 Combined Gene Therapy to Reduce the Neuronal Damage in the Mouse Model of Focal Ischemic Injury

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Abstract
Research into stroke is driven by frustration over the limited available therapeutics. Targeting a single aspect of this multifactorial disease contributes to the therapeutic boundaries. To overcome this, we devised a novel multifactorial-cocktail treatment, using lentiviruses encoding excitatory amino acid transporter 2 (EAAT2), glutamate dehydrogenase 2 (GDH2), and nuclear factor E2-related factor 2 (Nrf2) genes, that acts synergistically to address the effected excito-oxidative axis. Here, we used the vasoconstrictor endothelin-1 (ET-1) to induce focal ischemic injury in mice by direct injection into the striatum. Mice treated with the mixture of these three genes show significant improvement in body balance, motor coordination, and decreased motor asymmetry compared to each gene separately. These results demonstrate that overexpression of the combined EAAT2, GDH2, and Nrf2 genes can provide neuroprotection after ischemic injury.

Keywords Stroke · Ischemic injury · Gene therapy · EAAT2 GDH2 · Nrf2

Introduction

The increasing prevalence of stroke and the limitations of the current therapeutic approaches put a load on society. Aside from the fact that the annual costs of stroke amount to billions of dollars, the increasing pressures on families and communities to provide care can be overwhelming (Donnan and Davis 2008; Stineman et al. 1997). After initial hospitalization and rehabilitation, 80% of stroke survivors rely on their family’s support for daily living (Han and Haley 1999; Anderson et al. 1995), making the search for new treatments imperative. Research is focusing on the possibilities to enhance neuroprotection following a stroke, targeting damaging processes such as excitotoxicity, oxidative stress, and inflammation as a new therapeutic approach for stroke (Chamorro et al. 2016; Lakhan et al. 2009a, b; Lo et al. 2003).

Stroke initiates a cascade of pathological processes which lead to permanent neuronal damage including excitotoxicity, oxidative stress, inflammation, ionic imbalance, blood-brain barrier (BBB) disruption, and apoptosis. The inflammatory response is a major player in the ischemic cascade and robust inflammation is elicited in the injured brain after ischemia (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. 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 (Dirnagl et al. 1999a, b). 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 and Feuerstein 1999; Lakhan et al. 2009a).

The main contributor to tissue damage and cell death after a stroke is glutamate-mediated neurotoxicity. Excessive accumulation of the excitatory neurotransmitter glutamate leads to a toxic increase in intracellular calcium, which activates multiple signaling pathways, eventually leading to necrosis.

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and apoptosis (Lai et al. 2014). A potential approach to prevent 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 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 a 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 in glutamate metabolism (Shashidharan et al. 1997; Hudson and Daniel 1993). Studies show that administration of GDH2 reduces glutamate bioavailability in neurons. Furthermore, GDH2 activity is decreased in multi-systemic neurological disorders (Plaitakis et al. 2000). A 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 major process in the ischemic cascade is the oxidative stress response. When an imbalance between the production of free radicals and endogenous scavenging capacity of cellular antioxidants is disturbed, damage to the tissue is unavoidable. Reactive oxygen species (ROS) and nitrogen molecules are related to tissue injury during ischemic stroke. ROSs are capable of initiating DNA single-strand breakage, leading to eventual severe energy depletion and necrotic-type cell death (Cuzzocrea et al. 2001; Choi et al. 2009). 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 the cellular defense. It enhances expression of protective enzymes and antioxidant response elements (ARE) and upregulates antioxidant genes, resulting in the reduction of the oxidative stress and inflammatory responses (Itoh et al. 1997; Nguyen et al. 2009; Suri et al. 2008; Ishii et al. 2000). Nrf2 regulates downstream antioxidative stress genes, such as NAD(P)H quinone oxidoreductase (NQO1), hemeoxygenase-1 (HO-1), and phase II detoxifying enzymes. In parallel, Nrf2 caused an increase in pro-inflammatory markers, such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), and interleukin-8 (IL-8).

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 also serve as a therapeutic target for stroke.

In this study, we investigated the effects of a novel multifactorial-cocktail treatment using lentiviruses encoding EAAT2, GDH2, and NRF2 genes as a potential future treatment of stroke. We have previously shown the effects of the three genes in ALS mice. The genes acted synergistically in reducing extracellular glutamate and glutamate availability while improving the metabolic state and the antioxidant and inflammatory responses (Benkler et al. 2016). Here, we extend our study and examine the possible efficacy of EAAT2, GDH2, and NRF2 treatment on functional outcome in mice focal ischemic injury and reveal possible underlying mechanisms of action.

**Methods and Materials**

**Ethics Statement**

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

**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 cytomagelovirus (CMV) promoter from pIRES2/AcGFP1 cDNA (Clontech, Palo Alto, CA, USA) was cloned into pENTR™-TOPO® (Invitrogen). To overexpress 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 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 1 ng of p24 considered equivalent to $1.25 \times 10^7$ lentiviral particle (LP), and 1 infectious unit considered equivalent to 1 in every 1000 LPs.

**Animals**

C57BL6 male mice at the age of 10 weeks were purchased from Harlan, Israel. 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 and then randomly divided into experimental groups ($n = 10$), 3 genes (mix of EAAT2, GDH2, NRF2) with ET-1 injection, GFP with ET-1 injection, EAAT2 with ET-1 injection, GDH2 with ET-1 injection, NRF2 with ET-1 injection, GFP with saline injection (sham-operated), and untouched (no surgery performed).

**Surgical Procedure and Treatment**

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 coordinates (relative to bregma) +0.5 mm anteroposterior, +1.9 mm mediolateral, and −2.9 mm dorsoventral. Mice received either lentiviruses carrying GFP (control); lentiviruses carrying only one of the genes EAAT2, GDH2, and NRF2; or a mixture of all three genes. Based on our previous studies (Benkler et al. 2016, Glat et al. 2016), we used a total viral load of $5 \times 10^8$ infectious viral particles administered (1.5 μl total, an infusion rate of 0.3 μl/min). The needle was left in place for three additional minutes before withdrawal, and the incision was sutured.

The vasoconstrictor Endothelin-1 (ET-1) was used to induce focal ischemic injury in mice 72 h after lentiviral administration. Mice were injected with 1 μl or 2 μl 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). Animals in this model display significant long-term neurological deficits, associated with excitotoxicity, inflammatory response, and oxidative stress (Kurosawa et al. 1991; Nguemen et al. 2015; Fuxe et al. 1989; Horie et al. 2008). The needle was left in place for three additional minutes before withdrawal, and the incision was sutured. Sham-operated mice were treated identically but received injections of sterile saline (instead of ET-1).

**Behavioral Tests and Analysis**

Behavioral tests were performed at 2 and 7 days after ischemic induction in order to measure motor function. The cylinder test, which measures forelimb use during vertical exploration, was performed as previously described (Schallert et al. 2000). 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 assesses motor coordination and balance. The elevated plus maze is generally used for the assessment of anxiety-related behavior. A plus-shaped maze containing two dark and enclosed arms and two lit and open arms, elevated 100 cm above the ground, will be used. The arms are $30 \times 5$ cm with a $5 \times 5$ cm center area, and the walls of the closed arms are 40 cm high. Mice will be placed in the center of the maze, tracked for 5 min with a video camera, and then returned to their home cage. Time spent in the open arms, numbers of entries to the open arms, and latency to enter the open arms will be scored using Ethovision video tracking system.

**Tissue Processing and Histology**

Four weeks after ET-1 administration, animals were anesthetized with ketamine-xylazine and transected 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 h and then incubated overnight at 4 °C with the following primary antibodies: rat anti-GFAP (1:150, Invitrogen) and 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, Invitrogen) for 1 h. The nuclei were stained with DAPI (1:1000, Sigma-Aldrich). For microscopic analysis, a Zeiss LSM 510 confocal laser scanning microscope was used (Carl Zeiss Inc., Thornwood, NJ, USA). The intensity of fluorescence was measured at the injection site using 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.

**Statistical Analysis**

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

**Results**

**Mouse Model of Focal Ischemic Injury**

In this set of experiments, two different concentrations of Endothelin-1 (ET-1) were used to learn the effects of the treatment on behavioral outcome in several stroke severities. We
used a milder form of stroke (1 μl, 1 mg/ml) and a more severe form of stroke (2 μl, 1 mg/ml) to test our hypothesis. Mice showed significant motor deficits as measured by the behavioral tests following ET-1-induced focal ischemic injury compared to sham-operated mice.

**Lentiviruses Carrying the Nrf2, GDH2, and EAAT2 Genes or GFP Infect Cells**

The tissue was examined after lentivirus administration to make sure that cells were infected. Cells in the injection area infected by the virus consequently overexpress the related proteins. The injected viral vectors contain a blasticidin-S deaminase (BSD) component which is expressed only in the infected cells. Immunohistochemistry showed expression of BSD in the trajectory of the needle insertion into the brain (in the treatment group, Fig. 1a) or GFP (in the control group, Fig. 1b). This suggests that the viral vectors infected the cells and therefore the cells are overexpressing the desired genes.

**Combined Treatment with GDH2, NRF2, and EAAT2 Genes Improve Functional Outcome in the Mouse Model of Focal Ischemic Injury**

To understand the effect of the gene mixture using a milder form of stroke (1 μl of ET-1, 1 mg/ml), lentiviruses carrying the GDH2, NRF2, and EAAT2 genes were injected to the right striatum 72 h prior to ischemic induction (Fig. 2). Baseline measurements were taken before the surgery and mice from all groups showed similar results. Two days after the ischemic injury, mice treated with the mixture of the three genes showed significant improvement in body balance and motor coordination, evident by the elevated bridge test (three genes, 7.48 s ± 0.49 s; GFP, 8.49 s ± 1.42 s; p < 0.005, Fig. 3a). In the cylinder test, less motor asymmetry was observed compared to the control group 2 days after the ischemic damage (three genes, 0.06 ± 0.03; GFP, 0.24 s ± 0.05; p < 0.05, Fig. 3b).

Next, we continued to explore the effect of the lentiviral vector carrying the three genes in a more severe form of stroke (2 μl of ET-1, 1 mg/ml). We were also interested in discovering the effect of each gene separately, compared to the three-gene mixture, in order to evaluate the synergistic effect of the treatment. In a more severe form of stroke (2 μl of ET-1, 1 mg/ml), the treatment group showed significant improvement in the cylinder test 7 days after ischemic injury compared to GFP control group (three genes, 0.018 ± 0.07; GFP, 0.23 ± 0.02; p < 0.05, Fig. 4b). An improvement was also significant in the elevated bridge test. Mice that received the three-gene treatment spent less time crossing the bridge than mice that received GFP (three genes, 6.19 s ± 0.15 s; GFP, 8.36 s ± 0.29 s; p < 0.05, Fig. 4a). We also found that the mixture of the three genes had a more significant effect on the functional outcome than 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 the mixture of the three genes together elicited a stronger outcome after ischemic injury (three genes, 6.19 s ± 0.15 s; GDH2, 6.9 s ± 0.17 s; NRF2, 6.7 s ± 0.37 s; EAAT2, 7.3 s ± 0.06 s; Fig. 4a). The mixture of three genes also attenuated motor asymmetry in a more significant manner as shown by the cylinder test (three genes, 0.018 ± 0.07; GDH2, 0.147 ± 0.01; NRF2, 0.142 ± 0.03; EAAT2, 0.216 ± 0.02; Fig. 4b).

**Effect of Overexpression of Nrf2, GDH2, and EAAT2 Genes on the Inflammatory Response after Focal Ischemic Injury**

Following the behavioral tests, we wanted to understand the effect of the treatment on inflammation processes in the ischemic area after stroke induction. 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 three genes. GFAP levels were lower in the brains of mice that received the three-gene mixture compared to GFP (three genes, 359.4 ± 17.68; GFP, 490.8 ± 75.79; sham, 211.6 ± 37.67; p < 0.05, Fig. 4b). This decrease in reactive astrocytes in the ischemic area suggests a reduction in the inflammatory response.
Discussion

The present study shows the benefits of overexpression of EAAT2, GDH2, and NRF2 genes on focal ischemic injury in mice. We induced an ischemic injury using the vasoconstrictor Endothelin-1 (ET1) as a model of stroke (Horie et al. 2008). Motor function was improved in the treated group compared to control following an ischemic injury. Previous work in our lab showed that this multifactorial-cocktail treatment provides protection and promotes survival in mouse models of neurodegenerative diseases with severe motor dysfunction including ALS and MSA (Benkler et al. 2016, Glate et al. 2016). This study expands these results, showing that treatment with lentiviruses carrying the EAAT2, GDH2, and NRF2 genes improve functional outcome after ischemic injury.

The complex role of astrocytes after a brain injury such as ischemic stroke gives rise to evidence of both beneficial and deleterious roles of astrocytes in stroke pathology. Although there is evidence of the beneficial effects of some aspects of astrogliosis after stroke, the major body of evidence still remains clear about the negative influences. A recent publication shows that after ischemic stroke, astrocytes release proinflammatory agents that recruit immune cells and initiate the formation of a glial scar. Li et al. (2017) report that astrocyte-derived IL-15 is a major driver of tissue damage
and poor outcome after cerebral ischemia. This recent evidence demonstrates that astrocytes are a major source of IL-15 in the CNS after an ischemic stroke that aggravates the state of the tissue post stroke. Begum et al. (2018) show that a component of the astrocyte (NHE1: Na+/H+ exchanger isoform 1) causes ionic dysregulation under ischemic conditions. Selective deletion of this component in astrocytes demonstrated less cellular hypertrophy, less peri-lesion gliosis, and reduced cerebral microvessel damage and blood-brain barrier (BBB) injury in ischemic brains. Laterza et al. (2017) showed that blocking monocyte recruitment reduced astrocyte activation in the SVZ and striatum, which could contribute to the improved neuroblast survival. A similar decrease of astrocyte activation was found in and around human induced pluripotent stem cell (iPSC)-derived NSPCs transplanted into the striatum at 1 week after stroke in monocyte-depleted mice.

The growing frequency of stroke and the restrictions of the current therapeutics highlight the need for new methods to reduce stroke-related disability. The type of gene therapy described in this study may be such a method. Due to the fact that we combined three genes that act synergistically in the damaged area (Benkler et al. 2016), several protective pathways can be triggered simultaneously to handle the damaging effects of oxidative stress, neurotoxic insult, and inflammation. The reduction of these responses leads to neuroprotection and minimizes neuronal damage (Chamorro et al. 2016; Cuzzocrea et al. 2001). Here, we show the synergistic effect yet again and conclude that the mixture of the three genes works more efficiently than each gene separately in improving motor capabilities after ischemic injury.

The inflammatory response plays a key role after injury, contributing to the secondary brain damage after stroke (Lakhan et al. 2009a, b; Barone and Feuerstein 1999; Dirnagl et al. 1999a, b). This suggests that reducing inflammation may be useful after brain injury. A causal link has been established between astrocytic activation in the peri-infarct area and the occurrence of delayed infarct expansion (Matsui et al. 2002; Murphy 2000). Here, we show a decrease in reactive astrocyte expression after the treatment, suggesting that the treatment helps attenuate the inflammatory response and thereby promotes functional recovery. Furthermore, when activated, Nrf2 specifically targets genes bearing an antioxidant response element within their promoters which influence the inflammatory response (Lakhan et al. 2009a, b). 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; Dirnagl et al. 1999a, b).

Nevertheless, research using animal models has its limitations. Although the animals in this model display significant neurological deficits that are associated with the regarded damaging processes (Kurosawa et al. 1991; Nguemeni et al. 2015; Fuxe et al. 1989; Horie et al. 2008), 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). Further studies using the MCAo model can help determine the clinical relevance of these results. Additionally, though lentiviruses are currently used in several clinical trials, other delivery methods such as adenovirus should be tested for safety purposes.

The present study offers a proof of concept to evaluate the benefits of overexpression of the three genes on recovery after ischemic injury. Because of technical limitations in using lentiviruses, the injection of the viral vectors was done before ischemic induction. It takes time for the viruses to infect the cells and for the genes to be integrated and expressed in the cells. We show that overexpression of EAAT2, GDH2, and Nrf2 at close proximity to the induction of ischemia improved motor function after ischemic injury. However, we cannot exclude a protective effect. A further experiment was done in our lab to evaluate the effect of the treatment after the induction of stroke, with no significant results (data not

![Fig. 4](https://example.com/fig4.png)

**Fig. 4** 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). b Viral vector injection into the right striatum led to a decrease in inflammation in the treated group compared to control, as indicated by GFAP staining for astrocytes (scale bar 20 μm; data are given as mean ± SEM).
shown). This might be due to the fact that the ischemic cascade is immediate and the effect of lentiviral-based treatment is delayed. Also, it is possible that the effect was not strong enough to elicit a significant result. Future experiments should test the effect of overexpression of these genes after ischemic injury by using different concentrations and different delivery methods with more immediate options of expression.

**Conclusion**

The results of this study show that lentivirus-mediated gene delivery of EAAT2, GDH2, and NRF2 genes enhances functional recovery and induces neuroprotection after focal ischemic injury. The data suggest a possible basis for clinical application, reducing long-term immobility and disability in stroke patients.

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**Compliance with Ethical Standards**

**Competing Interests** DO holds several patents related to gene therapy in neurodegenerative diseases. All were assigned to “Ramat at Tel Aviv University.” DO is a consultant to “Brainstorm Cell Therapeutics.” The other authors have nothing to disclose.

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