TEL-AVIV UNIVERSITY
SACKLER SCHOOL OF MEDICINE
GRADUATE SCHOOL

DEPARTMENT OF HUMAN MOLECULAR GENETICS AND BIOCHEMISTRY

Development of a novel gene therapy approach for neuroprotection in models of Amyotrophic Lateral Sclerosis

THESIS SUBMITTED FOR THE DEGREE "DOCTOR OF PHILOSOPHY"

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I.D.034700492

SUBMITTED TO THE SENATE OF TEL AVIV UNIVERSITY
JULY 2013

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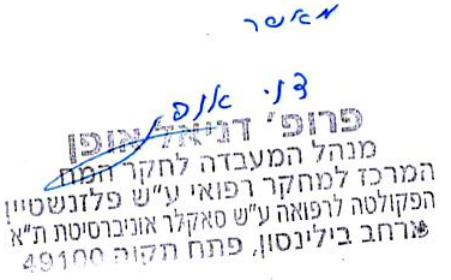

אוניברסיטת תל-אביב

הפקולטה לרפואה ע"ש סאקלר

המדרשה לתארים מתקדמים

החוג לביוכימיה קלינית וגנטיקה של האדם

פיתוח אסטרטגית טיפול גנטי חדשה המקנה הגנה נוירופרוטקטיבית במודלים של מחלת ניוון השרירים

חיבור לשם קבלת התואר "דוקטור בפילוסופיה"

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יולי 2013

עבודה זו בוצעה בהנחייתו של

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הפקולטה לרפואה ע"ש סאקלר, אוניברסיטת ת"א

Acknowledgments

I would like to take this unique opportunity in order to thank some of the people that were instrumental in helping me reach this goal.

First and foremost I would like to convey my highest appreciation to my advisor Prof. Daniel Offen, who was good enough to invite me to his lab as a very young BSc. student and exposed me to the world of research as a whole and to the field of ALS and neurodegenerative diseases. Dani, you guided me through my first steps in the world of medical research and for that I am very grateful. You were a wonderful advisor and teacher and always allowed me to explore my ideas, however bizarre they might have seamed and supported me in them wholeheartedly. I will cherish the guidance and insight you have provided me during my formidable years as a researcher those that I have spent at your lab, for many years to come.

Thank you.

I would also like to convey my gratitude to Dr. Tali Ben-Zur and Ms. Yael Barhum for their invaluable support based on their vast experience as researchers. You provided me with insight and knowledge that was vital for me as a young budding researcher. You were both always ready to teach me and support me in all that I needed and always happy to lend a helping hand. You will not be soon forgotten. I would also like to thank all the members of the lab, past and present for making the time I spent at the lab, truly years to remember.

Last but most certainly not least, on a personal note, I would also like to thank my family, my two wonderful sisters and most of all my parents, getting me here, particularly from where I started, was not an easy task, I have no doubt that without your help and support, perseverance, inspiration and patience to no end I would not have made it here.

Thank you.

Thank you all, this would not have been possible without you.

With all my gratitude,

Chen Benkler.

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A.A	Amino Acid
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
AMPA	(2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
ANOVA	Analysis of variance
ARE	Antioxidant response element
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CEF	Ceftriaxone
CMV	Cytomegalovirus
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal fluid
DAPI	4,6-diamino-2-phenylindole
DCN	Deep cerebellar nuclei
DMEM	Dulbecco’s Modified Eagle’s Medium
DMEM:F-12	Dulbecco's modified eagle medium 1:1 plus Ham's F12
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EAAT1	Excitatory amino acid transporters 1
EAAT2	Excitatory amino acid transporters 2

EGF	Epidermal growth factor
EMG	Electromyography
ESCs	Embryonic stem cells
fALS	Familial Amyotrophic Lateral Sclerosis
FBS	Fetal bovine serum
FDA	Food and Drug Administration
G5 supplement	A cocktail of growth factors
GABA	Gamma-aminobutyric acid
GDH1	Glutamate dehydrogenase 1
GDH2	Glutamate dehydrogenase 2
GDNF	Glia derived neurotrophic factor
GDNF:TTC	Fusion protein of GDNF and TTC
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLAST	Glutamate and aspartate transporter
Gliotransmitters	Transmitters released from glia as opposed to neurotransmitters
GLT-1	Glutamate transporter 1
GluR2	Glutamate receptor 2
GRP	Glial restricted precursors
GS	Glutamine synthetase
HBSS	Hanks’ Balanced Salts Solution
HD	Huntington's disease
HIV-1	Human immunodeficiency virus
hMNPs	Human motor neuron progenitors
hMSCs	Human mesenchymal stem cells

hNSCs	Human neuronal stem cells
HO-1	Heme oxygenase-1
HSCs	Hematopoietic stem cells
hVEGF	Human vascular endothelial growth factor
ICV	Intracerebroventricular
IGF1	Insulin like growth factor 1
IGF-1:TTC	Fusion protein of IGF-1 and TTC
IM	Intramuscular
iNOS	Inducible nitric oxide synthase
iPS	Induced pluripotent stem
LP	Lentiviral particle
LPS	Lipopolysaccharide
LTR	Long terminal repeats
LV	Lentivirus
MOI	Multiplicities of infection
MSCs	Mesenchymal stem cells
NOS	Nitric oxide synthase
NPCs	Neuronal progenitor cells
NRF2	Nuclear factor (erythroid-derived 2) related factor 2
NSC-34	Neuroblastoma (N18TG2) x spinal cord hybrid cell line number 34
NSC-34D	Differentiated neuroblastoma (N18TG2) x spinal cord hybrid cell line number 34
NSCs	Neuronal stem cells
P0	Passage 0

P1	Passage 1
PBS	Phosphate buffer saline
PD	Parkinson's disease
PFA	Paraformaldehyde
qRT-PCR	quantitative reverse transcription polymerase chain reaction
rAAV	Recombinant Adeno-associated virus
RAd	Recombinant adenovirus
RNAi	RNA interference
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
sALS	Sporadic Amyotrophic Lateral Sclerosis
SEM	Standard error of the mean
SOD1	Superoxide dismutase-1
SOD1 G93A	Superoxide dismutase-1 in which the amino acid glycine is replaced by alanine at position 93
SPN	Streptomycin, Penicillin and Nystatin mixture
TARDBP	TAR-DNA binding protein TDP-43
TNFα	Tumor necrosis factor α
t-PDC	L-trans-Pyrrolidine-2,4-dicarboxylic acid
TTC	Tetanus toxin heavy chain
VAPB	vesicle associated membrane protein
WT	Wild-type

Chen Benkler; PhD thesis:

"Development of a novel gene therapy approach for neuroprotection in models of Amyotrophic Lateral Sclerosis"

Supervisor: Prof. Daniel Offen

Abstract

Amyotrophic Lateral Sclerosis (ALS) is a fatal, rapidly progressive, neurodegenerative disease, in which relentless degeneration of motor neurons leads to progressive paralysis and ultimately death due to respiratory failure within two to five years of clinical onset. Despite extensive efforts the underlying cause of ALS and the path of neurodegeneration remain elusive. This led to the proposal of numerous hypotheses regarding disease pathogenesis and pathophysiology. Today it is generally believed that ALS is a multifactorial and multisystemic condition that arises from the combination of several of these hypothesized mechanisms. These include; deficits in neurotrophic factors, toxic intracellular protein misfolding and aggregations, mitochondrial dysfunction, axonal impairment due to neurofilament accumulation, neuroinflammation, genetic factors and iron accumulation.

Perhaps two of the most prominent hypotheses regarding ALS progression involve glutamate excitotoxicity as well as glutamate dependent and independent oxidative stress induced damage.

The selective motor neuron death led researchers to explore cell autonomous mechanisms, however, studies performed in the ALS mouse model suggest that non-neuronal cells such as astrocytes might be at play. Astrocyte activation occurs in response to central nervous system (CNS) insult and is considered a double edged sword in many pathological conditions. In this work we describe for the first time several deficits in the astrocytic response to activation in astrocytes derived from the ALS mouse model, which is based on the human disease causing mutation G93A in the SOD1 enzyme. We found that SOD1 G93Aastrocytes exhibit a reduced glutamatergic and trophic response to specific activations compared to their wild-type counterparts. Wild-type

astrocytes exhibited a robust response when activated *in vitro* with lipopolysaccharide (LPS), G5 or treated with ceftriaxone in several parameters evaluated. These parameters include increased expression of GLT-1 and GLAST the two major astrocytic glutamate transporters, accompanied by a marked increase in the astrocytic glutamate clearance and up-regulation of neurotrophic factor expression. However, not only do un-treated SOD1 G93A astrocytes take up glutamate less efficiently, but in response to activation they show no further increase in any of the glutamatergic parameters evaluated.

Furthermore, activation of wild-type astrocytes, but not SOD1 G93A astrocytes, improved their ability to protect the motor neuron cell line NSC-34 from glutamate induced excitotoxicity. The data we collected from astrocytes of the SOD1 G93A ALS mouse model indicates that altered astrocyte activation may well be pivotal to the pathogenesis of ALS.

Unfortunately, currently available therapies, for neurodegenerative diseases in general and particularly ALS leave much to be desired, as they provide only symptomatic alleviation, which is often accompanied by severe side effects. Far worse is the fact that it appears that none of these treatments effect the degenerative progression, imploring us to explore new alternatives. Riluzole the only currently available FDA approved drug for treating ALS prolongs the patients' survival by 2-3 months with little effect on the diseases progression.

Based on the astrocytic dysfunctions we identified in the early stages of this work, we devised an alternative therapeutic strategy. This therapeutic alternative attempts to compensate for these astrocytic dysfunctions by enhancing the cellular anti-oxidative response, improving the cellular glutamate metabolism and sufficiently reducing extracellular glutamate levels around the susceptible neurons affected by glutamate excitotoxicity thus attempting to hinder the neurodegenerative progression.

We chose to attempt to provide such neuronal protection using genetically engineered lentiviral vectors containing three genes, each addressing one of these three elements; Excitatory amino acid

transporter 2 (EAAT2), Glutamate dehydrogenase (GDH2) and nuclear factor (erythroid-derived 2) related factor 2 (NRF2). We found that treatment with lentiviral vectors containing these three genes presented with a therapeutic effect *in vitro* as well as in the ALS mouse model SOD1 G93A. *In vitro* transfection of astrocytes with these three genes significantly increased the neuroprotection they provided to cells of the motor neuron cell line NSC-34from excito-oxidative insult.

Furthermore, we demonstrated that both *in vitro* and *in vivo* a synergistic relationship exists between the three genes, and combined treatment with all three genes provided a significantly stronger effect than that obtained by treatment with each individual gene.

Injection of these three genes into the cisterna magna and hind limb muscles of the SOD1 G93A ALS mouse model we found that it exerts an extremely beneficial therapeutic effect in every parameter evaluated. Our treatment delayed weight loss, preserved hindlimb reflex and motor function as well as protected the overall neurological function. Our treatment significantly delayed the onset of symptoms by 81% in males and 96% in females. Furthermore, our treatment was able to prolong the survival of ALS mice by 120% in males and 136% in females from the onset of symptoms.

This study started as an in depth investigation into the unknown mechanism of ALS, building on the resulting understandings we devised a therapeutic strategy. We found that this novel therapeutic approach had a remarkable therapeutic effect in the SOD1 G93A ALS mouse.

Neurodegenerative disorders represent some of modern medicines greatest challenges, as most remain not only incurable but mostly untreatable. Looking ahead we hope that our therapeutic approach might provide a novel strategy that could someday be translated to people and help slow down the disease progression and alleviate the symptoms of patients suffering from ALS and potentially other neurodegenerative diseases involving glutamate excitotoxicity and oxidative stress.

חן בנקלר; עבודת דוקטורט:

"פיתוח אסטרטגית טיפול גנטי חדש המקנה הגנה נוירופרוטקטיבית במודלים של מחלת ניוון השרירים"

מנחה: פרופ' דניאל אופן

תקציר

מחלת ניוון השרירים ALS הינה מחלה ניוונית מתקדמת וסופנית, שבה דגנרציה פרוגרסיבית של נוירונים מוטוריים מובילה לשיתוק ההולך ומחמיר עד שלבסוף מוביל למוות כתוצאה מכשל נשימתי, תוך כ 2-5 שנים מרגע הופעת הסימפטומים הקליניים. למרות המאמצים הרבים שהושקעו הגורמים למחלה ואופן תמותת הנוירונים נותרו בגדר תעלומה. חוסר ידיעה זה הוביל לפיתוחן של מספר תיאוריות הדנות באופן התפתחות המחלה ובפאטופיזיולוגיה שלה.

כיום מקובלת הדעה לפיה מחלת ה ALS, הינה מחלה מולטי-פקטוריאלית המערבת מספר מערכות פיזיולוגיות שונות, ונובעת משילוב של אלמנטים ממספר תיאוריות שונות. תיאוריות אלו כוללות: חוסר של חומרים נוירוטרופיים, ליקויים בקיפול חלבונים ויצירת אגרגטים תוך תאים רעילים, ליקויים בתפקוד המיטוכונדריות, הצטברות נוירופילמנטים באקסונים של הנוירונים המובילים לפגיעות בתפקוד האקסונים, דלקת נוירונלית, גורמים גנטיים והצטברות משקעי ברזל.

יתכן והתיאוריות המקובלות ביותר כיום הן אלו המערבות רעילות של גלוטמט, ונזקי חמצון אשר תלויים או בלתי תלויים בגלוטמט.

דפוס תמותת התאים במחלה אשר פוגעת בראש ובראשונה בנוירונים הובילה את החוקרים לבחון במשך שנים רבות בעיקר מנגנונים ודפוסי מחלה תלויי נוירונים בלבד. למרות זאת בשנים האחרונות עולה ממחקרים שבוצעו בחיות מודל למחלת ה ALS, כי יתכן ומעורבותם של תאים לא נוירונליים, כגון אסטרוציטים חיונית להתפתחותה המלאה של המחלה. במערכת העצבים המרכזית, אסטרוציטים מאוקטבים בתגובה לנזק ולחץ סביבתי. אקטיבציה זו נחשבת כיום לחרב פיפיות במגוון רחב של מצבים פתולוגיים. בעבודה זו אנו מאפיינים לראשונה מספר ליקויים בתגובת האקטיבציה של אסטרוציטים, שנלקחו מעכברי מודל למחלת הALS, אשר מבטאים את המוטציה G93A בגן SOD1.

מצאנו כי בתגובה לאקטיבציה במספר אקטיבטורים (G5, ליפופולי-סכרידים וצפטריאקסון), אסטרוציטים אשר מבטאים את המוטציה SOD1 G93A, הינם בעלי תגובה פחותה במספר פרמטרים גלוטמטרגיים ונוירו-טרופיים בהשוואה לאסטרוציטים אשר לא מבטאים את המוטציה. בניגוד לאסטרוציטים בריאים, אסטרוציטים המבטאים את המוטציה SOD1 G93A לא מעלים את כמות הפרשת החומרים הנוירוטרופיים. יתר על כן, לא רק שאסטרוציטים המבטאים את המוטציה SOD1 G93A, הינם בעלי קצב שאיבת גלוטמט התחלתי הנמוך מזה של אסטרוציטים בריאים. בניגוד לאסטרוציטים הבריאים הם גם לא מעלים את רמות הביטוי של התעלות האסטרוציטיות שואבות הגלוטמט (GLT-1 ו GLAST) ובהתאם לכך גם לא את קצב שאיבת הגלוטמט שלהם.

תגובה זו של האסטרוציטים המבטאים את המוטציה SOD1 G93Aמובילה לכך שבניגוד לאסטרוציטים אשר לא מבטאים את המוטציה SOD1 G93A, האסטרוציטים המוטנטיים לא מגנים טוב יותר על תאים מקו התאים הנוירונלי המוטורי (NSC-34), מפני נזקי גלוטמט בעקבות אקטיבציה. ממצאים אלו מצביעים על היתכנותה של מעורבות קריטית של ליקויים בתגובת אסטרוציטים לאקטיבציה בהתפתחות והתהוות מחלת ה ALS.

למרבה הצער, טיפולים הזמינים כיום לטיפול במחלות ניוון של מערכת העצבים ככלל ולמחלת ה ALS בפרט מסוגלים להקנות במקרה הטוב ביותר הקלה סימפטומטית המלווה לרוב בתופעות לוואי קשות. חמורה מכך היא העובדה כי מרביתם המכריע של טיפולים אלו לא מסוגלים להאט את קצב התדרדרות המחלה. כול אלו מפצירים בנו לנסות ולחפש אסטרטגיות טיפוליות אלטרנטיביות. רילוזול, התרופה היחידה המאושרת כיום לטיפול ב ALS, מסוגלת להאריך את חיי החולים בכ 2-3 חודשים, אך אין ביכולתה להאט את קצב התדרדרות המחלה.

בהתבסס על הליקויים האסטרוציטיים שאפיינו בשלבים מוקדמים של מחקר זה, הגינו אסטרטגיה טיפולית חדשה למחלת ה ALS. אסטרטגיה זו פועלת בכדי לפצות על הליקויים האסטרוציטיים, שאפיינו בכך שהיא מגבירה את תגובת ההגנה התאית מפני נזקי חמצון, ובד בבד מקטינה את רמות הגלוטמט הסינפטיות בעודה מגבירה את יכולת פירוק הגלוטמט התאי, ובכך מנסה להגן על הנוירונים הרגישים לנזקים אלו, אשר מתים במחלה, מפני תמותה והתנוונות.

אנחנו בחרנו לנסות ולהקנות הגנה נוירונלית זו על ידי הנדסת 3 לנטי-וירוסים, אשר כול אחד מהם מבטא גן אחד המסוגל לפצות על אחד משלושת הליקויים: EAAT2, תעלה השואבת גלוטמט מהמרווח הסינפטי, גלוטמט דה-הידרוגנאז המסוגל לפרק גלוטמט תוך תאי, ו NRF2 המהווה פקטור שעתוק המפעיל מגוון גנים המשתתפים בתגובה התאית כנגד גורמי חמצון.

במחקר זה מצאנו כי טיפול באמצעות שלושת הוירוסים האלו, הינו בעל אפקט טיפול הן בתרביות תאים והן במודל העכברי למחלת ה ALS. הדבקת אסטרוציטים בשלושת הגנים האלו שיפרה משמעותית את יכולת ההגנה של האסטרוציטים כלפי תאים מקו התאים הנוירונלי המוטורי (NSC-34), מפנינזק המשלב עקה חמצונית ורעילות גלוטמטרגית.

יתרה מכך, אנו מראים כי שלושת גנים אלו פועלים יחד בצורה סינרגיסטית בכדי להקנות את ההגנה שלהם בתרביות תאים ובמודל העכברי למחלה כאחד.

כאשר יישמנו את הטיפול הזה במודל העכברי למחלת ה ALS, מצאנו כי לטיפול זה אפקט טיפולי מגן בכל אחד מהפרמטרים הנבדקים. הטיפול שלנו מסוגל לדחות את איבוד המשקל של החיות, לשמר את הרפלקסים של הרגליים האחוריות של העכברים ואת התפקוד המוטורי. כמו כן, הטיפול שלנו משמר את המצב הנוירולוגי הכולל של החיה. הטיפול שלנו דחה משמעותי את גיל הופעת הסימפטומים הקליניים של החיה (ב 81% בזכרים וב 96% בנקבות), וכן האריך משמעותית את חיי העכברים מרגע הופעת הסימפטומים (ב 120% בזכרים וב 136% בנקבות).

מחקר זה התחיל בחקירה מעמיקה של המנגנונים הלא ידועים האחראיים להתפתחות מחלת ה ALS, מתוך תובנה זו פיתחנו אסטרטגיה טיפולית חדשה למחלה. מצאנו כי אסטרטגיה זו הינה בעלת אפקט טיפולי מגן משמעותי מאוד במודל העכבר של המחלה.

המחלות הנוירודגנרטיביות מהוות את אחד האתגרים הגדולים של הרפואה המודרנית, היות ולא רק שאת רובם לא ניתן לרפא, אך יתרה מכך ברובם, גם לא ניתן לטפל. במבט על העתיד, אנו מקווים כי ביום מן הימים ניתן יהיה לתרגם את הטיפול שלנו מעכברים לאנשים,ובכך להאט את קצב התפתחות המחלה, ולעזור להקל על הסימפטומים של אנשים החולים במחלת ה ALS.

Introduction

1. Amyotrophic Lateral Sclerosis

1.1 General

Amyotrophic Lateral Sclerosis (ALS) also known as Lou Gehrig's disease is a fatal, rapidly progressive, neurodegenerative disease characterized by loss of motor neurons in the motor cortex, brainstem and spinal cord. This motor neuron degeneration results in weakness, muscle atrophy, fasciculations and paralysis, a process culminating in death due to respiratory failure (Charles, et al., 2001; Oliveira, et al., 2009). The death of the upper motor neurons, found in the motor cortex in the brain, leads to the spasticity, hyperexcitability of reflexes and the appearance of pathological reflexes, such as the Babinski sign. Whereas the death of the lower motor neurons, which are found in the brain stem and in the spinal cord accounts for the weakness and atrophy of the muscles leading to the progressive paralysis (Acsadi, et al., 2002; Aguilar, et al., 2007; Bruijn, et al., 2004; Lev, et al., 2009; Mohajeri, et al., 1999; Offen, et al., 2009; Séverine, et al., 2006).

ALS has an estimated uniform prevalence of 4–6 cases per 100,000 in Western countries, where it is 50–100 times higher than elsewhere in the world (Cronin, et al., 2008). The onset of ALS is most common in midlife between 45 and 65 years of age, with a typical disease course of 1–5 years. In most cases this typical progression consists of two phases, rapid functional deterioration occurs during the first 1-3 years and the loss of critical physiological functions such as respiration lead to death generally within 3-5 years of clinical onset (Charles, et al., 2001; Oliveira, et al., 2009).

Despite extensive efforts the underlying cause of ALS and the path of neurodegeneration remain elusive. This led to the proposal of numerous hypotheses regarding disease pathogenesis and pathophysiology. Today it is generally believed that ALS is a multifactorial and

multisystemic condition that arises from the combination of several of these hypothesized mechanisms. These include; deficits in neurotrophic factors, toxic intracellular protein misfolding and aggregations, mitochondrial dysfunction, axonal impairment due to neurofilament accumulation, neuroinflammation, genetic factors and iron accumulation (Benkler, et al., 2013; Cozzolino, et al., 2008; Cronin, et al., 2008; Lev, et al., 2009; Offen, et al., 2009; Rothstein, 2009; Urushitani, et al., 2007; Van Damme, et al., 2005; Xiao, et al., 2006).

Perhaps two of the most prominent hypotheses regarding ALS progression involve glutamate excitotoxicity and glutamate dependent and independent oxidative stress induced damage (Barber, et al., 2010; Bogaert, et al., 2010; Coyle, et al., 1993; Kruman, et al., 1999; Lynch, et al., 2002; Rival, et al., 2004; Rothstein, 2009; Shaw, et al., 2000; Tolosa, et al., 2011; Van Damme, et al., 2005, Van Den Bosch, et al., 2006, Figure 1).

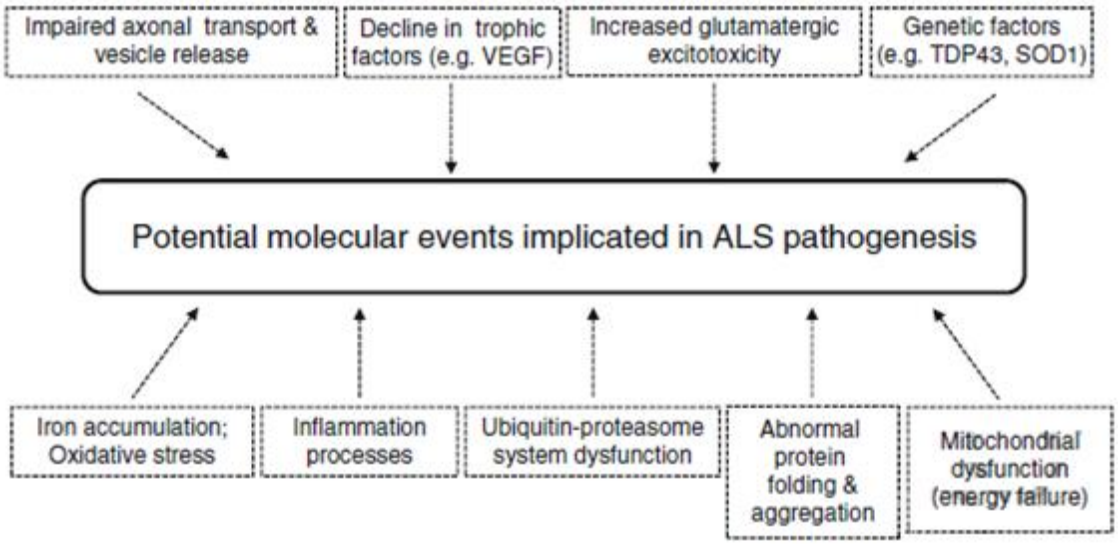

Figure 1. A schematic illustration of the potential diverse molecular events implicated in the pathogenesis of both sporadic and familial ALS forms that may involve in motor neuronal degeneration. *ALS is a multifactorial and multisystemic disease that arises from a combination of several mechanisms, which act through concurring damage inside motor neurons and their neighboring non-motor cells. These include; increased glutamate excitotoxicity, oxidative stress damage, deficits in neurotrophic factors protein, misfolding and aggregation, mitochondrial malfunction, impaired axonal transport and vesicle release, neuroinflammation, genetic factors and iron accumulation (Benkler, et al., 2013).*

1.2 Genetic factors

The vast majority of ALS cases have no known familial history and are classified as sporadic (sALS), the remaining approximate 10% of ALS cases are associated with familial genetic factors (fALS). 20% of those fALS cases are attributed to mutations in the zinc/copper superoxide dismutase-1 (SOD1) gene (Valdmanis, et al., 2009; Wijesekera, et al., 2009). To date, over 100 mutations associated with fALS have been mapped to the SOD1 gene. One of the most prevalent SOD1 mutations is the G93A variant, in which the amino acid glycine is replaced by alanine at position 93. This information was used in developing animal models of ALS; the first and most commonly used model is of transgenic rodents harboring the G93A mutation. These animals develop a pathophysiological condition resembling ALS, presenting with hind limb weakness at 3–4 months of age and progressive paralysis culminating in death at the age of 4–5 months. The pathological features of the disease resemble human neurologic diseases, including loss of large spinal cord motoneurons and robust spinal astrogliosis and microgliosis (Bendotti, et al., 2004; Cozzolino, et al., 2008; Gruzman, et al., 2007; Mohajeri, et al., 1999; Redler, et al., 2012; Turner, et al., 2008, Figure 2).

Other genes linking to fALS include alsin (ALS2), senataxin (ALS4), vesicle associated membrane protein (VAPB, ALS8), angiogenin and a mutation in the p150 subunit of dynactin (Wijesekera, et al., 2009). Recently, mutations in TARDBP gene, encoded the TAR-DNA binding protein TDP-43, and the hexanucleotide repeats in a non-coding region of C9ORF72, a protein whose function and structure are still un-known have been reported as associated with fALS and sALS as well as in frontotemporal dementia. Do date the C9ORF72 repeat is the most common genetic factor associated with sporadic ALS, however, the function of the protein and the path by which the mutation causes the disease remains un-known (Millecamps, et al., 2012 ; Polymenidou, et al., 2012; Wijesekera, et al., 2009). The

clinical and pathophysiological similarities between the sporadic and familial forms of ALS suggest a possible common pathogenesis (Benkler, et al., 2010; Offen, et al., 2009; Mohajeri, et al., 1999; Nassif, et al., 2010 ; Strong, et al., 2005) though fALS tends to develop earlier in life and present with an accelerated disease progression (Valdmanis, et al., 2008).

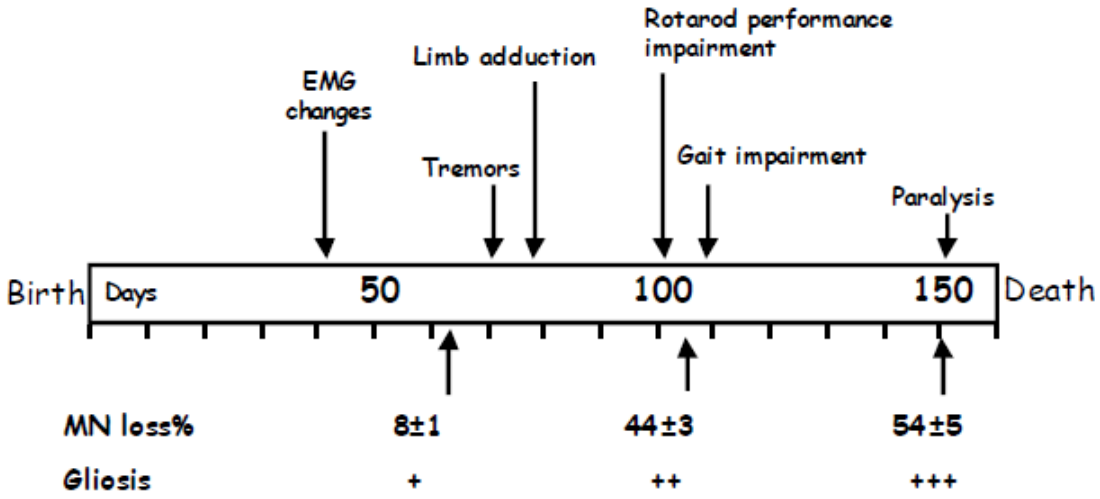

Figure 2. General scheme of the disease progression in the SOD1 G93A mouse model of ALS. *Motor neuron loss starts at a very young and asymptomatic age. Pre clinical functional signs begin with neuronal conductivity changes that can be measured by EMG, first appearing at about 40 days of age. The first clinical signs of motor deterioration become visible around the age of 70-80 days when the mouse's hind limbs start to tremble. The symptoms slowly progress, with motor function impairments becoming significantly more pronounced at 100-120 days and full clinically visible symptoms appear at the age of 140 days. From the onset of clinical symptoms the disease progresses very quickly and death occurs approximately at 150-155 days of age (Bendotti, et al., 2004; Cozzolino, et al., 2008; Gruzman, et al., 2007; Mohajeri, et al., 1999; Redler, et al., 2012; Turner, et al., 2008)*

1.3 ALS as a multifactorial disease

After nearly 20 years of research into the molecular bases of ALS the involvement of many different factors, mechanisms and pathways has been established, yet the initiating cause as well as which of these mechanisms are secondary or compensatory effects remains to be determined. In this section I will further elaborate on several of the more commonly expected mechanisms.

1.3.1 **Glutamate excitotoxicity**

Glutamate is the main excitatory amino acid (a.a) neurotransmitter in the human central nervous system (CNS). It plays a major role in learning, development, synaptic plasticity, cognitive functions and behavior (Danbolt, 2001; Maragakis, et al., 2001; Mattson, 2008; Molz, et al., 2008). However, when glutamate in not properly controlled it may lead to detrimental results. Overstimulation of glutamate receptors leads to neuronal degeneration, a process commonly referred to as glutamate excitotoxicity (Attwell, 2000; Bogaert, et al., 2010; Brown, 2000 ; Danbolt, 2001; de Hemptinne, et al., 2004 ; Foran, et al., 2009; Lee, et al., 2007 ; Mattson, 2008; Maragakis, et al., 2001; Sheldon, et al., 2007 , Sonnewald, et al., 2002; Van Damme, et al., 2005). Accumulating evidence implicates glutamate excitotoxicity not only in the pathophysiology of ALS but further in several other acute and chronic neurological conditions including; cerebral ischemia, traumatic brain injury and hypoglycemic brain damage (Foran, et al., 2009; Greenamyre, 1986; Maragakis, et al., 2001; Szatkowski, et al., 1994 ; Van Damme, et al., 2005) Huntington's disease (HD), Parkinson's disease (PD), Alzheimer's disease (AD) and epilepsy (Allen, et al., 2004; Attwell, 2000; Danbolt, 2001; de Hemptinne, et al., 2004 ; Doraiswamy, 2003; Estrada-Sánchez, et al., 2009; Foran, et al., 2009; Mattson, 2008; Maragakis, et al., 2001; Meldrum, 2000, Palmer, et al., 1986 ; Portera-Cailliau, et al., 1995; Sheldon, et al., 2007 ; Senkowska, et al., 2003 ; Tanaka, et al., 1997 ; Thomas, et al., 2003 ; Trotti, et al., 1998 ; Van Damme, et al., 2005; Vernon, et al., 2007).

Glutamate excitotoxicity constitutes one of the currently most prominent pathophysiological hypothesis explaining ALS progression (Foran, et al., 2009; Shaw, et al., 1997).

Under normal conditions, extracellular glutamate is kept at low concentrations, despite the much higher intracellular glutamate concentration and its frequent release at

glutamatergic synapses. Regulation of extracellular glutamate concentrations is attributed mostly to the astrocytic high affinity excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2) also known in rodents as GLAST and GLT-1 respectively. These transporters allow astrocytes to remove glutamate from the synaptic cleft, thus reducing glutamate receptor overstimulation (Allen, et al., 2004; Anderson, et al., 2000 ; Attwell, 2000; Danbolt, 2001; Lee, et al., 2004; Levy, et al., 1998; Meldrum, 2000; Sheldon, et al., 2007 ; Velasco, et al., 2003 ; Zerangue, et al., 1996).

These transporters stand at the heart of the ALS excitotoxic hypothesis, abnormal glutamate metabolism accompanied by selective loss of the astroglial glutamate transporter-1 GLT-1 (and its human counterpart EAAT2) were observed in sporadic and familial ALS patients as well as in mutant SOD1 animal models (Bendotti, et al., 2001; Rothstein, et al., 1992; Rothstein, et al., 1995). Furthermore, both *in vitro* and *in vivo* experiments demonstrate that selective loss of GLT-1 can lead to motor neuron degeneration (Rothstein, et al., 1996).

In 2003, mice overexpressing the EAAT2 gene were crossbred with SOD1 G93A mice (Guo, et al., 2003). EAAT2 overexpression in these mice increased the glutamate uptake abilities of synaptosomes obtained from cerebral cortices, subcortical forebrains and spinal cords. This increase in the cellular glutamate uptake capacity delayed spinal motoneuron death and preserved motor function, however, it did not postpone the onset of paralysis nor did it prolong life span. In this study (Guo, et al., 2003) the EAAT2 gene was coupled with the astrocytic glial fibrillary acidic protein (GFAP) promoter. The promoter activity only increases as the disease progresses thus hindering the potential protective effect of EAAT2. This may account for the partial protection observed.

A different study examined the complementary effect by crossbreeding SOD1 G93A mice with EAAT2 heterozygote mice (SOD1 G93A::EAAT2 (+/-)). These mice suffer from an increased rate of motor decline, earlier motoneuron loss and a reduction in life span

compared to SOD1 G93A mice (Pardo, et al., 2006). EAAT2 (+/-) mice do not develop motor neuron disease (Kiryk, et al., 2008), however in rats, chronic intra-ventricular administration of EAAT2 antisense oligonucleotides not only increases extracellular glutamate concentrations, but further results in a progressive motor neuron syndrome, manifesting in slowness of hind limb movement and progressing to hind limb paresis within 7 days of treatment. Pathological alterations could be further detected at the cellular level, where neuronal pathology, resembling excitotoxic damage, mainly cytoplasmic vacuolization and dendritic swelling, was observed (Rothstein, et al., 1996). Similar results were obtained with chronic intra-ventricular administration of EAAT1 antisense oligonucleotides (Rothstein, et al., 1996).

Several pharmacological substances, such as the immunophilin ligand GPI-1046, were recently noted for their ability to increase EAAT2 expression and improve glutamate uptake. Chronic treatment with GPI-1046, was reported to improve the survival of SOD1 G93A mice, but had no effect on disease onset and clinical progression (Ganel, et al., 2006).

In a screening study of 1040 pharmacological substances the β-lactam antibiotic Ceftriaxone was found to increase astrocyte mediated glutamate transport by stimulating the expression of GLT-1 (Rothstein, et al., 2005). Chronic Ceftriaxone treatment upregulated GLT-1mRNA levels, increased overall survival, significantly delayed loss of muscle strength and body weight, reduced motoneuron loss and decreased gliosis formation in the ALS SOD1 G93A mouse model (Rothstein, et al., 2005). Unfortunately, recent clinical trials using Ceftriaxone failed to show a therapeutic effect in patients (ClinicalTrials.gov identifier NCT00349622). More recently, a similar screening study, identified Harmine, a naturally occurring β-carboline alkaloid, as a very potent activator of the GLT-1 promoter. In vivo evaluation of Harmine yielded similar results to those obtained with ceftriaxone (Li, et al., 2011). In an effort to find compounds that can induce translation of EAAT2 transcripts, high-

throughput screening was used to evaluate approximately 140,000 compounds. This study identified 61 compounds with the ability to produce a dose-dependent increase in EAAT2 protein levels (Colton, et al., 2010).

Perhaps the most compelling indication implicating glutamate excitotoxicity in the pathophysiology of ALS stems from Riluzole, the only FDA approved ALS treatment. Riluzoles modest effect of prolonging ALS patients' survival by 2-3 months is attributed to its anti- excitotoxic effect, which stems from an inhibitory effect on glutamate release, inactivation of voltage-dependent sodium channels, and interference with intracellular events that follow transmitter binding at excitatory amino acid receptors. (Koh, et al., 2004; Koh, et al., 2006; Weinreb, et al., 2009; Xu, et al., 2006).

1.3.2 **Oxidative stress**

Oxidative stress arises from an imbalance between the production and accumulation of reactive oxygen and nitrogen species (ROS/RNS) and the cellular ability to detoxify and repair the damage caused by them. Neurons are particularly susceptible to oxidative stress due to the high rate of oxidative metabolism in the brain and the relatively low level of antioxidant enzymes. Furthermore, motor neurons are not replaced during the animals' lifespan and must deal with the lifelong accumulation of oxidative damage (Barber, et al., 2010 ; Wang, et al., 2010).

During the past few years the involvement of oxidative stress in ALS has been demonstrated many times. Evidence of enhanced oxidative stress have been recorded in the motor cortex and in the spinal cords of sporadic and familial ALS patients as well as in animal models (Abe, et al., 1995 ; Beal, et al., 1997 ; Bogdanov, et al., 2000 ; Ferrante, et al., 1997 ; Kruman, et al., 1999 ; Shaw, et al., 1995 ; Wootz, et al., 2004).

The involvement of oxidative stress in ALS is currently attributed to 2 separate pathways; glutamate dependant and independent oxidative insult.

Hyper-stimulation of glutamate receptors leads to increased Ca^{+2} influx into the cell which in turn leads to a cascade of intracellular responses including increased ROS production (Carriedo, et al., 2000 ; Lynch, et al., 1994 ; Lynch, et al., 2002 ; von Lewinski, et al., 2005). This effect is dramatically enhanced in the context of ALS, motor neurons are selectively more vulnerable to the glutamate receptor AMPA mediated cell death compared to other neuronal cell types. Under normal conditions AMPA receptors containing at least one GluR2 subunit have low Ca^{+2} permeability. However, this might not be the case in ALS as altered GluR2 post-transcriptional editing was observed in sporadic ALS patients. This altered form of GluR2 was later found to be extremely more permeable to Ca^{+2}, resulting in higher ROS production in response to glutamate receptor activation (Barber, et al., 2010; Coyle, et al., 1993; Hideyama, et al., 2010 ; Hideyama, et al., 2012 ; Kawahara, et al., 2004 ; Kruman, et al., 1999; Kwak, et al., 2005 ; Lynch, et al., 2002; Rival, et al., 2004; Shaw, et al., 2000, Van Damme, et al., 2005; Van Den Bosch, et al., 2006).

Recently, expression of mutant SOD1 in motor neuron like cells was shown to reduce the expression of anti-oxidant genes. Similar results were obtained when motor neurons isolated from spinal cord sections of two familial patients were evaluated. One of the genes that was dramatically down-regulated was the nuclear factor (erythroid-derived 2) related factor 2 (NRF2). NRF2 is a transcription factor which activates genes containing the antioxidant response element (ARE), thus constituting a major node in the cellular anti-oxidative response (Hybertson, et al., 2011; Vargas, et al., 2009). It has been previously shown that activation of NRF2 specifically in astrocytes protects neurons from a variety of *in vitro* insults as well as conveys protection in an ALS mouse model (Calkins, et al., 2010; Kraft, et al., 2004; Shih, et al., 2003; Vargas, et al., 2006; Vargas, et al., 2008; Vargas, et al., 2009).

2. Non cell autonomous involvement in ALS

2.1 General

The selective motor neuron death occurring in ALS led researchers to explore cell autonomous mechanisms. However, studies performed in the SOD1 fALS mouse model suggest that non-neuronal cells such as astrocytes and microglia might be at play (Beers, et al., 2006; Boillée, et al., 2006; Clement, et al., 2003; Di Giorgio, et al., 2007; Ilieva, et al., 2009; Yamanaka, et al., 2008).

The involvement of non-cell autonomous mechanisms was first consolidated by ineffective efforts to develop animal models of ALS. Under the cell autonomous hypothesis many attempts were made to develop animal models of ALS where the mutant SOD1 gene was expressed selectively in neurons. These efforts generally did not produce disease formation, when the disease mutation was mostly but not exclusively restricted to neurons, animals expressing very high levels of the mutation did develop disease symptoms, but at a much later stage without reaching the full extent of the disease outcome (Jaarsma, et al., 2008 ; Lino, et al., 2002 ; Pramatarova, et al., 2001). Furthermore, reducing the expression of mutant SOD1 selectively in motor neurons significantly slowed disease onset but had very little effect on the rate of disease progression after the initial onset (Boillée, et al., 2006; Ralph, et al., 2005 ; Yamanaka, et al., 2008).

On the other hand, reducing the expression of mutant SOD1 selectively in microglia and astrocytes drastically delayed disease progression with a less marked effect on disease onset (Boillée, et al., 2006; Wang, et al., 2009 ; Yamanaka, et al., 2008). The involvement of other non-neuronal cell types in ALS is slightly more controversial with: muscle cells and Schwann cells considered strong candidates for possible involvement in the non-cell autonomous

effects in ALS (Dobrowolny, et al., 2008 ; Holzbaur, et al., 2006 ; Lobsiger, et al., 2009 ; Miller, et al., 2006 ; Towne, et al., 2008 ; Turner, et al., 2010 ; Wong, et al., 2010).

2.2 Astrocytes

Astrocytes were once thought of as simply the structural background of the neuronal system, later studies called this theory into question and astrocytes are now considered integral and crucial units of CNS. Astrocytes are highly versatile cells that can adapt their performance and function in response to changes in their micro and macro environments (Molofsky, et al., 2012). They are strategically located between vascular and neural elements enabling them to act as mediators between these two systems, regulating the neuronal availability of metabolites and oxygen (Tsacopoulos, et al., 1996). The interaction between astrocytes and neurons is vast and complex, they provide metabolic and neurotrophic support but through a large repertoire of receptors and transporters they can influence the synaptic micro-environment and neuronal signaling. Astrocyte receptors also respond to the spillover of neurotransmitters during intense synaptic activity by inducing Ca^{+2} astrocytic signaling. This signaling causes the release of gliotransmitters (transmitters released from glia as opposed to neurotransmitters) which in turn effects the neuronal electrical excitability and synaptic transmission (Angulo, et al., 2004 ; Danbolt, 2001; Fellin, et al., 2004 ; Fiacco, et al., 2007 ; Jourdain, et al., 2007 ; Kozlov, et al., 2006 ; Navarrete, et al., 2008 ; Pasti, et al., 1997 ; Perea, et al., 2007 ; Rothstein, et al., 1996).

The involvement of astrocytes in ALS is currently attributed to two potential pathways, either gain of new toxic functions (Di Giorgio, et al., 2008 ; Foran, et al., 2011 ; Fritz, et al., 2013 ; Haidet-Phillips, et al., 2011 ; Nagai, et al., 2007) or loss of normal neuroprotective functions, among those are secretion of neurotrophic factors, maintenance of synaptic glutamate homeostasis and modulation of the neuronal susceptibility to glutamate

excitotoxicity. Increasing evidence indicate astrocytic dysfunction in all three mechanisms may well be instrumental in the pathogenesis of ALS (Van Den Bosch, et al., 2008; Staats, et al., 2009).

One of the most characterized astrocytic dysfunctions in ALS is loss of the glutamate transporter EAAT2. Evidence of pathologies involving EAAT2 were observed in sporadic and familial ALS patients as well as in mutant SOD1 animal models (Bendotti, et al., 2001; Rothstein, et al., 1992; Rothstein, et al., 1995). In sALS patients infected areas of the CNS showed up to 95% lost EAAT2 protein expression and function (Bristol, et al., 1996).

2.3 Astrocyte activation

Astrocyte activation occurs in response to central nervous system (CNS) insult and is considered a double-edged sword in many pathological conditions including brain trauma, ischemia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease and ALS (De Lanerolle, et al., 2010; Maragakis, et al., 2006; Myer, et al., 2006; Pekny, et al., 2005; Seifert, et al., 2010; Swanson, et al., 2004). On the one hand, astrocyte activation has been associated with a variety of deleterious effects ranging from loss of normal astrocytic function to secretion of toxic and pro-apoptotic factors, such as; glutamate, nitric oxide synthase (NOS) and tumor necrosis factor α (TNFα) (Barbeito, et al., 2004; Eddleston, et al., 1993; Hamby, et al., 2010).

On the other hand astrocytes have been reported to express several protective growth factors in response to CNS injury and neurological conditions, including brain derived neurotrophic factor (BDNF), glia derived neurotrophic factor (GDNF), insulin like growth factor 1 (IGF1) and ciliaryneurotrophic factor (CNTF) (Garcia-Estrada, et al., 1992; Lee, et al., 1997; Oyesiku, et al., 1997; Sato, et al., 2009; Sendtner, et al., 1990; Tokumine, et al., 2003; Yamaguchi, et al., 1991). All four aforementioned neurotrophic factors were found to

protect neurons *in vitro* and *in vivo* in several models of neurodegenerative diseases including ALS and are currently been pursued in clinical trials (Benkler, et al., 2010; Dawbarn, et al., 2003; Ekestern, 2004; Ochs, et al., 2000; Sakowski, et al., 2009).

Another potential promising neuroprotective property of astrocyte activation is the recently described increase in GLT-1 expression, a similar phenomena has also been described in chemically activated microglia (Persson, et al., 2005; O'Shea, et al., 2006). This aspect of astrocyte activation is especially interesting in regards to ALS where impairments in GLT-1 are well documented (Bendotti, et al., 2001; Rothstein, et al., 1992; Rothstein, et al., 1995; Rothstein, et al., 1996). The protective or harmful nature of the astrocytic response to activation depends on its cause, duration and microenvironment.

Astrocyte activation and glutamate excitotoxicity are well accepted features of ALS. However, the response of mutant SOD1 astrocytes to activation regarding glutamatergic parameters appears to be missing from the literature. In this study, we compare the effect activation exerts upon astrocytes derived from the mutant SOD1 G93A ALS mouse model and their wild-type counterparts. Astrocytes were activated using the bacterial endotoxin lipopolysaccharide (LPS) or a cocktail of growth factors (G5 supplement). As the β-lactam antibiotic ceftriaxone (CEF) has been reported to increase both GLT-1 expression and glutamate uptake as well as improve the overall survival of SOD1 G93A mice (Rothstein, et al., 2005) its effect was also evaluated as a method of specifically activating the astrocytic glutamatergic pathway.

In this study we identified for the first time several key dysfunctions in the SOD1 G93A astrocytic response to activation. Astrocytes derived from the SOD1 G93A ALS mouse model exhibit a reduced glutamatergic and trophic response to activation compared to their wild-type counterparts. Activation of wild-type astrocytes increased the expression of GLT-1 and GLAST the two major astrocytic glutamate transporters, accompanied by a marked

increase in the astrocytic glutamate clearance and up-regulation of neurotrophic factor expression. However, not only do un-treated SOD1 G93A astrocytes take up glutamate less efficiently, but in response to activation they show no further increase in any of the disease related parameters evaluated. Furthermore, activation of wild-type astrocytes, but not SOD1 G93A astrocytes, improved their ability to protect the motor neuron cell line NSC-34 from glutamate induced excitotoxicity. Our data indicates that altered astrocyte activation may well be pivotal to the pathogenesis of ALS.

3. Gene therapy in ALS

3.1 General

Treatment of ALS has been fueled in part by frustration over the shortcomings of the currently available drugs, at most, these drugs provide some symptomatic elevation, but are incapable of slowing down disease progression and neuronal degeneration. Currently, over 150 different potential therapeutic agents or strategies have been tested in transgenic ALS mice according to published reports (Turner, et al., 2008). The frustration with the limited results achieved using classic pharmacological treatment strategies led many researchers to explore alternative non-pharmacological approaches. In recent years, gene therapy has emerged as one of the leading non-conventional therapeutic strategies.

The concept of gene therapy is based on insertion of exogenous genes or DNA fragments into an individual's cells, tissue or system in an attempt to treat a disease.

3.2 Gene therapy approaches

Genetic manipulation can be achieved by employing numerous strategies and using a wide variety of pathways and techniques. The classical methods of gene manipulation are still the main methods being currently used, however, during the past few years many advances

and improvements to the gene delivery mechanisms and to these classical methods have been made.

In the clinical respect genetic manipulation can be divided into two major categories, indirect *ex vivo* gene delivery by transplanting genetically manipulated cells and direct *in vivo* gene delivery by injecting genetically manipulated vectors directly into the target area. Whereas *ex vivo* gene delivery can be mediated using a wide verity of delivery vectors and mechanisms, direct *in vivo* gene delivery generally relies on the use of viral vectors. Several viral vector systems currently exist each with its own unique qualities and advantages. The properties of some of the leading viral vector delivery systems are summarized in Table 1.

Table 1. Summary of viral vector properties.

Vector	Insert (kb)	Tropism	In host localization	Expression duration	Immunogenicity	Efficiency	Advantages	Limitations
Retrovirus	**8**	**Dividing cells**	**Integrated**	**Lifelong**	**Low –can be repeatedly administered**	**High**	**Long term expression**	**Random genomic integration**
Adenovirus	**4-25**	**Dividing and non-dividing**	**Non-integrated**	**Several months**	**High – single administration only**	**High**	**High insert capacity**	**Immunogenicity, transient expression, several infection cycles**
Adeno Associated Virus	**5**	**Dividing and non-dividing**	**Non-integrated**	**Several months**	**Relatively high – limited administration number**	**High**	**Transient expression increases clinical safety, non-inflammatory**	**Transient expression, limited number of administrations**
Lentiviral based vector	**8**	**Dividing and non-dividing**	**Integrated (non-integrated variants developed)**	**Lifelong (non integrated variants-several months)**	**Low – can be repeatedly administered**	**High**	**Long term expression and broad range infectivity single infection cycle**	**Random genomic integration**

In chronic diseases there is a clear advantage to utilizing vectors with either long term expression capacities or sufficiently low immunogenic response that would allow a high

number of repeated administrations. This aspect has higher yet significance when the target cells or tissues for gene delivery are highly proliferative tissues, as in this case the cells are capable of maintaining non integrated foreign DNA for a limited number of cell divisions and thus for a limited amount of time. For example, in the case of astrocytes, particularly diseases such as ALS, which involve high levels of astrocyte proliferation and astrogliosis (Schiffer, et al., 2004), there would be a clear advantage to selecting a lentiviral vector. This vector will allow incorporation of the target DNA into the genome that way inserted genes will not be lost during the massive astrocyte proliferation occurring during the later stages of the disease. Lentiviral vectors will also allow us to deliver the genes of interest multiple times and in multiple locations across years of treatment.

3.3 Lentiviral vectors

Lentiviruses are the most complex viruses in the retroviral family and are particularly suitable for CNS related genetic therapy. Lentiviral vectors have relatively large RNA packing capacity. They can efficiently transfect not only dividing but also non-dividing cells. Lentiviral vectors can reverse transcribe their RNA into the host cell's DNA, thus promoting stable and long term expression of the gene of interest (Alisky, et al., 2000; Cockrell, et al., 2007; Lewis, et al., 1992; Naldini, et al., 1996). Recombinant lentiviral vectors for gene therapy are produced by removal of as much of the viral genes as possible, then the desired expression cassette is inserted. Recombinant lentiviral vectors incapable of producing a functional envelope can be pseudotyped with different viruses to selectively direct the infection to the desired cellular type and prevent secondary infection (Azzouz, 2006; Cronin, et al., 2005). Most recombinant lentiviral vectors used today are based on the human immunodeficiency virus (HIV-1) that has been purged of most of its viral DNA, including the

genes necessary for production of the viral capsid, thus capable of only a single infection cycle.

The principle underlying these vectors is based on the assistance of "helper" cells. The viruses are produced in the "producer/helper" cells, these cells are transfected simultaneously with several plasmids that include the target gene and the trans-acting elements necessary to produce and pseudotype the infectious virus. However the viruses produced only contain the genetic material that has been placed between two packaging signals, that is, only the gene of interest, thus producing recombinant lentiviral vectors with a single infection cycle (Wiznerowicz, et al., 2005).

Recombinant lentiviral vectors are RNA based and consist two identical copies of single stranded positive sense RNAs. The helper plasmids express only 3 HIV-1 genes, gag, pol and rev. The envelope gene is derived from a different virus and selected to fit the desired target cell type. The VSV-G gene from Vesicular Stomatitis Virus is a common envelop selection (Trono, 2000). The gag gene encodes the capsid's core protein (p24) containing the matrix and nucleocapsid elements that are cleavage products of the gag precursor protein. The pol gene codes for the viral protease, reverse transcriptase and integrase enzymes derived from gag-pol precursor gene. The rev gene encodes a post-transcriptional regulator necessary for efficient gag and pol expression. The envelope gene encodes the envelope glycoprotein which mediates viral entry. An important feature of the retroviral genome is the presence of LTRs at each end of the genome. These sequences facilitate the initiation of viral DNA synthesis, moderate integration of the pro-viral DNA into the host genome, and act as promoters in regulation of viral gene transcription. Only the gene of interest, and none of these 4 genes is flanked by the long terminal repeats (LTR) packaging signal thereby maximizing safety.

3.4 In vivo lentiviral manipulation

One of the biggest challenges in the field of gene therapy is successful delivery of the gene of interest into the target tissue in a manner that would provide long term expression with minimal immunological response. In the case of direct *in vivo* gene delivery, lentiviral vectors provide particularly suitable candidates for gene delivery. Over the past decade lentiviral gene delivery has proven itself tried and true for gene delivery in many different animal models. Prolonged transgenes expression has been reported for various cell types including: astrocytes, neurons, hepatocytes, hematopoietic stem cells, retinal cells, dendritic cells and myocytes (Demaison, et al., 2002; Kafri, et al., 1997; Naldini, et al., 1996 ; Pertusa, et al., 2008; Poeschla, et al., 1998 ; Zufferey, et al., 1998).

Genomic integration is considered to slightly enhance the risk factor of using lentiviral vectors. However, despite this apparent added risk, integrating lentiviral vectors were found to have low oncogenic potential, hematopoietic stem cell gene transfer in a tumor-prone mouse model has revealed that prototypical lentiviral vectors have lower oncogenic potential than conventional retroviral vectors (Montini, et al., 2006). Similarly, in a sensitive in vitro immortalization assay to quantify the risk of hematopoietic cell transformation, the insertion pattern of lentiviral vectors was found to be approximately 3-fold lower than that mediated by retroviral vectors to trigger transformation of primary hematopoietic cells (Modlich, et al., 2009).

Furthermore, additional research is being conducted to lower these risks even further by developing non-integrating lentiviral vectors as well as lentiviral vectors with directed site integration.

4. Treatment strategies for ALS

4.1 General

Despite extensive efforts and the many advances that have been made in the field over the past few years there is still only one drug approved by the FDA for treating ALS. Unfortunately, this drug prolongs the survival of patients by only 2-3 months and whether it provides any symptomatic alleviation is still under debate (Bensimon, et al., 1994; Lacomblez, et al., 1996, http://dailymed.nlm.nih.gov/dailymed/lookup.cfm?setid=e49d207d-8e32-4183-94ba-cd264f124090).

Fueled by frustration at the limitations of currently available treatments for ALS, the scientific and medical communities forge onwards in the search for new and improved treatments. To date, numerous therapeutic agents and strategies have been evaluated in ALS mice and in clinical trials, these include; conventional pharmacological therapies, gene and cell therapies, immunizations and dietary or lifestyle regimes (Turner, et al., 2008).

4.2 Pharmacological treatments

The pharmacotherapy spectrum encompasses many approaches such as antioxidants, anti-excitotoxic agents, anti-aggregation compounds, anti-apoptotics, anti-inflammatories and neurotrophic agents. Regrettably, most of the candidates have been reported to have limited therapeutic benefits and at best modest effects on survival of ALS patients.

For example, antioxidant treatments such as vitamin E, N-acetylcysteine, and L-methionine demonstrated to delay the disease onset and progression in an ALS animal model (Pioro, 2000), but show a lack of clinical efficacy in randomized controlled trials in 830 ALS patients (Orrell, et al., 2007). Dexpramipexole is the R+ enantiomer of pramipexole and has

strong antioxidant effects, in part by targeting preservation of mitochondria function by reducing apoptosis (Cheah, et al., 2010) and showed very promising results in phase II clinical trials (Cudkowicz, et al., 2011). Unfortunately, even this treatment which was considered the best antioxidant candidate so far has recently failed in phase III clinical trials (ClinicalTrials.gov Identifier: NCT01281189). Similarly, systemic administration of the antiapoptotic compound, minocycline, a second-generation tetracycline antibiotic, that can delay the symptom onset in ALS animal model (Van Den Bosch, et al., 2002), has been reported to fail in a multicenter, phase III, randomized, controlled trial in more than 400 ALS patients (Gordon, et al., 2007). Similar results were obtained with many other antiapoptotic drugs (Turner, et al., 2008).

Abnormal protein metabolism and protein aggregates are hallmarks of ALS pathology (Kabashi, et al., 2006; Strong, et al., 2005; Ticozzi, et al., 2010). However, there is an ongoing debate as to whether these aggregates play a key role in pathogenesis, are harmless byproducts of the degeneration process, are beneficial (via the sequestration of toxic proteins), or are harmful (via the sequestration of proteins essential to normal cellular function) (Bruijn, et al., 2004; Kabashi, et al., 2006; Redler, et al., 2012; Strong, et al., 2005; Ticozzi, et al., 2010). The effect of treatments targeting aggregates appears to be equally inconclusive (Turner, et al., 2008).

Another common feature of ALS is neuroinflammation which renders it a popular therapeutic target. Many anti-inflammatory and immunosuppressing agents have been studied for their potential therapeutic effect in ALS including; corticosteroids, plasmapheresis, intravenous immunoglobulin, cyclophosphamide, and cyclosporine, unfortunately, all of which failed to alter disease progression (Appel, et al., 1988; Baumann, 1965; Brown, et al., 1986; Meucci, et al., 1996; Monstad, et al., 1979).

Glutamate excitotoxicity is one of the currently most prominent pathophysiological hypothesis explaining ALS progression (Foran, et al., 2009; Shaw, et al., 1997). It is therefore no surprise that many anti-excitotoxic agents have been studied in the context of ALS treatment. Most antiglutamate drugs are antiseizure medications that have antiglutamate properties and include topiramate (has four main properties: inhibition of the enzyme voltage-dependent sodium channels, inhibition of carbonic anhydrase, enhancement of some GABA-A receptors, and antagonism of glutamate receptors (Bialer, 2012; Cudkowicz, et al., 2003; Maragakis, et al., 2003), gabapentin (has a duel mechanism of action by inhibiting voltage gated calcium channels and reducing glutamate synthesis (Kaufmann, et al., 2003; Miller, et al., 2001)) and lamotrigine (a sodium channel blocker that also inhibits the release of glutamate and aspartate (Eisen, et al., 1993; Pagani, et al., 2011; Polymenidou, et al., 2011; Ryberg, et al., 2003)) but none have demonstrated survival benefit. Other drugs with antiglutamate proprieties, including dextromethorphan and memantine, failed to show improvement in survival (de Carvalho, et al., 2010; Gredal, et al., 1997).

Another anti-excitotoxic approach is to increase EAAT2 expression thus improving glutamate uptake and reducing excitotoxicity. Several pharmacological substances, such as the β lactam antibiotic Ceftriaxone and the immunophilin ligand GPI-1046, were recently noted for their EAAT2 upregulating abilities.

Chronic treatment with GPI -1046, was reported to improve the survival of SOD1 G93A mice, but had no effect on disease onset and clinical progression (Ganel, et al., 2006).

In a screening study of 1040 pharmacological substances ceftriaxone was found to increase astrocyte mediated glutamate transport by stimulating the expression ofGLT-1 (Rothstein, et al., 2005). Furthermore, the use of ceftriaxone in the SOD1 G93A mouse model of ALS prolonged

overall survival and upregulatedGLT-1mRNA levels (Rothstein, et al., 2005). More recently, a similar screening study, identified Harmine, a naturally occurring beta-carboline alkaloid, as a very potent activator of the GLT-1 promoter. In vivo evaluation of Harmine yielded similar results to those obtained with ceftriaxone (Li, et al., 2011). In an effort to find compounds that can induce translation of EAAT2 transcripts, high-throughput screening was used to evaluate approximately 140,000 compounds. This study identified 61 compounds with the ability to produce a dose-dependent increase in EAAT2 protein levels (Colton, et al., 2010).

Although Ceftriaxone yielded very encouraging results in ALS mice as well as positive preliminary results in phase II clinical studies (Berry, et al., 2013; Rothstein, et al., 2005) recently failed to show positive comes in phase III (ClinicalTrials.gov identifier NCT00349622).

The crowning glory of anti-excitotoxic treatment for ALS to date is Riluzole.

4.2.1 **Riluzole**

Riluzole is the only drug for the treatment of ALS approved by the US Food and Drug Administration (FDA). Riluzole's mode of action is un-known, but its pharmacological properties indicate the potential involvement of several mechanisms of action (Table 2, Cheah, et al., 2010). It is currently believed that its effect is predominantly due to its anti-excitotoxic properties, which are divided into 3 mechanisms of action. 1) An inhibitory effect on glutamate release, 2) inactivation of voltage-dependent sodium channels, and 3) ability to interfere with intracellular events that follow transmitter binding at excitatory amino acid receptors.

Table 2. Summary of the Known Mechanisms of Action of Riluzole

Mechanism of Action
Inhibiting glutamatergic neurotransmission: • Potential interactions with the NMDA glutamate receptor (pre and post synaptic) • Potential interactions with AMPA/Kainate receptors • Enhance glutamate uptake from the synaptic cleft • Inhibit glutamate and aspartate release
Ca^{+2} channel blockade
Na^{+} channel blockade: • Site of action: α sub-unit • Antagonist of persistent Na^{+} current • Blockade of transient Na^{+} channel
GABAergic mechanisms: • Reduced uptake of GABA from neuronal synapse • Potentiation of $GABA_A$ receptor affinity for GABA • Demonstration of general anesthetic properties at high doses • Restoration of cortical inhibitory patterns in ALS patients
Miscellaneous actions: • Noncompetitive antagonism of protein kinase C • Inhibition of pertussis toxin-sensitive and cholera toxin-sensitive G- proteins • Antagonism of neuronal nitric oxide synthase

Regrettably, Riluzole has very limited therapeutic benefits it prolongs the survival of patients by only 2-3 months and whether it provides any symptomatic alleviation is still under debate (Bensimon, et al., 1994; Lacomblez, et al., 1996, http://dailymed.nlm.nih.gov/dailymed/lookup.cfm?setid=e49d207d-8e32-4183-94ba-cd264f124090).

Much like the situation in the clinic, when evaluated in animal models Riluzole yielded very limited beneficial outcomes. Depending on the experimental design treatment with Riluzole increased the survival of mice by 9.4-10.7% of the overall lifespan which correlates

with 17-30% increased survival from the onset of symptoms. Motor function was preserved for 7.5-13% of overall life span that is 22-24% from the onset of symptoms. Treatment with Riluzole also had a mild beneficial effect on preserving body weight, presenting with delayed weight loss of 5.5% of overall lifespan and 17% from symptom onset. When administered prior to symptom onset, Riluzole did not delay the onset of symptoms in any of the experimental designs (Del Signore, et al., 2009; Gurney, et al., 1996; Gurney, et al., 1998; Waibel, et al., 2004, Table 3).

Table 3. Summary of the effect of Riluzole in animal models.

	Percent of beneficial effect from overall lifespan	**Percent of beneficial effect from symptom onset**
Survival	9.4-10.7	17-30
Symptom onset	No effect	No effect
Motor function	7.5-13	22-24
Body weight	5.5	17

4.3 Genetic and cellular therapeutic approaches in ALS patients and animal models

Coming to terms with the potential limitations of the traditional pharmaco-therapeutic approaches researchers turned to alternative therapeutic strategies, gene and cell therapy are two of the leading alternatives investigated. Over the past few years, numerous experiments were

performed taking advantage of the advances in these fields. Some of these experiments performed in animal models or adapted to clinical trials are summarized in

Figure *3* and Table 4. This field can be rudimentarily divided into three categories; 1) Cell replacement strategies in which either the effected neurons or cells of their immediate environment are attempted to be replaced with supposedly health cells. 2) Transplantation of non-endogenous cells or genetically enhanced cells that are believed to exert a therapeutic effect. 3) Direct gene manipulation in the effected individual, this consists of introduction, enhanced expression or silencing of genes in the affected areas.

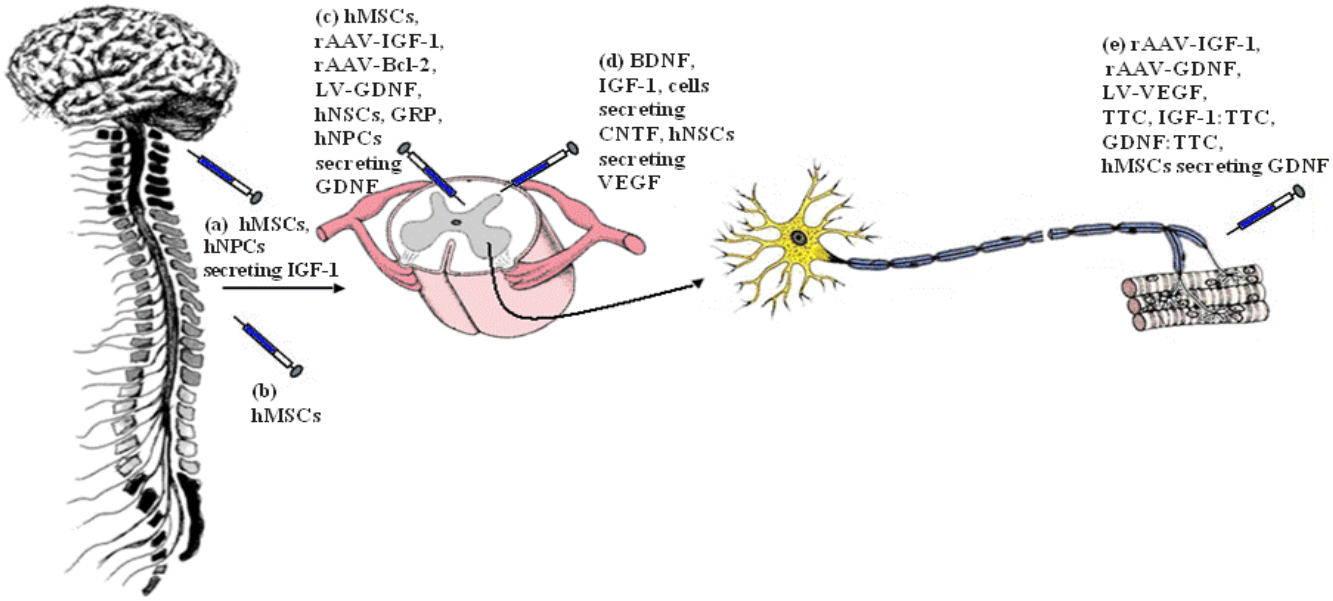

Figure 3. A schematic representation of methods allowing local delivery of neurotrophic factors and cellular transplantations performed in ALS animal models and patients. *(a) Represents the cisterna magna. (b) Refers to the CSF. (c) Concerns intraspinal treatment. (d) Indicates the spinal intrathecal space. (e) Pertains to the intramuscular space. Abbreviations: Human mesenchymal stem cells (hMSCs), human neuronal progenitor cells (hNPCs), recombinant Adeno-associated virus (rAAV), lentivirus (LV), human neuronal stem cells (hNSCs), glial restricted precursors (GRP), tetanus toxin heavy chain (TTC), fusion protein of IGF-1 and TTC (IGF-1:TTC), fusion protein of GDNF and TTC (GDNF:TTC).(Benkler et al .2010)*

4.3.1 **Motor neuron replacement therapies**

Motor neurons can be effectively derived from embryonic stem cells (ESCs), neuronal stem cells (NSCs) and induced pluripotent stem (iPS) cells (Dimos, et al., 2008; Harper, et al., 2004; Lai, et al., 1997). The strategy for treatment is to use these cells to replace damaged neurons in ALS disease. In several studies of neuronal transplantations, performed in mice and rat models of ALS, a delay in disease onset and a mild prolongation of life expectancy have been observed (Garbuzova-Davis, et al., 2002; Lai, et al., 1997; Willing, et al., 2001).

It has been further shown that human NSCs or human embryonic stem cell-derived motor neuron progenitors (hMNPs) grafted into the lumbar spinal cord of SOD1 G93A animals, survive after transplantation and they present with predominantly neuronal differentiation at the transplantation site. The grafts secrete functional levels of neurotrophic factors, while forming functional synapses with host motor and non-motor neurons. However, the differentiated neurons do not develop into large motor neurons, but are predisposed to a GABAergic or young neuronal phenotype, implying that the therapeutic effect observed was quite possibly attributed to factors other than motor neuron replacement. A mild delay in disease onset and prolongation of life expectancy has been observed (Xu, et al., 2006; Xu, et al., 2009).

The limited in vivo axonal growth rate of grafted motoneurons derived from ESC (Harper, et al., 2004) hinders their viability as candidates for replacement therapy in neurodegenerative diseases such as ALS. The observed therapeutic effects of neuronal stem cells in these experiments could be attributed to their neuron to neuron trophic functions, such as buffering glutamate levels and neurotrophic factor delivery (Sharma, 2007; Woo, et al., 2007; Xu, et al., 2006). In this case, whether motor neurons are best suited to provide such effects, or whether other cell types, such as astrocytes or microglia might provide a more potent tool, must be discussed.

4.3.2 **Astroglial replacement therapies**

Astrocytic abnormalities participate in the degeneration occurring in ALS (Luo, et al., 2007; Nagai, et al., 2007 ; Pramatarova, et al., 2001 ; Yamanaka, et al., 2008). Furthermore, astrocytes possess potent neuroprotective potential (Maragakis, et al., 2006). Taken together, these attributes render astrocytes interesting targets in ALS related therapeutic strategies.

Transplantation of healthy rat and mice glial restricted precursors (GRPs) around cervical spinal cord respiratory motor neuron pools of SOD1 G93A rats, extended survival and disease duration, attenuated motor neuron loss and moderated the decline in forelimb motor and respiratory functions. These effects correlated with reduced microgliosis at the transplantation site and were severely hindered when there was an absence of GLT1, the glutamate transporter, in the cells grafted (Lepore, et al., 2008; Maragakis, et al., 2005). These results suggest some potential for glial replacement therapies that should be further investigated.

4.3.3 **Microglia replacement therapies**

Neurons and astrocytes are not the only cells suspected as impaired in ALS. Microglia specifically, and the immune system in general have been implicated in disease pathophysiology (Demestre, et al., 2005; Engelhardt, et al., 2005; Hall, et al., 1998; Henkel, et al., 2009; Sargsyan, et al., 2005). $PU.1^{-/-}$ mice are impaired in their ability to develop myeloid and lymphoid cells and therefore lack CNS microglia. These mice were crossbred with SOD1 G93A mice and subsequently transplanted with donor wild type bone marrow cells. Donor cells differentiated into microglia and integrated into the CNS. Grafted mice benefited from reduced motoneuron degeneration, ameliorated disease progression and prolonged survival compared to mice transplanted with SOD1 G93A bone marrow cells and SOD1 G93A non-crossbred and non-transplanted mice (Beers, et al., 2006). Similar results

were obtained when SOD1 G93A mice were irradiated and then intra-bone marrow grafted with wild type bone marrow cells (Ohnishi, et al., 2009) and when SOD1 mice retro-ocularly received human umbilical cord blood cells (Ende, et al., 2000). These findings prompted a clinical trial where 6 sporadic ALS patients underwent full body irradiation and were then infused with a siblings' hematopoietic stem cells (HSCs). Unfortunately, clinical benefits were not evident. Nevertheless, the experiment was not completely unsuccessful: grafted cells were found in post mortem autopsy brain and spinal cord samples. Biases in HSCs engraftment were observed, indicating that the cells gravitate towards areas of motoneuron injury and neuroinflammation (Appel, et al., 2008).

It is possible that with alternative grafting techniques or cellular genetic engineering microglial cells therapeutic potential might be enhanced.

4.3.4 **Human mesenchymal stem cells**

Another potential cellular source for motor neuron protection is bone marrow derived mesenchymal stem cells (MSCs). MSCs are self-renewing precursors that can be differentiated *in vitro* to give rise to cells from multiple lineages, including but not limited to neuron, oligodendrocyte and astrocyte like cells (Bahat-Stroomza, et al., 2009; Sanchez-Ramos, et al., 2000; Suzuki, et al., 2004). The use of MSCs as the cellular source for stem cell based treatments not only circumvents ethical concerns but they are also candidates for autologous transplantation.

Several studies have shown that human MSCs (hMSCs), survive after transplantation into the lumbar spinal cord, the cisterna magna and the CSF for long periods of time, show limited migration into and within the spinal cord, extend survival, reduce neuroinflammation; astrogliosis and microgliosis, perform moderate differentiation into neurons and astrocytes

and ameliorate disease symptoms in mice and rat SOD1 G93A models (Boucherie, et al., 2009; Kim, et al., 2010; Vercelli, et al., 2008).

A different approach utilizes irradiation in order to improve hMSC engraftment into the brain and muscles (Francois, et al., 2006). Intravenous administration of hMSC in irradiated pre-symptomatic SOD1-G93A mice has yielded similar results to those previously mentioned (Zhao, et al., 2007).

Mazzini L, et al. performed two clinical trials (Appel, et al., 2008; Mazzini, et al., 2008)in which MSC were autologously transplanted intra-parenchymally at the thoracic level of the spinal cord of ALS patients. A total of 19 patients have been grafted in both experiments. Patients were monitored for up to 4 years and no significant acute or late side effects were evident. In most cases, no modifications of spinal cord volume or other signs of spinal or brain abnormal cell proliferation, were observed. However, in 4 of the cases the spinal cord was tethered anteriorly and posteriorly by postoperative scarring, producing marked distortion of the cord due to traction. In the first trial, the linear decline of the forced vital capacity was significantly moderated in 4 of the 9 transplanted patients. However, in the second trial, slower deterioration was observed in only 2 of the 10 grafted patients. The results suggest that hMSC might be suggested as candidates for stem cell based treatment of ALS. Moreover, intraspinal injection of MSCs showed no long term adverse outcome. Additional clinical trials have recently been launched.

Several mechanisms have been proposed to explain the neuroprotective effect observed in transplantations of native and differentiated MSC including cell replacement, immunomodulation and trophic factor delivery. hMSC can be further utilized as a platform for delivering neurotrophic factors to target areas within the brain.

4.3.5 **Genetically altered cells**

Using gene therapy technology to enhance the neuroprotective performance of stem cells prior to their transplantation might offer a potential therapeutic tool.

Neuronal progenitor cells (NPCs), NSCs and hMSCs are only a small portion of the cellular sources that can be utilized for this purpose (de Hemptinne, et al., 2004 ; Hwang, et al., 2009; Klein, et al., 2005; Park, et al., 2009; Pineda, et al., 2007; Suzuki, et al., 2007; Suzuki, et al., 2008). A vast variety of genes could also be considered as genetic targets for cellular enhancement, the most prominent of which are neurotrophic factors and glutamate transporters.

Delivery of appropriate neurotrophic factors to the immediate environment of afflicted neurons in ALS might improve their survival and thus ameliorate disease progression and symptoms. In ALS animal models depending on the site and rout of delivery, BDNF, GDNF, VEGF, CNTF or IGF-1 had varying abilities to delay disease onset, delayed disease progression and improved the overall life span (Aebischer, et al., 1996a; Aebischer, et al., 1996b; Bogaert, et al., 2009; Borasio, et al., 1998; Cunningham, et al., 2002; Hwang, et al., 2009; Kliem, et al., 2011; Lai, et al., 1997; Lambrechts, et al., 2006; Nagano, et al., 2005, Park, et al., 2009; Patel, et al., 2005; Pineda, et al., 2007; Rizvanov, et al., 2008; Slevin, et al., 2007; Sorenson, et al., 2008; Suzuki, et al., 2008; Tolosa, et al., 2011; Vergani, et al., 1997; Vergani, et al., 1999, Wang, et al., 2007; Zheng, et al., 2004; Zurn, et al., 2000). Despite the promising results in animal models clinical trials encountered numerous problems and adverse events and yielded very limited results (ACTS, 1996; Appel, et al., 2008; Aebischer, et al., 1996a; Aebischer, et al., 1996b; Beck, et al., 2005; Borasio, et al., 1998; Lopez-Gonzalez, et al., 2009; Mazzini, et al., 2008; Mazzini, et al., 2009, Miller, et al., 1996a; Miller, et al., 1996b; Nagano, et al., 2005; Ochs, et al., 2000; The BDNF Study, 1999; Zurn, et al., 2000).

A different approach to genetically engineered cells considers increasing glutamate removal from the neuronal environment rather than infusion of neurotrophic factors. Crossbreeding of SOD1 G93A mice with astrocyte overexpressing human EAAT2 mice, improved motor performance, protected motoneurons from glutamate induced excitotoxicity but did not delay the onset of paralysis nor did it prolong life span (Guo, et al., 2003).

4.3.6 **Direct gene manipulation**

Strategies of direct gene manipulation have been discussed at length in a previous section. Recombinant adeno-associated viruses were used as a vector to deliver several neurotrophic factors to various locations in animal models of ALS. This treatment delayed disease onset, prolonged life and moderated disease progression (Dodge, et al., 2010; Franz, et al., 2009; Kaspar, et al., 2003; Lepore, et al., 2007; Wang, et al., 2002). Neurotrophic factors are not the only genes that can be delivered using viral vectors. Bcl-2 an anti-apoptotic protein has previously been delivered to the lumbar spinal cord of SOD1 G93A mice using a recombinant adeno-associated viral construct, this to delayed disease onset and significantly increased the number of surviving motoneurons at disease end-stage, but was ultimately unable to prolong survival (Azzouz, et al., 2000). This represents an interesting alternative for gene selection in recombinant adeno-associated virus based gene therapy in ALS. However, when considering this approach we must be extremely vigilant regarding the transfected cells, as the effect of Bcl-2 might be a double edged sword, protective in non-dividing, degeneration prone motoneurons, yet potentially oncogenic in other cells.

Lumbar spinal cord and facial nucleus injections of recombinant lentiviral vectors incorporating the GDNF gene were found to significantly rescue motoneurons in the facial nucleus, but did not prevent spinal motoneuron loss, despite robust intraspinal GDNF

expression in SOD1 G93A mice and facial motor neurons axotomy mice models (Guillot, et al., 2004; Hottinger, et al., 2000).

Injection of a recombinant rabies-G pseudotyped lentiviral vector based on the equine infectious anemia virus encoding the human vascular endothelial growth factor (hVEGF) gene at several muscle terminals in SOD1 G93A mice, promoted hVEGF expression in spinal cord motoneurons, delayed disease onset, slowed disease progression and prolonged survival (Azzouz, et al., 2004).

4.3.7 **Gene silencing**

Gene therapy technologies can be used not only for delivery of potential therapeutic genes but could further be used for silencing of potentially pathogenic genes (Figure 4). RNA interference (RNAi) is a process by which non coding RNA mediates sequence specific gene regulation and silencing. Several forms of fALS are caused by dominant, gain of function genetic mutations. In these cases silencing the mutant gene might reduces its toxic effect and thus delay the disease progression. A potent proof of concept emerged from the development of transgenic mice harboring an anti-SOD1 small interference RNA gene, cross breeding of these mice with SOD1 G93A mice prevented the clinical development of the disease (Saito, et al., 2005). Several studies further demonstrated the ability to suppress mutant SOD1 products and in most cases delay clinical disease progression in rodent models of ALS. There are several available methods of RNAi delivery, injecting RNAi carrying recombinant adenoviruses directly into large nerve trunks such as the sciatic nerve exposes the viruses to a large number of axons thus reaching a large population of motor neurons. Wu et al. (2009) found that such injections extended the life span of SOD1 G93A mice. Intraspinal injections of a lentiviral vector that results in specific RNAi mediated silencing of SOD1 substantially delayed disease onset and progression rate in SOD1 G93A mice (Raoul, et al., 2005).

Similarly, intramuscular injections of lentiviral vectors carrying RNAi molecules specifically targeting the SOD1 gene preserved motor function, considerably delayed disease onset and significantly prolonged the survival of SOD1 G93A mice (Ralph, et al., 2005). Other molecular pathogenic targets, which could also apply to the sporadic patients, have also yielded positive results. Silencing the death signaling p75 neurotrophin receptor through systemic intraperitoneal administration as well as intrathecal administration of anti-Fas RNAi significantly delayed motor injury and prolonged the survival of SOD1 G93A mice (Locatelli, et al., 2007; Turner, et al., 2003). To realize the full therapeutic potential of siRNA for neurodegenerative diseases, researchers need to overcome many hurdles and challenges such as selecting suitable tissue-specific delivery vectors, minimizing the off-target effects and reducing side effects. Furthermore, with RNAi therapies safety and efficiency must be demonstrated for every desired mode of delivery individually.

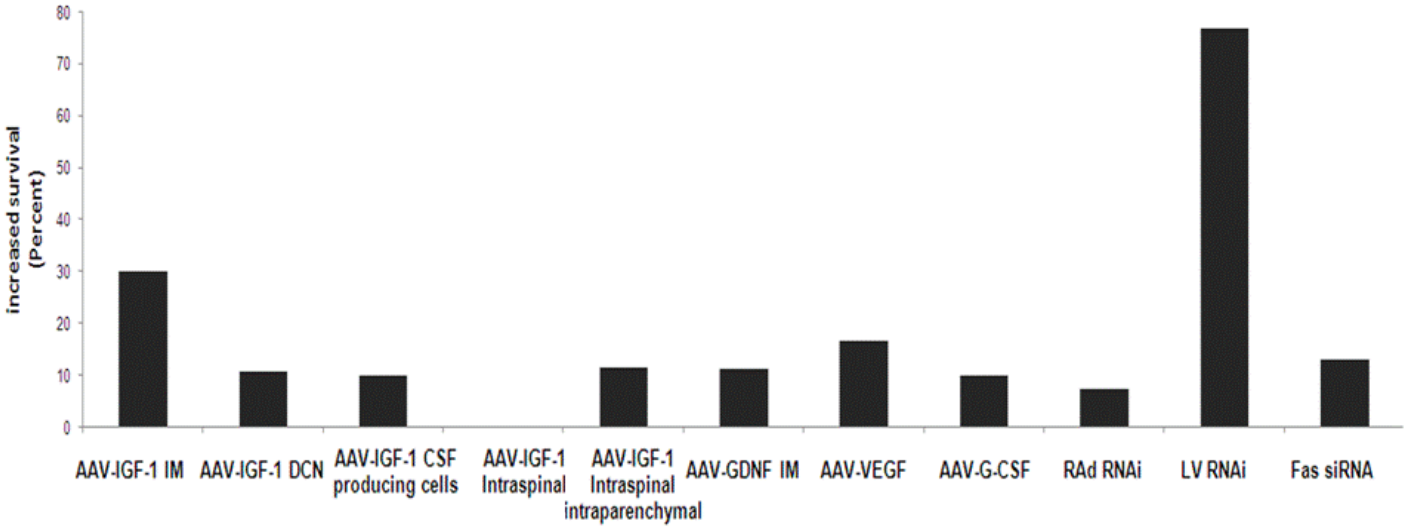

Figure 4. Survival effect of gene therapy in SOD1 transgenic mice. *Graph demonstrates the effect on lifespan of several gene therapy techniques. AAV-IGF-1 IM (intramuscular,(Kaspar, et al., 2003)), AAV-IGF-1 DCN (deep cerebellar nuclei, (Dodge, et al., 2008)), AAV-IGF-1 CSF producing cells (Dodge, et al., 2010), AAV-IGF-1 Intra spinal (Franz, et al., 2009), AAV-IGF-1 Intraspinal intraparenchymal (in males only, (Lepore, et al., 2007)), AAV-GDNF IM (Hall, et al., 1998; Xu, et al., 2006) , AAV-VEGF (CSF producing cells, (Dodge, et al., 2010), LV-VEGF (IM, (Azzouz, et al., 2004)), AAV-G-CSF(Henriques, et al., 2011), recombinant adenovirus (RAd) RNAi (Wu, et al., 2009), LV RNAi (Ralph, et al., 2005), Fas siRNA (Locatelli, et al., 2007).*

Table 4. An overview of several genetic and cellular therapeutic approaches relating to ALS patients and animal models

Method of treatment	Treated group	Method of delivery	Effect	Reference
Recombinant methionyl BDNF	Clinical trials ALS patients.	Intrathecal administration	No clinical or survival benefits.	(Beck, et al., 2005; Ochs, et al., 2000; The BDNF Study, 1999)
Recombinant IGF-1	Clinical trials ALS patients.	Subcutaneous administration	American trial: slower progression of functional impairment. European trial: no clinical benefits.	(Borasio, et al., 1998, Lopez-Gonzalez, et al., 2009)
Recombinant IGF-1	Clinical trials ALS patients.	Intrathecal infusion	Modest beneficial effects.	(Nagano, et al., 2001)
Recombinant human CNTF	Clinical trials ALS patients.	Subcutaneous injections	No beneficial clinical results, high dose CNTF administration was accompanied by severe adverse reactions and an increased number of deaths.	(ACTS, 1996; Miller, et al., 1996a; Miller, et al., 1996b)
Human MSCs	Clinical trials ALS patients.	Intraparenchymal autologous transplantation at the thoracic level of the spinal cord	Contradicting results of ether slight clinical improvement or no clinical benefits.	(Mazzini, et al., 2008; Mazzini, et al., 2009)
Human HSCs	Clinical trials sporadic ALS patients	Post-irradiation transplantation with a sibling's HSCs	No clinical benefits.	(Appel, et al., 2008)
Baby hamster kidney cells overexpressing CNTF	Clinical trials ALS patients.	Intrathecal transplantation of membrane encapsulated cells	Clinical benefits have not been reported.	(Aebischer, et al., 1996a; Aebischer, et al., 1996b; Zurn, et al., 2000)
rAAV-IGF-1	SOD1 G93A mice	rAAV-IGF-1 at respiratory and motor limb muscles or deep cerebellar nuclei	Delayed disease onset, prolonged life and moderated disease progression.	(Kaspar, et al., 2003; Dodge, et al., 2008)
rAAV-IGF-1	SOD1 G93A mice	rAAV-IGF-1 Spinal cord infection	Delayed disease onset, weight loss, decline in hindlimb grip strength and increased survival was observed in male but not female mice.	(Franz, et al., 2009; Lepore, et al., 2007)
rAAV-IGF-1 or rAAV-VEGF	SOD1 G93A mice	rAAV-IGF-1 or rAAV-VEGF intracerebroventricular (ICV) administration	Delayed motor decline and moderately extended the survival. Combined IGF-1 and VEGF administration yielded no add neuroprotection	(Dodge, et al., 2010)
rAAV-GDNF	SOD1 G93A mice	rAAV-GDNF at respiratory and motor limb muscles	Delayed disease onset, prolonged life and moderated disease progression.	(Wang, et al., 2002)
rAAV-GDNF	SOD1 G93A mice	rAAV-GDNF intramuscularly injecting	Delayed disease onset, amelioration of disease progression and prolongation of life expectancy.	(Wang, et al., 2002)
rAAV-G-CSF	SOD1 G93A	rAAV-G-CSF	Protected motor neurons,	(Henriques, et

or direct G-CSF administration	mice	intramuscular injection or subcutaneous G-CSF administration	ameliorated functional outcome and prolonged life.	al., 2011; Pitzer, et al., 2008)
rAAV-G-CSF	SOD1 G93A mice	rAAV-G-CSF intraspinal administration	Improved motor function, delayed disease progression and increased survival by 10% more than when G-CSF was subcutaneously administrated or rave-G-CSF was intramuscularly injected	(Henriques, et al., 2011)
rAAV-Bcl-2	SOD1 G93A mice	Lumbar spinal cord rAAV-Bcl-2 infection	Delayed disease onset and preservation of spinal motoneurons.	(Azzouz, et al., 2000)
LV-GDNF	SOD1 G93A mice and facial motor neuron axotomy model.	LV-GDNF lumbar spinal cord and facial nucleus injections	Rescue of motoneurons in the facial nucleus. No effect on spinal motoneurons.	(Guillot, et al., 2004; Hottinger, et al., 2000)
LV-VEGF	SOD1 G93A mice	LV-VEGF infection at muscle terminals	Delayed disease onset, slower disease progression and prolonged survival.	(Azzouz, et al., 2004)
Human NSCs	SOD1 G93A rats	Lumbar spinal cord transplantation	Delayed disease onset and prolongation of life expectancy.	(Xu, et al., 2006; Xu, et al., 2009)
Glial restricted precursors	SOD1 G93A rats	GRP transplantation at cervical spinal cord respiratory motor neuron pools	Extended survival and disease duration, attenuated motor neuron loss and moderated decline in forelimb motor and respiratory functions.	(Lepore, et al., 2008; Maragakis, et al., 2005)
Human MSCs	SOD1 G93A mice and rats	Lumbar spinal cord, cisterna magna and CSF transplantations	Extended survival, reduced neuroinflammation; astrogliosis and microgliosis and ameliorated disease symptoms.	(Boucherie, et al., 2009; Kim, et al., 2010; Vercelli, et al., 2008)
Human MSCs	SOD1 G93A mice	Post irradiation intravenous administration	Delayed disease onset, delayed disease progression and increased lifespan.	(Zhao, et al., 2007)
Lin-c-kit positive bone marrow cells	SOD1 G93A mice	Systemic intravascular injection	Delayed motor degeneration, reduced microgliosis delayed onset of the early symptomatic disease stage, slowed disease progression and extended the survival of SOD1-G93A mice by 16–17 days.	(Corti, et al., 2010)
Bone marrow cells	A cross-breed of PU.1-/- mice and SOD1 G93A mice	Bone marrow transplantation	Reduced motoneuron degeneration, ameliorated disease progression and prolonged survival.	(Beers, et al., 2006)
Bone marrow cells	SOD1 G93A mice	Post-irradiation intra-bone marrow grafts	Slowed disease progression and extended survival.	(Klein, et al., 2005)
Human umbilical cord	SOD1 G93A mice	Retro-ocular transplantation	Delayed disease onset and extended survival.	(Ende, et al., 2000)

blood cells				
Human umbilical cord blood cells	SOD1 G93A mice	Intraspinal administration	Transplantation before symptom onset (day 40) slightly enhanced survival and preserved motor function in a gender bias manor. transplantation after symptom onset (day 90) had no such effect	(Knippenberg, et al., 2011)
Human cord blood mononuclear cells	SOD1 G93A mice	Intracerebroventricular (ICV) administration	Significantly ameliorated symptoms progression and motor decline as well as significantly prolonged survival	(Bigini, et al., 2011)
Human NPCs secreting GDNF	SOD1 G93A mice and rats	Spinal cord grafts	Delayed disease onset but did not extend survival.	(Klein, et al., 2005; Park, et al., 2009; Suzuki, et al., 2007)
Human MSCs secreting GDNF	SOD1 G93A rats	Muscle transplantations	Delayed disease progression and extended overall life span.	(Suzuki, et al., 2008)
Human NSCs overexpressing VEGF	SOD1 G93A mice	Intrathecal transplantation into the lumbar spinal cord	Delayed disease onset, slower decline of motor function and prolonged survival.	(Hwang, et al., 2009)
Human NPCs secreting IGF-1	SOD1 G93A mice	Intrathecal transplantation into the cisterna magna	Enhanced motor neuron survival, but with no functional improvement or extension of life span.	(Park, et al., 2009)
Recombinant adenoviruses with anti SOD1 RNAi properties	SOD1 G93A mice	Direct nerve injection	Extended the life span of SOD1 G93A mice.	(Wu, et al., 2009)
LV with anti SOD1 RNAi properties	SOD1 G93A mice	Intraspinal injections	Delayed disease onset and progression rate.	(Raoul, et al., 2005)
LV carrying RNAi molecules specifically targeting the SOD1	SOD1 G93A mice	Intramuscular injections	Preserved motor function, delayed disease onset and prolonged survival.	(Ralph, et al., 2005)
Antisense peptide nucleic acid targeting p75	SOD1 G93A mice	Systemic intraperitoneal delivery	Delayed motor decline and prolonged survival	(Turner, et al., 2003)
Anti-Fas RNAi	SOD1 G93A mice	Intrathecal administration	Delayed motor injury and extension of life span	(Locatelli, et al., 2007)

Abbreviations: Mesenchymal stem cells (MSCs), neuronal progenitor cells (NPCs), neuronal stem cells (NSCs), glial restricted precursors (GRP), recombinant Adeno-associated virus (rAAV), lentivirus (LV), tetanus toxin heavy chain (TTC), fusion protein of IGF-1 and TTC (IGF-1:TTC), fusion protein of GDNF and TTC (GDNF:TTC)(Benkler et al. 2010).

5. A novel therapeutic approach for treating ALS

In the previous section we described at length the available treatments for ALS as well as the most common therapeutic strategies. As we can now clearly deduce, currently available therapies for ALS leave much to be desired, as they provide at best only symptomatic alleviation, which is often accompanied by severe side effects. Far worse is the fact that it appears that none of these treatments effect the degenerative progression, imploring us to explore new alternatives.

In this study we identified for the first time several key dysfunctions in the SOD1 G93A astrocytic response to activation. These dysfunctions may well be pivotal to the pathogenesis of ALS. Based on these newly discovered astrocytic dysfunctions we devised an alternative therapeutic strategy.

These dysfunctions we identified include reduced capacities to increase neurotrophic factor mRNA synthesis, lack of increase in glutamate uptake in response to activation and significantly reduced neuroprotective capacities as well as lack of increase in the neuroprotective potential in response to activation. We proposed that the reduced glutamatergic and trophic response of astrocytes to activation in ALS, may, over time, lead to disruption of glutamate homeostasis and accumulative CNS damage, thus facilitating motor neuron degeneration.

The molecular pathogenesis of ALS is poorly understood, contributing to the lack of effective system-based therapies to treat this disease. Investigations have inferred that ALS is a multifactorial and multisystemic disease that arises from a combination of several

mechanisms, which act together in tandem through concurring damage inside motor neurons and their neighboring non-motor cells.

Accepting the multifactorial and multisystemic nature of ALS was a crucial step in devising our therapeutic approach. We believed that in order to effectively treat a multifactorial disease, we also needed a multifactorial treatment, one that would address several different pathways involved in the disease progression. For our treatment, we selected 3 key pathways believed to be crucial elements in the pathophysiology of ALS. These 3 elements of our treatment are to work separately as well as synergistically and influence a broad range of deleterious effects involved in the motor neuron degeneration pathway thus exerting their therapeutic effect.

The three key pathways we selected were; glutamate uptake, glutamate metabolism and oxidative stress which together address the band and width of the excito-oxidative axis. For each pathway we selected a single gene located high at the top of its pathway, in a way that would allow us to influence the entire pathway by addressing that particular gene. EAAT2 was assigned to the glutamate uptake pathway, overexpression of EAAT2 would reduce the amount and duration of synaptic glutamate thus reducing excitotoxicity. GDH2 was assigned to the glutamate metabolism pathway, overexpression of GDH2 would reduce the systemic glutamate bioavailability also reducing excitotoxicity. The secondary damage of glutamate excitotoxicity is mediated by oxidative stress. The oxidative stress pathway was allocated the NRF2 gene, a key regulator of the entire cellular anti-oxidant and anti-inflammatory pathways.

5.1 Excitatory Amino Acid Transporter 2

The importance of glutamate, the destructive potential of glutamate excitotoxicity and the importance of maintaining low levels of synaptic glutamate have been described in detail in section 1.3.1 glutamate excitotoxicity.

The task of ensuring timely and effective removal of synaptic glutamate falls to the major astrocytic glutamate uptake transporters; the excitatory amino acid transporter 2 (EAAT2) and its murine counterpart GLT-1 (glutamate transporter 1) and the less widespread as well as less active excitatory amino acid transporter 1 (EAAT1, and its murine counterpart GLAST glutamate aspartate transporter).

There were many reasons that influenced our decision to select EAAT2 as our glutamate uptake pathway regulator. EAAT2 is one of the most potent glutamate transporters, it has exceptionally high affinity to glutamate and can take up extracellular glutamate even at very low extracellular concentrations and high intracellular concentrations. EAAT2 is believed to be a more efficient transporter than EAAT1 due to lower glutamate independent leak as well as higher transport probability. Under natural conditions, most of the glutamate uptake is performed by the EAAT2 channels (Allen, et al., 2004; Anderson, et al., 2000 ; Attwell, 2000; Danbolt, 2001; Lee, et al., 2004; Levy, et al., 1998; Meldrum, 2000; Sheldon, et al., 2007 ; Velasco, et al., 2003 ; Zerangue, et al., 1996). Furthermore, the involvement of EAAT2 in ALS unlike other glutamate transporters has been well established. Selective loss of EAAT2 was reported in sporadic and familial ALS patients as well as in mutant SOD1 animal models (Bendotti, et al., 2001; Rothstein, et al., 1992; Rothstein, et al., 1995). Both *in vitro* and *in vivo* experiments demonstrate that selective loss of GLT-1 can lead to motor neuron degeneration (Rothstein, et al., 1996). Moreover, pharmacologically induced upregulation of EAAT2 was shown to improve the overall status of ALS modeling mice (Rothstein, et al., 2005).

5.2 Glutamate Dehydrogenase 2

The complementary function to reducing synaptic glutamate availability is reducing the systemic bioavailability of glutamate. Following uptake into astrocytes glutamate can be metabolized by three different enzymes; glutamine synthetase and glutamate dehydrogenase 1 and 2. Glutamine synthetase metabolized glutamate into glutamine, which is then exported from the astrocyte to the synaptic cleft where it is taken up by neurons, metabolized to glutamate that can then be re-used in synaptic transmission (Eid, et al., 2004; Palmada, et al., 1998; Stanimirovic, et al., 1999).

Glutamate dehydrogenase 1 and 2 (GDH1, GDH2) convert glutamate into α-ketoglutarate through a process of oxidative deamination. (Mastorodemos, et al., 2005; Palmada, et al., 1998; Plaitakis, et al., 2001; Spanaki, et al., 2010). Unlike glutamine synthetase, glutamate dehydrogenase enzymes metabolize glutamate into a substrate that is not then re-metabolized into glutamate thus reducing the availability of glutamate. Furthermore, glutamate dehydrogenase enzymes metabolize glutamate into a substrate that is then further metabolized in the energy production processes into lactate that is then transported into neurons and fuels the neuronal energy production process. This provides glutamate dehydrogenase enzymes with a dual function; reducing glutamate bioavailability while improving the astrocytic and neuronal energetic state (Mastorodemos, et al., 2005; Palmada, et al., 1998; Plaitakis, et al., 2001; Spanaki, et al., 2010). Both of these functions were extremely desirable in our therapeutic strategy. We chose to use GDH2 rather than GDH1 due to two major properties unique to GDH2. 1) While GDH1 is a housekeeping gene, GDH2 is specific to the brain and testicular tissues and is highly expressed in astrocytes rendering it more compatible with high astrocytic overexpression. 2) GDH2 is not hindered by the presence of GTP and therefore can metabolize glutamate even when the cellular energy levels are high and when glutamate levels are relatively low, this function

also allows GDH2 to export higher levels of lactate in neurons and provide better metabolic and energetic support (Mastorodemos, et al., 2005; Palmada, et al., 1998; Plaitakis, et al., 2001; Spanaki, et al., 2010).

5.3 Nuclear factor (erythroid-derived 2) related factor 2

Glutamate excitotoxicity causes a cascade of effects harmful to motor neurons. One of the most potent players in this cascade is oxidative stress (Kruman, et al., 1999; Lynch, et al., 2002; Rival, et al., 2004; Shaw, et al., 2000; Van Damme, et al., 2005; Van Den Bosch, et al., 2008)

Nuclear factor (erythroid-derived 2) related factor 2 (NRF2) is a transcription factor which activates genes containing the antioxidant response element (ARE), thus constituting a major node in the cellular anti-oxidative response (Hybertson, et al., 2011; Vargas, et al., 2009).

NRF2-ARE binding regulates the expression of more than 200 genes involved in the cellular antioxidant and anti-inflammatory defense such as phase 2 detoxification enzymes (NAD(P)H quinoneoxyreductase, glutathione), enzymes which are necessary for glutathione biosynthesis, extracellular superoxide dismutase, glutamate-6-phosphate-dehydrogenase, heat shock proteins and ferritin, as well as pro and anti inflammatory enzymes such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and heme oxygenase-1 (HO-1) (Kotlo, et al., 2003; Shih, et al., 2003; van Muiswinkel, et al., 2005). NRF2 has also been reported to regulate the expression of genes promoting mitochondrial biogenesis such as mitochondrial transcription factors (TFAM) and is therefore directly involved in mitochondrial preservation (Yenki, et al., 2013).

From this we can deduce that similar to the situation observed in GDH2, NRF2 also presents with a dual mechanism of action, not only does it activate genes in the cellular anti-oxidant response but also effects the cellular anti-inflammatory responses.(Calkins, et al., 2010; Hybertson, et al., 2011; Innamorato, et al., 2008; Kraft, et al., 2004; Kumar, et al., 2012; Rojo, et al., 2010; Shih, et al., 2003; Vargas, et al., 2006; Vargas, et al., 2008; Vargas, et al., 2009). Oxidative stress and neuroinflammation are considered major participants in the pathophysiology of ALS, both on their own and in respect to glutamate excitotoxicity. (Barber, et al., 2010; Coyle, et al., 1993; Kruman, et al., 1999; Lynch, et al., 2002 ; Rival, et al., 2004; Shaw, et al., 2000; Tolosa, et al., 2011; Van Damme, et al., 2005; Van Den Bosch, et al., 2006).

Reduced NRF2 mRNA and protein levels were observed in postmortem specimens of motor neurons in the primary motor cortex and the spinal ventral horn of sporadic ALS patients (Sarlette, et al., 2008). Moreover, neuronal and astroglial primary cultures from NRF2 knockout mice are more vulnerable to oxidative stress than wild-type cells, while overexpression of NRF2 increases resistance against oxidative and excitotoxic stimuli (Calkins, et al., 2005; Kraft, et al., 2004; Shih, et al., 2005). Furthermore, it has been previously shown that activation of NRF2 specifically in astrocytes protects neurons from a variety of *in vitro* insults as well as conveys protection in an ALS mouse model (Calkins, et al., 2010; Kraft, et al., 2004; Shih, et al., 2003; Vargas, et al., 2006; Vargas, et al., 2008; Vargas, et al., 2009).

Taken together all these elements render NRF2 a potent effector of the oxidative stress pathway and therefore we believed that NRF2 possesses a strong therapeutic potentially.

5.4 Combined EAAT2, NRF2 and GDH2 Lentiviral based treatment

In order to evaluate the therapeutic potential of EAAT2, NRF2 and GDH2 we first had to overcome the obstacle of successful gene delivery. Based on the information discussed in section 3.2 gene therapy approaches, we chose to incorporate our genes into lentiviral vectors.

We then evaluate the therapeutic potential of our novel approach in several models of ALS, including primary cultures and SOD1 G93A ALS mice. In the present study we found that treatment with lentiviral vectors containing EAAT2, NRF2 and GDH2 shows a therapeutic effect in cellular systems as well as in the ALS mouse model SOD1 G93A. Furthermore, we demonstrated that both *in vitro* and *in vivo* a synergistic relationship exists between the three genes. When evaluating our treatment in the SOD1 G93A ALS mouse model we found that it exerts an extremely beneficial therapeutic effect. Our treatment delays weight loss, preserves hindlimb reflex and motor function as well as protects neurological function. Our treatment significantly delayed the onset of symptoms and prolonged survival by approximately 125% from the onset of symptoms.

ALS is a dreadful disease with treatment options currently being extremely limited. Our study represents an entirely new therapeutic strategy for treating ALS. We hope that this therapeutic strategy might be the first step towards developing a functional treatment that might someday help slow the disease progression and alleviate the symptoms of patients suffering from ALS.

Research aims

- To identify astrocytic functions compromised in ALS and effect disease pathogenesis.
- To improve motor function, delay symptom onset and increase survival in a mouse model of ALS by compensating for the astrocytic deficits we identified.
- To genetically enhance the ability of the environment surrounding motor neurons, affected in ALS to deal with glutamate excitotoxicity and its secondary damage, thus provide symptomatic alleviation and prolong life expectancy of SOD1 G93A mice.

Specific aims

- To compare various astrocytic functions between WT and SOD1 G93A so as to identify new astrocytic functions that are compromised in ALS.
- To design and generate lentiviral vectors harboring the genes EAAT2, NRF2 and GDH2.
- To evaluate the possible In Vitro neuroprotective effect excreted by the different genetic combinations and determine the most effective therapeutic combination of one, two or all three of these genes.
- To directly inject the selected therapeutic combination of lentiviruses into the cerebrospinal fluid (CSF) and hindlimb muscles of SOD1 G93A mice and evaluate their possible *in vivo* neuroprotective effect using behavioral and survival parameters.

Materials

1. Table 5. Tissue culture reagents

Material	Manufacturer	Website	Catalogue #
Dulbecco's modified Eagle medium (DMEM) with D-Glucose 4500mg/l	Biological Industries, BeitHaemek, Israel	http://www.bioind.com	01-055-1
Dulbecco's modified eagle medium 1:1 plus Ham's F12 (DMEM:F-12)	Biological Industries, BeitHaemek, Israel	http://www.bioind.com	01-170-1
Fetal bovine serum (FBS), Heat inactivated	Biological Industries, BeitHaemek, Israel	http://www.bioind.com	04-121-1
L-Glutamine 200mM	Biological Industries, BeitHaemek, Israel	http://www.bioind.com	03-020-1
Penicillin 10000 U/ml / streptomycin 10 mg/ml / nystatin 1250 U/ml (PSN)	Biological Industries, BeitHaemek, Israel	http://www.bioind.com	03-032-1
Modified Eagle's medium non-essential amino acids (X100)	Biological Industries, BeitHaemek, Israel	http://www.bioind.com	01-340-1
Phosphate buffer saline (PBS)	Biological Industries, BeitHaemek, Israel	http://www.bioind.com	02-020-1
Trypsin (0.25%) EDTA (0.05%)	Biological Industries, BeitHaemek, Israel	http://www.bioind.com	03-052-1
Water- Cell Culture Grade	Biological Industries, BeitHaemek, Israel	http://www.bioind.com	03-055-1
DNAse	Sigma	http://www.sigmaaldrich.com	DN25
poly-D-Lysine (mw 70-150,000)	Sigma	http://www.sigmaaldrich.com	P6407
Lipopolysaccharide (LPS)	Sigma	http://www.sigmaaldrich.com	L2880
G5-supplement (diluted 1:100 [final concentrations: 5 µg/ml insulin, 50 µg/ml human transferrin, 5.2 ng/ml selenite, 1 µg/ml biotin, 3.6 ng/ml hydrocortisone, 5 ng/ml basic fibroblast growth factor (bFGF) and 0.01µg/ml epidermal growth factor (EGF)])	Invitrogen	http://www.invitrogen.com	17503-012
Ceftriaxone	Sigma	http://www.sigmaaldrich.com	C5793
G418	Sigma	http://www.sigmaaldrich.com	A1720
Hygromycin B	Sigma	http://www.sigmaaldrich.com	H3274
Doxycyclinehyclate	Sigma	http://www.sigmaaldrich.com	D9891
Glutamate	Sigma	http://www.sigmaaldrich.com	G8415
H_2O_2	Merck-Millipore	http://www.merckmillipore.com	107210
Alamar Blue®	AbD Serotec	http://www.abdserotec.com	BUF012B
Sodium pyruvate (X100)	Biological Industries, BeitHaemek, Israel	http://www.bioind.com	03-042-1
Polybrenne	Sigma	http://www.sigmaaldrich.com	H9268

2. Tissue culture media

2.1 Table 6. Astrocytic, NSC-34 and 293T growth medium

Material	Concentration
Dulbecco's modified Eagle medium (DMEM)	
L-Glutamine	2mM
SPN [streptomycin (10mg/ml) / penicillin (10,000U/ml) / nystatin (1,250U/ml)]	1%
Fetal bovine serum (FBS)	10%

2.2 Table 7. NSC-34 Differentiation medium

Material	Concentration
Dulbecco's modified eagle medium 1:1 plus Ham's F12 (DMEM:F-12)	
L-Glutamine	2mM
SPN [streptomycin (10mg/ml) / penicillin (10,000U/ml) / nystatin (1,250U/ml)]	1%
Fetal bovine serum (FBS)	1%
Modified Eagle's medium non-essential amino acids (X100)	1%

2.3 Table 8. NSC-34 over-expressing WT or mutant SOD1 gene growth medium

Material	Concentration
Dulbecco's modified Eagle medium (DMEM)	
L-Glutamine	2mM
SPN [streptomycin (10mg/ml) / penicillin (10,000U/ml) / nystatin (1,250U/ml)]	1%
Fetal bovine serum (FBS)	10%
G418	700µg/ml
Hygromycin B	200µg/ml
Doxycycline	1µg/ml for 24 hours – for transgene induction

2.4 Table 9. NSC-34 over-expressing WT or mutant SOD1 gene differentiation medium

Material	Concentration
Dulbecco's modified eagle medium 1:1 plus Ham's F12 (DMEM:F-12)	
L-Glutamine	2mM
SPN [streptomycin (10mg/ml) / penicillin (10,000U/ml) / nystatin (1,250U/ml)]	1%
Fetal bovine serum (FBS)	1%
Modified Eagle's medium non-essential amino acids (X100)	1%
G418	700µg/ml
Hygromycin B	200µg/ml
Doxycycline	1µg/ml for 24 hours – for transgene induction

2.5 Table 10. 293T transfection medium

Material	Concentration
Opti-MEM medium	
L-Glutamine	2mM
Fetal bovine serum (FBS)	10%

2.6 Table 11. 293T viral production medium

Material	Concentration
Dulbecco's modified Eagle medium (DMEM)	
L-Glutamine	2mM
Fetal bovine serum (FBS)	10%
Modified Eagle's medium non-essential amino acids (X100)	1%
Sodium pyruvate (X100)	1%

2.7 Table 12. Cell lines

Cell line	Source	Manufacturer	Reference	Catalogue #
NSC-34	Neuroblastoma (N18TG2) x spinal cord hybrid	Developed by Dr. Neil Cashman (University of Toronto)	(Cashman, et al., 1992)	–
293T	Human embryonic kidney	ATCC	http://www.ATCC.com	CRL-11268

3. Table13. Antibodies

Antibody	Dilution	Manufacturer	Website	Catalog#
Rabbit α GFAP (immunocytochemistry)	1:500	DAKO	http://www.dako.com	Z0334
Mouse α GFAP (Western blot)	1:2,500	DAKO	http://www.dako.com	M0761
Mouse α S100β	1:500	Sigma	http://www.sigmaaldrich.com	S2532
Rabbit α GS (immunocytochemistry)	1:500	Sigma	http://www.sigmaaldrich.com	G2781
Rabbit α GS (Western blot)	1:10,000	Sigma	http://www.sigmaaldrich.com	G2781
Mouse α vimentin	1:500	Sigma	http://www.sigmaaldrich.com	V2258
Rabbit α GLT-1	1:1,000	Santa Cruz biotechnology	http://www.scbt.com	SC-15317
Rabbit α GLAST	1:500	Abcam	http://www.abcam.com	Ab416
Rabbit α GDH2	1:500	Proteintech	http://www.ptglab.com	14462-1-AP
Goat α GFP (immunocytochemistry)	1:500	Santa Cruz biotechnology	http://www.scbt.com	SC-5384
Goat α GFP (Western blot)	1:100	Santa Cruz biotechnology	http://www.scbt.com	SC-5384
Mouse α β-actin	1:10,000	Sigma	http://www.sigmaaldrich.com	T8660
Alexa 488 conjugated goat anti rabbit	1:500	Molecular probes	http://www.invitrogen.com	A11008
Alexa 568 conjugated goat anti mouse	1:500	Molecular probes	http://www.invitrogen.com	A11031
Alexa 488 conjugated donkey anti goat	1:500	Molecular probes	http://www.invitrogen.com	A11055
Goat α Rabbit IRDye 800CW	1:10,000	LI-COR	http://www.licor.com	926-32211
Goat α Rabbit IRDye 680RD	1:10,000	LI-COR	http://www.licor.com	926-32221
Goat α Mouse IRDye 800CW	1:10,000	LI-COR	http://www.licor.com	926-32219
Goat α Mouse IRDye 680RD	1:10,000	LI-COR	http://www.licor.com	926-32220
Donkey α Goat IRDye 800CW	1:10,000	LI-COR	http://www.licor.com	926-32214

4. Table 14. List of primers

Gene	Forward	Reverse
IL2	CTAGGCCACAGAATTGAAAGATCT	GTAGGTGGAAATTCTAGCATCATCC
Human SOD1 G93A	CATCAGCCCTAATCCATCTGA	CGCGACTAACAATCAAAGTGA
BDNF	CAAACAAGACACATTACCTTCCTGC	CTTCTCACCTGGTGGAACATTG
GDNF	CCGCTGAAGACCACTCCCT	TAATCTTCAGGCATATTGGAGTCACT
GLT-1	CAGTGCTGGAACTTTGCCTG	GGCTATGAAGATGGCTGCCA
GLAST	CACTGCTGTCATTGTGGGTACA	TTATACGGTCGGAGGGCAAA
GS	GGTGCAGGCTGCCATACC	TGGCCTCCTCAATGCACTTC
Human NRF2	AACCAGTGGATCTGCCAACTACTC	CTGCGCCAAAAGCTGCAT
HumanEAAT2	TTGGCTAGAGGAACCCAAG	CAGGATGACACCAAACACCGT
GAPDH	CCATGGAGAAGGCTGGGG	CAAAGTTGTCATGGACC

5. [^{3}H] D-aspartate uptake assay

5.1 Table 15. [^{3}H] D-aspartate uptake assay materials

Material	Manufacturer	Website	Catalog #
HEPES	Sigma	http://www.sigmaaldrich.com	H4034-10.G
KCl	Sigma	http://www.sigmaaldrich.com	p-4504
KH_2PO_4	Sigma	http://www.sigmaaldrich.com	60220
$CaCl_2$	Sigma	http://www.sigmaaldrich.com	C5670
MgSO4	Sigma	http://www.sigmaaldrich.com	M7774
D-Glucose	Sigma	http://www.sigmaaldrich.com	G7528
NaCl	Merck-Millipore	http://www.merckmillipore.com	K17887500
Choline Cloride	Sigma	http://www.sigmaaldrich.com	C7527
[^{3}H] D-aspartate	American radiolabeled chemicals	www.arc-inc.com	ART-0212
D-aspartate	Sigma	http://www.sigmaaldrich.com	219096
L-trans-Pyrrolidine-2,4-dicarboxylic acid (t-PDC)	Sigma	http://www.sigmaaldrich.com	P7575
NaOH	Merck-Millipore	http://www.merckmillipore.com	106498

5.2 Table 16. Na^+ Krebs buffer

Material	Concentration
HEPES (pH 7.4)	25 mM
KCl	4.8 mM
KH_2PO_4	1.2 mM
$CaCl_2$	1.3 mM
MgSO4	1.2 mM
D-Glucose	6 mM
NaCl	140 mM

5.3 Table 17. Na^+ free Krebs buffer

Material	Concentration
HEPES (pH 7.4)	25 mM
KCl	4.8 mM
KH_2PO_4	1.2 mM
$CaCl_2$	1.3 mM
MgSO4	1.2 mM
D-Glucose	6 mM
Choline Cloride	120 mM

6. Table 18. Materials used for vector preparation

Material	Manufacturer	Website	Catalogue #
Ampicilin	Sigma	http://www.sigmaaldrich.com	A0166
Chloramphenicol	Sigma	http://www.sigmaaldrich.com	857440
Kanamycin	Sigma	http://www.sigmaaldrich.com	K-1377
LB	Sigma	http://www.sigmaaldrich.com	L-3022
LB AGAR	Sigma	http://www.sigmaaldrich.com	L-2897
LR Clonase™ Plus Enzyme Mix	Invitrogen	http://www.invitrogen.com	Part of kit K5910-00
One Shot® Stbl3™ Chemically Competent *E. coli*	Invitrogen	http://www.invitrogen.com	Part of kit K5910-00
Plasmid Midi Prep Kit	QIAGEN	http://www.qiagen.com	12143
Plasmid Mini Prep Kit	QIAGEN	http://www.qiagen.com	27104
ViraPower™ PromoterlessLentiviral Gateway® Expression Kit	Invitrogen	http://www.invitrogen.com	K5910-00
Water, DEPC treated	Biological Ind.	http://www.bioind.com	01-852-1A

7. Table 19. List of vectors

Vector name	Manufacturer	Website	Catalog#
pCR®8/GW/TOPO®	Invitrogen	http://www.invitrogen.com	K2500-20
pLenti6/R4R2/V5-DEST	Invitrogen	http://www.invitrogen.com	Part of kit K5910-00
pENTER-5'-CMV	Invitrogen	http://www.invitrogen.com	Custom ordered
pENTER-EAAT2	ImaGenes	http://www.imagenes-bio.de	IOH42832
pENTER-NRF2	ImaGenes	http://www.imagenes-bio.de	IOH14493
pENTER-GDH2	ImaGenes	http://www.imagenes-bio.de	IOH27063
pENTER-AcGFP	Invitrogen	http://www.invitrogen.com	Custom ordered

8. Table 20. Materials used for virus production

Material	Manufacturer	Website	Catalogue #
293T	ATCC	http://www.ATCC.com	CRL-11268
Lipofectamine 2000	Invitrogen	http://www.invitrogen.com	11668-019
Opti-MEM medium	Invitrogen - Gibco	http://www.invitrogen.com	31985-047
0.45µm PVDF filters	Millipore	http://www.millipore.com	SLHV033NS
Polybrenne	Sigma	http://www.sigmaaldrich.com	H9268
Polyallomer ultra centrifuge tubes	Beckman	https://www.beckmancoulter.com	326823
Hanks' Balanced Salts Solution (HBSS)	Biological Ind.	http://www.bioind.com	02-016-1
Lenti-X p24 Rapid Titer Kit	Clontech	http://www.clontech.com	632200

9. Table 21. Other materials

Material	Manufacturer	Website	Catalogue #
Cell culture flasks	Corning	http://www.corning.com	3290
Tissue culture dishes	Corning	http://www.corning.com	3516, 3524, 3596
Paraformaldehyde (PFA)	Sigma	http://www.sigmaaldrich.com	P6148
Ethanol 70%	Gadot	http://www.gadot.com	8832
Goat serum	Biological industries	http://www.bioind.com	04-009-1B
Bovine serum albumin (BSA)	Sigma	http://www.sigmaaldrich.com	A-4503
BCA protein assay kit	Pierce	http://www.piercenet.com	23225
TritonX-100 (octylphenoxypolyethoxyethanol)	Sigma	http://www.sigmaaldrich.com	T-6878
Dimethyl sulfoxide (DMSO)	Sigma	http://www.sigmaaldrich.com	D-5879
Alamar blue 10%	AbDSerotec	http://www.abdserotec.com	BUF012B
4',6-Diamidino-2-phenylindole Dihydrochloride (DAPI, 10 μg/ml)	Sigma	http://www.sigmaaldrich.com	D9542
D-Tail DNA extraction kit	Syntezza Bioscience	http://www.syntezza.com	DE-11
Saline	Teva	http://www.tevagenerics.com	AWB1324
TriReagent	Sigma	http://www.sigmaaldrich.com	T9424
SuperScript III Reverse Transcriptase kit	Invitrogen	http://www.invitrogen.com	18080-051
Platinum Sybr green qPCRsupermix	Invitrogen	http://www.invitrogen.com	11744-500
Sucrose	Frutarom	http://www.frutarom.com/	2355538000
Tris/HCl (pH 6.8)	USB	http://www.usbweb.com/	75825
EDTA	Merck-Millipore	http://www.merckmillipore.com	108418
Digitonin	Sigma	http://www.sigmaaldrich.com	D141
Dithiothreitol (DTT)	Invitrogen	http://www.invitrogen.com	Y0012
Phenylmethylsulfonylfluoride	Sigma	http://www.sigmaaldrich.com	P7626
Complete protease inhibitor cocktail	Roche Diagnostics	http://www.roche.com	12505200
Nitrocellulose membranes	GE healthcare life sciences	http://www.gelifesciences.com	10401180
12 well transwells	BD Falcon	http://www.bdbiosciences.com	353494
12 well transwell plates	BD Falcon	http://www.bdbiosciences.com	353503
Nuclear Extract Kit	Active motif	http://www.activemotif.com	40010
TransAM Nrf2	Active motif	http://www.activemotif.com	50296
GoTaq green PCR master mix	Promega	http://www.promega.com	M7123
2-methylbutane	Sigma	http://www.sigmaaldrich.com	M6250
Fluoromount-G,	SouthernBiotech	http://www.southernbiotech.com	0100-01

10. Table 22. Equipment and software

Instrumentation	Manufacturer
ABI Prism 7000 sequence detection system	Applied Biosystems, Foster City, CA, USA
Autoclave	Tuttnauer, USA
BX52TF normal & fluorescent light source microscope	Olympus, Tokyo, Japan
Centrifuge	Ohermle Z 360 K
Centrifuge 5403 or 5417C or 5810R	Eppendurf 5403, Hamburg, Germany
Centrifuge RC5C plus	Sorvall instruments, Newtown, CT, USA
Ultra centrifuge optima-L-90K – 32SwTi adapter	Beckman coulter, NyonSwitzerland
Cryostat	Leica, Wezlar, Germany
DP50-CU microscope digital camera	Olympus, Tokyo, Japan
Electrophoresis power supply model 3000Xi	BioRad, Japan
Gel box for Electrophoresis	BioRad, Japan
Ice machine	Scotsman AF-10, Italy
Incubator	Tuttnauer, USA
IX70-S8F2 normal & fluorescent light source microscope	Olympus, Tokyo, Japan
Laminar hood – Class II	Nuaire, Plymouth, MN, USA
Light-microscope BH-2	Olympus, Japan
Odyssey	LI-COR, Lincoln, NE, USA
PCR device	Biometra, Goettingen, Germany
pH Meter MP220	MettlerToledo,Switzerland
Quantity One®	BioRad, Japan
Rotarod	San Diego instruments, San Diego, CA, USA
StepOnePlus™ Real Time PCR System and software	Applied biosystems, Foster City, California, U.S.A
Spectrophotometer ND-1000	Nano-drop, Wilmington, DE, USA
Spectrophotometer PowerWaveXmicroplate reader	BIO-TEK instruments. Inc, VT, USA
Horizontal orbital shakerSf-670, class 2.0	MRC, Holon, Israel
-80^{o}C RevcoFreezer	Thermo Fisher Scientific Inc.Waltham, MA, USA
Odyssey Infrared Imager , 9120	Li-cor® biosciences, Lincoln, Nebraska USA
Odyssey 2.1 software	Li-cor® biosciences, Lincoln, Nebraska USA

Methods

1. Primary cultures of mice cortical astrocytes

1.1 Identification of Transgenic Mice Expressing the Human Mutant Superoxide Dismutase 1

Mice overexpressing the human mutant superoxide dismutase 1 (SOD1 G93A) were purchased from Jackson Laboratories (Bar Harbor, ME). The SOD1 G93A transgenic mouse model was developed and characterized as a model for ALS by Gurney et al. (1994). The animals were housed in standard conditions: constant temperature (22±1C), humidity (relative, 40%), and a 12-h light/dark cycle and were allowed free access to food and water. This model exhibits a dominant gain of function effect, therefore, male mice with hemizygous background were bred with control C57Bl females so that each litter would generate hemizygous SOD1 G93A transgenic mice and littermate controls. Newborn mice were genotyped by PCR analysis using the following primers; IL2 primers: CTAGGCCACAGAATTGAAAGATCT and GTAGGTGGAAATTCTAGCATCATCC and the human SOD1 G93A primers: CATCAGCCCTAATCCATCTGA and CGCGACTAACAATCAAAGTGA. Genomic DNA was extracted from tail biopsies using the D-Tail DNA extraction kit (Syntezza Bioscience, Jerusalem, Israel). The experiments were performed in accordance with local and international regulations, every effort was made to reduce the number of animals used and to minimize their suffering. For all studies, control primary cultures were obtained from littermates negative for the transgene to insure the use of an appropriate control.

2. Primary cortical astrocyte preparation and culturing

Astrocyte primary cultures were prepared from the cortex of newborn mice as previously described (Aprico`, et al., 2004), with minor changes. The cortex tissue was dissected from newborn mice (postnatal Days 1–3) and the meninges removed. Purified tissues were mechanically dissociated in cold PBS (Biological Industries, BeitHaemek, Israel) and digested by incubation with 1/5 V/V trypsin (Biological Industries, BeitHaemek, Israel) for 10 min at 37ºC. The reaction was stopped by the addition of complete medium (Dulbecco's Modified Eagle's Medium (DMEM); Biological Industries, BeitHaemek, Israel), supplemented with 10% fetal bovine serum (Biological Industries, BeitHaemek, Israel), 100μg/ml streptomycin, 100 U/ml penicillin, 12.5 units/ml nystatin (SPN; Biological Industries, BeitHaemek, Israel) and2 mm L-Glutamine (Biological Industries, BeitHaemek, Israel) and 50 μg/ml DNAse (Sigma, St. Louis, MO). At this point, the tissue was mechanically dissociated once more to ensure full dissociation into single cells. The cells were washed with complete medium once and centrifuged at 1,100 RPM for 7 min. The supernatant was removed and the pellet was resuspended in complete medium. The cells were plated at a density of 10^4 cells/cm^2. Cell cultures were grown in complete medium and maintained at37ºC and 5% CO_2. The cells were allowed to proliferate until confluence was achieved, at this point microglia cells were eliminated by shaking at 250 rpm for 18 h on a horizontal orbital shaker followed by removal of the microglia containing medium. After a few hours the cells were either infected with viruses and used without passaging (P0)or trypsinized and replated and then used at this passage (P1). Astrocytes used in all studies were from P0 or P1.

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3. Immunocytochemistry.

Primary cortical astrocyte cultures were plated on poly-D-Lysine (mw 70-150,000, Sigma) coated 96 well plates (Corning Inc. Corning, New York, U.S.A) when indicated, the cell cultures were activated 48 hours prior to evaluation. Three activation methods were used; 1. Activation with the bacterial endotoxin lipopolysaccharide (LPS, 1μg/ml, Sigma), this concentration is commonly used to activate a variety of cells including astrocytes (Chung, et al., 1990; Holst, et al., 1996; Lin, et al., 2008; O'Shea, et al., 2006; Zagami, et al., 2005). 2. Activation with the G5-supplement; G5 was diluted 1:100 [final concentrations: 5 μg/ml insulin, 50 μg/ml human transferrin, 5.2 ng/ml selenite, 1 μg/ml biotin, 3.6 ng/ml hydrocortisone, 5 ng/ml basic fibroblast growth factor (bFGF) and 0.01μg/ml epidermal growth factor (EGF)]. As previously reported, this supplement proved effective in triggering the in vitro activation of astrocytes (Michler-Stuke, et al., 1984; Vermeiren, et al., 2005). 3. Activation with the β-lactam antibiotic ceftriaxone (10μM, Sigma, Lee, et al., 2008; Rothstein, et al., 2005). The cells were fixed with 4% paraformaldehyde and were then incubated in a blocking and permeabilizing solution (5% goat serum, 1% BSA and 0.5% Triton X-100) for 1 hour. The cells were stained with rabbit anti-glial fibrillary acidic protein (GFAP; 1:500, DAKO, Glostrup, Denmark), mouse anti-S100 β (1:500, Sigma), rabbit anti glutamine synthetase (GS; 1:500, Sigma) or mouse anti vimentin (1:500, Sigma) at 4ºC overnight. After washing with PBS, the cells were incubated with secondary antibodies for 1 hour at room temperature. Secondary antibodies were goat anti-rabbit Alexa-488 (1:500, Molecular Probes, Invitrogen, Carlsbad, CA, U.S.A) and goat anti-mouse Alexa-568 (1:500, Molecular Probes, Invitrogen). Nuclear DNA was stained by 4,6-diamino-2-phenylindole (DAPI, 1:1,000, Sigma). Cells were photographed by fluorescence Olympus IX70-S8F2 microscope with a fluorescent light source (excitation wavelength, 330–385nm; barrier filter, 420 nm) and a U-MNU filter cube (Olympus).

4. RNA isolation and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Total RNA was isolated from primary cortical astrocyte cultures derived from wild-type and SOD1 G93A mice using a commercial reagent TriReagent™ (Sigma) and the manufacturer's recommended procedure. The amount of RNA was determined spectrophotometrically using the ND-1000 spectrophotometer (Nano-drop). RNA quality was verified by measuring OD260/OD280 ratio. RNA was stored at −80ºC until used. First-strand cDNA synthesis was carried out using the commercial SuperScript™ III Reverse Transcriptase kit (Invitrogen) and the manufacturer's recommended procedure. Samples were stored at −20ºC until used. Real-time semi quantitative PCR of the desired genes was performed in a StepOnePlus™ Real Time PCR System (Applied biosystems, Foster City, California, U.S.A) using Sybr green PCR master mix (Applied biosystems) and the following primers: BDNF: CAA ACA AGA CAC ATT ACC TTC CTG C Forward, BDNF: CTT CTC ACC TGG TGG AAC ATT G Reverse, GDNF: CCG CTG AAG ACC ACT CCC T Forward, GDNF: TAA TCT TCA GGC ATA TTG GAG TCA CT Reverse, GLT-1: CAG TGC TGG AAC TTT GCC TG Forward, GLT-1: GGC TAT GAA GAT GGC TGC CA Reverse, GLAST: CAC TGC TGT CAT TGT GGG TAC A Forward, GLAST: TTA TAC GGT CGG AGG GCA AA Reverse, GS: GGT GCA GGC TGC CAT ACC Forward, GS: TGG CCT CCT CAA TGC ACT TC Reverse, And GAPDH: CCA TGG AGA AGG CTG GGG Forward , GAPDH: CAA AGT TGT CAT GGA CC Reverse

GAPDH served as an internal control as it is considered a valid reference gene for transcription profiling. Data was calculated as the ratio of mean threshold targeted gene expression to GAPDH. For each gene, the specificity of the PCR product was assessed by verifying a single peak on melting curve analysis. When indicated, the cell cultures were

activated 24 hours prior to evaluation. At least three independent experiments were performed, each in triplicate.

5. Protein extraction and Western blotting.

Proteins were extracted from primary cortical astrocyte cultures. The cells were washed twice with PBS and resuspended in a lysis buffer containing 250mM sucrose, 25 mM Tris/HCl, pH 6.8, 1 mM EDTA, 0.05% digitonin (a mild nonionic detergent used to solubilize receptors and permeabilize cellular and nuclear membranes), 1 mM dithiothreitol (DTT),0.1 mM phenylmethylsulfonylfluoride, 1:100 V/V complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Samples were centrifuged at 13,000g for 3 min at4ºC. Supernatants were stored at −80ºC until used. Proteins were separated by 12% sodiumdodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were probed overnight at 4ºC with the following primary antibodies; rabbit anti-GLT-1 (1:1,000, Santa Cruz Biotechnology, Inc. Santa Cruz, California U.S.A), rabbit anti-GLAST (1:500, abcam®, Cambridge, U.K), mouse anti-GFAP (1:2,500, DAKO) and rabbit anti-GS (1:10,000, Sigma). Following wash, membranes were incubated with secondary antibodies: goat anti-mouse IRDye®800CW or 680CW (1:10,000, infra-red dye, Li-cor® biosciences, Lincoln, NE, U.S.A.) or with secondary antibodies goat anti-rabbit IRDye®800CW or 680CW (1:10,000, infra-red dye, Li-cor® biosciences) for 1 hour at room temperature. The membranes were then developed with Odyssey Infrared Imager (model 9120, Li-cor® biosciences). As a control for protein loading, blots were subsequently probed for mouse anti β-actin (1:10,000; Sigma) using the same procedures. Data was calculated as the ratio of mean target gene intensity to β-actin intensity.

Densitometric analysis of Western blots was performed using Odyssey 2.1 software (Li-cor® biosciences) to measure the area and density of protein bands. When indicated, the cell

cultures were activated 48 hours prior to evaluation. At least three independent experiments were performed.

6. [^{3}H] D-aspartate uptake.

For uptake assays, primary cultured astrocytes were grown on poly-D-lysine (mw 70-150,000, Sigma) coated 24well plates (Corning Inc. Corning, New York, U.S.A). Cells were rinsed twice with 0.5ml of pre-heated Krebs buffer (25 mM HEPES pH 7.4, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 6 mM glucose and 140 mM NaCl).When indicated Na^+ free Krebs buffer was used (in which NaCl was replaced by choline chloride at the same osmolarity). As in the majority of studies concerning glutamate transporters, L-glutamate was substituted with aspartate, a transportable analogue, which does not interact with glutamate receptors and is not metabolized. Astrocyte cultures were incubated for 20 minutes at 37ºC with radiolabeled aspartate ([^{3}H]D-aspartate, 40nM, American radiolabeled chemicals, inc. St. Louis, MO, U.S.A) and unlabeled aspartate (Sigma) mixture in Krebs buffer to obtain a final concentration of 40 μM for single concentration assays or a range of concentrations of 5–1000 μM for saturation assays. These mixed solutions are referred to as [^{3}H] D-aspartate throughout the manuscript. When indicated, the inhibitor L-trans-Pyrrolidine-2,4-dicarboxylic acid (t-PDC, 0.314μM, Sigma) was added 15 minutes before the addition of [^{3}H] D-aspartate. Uptake was terminated by rinsing the cells three times with cold Na^+ free Krebs buffer. Cells were lysed in 1M NaOH and radioactivity was determined by liquid scintillation counting of the cell lysate. A fraction of the lysate was also used to determine the protein concentration. When indicated, the cell cultures were activated 48 hours prior to evaluation. At least three independent experiments were performed, each in duplicates or triplicate.

7. Co-culturing of primary cortical astrocytes with differentiated cells of the motor neuron cell line NSC-34

7.1 Differentiation of the motor neuronal cell line NSC-34

NSC-34 is a neuroblastoma (N18TG2) x spinal cord hybrid cell line. These cells closely resemble motor neurons in many morphological and physiological aspects and are widely used as a model system for motor neurons (Cashman, et al., 1992; Eggett, et al., 2000; Matusica, et al., 2008; Weishaupt, et al., 2006). These cells were maintained in complete medium (Dulbecco's Modified Eagle's Medium (DMEM; Biological Industries), supplemented with 10% fetal bovine serum (Biological industries), 100 μg/ml streptomycin, 100 U/ml penicillin, 12.5 units/ml nystatin (SPN; Biological industries) and 2 mM L-Glutamine (Biological industries) at 37ºC and 5% CO_2. NSC-34 differentiation was performed in accordance with previously established differentiation protocols (Cashman, et al., 1992; Eggett, et al., 2000; Matusica, et al., 2008; Weishaupt, et al., 2006). Differentiation entailed replacing the complete medium with differentiation medium (Dulbecco's Modified Eagle's Medium 1:1 plus Ham's F12 (DMEM:F-12, Biological Industries), supplemented with 1% fetal bovine serum (Biological industries), 100 μg/ml streptomycin, 100 U/ml penicillin, 12.5 units/ml nystatin (SPN; Biological industries), 2 mM L-Glutamine (Biological industries) and 1% modified Eagle’s medium non-essential amino acids (Biological industries). NSC-34 cells were considered fully differentiated after exposure to differentiation medium for 10 days providing that the NSC-34 cells also exhibited morphological changes consistent with differentiation upon microscopic evaluation (Olympus IX70-S8F2 microscope). Differentiated NSC-34 (NSC-34D) cells survive in differentiation medium and could be serially passaged at least three times without loss of viability.

7.2 Co-culturing of astrocytes and NSC-34 cells

For co-culture experiments, astrocytes were plated on the top deck of 12 well transwells (BD Falcon™, Becton, Dickinson and Company (BD), Franklin Lakes, New Jersey, U.S.A) The NSC-34D cells were plated on the bottom deck of poly-D-lysine (mw 70-150,000, Sigma) coated 12 well plates (BD Falcon™). When indicated, astrocyte cultures were activated 48 hours prior to co-culturing. To achieve co-culturing the astrocyte-containing transwells were inserted into the NSC-34D containing 12 well plates. This transwell system was subsequently exposed for 24 hours to 4mM glutamate (Sigma) for single concentration experiments or to a range of concentrations of 1–7 mM glutamate (Sigma) for dose-dependency studies. Astrocyte transwells were then removed to allow evaluation of NSC-34D viability. Cellular viability was assessed using alamar Blue® (Invitrogen). At least six independent experiments were performed.

8. Generation of lentiviral vectors

pLenti6.3-CMV-EAAT2, pLenti6.3-CMV-NRF2, pLenti6.3-CMV-GDH2 and pLenti6.3-CMV-AcGFP2 were constructed using ViraPower™ Promoterless Lentiviral Gateway® Kit. (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Briefly, the pENTER-EAAT2, pENTER-NRF2, pENTER-GDH2 and pENTER-AcGFP plasmids were purchased from ImaGenes (ImaGenes, Berlin, Germany). The pLenti-DEST destination plasmid and the pENTER-5'-CMV plasmid were purchased from (Invitrogen, Carlsbad, CA, USA). The pENTER-Gene plasmid, pENTER-5'-CMV promoter plasmid and the pLenti-DEST destination plasmid were recombined using the LR Clonase II plus enzyme (Invitrogen, Carlsbad, CA, USA). This enzyme displaced the fragments flagged by the recombination sequences to produce the final pLenti6.3-CMV-GENE construct. For each

reaction sample, a plasmid containing the CMVpromoter was incubated overnight at room temperature with a plasmid containing the DNA fragment, the destination plasmid and their combination enzyme, LR clonase (Invitrogen Carlsbad, CA, USA). Following recombination, the expression constructs were transformed into the One Shot® Stbl3™ Competent E. Coli (Invitrogen): 4 µl of the recombination reaction were added to One Shot® Stbl3™ Competent E. coli and were incubated for 30 minutes on ice. After incubation, the mix was transferred to 42ºC for 30 seconds and then for 2 minutes on ice. 250 µl of SOC medium was added to the mix and incubated at 37ºC. After one hour the mix was placed on LB agar plates with ampicillin (Sigma- Aldrich, St. Louis, MO, USA) for 24 hours at 37ºC. On the following day, one colony was picked and DNA was produced using a midi kit. Expression clones were sequenced to confirm both the CMV promoter and the expression gene presence. The vector also contains the Blasticidin resistance marker to select the cells expressing the vector.

9. Production of lentiviral vectors

The viruses were produced using the ViraPower Lentiviral Expression System (Invitrogen) according to the manufacturer's instructions. Expression constructs (3µg) were co-transfected with the packaging plasmids (9µg): pLP1, pLP2, and pLP/VSVG using Lipofectamine 2000 (Invitrogen) into the 293T producer cell line which stably expresses the SV40 large T antigen. The expression constructs and the packaging plasmids were co-diluted into 1.5ml of Opti- MEM (Biological Industries). Lipofectamaine 2000 (Invitrogen) was separately diluted in 1.5ml Opti-MEM. After 5 minutes of room temperature incubation, the diluted mix and the DNA with the diluted Lipofectamine 2000 were combined and incubated for 20minutes at room temperature. After incubation, the 3 ml of complexes were added to culture plates containing 293T cells and 5ml OptiMem medium (10% FBS, 2mM L-Glutamine). The following day, the medium was replaced with 5ml medium (DMEM

containing 10% FBS, 2 mM L-Glutamine and 1% sodium pyruvate). The medium containing the viral particles was collected 48 or 72 hours after transfection, filtered through 0.45µm PVDF filters (Millipore, Billerica, MA. USA) followed by ultra centrifugation of the virus containing medium to concentrate the viruses. The virus containing medium was dispensed into Beckman polyallomer conical centrifuge tubes (Beckman, Brea CA. USA) and the tubes were measured and balanced. The tubes were placed into the spinning buckets using the 32SwTi rotor and adaptor. The tubes contain the virus were centrifuged for 2 hours at 20,000 RPM at 4ºC under vacuum conditions. The pellets were re-suspended in 300-1000µl of Hanks' Balanced Salts Solution (HBSS, Biological industries).

9.1 Tittering lentiviral vectors

The lentiviral load was determined using the Lenti-X p24 Rapid Titer Kit and the manufacturer's recommended procedure. The viral titter 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 $1.25X10^7$lentiviral particle (LP). And 1 infectious unit considered equivalent to 1 in every 1000 LP's.

10. Transduction of astrocytes with lentiviral vectors

Cortical astrocyte cultures were produced and cultured as described above (2. Primary cortical astrocyte preparation and culturing). When the cultures reached 90% confluence the flasks were shaken at 250 rpm for 18 hours on a horizontal orbital shaker followed by removal of the microglia containing medium.

48 hours after shaking the astrocytes were transduced at multiplicities of infection (MOI) 10 for expression evaluation experiments and at MOI 25 for functional and co-culturing experiments with lentiviruses containing EAAT2, NRF2, GDH2 or GFP in the presence of 6μg/ml Polybrenne (Sigma).

10.1 RNA isolation and real-time quantitative reverse transcription polymerase chain reaction

RNA isolation and qRT-PCR were performed as described above (in methods section 4. RNA isolation and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). For these experiments, RNA was collected 96 hours after transduction. The following primers were used: NRF2: AACCAGTGGATCTGCCAACTACTC Forward, NRF2: CTGCGCCAAAAGCTGCAT Reverse, EAAT2: TTGGCTAGAGGAACCCAAG Forward, EAAT2: CAGGATGACACCAAACACCGT Reverse.

GAPDH served as an internal control as it is considered a valid reference gene for transcription profiling. Data was calculated as the ratio of mean threshold targeted gene expression to GAPDH. For each gene, the specificity of the PCR product was assessed by verifying a single peak on melting curve analysis. At least three independent experiments were performed.

10.2 Protein extraction and Western blotting

Protein expression and western blotting was performed as described above (in methods section5. Protein extraction and Western blotting.). For these experiments, Protein was collected 96 hours after transduction. The following anti-bodies were used: primary antibodies; Rabbit α GDH2 (1:500, Proteintech), Goat α GFP (1:100, Santa Cruz

biotechnology). Secondary antibodies were secondary antibodies: goat anti-Rabbit IRDye®800CW or Donkey α Goat IRDye 800CW (1:10,000, infra-red dye, Li-cor® biosciences).

As a control for protein loading, blots were subsequently probed for mouse anti β-actin (1:10,000; Sigma) using the same procedures. Data was calculated as the ratio of mean target gene intensity to β-actin intensity. Densitometric analysis of Western blots was performed using Odyssey 2.1 software (Li-cor® biosciences) to measure the area and density of protein bands. At least three independent experiments were performed.

10.3 Evaluating transgene function in transduced astrocytes

To evaluate whether transduction of astrocytes with NRF2 increased the function of the NRF2 protein we evaluated the amount and function of NRF2 protein in the cell nucleus using the Active motif Nuclear Extract Kit and TransAM NRF2 activity kit following the manufacturer's recommended protocol. For the Nuclear Extract Kit only the nuclear fraction was collected and the samples were maintained at -80^{o}C until use. The protein concentration was determined using the BCA protein assay kit (Pierce) following the manufacturer's recommended protocol. An equal total amount of nuclear protein was loaded into the TransAM NRF2 activity kit for each sample that was equal to the amount used for the positive control. The final activity was determined using the manufacturer's recommended protocol.

To evaluate whether transduction of astrocytes with EAAT2 increased the function of the EAAT2 protein we evaluated glutamate uptake using the [^{3}H] D-aspartate assay. The assay was performed as described above (in methods section 6. For these experiments the astrocytes were transduced 96 hours prior to functional evaluation. When appropriate,

cellular viability was assessed 96 hours after transduction using the alamar Blue® (Invitrogen) assay. All experiments were performed at least in three independent biological replications.

11. Excito-oxidative stress

11.1 Differentiation of the motor neuronal cell line NSC-34 and induction of transgene expression

Stable NSC-34 lines over-expressing the WT or mutant form of the SOD1 gene were differentiated as described above (in methods section 7.1Differentiation of the motor neuronal cell line NSC-34). Prior to performing the experiments transgene expression was induced by exposure to doxycycline (1μg/ml) for 24 hours, as the transgene was fused to the GFP gene transgene expression was determined by microscopic evaluation for GFP fluorescence (Olympus IX70-S8F2 microscope).

11.2 Induction of excitotoxicity and oxidative stress

Astrocyte and NSC-34D cells were produced, cultured and maintained as described above (in methods section 2. Primary cortical astrocyte preparation and culturing and section 7.1. Differentiation of the motor neuronal cell line NSC-34). For these experiments, astrocyte cells were plated on 96 well plates (Corning) 4 days prior to exposure to the toxin. The NSC-34D cells were plated on poly-D-lysine (Sigma) coated 96 well plates (Corning) and transgene expression was induced by exposure to doxycycline (1μg/ml) for 24 hours. Astrocyte cultures were exposed to a range of 0-75nM H_2O_2 or to 4mM glutamate for 16 hours. Mutant and WT SOD1 overexpressingNSC-34D cells were exposed for 16 hours to a range of glutamate (0mM-9mM) or hydrogen peroxide (0-50nM) concentrations. For mixed excito-oxidative stress experiments NSC-34D cells were exposed to a range of hydrogen

peroxide (0-50nM) concentrations combined with a single concentration of glutamate (2,3,4 or 5mM of glutamate) for 16 hours. Cellular viability was assessed using alamar Blue® (Invitrogen). At least three independent experiments were performed.

12. Co-culturing of primary cortical astrocytes with differentiated cells of the motor neuron cell line NSC-34 overexpressing the WT or mutated SOD1 gene

Astrocyte and NSC-34D cells were produced, cultured and maintained as described above (in methods section 2. Primary cortical astrocyte preparation and culturing and section 7.1.Differentiation of the motor neuronal cell line NSC-34). For co-culture experiments, astrocytes were plated on the top deck of 12 well transwells (BD Falcon™, Becton, Dickinson and Company (BD), Franklin Lakes, New Jersey, U.S.A) The NSC-34D cells were plated on the bottom deck of poly-D-lysine (mw 70-150,000, Sigma) coated 12 well plates (BD Falcon™) and transgene expression was induced 24 hours prior to combination of the co-culture system. When indicated, astrocyte cultures were transduced with lentiviral constructs expressing EAAT2, NRF2, GDH2, GFP or different combinations of the genes 5 days prior to combination of the co-culture system. To achieve co-culturing the astrocyte-containing transwells were inserted into the NSC-34D containing 12 well plates. This transwell system was subsequently exposed for 16 hours to 3.5 mM Glutamate combined with 17.5 nM H_2O_2. Astrocyte transwells were then removed to allow evaluation of NSC-34D viability separately from the astrocyte viability. Cellular viability was assessed using alamar Blue® (Invitrogen). At least three independent experiments were performed.

13. Applying lentiviral vectors into the SOD1 G93A ALS mouse model

13.1 Identification of Transgenic Mice Expressing the Human Mutant SOD1

Mice were housed, bread and identified as described above (in methods section 1.1. Identification of Transgenic Mice Expressing the Human Mutant Superoxide Dismutase 1).

For these experiments the mice were genotyped at 3–4 weeks of age. When SOD1 mice became weak, food pellets were supplied inside the cage. Newborn litters were selected randomly for inclusion in control or treatment groups. The experimental design maintained a studmate distribution into the control or treatment groups. We also evaluated the date of death of the male founders as an indication of the expected disease severity and mutation copy number and insured that all the animals used in the behavioral experiments came from founders of similar behavioral traits. All experiments were performed by a blinded observer. All experimental protocols were approved by the Tel Aviv University Committee of Animal Use for Research and Education.

13.2 Immunohistochemistry

13.2.1 Tissue preparation

Mice were sacrificed and their tissues harvested. Spinal cord samples were then immersed in 4% paraformaldehyde for 24 h at 4 °C followed by then the spinal cords were washed with PBS and the cord removed from the spinal bones. The cords were then immersed in 30% sucrose for an additional 48 h. The cords were frozen in chilled 2-

methylbutane (Sigma-Aldrich), stored at −70 °C, and subsequently sectioned into slices measuring 5 μm.

13.2.2 **Immunohistochemistry**

Slides were incubated with blocking solution (5% goat/donkey serum, 1% BSA, 0.5% Triton X-100 in PBS) for 1 hour before overnight incubation at 4°C with the Goat anti GFP (1:500 in blocking solution, Santa Cruz) primary antibody. Thereafter, sections were incubated with the Alexa 488 donkey anti Goat (1:500 in PBS, Invitrogen) secondary antibody for 1 hour at room temperature. The sections were then mounted with fluorescent mounting solution (Fluoromount-G, Southern Biotech), covered with a cover slide, and sealed. Digital images were obtained with a fluorescence Olympus BX52TF microscope.

13.3 Lentiviral administration

At the age of 65 days SOD1 G93A mice received either lentiviruses carrying GFP, or one of the three genes or a mixture of lentiviruses carrying EAAT2, NRF2 and GDH2. A non-viral saline control was also evaluated. All mice were injected both intra-cisternaly and intra-muscularly into the gastrocnemius muscles of both hind limbs. Mice received a total viral load of $5X10^8$ infectious viral particles in 200μl, half of the viral load was administered intra cisternaly and a quarter into each hind limb. Unless otherwise specified a minimum of 12 animals per group per gender was maintained.

14. Neurological score evaluation by ladder testing of SOD1 mice

To evaluate the animal's overall neurological state we performed a neurological scoring based on the ladder test which is an extension of the hanger test. The ladder is placed at a 45 degree angle, after a brief training period, when placed on the ladder healthy mice quickly and efficiently climb up the ladder. As the disease progresses the animals' ability to climb is hindered beginning with leg tremors and developing up until the point where the mice can simply not climb up the ladder at all. From the age of 90 days the mice were evaluated for their neurological score twice weekly by a blinded observer. The mice were scored based on their performance on the ladder test with a score of 12 representing completely healthy mice and 0 correlating with disease end stage. The score was determined using the guidelines described in Table 23.

Table 23. Guidelines for neurological scoring of mice using the ladder test

Score	Behavioral manifestation
0	Pre-symptomatic, no visible difficulties climbing the ladder
1	Early stage tremors, climbing speed not affected
2	Increased tremors, difficulty in closing hind leg fingers around the stages of the ladder
3	One of the hind legs begins to occasionally miss the stages of the ladder, climbing speed not affected
4	Both of the hind legs begin to occasionally miss the stages of the ladder, climbing speed not affected
5	One of the hind legs occasionally misses the stages of the ladder, climbing speed affected
6	Both of the hind legs occasionally misses the stages of the ladder, climbing speed affected
7	Climbing speed clearly affected, medium to slow speed, hind legs show symptoms
8	Slow climbing speed, symptoms clearly visible in hind legs but not front legs
9	Slow climbing speed, symptoms visible in hind legs as well as front legs
10	The mouse climbs up the ladder very slowly and misses the stages of the ladder with all legs
11	The mouse barely climbs up the ladder
12	The mouse does not move once placed on the ladder

15. Weight measurement of SOD1 mice

One of the hallmarks of ALS progression and the best predictor of clinical translatability of an ALS based treatment is preserving body weight. From the age of 40 days the animals were weighed weekly. The animal's weight at the time of death was recorded and used as the animal's terminal weight.

16. Hindlimb reflex measurement of SOD1 mice

When lifted by their tails, healthy mice reflexively extend their legs backwards to improve their balance. As the disease progresses SOD1 G93A ALS mice begin to lose their ability to extend their legs and this reflexive extension is gradually lost. From the age of 90 days the mice were evaluated weekly for their hind leg reflexes by a blinded observer. Each leg was given a score of 0 to 100 (100 being fully extended leg, 75 represents extension of the leg of no more then $^{3}/_{4}$ of full extension, 25 represents extension of the leg of no more then half of full extension, 25 represents extension of the leg of no more then $^{1}/_{4}$ of full extension, and 0 no extension reflex observed). As the disease progresses bilaterally but not symmetrically, the animals score was the combined score given to each leg.

17. Motor function measurement by rotarod of SOD1 mice

Adult wild-type mice are able to remain balanced on a rod rotating at 12.5 rpm for up to 4 min, following a brief training period. As SOD1 mice weaken, however, they lose this balancing ability and fall off the rod after progressively shorter periods of time. We analyzed

the abilities of mice to perform this task. All subjects were tested on a weekly basis starting at 90 days of age after three sessions of training. We averaged three consecutive runs on the rotarod from each session and compared the performance of the groups.

18. Symptom onset evaluation of SOD1 mice

The clinical condition of the mice was monitored daily starting at 40 days. The onset of clinical signs was scored by following the animal's weight loss. The age of clinical onset was determined by the age (in days) at which the mice loss 5% of their body weight (compared to peak weight).

19. Survival evaluation of SOD1 mice

The clinical condition of the mice was monitored daily starting at 40 days of age. Examining the mice for the loss of righting reflex determined the end stage of the disease. The mice were sacrificed if they could not right themselves within 30 seconds when placed on either side on a flat surface as requested by the Tel Aviv University Committee of Animal Use for Research and Education. The age (in days) at which the mice were sacrificed represented the age of death and the extent of survival in accordance with currently accepted practices.

20. Statistics

Statistical analysis of the data sets was carried out with the aid of SPSS for windows (version 10.0.1).

Statistical significance for figures 6, 7, 8B-C, 10B-G,15, 16, 21, 22 and 27-30 was determined by one way (or two way as appropriate) analysis of variance (ANOVA) with repeated measures followed by Dunnett's post hoc test. Statistical significance for figures 26, 31C-D, 32B, C, E and F was determined by one-way ANOVA followed by Dunnett's post hoc test. Statistical significance for figures 8A, 10A, 17 and 19 was evaluated in point to point comparison in which the two-tailed student's t test was used. For determining the statistical significance of figures 13 and 14 we used the two-tailed student's t test.

For determining the statistical significance for the survival and symptom onset tests in figures 31A, B and 32A, D the Kaplan- Mayer test was employed. In all experiments for direct comparison between the wild-type control and theSOD1 G93A control groups the student's t test was used. Results are expressed as means ±standard error of the mean (SEM) and error bars represent SEM. Significance was considered for $p<0.05$.

Results

1. Altered astrocytic response to activation in SOD1 G93A mice and its implications on amyotrophic lateral sclerosis pathogenesis

Most of the data described in this section was published in Benkler, Ben Zur, Barhum and Offen, 2013 in Glia Journal. Despite the publication all of the data described in this section was produced and written by Chen Benkler, any and all data generated or written by others involved in the publication was omitted from this thesis.

1.1 Cellular and morphological characterization of astrocyte enriched cultures

Astrocyte enriched primary cultures were generated from postnatal day 1-3 wild-type and SOD1 G93A mice. The use of appropriate culture medium combined with a vigorous mechanical dissociation step promoted the enrichment of astrocytes and the elimination of microglia cells from the culture. To evaluate the purity of our cultures we employed an extensive marker analysis and found high levels of well-known astrocytic markers including glial fibrillary acidic protein (GFAP), the calcium binding protein S100β, vimentin and glutamine synthetase (GS) (Figure 5A-D). Astrocyte enriched cultures were found to contain less than 4% microglia cells (as assessed by immunocytochemical evaluation for CD11B, Figure 5E). The cultures obtained from transgenic animals were morphologically indistinguishable from control cultures. Prior to activation, both wild-type and transgenic astrocyte cultures maintained a polygonal flat morphology.

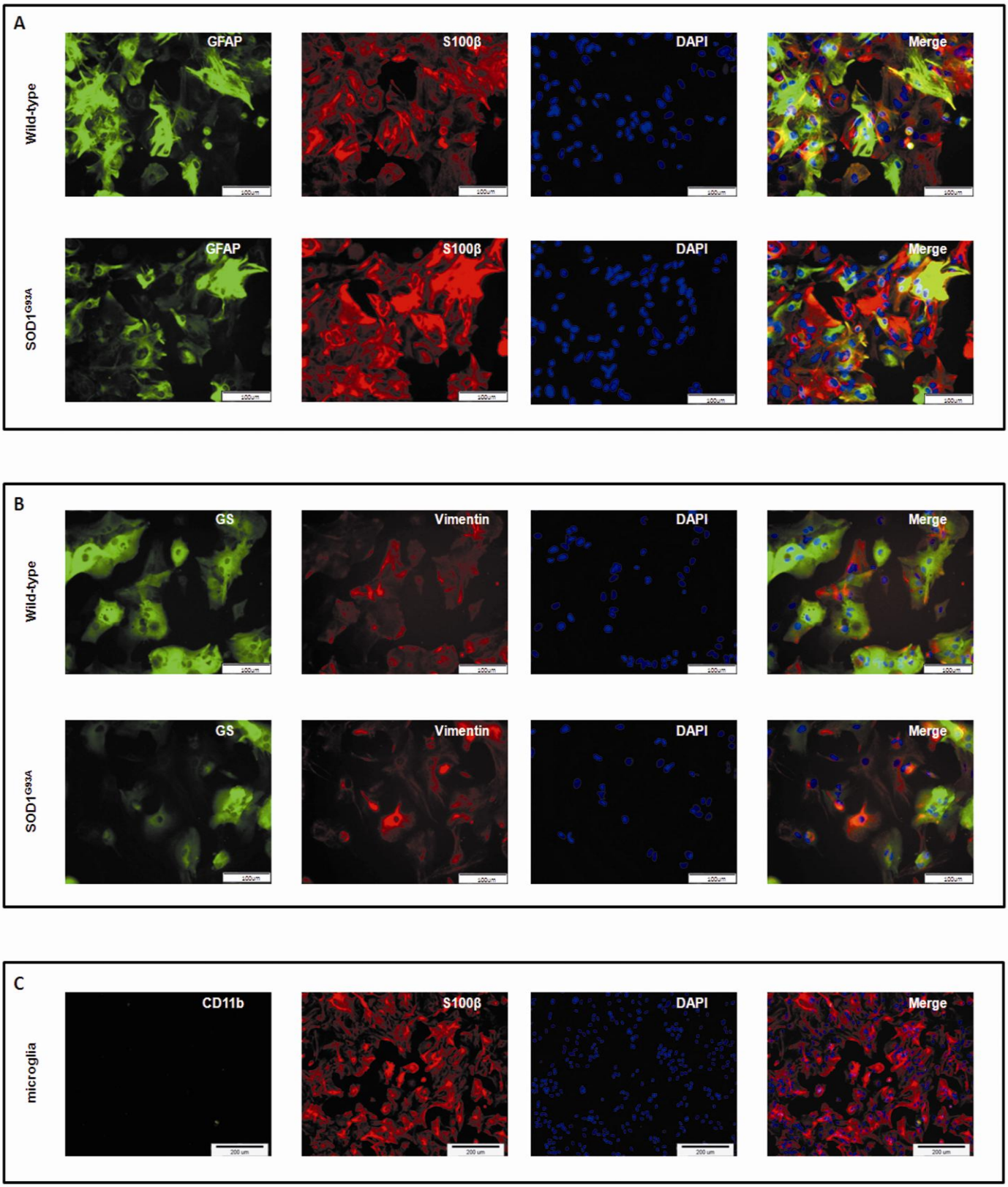


Figure 5. Morphological characterization of primary astrocyte cultures. *(A), immunostaining of wild-type and SOD1 G93A astrocytes for GFAP (green), S100β (red), nuclei are marked with DAPI (blue) , Scale bar: 100µm. (B), immunostaining of wild-type and SOD1 G93A astrocytes for GS (green), vimentin (red), nuclei are marked with DAPI (blue), Scale bar: 100µm. (C), immunostaining for the microglial marker CD11B (green), nuclei are marked with DAPI (blue), Scale bar: 200µm.*

1.2 Activation induces different expression of several key genes in wild-type and SOD1 G93A astrocytes

To characterize the unique effect activation might exert upon SOD1 G93A astrocytes, we first evaluated selected features of their individual genetic profiles following activation. To this end we selected 3 specific activators the bacterial endotoxin lipopolysaccharide (LPS) which activates a variety of cell types, the G5-supplement, a cocktail of growth factors which specifically activates astrocytes (Michler-Stuke, et al., 1984; Vermeiren, et al., 2005). As the β-lactam antibiotic ceftriaxone (CEF) has been reported to increase both GLT-1 expression and glutamate uptake as well as improve the overall survival of SOD1 G93A mice (Rothstein, et al., 2005) its effect was also evaluated. The expression levels of 5 major astrocytic genes were ascertained using quantitative real time PCR (qRT-PCR).

Secretion of neurotrophic factors is an important neuroprotective function performed by astrocytes. We first evaluated the transcription of BDNF and GDNF, two neurotrophic factors with well-known motor neuron protective properties (Benkler, et al., 2010; Ekestern, 2004; Gould, et al., 2011). Activation with G5, LPS and CEF elevated both BDNF and GDNF transcription in astrocytes derived from wild-type mice (BDNF G5: 2.89±0.5, LPS: 2.37±0.2, CEF: 5.29±0.8, GDNF G5: 2.90±0.5, LPS: 16.33±1.1, CEF: 9.78±1.2 fold of un-activated astrocytes, $p<0.05$, Figure 6A-B). Contrary to the response of wild-type astrocytes, activation of SOD1 G93A astrocytes with all three activators reduced the expression of both BDNF (G5: 0.05±0.02, LPS: 0.11±0.04, CEF: 0.06±0.01 fold of un-activated wild-type astrocytes, $p<0.05$, Figure 6A) and GDNF with the exception of activation with LPS which had no effect on GDNF transcription levels (G5: 0.16±0.02, CEF: 0.19±0.07 fold of un-activated wild-type astrocytes, $p<0.05$, Figure 6B). Furthermore, basal expression levels of BDNF and GNDF in

un-activated SOD1 G93A astrocytes were significantly lower than those found in their wild-type counterparts (BDNF: 0.34±0.04, GDNF: 0.79±0.08, $p<0.05$, Figure 6A-B).

Glutamate mediated excitotoxicity due to impaired astrocytic glutamate clearance constitutes one the currently most prominent pathophysiological hypothesis explaining the progression of ALS (Bogaert, et al., 2010; Cleveland, et al., 2001; Danbolt, 2001; Oliveira, et al., 2009; Van Damme, et al., 2005). We evaluated the mRNA transcription levels of 3 key components of the glutamate-glutamine cycle; the major astrocytic high affinity glutamate transporters GLT-1 and GLAST (glutamate-aspartate transporter) as well as the metabolic enzyme glutamine synthetase which converts glutamate into glutamine. These elements are essential for glutamatergic transmission and play an important role in preventing excitotoxicity (Bos, et al., 2006; Danbolt, 2001; Fray, et al., 2001; Hertz, 1979; Sonnewald, et al., 2002). We found that glutamine synthetase was similarly expressed in wild-type and SOD1 G93A astrocytes and maintained a similar response to activation. Interestingly, activation with G5 and CEF up-regulated glutamine synthetase expression (wild-type G5: 1.60±0.08, CEF: 1.57±0.1 and SOD1 G93A G5: 1.77±0.1, CEF: 1.26±0.06 fold of un-activated wild-type astrocytes, $p<0.05$, Figure 6C), whereas exposure to LPS resulted in slightly reduced mRNA levels (wild-type: 0.9±0.02, SOD1 G93A: 0.41±0.09 fold of un-activated wild-type astrocytes, $p<0.05$, Figure 6C). Further analysis revealed increased transcription of the 2 major astrocytic glutamate transporters in wild-type astrocytes in response to activation. GLAST levels were most prominently elevated by exposure to CEF but were unaltered in the presence of G5, whereas GLT-1 levels were elevated by exposure to all three activators (GLAST LPS: 1.91±0.16, CEF: 2.54±0.12. GLT-1 G5: 1.74±0.09, LPS: 2.46±0.18, CEF: 2.56±0.3 fold of un-activated wild-type astrocytes, $p<0.05$, Figure 6D-E). Astrocytes derived from SOD1 G93A mice exhibited reduced initial GLT-1 and GLAST mRNA levels (83±6.7% and 57±1.5% of wild-type expression levels respectively $p<0.05$, Figure 6D-E). Remarkably, contrary to wild-type cells in astrocytes derived from SOD1 G93A GLT-1 levels could only be elevated by activation with LPS and reach half (53.25±8.5% $p<0.05$, Figure 6D) of those found in wild-type LPS activated astrocytes. GLAST levels remained unaltered in response to all three activators used (Figure 6E).

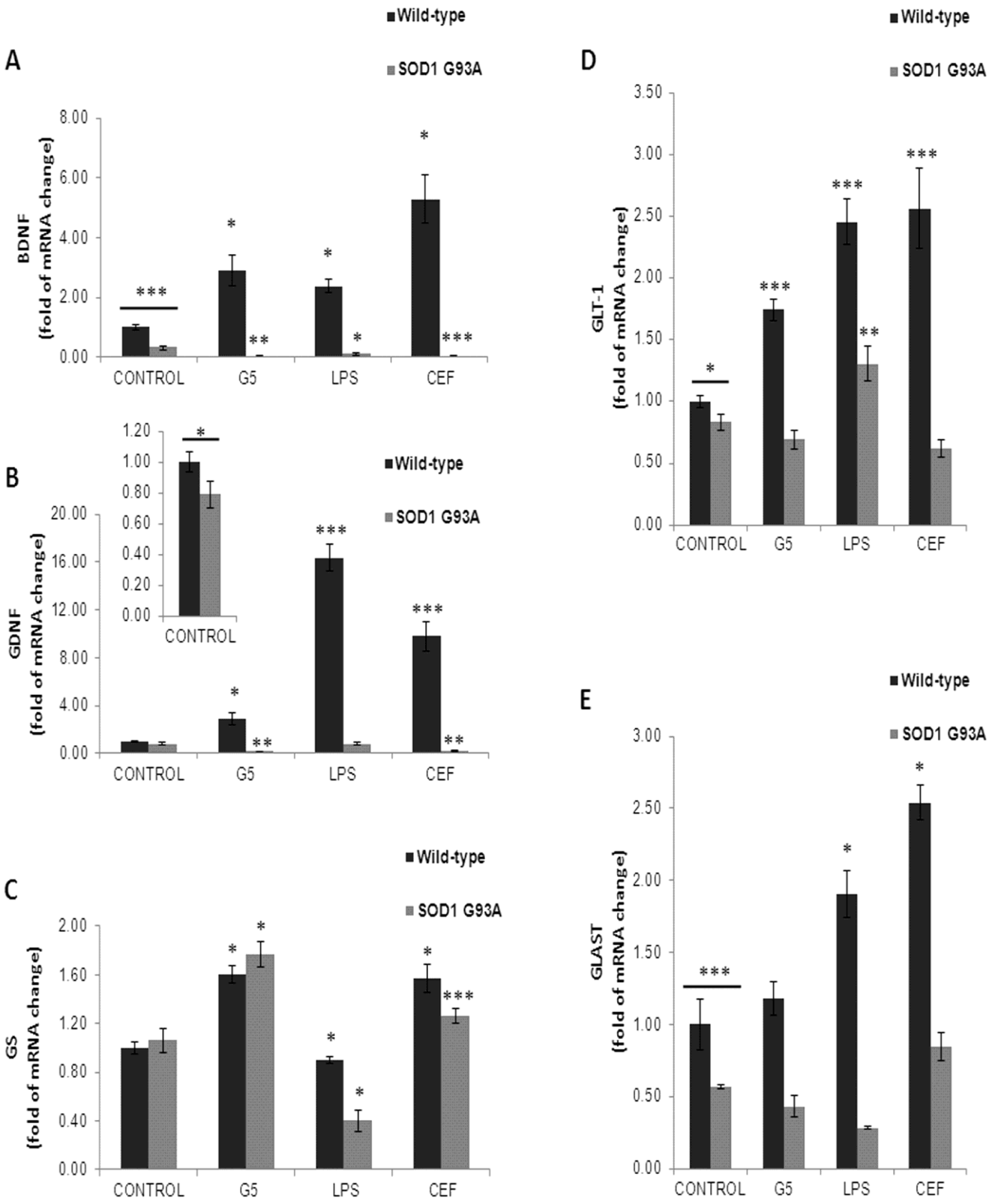


Figure 6. Evaluated mRNA levels in astrocytes derived from wild-type and SOD1 G93A mice following 24 hour activation with G5 (1:100), LPS (1µg/ml) and CEF (10µM). *mRNA levels of 5 key genes; BDNF (**A**), GDNF (**B**), GS (**C**), GLT-1 (**D**), GLAST (**E**). *p< 0.05; **p< 0.01, ***p<0.001 as compared to control.*

1.3 Activation increases GLT-1 and GLAST protein expression less potently in SOD1 G93A astrocytes

The up-regulation of GFAP is a well characterized feature of astrocyte activation. We found that all three activators significantly increased GFAP protein expression levels in both wild-type and SOD1 G93A astrocytes (Wild-type G5: 199.79±14.39%, LPS: 187.15±7.39%, CEF: 162.89±7.93%, SOD1 G93A G5: 171.56±14.01%, LPS: 159.92±14.83%, CEF: 158.87±16.72%percent of un-activated wild-type astrocytes, $p<0.05$, Figure 7A).

Much like the situation observed in GFAP immunoreactivity, GS expression levels responded similarly to activation in astrocytes derived from wild-type and SOD1 G93A astrocytes. GS immunoreactivity levels remained impervious to activation with LPS and CEF. However, it is noteworthy that upon activation with G5 Glutamine Synthetase levels increased similarly in both wild-type and SOD1 G93A cultures (964.28±4.50% and 910.04±43.13%percent wild-type immunoreactivity respectively, $p<0.01$, Figure 7B).

Regarding the astrocytic glutamate transporters, our data revealed that the alterations observed in GLT-1 and GLAST transcription levels were reflected in their protein levels. GLT-1 and GLAST immunoreactivity increased in wild-type astrocytes following activation with G5, LPS and CEF (GLT-1 G5: 133.21±10.31%, LPS: 140.98±6.88%, CEF: 228.16±14.33% GLAST G5: 148.10±2.05%, LPS: 141.20±4.20%, CEF: 213.29±6.15%percent of wild-type immunoreactivity, $p<0.05$, Figure 7C-D). GLT-1 and GLAST immunoreactivity was significantly lower in un-activated SOD1 G93A astrocytes compared to their wild-type counterparts (79.68±7.68% and 65.60±7.18%percent of wild-type immunoreactivity respectively, $p<0.05$, Figure 7C-D) and were un-altered in response to activation.

This data highlights a potential dysfunction in the SOD1 G93A astrocytic response to stress induced activation signals as well as impairments in their glutamate glutamine handling.

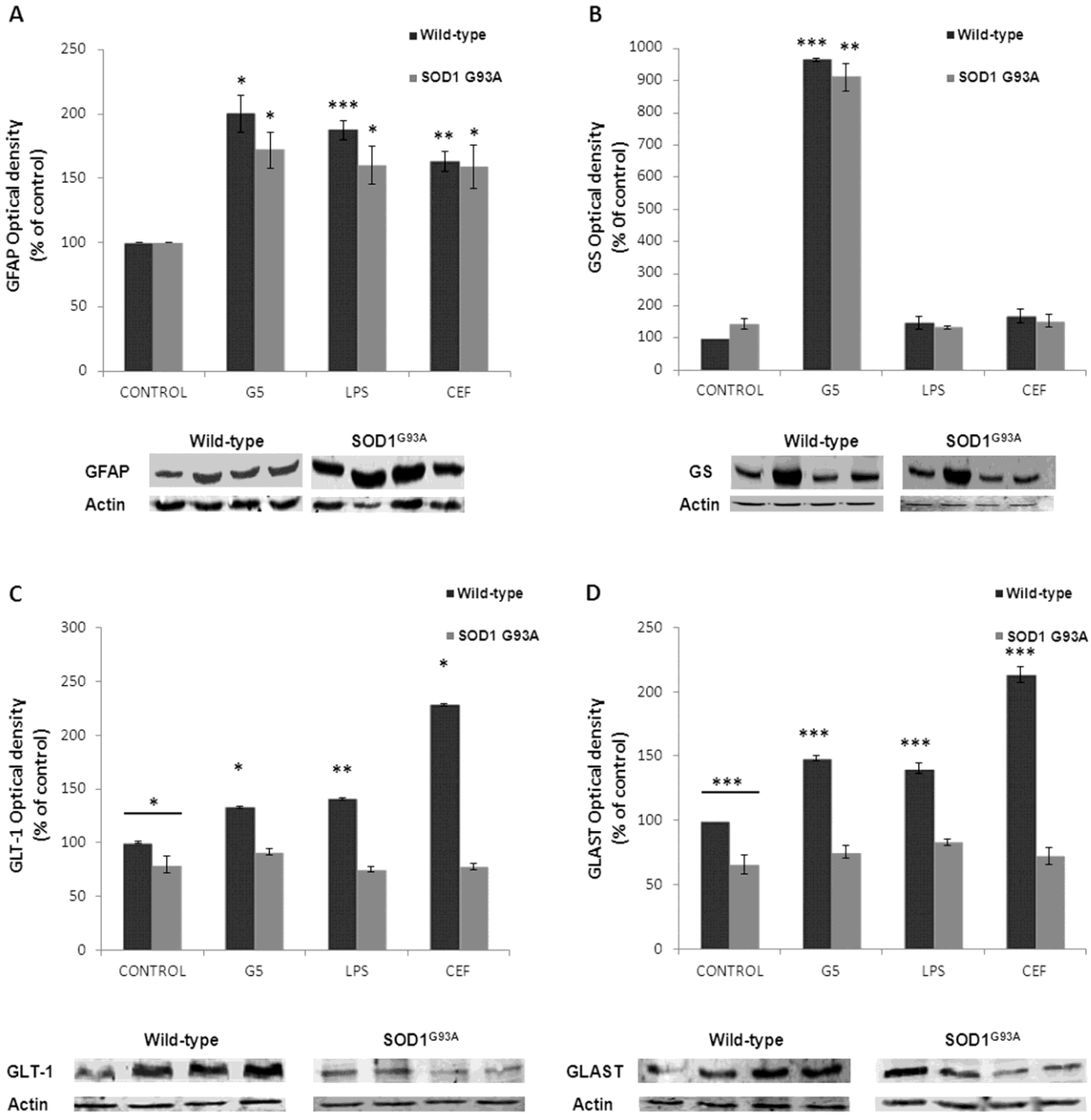


Figure 7. Modulation of GFAP (A), GS (B), GLT-1 (C) and GLAST (D) protein levels by activation in wild-type and SOD1 G93A astrocytes. *Cell cultures were activated with G5 (1:100), LPS (1μg/ml) and CEF (10μM) for 48 hours. Upper panels show the quantification of protein levels by densitometry of immunoreactivity compared to β- actin which served as an internal protein control. Lower panels show representative western blot membranes. *p< 0.05; **p< 0.01, ***p<0.001 as compared to control.*

1.4 Loss of glutamate uptake enhancement by activation in SOD1 G93A astrocytes

Activation induced the up-regulation of the high affinity glutamate transporters GLT-1 and GLAST transcript and protein levels. To further evaluate whether this up-regulation has a functional effect on the astrocytic glutamate clearance potential, we performed [^{3}H] D-aspartate based saturation kinetics and glutamate uptake assays. Both assays utilized [^{3}H] D-aspartate as it is a commonly used non-metabolized transportable glutamate analog which does not interact with glutamate receptors.

Un-activated astrocytes derived from SOD1 G93Amice demonstrated a significantly lower maximal velocity (V_{max}) of substrate uptake compared to their wild-type counterparts (V_{max} wild-type: 6.45±0.09, SOD1 G93A: 3.51±0.08 nmol/min/mg, $p<0.01$, Figure 8A). Glutamate transporters activity was considerably reduced in the absence of Na^+ and in the presence of the glutamate uptake inhibitor t-PDC (wild-type Na^+: 97.50±1.04%, t-PDC: 99.03±0.30%. SOD1 G93A Na^+ 96.60±1.04%, t-PDC: 98.52±0.39% reduction in substrate uptake. $p<0.001$, Figure 8B). Activation of wild-type astrocytes with G5, LPS or CEF induced a robust increase in substrate uptake (G5: 153.83±11.30%, LPS: 175.30±20.51% and CEF 200.20±16.67% of un-activated astrocytes, $p<0.05$, Figure 8C). This increase paralleled the previously observed increase in GLT-1 and GLAST mRNA and protein levels. However, not only do un-activated SOD1 G93A astrocytes take up substrate less efficiently (73.31±7.95% of wild-type astrocytes, $p<0.05$), but unlike their wild-type counterparts, in response to activation they show no further increase in substrate uptake (Figure 8C).

This observed inability of SOD1 G93A astrocytes to increase substrate uptake in response to activation signals might indicate a potential pathway contributing to the development of glutamate induced excitotoxicity in the SOD1 G93A ALS mouse model.

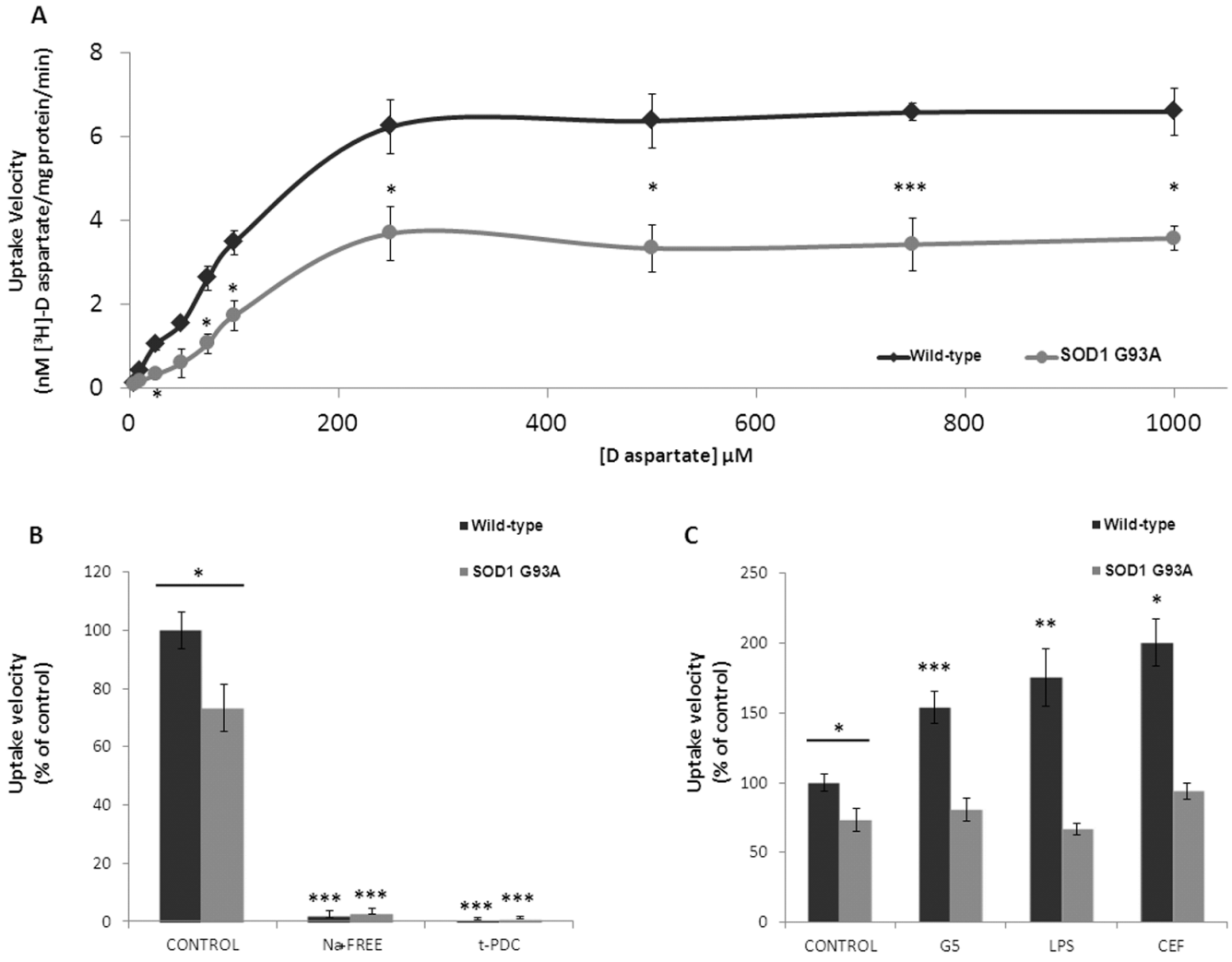


Figure 8. Activation enhanced [^{3}H] D-aspartate uptake in astrocytes derived from wild-type but not SOD1 G93A mice. *(A), Saturation curves for [^{3}H] D-aspartate (5-1000 µM) uptake into astrocyte cultures prepared from wild-type and SOD1 G93A mice. (B), Uptake of [^{3}H] D-aspartate (40 µM) in wild-type and SOD1 G93A astrocyte cultures and the effect of uptake inhibition by incubation in sodium-free buffer (Na^+ free) or by exposure to the uptake inhibitor t-PDC (0.314µM). (C), The effect of activation with G5 (1:100), LPS (1µg/ml) and CEF (10µM) for 48 hours on the [^{3}H] D-aspartate (40 µM) uptake in wild-type and SOD1 G93A astrocyte cultures. *p< 0.05; **p< 0.01, ***p<0.001 as compared to control.*

1.5 Activation improves the neuroprotective potential of wild-type but not SOD1 G93Aastrocytes

NSC-34 is a neuroblastoma (N18TG2) x spinal cord hybrid cell line. These cells closely resemble motor neurons in many morphological and physiological aspects and are widely used as a model system for motor neurons (Cashman, et al., 1992; Eggett, et al., 2000;

Matusica, et al., 2008; Weishaupt, et al., 2006). Previous studies of NSC-34 cells established a differentiation protocol capable of enhancing their motor neuron traits as well as potentiating their sensitivity to glutamate induced excitotoxicity (Cashman, et al., 1992; Eggett, et al., 2000; Matusica, et al., 2008; Weishaupt, et al., 2006).

During the differentiation procedure NSC-34 cells undergo morphological changes that increase their phenotypic resemblance to motor neurons, this change is accompanied by reduced cellular proliferation and marks the end of the first stage of differentiation (Figure 9).

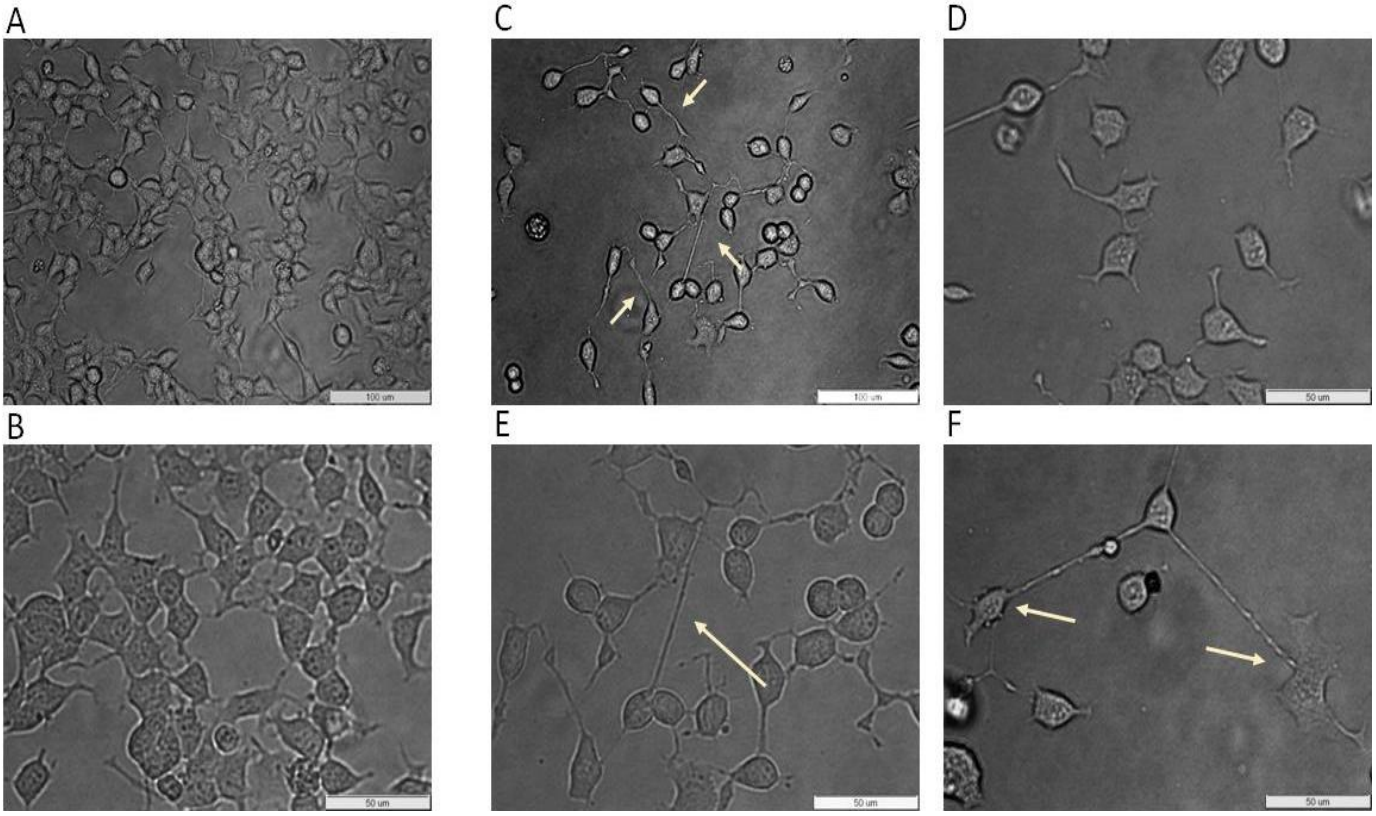


Figure 9. Differentiation of the motor neuron like cell line NSC-34 increases their motor neuronal traits. *(A, B) NSC-34 cell prior to differentiation present with a high proliferation rate with only few cells developing neuronal processes. (C-F) Following differentiation NSC-34 cells show typical morphological changes consistent with motor neuron maturation with many cells extending long neuronal processes. Some cells even begin forming complex neuronal networks connecting two or more cells (examples are indicated with arrows). A, C; Scale bar: 100µm. B, D-F; Scale bar: 50µm.*

To examine the sensitivity of NSC-34 cells to glutamate excitotoxicity both differentiated NSC-34 (NSC-34D) cells and non differentiated NSC-34 cells were exposed for 24 hours to a range of glutamate concentrations (1mM-7mM). Cellular viability following exposure to glutamate was assessed. A significant and dose dependent cell death was observed for NSC-34D cells beginning at 3mM, whereas NSC-34 cells were barely affected, even at 7mM glutamate (Figure 10A). Wild-type and SOD1 G93A astrocyte viability was not affected by exposure to 7mM glutamate or by activation (Figure 10B-C).

The astrocytic ability to perform glutamate uptake renders them suitable candidates for providing protection against glutamate neurotoxicity. To explore this possibility, wild-type and SOD1 G93A astrocytes were activated for 48 hours with G5, LPS or CEF prior to incubation with NSC-34D. This transwell co-culture system was then immediately challenged with 4mM glutamate for 24 hours. Astrocytes were then removed to enable the evaluation of the viability of the motor neuron cell line (NSC-34D) without interference from the astrocytic viability signals. The presence of both wild-type and transgenic astrocytes significantly reduced glutamate induced NSC-34D injury (63.94±1.39% survival of NSC-34D cells alone, 80.17±2.24% survival of NSC-34 cells co-cultured with wild-type astrocytes and 77.49±1.22% survival of NSC-34 cells co-cultured with SOD1 G93A astrocytes, $p<0.01$, Figure 10D). Activation of wild-type astrocytes with LPS or CEF but not G5 significantly improved their protective potential (LPS: 90.50±1.37%, CEF: 94.56±0.74% and G5: 82.79±1.12% compared to 80.17±2.24% survival provided by non-activated astrocytes, $p<0.05$, Figure 10D-G). The activation induced increase in the astrocytic protective potential stands in direct correlation with the activators effect on the

astrocytic glutamate uptake abilities as well as the GLT-1 and GLAST mRNA and protein levels. On the other hand, when evaluating the effect of activation on astrocytes derived from SOD1 G93A mice, activation with all three activators did not potentiate their protective potential (Figure 10D-G).

To investigate the extent to which glutamate uptake participated in the astrocytic neuroprotection we added the glutamate uptake inhibitor t-PDC to the astrocyte-NSC-34D co-culture system during the glutamatergic insult. Inhibiting glutamate uptake reduced the protection provided by wild-type and transgenic astrocytes by approximately 60% (inhibited astrocytes provide 34.76±4.98% (wild-type) and 39.25±3.83% (SOD1 G93A) of the added protection provided by uninhibited un-activated wild-type astrocytes, $p<0.05$, Figure 10D). Furthermore, exposure to t-PDC completely abolished the increase in neuroprotection induced by activation as well as the difference between wild-type and SOD1 G93A astrocytes (Figure 10E-G). These results suggest that the entire increase in the neuroprotective potential of activated wild-type astrocytes is attributed to their increased glutamate clearance potential.

Taken together these results indicate a severe impairment in SOD1 G93A astrocytes response to activation which may well play a crucial role in the pathogenesis of ALS.

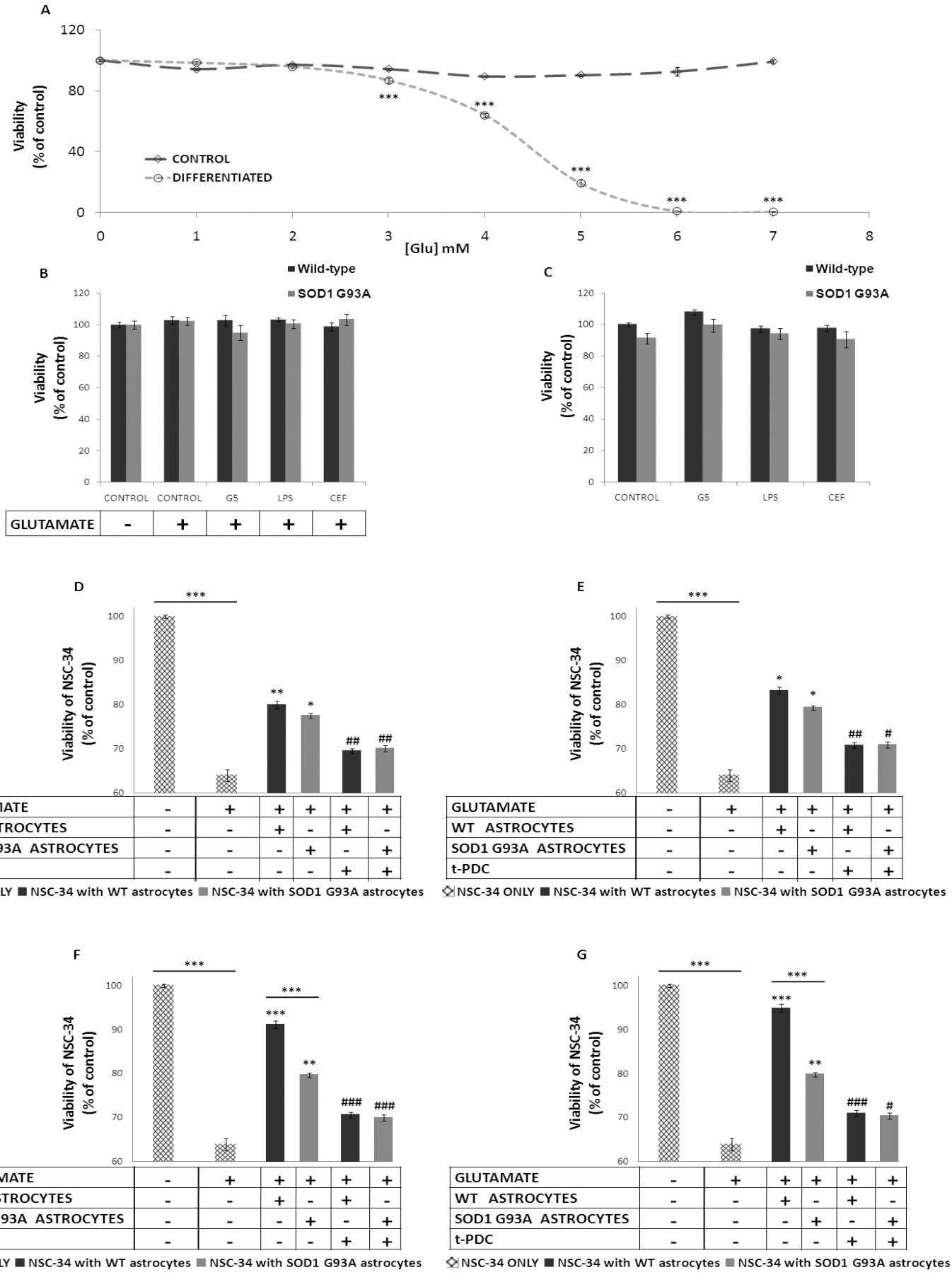


Figure 10. Activation increases the neuroprotection provided by wild-type but not SOD1 G93A astrocytes. *(A) Effect of exposure to 1-7mM glutamate on differentiated NSC-34 cells (NSC-34D) and non-differentiated NSC-34 cells. Differentiated cells are significantly more sensitive to glutamate excitotoxicity than non-differentiated cells under these conditions. Astrocyte viability is not affected by activation (B) or by exposure to 7mM glutamate (C). NSC-34D cells were co-cultured with un-activated (control, D), G5 activated (E), LPS activated (F) or CEF activated (G) astrocytes. Astrocytes were activated for 48 hours and were then inserted into the co-culture system which was immediately challenged with 4mM glutamate for 24 hours in the absence or presence of the glutamate uptake inhibitor t-PDC (0.314μM). The astrocytes were then removed from the co-culture system and the viability of the NSC-34D cells was evaluated. Percent of cell viability was calculated as the percentage of NSC-34D viability exposed to activated or un-activated astrocytes and 4mM glutamate as compared to NSC-34 cell viability when not exposed to astrocytes or glutamate. *p< 0.05; **p< 0.01, ***p<0.001 as compared to control. In co-culture experiments, # p< 0.05; ## p< 0.01; ### p< 0.001 for astrocytes exposed to t-PDC compared to astrocytes not exposed to t-PDC.*

2. Developing a therapeutic strategy for treating SOD1 G93A ALS mice

Based on our earlier result and the astrocytic deficits first defined by us (Benkler, et al., 2013), we devised a new therapeutic strategy for treating ALS. Our therapeutic strategy was designed to attempt to compensate for those astrocytic deficits. To achieve this goal we selected three pathways severally affected in ALS. For each pathway we selected a single key gene that could affect the entire pathway. The first gene selected was EAAT2 the major astrocytic glutamate uptake transporter, enhancing the presence of this gene in cell populations in the vicinity of the neurons affect in ALS can reduce the synaptic glutamate availability, thus reducing the glutamate excitotoxic levels in the neuronal microenvironment (Bogaert, et al., 2010; Cleveland, et al., 2001; Danbolt, 2001; Oliveira, et al., 2009; Van Damme, et al., 2005). To influence the neuronal environment in a more systemic way we selected theGDH2 gene. The GDH2 enzyme converts glutamate into α keto-glutarate involved in the metabolic pathway, thus depleting the systemic glutamate bio-availability as well as increasing the metabolic energetic state (Palmada, et al., 1998; Plaitakis, et al., 2001; Spanaki, et al., 2010; Mastorodemos, et al., 2005). The combined use of these two genes can significantly influence the potential excitotoxic damage inflicted upon motor neurons during the disease progression.

Glutamate excitotoxicity causes a cascade of effects harmful to motor neurons. One of the most potent players in this cascade is oxidative stress (Lynch, et al., 2002; Van Damme, et al., 2005; Van Den Bosch, et al., 2006; Shaw, et al., 2000; Rival, et al., 2004; Kruman, et al., 1999). Therefore we believed that NRF2 also has a strong therapeutic potential. NRF2 is a major transcription factor involved in the cellular anti-oxidant and anti-inflammatory

responses (Hybertson, et al., 2011; Vargas, et al., 2006; Vargas, et al., 2008; Vargas, et al., 2009; Shih, et al., 2003.; Calkins, et al., 2010; Kraft, et al., 2004; Innamorato, et al., 2008; Rojo, et al., 2010; Kumar, et al., 2012).Oxidative stress and neuroinflammation are considered major participants in the pathophysiology of ALS, both on their own and in respect to glutamate excitotoxicity (Lynch, et al., 2002; Van Damme, et al., 2005; Van Den Bosch, et al., 2006; Shaw, et al., 2000; Rival, et al., 2004; Kruman, et al., 1999; Coyle, et al., 1993; Tolosa, et al., 2011; Barber, et al., 2010).

We hypnotized that these three genes will work together in a synergistic manner and provide strong neuroprotection to the motor neurons degenerating during the progression of ALS.

3. Creation and evaluation of lentiviral constructs engineered to harbor the genes: EAAT2, NRF2 and GDH2

3.1 Generation of lentiviral vectors

In order to evaluate our hypothesis that overexpression of EAAT2, NRF2 and GDH2 might have therapeutic potential in *in vitro* and *in vivo* models of ALS, we first had to overcome the obstacle of successful gene delivery. Several studies have reported that lentiviral vectors are one of the most potent methods of delivering genes and providing long lasting transgene expression in numerous *in vitro* and *in vivo* systems (Clements, et al., 2006; Gjertsson, et al., 2009; Liao, et al., 2008; Van Damme, et al., 2005; McMahon, et al., 2006.)

Furthermore, these studies indicated that the transduction procedure itself did not affect the basic cellular processes other than the added expression of the transgene.

We wanted to allow our gene to be constitutively and massively over-expressed, to this end we selected to express our genes under the control of the enhanced cytomegalovirus (CMV) promoter. This promoter has been reported to induce extremely high expression rates in cellular systems compared to other promoters (Clements, et al., 2006). The viruses were pseudotyped with the vesicular stomatitis virus G protein and cloned into the pLenti6.3lentiviral production system. We constructed one reporter vector expressing the GFP gene (pLenti6.3-CMV-AcGFP) and 3 expression vectors, one for each potentially therapeutic gene EAAT2 (pLenti6.3-CMV-EAAT2), NRF2 (pLenti6.3-CMV-NRF2) and GDH2 (pLenti6.3-CMV-GDH2, Figure 11)

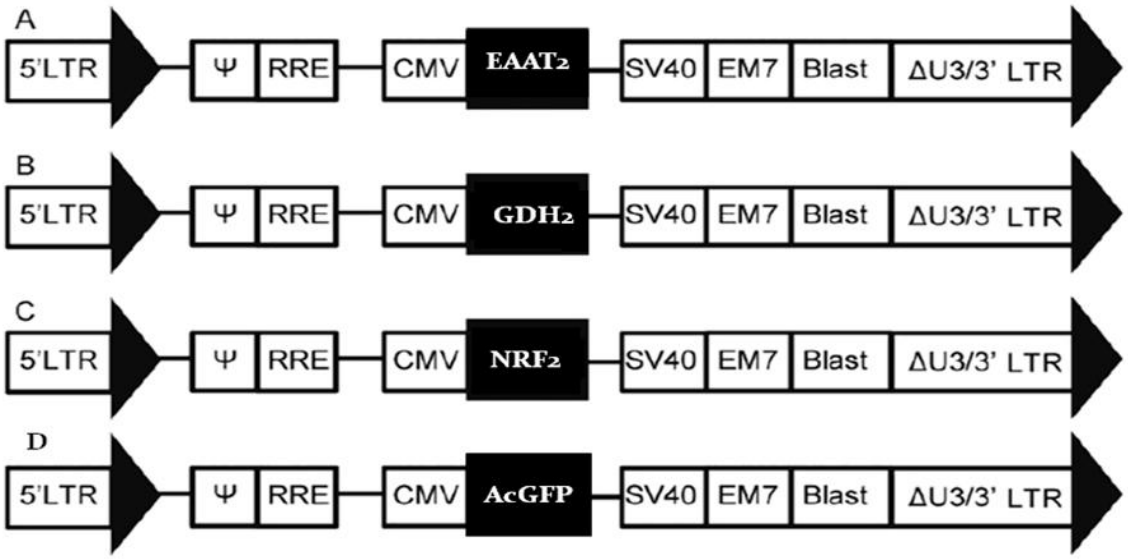


Figure 11. Schematic diagram of lentiviral vectors. An illustrating scheme for the different vectors. *All of the vectors are self inactivating vectors due to deletions in the U3 region of the long terminal repeat (ΔU3) and contain the packing signal (Ψ) and the complete RRE (Rev response element) to facilitate RNA export. Expression of the genes is controlled by the CMV promoter.*

Production of these lentiviral vectors entailed a complicated construction step, involving 3 different plasmids, 1) pEnter containing the gene of interest flagged by the attL1 and attL2

recombination sequences. 2) pEnter 5' promoter, containing the CMV promoter sequence flagged by the attL4 and attR1 recombination sequences. 3) The pLenti-DEST destination plasmid containing the attR4 and attR2 recombination sequences. These 3 plasmids were recombined using the LR Clonase II plus enzyme. This enzyme displaced the fragments flagged by the recombination sequences in a manner determined by the sequence attL1 with attR1 and so forth to produce the final pLenti6.3-CMV-GENE construct (Figure 12). Production of the viruses was performed by co-transfection of these constructs with 3 helper plasmids (pLP1, pLP2 and pLP/VSVG) into the 293T producer cell line which stably expresses the SV40 large T antigen.

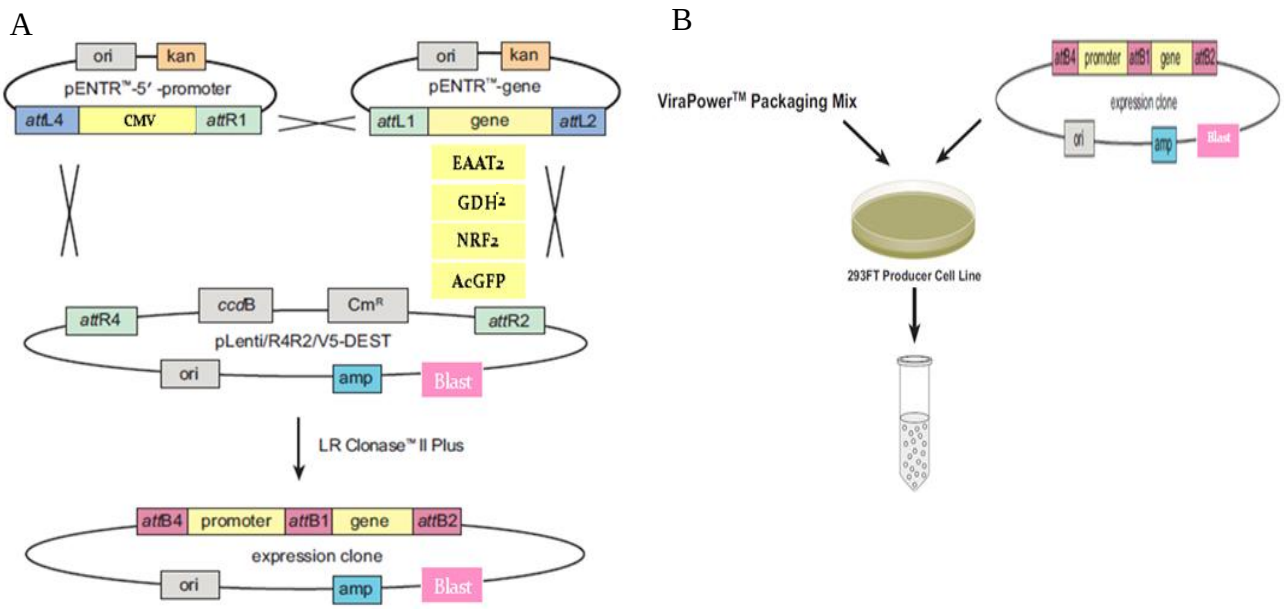


Figure 12. Schematic representation of the lentiviral vectors production process. *(A) An illustrating scheme for the creation of the different destination plasmids. (B) Schematic representation of the virion production.*

3.2 Transduction of astrocytes with EAAT2, NRF2 or GDH2 enhances the expression and function of the gene of interest

Prior to utilizing the lentiviruses in any functional experiments, we first sought to determine the transgene expression levels in transduced cells. Following transduction of primary astrocytic cultures with EAAT2, NRF2 and GDH2 at MOI 10 total RNA and protein were extracted and the genes expression levels were determined by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot analysis. As the GDH gene has no introns it is not suitable for qRT-PCR and was only quantified using western blot analysis. We found a substantial increase in the expression levels of all four genes compared to control (EAAT2 37.57±2.57 fold, NRF2 968.76±18.99 fold, GDH2. 88±0.45 fold of control expression levels, GFP levels were only detectable in cells infected with GFP viruses. Figure 13 and Figure 14)

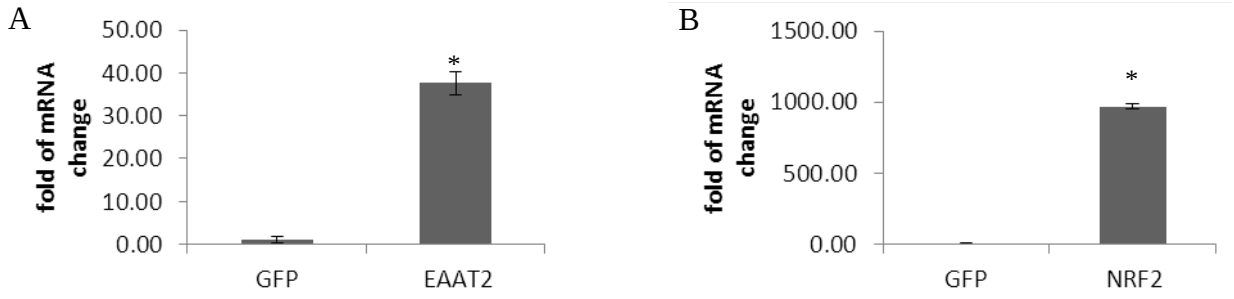


Figure 13. Evaluated mRNA levels in astrocytes infected with lentiviral vectors containing EAAT2, NRF2 or GFP 96 hours after infection.*mRNA levels of EAAT2 (A) and NRF2 (B). *p< 0.005 compared to control as determined by the students t-test. Error bars represent standard error.*

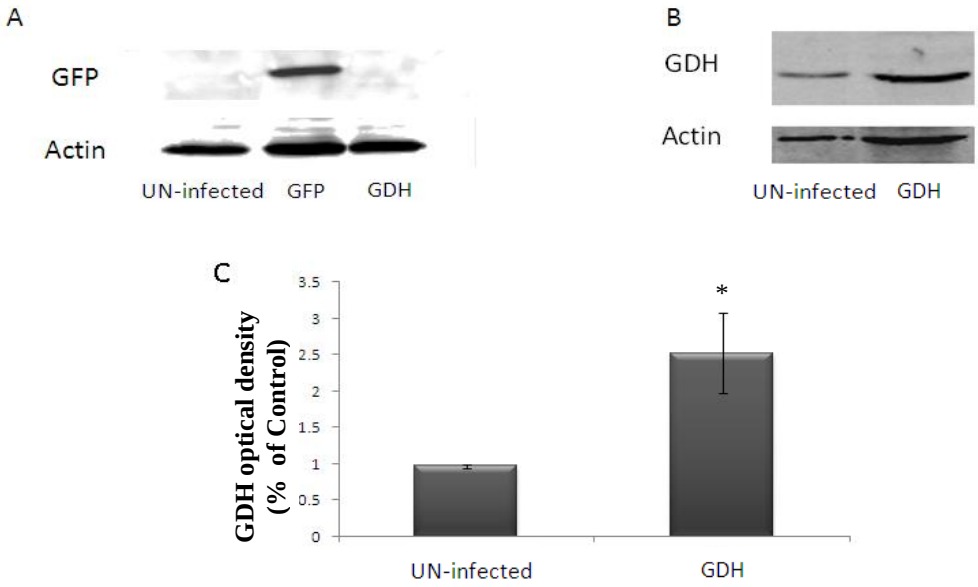


Figure 14. Evaluated protein levels in astrocytes infected with lentiviral vectors containing GDH or GFP 96 hours after infection. *(A) Representative western blot membrane of un-infected astrocytes compared to astrocytes infected with lentiviral vectors containing GFP or GDH stained for GFP. (B) Representative western blot membrane of un infected astrocytes and astrocytes infected with lentiviral vectors containing GDH 96 hours after transfection. (C) Quantification of protein levels by densitometry of immunoreactivity of GDH compared to β- actin which served as an internal protein control. *p< 0.05 compared to control as determined by the students t-test. Error bars represent standard error.*

To further evaluate the integrity and functionality of the transduced cells, we also employed an evaluation of the function of the gene of interest. The NRF2 transcription factor binds to the Anti-Oxidant Response Element (ARE) sequence so as to enhance the expression of those genes. In order to evaluate the function of cells transduced with viruses containing the NRF2 gene we utilized a Luciferase based assay, in which the Luciferase gene is controlled by a promoter containing the ARE sequence, quantifying the luminescence of Luciferase enables us to determine functionality of NRF2. In this assay we use only nuclear extracts to insure that the Luciferase luminescence is a result of functional NRF2 which has already relocated to the nuclease rather than an indication of increased cytoplasmic and non functional NRF2 levels.

We found that transduction of wild-type (WT) and SOD1 G93A astrocytes with NRF2 significantly increases Luciferase luminescence (WT-NRF2 3.13±0.3 fold of WT control,

SOD1 G93A-NRF2 2.38±0.17 fold of mutant SOD1 control). Furthermore, we found that transduction of either WT or SOD1 G93A astrocytes with viruses containing the GFP gene did not alter NRF2 function (Figure 15A).

We next sought to evaluate the effect transduction of astrocytes with lentiviruses containing EAAT2 can enhance glutamate uptake. Whereas transduction of both WT and SOD1 G93A astrocytes with GFP had no effect on EAAT2 function, transduction with lentiviruses containing EAAT2 significantly increased glutamate uptake velocity (WT-EAAT2 299.72±16.85%, SOD1 G93A 283.07±26.41% of WT control, Figure 15B)

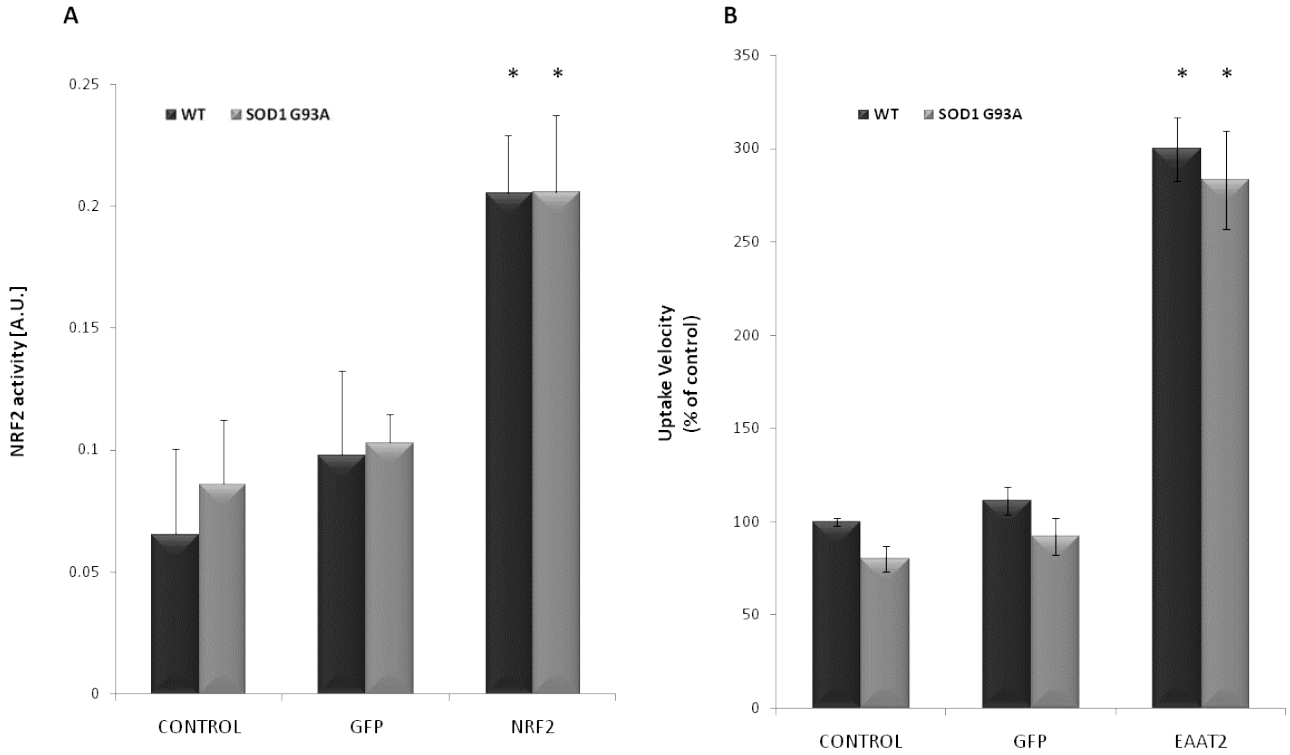


Figure 15. Transduction of astrocytes with lentiviruses increases the function of the gene of interest. *NRF2 activity 5 days after transduction (A). Uptake velocity 5 days after transduction (B). *p< 0.01 compared to control as determined by ANOVA with repeated measures. Error bars represent standard error.*

Cellular viability is another essential cellular function that could be affected by the transduction procedure. Impaired cellular viability has the potential to significantly damage the cellular neuroprotective capacity. In order to evaluate this risk, we compared the cellular

viability of transduced and non-transduced astrocytes using the AlamarBlue test. We found that transduction of astrocytes with; EAAT2, NRF2, GDH2 or GFP or their combination did not hinder their viability (Figure 16)

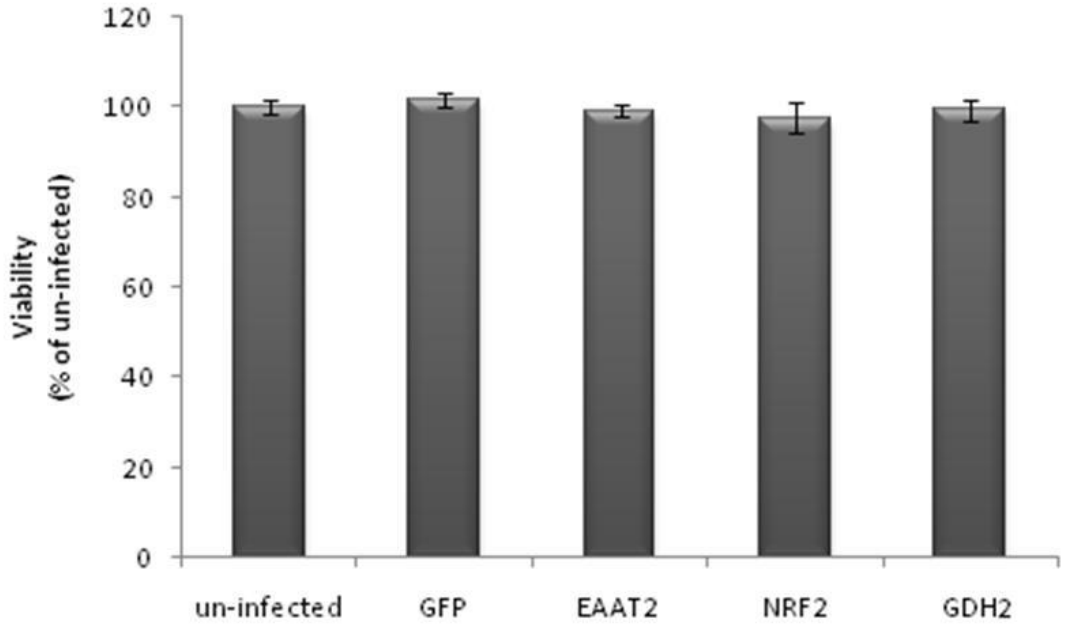


Figure 16. Astrocyte viability is not affected by infection with lentiviral vectors containing GFP, EAAT2, NRF2 or GDH2. *Error bars represent standard error.*

4. Expression of the genes EAAT2, NRF2 and GDH2 in astrocytes increases their neuroprotective capacities

In an attempt to determine the most neuroprotective combination of genes prior to *in vivo* experiments as well as verify the synergistic relationship between the genes, we conducted *in vitro* experiments in primary cortical astrocyte cultures and in cells of the motor neuron like cell line NSC-34. To most closely mimic the conditions occurring in the SOD1 G93A mice we concentrated on cells expressing the SOD1 G93A mutation for both astrocytes and neurons.

4.1 Characterization of astrocytic and neuronal sensitivity to glutamate excitotoxicity and oxidative stress

4.1.1 Astrocytic sensitivity to glutamate excitotoxicity and oxidative stress

The astrocytic sensitivity to oxidative stress was determined using the AlamarBlue viability test. Astrocytes were exposed to a range of hydrogen peroxide concentrations and their viability was measured. A significant and dose dependent cell death was observed for WT astrocytes beginning at a concentration of 40nM H_2O_2. SOD1 G93A astrocytes were slightly more sensitive to oxidative stress with reduced cellular viability commencing at a concentration of 30nM H_2O_2 (Figure 17). As previously shown (Figure 10 and Benkler, et al., 2013), we have already established that astrocyte viability is not effected by relatively high and extremely neurotoxic glutamate concentrations.

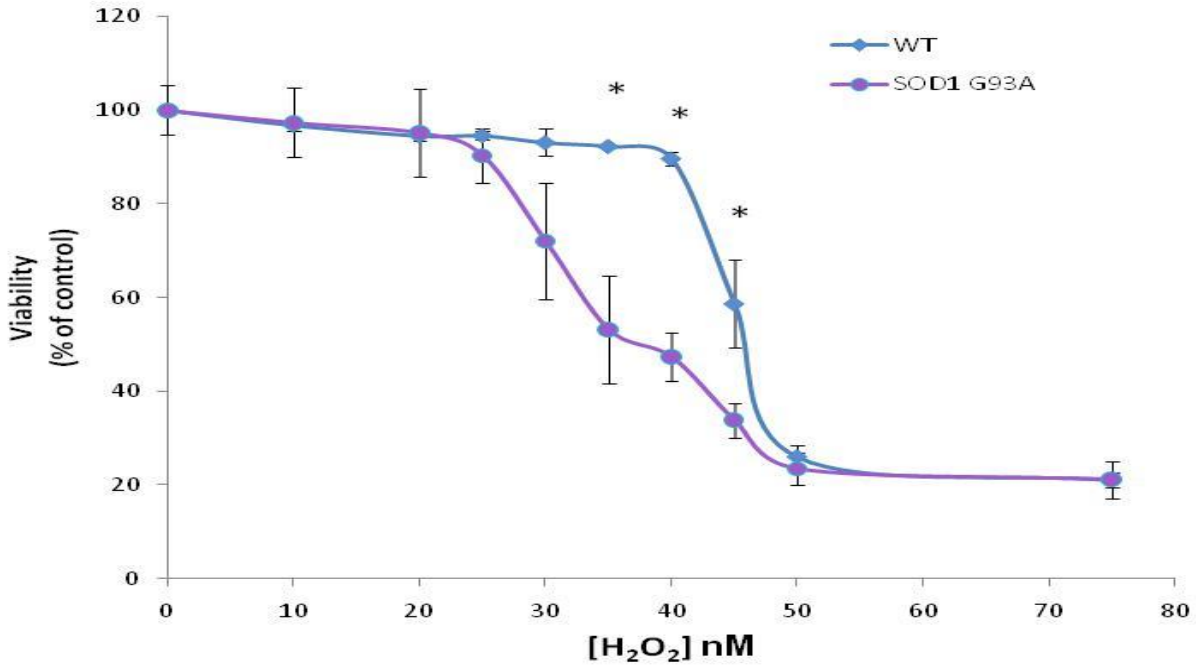


Figure 17. Effect of exposure to 0-75nM H_2O_2 on WT and SOD1 G93A astrocytes. *Astrocytes derived from SOD1 G93A mice exhibit slightly elevated sensitivity to H_2O_2 compared to their WT counterparts. *$p < 0.05$ compared to control. Error bars represent standard error.*

4.1.2 **Neuronal sensitivity to excito-oxidative stress**

To most closely resemble the conditions occurring in the SOD1 G93A mice we decided to use cells of the motor neuron like cell line NSC-34 that express the mutated form of SOD1. These cells can be induced to overexpress the SOD1 G93A mutant, as a control to these cells we selected NSC-34 cells that can be induced to overexpress the WT form of the SOD1 enzyme. Prior to induction only very few NSC-34 cells express the transgene (Figure 18A-C). However, 24 hours after exposure to doxycycline we can see extremely high SOD1 G93A expression levels (Figure 18 D-F)

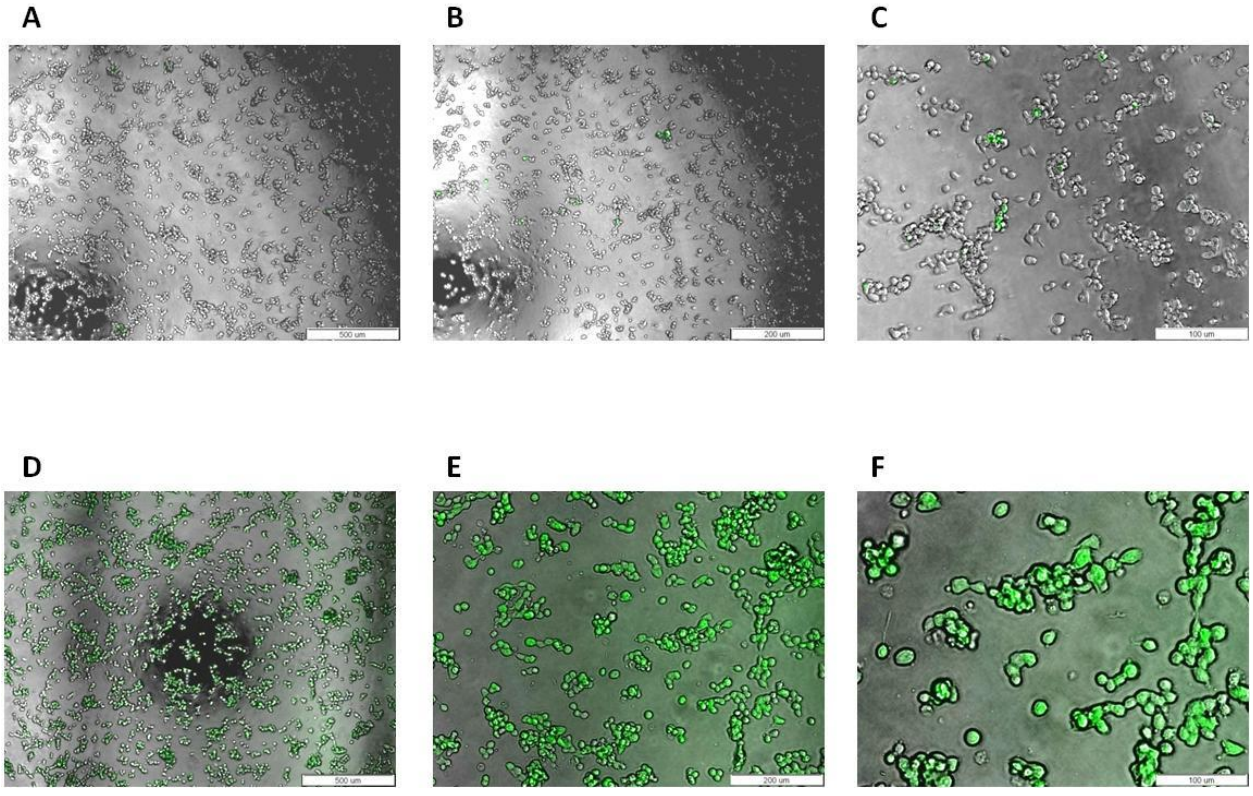


Figure 18. Induction of SOD1 G93A in NSC-34 cells. *In these NSC-34 cells the SOD1 G93A transgene is coupled to the GFP protein, before induction we can see relatively low leakage levels as GFP can be seen in only a few cells (A-C). 24 hours after induction with doxycycline (1μg/ml) the expression levels of the GFP coupled transgene significantly increase (D-F). (A, D) scale bar: 500μm, (B, E) scale bar: 200μm, (C, F) scale bar: 100μm.*

We previously established that differentiation of NSC-34 cells enhances the cellular motor neuron like properties as well as their sensitivity to glutamate toxicity (Figure 9, Figure 10 and Benkler, et al., 2013). Therefore in the following experiments we only used differentiated cells.

To examine the sensitivity of differentiated NSC-34 cells to glutamate excitotoxicity and oxidative stress both mutant and WT SOD1 overexpressing cells were exposed for 16 hours to a range of glutamate (0mM-9mM) or hydrogen peroxide (0-50nM) concentrations. Cellular viability following exposure to insult was assessed. A significant and dose dependent cell death was observed for mutant and WT SOD1 overexpressing cells beginning at 3mM glutamate and 10nM H_2O_2 (Figure 19A, B).

It is currently thought that the pathophysiology of ALS involves glutamate excitotoxicity as well as glutamate dependent and independent oxidative stress, to more closely model this effect we used a system that inflicts glutamate excitotoxic insult and oxidative stress (excito-oxidative insult). To evaluate the sensitivity of WT and mutant SOD1 overexpressing NSC-34 cells to this form of insult we exposed the cells to a range of glutamate concentrations (2-5mM) as well as a range of H_2O_2 concentrations (0-50nM) simultaneously (Figure 19 C-F). Applying combined excito-oxidative insult can lead to similar cell death levels as the individual insults but at lower concentrations of each individual insult. SOD1 G93A overexpressing NSC-34 cells were slightly more sensitive to the combined insult compared to their WT controls, particularly at the higher H2O2 concentrations (Figure 19 C-F).

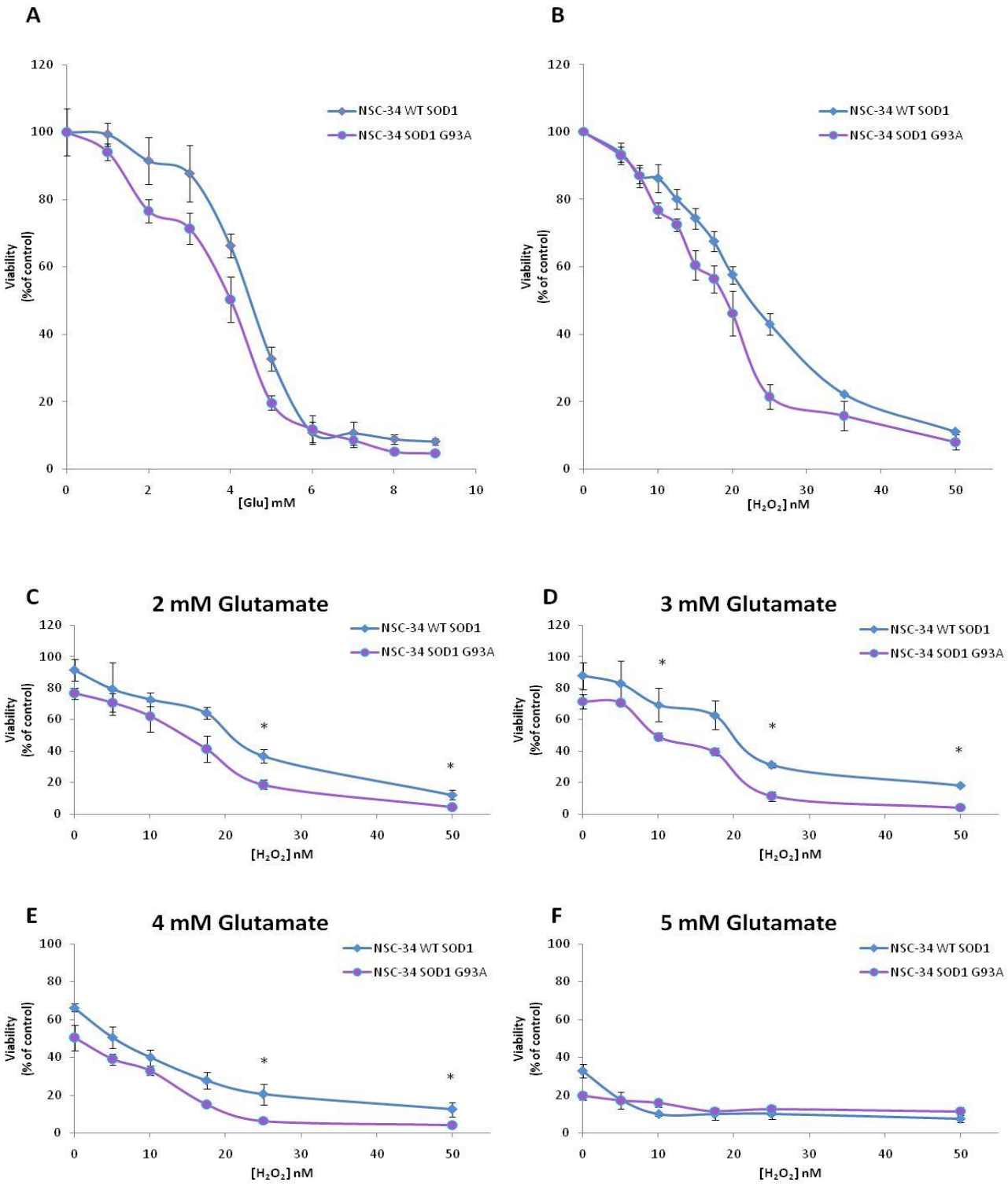


Figure 19. Differentiated NSC-34 cells are sensitive to glutamate excitotoxicity, oxidative stress and excito-oxidative insult*. Effect of exposure to 0-9mM glutamate (A) or 0-50nM H2O2 (B) on differentiated NSC-34 cells induced to express the WT or mutated form of the human SOD1 enzyme. Effect of exposure to excito-oxidative insult on the different NSC-34 cells a range of 0-50nM H2O2 concentrations was used coupled with 4 different glutamate concentrations 2mM (C), 3mM (D), 4mM (E), 5mM (F).NSC-34 cells expressing the SOD1 G93A gene are slightly more sensitive to excito-oxidative stress at high H_2O_2 concentrations.*p< 0.05 compared to control. Error bars represent standard error.*

4.2 Expression of the genes EAAT2, NRF2 and GDH2 in SOD1 G93A astrocytes synergistically increases their neuroprotective capacities

To examine our hypothesis that the three genes we selected; EAAT2, NRF2 and GDH2 could have a neuroprotective therapeutic effect we performed *in vitro* neuroprotection experiments. To most closely simulate the disease conditions occurring in the SOD1 G93A mice, we choose to challenge our motor neuron like cells with a mixed excito-oxidative insult and attempt to protect them using astrocytes derived from SOD1 G93A mice. For the neuronal cultures we selected differentiated NSC-34 cells overexpressing either the WT or the mutated form of the SOD1 gene.

The astrocytes were first plated on the upper level of the transwell co-culture system. The following day, the astrocytes were transduced with GFP, EAAT2, NRF2, GDH2 or different combinations of those genes. The astrocytes were allowed 5 days prior to further evaluation to insure proper transgene expression. Differentiated WT or mutant SOD1 overexpressing NSC-34 cells were plated on the bottom well 24 hours prior to exposure to the insult. At this point astrocyte transwells were transferred into the wells containing the NSC-34 cells and this co-culturing transwell system was exposed to excito-oxidative stress for 16 hours. Then the transwell containing the astrocytes was separated from the NSC-34 cells and the neuronal or astrocytic viability could be assessed separately (Figure 20).

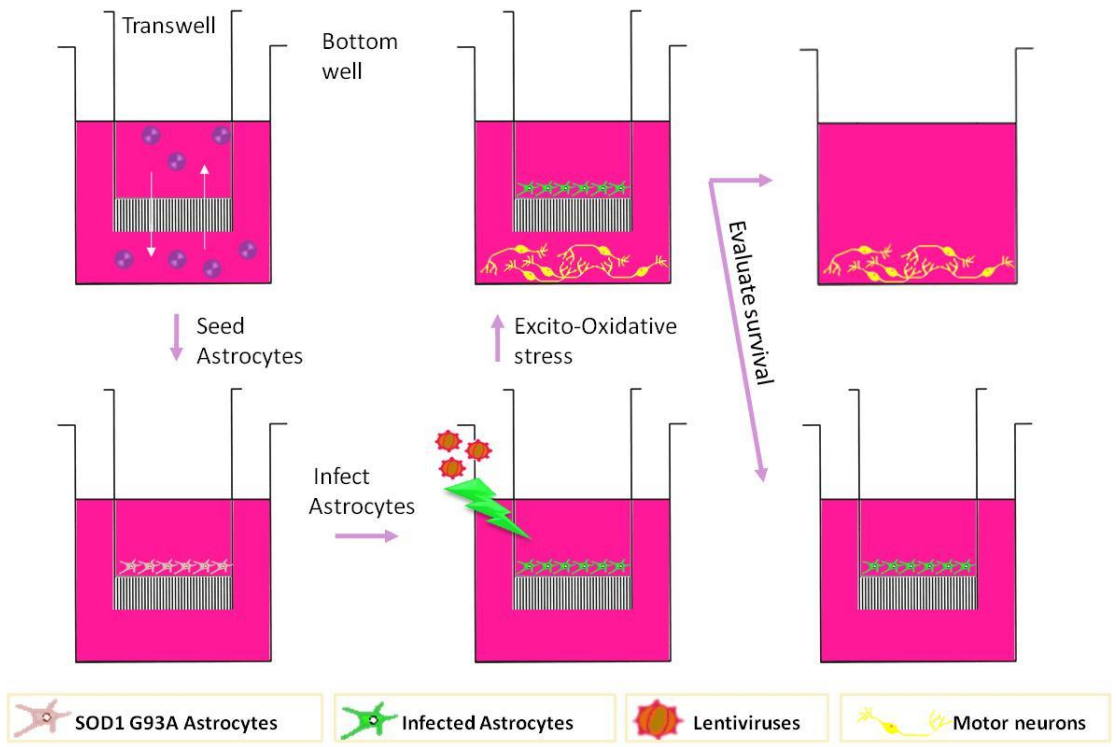


Figure 20. A schematic representation of the co-culturing transwell system used to evaluate the neuroprotective effect of lentiviral infected astrocytes. *Astrocytes were infected with GFP, EAAT2, NRF2, GDH2 or different combinations of these genes.*

To explore the neuroprotective capacities of SOD1 G93A astrocytes we selected to expose the co-culture transwell system to a combination of excitatory and oxidative insults (excito-oxidative insult) at a concentration that significantly hindered the neuronal viability (3.5 mM glutamate combined with 17.5 nM H_2O_2, SOD1 G93A-NSC-34; 44.42±0.33% of control viability and WT SOD1-NSC-34; 46.07±0.70% of control viability, Figure 21A-C). Co-culturing both WT and mutant SOD1 overexpressing NSC-34 cells with naïve or GFP transduced astrocytes slightly increased their viability (naïve astrocytes 55.31±1.60%, 51.08±0.95% of control viability respectively, GFP transduced astrocytes 56.08±1.67%, 51.28±2.72% of control viability respectively, Figure 21A-C). Transduction of the SOD1 G93A astrocytes with one of the three genes, EAAT2, NRF2, GDH2 slightly elevated their neuroprotective potential by an additional 5-10% compared to naïve astrocytes, this neuroprotective effect was similar to that achieved by co-culturing the neuronal cells with WT rather than SOD1 G93A astrocytes (WT SOD1 overexpressing NSC-34; 9.74±2.54%, 12.18±4.47%, 6.49±1.64% and 9.12±2.89% added neuroprotection on-top of the protection provided by naïve-un-transduced astrocytes respectively. SOD1 G93A overexpressing NSC-34;6.64±0.41%, 8.58±3.12%, 5.02±1.73% and 6.71±1.37% added neuroprotection on-top of

the protection provided by naïve-un-transduced astrocytes respectively. Figure 21). Transduction of the astrocytes with different combinations of two of the three genes increased their neuroprotective effect even further (WT SOD1 NSC-34; EAAT2 with NRF2 15.60±1.94%, EAAT2 with GDH2 22.53±0.76% and NRF2 with GDH2 9.41±2.81% added neuroprotection on-top of the protection provided by naïve-un-transduced astrocytes. SOD1 G93A NSC-34; EAAT2 with NRF2 12.13±2.72%, EAAT2 with GDH2 10.80±0.61% and NRF2 with GDH2 10.07±0.54% added neuroprotection on-top of the protection provided by naïve-un-transduced astrocytes. Figure 21).

However, by far, the most neuroprotective effect was achieved by transduction of the astrocytes with all three genes simultaneously, with the neuronal survival reaching approximately 90% viability (WT SOD1 NSC-34; 96.75±1.32%, SOD1 G93A NSC-34; 88.26±0.32% compared to neuronal cells not exposed to excito-oxidative insult, Figure 21 A-C).

We found that the neuroprotection provided by the differently transduced astrocytes was very similar when they were protecting either WT or mutant SOD1 overexpressing astrocytes, with SOD1 G93A overexpressing NSC-34 cells being slightly more sensitive to the excito-oxidative stress (SOD1 G93A-NSC-34; 44.42±0.33% and WT SOD1-NSC-34; 46.07±0.70% of control viability, Figure 21 C, F)

In these experiments we could clearly establish for the first time the dramatic synergistic relationship existing between these three lentiviral genetic constructs. This could be determined by comparing the increase in the neuroprotective potential of astrocytes provided by each of the individual genes; EAAT2, NRF2 and GDH2 to the increase in neuroprotective potential provided by the combination of all three genes (WT SOD1 NSC-34; 9.74±2.54%, 6.93±3.87%, 6.49±1.64% compared to 41.44± 1.32% added neuroprotection on-top of the protection provided by naïve-un-transduced astrocytes respectively. SOD1 G93A NSC-34; 6.64±0.41%, 8.58±3.12%, 5.02±1.73% compared to 37.17±0.32% added neuroprotection on-top of the protection provided by naïve-un-transduced astrocytes respectively. Figure 21 D-F). The increase in neuroprotection exerted by astrocytes transduced with all three genes far exceeded the sum of that exerted by each individual gene, as well as that achieved by any partial combination between the three genes (Figure 21 D-F).

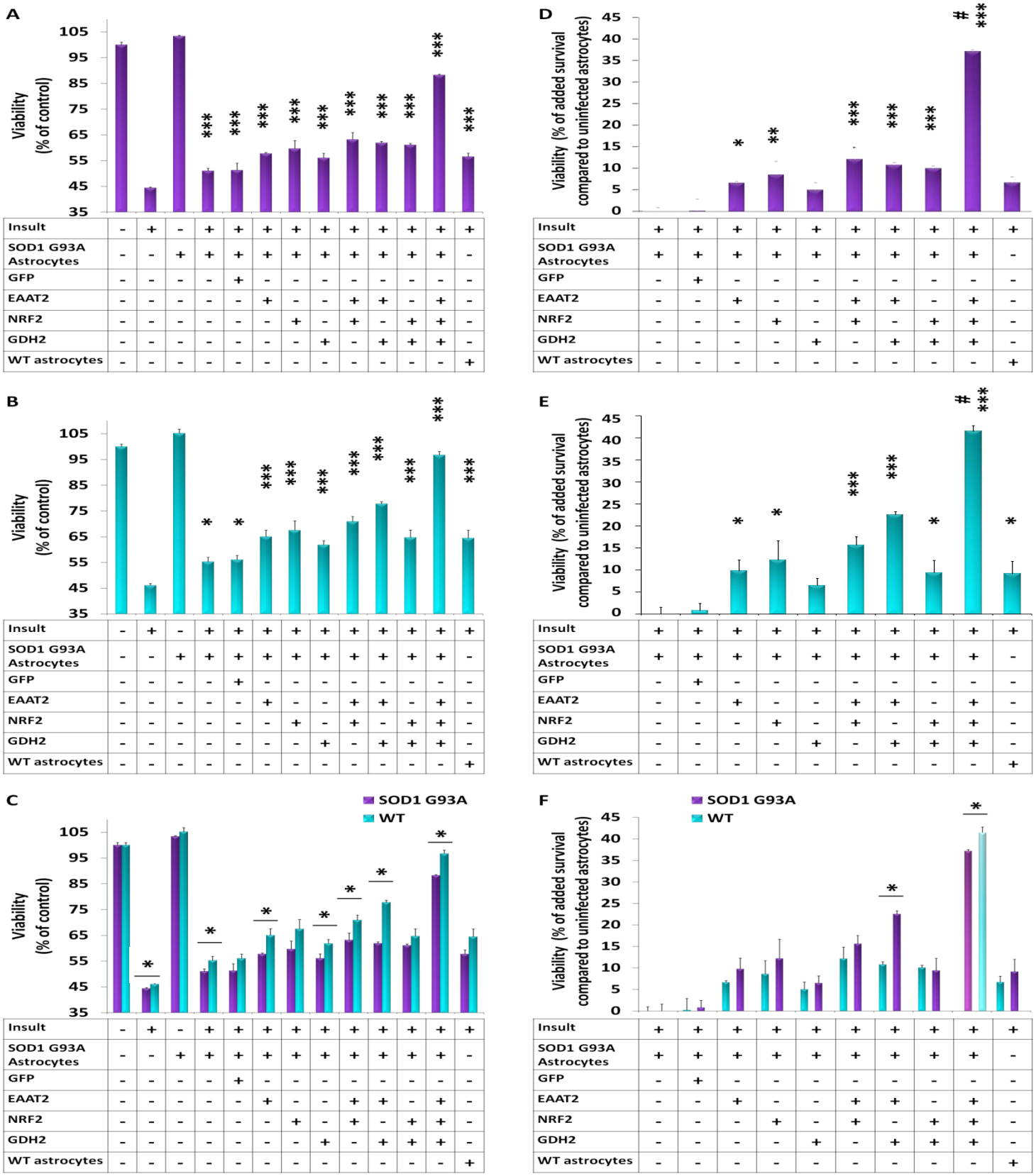
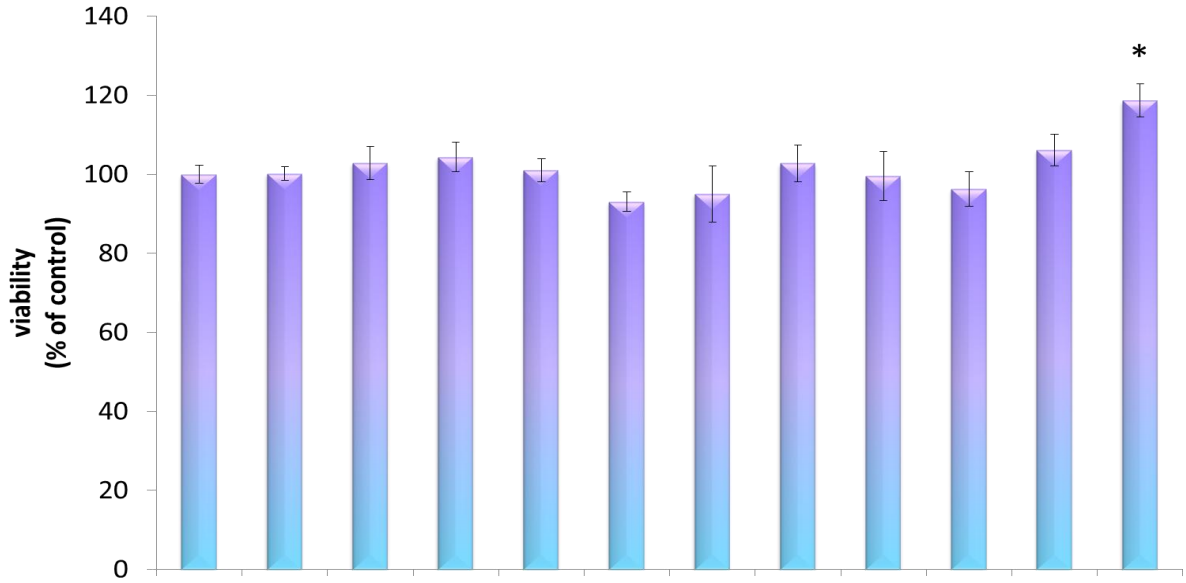


Figure 21. Expression of the genes EAAT2, NRF2 and GDH2 in SOD1 G93A astrocytes increases their neuroprotective capacities in a synergistic fashion. *The neuroprotective effect of astrocytes transduced with different combinations of the EAAT2, NRF2 and GDH2 genes. Transduced astrocytes were co-cultured with NSC-34 cells overexpressing SOD1 G93A (A) or WT SOD1 (B) and subsequently challenged with excito-oxidative insult (3.5mM Glutamate combined with 17.5nM H_2O_2) for 16 hours. Following the insult the neuronal viability was assessed. (C) A comparison between the neuroprotection provided by transduced astrocytes to NSC-34 cells overexpressing SOD1 G93A or WT SOD1. Evaluation of the synergistic effect provided by transduction of astrocytes with the different genetic combinations. The percent of neuroprotection added to the neuroprotection capacities of naïve astrocytes by transduction with different combinations of the EAAT2, NRF2 and GDH2 genes, the astrocytic neuroprotective effect was evaluated for NSC-34 cells overexpressing SOD1 G93A (D) or WT SOD1 (E). A comparison between the added neuroprotection provided by transduced astrocytes to NSC-34 cells overexpressing SOD1 G93A or WT SOD1.Percent of cell viability was calculated as the percentage of NSC-34D viability exposed to transduced astrocytes and insult as compared to NSC-34 cell viability when not exposed to astrocytes or insult. *p< 0.05, **p< 0.01,*** p< 0.001; as compared to control (A-C, NSC-34 cells with insult, D-F, NSC-34 cells with insult and Naïve astrocytes) In the synergistic experiments, # p< 0.05 as compared to astrocytes transduced with two genes).*

We next sought to insure that the different effect provided by astrocytes transduced with all the different genetic combinations was directly related to their neuroprotective capacities and not to any astro-protective properties the three genes might have. To achieve this goal we next evaluated the effect of the excito-oxidative insult used in the co-culturing experiments with or without transduction of the astrocytes. We found, that transduction of the astrocytes with the viral vectors had no effect on the astrocytic viability, nor did exposure to the excito-oxidative insult in any of the groups evaluated. The only group that showed slightly elevated astrocytic viability following the excito-oxidative insult was that of astrocytes derived from WT mice compared to the rest of the groups that were derived from SOD1 G93A mice (118.66±12.67% viability compared to control, Figure 22).



Insult	-	+	-	+	+	+	+	+	+	+	+	+
SOD1 G93A Astrocytes	+	+	+	+	+	+	+	+	+	+	+	-
GFP	-	-	+	+	-	-	-	-	-	-	-	-
EAAT2	-	-	-	-	+	-	-	+	+	-	+	-
NRF2	-	-	-	-	-	+	-	+	-	+	+	-
GDH2	-	-	-	-	-	-	+	-	+	+	+	-
WT astrocytes	-	-	-	-	-	-	-	-	-	-	-	+

Figure 22. The astrocytic viability is not affected by transduction or excito-oxidative stress. *The viability of WT or SOD1 G93A astrocytes in the presence of excito-oxidative insult (3.5mM glutamate combined with 17.5mM H2O2), in response to the transduction procedure or a combination of the two.*p< 0.05 compared to control. Error bars represent standard error.*

5. Treating SOD1 G93A ALS mice with the viral vectors engineered to harbor the genes: EAAT2, NRF2 and GDH2

5.1 Injection of genetically engineered lentiviral vectors

In order to evaluate the therapeutic potential of overexpressing EAAT2, NRF2 and GDH2 we first had to overcome the obstacle of successful gene delivery. The first hurdle we had to circumvent was establishing an efficient mode of lentiviral administration. To this end we selected two administration locations with high dispersal potential; direct intra-cisternal injection and intra-muscular injection. The intra-cisternal rout delivers the viruses into the animals CSF, the viruses are then carried with the CSF allowing them to infect cells dispersed throughout the brain and spinal cord. To increase the penetration potential of the viruses, intra-cisternal injections were accompanied by pre-treatment with mannitol, a substance that when given at high dose opens the blood brain barrier and has been previously reported to increases the lentiviral infectious zone (Fu, et al., 2007; Louboutin, et al., 2012).On the other hand, intra muscular injections have the potential of directed and more localized viral expression. When delivered into the muscle, the mostly neurotropic lentiviruses more potently infect the nerve terminals innervating the injected muscle rather than the muscle itself. The viruses are then retrogradely transported to the neuronal cell body where they can integrate into the cellular DNA. This mode of delivery leads to the forming of select smaller motor neuron pools with higher local transduction efficiency compared to intra cisternal injection. To evaluate the dispersal of the lentiviruses, animals were injected both intra-cisternaly and intra-muscularly with GFP carrying lentiviruses. The mouse spinal cords were

harvested 70 days after the administration and stained for GFP We found GFP expressing cells dispersed throughout the spinal cord (Figure 23).

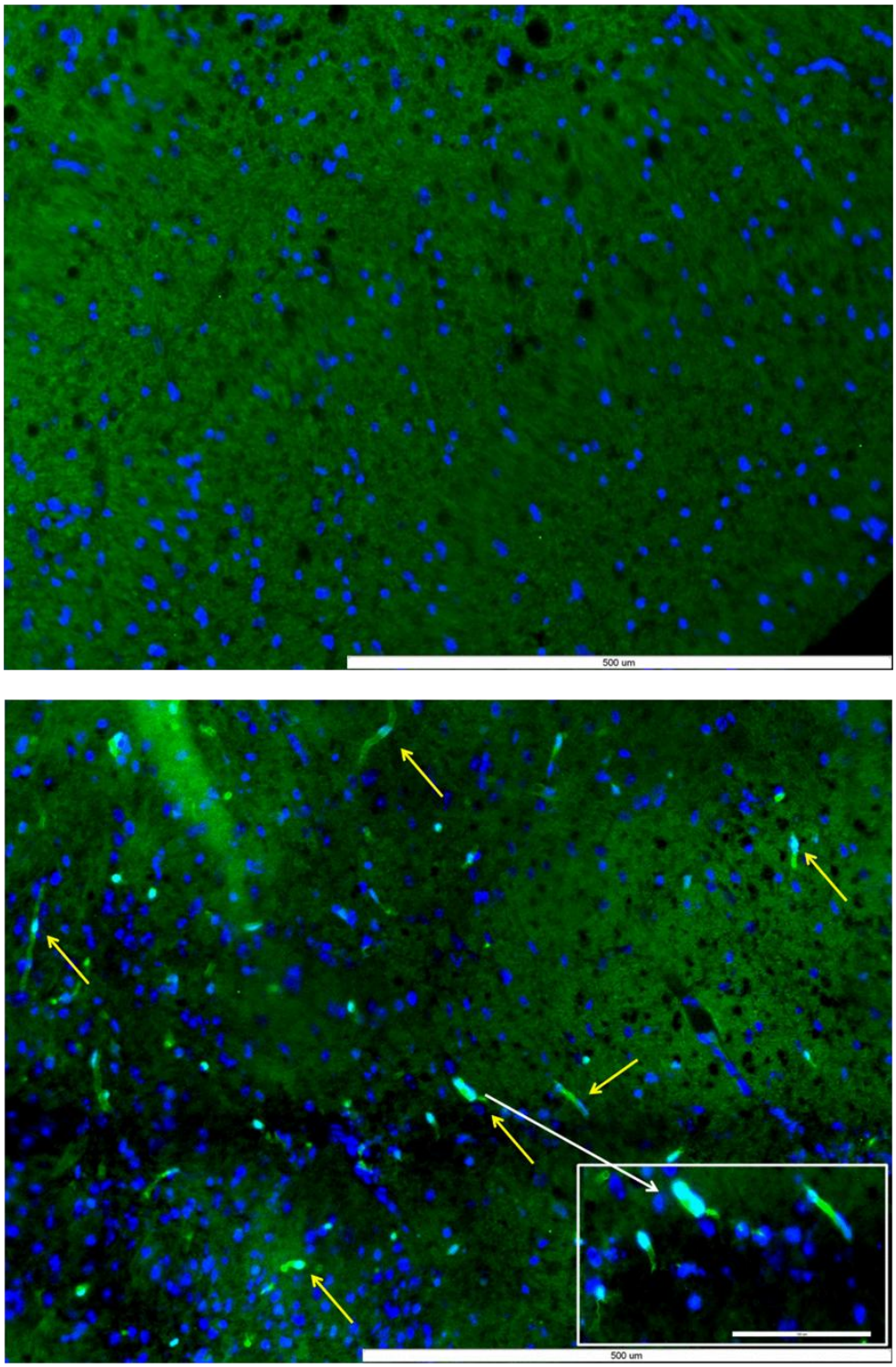


Figure 23. Injection of lentiviral vectors containing GFP and dispersal through the spinal cord.*(A) A representative image of a spinal cord cross-section of a control mouse that received saline injections. (B)A representative image of a spinal cord cross-section of a mouse that received GFP injections, the arrows points to a group of cells expressing GFP. The inlay image is a higher resolution magnification of the image. Scale bars are 500μm, the scale bare of the inlaid image is 100μm.*

5.2 Evaluating the therapeutic potential of genetically engineered lentiviral vectors in an ALS mouse model

At this stage we wanted to assess the *in vivo* effect of our constructs thus evaluating the potential neuroprotective effect our gene therapy might have in an animal model of ALS. To this end we established a colony of SOD1 G93A mice (Figure 24). The mice randomly distributed into groups differentiating between genders. The mice received either different lentiviral combinations or a non-viral saline solution. All mice were injected both intra-cisternaly and intra-muscularly.

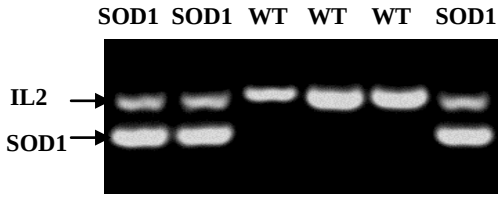


Figure 24. Identification of mice harboring the human SOD1 G93A gene. *Newborn mice were genotyped by PCR analysis using IL2 and human SOD1 G93A primers. Mice carrying the human SOD1 G93A gene appear with two positive bands, one belonging to each gene amplified. Wild type littermates are only positive for the IL2 gene and appear with only one band.*

In this ALS mouse model the pre-clinical symptoms such as reduced electrical nerve conductivity begin at the age of 40 days postnatal. The symptoms slowly progress, motor function begins to deteriorate at 120 days and full clinically visible symptoms appear at the age of 140 days. From the onset of clinical symptoms the disease progresses very quickly and the mice die at an average age of 155 days (Gurney, et al., 1994; Shibata, 2001; Heiman-Patterson, et al., 2011; Dadon-Nachum, et al., 2011; Hegedus, et al., 2009; Mohajeri, et al., 1999; Gruzman , et al., 2007; Turner , et al., 2008).

We decided to administer the treatment at the age of 65 days, the transgene expression slowly builds up for approximately 10-15 days (Figure 25).

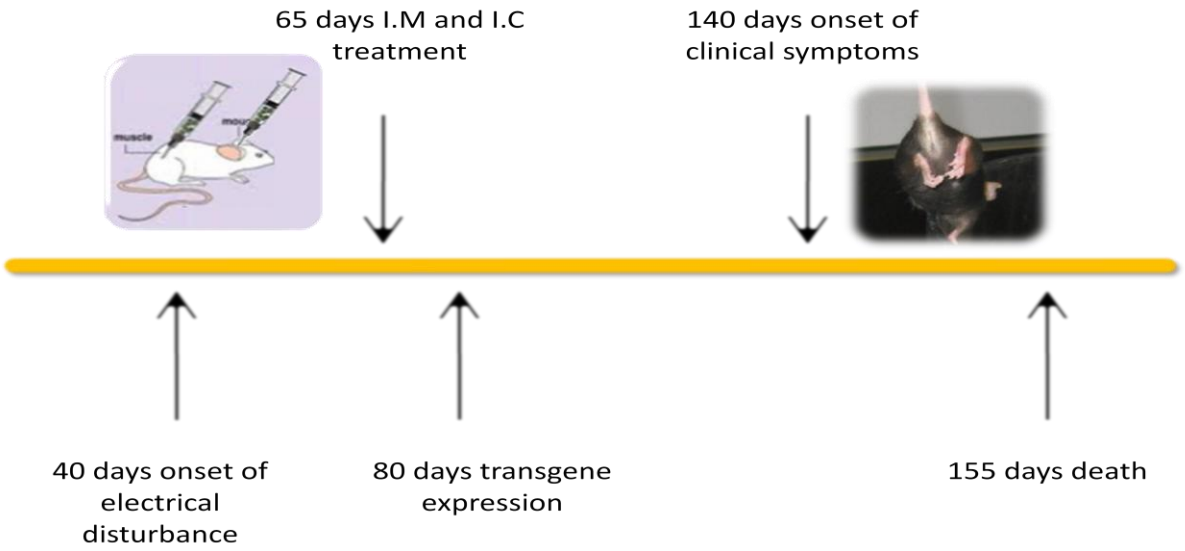


Figure 25. A schematic representation of the experimental design depicting the administration of treatment in relation to the disease progression.

5.2.1 **Combined treatment with EAAT2, NRF2 and GDH2 lentiviruses has a synergistic effect**

We next sought to evaluate whether the synergistic effect that we observed in our *in vitro* experiments also exists in treated mice and determine the treatment with the highest therapeutic potential. To this end we divided the mice into 12 groups, differentiating between genders, each one of the groups was treated with either Saline (male n=6, female n=5), GFP (male n=5, female n=8), EAAT2 (male n=7, female n=8), NRF2 (male n=4, female n=7), GDH2 (male n=4, female n=5) or a mixture of all three genes (MIX, male n=6, female n=9). The neurological score and weight of the male and female groups was recorded at the age of 17 weeks and 18 weeks respectively. We found that treatment with GFP or each of the

individual genes separately was not sufficient to provide a therapeutic effect. However, treatment with all three genes combined had the ability to preserve the animals' neurological score and weight. On average, male mice treated with all three genes had a neurological score 2.25±0.28 points higher and weighed 6.05±0.33% more than their saline treated counterparts (Figure 26 A, C). Whereas female mice, treatment with all three genes had a neurological score 1.5±0.26 points higher than their saline treated counterparts (Figure 26 B). However, as at this age female mice have not yet started the accelerated weight loss phase the weight of treated females was not significantly different than their saline treated counterparts (at this age we could only observe a trend towards weight preservation, Figure 26 D).

The synergistic relationship demonstrated in this result is strengthened even further by this particular experimental design in which the mixed treatment group that received all three genes was treated with a total viral amount equivalent to that given for each individual gene, leading to a situation in which this group only received a third of the amount of each gene given to the individual gene groups.

Taken together these results suggest a strong therapeutic potential of combined treatment with lentiviruses genetically engineered to express the genes; EAAT2, NRF2 and GDH2.

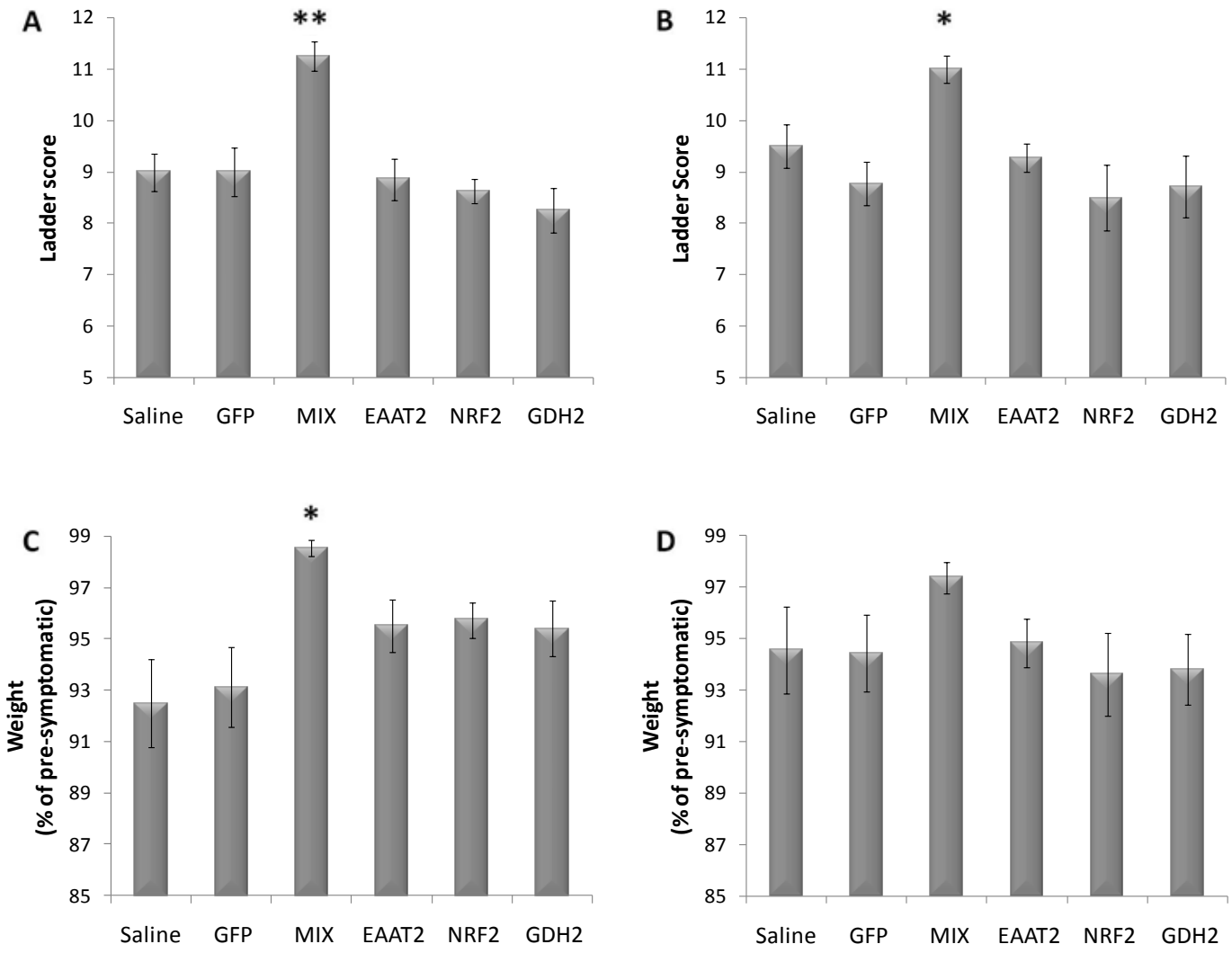


Figure 26. Combined treatment with EAAT2, NRF2 and GDH2 has a therapeutic effect not achieved by treatment with each gene individually. *Combined treatment preserved male (A) and female (B) neurological score and body weight (C, D respectively). *P<0.05, **P<0.001 as determined by ANOVA.*

5.2.2 **Treatment with EAAT2, NRF2 and GDH2 lentiviruses preserves body weight**

To evaluate the potential relevance of our treatment for clinical use we aimed at evaluating several key disease parameters. To this end we selected 150 mice of our SOD1 G93A colony, in accordance with our privies study design (Figure 25), the mice were

randomly distributed into 12 groups, 6 groups of male mice and 6 groups of female mice preserving a stud-mate design. At the age of 65 days mice received either lentiviruses carrying GFP, or one of the three genes or a mixture of lentiviruses carrying EAAT2, NRF2 and GDH2. A non-viral saline control was also evaluated. All mice were injected both intra-cisternaly and intra-muscularly and a minimum of 12 animals per group was maintained.

A treatments influence on the animal's body weight is considered to be one of the best predictors of clinical success in the pre-clinical stage of any ALS treatment. Mice treated with saline or GFP lost 15% of their body weight at the age of 20 and 21 weeks in males respectively and 22 weeks in both female groups. Our treatment was able to significantly delay the loss of body weight in both male and female mice reaching the same level of body weight loss at the age of 23 and 24 weeks, respectively (Figure 27).

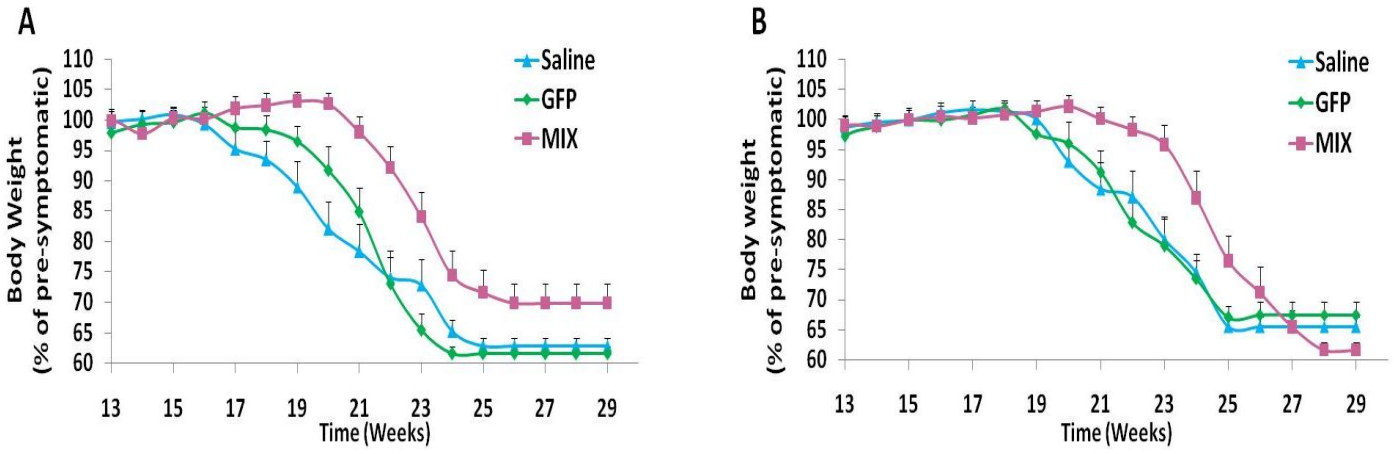


Figure 27. Treatment with EAAT2, NRF2 and GDH2 containing lentiviruses preserves body weight in SOD1 G93A ALS mice. *The mice were injected with saline, lentiviruses containing GFP or a mixture of three types of lentiviruses each containing one of the three genes; EAAT2, NRF2 or GDH2 (MIX). Male (A) and female (B) body weight loss during disease progression (for MIX, $P<0.001$ as determined by two way ANOVA for repeated measures, $12<n>17$ animals per group per gender).*

5.2.3 **Treatment with EAAT2, NRF2 and GDH2 lentiviruses reduces the loss of hindlimb reflex**

When healthy mice are lifted by their tail they reflexively extend their legs backwards to improve their balance. As the disease progresses their ability to extend their legs decreases until they can no longer extend them at all. Mice treated with saline or GFP lost 50% of their hindlimb reflex between the ages of 17to18 weeks in males, and 18-19 weeks in females. Our treatment was able to significantly delay the loss of hindlimb reflex in both male and female mice reaching the 50% loss of reflex between the ages of 20 and 21 weeks for males and 22 to 23 weeks in females (Figure 28).

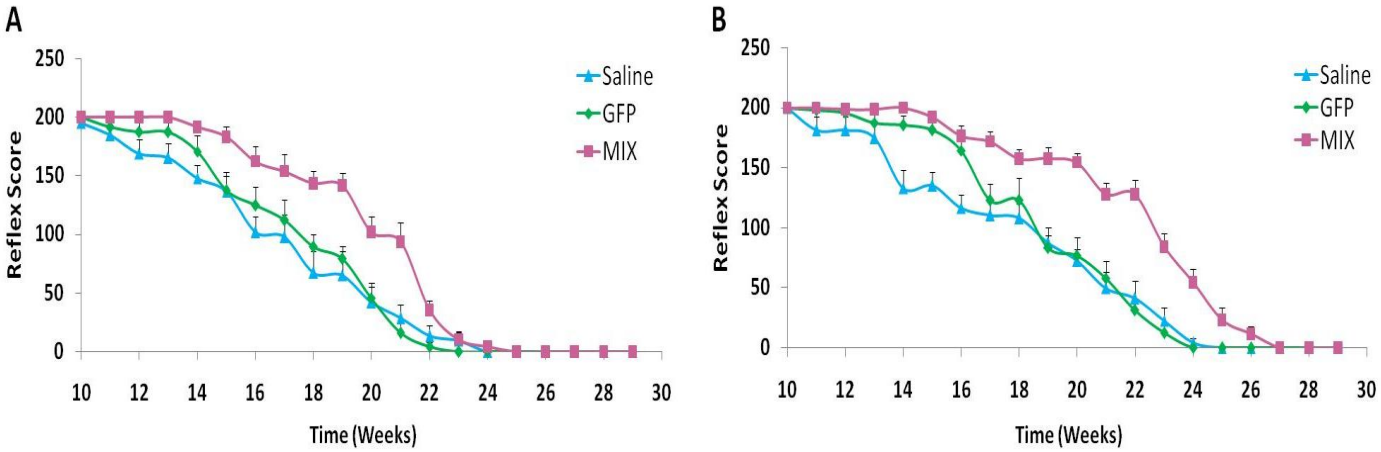


Figure 28. Treatment with EAAT2, NRF2 and GDH2 containing lentiviruses reduces the loss of hindlimb reflexes in SOD1 G93A ALS mice. *The mice were injected with saline, lentiviruses containing GFP or a mixture of three types of lentiviruses each containing one of the three genes; EAAT2, NRF2 or GDH2 (MIX). Male (A) and female (B) hindlimb reflex loss during disease progression (for MIX, P<0.001 as determined by two way ANOVA for repeated measures. 12<n>17 animals per group per gender).*

5.2.4 **Treatment with EAAT2, NRF2 and GDH2 lentiviruses protects motor function**

We next evaluated the effect our treatment has on motor performance. Motor function was determined by Rotarod measurement. In a similar fashion to the results observed so far, we found that our treatment was able to significantly slow down the deterioration of motor function. Mice treated with saline or GFP reached 65% of their pre-symptomatic motor capabilities at the age of 17 to 18 weeks in males respectively and 19 weeks in both female groups. Our treatment was able to significantly delay this loss in both male and female mice reaching the 65% mark at the ages of 20 and 22 weeks respectively (Figure 29).

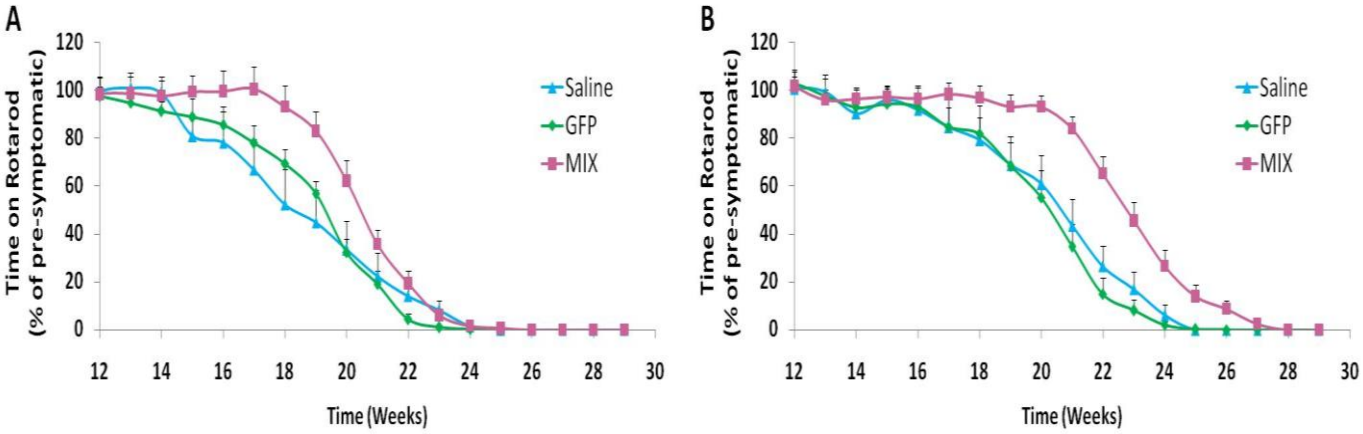


Figure 29. Treatment with EAAT2, NRF2 and GDH2 containing lentiviruses protects motor function in SOD1 G93A ALS mice. *The mice were injected with saline, lentiviruses containing GFP or a mixture of three types of lentiviruses each containing one of the three genes; EAAT2, NRF2 or GDH2 (MIX). Male (A) and female (B) motor function deterioration during disease progression as determined by Rotarod (for MIX, $P<0.001$ as determined by two way ANOVA for repeated measures. $12<n>17$ animals per group per gender).*

5.2.5 **Treatment with EAAT2, NRF2 and GDH2 lentiviruses preserves neurological score**

The parameters evaluated up until this point are rather rigid parameters. We next sought to evaluate a more refined parameter of the disease progression. Use of such a parameter would allow us to observe the progression of the disease in a more sensitive way and observe

more subtle changes. One such parameter is evaluation of mice neurological score using the ladder test. Unlike the other parameters evaluated the sensitivity of this test enabled us to start observing symptomatic changes in the animals at the much earlier age. Mice treated with saline or GFP reached a neurological score of 8 indicating clear neurological deficits at the age of 16, 17.5 weeks in males and 17.5, 18 weeks in females respectively. Our treatment was able to significantly delay this loss in both male and female mice reaching similar neurological deficits at the ages of 19 and 22.5 weeks respectively (Figure 30).

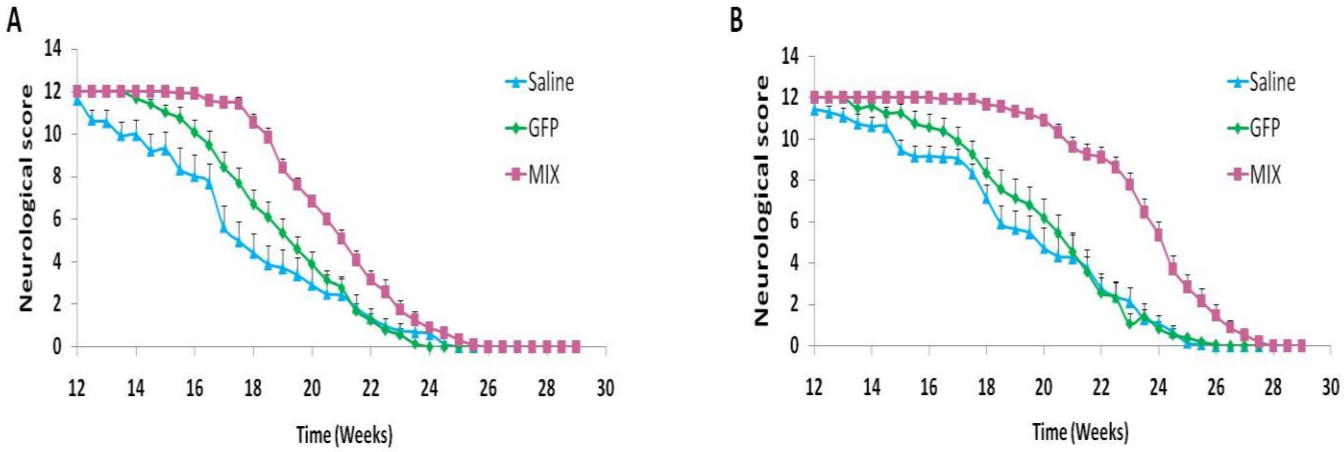


Figure 30. Treatment with EAAT2, NRF2 and GDH2 containing lentiviruses preserves neurological score in SOD1 G93A ALS mice. *The mice were injected with saline, lentiviruses containing GFP or a mixture of three types of lentiviruses each containing one of the three genes; EAAT2, NRF2 or GDH2 (MIX). Male (A) and female (B) neurological score deterioration during disease progression (for MIX, $P<0.001$ as determined by two way ANOVA for repeated measures. $12<n>17$ animals per group per gender).*

5.2.6 Treatment with EAAT2, NRF2 and GDH2 lentiviruses delays the onset of symptoms

In this ALS mouse model pre-clinical symptoms begin at an early age, whereas the onset of clinical symptoms occurs at a much later stage. We can improve the animals' quality of life by delaying the onset of clinical symptoms. We defined the onset of symptoms as a 5% reduction in the animals' mass. In male mice symptoms were first observed at 131.69±5.24

and 133.46±5.24 days in mice treated with saline or GFP. However, symptom onset was significantly delayed in males treated with all three genes and began at the age of 144.92±2.88 days. In female mice the symptoms begin at a slightly older age, females treated with saline or GFP showed symptoms at 142.46±3.90 and 144.13±3.53 days respectively. Our treatment significantly delayed the onset of symptoms with females treated with all three genes developing symptoms at 157.70±2.85 days (Figure 31).

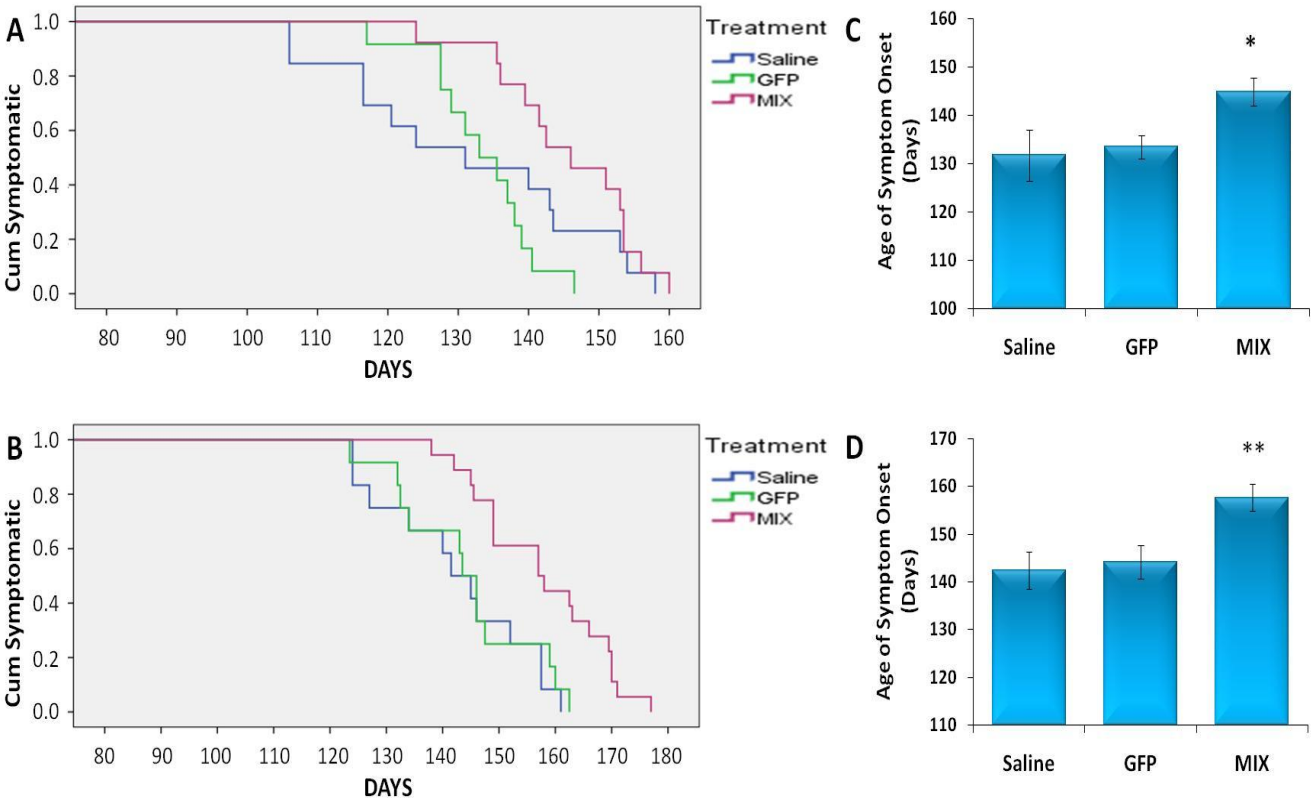


Figure 31. Treatment with EAAT2, NRF2 and GDH2 containing lentiviruses delays the onset of symptoms in SOD1 G93A ALS mice. *The mice were injected with saline, lentiviruses containing GFP or a mixture of three types of lentiviruses each containing one of the three genes; EAAT2, NRF2 or GDH2 (MIX). Onset of symptoms in male (A) and female (B) mice (for MIX $P<0.05$ as determined by Kaplan-Meier). Male (C) and female (D) mean age of symptom onset (*$P<0.05$, **$P<0.01$ as determined by ANOVA). $12<n>17$ animals per group per gender.*

5.2.7 **Treatment with EAAT2, NRF2 and GDH2 lentiviruses prolongs survival**

One of the most important parameters in the evaluation of the potential clinical relevance of treatments for ALS is whether the treatment can increase the animals' life span. Our treatment significantly prolonged survival in male mice by 18.96±4.81 and 12.5±2.40 days compared to mice treated with saline or GFP respectively (Figure 32 A, B). This correlates with a 119.66% increased survival from the onset of symptoms in treated mice (compared to saline, Figure 32C).

In females, the survival was increased even further, with our treatment prolonging survival by 21.31±4.32 and 20.83±3.17 days compared to mice treated with saline or GFP respectively (Figure 32 D, E). This correlates with a 136.27% increased survival from the onset of symptoms in treated mice (compared to saline, Figure 32 F).

Taken together these results show that our novel treatment strategy has a remarkable therapeutic potential in the SOD1 G93A mouse model of ALS, we hope that these results might indicate the potential relevance of our treatment for future clinical use.

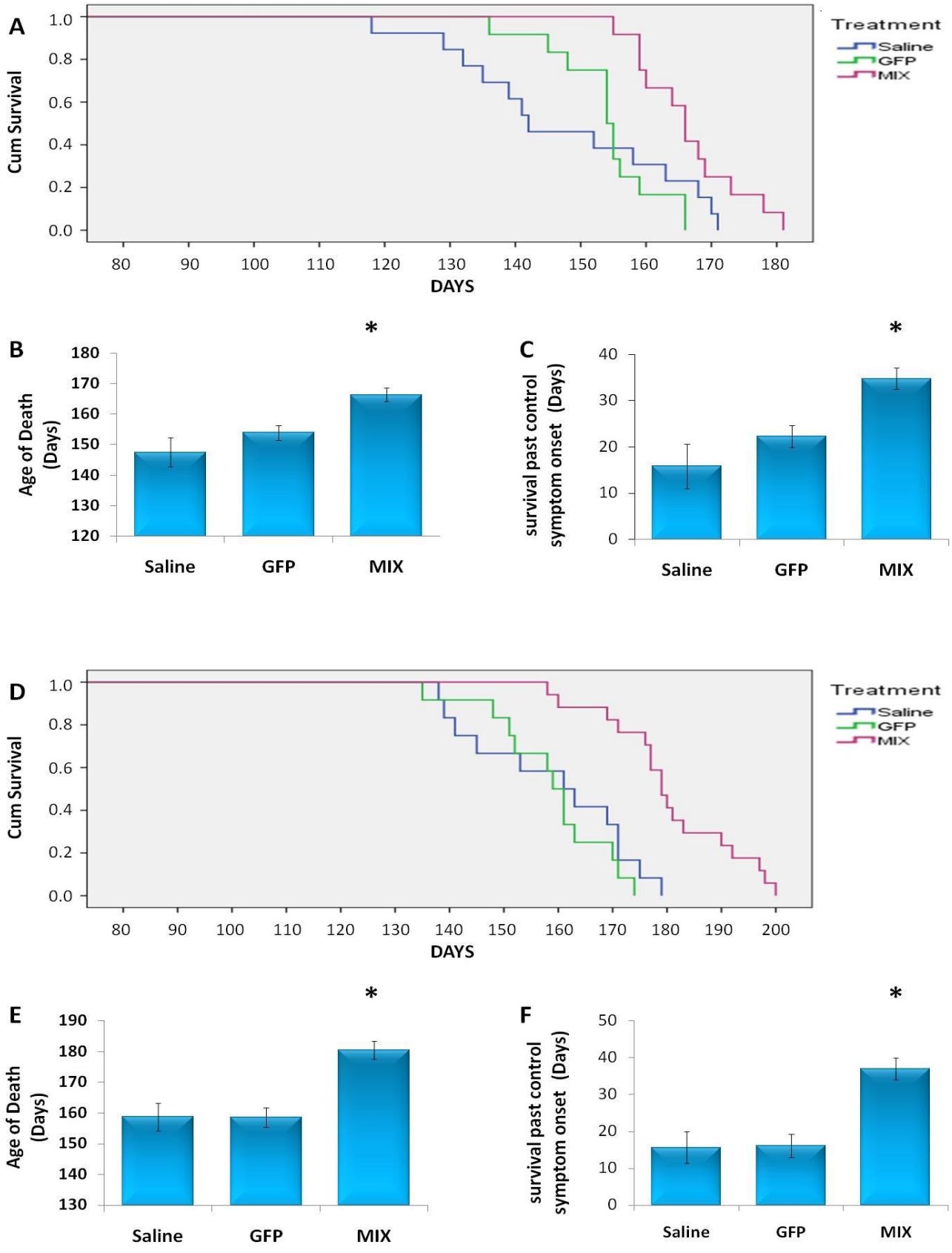


Figure 32. Treatment with EAAT2, NRF2 and GDH2 containing lentiviruses prolongs the survival of SOD1 G93A ALS mice. *The mice were injected with saline, lentiviruses containing GFP or a mixture of three types of lentiviruses each containing one of the three genes; EAAT2, NRF2 or GDH2 (MIX). Survival of male (A) and female (D) mice (for MIX $P<0.005$ as determined by Kaplan-Meier). Male (B) and female (E) mean age at death (*$P<0.001$ as determined by ANOVA). Average number of days the male (C) and female (F) mice survived past the symptom onset of saline treated mice (*$P<0.001$ as determined by ANOVA). $12<n>17$ animals per group per gender.*

Discussion

1. General

ALS is a dreadful disease due to its relentlessly progressive degeneration of motor neurons and extremely limited treatment options. The object of this study was to develop a novel therapeutic strategy for treating ALS. This therapeutic strategy was designed to emerge from identification of previously un-known astrocytic functions compromised in ALS and involved in the disease pathophysiology. In the early stages of this study we described for the first time several such astrocytic dysfunctions. Our novel treatment strategy was based on an attempt to compensate for these astrocytic functions that we identified as compromised.

For our treatment, we selected 3 key pathways believed to be crucial elements in the pathophysiology of ALS. These 3 elements of our treatment work synergistically and influence a broad range of deleterious effects involved in the motor neuron degeneration pathway thus exerting their therapeutic effect. We selected to focus on the EAAT2, GDH2 and NRF2 genes. Together these genes address the band and width of the excito-oxidative axis affecting many of the degenerative processes involved in ALS. These genes were engineered into lentiviral vectors that were used as a platform to deliver the genes into the CNS.

Our findings show the importance of the excito-oxidative axis in the pathophysiology of ALS in the SOD1 G93A mouse model, and that together EAAT2, GDH2 and NRF2 have a synergistic neuroprotective effect capable of significantly prolonging the survival (by up to 136% from symptom onset) as well as protecting the neurological state and motor function of ALS mice.

2. Altered astrocytic response to activation in SOD1 G93A mice and its implications on ALS pathogenesis

Despite extensive efforts the underlying cause of neurodegeneration and the path of motor neuron death in ALS remain elusive. However, over the past few years, accumulating evidence indicates that the pathogenesis of ALS entails more than the simple dysfunction of motor neurons, but further involves non-neuronal cells such as astrocytes and microglia (Ilieva, et al., 2009; Lasiene, et al., 2011). In fact, the role of astrocytes in ALS pathology is now widely recognized (Ilieva, et al., 2009; Lasiene, et al., 2011) and several astrocytic functions have been described as compromised in both ALS patients and animal models (Barbeito, et al., 2004; Ilieva, et al., 2009; Vargas, et al., 2010). Reactive astrocytes were once thought of as detrimental agents responsible for neuronal demise, however, recent studies challenge this view and they are now considered a double-edged sword in many pathological conditions with innate neuroprotective capabilities (Eddleston, et al., 1993; Hamby, et al., 2010; Pekny, et al., 2005). Reduced activation or dysfunction of reactive astrocytes has the potential to contribute to, or be the primary cause of CNS pathologies, either through loss of normal astrocytic function or gain of neurotoxic effects. Moreover, astrocytes that fail to gain the added neuroprotective functions induced by activation render the neurons under their protection more susceptible to injury and over time promote accumulative damage which might lead to progressive neurodegeneration. In this study we identify altered astrocytic response to activation as an unexpected potential pathway that may contribute to the pathogenesis of ALS.

In the present study we evaluated the response of SOD1 G93A and wild-type astrocytes to chemical activation. To this end, we selected three treatments, the bacterial endotoxin LPS, which is known to cause acute astroglial and microglial activation, the G5-supplement a

cocktail of growth factors designed to specifically activate astrocytes in a manner resembling mature type-II astrocytes (Michler-Stuke, et al., 1984; Vermeiren, et al., 2005). The third activator selected was the β-lactam antibiotic ceftriaxone, which specifically affects the astrocytic glutamatergic pathway. In a screening study of 1040 pharmacological substances, ceftriaxone was found to increase astrocyte glutamate transport by stimulating the expression of GLT-1 and prolonged the overall survival of SOD1 G93A mice (Rothstein, et al., 2005).

In this study we chose to use cultured astrocytes derived from newborn mice, although at this age, SOD1 G93A mice demonstrate no *in vivo* disease hallmarks. However, it has been previously shown that *in vitro* SOD1-linked alterations in astrocyte function appear even at this early stage (Di Giorgio, et al., 2007; Hedlund, et al., 2008; Scorisa, et al., 2010; Vargas, et al., 2008; Vermeiren, et al., 2006). Such astrocytes were found to induce motor neuron mitochondrial deficits (Bilsland, et al., 2008), they demonstrate a significant elevation in neuroinflamatory markers (Hensley, et al., 2006) and even release factors selectively toxic to motor neurons (Nagai, et al., 2007). Furthermore, these astrocytes show many of the molecular markers and functional properties of mature astrocytes (Falsig, et al., 2006; Ferraiuolo, et al., 2011; Hensley, et al., 2006; Kuno, et al., 2006; Saura, 2007). Using this system of astrocytes derived from newborns enables us to isolate the most primary astrocytic dysfunctions, which have the greatest potential of influencing not only disease progression but also disease initiation and pathogenesis.

Little is known about alterations in glutamate transporter expression and function in response to reactive changes in glia which occur in a range of acute and chronic neurodegenerative conditions (Doble, 1999; Lipton, et al., 1994). It has been recently demonstrated that astrocyte activation or treatment with inflammatory mediators, growth factors or co-culturing with neurons increased the expression and function of the glial glutamate transporters (Cassina, et al., 2005; Figiel, et al., 2003; Okada, et al., 2005; O'Shea,

et al., 2006; Schlag, et al., 1998; Vermeiren, et al., 2005). However, the effect of these treatments on astrocytes derived from animal models of ALS, where the involvement of glutamate excitotoxicity is widely accepted appears to be sorely missing from the literature.

In this study we addressed several aspects of this issue. We observed that although activation of wild-type astrocytes with G5, LPS and ceftriaxone increased the glutamate transporters GLT-1 and GLAST transcript and protein levels, activation of SOD1 G93A astrocytes yielded no such increase.

In accordance with the increased mRNA and protein levels of the astrocytic glutamate transporters, activation of wild-type astrocytes with all three activators significantly enhanced substrate uptake. On the other hand, not only do SOD1 G93A astrocytes exhibit reduced substrate uptake and saturation kinetics compared with their wild-type counterparts, but they show no further increase in substrate uptake in response to activation. This results in a considerably reduced potential for glutamate clearance provided by SOD1 G93A astrocytes under activation and neuronal stress conditions.

It is suggested that chronic glutamate neurotoxicity due to non-effective glutamate uptake participates in various pathological conditions (Danbolt, 2001; Sonnewald, et al., 2002)including the selective loss of motor neurons in ALS (Rothstein et al., 1992; 1996). Therefore, it stands to reason that increasing glutamate clearance might protect motor neurons damaged in ALS from excitotoxicity. Here we found that enhancing glutamate uptake by activation with LPS and CEF improved the protection provided by wild-type astrocytes towards the motor neuron cell-line NSC-34 from glutamate induced excitotoxicity. However, the impaired capacity of SOD1 G93A astrocytes to gain the neuroprotective functions induced by activation rendered the NSC-34 cells under their protection far more susceptible to glutamate neurotoxicity. Furthermore, by inhibiting glutamate uptake, we found that approximately 60% of the protection provided by astrocytes was attributed to

glutamate clearance. Furthermore, we found that the entire increase in the neuroprotective potential of wild-type astrocytes in response to activation resulted from their increased glutamate clearance capacities.

Taken together these results suggest that the protective potential of SOD1 G93Aastrocytes towards motor neurons is extremely compromised. This is due not only to the limited up-regulation of glutamate clearance in response to stress signals from their surroundings but also to decreased BDNF and GDNF transcription. The combination of the neuroprotective effect of LPS observed here (and elsewhere, (Faulkner, et al., 2004; Fischer, et al., 2011)) and its neurotoxic effect reported by others (Cassina, et al., 2002; Skaper, et al., 1995), stress the extreme importance of the activations timing, duration and nature. It further emphasizes how narrow the border between beneficial and detrimental astrocyte activation might be.

Our results up until this point suggested to us that altered astrocyte activation may well be pivotal to the pathogenesis of ALS. We postulated that improving the astrocytic response to activation or forcing the astrocytes to constantly imitate a protective astrocytic activated state could possibly be a first step towards slowing the disease progression and alleviating the symptoms of patients suffering from ALS.

3. Alternative strategies to compensate for the activation related astrocytic functions compromised in ALS

Based on our results described in the previous section we believed that compensating for several of the astrocytic functions compromised in ALS possesses a tremendous therapeutic potential. However, in order to tap into this therapeutic potential we had to overcome two major and not so simple hurdles.

First we had to select the appropriate compromised astrocytic functions to compensate for, those that would yield the most effective results. Building on the results we achieved in this study so far we believed that forcing the endogenous astrocytes to constitutively express the neuroprotective genes upregulated by activation, using genetic manipulation would yield cells that persistently imitate the protective astrocytic activated state could expert an extremely therapeutic effect.

Next we had to overcome the obstacle of successfully delivering the compensating agents into the target areas located throughout the CNS. There were many alternative strategies for gene delivery available to us, each with its own unique set of advantages and disadvantages.

One of the most potent strategies for gene delivery into animal models is directly engineering the entire mouse genome to express a particular gene, in specific target cells or tissues. For example, cross breeding of mice from two ALS models with mice over expressing NRF2 under the astrocyte specific GFAP promoter, significantly delayed the onset of symptoms and prolonged the survival of ALS mice (Vargas, et al., 2008). Unfortunately, although extremely efficient in animal models this strategy cannot be translated into clinical trials, therefore limiting its viability to proof of concept experiments rather than translational studies.

The remaining alternative gene therapy strategies consist mostly of two alternative pathways, *ex vivo* gene delivery based on transplantation of genetically altered cells and direct *in vivo* gene delivery using viral vectors. Where *ex vivo* gene delivery requires administration of exogenous cells, the direct *in vivo* delivery takes advantage of the endogenous cellular system already in place. This strategy circumvents the need for transplanted cells to migrate towards the site of injury and there to form functional units. In a disease such as ALS where the treatment needs to reach numerous targets spread

throughout the CNS, building on the pre-existing cellular network facilitates wide distribution thus facilitating the therapeutic effect.

In recent years accumulating evidence highlighted the potential of lentiviral vectors and placed them at the forefront of gene delivery. These vectors have many advantages, they can transfect both dividing and non-dividing cells, their integration into the host genome insures long term and stable expression, their large packaging capabilities afford them with the ability to deliver complex or polycistronic constructs, moreover, lentiviruses can be pseudotyped with various distinct viral envelopes that can determine the exact cell populations that would be infected with the virus. Furthermore, lentiviral vectors have been successfully used in several laboratory, animal and preclinical studies (Dropulić, 2011; Durand, et al., 2011; Picanco-Castro, et al., 2012; Sakuma, et al., 2012; Yi, et al., 2011). In mouse models of ALS lentiviral vectors were used to deliver therapeutic genes as well as anti SOD1 RNAi that silence the mutated gene (Azzouz, et al., 2004; Guillot, et al., 2004; Hottinger, et al., 2000; Ralph, et al., 2005 ; Raoul, et al., 2005). In both cases treatment with lentiviral vectors yielded beneficial results and sustained periods of transgene expression. Although not yet in ALS, but in several other conditions including; X-linked severe combined immunodeficiency in newly diagnosed infants, Adenosine deaminase deficiency and non-Hodgkin lymphoma, clinical trials using lentiviral vectors as a bases of ex-vivo treatments have commenced (ClinicalTrials.gov identifiers; NCT01512888, NCT01380990, NCT01815749).

Following in the footsteps of the great experiments performed before us, we two selected to use lentiviral vectors as our gene delivery platform with high hopes.

4. Combined administration of lentiviral vectors containing; EAAT2, NRF2 and GDH2 as a novel therapeutic strategy for treating ALS

4.1 General

Since it was first described in 1869 by the French neurologist, Jean Martin Charcot many advances have been made in the field of ALS research. The symptoms of the disease have been well characterized, several genes leading to the familial form of ALS have been uncovered and the progressive pattern of neuronal degeneration has been established. Unfortunately, despite the advances that have been made and the decades of research, the underlying cause of ALS and the path of motor neuron degeneration have yet to be elucidated. This may be due to the extremely complex nature of the disease, it is now believed that ALS is a multifactorial multisystemic condition, in which numerous pathogenic pathways are involved.

We believed that in order to effectively treat a multifactorial disease we had to tailor a multifactorial treatment that would be capable of addressing several factors influencing the disease progression. In certain diseases the concept of combined treatment with multiple factors might be controversial as a simple one mechanism treatment is considered easier to prove and safer to administer. However, as researchers discovered for other fields quite some time ago, that is not always the case. In the field of genetics for example it is now well accepted that for most conditions it is not simply one gene for one disease but a far more complicated array of events and genetics. We applied this logic to our treatment as well, ALS

is a multifactorial disease in which the strategy of one simple treatment for the disease has yielded limited results over the past few decades. We thought that perhaps it was time to attempt a broader view treatment strategy and the best way to approach such a treatment, where one element is simply not sufficient is to select therapeutic elements that can work synergistically to achieve a goal that they can simply not achieve on their own. The validity of this approach was later demonstrated, in this study, when we established the extremely significant and rather potent of synergistic effect provided by combined mixed treatment with all three genes compared to their relative singular effect in culture as well as animal models of ALS.

4.2 EAAT2, NRF2 and GDH2 work together synergistically to exert their potent neuroprotective effect

Our treatment was devised as an attempt to compensate for some of the major astrocytic deficits we observed in our earlier research. The three key pathways we selected were; glutamate uptake, glutamate metabolism and oxidative stress which together address the band and width of the excito-oxidative axis. For each pathway we selected a single gene located high at the top of its pathway, in a way that would allow us to influence the entire pathway by addressing that particular gene. EAAT2 was assigned to the glutamate uptake pathway, overexpression of EAAT2 would reduce the amount and duration of synaptic glutamate thus reducing excitotoxicity. GDH2 was assigned to the glutamate metabolism pathway, overexpression of GDH2 would reduce the systemic glutamate bioavailability also reducing excitotoxicity. The secondary damage of glutamate excitotoxicity is mediated by oxidative

stress. The oxidative stress pathway was allocated the NRF2 gene, a key regulator of the entire cellular anti-oxidant and anti-inflammatory pathways.

Each one of these genes was carefully selected for its potential therapeutic effect as well as the potential of their combined synergistic effect. Taking all these things into account we selected to continue our study by genetically engineering three lentiviral constructs each containing one individual gene (EAAT2, NRF2 and GDH2) accompanied by a forth lentiviral construct containing GFP as the most closely resembling control. The first set of our experiments offered a simple and straight forward method to test the therapeutic effect of these three genes individually as well as synergistically. This was achieved by evaluating the effect of our lentiviral vectors independently and comparing it to the combined effect of mixed treatment with different combinations of these three genes.

Using astrocytes derived from SOD1 G93A ALS mice that were infected with lentiviral vectors that induce over-expression of EAAT2, NRF2 and GDH2 we evaluated the neuroprotective potential our three genes exert towards the motor neuron like cell line that overexpresses SOD1 G93A. We found that although each of the genes we selected on its own increased the astrocytic neuroprotective potential, their individual effect, as well as the effect of every possible partial combination could not possibly compare to the combined synergistic neuroprotective effect provided by all three genes put together. We thought that this increased potential of the synergistic effect well outweighs the potential complications that can arise from the combination. This belief was then strengthened even further when we evaluated the results obtained from SOD1 G93A mice. Treatment with each of the individual genes was not sufficient to exert a therapeutic effect whereas combined treatment yielded extremely positive results.

It is noteworthy that overexpression of NRF2 on its own in all of the animals astrocytes as reported by Vargas et al. to significantly delayed the onset of symptoms and prolonged the survival of ALS mice (Vargas, et al., 2008) an effect not observed in our case with NRF2 on its own. We speculate that this may be due to the extreme robustness of NRF2 expression in the Vargas et al. experiment where NRF2 was expressed in every astrocytic cell which far exceeds the NRF2 expression levels obtained in our experiment. This result however does not diminish the observed synergistic effect described in our treatment as the amount of lentiviruses containing NRF2 in the group treated with all three genes was a third of the total NRF2 containing lentiviruses in the NRF2 only group. Moreover, unlike the system employed in the Vargas et al. experiment that demands manipulation of the entire individuals' genome our system has the potential to someday be translated to clinical applications. Furthermore, robust NRF2 over- expression and over-activation as well as the use of pharmacological drugs directly enhancing the activity of NRF2 has raised some concerns as this response is a non physiological response in which regardless of the cellular condition the cell would be more resistant to oxidative stress and oxidative stress induced death. Where this effect might be safe in motor neurons in a systemic respect it might be slightly more dangerous, shifting the balance of cellular life and death towards life in a potentially carcinogenic path. Our treatment on the other hand only increases the bio-availability of NRF2 making it more ready and available for action, but it does not affect the regulatory system that prevents unprovoked NRF2 activation. This increases the safety of our approach.

Unlike the situation with NRF2, genetic astrocytic overexpression of EAAT2 on its own was not sufficient to postpone the onset of paralysis nor did it prolong the life span of ALS mice (Guo, et al., 2003). This study supports our results in that in this study, like in our

study EAAT2 overexpression on its own was not sufficient to provide a therapeutic effect whereas adding EAAT2 to our treatment regime increased the synergistic therapeutic effect of the treatment. Although Guo et al. results of genetic astrocytic overexpression of EAAT2 (Guo, et al., 2003) supported our results, we had much to learn from it as we designed our treatment. In that study (Guo, et al., 2003) the EAAT2 gene was coupled with the week astrocytic GFAP promoter. The promoter activity only increases as the disease progresses thus hindering the potential protective effect of EAAT2 this may account for the partial protection observed. This limitation on the therapeutic potential of our treatment concerned us, as we aimed to achieve the strongest therapeutic effect possible. To circumvent this potential issue we coupled all three of our genes with the strong constitutive CMV promoter thus enhancing the transgenes expression and potentiating its therapeutic effect.

4.3 Combined EAAT2, NRF2 and GDH2 possesses a unique and significant therapeutic potential in ALS animal models

In light of the above described synergistic effect and the potent neuroprotective potential we observed, we next sought to evaluate whether our novel therapeutic strategy could also manage to protect degenerating motor neurons in the severe motor neuron pathology model of transgenic SOD1 G93A ALS mouse model.

Lentiviral vectors genetically engineered to induce overexpression of EAAT2, NRF2 and GDH2 were injected into the cisterna magna and hind limb muscles of 60 day old SOD1 G93A ALS mice. The intra cisternal route of administration facilitated lentiviral spinal cord transduction, whereas the muscle pathway allowed for retrograde transport of lentiviral vectors through the neuronal terminals innervating the muscle (Fu, et al., 2003; Fu, et al., 2007; Louboutin, et al., 2012; Mazarakis, et al., 2001; Mentis, et al., 2006).

We found that treatment with all three lentiviruses preserved body weight, reduced the loss of hindlimb reflexes, protected motor function and neurological score in both male and female SOD1 G93A ALS mice. These parameters are indicators of improved quality of life provided by our treatment. Our data further demonstrates that our treatment was able to significantly delay the onset of symptoms and prolong the lifespan of male and female SOD1 G93A ALS mice.

Throughout our animal experiments we observed an interesting phenomena. We found that in every parameter evaluated, the effect of our treatment was significantly more pronounced in female subjects, with most parameters being preserved for an entire week longer in females than in males. Different responses to treatment as well as a different pattern of disease progression between genders is a known phenomenon in ALS research. This difference can sometimes extend even to the point at which treatment would affect only one gender while exerting no effect on the other gender (Acevedo-Arozena, et al., 2011; Alves, et al., 2011; Heiman-Patterson, et al., 2005; Heiman-Patterson, et al., 2011; Veldink, et al., 2003; Vercelli, et al., 2008).

4.3.1 **How does our treatment compare to currently available treatments for ALS?**

In order to evaluate the relevance of our treatment for clinical use, we wanted to compare the therapeutic effect of our treatment to Riluzole, the only FDA approved drug currently available for treating ALS. Unfortunately, with our animal experiments totaling over 200 animals and more than 6 different treatment groups, we could not incorporate an

experimental arm of Riluzole treatment into our experimental paradigm. However, we did not want to completely disregard this aspect. To this end, we conducted a literary comparison between the results published for ALS mice treated with Riluzole and the results obtained in our research. We found that in many parameters, our novel therapeutic strategy rivals or exceeds the results reported for Riluzole (Table 24). Our treatment is still a long way away from actual potential clinical use. However, this first preliminary comparison combined with the extremely significant results obtained in our research dose indeed highlight the importance of our discovery as well as the therapeutic potential and perhaps the clinical relevance of our novel therapeutic strategy in the treatment of ALS.

Table 24. A literary comparison between the results published for Riluzole and those obtained in this study for our treatment in mice models of ALS.

	Riluzole (Percent of beneficial effect from symptom onset)	**Our Treatment** (Percent of beneficial effect from symptom onset)
Survival	17-30%	120-136%
Symptom onset	No effect	81-96%
Motor function	22-24%	41-82%
Body weight	17%	87-90%

This table is based on the results published for experiments performed in mouse models of ALS for the effectiveness of treatment with Riluzole (Del Signore, et al., 2009; Gurney, et al., 1996; Gurney, et al., 1998; Waibel, et al., 2004).

5. Conclusions

Neurodegenerative disorders represent some of modern medicines greatest challenges as most remain not only incurable but mostly untreatable.

Our results demonstrate that treatment with EAAT2, NRF2 and GDH2 possesses a strong neuroprotective potential in both in vitro and in vivo models of excito-oxidative toxicity and the rapidly progressive neurodegenerative disease ALS. Furthermore our results demonstrate the importance of the synergistic effect provided by the combined treatment of these three genes as well as strengthen the multifactorial treatment for a multifactorial disease therapeutic concept.

We hope that our study might provide a novel therapeutic strategy for slowing disease progression and alleviating the symptoms of patients suffering from ALS and potentially other neurodegenerative diseases involving excito-oxidative toxicity.

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