2+ -activated K+ channel (BK channel), can be activated by both membrane depolarization and intracellular Ca2+ ions (Calderone 2002). BK channels play an important role in stress conditions, as they appear to rescue neurons affected by different insults (Sausbier et al. 2004; Kulawiak et al. 2008), and more specifically, improve the survival of neurons exposed to ischemic conditions, since their activation tends to reduce cellular excitability (Rundén-Pran et al. 2002). Cereblon (CRBN) protein regulates the assembly and neuronal surface expression of the BK channels (Jo et al. 2005; Higgins et al. 2008). A direct interaction of CRBN with the cytosolic C-terminus of the BK channel reduces the surface expression of functional channels and therefore, CRBN plays a role in modulating neuronal BK channel activity (Jo et al. 2005). Another type of potassium channel implicated in ischemia is the Kvα4 (Shal-related) voltage-gated rapidly inactivating A-type potassium channels (A type/kv4 channels). These channels mediate rapidly inactivating A-type (IA) currents and recover from inactivation at a faster rate than other Kv channels (Serôdio & Rudy 1998; Shibata et al. 2000). Research show that A-type channels may be involved in reducing ischemic injury damage. Hypoxia exposure induces inhibition of A-type currents (IA) and downregulation of A-type channels (Liu et al. 2014). This downregulation of IA channels increases neuronal excitability, and the resulted abnormal hyper-excitability promotes tissue damage and high susceptibility to seizures after ischemia (Lei et al. 2016; Bernard 2011). Accordingly, upregulation of A-type currents (IA) after ischemia correlates with higher resistance of cells to ischemic insult by decreasing excitotoxicity (Deng et al. 2011).

Furthermore, neurons lacking Kv4 channel show increased vulnerability to ischemic insults and are more sensitive to ischemic cell death. This suggests a protective role of A-type channels against ischemia (Deng et al. 2011; Shah & Aizenman 2014). The protein Kcnip4 (Potassium Voltage-Gated Channel Interacting Protein 4) is a regulatory subunit of Kv4 channels that mediate the neuronal IA currents. Kcnip4 modulates channel expression, gating characteristics, and inactivation kinetics of the channel (Schwenk et al. 2008; Jerng & Pfaffinger 2008; Holmqvist et al. 2002). Unlike other K-channel interacting proteins, Kcnip4 largely reduces surface expression of the Kv4 channel complexes (Schwenk et al. 2008). It exhibits a unique N terminus that suppress Kv4 function resulting in A-type current suppression (Jerng & Pfaffinger 2008), suggested to occur via ER retention and enhancement of Kv4 closed-state inactivation (Tang et al. 2013).

The inflammatory response is also a major player in the ischemic cascade. Within minutes a robust inflammatory response is elicited in the injured brain (Muir et al. 2007). This involves the activation of glial cells including microglia and astrocytes and infiltration of immune cells through the BBB (Wang et al. 2007). Secretion of inflammatory cytokines and chemokines, and the accumulation of immune cells in the injured brain region facilitate the inflammatory response (Arvin et al. 1996). The activation of glial cells can last for several months. An acute inflammatory response aggravates tissue injury during ischemic stroke contributing to secondary brain damage (U Dirnagl, Iadecola & M. A. Moskowitz 1999). Astrocytes may contribute to damage by sending pro-apoptotic signals to healthy tissue, and inhibiting regeneration by inducing the formation of the glial scar (Anderson et al. 2003). Focusing on these harmful processes may provide opportunities for novel treatment (Barone & Feuerstein 1999; Lakhan et al. 2009b).

Targeting all of these elements may help improve stroke outcome and introduce new therapeutic options. This work shows two options for such targets. First approach is peptide therapy using ND-13 treatment, a peptide based on the protein DJ-1 (also known as PARK7). DJ-1 has diverse function such as preserving mitochondrial function, regulating kinase pathways and acting as a transcriptional regulator affecting anti-oxidant genes (Kaneko, Tajiri, et al. 2014b; Milani et al. 2013; Hao et al. 2010; Kahle et al. 2009; Waak et al. 2009; Pantcheva et al. 2014). It has been shown that parkin and DJ-1 interact under oxidative stress conditions, causing an increase in the steady-state levels of DJ-1, and thus decreasing oxidative stress (van der Merwe et al. 2015; Jin et al. 2007; Moore et al. 2005). Pink1 and DJ-1 are also recruited when the mitochondrial membrane potential has decreased, resulting in an increase in cell viability (van der Merwe et al. 2015; Hao et al. 2010; Tang et al. 2006). DJ-1 also provides protection against excitotoxicity and ischemic brain injury (Kitamura et al. 2011; Aleyasin et al. 2007a). ND-13 is a 20 amino acid peptide composed of 13 amino acids DJ-1-derived peptide, attached to 7 amino acids derived from the TAT cell penetrating peptide (CPP). TAT-derived CPP's have

previously been shown to facilitate the delivery into the central nervous system (Doeppner et al. 2010; Tao et al. 2008; Gou et al. 2010).
ND-13 has been shown to protects cells and promote survival in various animal models. Furthermore, ND-13 protects cells against oxidative and neurotoxic insults, reducing reactive oxygen species (ROS) accumulation, and activating protective factors that increase cell survival e (Lev et al. 2015; Glat et al. 2016).
Second approach is gene therapy using lentiviruses encoding EAAT2, GDH2 and Nrf-2 genes as a potential future treatment of stroke. Research show that these genes act synergistically to address the harmful processes occurring after stroke, reducing extracellular-glutamate and glutamate availability while improving the metabolic state and the anti-oxidant and inflammatory responses. This strategy has yielded impressive results in various animal models of neurological disorders (Benkler et al. 2016, Glat et al. in print)preparation)

The increasing prevalence of stroke and the limitations of the current therapeutic approaches puts a load on society. Aside from the fact that the annual costs of stroke amount to billions of dollars (in medical costs and costs due to lost productivity), The increasing pressures on families and communities to provide care can be overwhelming (Donnan et al. 2008; Stineman et al. 1997). After initial hospitalization and rehabilitation, 80% of stroke survivors return to the community, relying on their family's support for daily living (Han & Haley 1999; Anderson et al. 1995). This makes the search for new treatments for stroke imperative. Research is focusing on the possibilities to enhance neuroprotection following stroke, targeting damaging processes such as excitotoxicity, oxidative stress and inflammation as new therapeutic approach for stroke and strategies that combine promotion of tissue integrity and neuroprotection can make valid therapeutic targets (Chamorro et al. 2016; Lakhan et al. 2009b; Lo et al. 2003b).
The aim of the present work is to examine two novel approaches for treatment after stroke. We wanted to study the possible efficacy of peptide and genetic treatments on functional outcome and recovery following focal ischemic injury in mice. A large number of processes contribute to the outcome of stroke, and both treatments relay on the regulation of those processes in an attempt to enhance neuroprotection. It was also sought out to investigate possible underlying mechanisms of action that mediate the effect.

Materials and Methods

Ethics statement

All experimental procedures were approved by the Tel Aviv University Committee of Animal Use for Research and Education. All surgeries were performed under subcutaneous injections of a mixture of ketamine (100mg/kg) and xylazine (8mg/kg) anesthesia, and all efforts were made to minimize suffering.

DJ-1 based peptide: ND-13

ND-13 is a 20 amino acid peptide composed of 13 amino acids DJ-1-based peptide, attached to a TAT-derived 7 aa sequence. The ND-13 sequence is YGRKKRRKGAEEMETVIPVD (Lev et al. 2015; Glat et al. 2016). The ND-13 was synthesized and provided by China Peptides (China).

Lentiviral Vector Cloning

The lentiviral vectors LV-EAAT2, LV-NRF2, LV-GDH2, and LV-GFP were constructed using the ViraPower Promoterless Lentiviral Gateway® Kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. The cloning was done by the lab technician in our lab. The cytomegalovirus (CMV) promoter from pIRES2/AcGFP1 cDNA (Clontech, Palo Alto, CA, USA) was cloned into pENTR™5′-TOPO® (Invitrogen). To over-express the three genes, we used plasmids procured from source bioscience (http://www.lifesciences.sourcebioscience.com): EAAT2 (IOH:42832), NRF2 (IOH14493), and GDH2 (IOH27063). GFP gene was used as a control vector (LV-GFP). The constructs were cloned into the pCR®8/GW/TOPO® (Invitrogen). The final expression constructs were obtained by recombination of the entry clone harboring the CMV promoter, the entry clone harboring the expression gene of interest, and pLenti6/R4R2/V5-DEST (Invitrogen). The ability of the LV vectors to express the gene of interest was evaluated using real-time PCR and protein functional tests. The lentiviral load was determined using the Lenti-X p24 Rapid Titer Kit and the manufacturer's recommended procedure. The viral titer was determined using the resulting p24 standard curve and the sample OD. The p24 content was converted into infectious units using the manufacturer's recommended formulas, with 1ng of p24 considered equivalent to 1.25×107 lentiviral particle (LP), and 1 infectious unit considered equivalent to 1 in every 1000 LPs.

Animals

C57BL6 male mice at the age of 10-12 weeks were purchased from Harlan, Israel. Transgenic DJ-1 knockout mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were placed in a light-controlled environment (12-h light/dark cycle) and housed in individually ventilated cages (IVC) with free access to food and

water. Animals were acclimatized for 1 week prior to experimentation then randomly divided into experimental groups.

Surgical procedure and treatment

Focal ischemic injury

The vasoconstrictor Endothelin-1 (ET-1) is increasingly being used to induce focal ischemic injury in rodents as a preclinical model of stroke. Animals in this model, display significant long-term neurological deficits, associated with excitotoxicity, inflammatory response and oxidative stress (Kurosawa et al. 1991; Nguemeni et al. 2015; Fuxe et al. 1989; Horie et al. 2008). Mice were anesthetized with ketamine and xylazine (100 mg/kg and 8 mg/kg, respectively) and placed in a stereotaxic frame. All injections were to the right striatum at the following coordinates (relative to bregma): +0.5 mm anterioposterior, +1.9 mm mediolateral, −2.9 mm dorsoventral (Fig 1A).

For the peptide therapy (ND-13) experiments: 5 µl of the vasoconstrictor endothelin-1 (ET-1, 0.2 mg/ml dissolved in sterile saline, Calbiochem, CA, USA) where injected into the right striatum (infusion rate 0.3 µl/min).

For the gene therapy (3-genes) experiments: mice were injected with 1 µl or 2µl of ET-1 into the right striatum at the same coordinates (1 mg/ml dissolved in sterile saline, infusion rate 0.3 µl, Calbiochem, CA, USA) 72h after lentiviral administration.

In both cases the needle was left in place for 3 additional minutes before withdrawal, and the incision was sutured. Sham operated mice were treated identically except they received injections of sterile saline (instead of ET-1).

Treatment

For the peptide therapy (ND-13) experiments: ND-13 (1mg/ml dissolved in sterile saline) or saline (as control) were administered subcutaneously twice a day for five days following surgery, starting 3 hours after ET-1 injection.

For the gene therapy (3-genes) experiments: mice received either lentiviruses carrying EAAT2, GDH2, and NRF2 genes, lentiviruses carrying only one of the genes, or lentiviruses carrying GFP (as control). Lentiviruses were injected stereotaxicly into the right striatum at the same coordinates (see above). A total viral load of 5×10^8 infectious viral particles was administered (1.5µl total, infusion rate 0.3 µl/min). The needle was left in place for 3 additional minutes before withdrawal, and the incision was sutured.

Behavioral Tests and Analysis

Behavioral tests were performed at 2, 7, 14 and 21 days after ischemic induction.

The cylinder test: the test measures forelimb use during vertical exploration. Test was performed to as previously described (Schallert et al. 2000). The number of wall contacts with each forelimb during rearing was recorded. The final score was calculated as follows: non-impaired forelimb movement − impaired forelimb movement / total (non-impaired + impaired + both forelimb movements).

The Elevated bridge test: the test measures motor coordination and balance. Test was performed as previously described (Brooks 2011). Mice were trained over four consecutive days. On the first day animals were habituated to the goal box for 3 min. On the second and third day mice were trained to enter the goal box from different distances on the bridge. On the fourth day mice were placed on the extreme end of the beam, facing away from the box. Each test consisted of three measurements. The score represents the average time it took the animal to cross the bridge and get into the goal-box (in seconds).
The Pole test: the test assesses the mouse locomotor activity. the mice were placed head-upward on the top of a rough-surfaced vertical pole, and test was performed as previously described (Bouët et al. 2007). Each test consisted of three measurements. The score represents the average time it took the mouse to descend on the vertical pole.

Tissue Processing and Histology

For the peptide therapy (ND-13) experiments: global quantification of protein expression was done in the De Botton Institute for Protein Profiling (Weizmann institute, Israel). For the proteomic study, two experimental groups were generated as follows: (1) ND-13 treatment following Endothelin-1 injection into the right striatum, (2) Vehicle treatment following Endothelin-1 injection into the right striatum. Treatment was given 3h after surgery and twice a day for the next 2 days. Then, mice were sacrificed for striatal protein extraction. Total of 8 mice participated in the experiment in order to decrease intrinsic variability. The protein mix of each individual mouse was analyzed separately and only then averages of the animals in the same groups were calculated. Fold changes of protein expression between the tested groups was also calculated. Changes between groups were considered significant at ±2-fold change and $p<0.05$ (complete protocol in appendix 1).
For the gene therapy (3-genes) experiments: four weeks after ET-1 administration, animals were anesthetized with ketamine-xylazine and transcardially perfused with cold PBS followed by 4% PFA. The brains were then fixed with 4% PFA and equilibrated in 30% sucrose. Brains were sectioned (10 μm) using a cryostat and mounted directly onto slides for analysis. For immunohistochemistry, slides were incubated with blocking solution (5% goat serum, 1% BSA, 0.05% Triton-X in PBS) for 1 hr, and then incubated overnight at 4°C with the following primary antibodies: rat anti-GFAP (1:500, Invitrogen), rabbit anti-EAAT-2 (1:250, Santa-Cruz biotech), rabbit anti-NRF-2 (1:250, Santa-Cruz biotech), rabbit anti-GLUD-2 (GDH, 1:100, Proteintech), rabbit anti-BSD (1:500, Abnova). Then, sections were incubated with secondary antibodies: goat anti-rat Alexa Fluor 568 (1:500, Lifetech), and goat anti-rabbit Alexa 568 (1:500, Invitogen) for 1h. The nuclei were stained with DAPI (1:1000, Sigma-Aldrich).
For microscopic analysis, Zeiss LSM 510 confocal laser scanning microscope was used (Carl Zeiss Inc., Thornwood, NJ, USA). Intensity of fluorescence was measured at the injection site using ImageJ software (ImageJ software, U.S. NIH, Bethesda, MD, USA).

For negative control staining, only secondary antibodies were used. Four brains for each group were used for quantification. Results represent the average of each group.
For Triphenyl tetrazolium chloride (TTC) staining, animals were sacrificed two days after ET-1 administration. Immediately thereafter, brains were sectioned (2mm) and placed in 2% TTC (Sigma–Aldrich) for 15 min. slices were then fixed with 8% formalin for 24h.

Statistical Analysis

Statistical of the data sets were carried out using GraphPad Prism for Windows (GraphPad Software, CA, USA). Statistical significance was determined by one-way (or two-way as appropriate) ANOVA with repeated measures followed by Dunnett's post hoc test. Values are presented as mean ± SEM. The results were considered significant at minimal significance level of $p \leq 0.05$.

Fig 1

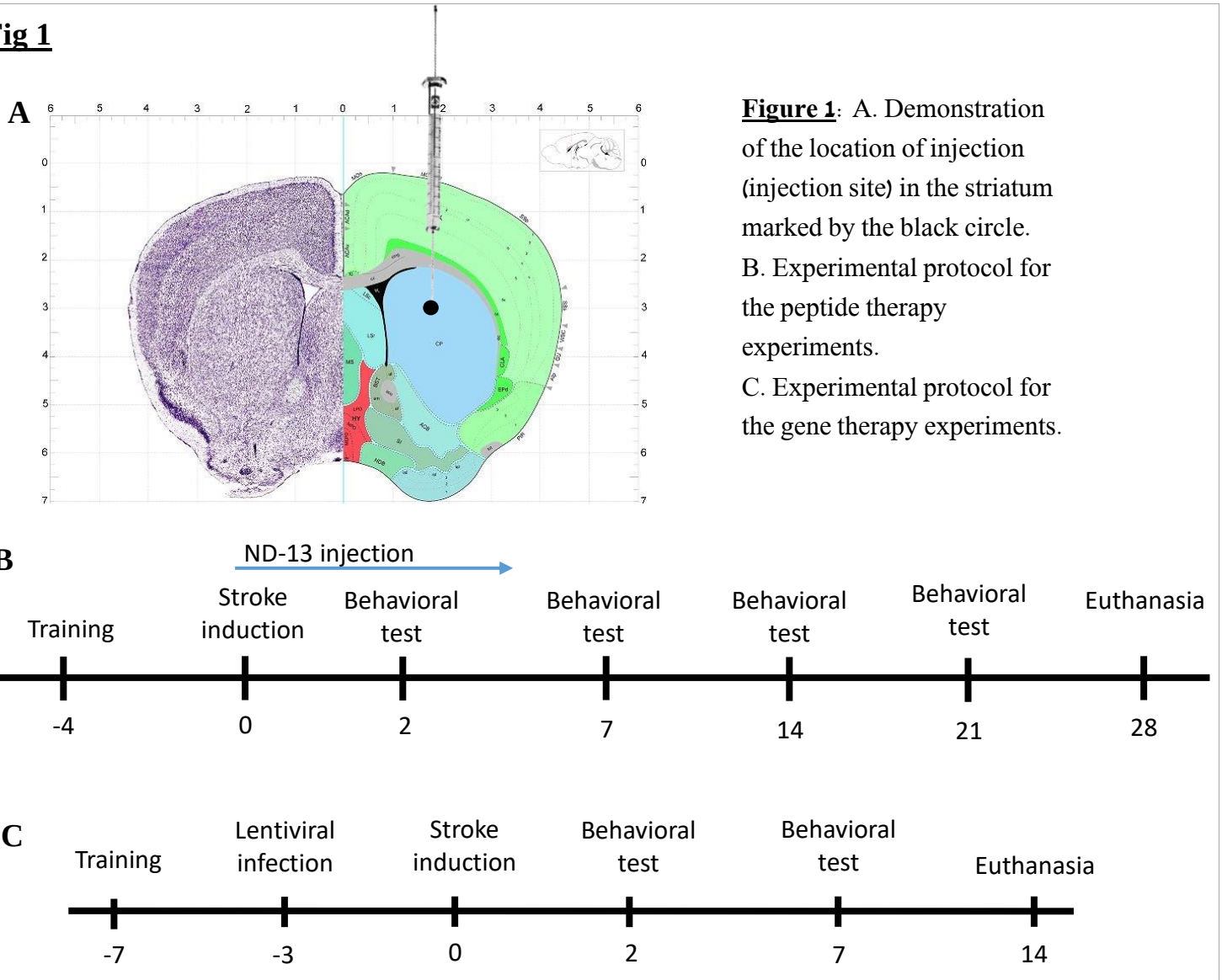

Figure 1: A. Demonstration of the location of injection (injection site) in the striatum marked by the black circle.
B. Experimental protocol for the peptide therapy experiments.
C. Experimental protocol for the gene therapy experiments.

Results

Peptide therapy

Mouse model of focal ischemic injury

The vasoconstrictor Endothelin-1 (ET-1) is increasingly being used to induce focal ischemic injury in rodents as a preclinical model of stroke. Animals in this model, display significant long-term neurological deficits, associated with excitotoxicity, inflammatory response and oxidative stress (Kurosawa et al. 1991; Nguemeni et al. 2015; Fuxe et al. 1989; Horie et al. 2008). In order to learn the effects of ND-13 after ischemic injury, 5 µl of ET-1 (0.2 mg/ml) was injected into the right striatum. Mice showed significant motor deficits as measured by the behavioral tests following ischemic injury compared to sham operated mice. Baseline measurements were done in order to make sure that the damage is due to the inflicted injury.

ND-13 improve motor function in mouse model of focal ischemic injury

After ET-1 was injected into the right striatum, mice were treated with ND-13 or vehicle (saline) twice a day for 5 days. Mice treated with ND-13 showed significant improvement in body balance and motor coordination as measured by pole and elevated bridge tests. The group treated with ND-13 improved by 30% in the elevated bridge test compared to the group treated with saline 2 days after injury (ND-13: 10.24 sec ± 0.57 sec; Vehicle: 14.17 sec ± 0.76 sec; $p<0.05$; Fig 2A). The improvement in the time taken the animals to cross the bridge and reach the goal-box was still significant 7 and 14 days from injury, compared to the group treated with vehicle, suggesting that ND-13 treatment enhances recovery after ischemic injury. Improvement was also observed in the pole test 2 days after injury, as the group treated with ND-13 descended the pole faster than the group treated with saline (ND-13: 6.26 sec ± 0.13 sec; Vehicle: 7.41 sec ± 0.64 sec; $p<0.05$, Fig 2B). In the following weeks after ischemia, the difference between the groups decreases, but the trend is still clear, and the animals treated with ND-13 perform better than those that received vehicle. ND-13 also attenuated motor asymmetry significantly in the cylinder test in the group treated, compared to non-treated group, 2 days after injury (ND-13: 0.10 ± 0.05; Vehicle: 0.30 sec ± 0.05; $p<0.05$, Fig 2C). The effect of ND-13 was still consistent 21 days from surgery, as ND-13 treated mice use both paws more equally.

Fig 2

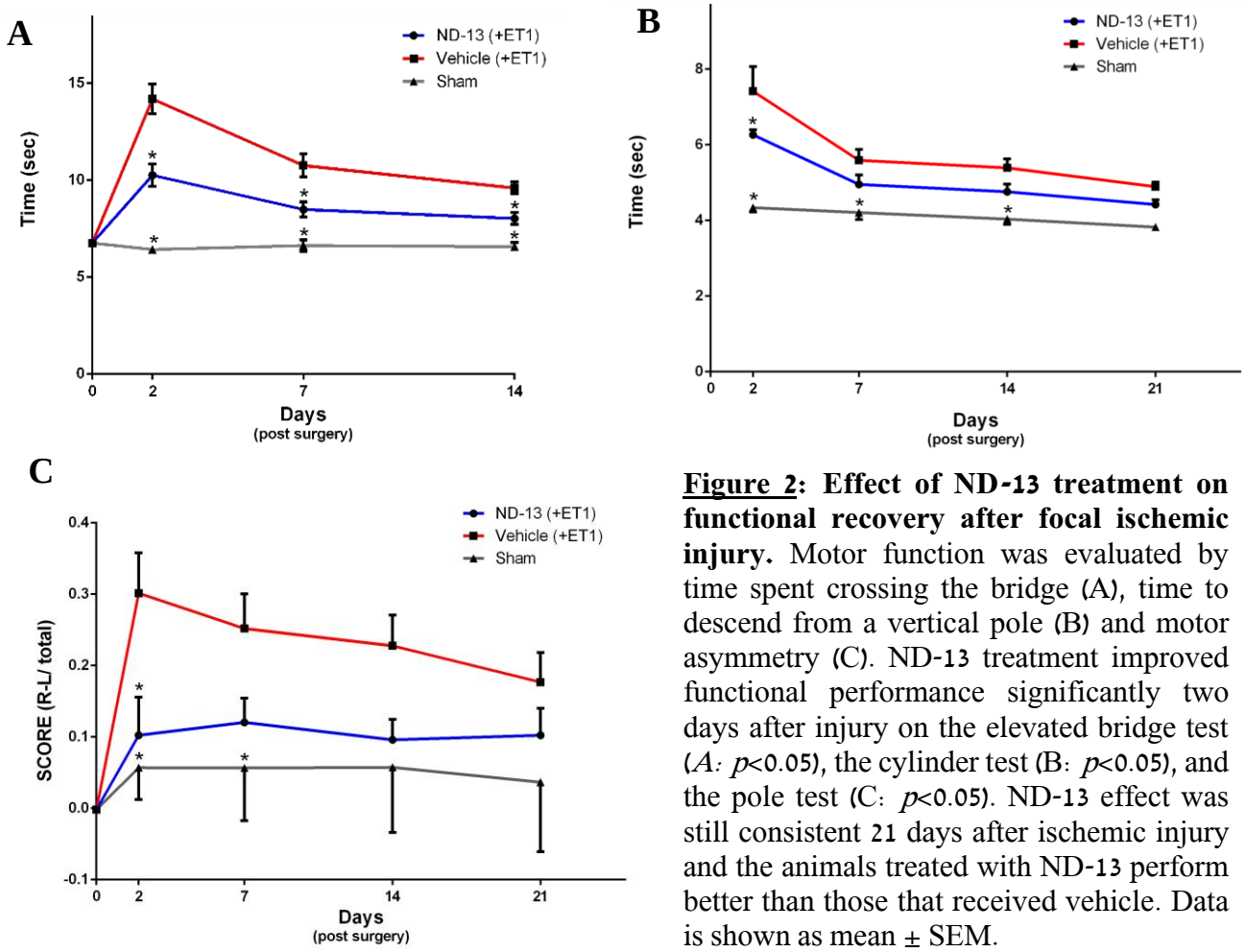

Figure 2: Effect of ND-13 treatment on functional recovery after focal ischemic injury. Motor function was evaluated by time spent crossing the bridge (A), time to descend from a vertical pole (B) and motor asymmetry (C). ND-13 treatment improved functional performance significantly two days after injury on the elevated bridge test (*A*: $p<0.05$), the cylinder test (B: $p<0.05$), and the pole test (C: $p<0.05$). ND-13 effect was still consistent 21 days after ischemic injury and the animals treated with ND-13 perform better than those that received vehicle. Data is shown as mean ± SEM.

DJ-1 KO mice show higher sensitivity and less spontaneous recovery after focal ischemic injury compared to C57BL mice

In order to evaluate the effect of DJ-1 deficiency on ischemic injury, DJ-1 KO mice where compared to C57BL mice. Both groups received ET-1 injection into the right striatum to induce focal ischemic injury and motor dysfunction. DJ-1 KO mice showed higher sensitivity to ischemic damage and slower recovery compared to C57BL mice. Motor function was reduced in the DJ-1 KO group by 21% as measured by the elevated bridge test 7 days after injury (DJ-1 KO: 8.75 sec ± 0.57 sec; C57BL: 6.89 sec ± 0.34 sec; $p<0.05$; Fig 3A). Recovery is measured by the improvement in time taken to cross the bridge across measurement days. Recovery was slower in DJ-1 KO mice, with only 5% recovery from day 2 to days 7 after injury, compared to C57BL mice that improved by 20% (Fig 3A). In the cylinder test the effect was not statistically significant but the same trend is still observed 2 days and 7 days after ischemic injury (Fig 3B).

ND-13 improves functional recovery of DJ-1 KO mice after induced ischemic injury

To study the activity of ND-13 in the absence of DJ-1, we used the endothelin-1 model on DJ-1 KO mice. After ET-1 injection into the right striatum, DJ-1 KO mice received ND-

13 or vehicle (saline) twice a day for 5 consecutive days. The group that received ND-13 treatment showed improvement in motor behavior even in the absence of endogenous DJ-1, compared to the control group. In the elevated bridge test, ND-13 treatment decreases time to cross the beam by 30% compared to vehicle treatment 7 days after injury (ND-13: 6.08 sec ± 0.27 sec; Vehicle: 8.75 sec ± 0.57 sec; $p<0.05$; Fig 4A). The effect was still significant two weeks after injury. In the cylinder test, DJ-1 KO mice treated with ND-13 showed a reduction in motor asymmetry in comparison to the control group 7 days after injury (ND-13: 0.058 ± 0.04; Vehicle: 0.188 ± 0.08; Fig 4B). Results were not significant but a trend is noticed.

Fig 3

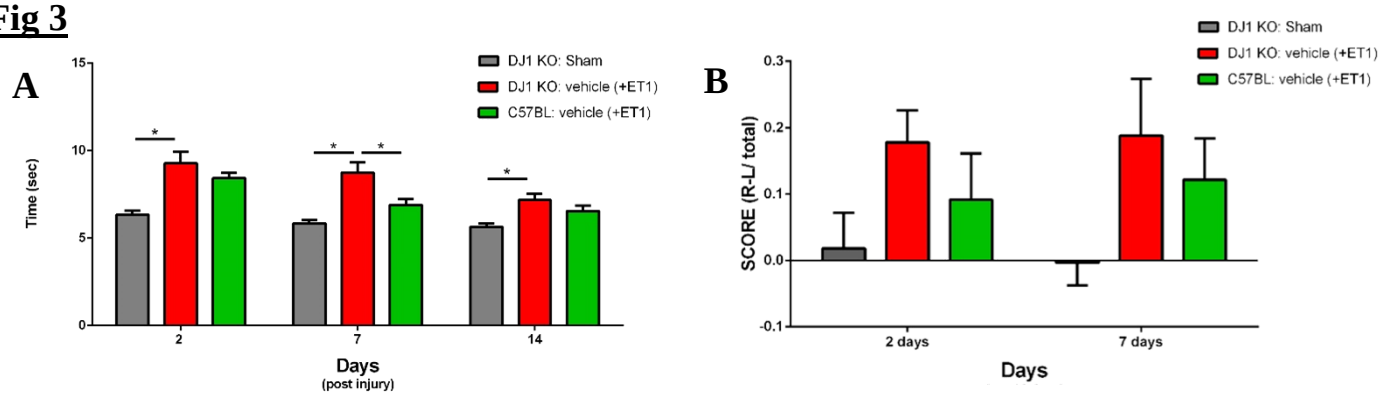

Figure 3: DJ-1 KO mice show higher sensitivity and less spontaneous recovery after ischemic injury compared to C57BL mice. DJ-1 KO mice are more sensitive to ischemic insult and show slower spontaneous recovery compared to C57BL mice, as shown by the elevated bridge test (A: $p<0.05$), and cylinder test (B). DJ-1 KO mice show only 5% recovery from day 2 to days 7 after injury, compared to C57BL mice that improved by 20% over the same period of time. Data is shown as mean ± SEM.

Fig 4

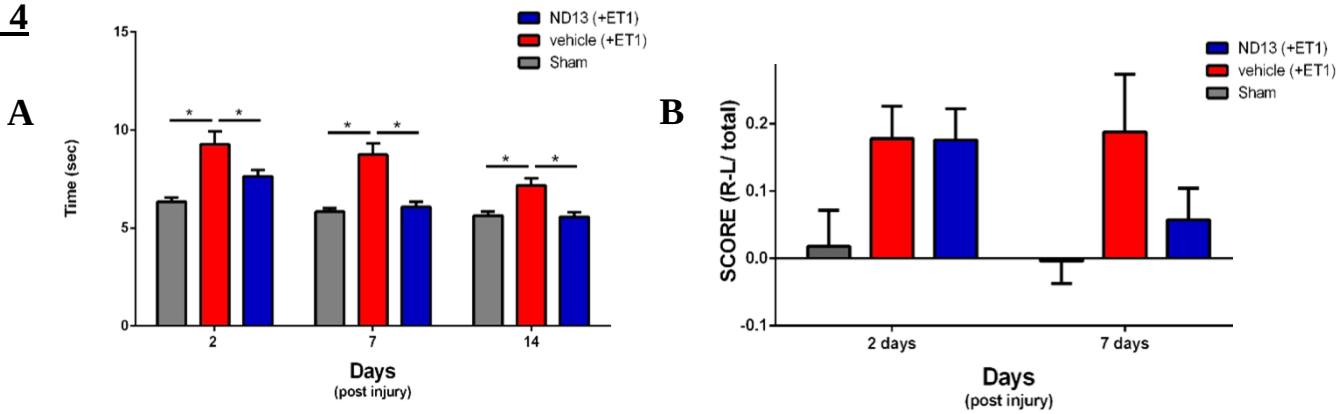

Figure 4: Effect of ND-13 treatment on functional recovery of DJ-1 KO mice after ET-1 induced focal ischemic injury. DJ-1 KO mice treated with ND-13 show improvement 2 days after injury on the elevated bridge test (A: $p<0.05$). This effect was consistent for at least two weeks following injury. Improvement was also noted in cylinder test 7 days after injury (B). Data is shown as mean ± SEM.

Proteomics analysis reveals protein level changes following ND-13 treatment

Experimental groups were tested for protein expression changes (see methods). Average expression for each protein and fold changes between the tested groups were calculated. Changes were considered significant only if $p<0.05$ and the fold change>2. Out of all proteins that were found in the analysis, expression levels of 39 proteins changed following ND-13 treatment compared to vehicle treatment. Here we focus on 7 proteins that have a link to the ischemic insult and may help explain some of the effects of the treatment.

Changes in proteins involved in oxidative stress response following ND-13 treatment

Previous reports show the effect of ND-13 on the mitochondria. ND-13 treatment helped preserve mitochondrial membrane potential, thus stabilizing mitochondrial function in the presence of 3NP toxin that inhibits succinate dehydrogenase activity (Glat et. al, 2016). We have identified an increase in the levels of mitochondrial protein succinate dehydrogenase assembly factor 4 (SDHAF4) following ND-13 treatment (ND-13: 1.13E+09 ±2.51E+07; Vehicle: 1.05E+07 ±2.31E+06; fold change: 107.4, $p<0.001$; Fig 5A). SDHAF4 has a protective role on the mitochondria against oxidative stress: It enhances mitochondrial succinate dehydrogenase (SDH) activity, promoting the block of the generation of excess reactive oxygen species (ROS). Furthermore, SDHAF4 mutants display neuronal dysfunction, neurodegeneration, and sensitivity to oxidative stress (Van Vranken et al. 2014). Our data indicates a higher degree of mitochondrial preservation after ND-13 treatment. SDH complex (also known as respiratory complex II), a part of the mitochondrial electron transport chain, elicits reduction of ubiquinone (coenzyme-Q) to ubiquinol, and therefore promote energy production and antioxidant protection (Rutter et al. 2010; Mellors & Tappel 1966; Mellors & Tappel 1966). We found a reduction in the expression levels of ubiquinone following ND-13 treatment, corresponding to SDH-complex activity (ND-13: 1.30E+08 ±1.97E+07; Vehicle: 4.64E+08 ±7.51E+07; fold change: 3.5, $p<0.01$; Fig 5B).

Another key component of the oxidative stress response is the antioxidant enzyme SOD1 (copper-zinc superoxide dismutase) that provides defense against reactive oxygen species (ROS), scavenging superoxide radicals (Girotto et al. 2014; Valentine et al. 2005). The activation of SOD1 is dependent on copper incorporation at the active site, a complex and highly regulated process (Goto et al. 2000; Beem et al. 1974). The final step in SOD1 maturation is the formation of homodimers. Copper homeostasis protein COMMD1 (copper metabolism Murr1 domain containing 1) regulates the activation of SOD1. COMMD1 impairs SOD1 activity by reducing the expression levels of enzymatically active SOD1 homodimers late in the post-translational maturation process (Vonk et al. 2010). We found that after ND-13 treatment, COMMD1 protein levels decreased compared to vehicle treatment (ND-13: 7.45E+05; Vehicle: 8.01E+06; fold change: 10.76, $p<0.05$). This downregulation of COMMD1 suggests less impairment of SOD1 activity.

These results are consistent with the role of DJ-1 against oxidative stress. Under ischemic condition, DJ-1 translocated into the mitochondrial inter membrane and protected electron transport chain enzymes against ROS. Translocation of DJ-1 into the mitochondria sequesters ROS-induced toxicity endogenously (Pantcheva et al. 2014). Furthermore, DJ-1-deficient cells showed a 19% decrease in SDH (complex II) activity (Shim et al. 2011). DJ-1 has also been reported to regulate SOD1 activity in its protective response to oxidative insult through several pathways (Wang et al. 2011; Milani et al. 2013; Girotto et al. 2014).

Changes in proteins involved in mitophagy regulation following ND-13 treatment

Under ischemic conditions, DJ-1 translocate into the healthy mitochondria and takes part in the early phase of neuroprotection against ischemic stroke, facilitate oxidative stress response (Kahle et al. 2009; Kaneko, Shojo, et al. 2014; Kaneko, Tajiri, et al. 2014b). DJ-1 also has an important role in elimination of damaged mitochondria and degradation of mitochondria (mitophagy). These processes are regulated by pink1, parkin and the expression profile of Miro proteins (see introduction). The degradation of Miro results in arrest of the mitochondrial movement and subsequently increases the clearance of damaged mitochondria (van der Merwe et al. 2015; Liu et al. 2012). We found that after ND-13 treatment, Mitochondrial Rho GTPase 2 (RHOT2, Miro2) protein expression levels decreased compared to vehicle treatment (ND-13: 2.49E+09 ±2.16E+08; Vehicle: 6.28E+09 ±1.02E+09; fold change: 2.5, $p<0.01$; Fig 5C). As a Miro protein, RHOT2 is involved in mitochondrial homeostasis and apoptosis, indicating dysfunctional mitochondria elimination following ND-13 treatment. Furthermore, USP35 (Ubiquitin Specific Peptidase 35), a mitochondrial deubiquitinating enzyme, can delay parkin mediated mitophagy. Upon mitochondrial depolarization, USP35 dissociates from damaged mitochondria, allowing parkin activity. In the absence of USP35, the mitophagy increases (Wang et al. 2015). We found a decrease in USP35 protein expression levels following ND-13 treatment (ND-13: 1.94E+09 ±2.23E+08; Vehicle: 4.61E+09 ±5.19E+08; fold change: 2.4, $p<0.01$; Fig 5D) corresponding to the increase in mitophagy.

Changes in proteins involved in potassium channel regulation following ND-13 treatment

The opening of K+ channels mediates feedback control of excitability in a variety of conditions. Voltage gated K+ channels help bring the activated plasma membrane more rapidly back toward its original negative potential (Alberts et al. 2002). The large-conductance voltage- and Ca2+-activated K+ channel (BK channel) improve the survival of neurons exposed to ischemic conditions, since their activation tends to reduce cellular excitability (Rundén-Pran et al. 2002). Cereblon protein (CRBN) interaction with the BK channel reduces the surface expression of functional channels. Therefore, CRBN plays a role in modulating neuronal BK channel activity (Jo et al. 2005). We have identified

a decrease in the levels of CRBN following ND-13 treatment (ND-13: 1.77E+07 ±3.55E+06; Vehicle: 1.19E+08 ±2.52E+07; fold change: 6.7, p<0.01; Fig 6A), suggesting an increase in the surface expression of functional KB channels that improve the survival of cells after ischemia.

Another type of potassium channel that may be involved in reducing ischemic injury damage is the Kvα4 (Shal-related) voltage-gated rapidly inactivating A-type potassium channels. upregulation of A-type currents (IA) after ischemia correlates with higher resistance of cells to ischemic insult by decreasing excitotoxicity (Deng et al. 2011). Kcnip4 largely reduces surface expression of the Kv4 channel complexes (Schwenk et al. 2008). We show that Kcnip4 levels decrease after ND-13 treatment (ND-13: 4.38E+08 ±4.19E+07; Vehicle: 1.52E+09 ±9.09E+07; fold change: 3.4, p<0.01; Fig 6B). That may suggest an increase in A-type channel expression and subsequent reduction in excitability and ischemic damage.

Fig 5

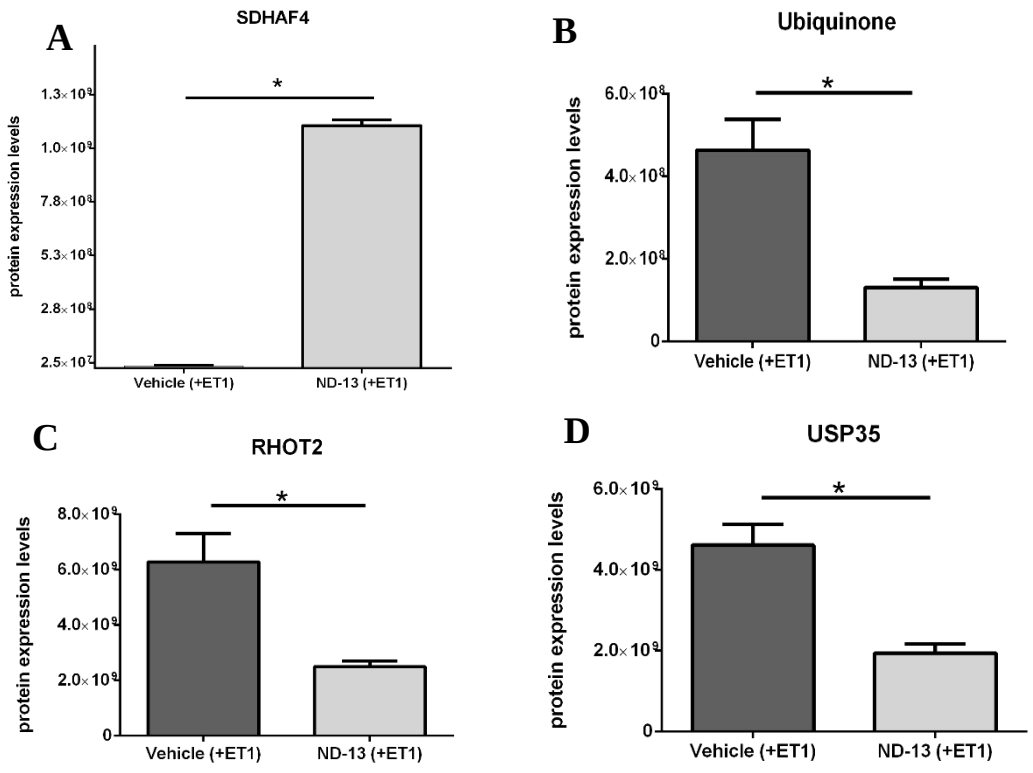

Figure 5: Changes in mitochondrial protein expression levels following ND-13 treatment. Ischemic mice treated with ND-13 show significant change in protein expression levels compared to mice treated with vehicle 2d after injury. SDHAF4 protein levels increased after ND-13 treatment (A: FC=107.4, p<0.001). Ubiquinone protein levels decreased after ND-13 treatment (B: FC=3.5, p<0.01). Rhot2 protein levels decreased after ND-13 treatment (C: FC=2.5, p<0.01). Usp35 protein levels decreased after ND-13 treatment (C: FC=2.4, p<0.01) Data is shown as mean ± SEM.

Fig 6

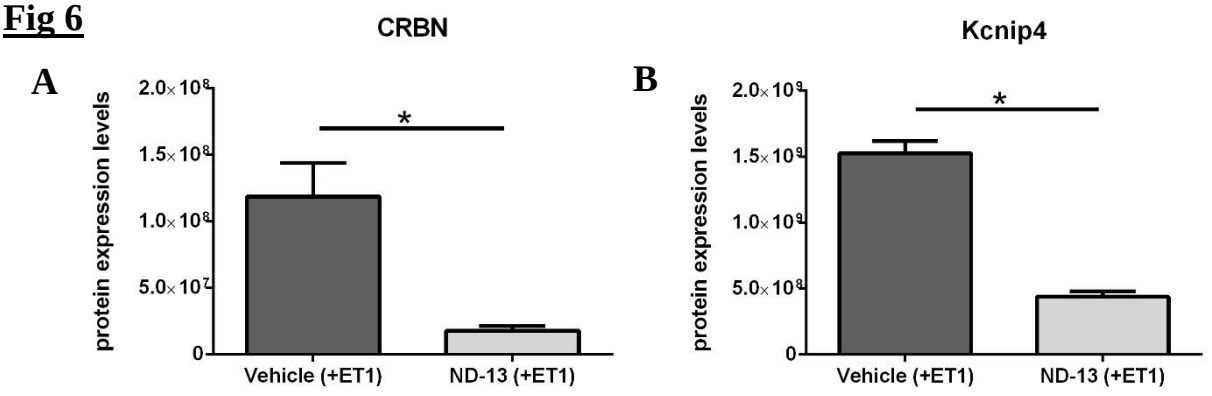

Figure 6: Changes in potassium channel regulators protein expression levels following ND-13 treatment. Ischemic mice treated with ND-13 show significant change in protein expression levels compared to mice treated with vehicle 2d after injury. CRBN protein levels decreased after ND-13 treatment (A: FC=6.7, $p<0.01$). Kcnip4 protein levels decreased after ND-13 treatment (B: FC=3.4, $p<0.01$). Data is shown as mean ± SEM.

Results – Combined gene therapy

Mouse model of focal ischemic injury

In this set of experiments, we wanted to decrease the injection volume of ET-1 while increasing the concentration in order to get a more specific and focal area of ischemia. The genetic treatment was given directly to the damaged area and therefore we wanted a more focused area of damage. Two different concentrations of ET-1 were used to in order to learn the effects of the treatment on behavioral outcome in several stroke severities. Mice showed significant motor deficits as measured by the behavioral tests following ET-1 induced focal ischemic injury compared to sham operated mice. TTC staining was used to show damaged area for the milder form of stroke (1 µl, 1 mg/ml, Fig 7A) and a more severe form of stroke (2µl, 1 mg/ml, Fig 7B) two days after ET-1 administration.

Lentiviruses carrying the Nrf2, GDH2 and EAAT2 genes or GFP infect cells in the injection site

In order to make sure our results are due to the effects of the treatment, we examined the tissue after lentivirus administration. Cells in the injection area should be infected by the virus and therefore overexpress the related proteins. The viral vectors that were injected contain a blasticidin-S deaminase (BSD) component which is expressed only in the infected cells. Using immunohistochemistry, we show an expression of BSD in the trajectory of the needle insertion into the brain (in the treatment group, Fig 7C), or GFP (in the control group, Fig 7D). Similarly, we show the protein expression around the trajectory of the needle for each gene in the right hemisphere (data shown in appendix 2). this suggests that the viral vectors infected the cells and therefore the cells in the injection site are overexpressing the desired genes.

Fig 7

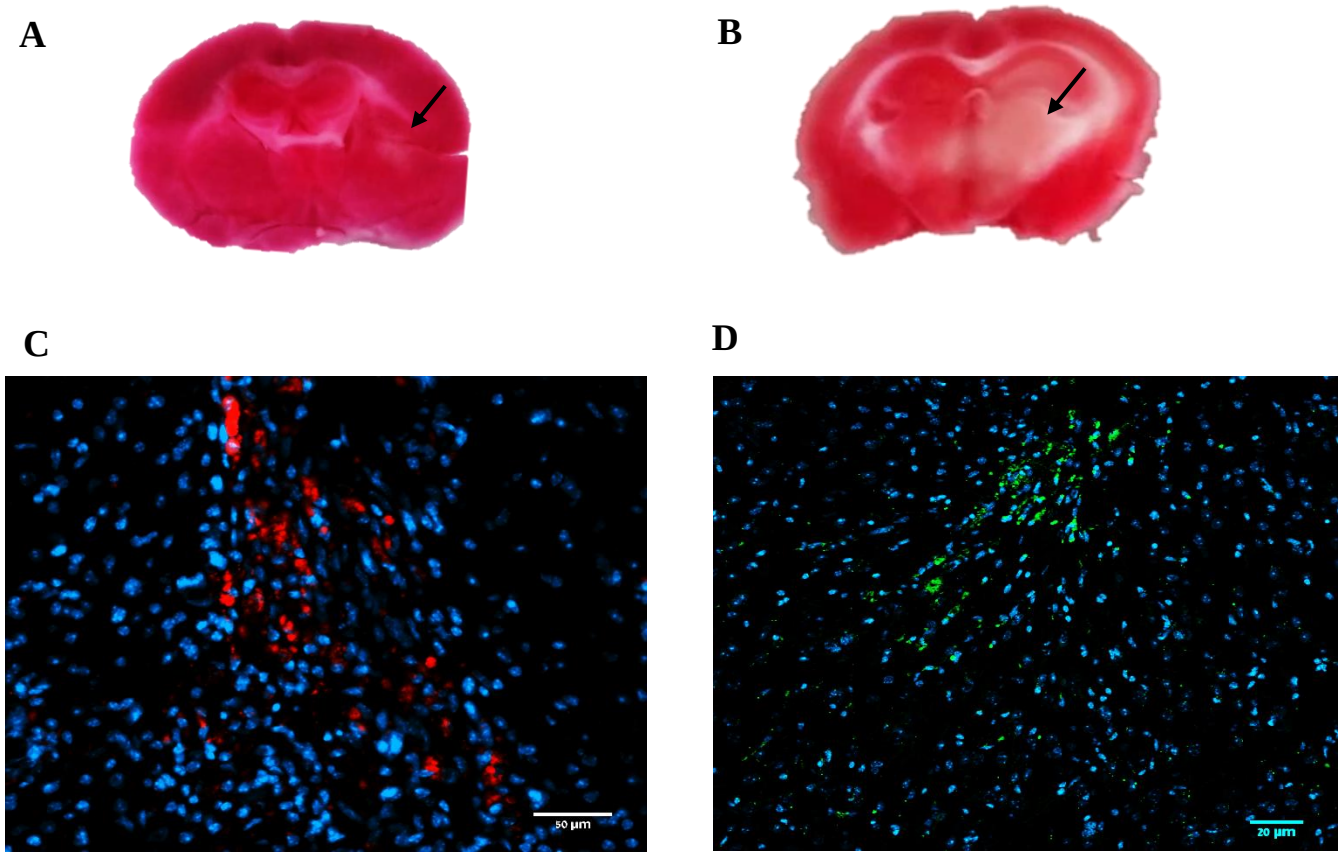

Figure 7: **A-B. Ischemic injury location**. The vasoconstrictor Endothelin-1 was injected into the right striatum to induce ischemia, in different quantity: (A) 1 µl, (B) 2µl (1 mg/ml). The ischemic area, not stained in red by TTC staining, is indicated by the arrows.

C-D. Evidence of viral infection. Viral infection shown by cells expressing BSD, a part of the lentiviral vector injected into the striatum of the treated group (A) or GFP, part of the lentiviral vector injected to the striatum of the control groups (B).

Combined treatment with GDH2, NRF2 and EAAT2 genes improve functional outcome in mouse model of focal ischemic injury in a synergistic manner

To understand the effect of the gene mixture using a milder form of stroke (1µl of ET-1, 1mg/ml) lentiviruses carrying the GDH2, NRF2 and EAAT2 genes were injected to the ischemic area. Improvement was observed 2 days after injury, as mice showed significant improvement in body balance and motor coordination, evident by the elevated bridge test (3 genes: 7.48 sec ± 0.49 sec; GFP: 8.49 sec ± 1.42 sec; $p<0.005$, Fig 8A). Baseline measurements were done before the surgery to show that the effect is really due to the treatment with the 3 genes. At baseline measurement of the elevated bridge test, mice from all groups have similar results. In the cylinder test, less motor asymmetry was observed compared to the control group 2 days after the ischemic damage (3 genes: 0.06 ± 0.03; GFP: 0.24 sec ± 0.05; $p<0.05$, Fig 8B).

Next, we continued to explore the effect of lentiviral vector carrying the 3 genes in a more severe form of stroke (2µl of ET-1, 1mg/ml).We were also interested in discovering the effect of each gene separately, compared to the 3 gene mixture, in order to evaluate the synergistic effect of the treatment, which was previously reported in ALS mouse model (Benkler et al. 2016). The 3 genes treatment also had an effect on a more severe form of stroke, and significant improvement was noted in the cylinder test compared to GFP control group (3 genes: 0.018 ± 0.07; GFP: 0.23 ± 0.02; p<0.05, Fig 8B) 7 days after stroke. Improvement was also significant in the elevated bridge test. Mice that received the 3 genes treatment spent less time crossing the bridge than mice that received GPF (3 genes: 6.19 sec ± 0.15 sec; GFP: 8.36 sec ± 0.29 sec; p<0.05, Fig 9A). Furthermore, the mixture of 3 genes had a more significant effect on functional outcome then each gene separately, suggesting a synergistic effect of these genes. In the elevated bridge test, some improvement was noted in treatment with each gene separately, but all 3 genes together elicited a stronger outcome after ischemic injury (3 genes: 6.19 sec ± 0.15 sec; GDH2: 6.9 sec ± 0.17 sec; NRF2: 6.7 sec ± 0.37 sec; EAAT2: 7.3 sec ± 0.06 sec; Fig 9A). The mixture of 3 genes also attenuated motor asymmetry in a more significant manner as shown by the cylinder test (3 genes: 0.018 ± 0.07; GDH2: 0.147 ± 0.01; NRF2: 0.142 ± 0.03; EAAT2: 0.216 ± 0.02; Fig 9B).

Fig 8

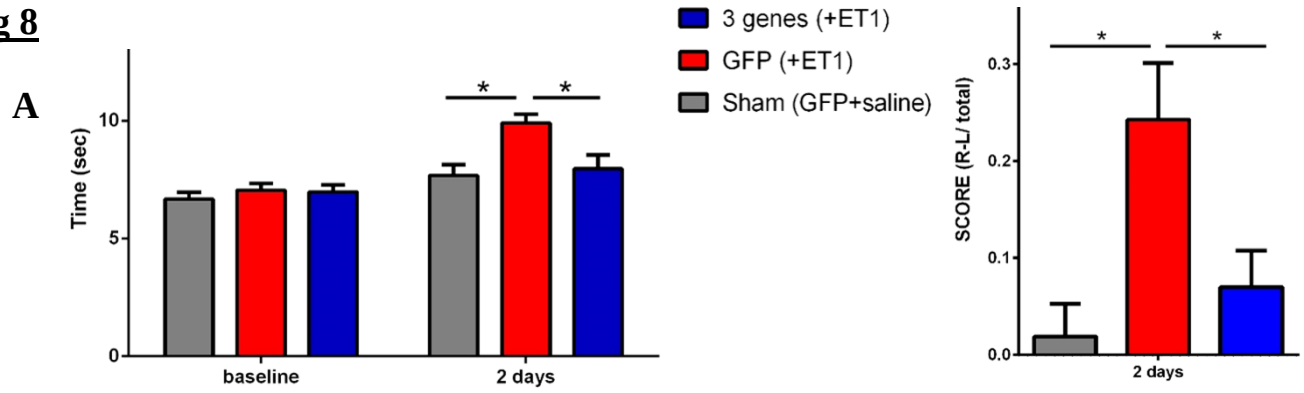

Figure 8: Effect of lentivirus carrying the Nrf2, GDH2 and EAAT2 genes on functional recovery after ET-1 induced mild focal ischemia. A-B. Motor function improvement was observed in ischemic mice treated with viral vector containing Nrf2, GDH2 and EAAT2 genes compared to mice treated with viral vector containing GFP. Motor function was evaluated by time spent crossing the bridge (A) and motor asymmetry (B). Treatment significantly improved functional performance 2 days after injury on the elevated bridge test (*A*: $p<0.05$) and the cylinder test (B: $p<0.05$). Data are given as mean ± SEM.

Fig 9

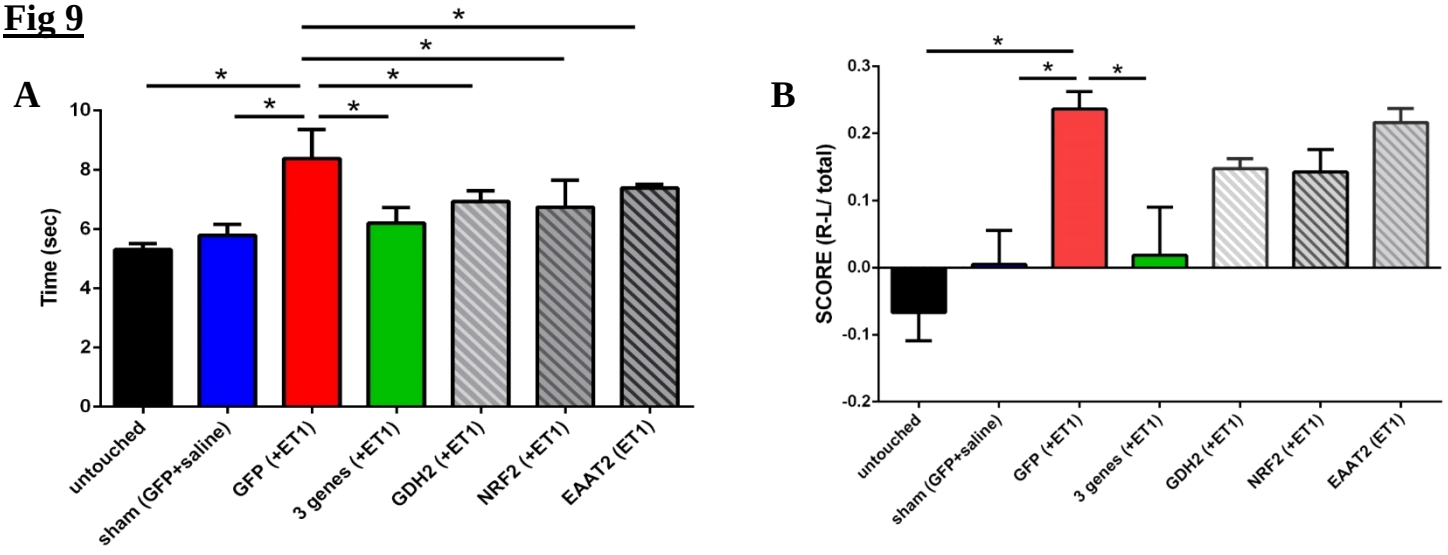

Figure 9: Effect of treatment with the Nrf2, GDH2 and EAAT2 genes and each gene separately on functional recovery of ischemic mice. A-B. Motor function improved after treatment with viral vector carrying Nrf2, GDH2 and EAAT2 genes compared to GFP in a more severe model of stroke. Time spent crossing the bridge was significantly reduced in the elevated bridge test compared to control (A) and motor asymmetry decreased in the cylinder test compared to control (B). Treatment with mixture of the 3 genes seems to have a greater effect on functional recovery in both cases. Greater improvement was shown after 3 genes mixed treatment compared to each gene separately in the elevated bridge test (*A*: $p<0.05$) and the cylinder test (B: $p<0.05$). Data are given as mean ± SEM.

Effect of overexpression of Nrf2, GDH2 and EAAT2 genes on the inflammatory response after focal ischemic injury

Next we wanted to understand the effect of the treatment on inflammation processes occurring in the ischemic area after stroke. Following ischemia, astrocytes are activated resulting in increased glial fibrillary acidic protein (GFAP) levels (Wang et al. 2007). Based on this knowledge, we used immunohistochemistry to search for reactive astrocytes with staining against GFAP protein. A significant decrease in GFAP expression was noted after treatment with the 3 genes. GFAP levels were lower in the brains of the mice that received 3 genes compared to GFP (3 genes: 359.4 ± 17.68; GFP: 490.8 ± 75,79; Sham: 211.6 ± 37.67; $p<0.05$, Fig 10B). This suggests a decrease in reactive astrocytes in the ischemic area that marks a reduction in the inflammatory response.

Fig 10

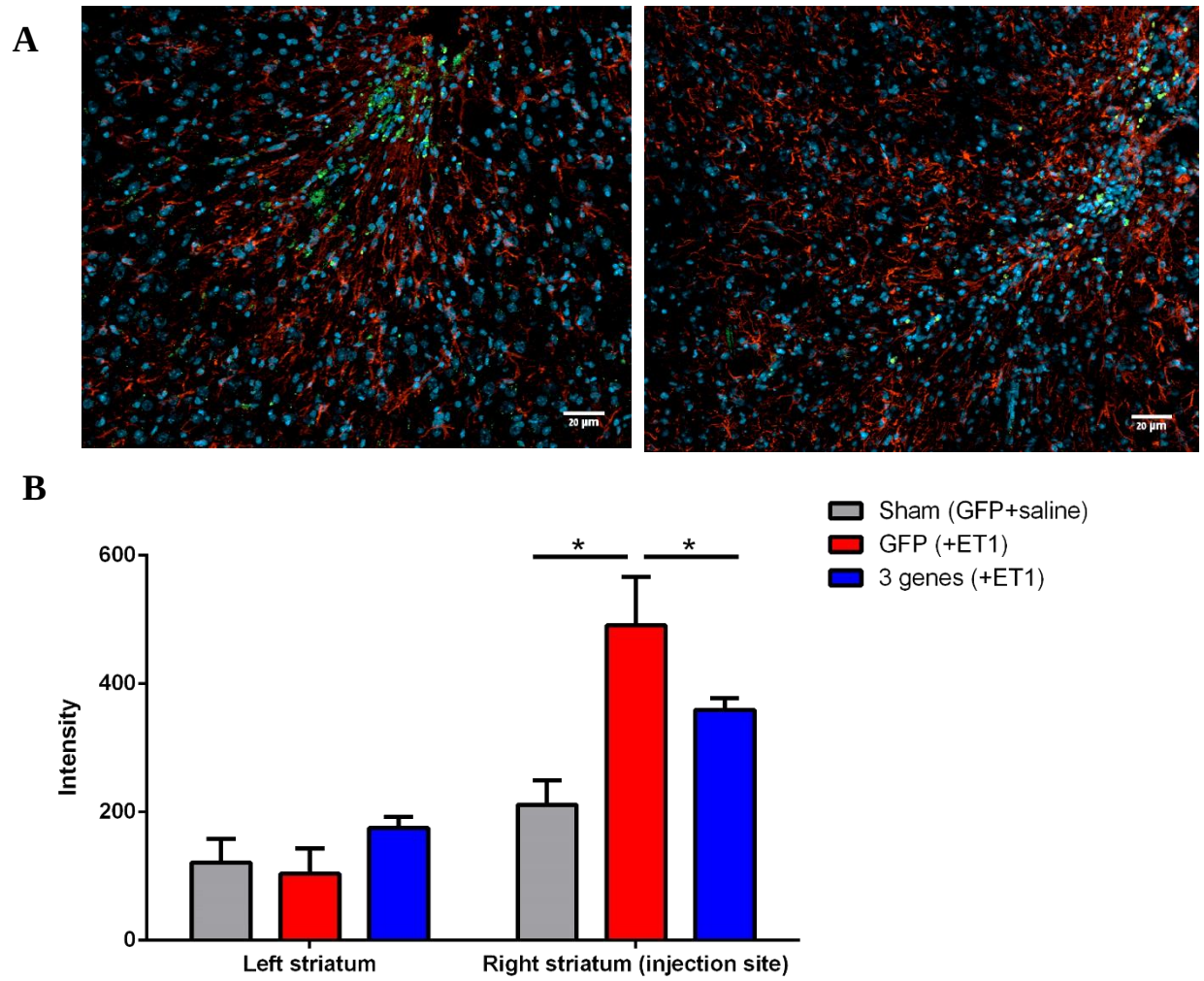

Figure 10: Effect of overexpression of Nrf2, GDH2 and EAAT2 genes on inflammation 28 days after ET-1 induced focal ischemia. A. representative images of GFAP (red) staining for astrocytes after focal ischemic injury in mice that received lentivirus carrying GFP gene (green). Scale bars: 20 µm. B. Viral vector injection into the striatum led to a decrease in inflammation as shown by GFAP staining for astrocytes. Data are given as mean ± SEM.

Discussion

The present work shows the benefits of treatment on focal ischemic injury in mice using two different approaches. The vasoconstrictor Endothelin-1 was used to induce ischemic injury in mice, causing tissue damage and motor dysfunction (Horie et al. 2008). In order to assess the effects of the treatments on motor function, behavioral tests and protein analysis were performed. In the peptide therapy approach, the DJ-1 based peptide, ND-13, was given to mice after ischemia. The motor capabilities significantly improved after ND-13 subcutaneous administration following stroke. In the gene therapy approach, the benefits of overexpression of the EAAT2, GDH2 and NRF2 genes were examined. Similarly, motor function was improved in the treated group compared to control after ischemia. Previous studies show both treatments provide protection and promotes survival in mouse models of neurodegenerative diseases with severe motor dysfunction including Parkinson's disease, ALS, and MSA (Glat, et al. 2016; Lev et al. 2015; Benkler et al. 2016). This study expands these findings, demonstrating that both approaches caused improved functional recovery after ischemic stroke by promoting tissue survival.

Ischemic stroke is a common cause of permanent disability in adults worldwide. Surviving the initial injury usually leads to a long-term loss or limitations of function and the need for long and agonizing rehabilitation (Langhorne et al. 2011; Wade 1992). Motor impairments are the most common impairments caused by stroke and affect an individual's ability to complete everyday activities and participate in everyday life situations (O'Dell et al. 2009; Langhorne et al. 2009). The growing frequency of stroke and the restrictions of the current therapeutics, emphasizes the need for new methods to reduce stroke-related disability. The types of approaches described here might provide such methods, because both treatments eventually cause reduction of the harmful processes initiated after stroke, leading to cell survival and improved recovery.

In the gene therapy approach, due to the fact that a combination of 3 genes that act synergistically in the damaged area was used (Benkler et al. 2016), several protective pathways can be triggered simultaneously, handling the damaging processes including oxidative stress, neurotoxic insult and inflammation. The reduction of these responses leads to neuroprotection and minimize neuronal damage (Chamorro et al. 2016; Cuzzocrea et al. 2001). The synergistic effect is shown here yet again, as the 3 genes work better together then each gene separately in improving motor capabilities after ischemia. Moreover, this study shows a decrease in reactive astrocyte expression following treatment with EAAT2, Nrf-2 and GDH2, suggesting that the treatment help attenuate the inflammatory response after focal ischemic injury, thereby promoting functional recovery. The inflammatory response plays a key role after injury, contributing to the

secondary brain damage after stroke (Lakhan et al. 2009b; Barone & Feuerstein 1999; Ulrich Dirnagl et al. 1999). A causal link has been established between astrocytic activation in the periinfarct area and the occurrence of delayed infarct expansion (Matsui et al. 2002; Murphy 2000). Additionally, Nrf2 specifically targets genes bearing an antioxidant response element within their promoters which influence the inflammatory response (Lakhan et al. 2009a). EAAT2 and GDH2 act to reduce extracellular-glutamate and glutamate availability reducing cell death caused by excitotoxicity after stroke, and therefore also influencing the inflammatory response (Chamorro et al. 2016; U Dirnagl, Iadecola & M. a Moskowitz 1999).

The study shows a proof of concept, evaluating the benefits of overexpression of the 3 genes on recovery after ischemia. Nevertheless, because of technical limitations in using lentiviruses, the injection of the viral vectors was done before ischemic induction. Overexpression of EAAT2, GDH2 and NRF2 at close proximity to the induction of ischemia synergistically improved motor function. Yet, a protective effect cannot be excluded. Further experiment was done to evaluate the effect of the treatment 1 hour after induction of stroke. No significant results were found, but a trend can be spotted (Data shown in appendix 3). This might be due to the fact that the ischemic cascade is immediate and the effect of lentiviral based treatment is delayed by a few days. Therefore, injection of the genes prior to the induction of ischemia show better results. However, based on the results of injection of the viruses 1h after the damage, we hypothesize that there is a potential therapeutic effect. Future experiments should test the effect of overexpression of these genes after stroke by using a different delivery method with more immediate options of expression. Also, though lentiviruses are currently used in several clinical trials, other delivery system such as adeno-associated virus should be tested for safety purposes.

In the peptide therapy approach, the study demonstrates a therapeutic effect of treatment with ND-13 as it reduces motor asymmetry, improves body balance and motor coordination. Further experiment on DJ-1 knock-out (KO) mice reveled that DJ-1 KO mice show higher sensitivity and less spontaneous recovery after striatal injury, consistent with the notion that DJ-1 participates in the endogenous neuroprotection after stroke. It has been reported that loss of DJ-1 increases the sensitivity to excitotoxicity after ischemia, whereas elevated expression of DJ-1 can reverse this sensitivity and provide further protection through alleviation of oxidative stress in various rodent models (Aleyasin et al. 2007b; Yanagisawa et al. 2008).Lev et al 2015) DJ-1 is detected immediately after stroke and efficiently translocated into the mitochondria and may contribute to mitochondria-mediated neuroprotection (Kaneko, Tajiri, et al. 2014a). Furthermore, oxidative stress induces the release of DJ-1 protein in reactive astrocytes, resulting in scavenge of free radicals and reduced cell injury (Yanagida et al. 2009). Our study shows that ND-13 provides compensation for DJ-1 deficits in DJ-1 KO mice,

suggesting that ND-13 works in a DJ-1 independent manner. That is, the presence of the endogenous DJ-1 protein is not required for ND-13 activity, and even in the absence of DJ-1 protein, ND-13 manages to improve motor function and recovery significantly after ischemic injury. These effects are mediated by regulation of different pathways as shown here by a detailed analysis of protein expression levels after ND-13 treatment. Many of the proteins found have a housekeeping function not directly connected to the ischemic insult and treatment. It is possible that the changes in expression levels were an indirect result of other processes and are not the first target. Further experiments would need to be performed in order to test that. However, the analysis did reveal some significant changes in regulatory proteins involved in major processes occurring after ischemic insult that have great implications on the recovery after ischemia. Protein expression levels of regulatory proteins involved in the oxidative stress and neurotoxicity responses were discovered. These responses are initiated after stroke leading to increased production of free radicals and reactive oxygen species (ROS) in the brain (Chehaibi et al. 2016; Guzik et al. 2003), as well as accumulation of glutamate and excessive activation of glutamate receptors (Coyle & Puttfarcken 1993b; Choi & Rothman 1990). Both responses, eventually leading to cell vulnerability and neuronal death, are sequential but also interacting processes, and the close relationship between these responses is well defined (Singh et al. 2003; Choi & Rothman 1990; Coyle & Puttfarcken 1993b; Vergun et al. 2001). Previous studies in our lab showed that ND-13 activates Nrf2, upregulates antioxidant genes and exerts neuroprotection (Lev et al. 2015). Here we show changes in the expression levels of several proteins involved in regulation of mitochondrial function in response to ND-13 administration. These changes can help promote the anti-oxidative stress response, preserve mitochondrial function and regulate the elimination of damaged mitochondria, which encourage cell survival. Other proteins found in the analysis regulate different types of potassium channels. Voltage gated potassium channels are major mediators of excitability in the brain, and help reduce membrane potential (Shah & Aizenman 2014). The observed downregulation of these proteins after ND-13 treatment, can lead to reduction in excitability of the tissue after stroke and consequently lessen tissue damage leading to improved function. Having said that, there is still not enough evidence of the mechanism of action of ND-13 and further research will have to be done to establish its effects. Also, in order to implement these findings in the clinic, further research is needed to determine the safety of ND-13, its stability, favorable way of administration, and the effective doses.

Research using animal models has its limitations. There are other models for stroke that are more similar to the clinical manifestations of human stroke, such as the middle cerebral artery occlusion (MCAo). The advantages of the ET-1 model over the MCAo are its simplicity, reliability and the option to choose the damage site. Animals in this model display significant long-term neurological deficits, associated with the regarded

damaging processes (Kurosawa et al. 1991; Nguemeni et al. 2015; Fuxe et al. 1989; Horie et al. 2008). However, because of its similarities to human stroke, further studies using the MCAo model are needed to determine the clinical relevance of the presented results. Also, experiments on other mammalian models can provide better understanding on the relevance to human patients.

In conclusion, the findings presented in this work propose new therapeutic targets for ischemic stroke. Both ND-13 treatment and lentivirus-mediated gene delivery of EAAT2, GDH2 and NRF2 genes enhance functional recovery and may play a role in neuroprotection after ischemic injury. The data has important implications and suggest possible basis for clinical application, reducing long-term immobility and disability that could benefit patients with ischemic stroke.

References

Alberts, B., Johnson, A. & Lewis, J., 2002. Ion channels and the Electrical Properties of Membrane. *Molecular Biology of the Cell*, pp.1–18.

Aleyasin, H. et al., 2007. The Parkinson's disease gene DJ-1 is also a key regulator of stroke-induced damage. *Proceedings of the National Academy of Sciences of the United States of America*, 104(47), pp.18748–53.

Anderson, C.S., Linto, J. & Stewart-Wynne, E.G., 1995. A Population-Based Assessment of the Impact and Burden of Caregiving for Long-term Stroke Survivors. *Stroke*, 26(5), pp.843–849.

Anderson, M.F. et al., 2003. Astrocytes and Stroke: Networking for Survival? *Neurochemical Research*, 28(2), pp.293–305.

Arvin, B. et al., 1996. The role of inflammation and cytokines in brain injury. *Neuroscience and Biobehavioral Reviews*, 20(3), pp.445–452.

Ashrafi, G. et al., 2014. Mitophagy of damaged mitochondria occurs locally in distal neuronal axons and requires PINK1 and Parkin. *The Journal of cell biology*, 206(5), pp.655–70.

Barone, F.C. & Feuerstein, G.Z., 1999. Inflammatory mediators and stroke: new opportunities for novel therapeutics. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, 19(8), pp.819–34.

Beem, K.M., Rich, W.E. & Rajagopalan, K. V, 1974. Total reconstitution of copper-zinc superoxide dismutase. *The Journal of biological chemistry*, 249(22), pp.7298–305.

Benkler, C. et al., 2016. Multifactorial Gene Therapy Enhancing the Glutamate Uptake System and Reducing Oxidative Stress Delays Symptom Onset and Prolongs Survival in the SOD1-G93A ALS Mouse Model. *Journal of Molecular Neuroscience*, 58(1), pp.46–58.

Bernard, C., 2011. Acquired Dendritic Channelopathy in Temporal. *Science*, 532(2004), pp.532–536.

Bouët, V. et al., 2007. Sensorimotor and cognitive deficits after transient middle cerebral artery occlusion in the mouse. *Experimental Neurology*, 203(2), pp.555–567.

Brooks, S.P., 2011. Neurological evaluation of movement disorders in mice. *Neuromethods*, 61, pp.65–86.

Bruno, V. et al., 2001. Metabotropic glutamate receptor subtypes as targets for neuroprotective drugs. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, 21(9), pp.1013–33.

Cai, Q. et al., 2012. Spatial parkin translocation and degradation of damaged mitochondria via mitophagy in live cortical neurons. *Current biology : CB*, 22(6), pp.545–52.

Calderone, V., 2002. Large-conductance, ca(2+)-activated k(+) channels: function, pharmacology and drugs. *Current medicinal chemistry*, 9(14), pp.1385–95.

Chamorro, Á. et al., 2016. Neuroprotection in acute stroke: targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. *The Lancet Neurology*, 15(8), pp.869–881.

Chan, P.H., 2001. Reactive Oxygen Radicals in Signaling and Damage in the Ischemic Brain. *Journal of Cerebral Blood Flow & Metabolism*, 21(1), pp.2–14.

Chao, N. & Li, S.-T., 2014. Synaptic and extrasynaptic glutamate signaling in ischemic stroke. *Current medicinal chemistry*, 21(18), pp.2043–64.

Chaudhry, F.A. et al., 1995. Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. *Neuron*, 15(3), pp.711–20.

Chehaibi, K. et al., 2016. Correlation of Oxidative Stress Parameters and Inflammatory Markers in Ischemic Stroke Patients. *Journal of Stroke and Cerebrovascular Diseases*.

Choi, D.W. & Rothman, S.M., 1990. The Role of Glutamate Neurotoxicity in Hypoxic-Ischemic Neuronal Death. *Annual Review of Neuroscience*, 13(1), pp.171–182.

Choi, K. et al., 2009. Oxidative stress-induced necrotic cell death via mitochondira-dependent burst of reactive oxygen species. *Current neurovascular research*, 6(4), pp.213–22.

Chu, K. et al., 2007. Pharmacological Induction of Ischemic Tolerance by Glutamate Transporter-1 (EAAT2) Upregulation. *Stroke; a journal of cerebral circulation*, 38(1), pp.177–82.

Coyle, J.T. & Puttfarcken, P., 1993. Oxidative stress, glutamate, and neurodegenerative disorders. *Science (New York, N.Y.)*, 262(5134), pp.689–95.

Cuzzocrea, S. et al., 2001. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. *Pharmacological reviews*, 53(1), pp.135–59.

Deng, P. et al., 2011. Up-regulation of A-type potassium currents protects neurons against cerebral ischemia. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, 31(9), pp.1823–35.

Dirnagl, U., Iadecola, C. & Moskowitz, M.A., 1999. Pathobiology of ischaemic stroke: an integrated view. *Trends in Neurosciences*, 22(9), pp.391–397.

Doeppner, T.R. et al., 2010. Protection of hippocampal neurogenesis by TAT-Bcl-xL after cerebral ischemia in mice. *Experimental Neurology*, 223(2), pp.548–556.

Donnan, G.A. et al., 2008. Stroke. *Lancet*, 371(9624), pp.1612–23.

Fisher, M. & Garcia, J.H., 1996. Evolving stroke and the ischemic penumbra. *Neurology*, 47(4), pp.884–8.

Fuxe, K. et al., 1989. Centrally administered endothelin-1 produces lesions in the brain of the male rat. *Acta Physiol Scand*, 137(1), pp.155–6.

Girotto, S. et al., 2014. DJ-1 Is a Copper Chaperone Acting on SOD1 Activation. *Journal of Biological Chemistry*, 289(15), pp.10887–10899.

Glat, M.J., Ben-Zur, T., Barhum, Y. & Offen, D., 2016. Neuroprotective Effect of a DJ-1 Based Peptide in a Toxin Induced Mouse Model of Multiple System Atrophy. *PloS one*, 11(2), p.e0148170.

Goto, J.J. et al., 2000. Loss of in Vitro Metal Ion Binding Specificity in Mutant Copper-Zinc Superoxide Dismutases Associated with Familial Amyotrophic Lateral Sclerosis. *Journal of Biological Chemistry*, 275(2), pp.1007–1014.

Gou, X. et al., 2010. TAT-NEP1-40 as a novel therapeutic candidate for axonal regeneration and functional recovery after stroke. *http://dx.doi.org/10.3109/10611861003733961*.

Guo, Y. et al., 2014. Pathophysiology and Biomarkers in Acute Ischemic Stroke – A Review. *Tropical Journal of Pharmaceutical Research*, 12(6), p.1097.

Guzik, T.J., Korbut, R. & Adamek-Guzik, T., 2003. Nitric oxide and superoxide in inflammation and immune regulation. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society*, 54(4), pp.469–87.

Han, B. & Haley, W.E., 1999. Family Caregiving for Patients With Stroke : Review and Analysis. *Stroke*, 30(7), pp.1478–1485.

Hao, L.-Y., Giasson, B.I. & Bonini, N.M., 2010. DJ-1 is critical for mitochondrial function and rescues PINK1 loss of function. *Proceedings of the National Academy of Sciences*, 107(21), pp.9747–9752.

Harvey, B.K. et al., 2011. Targeted over-expression of glutamate transporter 1 (GLT-1) reduces ischemic brain injury in a rat model of stroke. *PloS one*, 6(8), p.e22135.

Heiss, W.-D. et al., 1992. Progressive Derangement of Periinfarct Viable Tissue in Ischemic Stroke. *Journal of Cerebral Blood Flow & Metabolism*, 12(2), pp.193–203.

Higgins, J.J. et al., 2008. Dysregulation of large-conductance Ca2+-activated K+ channel expression in nonsyndromal mental retardation due to a cereblon p.R419X mutation. *Neurogenetics*, 9(3), pp.219–223.

Holmqvist, M.H. et al., 2002. Elimination of fast inactivation in Kv4 A-type potassium channels by an auxiliary subunit domain. *Proceedings of the National Academy of Sciences*, 99(2), pp.1035–1040.

Horie, N. et al., 2008. Mouse model of focal cerebral ischemia using endothelin-1. *Journal of neuroscience methods*, 173(2), pp.286–290.

Horn, J. & Limburg, M., 2001. Calcium antagonists for ischemic stroke: a systematic review. *Stroke; a journal of cerebral circulation*, 32(2), pp.570–6.

Hudson, R.C. & Daniel, R.M., 1993. L-glutamate dehydrogenases: distribution, properties and mechanism. *Comparative biochemistry and physiology. B, Comparative biochemistry*, 106(4), pp.767–92.

Ishii, T. et al., 2000. Transcription Factor Nrf2 Coordinately Regulates a Group of Oxidative Stress-inducible Genes in Macrophages. *Journal of Biological Chemistry*, 275(21), pp.16023–16029.

Itoh, K. et al., 1997. An Nrf2 / Small Maf Heterodimer Mediates the Induction of Phase II Detoxifying Enzyme Genes through Antioxidant Response Elements. , 322(236), pp.313–322.

Jerng, H.H. & Pfaffinger, P.J., 2008. Multiple Kv channel-interacting proteins contain an N-terminal transmembrane domain that regulates Kv4 channel trafficking and gating. *The Journal of biological chemistry*, 283(51), pp.36046–59. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18957440 [Accessed July 31, 2016].

Jin, J. et al., 2007. Identification of novel proteins associated with both alpha-synuclein and DJ-1. *Molecular & cellular proteomics : MCP*, 6(5), pp.845–59.

Jo, S. et al., 2005. Identification and functional characterization of cereblon as a binding protein for large-conductance calcium-activated potassium channel in rat brain. *Journal of Neurochemistry*, 94(5), pp.1212–1224.

Kahle, P.J., Waak, J. & Gasser, T., 2009. DJ-1 and prevention of oxidative stress in Parkinson's disease and other age-related disorders. *Free Radical Biology and Medicine*, 47(10), pp.1354–1361.

Kaneko, Y., Shojo, H., et al., 2014. DJ-1 ameliorates ischemic cell death in vitro possibly via mitochondrial pathway. *Neurobiology of disease*, 62, pp.56–61.

Kaneko, Y., Tajiri, N., et al., 2014. Oxygen-glucose-deprived rat primary neural cells exhibit DJ-1 translocation into healthy mitochondria: A potent stroke therapeutic target. *CNS Neuroscience and Therapeutics*, 20(3), pp.275–281.

Keller, A., et al., 2002. An explanation of the Peptide Prophet algorithm developed.

Anal. Chem, 74, pp.5383–5392.

Kim, Y. et al., 2008. PINK1 controls mitochondrial localization of Parkin through direct phosphorylation. *Biochemical and Biophysical Research Communications*, 377(3), pp.975–980.

Kitamura, Y. et al., 2011. Neuroprotective effect of a new DJ-1-binding compound against neurodegeneration in Parkinson's disease and stroke model rats. *Molecular Neurodegeneration*, 6(1), p.48.

Kulawiak, B. et al., 2008. BK channel openers inhibit ROS production of isolated rat brain mitochondria. *Experimental Neurology*, 212(2), pp.543–547.

Kurosawa, M. et al., 1991. Responses of blood flow, extracellular lactate, and dopamine in the striatum to intrastriatal injection of endothelin-1 in anesthetized rats. *Journal of cardiovascular pharmacology*, 17 Suppl 7, pp.S340–2.

Lai, T.W., Zhang, S. & Wang, Y.T., 2014. Excitotoxicity and stroke: identifying novel targets for neuroprotection. *Progress in neurobiology*, 115, pp.157–88.

Lakhan, S.E. et al., 2009. Inflammatory mechanisms in ischemic stroke: therapeutic approaches. *Journal of Translational Medicine*, 7(1), p.97.

Langhorne, P., Bernhardt, J. & Kwakkel, G., 2011. Stroke rehabilitation. *The Lancet*, 377(9778), pp.1693–1702.

Langhorne, P., Coupar, F. & Pollock, A., 2009. Motor recovery after stroke: a systematic review. *The Lancet Neurology*, 8(8), pp.741–754.

Lee, J., Giordano, S. & Zhang, J., 2012. Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. *The Biochemical journal*, 441(2), pp.523–40.

Lei, Z. et al., 2016. Reduced expression of IA channels is associated with post-ischemic seizures. *Epilepsy Research*, 124, pp.40–48.

Lev, N. et al., 2015. A DJ-1 Based Peptide Attenuates Dopaminergic Degeneration in Mice Models of Parkinson's Disease via Enhancing Nrf2 D. I. Finkelstein, ed. *PLOS ONE*, 10(5), p.e0127549.

Lin, C.-L.G. et al., 2012. Glutamate transporter EAAT2: a new target for the treatment of neurodegenerative diseases. *Future medicinal chemistry*, 4(13), pp.1689–700.

Lipton, P. et al., 1999. Ischemic cell death in brain neurons. *Physiological reviews*, 79(4), pp.1431–568.

Liu, S. et al., 2012. Parkinson's Disease–Associated Kinase PINK1 Regulates Miro Protein Level and Axonal Transport of Mitochondria G. P. Copenhaver, ed. *PLoS Genetics*, 8(3), p.e1002537.

Liu, Y.-Q. et al., 2014. Regulation of Kv4.2 A-Type Potassium Channels in HEK-293 Cells by Hypoxia. *Frontiers in cellular neuroscience*, 8(October), p.329.

Lo, E.H., Dalkara, T. & Moskowitz, M.A., 2003. Neurological diseases: Mechanisms, challenges and opportunities in stroke. *Nature Reviews Neuroscience*, 4(5), pp.399–414.

Martin, L.J. et al., 1998. Neurodegeneration in Excitotoxicity, Global Cerebral

Ischemia, and Target Deprivation: A Perspective on the Contributions of Apoptosis and Necrosis. *Brain Research Bulletin*, 46(4), pp.281–309.

Matsuda, N. et al., 2010. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *The Journal of Cell Biology*, 189(2), pp.211–221.

Matsui, T. et al., 2002. Astrocytic Activation and Delayed Infarct Expansion After Permanent Focal Ischemia in Rats. Part I: Enhanced Astrocytic Synthesis of S-100 beta in the Periinfarct Area Precedes Delayed Infarct Expansion. *Journal of Cerebral Blood Flow & Metabolism*, 22(6), pp.711–722.

Mellors, A. & Tappel, A.L., 1966a. Quinones and quinols as inhibitors of lipid peroxidation. *Lipids*, 1(4), pp.282–4.

Mellors, A. & Tappel, A.L., 1966b. The inhibition of mitochondrial peroxidation by ubiquinone and ubiquinol. *The Journal of biological chemistry*, 241(19), pp.4353–6.

van der Merwe, C. et al., 2015. Evidence for a common biological pathway linking three Parkinson's disease-causing genes: *parkin* , *PINK1* and *DJ-1*. *European Journal of Neuroscience*, 41(9), pp.1113–1125.

Milani, P. et al., 2013. SOD1 and DJ-1 converge at Nrf2 pathway: a clue for antioxidant therapeutic potential in neurodegeneration. *Oxidative medicine and cellular longevity*, 2013, p.836760.

Moore, D.J. et al., 2005. Association of DJ-1 and parkin mediated by pathogenic DJ-1 mutations and oxidative stress. *Human molecular genetics*, 14(1), pp.71–84.

Muir, K.W. et al., 2007. Inflammation and ischaemic stroke. *Current opinion in neurology*, 20(3), pp.334–42.

Murphy, A.N., Fiskum, G. & Beal, M.F., 1999. Mitochondria in Neurodegeneration: Bioenergetic Function in Cell Life and Death. *Journal of Cerebral Blood Flow & Metabolism*, 19(3), pp.231–245.

Murphy, S., 2000. Production of nitric oxide by glial cells: Regulation and potential roles in the CNS. *Glia*, 29(1), pp.1–13.

Narendra, D. et al., 2008. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *The Journal of Cell Biology*, 183(5), pp.795–803.

Narendra, D.P. et al., 2010. PINK1 Is Selectively Stabilized on Impaired Mitochondria to Activate Parkin D. R. Green, ed. *PLoS Biology*, 8(1), p.e1000298.

Nguemeni, C. et al., 2015. Time course of neuronal death following endothelin-1 induced focal ischemia in rats. *Journal of Neuroscience Methods*, 242, pp.72–76.

Nguyen, T., Nioi, P. & Pickett, C.B., 2009. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *The Journal of biological chemistry*, 284(20), pp.13291–5.

O'Dell, M.W., Lin, C.-C.D. & Harrison, V., 2009. Stroke rehabilitation: strategies to enhance motor recovery. *Annual review of medicine*, 60(1), pp.55–68.

Pantcheva, P. et al., 2014. The role of DJ-1 in the oxidative stress cell death cascade after stroke. *Neural regeneration research*, 9(15), pp.1430–3.

Pellegrini-Giampietro, D.E. et al., 1992. Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. *Proceedings of the National Academy of Sciences of the United States of America*, 89(21), pp.10499–503.

Plaitakis, A. et al., 2010. Gain-of-function variant in GLUD2 glutamate dehydrogenase modifies Parkinson's disease onset. *European journal of human genetics : EJHG*, 18(3), pp.336–41.

Plaitakis, A., Metaxari, M. & Shashidharan, P., 2000. Nerve Tissue-Specific (GLUD 2) and Housekeeping (GLUD 1) Human Glutamate Dehydrogenases Are Regulated by Distinct Allosteric Mechanisms : Implications for Biologic Function. , pp.1862–1869.

Rao, V.L. et al., 2001. Antisense knockdown of the glial glutamate transporter GLT-1 exacerbates hippocampal neuronal damage following traumatic injury to rat brain. *The European journal of neuroscience*, 13(1), pp.119–28.

Rothstein, J.D. et al., 1996. Knockout of Glutamate Transporters Reveals a Major Role for Astroglial Transport in Excitotoxicity and Clearance of Glutamate. *Neuron*, 16(3), pp.675–686.

Rundén-Pran, E. et al., 2002. BK channel activity determines the extent of cell degeneration after oxygen and glucose deprivation: a study in organotypical hippocampal slice cultures. *Neuroscience*, 112(2), pp.277–288.

Rutter, J., Winge, D.R. & Schiffman, J.D., 2010. Succinate dehydrogenase - Assembly, regulation and role in human disease. *Mitochondrion*, 10(4), pp.393–401.

Sausbier, M. et al., 2004. Cerebellar ataxia and Purkinje cell dysfunction caused by Ca2+-activated K+ channel deficiency. *Proceedings of the National Academy of Sciences of the United States of America*, 101(25), pp.9474–8.

Schallert, T. et al., 2000. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology*, 39(5), pp.777–787.

Schwenk, J. et al., 2008. NMR analysis of KChIP4a reveals structural basis for control of surface expression of Kv4 channel complexes. *The Journal of biological chemistry*, 283(27), pp.18937–46.

Serôdio, P. & Rudy, B., 1998. Differential expression of Kv4 K+ channel subunits mediating subthreshold transient K+ (A-type) currents in rat brain. *Journal of neurophysiology*, 79(2), pp.1081–1091.

Shah, N.H. & Aizenman, E., 2014. Voltage-Gated Potassium Channels at the Crossroads of Neuronal Function, Ischemic Tolerance, and Neurodegeneration. *Translational Stroke Research*, 5(1), pp.38–58.

Shalit, T. et al., 2015. MS1-based label-free proteomics using a quadrupole orbitrap

mass spectrometer. *Journal of Proteome Research*, 14(4), pp.1979–1986.

Shashidharan, P. et al., 1997. Nerve Tissue-Specific Human Glutamate Dehydrogenase that Is Thermolabile and Highly Regulated by ADP. *Journal of Neurochemistry*, 68(5), pp.1804–1811.

Sheng, Z.-H. & Cai, Q., 2012. Mitochondrial transport in neurons: impact on synaptic homeostasis and neurodegeneration. *Nature Reviews Neuroscience*, 13(2), p.77.

Shibata, R. et al., 2000. A-type K+ current mediated by the Kv4 channel regulates the generation of action potential in developing cerebellar granule cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 20(11), pp.4145–4155.

Shih, A.Y., Li, P. & Murphy, T.H., 2005. A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 25(44), pp.10321–35.

Shim, J.H. et al., 2011. The antioxidant Trolox helps recovery from the familial Parkinson's disease-specific mitochondrial deficits caused by PINK1- and DJ-1-deficiency in dopaminergic neuronal cells. *Mitochondrion*, 11(5), pp.707–715.

Singh, P. et al., 2003. Prolonged glutamate excitotoxicity: Effects on mitochondrial antioxidants and antioxidant enzymes. *Molecular and Cellular Biochemistry*, 243(1/2), pp.139–145.

Stineman, M.G. et al., 1997. A Prediction Model for Functional Recovery in Stroke. *Stroke*, 28(3), pp.550–556.

Surh, Y. et al., 2008. Nrf2 as a Master Redox Switch in Turning on the Cellular Signaling Involved in the Induction of Cytoprotective Genes by Some Chemopreventive Phytochemicals. , pp.1526–1539.

Takagi, T. et al., 2014. Temporal activation of Nrf2 in the penumbra and Nrf2 activator-mediated neuroprotection in ischemia-reperfusion injury. *Free radical biology & medicine*, 72, pp.124–33.

Takahashi, K., Foster, J.B. & Lin, C.-L.G., 2015. Glutamate transporter EAAT2: regulation, function, and potential as a therapeutic target for neurological and psychiatric disease. *Cellular and Molecular Life Sciences*, 72(18), pp.3489–3506.

Tang, B. et al., 2006. Association of PINK1 and DJ-1 confers digenic inheritance of early-onset Parkinson's disease. *Human molecular genetics*, 15(11), pp.1816–25.

Tang, Y.-Q. et al., 2013. Auxiliary KChIP4a suppresses A-type K+ current through endoplasmic reticulum (ER) retention and promoting closed-state inactivation of Kv4 channels. *The Journal of biological chemistry*, 288(21), pp.14727–41.

Tao, F., Su, Q. & Johns, R.A., 2008. Cell-permeable Peptide Tat-PSD-95 PDZ2 Inhibits Chronic Inflammatory Pain Behaviors in Mice. *Molecular Therapy*, 16(11), pp.1776–1782.

Valentine, J.S., Doucette, P.A. & Zittin Potter, S., 2005. Copper-zinc superoxide

dismutase and amyotrophic lateral sclerosis. *Annual review of biochemistry*, 74(1), pp.563–593.

Vergun, O. et al., 2001. Exploration of the role of reactive oxygen species in glutamate neurotoxicity in rat hippocampal neurones in culture. *The Journal of physiology*, 531(Pt 1), pp.147–63.

Vonk, W.I.M. et al., 2010. Cu,Zn superoxide dismutase maturation and activity are regulated by COMMD1. *Journal of Biological Chemistry*, 285(37), pp.28991–29000.

Van Vranken, J.G. et al., 2014. SDHAF4 promotes mitochondrial succinate dehydrogenase activity and prevents neurodegeneration. *Cell metabolism*, 20(2), pp.241–52.

Waak, J. et al., 2009. Regulation of astrocyte inflammatory responses by the Parkinson's disease-associated gene DJ-1. *The FASEB Journal*, 23(8), pp.2478–2489.

Wade, D.T., 1992. Measurement in neurological rehabilitation. *Current opinion in neurology and neurosurgery*, 5(5), pp.682–6.

Wang, Q., Tang, X.N. & Yenari, M.A., 2007. The inflammatory response in stroke. *Journal of neuroimmunology*, 184(1-2), pp.53–68.

Wang, Y. et al., 2015. Deubiquitinating enzymes regulate PARK2-mediated mitophagy. *Autophagy*, 11(4), pp.595–606.

Wang, Z. et al., 2011. DJ-1 modulates the expression of Cu/Zn-superoxide dismutase-1 through the Erk1/2-Elk1 pathway in neuroprotection. *Annals of Neurology*, 70(4), pp.591–599.

Weller, M.L. et al., 2008. Selective overexpression of excitatory amino acid transporter 2 (EAAT2) in astrocytes enhances neuroprotection from moderate but not severe hypoxia-ischemia. *Neuroscience*, 155(4), pp.1204–11.

Yamauchi, K. et al., 2016. A novel nuclear factor erythroid 2-related factor 2 (Nrf2) activator RS9 attenuates brain injury after ischemia reperfusion in mice. *Neuroscience*, 333, pp.302–310.

Yanagida, T. et al., 2009. Oxidative stress induction of DJ-1 protein in reactive astrocytes scavenges free radicals and reduces cell injury. *Oxidative medicine and cellular longevity*, 2(1), pp.36–42.

Yanagisawa, D. et al., 2008. DJ-1 protects against neurodegeneration caused by focal cerebral ischemia and reperfusion in rats. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, 28(3), pp.563–578.

Yang, C. et al., 2009. Curcumin upregulates transcription factor Nrf2, HO-1 expression and protects rat brains against focal ischemia. *Brain research*, 1282, pp.133–41.

Zhao, J. et al., 2006. Sulforaphane reduces infarct volume following focal cerebral ischemia in rodents. *Neuroscience letters*, 393(2-3), pp.108–12.

Appendix 1

Proteomics analysis protocol

Sample preparation

Samples were subjected to in-solution tryptic digestion using a modified Filter Aided Sample Preparation protocol (FASP). All chemicals are from Sigma Aldrich (unless stated otherwise). Sodium dodecyl sulfate buffer (SDT) included: 4%(w/v) SDS, 100mM Tris/HCl pH 7.6, 0.1M DTT. Urea buffer A (UA) contained: 8 M urea (Sigma, U5128) in 0.1 M Tris/HCl pH 8.5. Urea buffer B (UB) contained: 8 M urea in 0.1 M Tris/HCl pH 8.0. IAA solution: 0.05 M iodoacetamide in UA. Tissue was homogenized and dissolved in 100µL SDT buffer. Homogenate was centrifuged at 16,000 g for 10min. 100ug total protein were mixed with 200 µL UB and loaded onto 30 kDa molecular weight cutoff filters and centrifuged. 200 µl of UB were added to the filtering unit and centrifuged at 14,000 x g for 40 min. Proteins were alkylated by adding 100 µl IAA and incubating in the dark for 30 min, followed by 2 washes with Ammonium Bicarbonate. Trypsin was then added (50:1 protein amount:trypsin) and samples incubated at 37°C overnight. Additional amount of trypsin was added and incubated for 4 hours at 37°C. Digested proteins were then centrifuged and collected in a clean collecting tube. 50ul Nacl 0.5M was added to the filtering unit and centrifuged. Reaction was stopped by acidifying with 1% trifloroacetic acid. Peptides were desalted using HBL Oasis, Speed vac to dryness and stored in -80°C until analysis.

Liquid chromatography

ULC/MS grade solvents were used for all chromatographic steps. Each sample was loaded using split-less nanoUltra Performance Liquid Chromatography (10 kpsi nano-Acquity; Waters, Milford, MA, USA). The mobile phase was: A) H2O + 0.1% formic acid and B) acetonitrile + 0.1% formic acid. Dry peptides were dissolved in 97:3 water:acetonitrile (v/v) + 0.1% formic acid solution. Desalting of the samples was performed online using a reversed-phase C18 trapping column (180 µm internal diameter, 20 mm length, 5 µm particle size; Waters). The peptides were then separated using a T3 HSS nano-column (75 µm internal diameter, 250 mm length, 1.8 µm particle size; Waters) at 0.35 µL/min. Peptides were eluted from the column into the mass spectrometer using the following gradient: 4% to 20%B in 140 min, 20% to 90%B in 25 min, maintained at 90% for 5 min and then back to initial conditions.

Mass Spectrometry

The nanoUPLC was coupled online through a nanoESI emitter (10 µm tip; New Objective; Woburn, MA, USA) to a quadrupole orbitrap mass spectrometer (Q Exactive Plus, Thermo Scientific) using a FlexIon nanospray apparatus (Proxeon).

Data was acquired in DDA mode, using a Top20 method. MS1 resolution was set to 70,000 (at 400m/z) and maximum injection time was set to 20 msec. MS2 resolution was set to 17,500 and maximum injection time of 60 msec.

Data processing and analysis

Raw data was imported into the Expressionist® software (Genedata) and processed as described here (Shalit et al. 2015). The software was used for retention time alignment and peak detection of precursor peptides. A master peak list was generated from all MS/MS events and sent for database searching using Mascot v2.5 (Matrix Sciences). Data was searched against the mouse protein database from UniprotKB (http://www.uniprot.org/) appended with 125 common laboratory contaminant proteins. Fixed modification was set to carbamidomethylation of cysteines and variable modifications were set to oxidation of methionines and deamidation of N or Q. Search results were then filtered using the PeptideProphet (Keller, A. 2002) algorithm to achieve maximum false discovery rate of 1% at the protein level. Peptide identifications were imported back to Expressions to annotate identified peaks. Quantification of proteins from the peptide data was performed using an in-house script (Shalit et al. 2015). Data was normalized base on the total ion current. Protein abundance was obtained by summing the three most intense, unique peptides per protein. A Student's t-Test, after logarithmic transformation, was used to identify significant differences across the biological replica. Fold changes between treatments were calculated based on the ratio of arithmetic means of the replicate samples.

Appendix 2

Immunohistology of EAAT2, NRF2, and GDH2

Fig 11

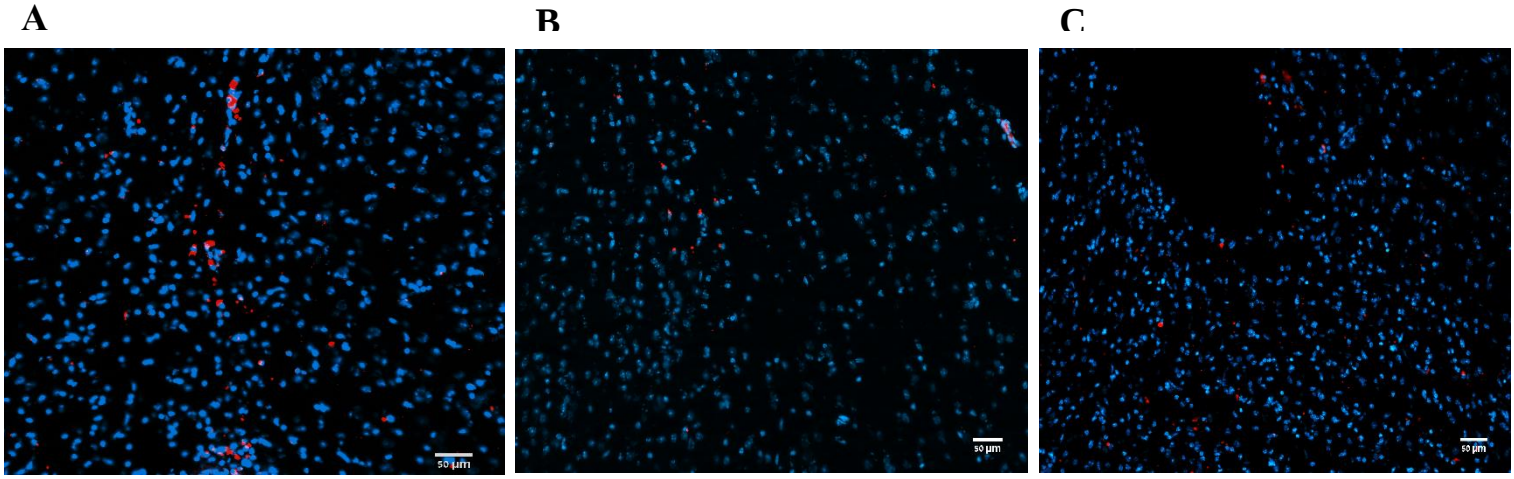

Figure 11: Immunohistology of EAAT2, NRF2, and GDH2. Expression of the proteins related to the 3 genes GDH2 (A), NRF2 (B) and EAAT2 (C), in the trajectory of the needle insertion into the right striatum. Scale bar 50 µm.

Appendix 3

Effect of lentiviruses carrying EAAT2, NRF2 and GDH2 or GFP injection 1h after focal ischemic injury induction

Fig 12

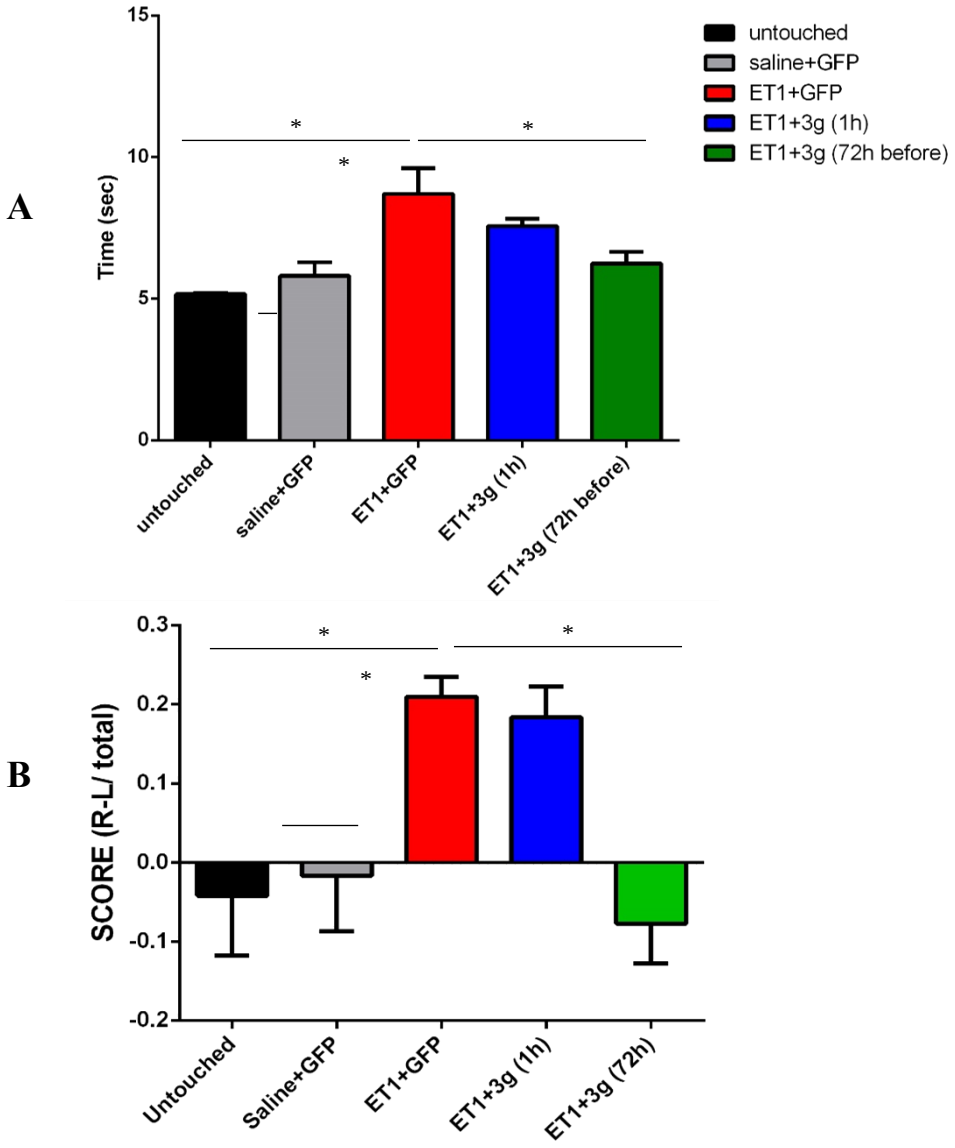

Figure 12: Effect of overexpression of Nrf2, GDH2 and EAAT2 genes on ET-1 induced focal ischemic injury. Injection of lentiviruses was tested in 2 time points: 72h before injury (shown in green) and 1h after injury (shown in blue). Significant improvement in time spent crossing the bridge (A) and the use of both hand more equally in the cylinder test (B) was observed in the group treated 72h before ischemic insult ($p>0.05$). Treatment 1h after damage also show some improvement but not sufficient for statistical significant. Data are given as mean ± SEM.