Universitat Autònoma de Barcelona

Gene therapy targeting neuregulins for the treatment of Amyotrophic Lateral Sclerosis

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INDEX

SUMMARY

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disorder with no effective treatment currently available. The molecular mechanisms that are involved in the motoneuron (MN) death are complex and include several MN dysfunctions, and contribution of surrounding cells such as microglia and astrocytes. Neuregulin 1 (NRG1) is a neurotrophic factor highly expressed in MNs and neuromuscular junctions that supports axonal and neuromuscular development and maintenance. Recent studies have suggested a crucial role for NRG1 and their ErbB receptors in ALS, particularly for isoform I (NRG1-I) in the collateral reinnervation process, and isoform III (NRG1-III) in the preservation of the MNs, opening a new window for developing novel ALS therapies. However, further studies are needed to clarify the role of the NRG1-ErbB pathway on MN survival and to provide the proof of concept of its therapeutical efficacy.

In the present thesis we have evaluated the therapeutic effect of NRG1 overexpression in the central and the peripheral nervous system. For this purpose, we first characterized the role of exogenous NRG1 using an *in vitro* model of spinal cord organotypic cultures (SCOC) subject to chronic excitotoxicity caused by DL-*threo*-β-hydroxyaspartic acid. Our results revealed that addition of recombinant human NRG1 (rhNRG1) to the medium significantly increased MN survival through the activation of ErbB receptors, which was blocked by addition of lapatinib, an ErbB inhibitor, and reduced microglial reactivity overcoming the excitotoxicity effects. rhNRG1 activated the pro-survival PI3K/AKT pathway and restored the autophagic flux in the spinal cord culture. Furthermore, addition of rhNRG1 to the medium promoted motor and sensory neurite outgrowth.

We have then directed gene therapies based on adeno-associated viruses to overexpress NRG1-I in the skeletal muscles, and NRG1-III in the spinal cord to preserve the MNs in the *in vivo* model of ALS, the $SOD1^{G93A}$ mice. Our results indicate that both gene therapies were able to preserve the neuromuscular function of the hindlimb muscles, improve the locomotor performance, increase the number of surviving MNs and reduce the astrocyte and microglial reactivity in the treated female $SOD1^{G93A}$ mice at the end-stage of the disease. Furthermore, in the spinal cord the NRG1-III/ErbB4 axis regulates MN excitability through the KCC2 transporter and reduces the expression of the MN vulnerability marker MMP-9.

NRG1-I expressed in the skeletal muscle signals with ErbB2 and 3 receptors present in terminal Schwann cells to promote axonal reinnervation. However, when we aimed to combine both viral-mediated therapies we did not find a synergic effect. Altogether, our results indicate that NRG1 isoforms play an important role on MN survival and that a viral-mediated overexpression may be considered as a potential novel therapy to treat ALS.

ABBREVIATIONS

AAV, adeno-associated vectors

ADAM, disintegrin and metalloproteinase

AKT, protein kinase B

ALS, amyotrophic lateral sclerosis

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid

ANG, angiogenin

ANOVA, analysis of variance

ASO, antisense oligonucleotide

BACE1, protease β-secretase 1

BDNF, brain-derived neurotrophic factor

C9ORF72, chromosome 9 open reading frame 72

ChAT, choline acetyltransferase

CMAP, compound muscle action potential

CNS, central nervous system

CNTF, ciliary neurotrophic factor

CRD, cysteine rich domain

CSF, cerebrospinal fluid

DNA, deoxyribonucleic acid

DRG, dorsal root ganglia

$EAAT_2$, excitatory aminoacids transporter 2

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

EMG, electromyography

ER, endoplasmic reticulum

ERAD, ER-associated degradation

ErbB, erythroblasticleukemia viral oncogene

ERK, extracellular signal-regulated kinases

fALS, familial amyotrophic lateral sclerosis

FTLD, frontotemporal lobar degeneration

G4C2, GGGGCC hexanucleotide

GDNF, glial-derived neurotrophic factor

GGF, glial growth factor

GM, gastrocnemius muscle

Grb2, growth factor receptor-bound protein 2

HBD, heparin-binding domain

HGF, hepatocyte growth factor

HRG, heregulin

HSP, heat shock protein

HSPGs, extracellular matrix heparan-sulfate proteoglycans

IBA-1, anti-ionized calcium binding adapter molecule 1

ICDs, intracellular domains

IF, immunofluorescence

IGF-1, insulin growth factor - 1

IHC, immunohistochemistry

IL-6, interleukin 6

IM, intramuscular

IN, intraneural

IP, intraparenchymal

iPSCs, induced pluripotent stem cells

IT, intrathecal

IV, intravenous

KAP3, kinesin-associated protein 3

LC3-II, Microtubule-associated protein 1A/1B-light chain 3 II

LTP, long-term depression

MAMs, mitochondria associated membranes

MEP, motor evoked potential

MGE, medial ganglionic eminence

MN, motoneuron

MND, motoneuron disease

MRI, magnetic resonance image

MSCs, mesenchymal stem cells

MUNE, motor unit number estimation

NAC, N-acetyl-L-cysteine

NMDA, N-methyl-D-aspartic acid

NRG1, neuregulin 1

NRG1-I, NRG1 type I

NRG1-III, NRG1 type III

NSCs, neural stem cells

NT-3, neurotrophin-3

PI3K, phosphoinositide 3-kinase

PL, plantar interossei muscle

PNS, peripheral nervous system

PRE084, 2-(4-morpholinethyl) 1-phenylcyclohexanecarboxylate

rhNRG1, recombinant human NRG1

RNA, ribonucleic acid

ROS, reactive forms of oxygen

sALS, sporadic amyotrophic lateral sclerosis

SCOC, spinal cord organotypic cultures

Sema3A, semaphorin 3ª

SH2, Src homology 2 domain

SMDF, sensory and motor neuron-derived factor

SMI-32, anti-neurofilament H non-phosphorylated

SOD1, superoxide dismutase 1

SZ, schizophrenia

TA, tibialis anterior muscle

THA, DL-*threo*-β-hydroxyaspartic acid

TMS, transcranial magnetic stimulation

TNF α, tumour necrosis factor α

TDP-43, TAR-DNA binding protein

VEGF, vascular endothelial growth factor

WB, western blot

INTRODUCTION

1.1 Amyotrophic lateral sclerosis

Motor neuron diseases (MND) are a group of progressive neurodegenerative disorders, which despite having different aetiology and clinical spectra contribute to a common end; the degeneration and loss of the upper and/or lower motor neurons (MN) (Mancuso & Navarro, 2015). The most common MND affecting adults is Amyotrophic Lateral Sclerosis (ALS), with an incidence of 1-5 per 100,000, but other frequent MND are Spinal Muscular Atrophy (SMA), affecting children, and progressive bulbar palsy.

Amyotrophic lateral sclerosis (ALS), also known as Charcot disease and Lou Gehrig's disease, is a heterogeneous neurodegenerative disorder with no cure currently available. This MND is characterized by the loss of MNs of primary motor cortex, brainstem and spinal cord (Wijesekera & Leigh., 2009). The name reflects both the degeneration of corticospinal MNs, the descending axons of which in the lateral spinal cord appear injured (lateral sclerosis) and the degeneration of spinal MNs, with secondary denervation and muscle wasting (amyotrophy) (Taylor et al., 2016). Around the areas of degeneration there is also a huge reactive response by the glial cells.

From 90 to 95% of the cases are sporadic (sALS) in which the aetiology is unknown, and the remaining 5-10% inherit the familiar form (fALS), associated with dominantly inherited adult onset ALS genes, being the most frequent the mutations in the superoxide dismutase 1 (SOD1), the TAR-DNA binding protein (TDP-43) and the hexanucleotide repeat expansions in chromosome 9 open reading frame 72 (C9ORF72) (Rosen et al., 1993; Shaw., 2005; Kabashi et al., 2008; Yokoseki et al., 2008; DeJesus-Hernandez et al., 2011; Renton et al., 2011). Both sALS and fALS forms course with degeneration of MNs leading to muscle atrophy and weakness, fasciculations and spasticity (Robberecht & Philips., 2013).

ALS has a clinical onset highly variable, but most frequently after the fourth decade of life; while juvenile ALS is rare. Patients develop a progressive weakness, as MN degeneration gradually spreads, axonal connections fail and denervation starts distally or proximally in the upper or lower limbs and reaching all muscles, including those related with breathing, speaking and swallowing , causing muscular atrophy and paralysis. Most patients with ALS die within 2 to 5 years after

symptoms onset due to respiratory failure (Robberecht & Philips., 2013).

Unfortunately, there is no effective therapy for this disorder. There are only two drugs approved for use in ALS. Riluzole is the most widely used drug therapy, but it only prolongs survival for 3-4 months (Doble., 1996). The second one is edaravone a drug that presumably works to mitigate oxidative injury in CNS neurons in a subgroup of ALS patients (Ludolph & Jesse., 2009; Edaravone ALS Study Group., 2017). Symptomatic and palliative measures (for example, feeding tube and respiratory support) are the pillar of management of this disorder (Pasinelli & Brown., 2006).

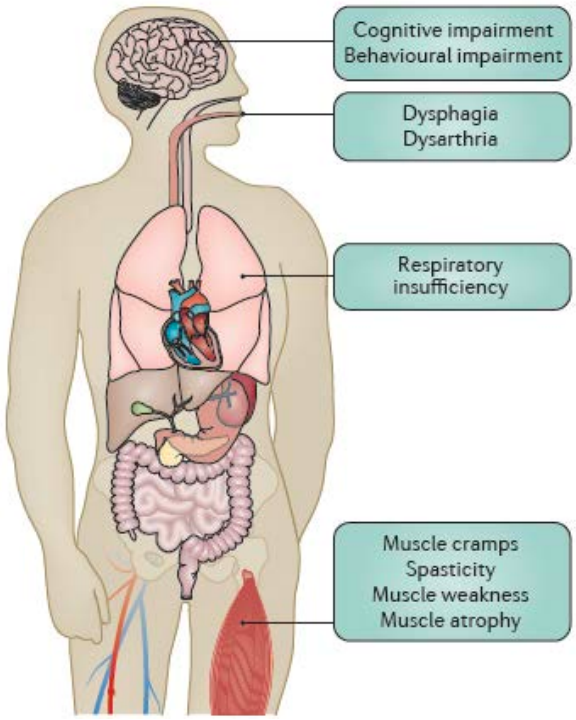

Figure 1. Clinical manifestations of ALS. Although motor manifestations such as muscle weakness and dysphagia (difficulty swallowing) are the main clinical manifestations of amyotrophic lateral sclerosis (ALS), up to half of patients have non-motor symptoms, such as cognitive impairment. Dysarthria; difficulty with speech (Extracted from Hardiman et al., 2017).

1.2 Diagnosis and evaluation of ALS

Clinical signs

ALS clinical manifestations can be highly variable depending on the involvement of different sets of MNs or regions of the body (Taylor et al., 2016). Therefore, patients affected may develop different forms, including: progressive muscular atrophy due to predominant loss of lower MNs (patients show flaccidity and muscle atrophy), primary lateral sclerosis due to mostly loss of upper MNs (development of prominent hyperreflexia and spasticity), bulbar ALS due to

degeneration of brainstem MNs (development of tongue atrophy with difficulty for swallowing and speaking), and pseudobulbar palsy due to cortical frontobulbar MNs degeneration (dysfunctional speech and swallowing) (Taylor et al., 2016).

Diagnostic criteria

ALS does not possess a definitive test for the diagnosis and therefore the procedure is based on excluding other possible causes depending on the symptoms and requires evidence of disease progression (Hardiman et al., 2017). Nowadays the diagnosis is based on the El Escorial and Airlie House criteria (Brooks et al., 2000). In agreement with this criteria, diagnosis needs a history of progressive weakness spreading within a region or to other regions, such as bulbar, cervical, thoracic or lumbar regions, with symptoms associated to loss of lower MNs and upper MNs.

Biomarkers

Since ALS is a clinical disease highly heterogeneous regarding manifestations and clinical course of the disease, in order to find suitable and effective therapeutic approaches for patients early diagnostic and prognostic biomarkers are needed. In this way, levels of neurofilament light polypeptide and phosphorylated neurofilament heavy polypeptide in the cerebrospinal fluid (CSF) can differentiate patients with ALS from others that mimic this disease with moderate sensitivity and specificity and correlate with disease progression (Gaiottino et al., 2013; Steinacker et al., 2016; Gaiani et al., 2017).

Electrophysiological tests

Another important issue is how motor units are vulnerable to the disease process. Electromyography tests in ALS patients showed that larger and stronger motor units are clearly more affected by the disease (Dengler et al., 1990). Also, histopathological studies confirmed a preferential degeneration of large MNs in ALS (Sobue et al., 1983). Because there is an absence of specific disease markers (Turner et al., 2009), clinical diagnostic criteria have been established based on the presence

and distribution of lower and upper MN involvement, including electrophysiological studies, to support the diagnosis (Brooks et al., 2000).

To study the lower MN involvement, motor nerve conduction studies are essential for the diagnosis of ALS. They permit to define and exclude other peripheral nerve, neuromuscular junction or muscle diseases that may mimic the ALS phenotype (Brooks et al., 2000). In ALS, the most important electrophysiological feature is the reduction in amplitude of the compound muscle action potential (CMAP). In contrast, the distal motor latency and the motor nerve conduction velocity persist close to normal, below 70% of upper or lower limits of normal due to loss of the fastest MNs (Cornblath et al., 1992; De Carvalho & Swash et al., 2000; Mills & Nithi., 1998). Furthermore, by the detection of partial conduction blocks motor nerve conduction studies can exclude multifocal motor neuropathies. It implies a marked reduction of proximal amplitude or area as compared with the distal ones (over 50%) in short segments (De Carvalho et al., 2001).

Needle electromyography (EMG) also shows evidence of lower MN dysfunction (Krarup et al., 2011). For diagnosis, EMS alterations must be observed in muscles innervated by MN from at least two of the following four CNS regions: brainstem (bulbar/cranial MN), cervical, thoracic and lumbosacral spinal MNs (Brooks et al., 2000; Mitsumoto et al., 2007). The EMG tests from ALS patients appear altered and show some particular characteristics: first, large motor unit action potentials of increased duration with an increased proportion of polyphasic components. Second, a reduced interference pattern with firing rates higher than 10 Hz. Third, unstable motor unit action potentials. And fourth, higher firing rates from the fast motor units, because of its increased susceptibility. Furthermore, in ALS patients we can observe a higher variability of the interspike interval of firing motor units, coherent with elevated excitability of the lower MNs (Piotrkiewicz & Hausmanowa-Petrusewicz., 2011). Muscle denervation activity is another indication of the ALS pathophysiology that is proved in the EMG tests. This appears in form of spontaneous fibrillation potentials and positive sharp-waves recorded in the muscles of ALS patients (Krarup et al., 2011). However, it is not a specific sign of ALS, since it can occur also in neuropathies and in myopathies associated with degeneration of muscle fibers. Abnormal fasciculation potentials are another

frequent EMG feature in ALS. Nevertheless, it does not appear in all the muscles in the ALS patients and can also be observed in normal muscles as benign fasciculations (Mancuso & Navarro, 2015).

Another useful electrophysiological technique is the motor unit number estimation (MUNE). It shows quantitative information about the number of MNs innervating a muscle. This technique can be evaluated using different methods (incremental method, multiple point stimulation, spike-triggered averaging, F-wave, and statistical method) and each one has advantages and limitations. It is essential for the estimation of the motor axonal loss in ALS (Bromberg & Brownell., 2008, Mitsumoto et al., 2007).

To evaluate the upper MN involvement recording of motor evoked potentials (MEPs) by transcranial magnetic stimulation (TMS) is a useful technique. It evaluates the neurophysiologic integrity of corticospinal motor pathways and therefore the detection of upper MN involvement even when patients do not show upper MN clinical signs (Mitsumoto et al., 2007). In addition, muscle biopsy may be used to demonstrate lower MN dysfunction in a region where clinical or electrophysiological data do not evidence involvement (Brooks et al., 2000).

Neuroimaging studies

Neuroimaging studies are another tool used for the ALS diagnosis, since they are important to exclude treatable structural lesions that mimic ALS producing upper and lower motor signs. Live-imaging fields such as Magnetic resonance image (MRI) studies have reported corticospinal tract degeneration in patients with ALS compared to controls (Bede & Hardiman., 2014; Schuster et al., 2015; Bede et al., 2016). Unfortunately, imaging studies cannot provide individualized data to be used as reliable biomarkers of upper MN dysfunction or cognitive impairment in patients with ALS (Hardiman et al., 2017).

1.3 ALS pathogenesis

The primary pathogenic mechanisms underlying ALS are likely to be multifactorial as it happens with other neurodegenerative disorders. A complex network between multiple processes not mutually exclusive (including genetic factors) contribute to MN degeneration (Shaw., 2005; Wijesekera & Leigh., 2009; Robberecht & Philips., 2013; Mancuso & Navarro., 2015). Traditionally, ALS has been "neuron-centric", but there has been emerging interest in the protective as well as deleterious roles that the non-neuronal neighbor cells have in the pathogenesis of MN injury (Song et al., 2012). Evidence has also provided that the final process of MN death is inclined to occur through a caspase dependent programmed cell death pathway, resembling apoptosis (Shaw., 2005).

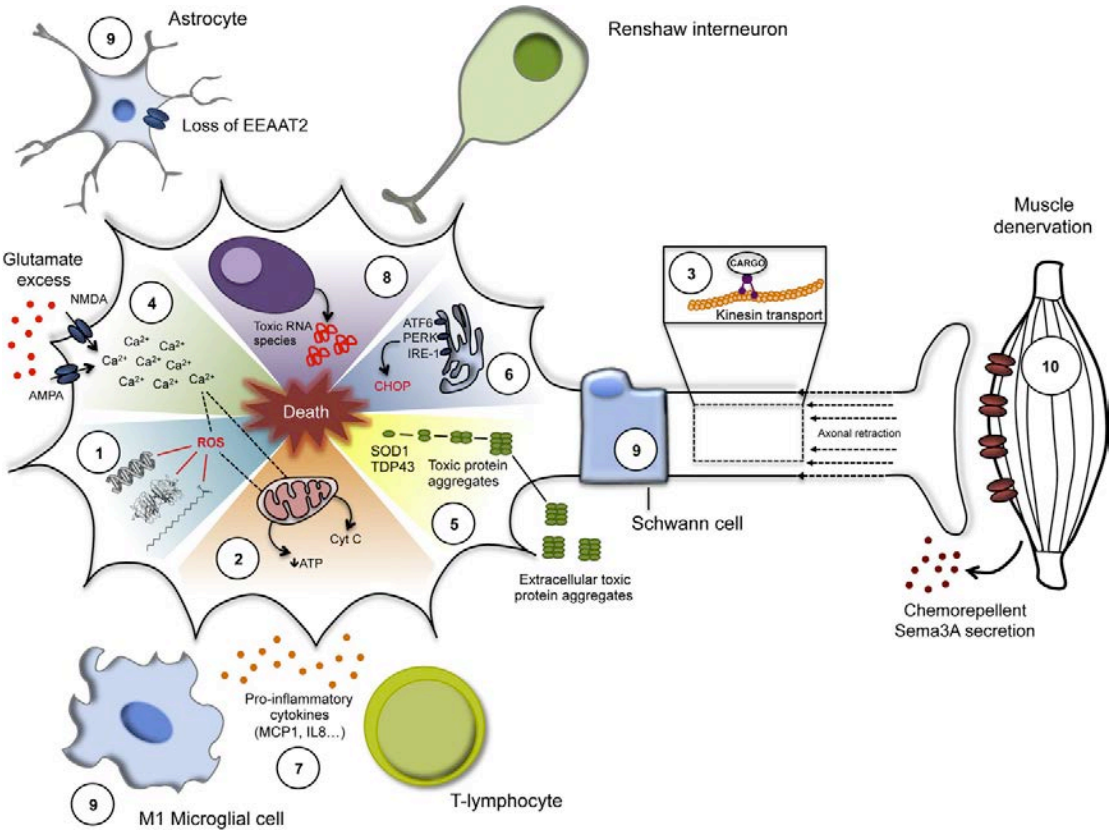

Figure 2. Main pathophysiological mechanisms contributing to motoneuron degeneration in ALS, including those involving the motoneuron itself and those related to the relation with accompanying glial cells and skeletal muscle. (1) Oxidative stress, (2) mitochondrial dysfunction, (3) impaired axonal transport, (4) excitotoxicity, (5) protein aggregation, (6) endoplasmic reticulum stress, (7) neuroinflammation, (8)

abnormal RNA processing, (9) non-neuronal cells, and (10) target muscle contribution (Extracted from Mancuso & Navarro., 2015).

Oxidative stress

Oxidative stress has generally been linked to neurodegeneration and occurs upon increased production of reactive oxygen species (ROS), which are mostly generated by leakage of excess electrons from an over-reduction state in impaired mitochondrial respiratory chains (Lenaz et al., 2002). Oxidative stress by itself may not be such harmful, but accumulation of ROS may be an important factor that reduces the ability of the cell to confront an underlying pathologic situation (Mancuso & Navarro., 2015).

Since mutations in the gene encoding for SOD1, a protein that has the capacity to catalyze the production of ROS, were associated with around 20% of fALS cases oxidative stress was targeted as a possible contributor to MN death. However further experiments show that MN death occurred due to an abnormal accumulation of SOD1 instead of a disruption of its dismutase activity; in other words, the mutant SOD1 protein exerts its detrimental effects through a toxic gain of function rather than a loss of function. Thus, mutated SOD1 have toxic effects on the cells degradation machinery, impairing its two major components: the proteasomal pathway and autophagy. Furthermore, SOD1 deletion in mice do not lead to MN degeneration (Reaume et al., 1996).

It has been reported that CSF and serum from ALS patients show increased concentrations of oxidative stress-damaged proteins (Shaw et al., 1995), lipids (Simpson et al., 2004), DNA (Bogdanov et al., 2000) and RNA species (Chang et al., 2008). Indeed, the mRNA species involved in mitochondrial electron transport chain, protein biosynthesis, folding, degradation pathways and cytoskeleton are more susceptible to oxidation (Chang et al., 2008). Strikingly, aberrant oxidation of wtSOD1 present in sALS patients provides pathological properties similar to those observed in mSOD1, such as the impairment of axonal transport (Bosco et al., 2010).

Excitotoxicity

Glutamate is the major excitatory transmitter in the human central nervous system (CNS), acting on ionotropic and metabotropic (G-protein coupled) receptors (Shaw., 2005). MNs are very sensitive to toxicity induced by calcium entry following excessive glutamate stimulation (Hardiman et al., 2017). An over-stimulation of these neuronal glutamate receptors, known as excitotoxicity, leads to neuronal dysfunction and ultimately to cellular death (Arundine & Tymianski., 2003; Shaw., 2005). This increased activation induces massive calcium influx that damages the cell through the activation of calcium-dependent proteases, lipases and nucleases (Mancuso & Navarro., 2015).

Excitotoxicity has been suggested to play a central role in ALS, as a contributory factor to MN degeneration (Heath & Shaw., 2002), although clear evidence that it is a primary disease mechanism is lacking. There are some key findings supporting the role of excitotoxicity in ALS physiopathology. The expression and function of the major glial glutamate reuptake transporter protein, excitatory amino-acid transporter ($EAAT_2$) is impaired in the CNS of MND patients; this could lead to a failure in the neurotransmitter clearance from the synaptic cleft, increasing sensitivity to glutamate (Bristol and Rothstein., 1996). Moreover, cerebrospinal fluid (CSF) levels of glutamate appear to be abnormally raised in MND patients (Perry et al., 1990; Shaw et al., 1995). Interestingly, riluzole is the only drug approved for the ALS treatment and achieves its benefits acting as an anti-excitotoxic drug (Ludolph & Jesse., 2009).

Among the possible explanations for the role of excitotoxic injury to MNs is that ALS-vulnerable spinal and brainstem MNs exhibit low endogenous calcium buffering capacity, that is 5-6 times lower than that found in ALS-resistant oculomotor MNs, and therefore making them more susceptible to excitotoxic insults (Alexianu et al., 1994). Also, overactivation of α-amino-3-hydroxy-5-methyl-4-isoxazole- propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors is contemplated as one of the main causes of excitotoxicity (Van Den Bosch et al., 2006). Indeed, Carriedo and collaborators (1996) revealed that MNs are especially vulnerable to AMPA-mediated excitotoxicity in vitro. Furthermore, excitotoxicity is closely related to other important pathogenic mechanisms of ALS, such as

intracellular calcium impairment with secondary activation of proteolytic and ROS-generating enzymes, and mitochondrial abnormalities (Arundine & Tymianski., 2003).

Finally, it has been demonstrated that mSOD1 has a role on the disruption of calcium homeostasis, since misfolded A4V mSOD1 aggregates form pores that integrate in the membrane allowing the influx of calcium (Allen et al., 2012). These results propose that mSOD1 can support the excitotoxic damage to MNs without the contribution of other glutamatergic-related elements.

Role of non-neuronal cells in ALS

Recently, there has been increasing evidence that non-neuronal cells, including activated microglia and astrocytes have a crucial role in the MN degeneration in ALS/MND (Ilieva et al., 2009; Valori et al., 2014). Several studies in mouse models have indicated that expression of mutant SOD1 in neurons alone is insufficient to cause MN degeneration and that participation of non-neuronal cells may be required. This was demonstrated generating a chimeric animal expressing mSOD1 in glial cells; in this case normal MNs developed ALS signs (Clement et al., 2003). Also, double transgenic mice were generated expressing the Cre-Lox recombination system to suppress mSOD1 expression in MNs or microglia. Deletion of mSOD1 in MNs showed delayed disease onset but no changes in the disease progression. In contrast, mSOD1 suppression from microglia and macrophages did not change the disease onset but rather significantly prolonged mice survival (Boillée et al., 2006a,b). Thus, mutant SOD1 may cause neurotoxicity indirectly by disturbing the function of non-neuronal cells, for example microglia.

In contrast, the generation of transgenic animals expressing selectively mSOD1 in astrocytes failed to induce MN degeneration (Gong et al., 2000). However, there is a body of evidence that astrocytes play a crucial role in ALS. Indeed, astrocytes extracted from post-mortem tissue of ALS patients induce a toxic effect to MNs. Strikingly, blocking mSOD1 expression in these ALS-astrocyte cultures produced significant neuroprotective effects (Haidet-Phillips et al., 2011). Also, astrocytes play a key role in the neuroinflammatory process by producing several

inflammatory signals. In the ALS mice model carrying a mutation of a glycine to alanine conversion at the 93rd codon of SOD1 gene ($SOD1^{G93A}$) astrocytes secrete inflammatory mediators such as prostaglandin E2 and nitric oxide, promoting a toxic effect on MNs (Haidet-Phillips et al., 2011).

Microglia has an essential role as resident immunocompetent and phagocytic cells within the CNS (Shaw., 2005). Activation is related with transformation to phagocytic cells able to deliver potentially cytotoxic molecules including ROS, nitric oxide, proteases and proinflammatory cytokines such as interleukin-1B, tumour necrosis factor α (TNF α) and interleukin 6 (IL-6) (Shaw., 2005). Proliferation of activated microglia is a well-known histological feature in the spinal ventral horn both in mutant SOD1 transgenic mice and in human ALS/MND (Kawamata et al., 1992; Alexianu et al., 2001). Also, microglia seems to mediate the toxicity to neurons in culture of CSF from patients with ALS/MND by releasing factors that promote glutamate toxicity (Tikka et al., 2002). Therefore, activated microglia can provoke significant damage on MNs, but as explained previously, their role is complex and they are capable of stimulating neuroprotective as well as neurotoxic effects (Shaw., 2005; Mancuso & Navarro., 2015).

Novel studies provide evidence indicating that oligodendrocytes have a crucial metabolic role to support neurons (Nave., 2010). Recently it was shown an extensive degeneration of grey matter oligodendrocytes in the spinal cord of $SOD1^{G93A}$ mice before disease onset (Kang et al., 2013). Furthermore, this study revealed that selective removal of mSOD1 from oligodendroglia significantly delayed disease onset and prolonged survival of the $SOD1^{G93A}$ mice.

Recent findings have also revealed that Schwann cells have a limited involvement in ALS. The expression of mutant $SOD1^{G93A}$ in Schwann cells did not produce differences in locomotion or neuronal degeneration, and these cells were resistant to mutant SOD1 aggregation (Turner et al., 2010). However, the perisynaptic Schwann cells have an essential role in guiding motor axonal sprouts to reinnervate denervated endplates in the $SOD1^{G93A}$ mice (Mancuso & Navarro., 2015). Indeed, collateral and terminal sprouting is an important compensatory mechanism which is found reduced during the ALS course (Shefner., 2006; Mancuso et al., 2011).

Mitochondrial disfunction

Mitochondria is a cellular organelle with essential cellular roles such as the generation of intracellular ATP, calcium homeostasis maintenance and intrinsic apoptosis regulation (Mancuso & Navarro., 2015). Recent studies have demonstrated the important role of mitochondria in the pathogenesis of neurodegenerative diseases (Lin & Beal., 2006), and changes in mitochondrial morphology and biochemistry have been observed in sporadic ALS patients and in SOD1 transgenic mice (Vande Velde et al., 2011; Magrané et al., 2014). Indeed in these mice vacuoles containing protein aggregates that include mutant SOD1 can be observed in the mitochondrial inter-membrane space, guiding to a disruption in protein import (Parone et al., 2013). Also, increased mutations of mitochondrial DNA associated to a reduced mitochondrial DNA and content and respiratory chain complexes activity have been described in the spinal cord of ALS patients (Hirano et al., 1984; Wiedemann et al., 2002) and in the skeletal muscle of ALS patients (Wiedemann et al., 1998).

Since the exact mechanism by which mSOD1 might impair mitochondrial function is not clear, several possible explanations have been hypothesized (Mancuso & Navarro., 2015). mSOD1 could aggregate into the mitochondrial intermembrane space blocking the TOM/TIM protein import machines, and therefore interfering with the mitochondrial protein import (Wong 1995; Liu et al., 2004). Also, mitochondrial respiration, electron transfer chain and ATP synthesis have been shown to be disrupted in SOD1 mice at disease onset (Jung et al., 2002; Mattiazzi et al., 2002) and there is controversy with changes in ATP synthesis (Browne et al., 2006; Damiano et al., 2006). Also, it has been reported that neuronal mitochondrial calcium buffering capacity is impaired before the disease onset in the brain and spinal cord of $SOD1^{G93A}$ mice (Damiano et al., 2006). This disruption of the calcium buffering in MNs could increase their susceptibility to altered calcium homeostasis associated with glutamate-mediated excitotoxicity (Parone et al., 2013).

Mitochondria are also essential to control apoptosis since the opening of the permeability transition pore and releasing the cytochrome C from the mitochondrial intermembrane space is crucial for the activation of the caspase cascade (Mancuso

& Navarro, 2015). mSOD1 aggregates may activate an intrinsic apoptosis by the release of cytochrome C to the cytoplasm (Pasinelli et al., 2004). Interestingly, when small peptides specifically block the mSOD1-BCLK-2 complex formation, the mitochondrial dysfunction is rescued (Tan et al., 2013).

Moreover, many cellular mechanisms that are impaired in ALS are regulated by a signalling between the endoplasmic reticulum and mitochondria (Hardiman et al., 2017). These associations can be disrupted by mutations in gens such as TARDBP and FUS (Stoica et al., 2014; 2016). Indeed, TDP43 accumulates in the mitochondria of ALS patients (Wang et al., 2016). Also, in models of C9ORF72 the dipeptide repeat protein poly(GR) may compromise mitochondrial function, promoting oxidative stress and DNA damage (Lopez-Gonzalez et al., 2016).

Impaired axonal transport

MNs have long axons in humans, particularly those innervating muscles of the limbs (Mancuso & Navarro., 2015). Therefore, these cells rely on an efficient intracellular transport that has anterograde (kinesin) and retrograde (dynein) components (Shaw., 2005). Many studies have shown an accumulation of neurofilaments in MN cell bodies in human patients, implying that axonal transport is disrupted in these cells (Hirano et al., 1984; Schmidt et al., 1987; Julien et al., 1997; 1998). Strikingly, an impairment of the organelle axonal trafficking has been described in ALS patients (Breuer et al., 1987). Also, mutations in the dynein-dynactin complex, responsible for the retrograde transport can cause a progressive MND in mice (LaMonte et al., 2002; Hafezparast et al., 2003).

The identification of mutations in genes such as DCTN, PFN1 and TUBA4A in ALS patients further indicates that impairment of axonal transport proteins is involved in ALS pathology (Puls et al., 2003; Wu et al., 2012: Smith et al., 2014).

In the SOD1 transgenic mice a cytoskeletal pathology resembling that observed in human ALS develops (Tu et al., 1996), as well as an impairment of the anterograde axonal transport and an accumulation of neurofilament (Zhang et al., 1997). The mechanism by which mSOD1 perturbs axonal transport is not fully understood but there is some evidence pointing towards the binding that misfolded

SOD1 has with kinesin-associated protein 3 (KAP3) in the spinal cord ventral horn of $SOD1^{G93A}$ mice. Therefore KAP3 protein which is responsible for binding cargos including choline acetyltransferase (ChAT), is sequestrated by SOD1, and this results in the reduction of ChAT in the terminal axons, causing a synaptic deficit and finally muscle denervation (Tateno et al., 2009). However, other studies suggest that axonal transport deficits are not dependent of MN degeneration in $SOD1^{G93A}$ and in $SOD1^{G85R}$ mice since the axons can survive despite significant axonal transport dysfunction (Marinkovic et al., 2012). Thus, to define the role of axonal transport dysfunction in ALS pathogenesis, further experiments should be performed.

Impaired protein homeostasis

In ALS, mutations in some genes lead to the misfolding of translated proteins that are accumulated in abnormal cellular localizations and end up disrupting the proteasomal or autophagic machinery of the MNs (Hardiman et al., 2017). Indeed, protein aggregates are a pathological indication of numerous neurodegenerative diseases, including both sporadic and familial ALS. For instance, SOD1 inclusions are present in motoneurons of familial and sporadic ALS patients (Shibata et al., 1994), and also in mSOD1 rodent models (Bruijn et al., 1998). Moreover, recent evidences show that misfolded SOD1 binds to the cytoplasmic surface of the ER integral membrane protein derlin-1 (Nishitoh et al., 2008), and this leads to the inhibition of ER-associated degradation (ERAD) the pathway for extraction and degradation of misfolded proteins from the ER. Also, relieving ER stress delays the progression of disease in an animal model of ALS (Saxena et al., 2009). Therefore, ER stress is another pathogenic mechanism implicated broadly in ALS.

TDP-43 is a nuclear protein, and mutations in its gene (TARDBP) are a rare cause of dominant ALS (Rutherford et al., 2008; Sreedharan et al., 2008). Interestingly, its loss is commonly seen in most cells containing TDP-43 positive cytoplasmic aggregates (Zhang et al., 2008). Indeed, TDP-43 inclusions can also form protein aggregates both in neuronal and non-neuronal cells in sALS patients and in most of the fALS patients but not in SOD1 related ALS (Neumann et al., 2006; Mackenzie et al., 2007). Thus, TDP-43 cytoplasm redistribution may be an early pathogenic event in ALS (Mancuso & Navarro., 2015). Also, both TDP43 and SOD1

are known substrates of autophagy, suggesting that defective autophagy contributes to the toxic accumulation of these proteins in ALS. In addition, another RNA-binding protein found in cytoplasmic inclusions in ALS patients is FUS (Groen et al., 2010; Hewitt et al., 2010).

Mutations in the intronic hexanucleotide repeat expansion in C9ORF72 can cause ALS, and lead to the formation of stable parallel unimeric and multimeric G-quadruplex structures which can interact with RNA processing factors (Reddy et al., 2013; Haeusler et al., 2014). Strikingly these mutations have recently been established as the most prevalent known cause of ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011). The normal function of this protein is still unknown, but it is structurally related to endosomal trafficking, interacting with GDP-GTP exchange factors for RAB GTPases (Levine et al., 2013). Also, C9ORF72 participates in the regulation of the autophagy initiation and loss of this function might be linked to the presence of ubiquitin-positive, p62 positive and TDP43 negative inclusions in extra-motor areas of the CNS in ALS associated with mutations in C9orf72 (Webster et al., 2016). Interestingly, mutations in ubiquilin 2 gene (UBQLN2) which has a role in the early steps of autophagy (Wong et al., 2014; Katsuragi et al., 2015; Hjerpe et al., 2016) can cause X-linked FALS or FALS-FLTD (Deng et al., 2011).

Another protein that has a key role in the endoplasmic reticulum, proteasomal degradation and autophagy is VCP (Meyer et al., 2012). VCP is a multifunctional ubiquitin-sensitive chaperone that unfolds proteins and disassembles complexes. Mutations in the gene coding for VCP can also cause ALS (Johnson et al., 2010), and were first identified in patients with inclusion body myopathy with early onset Paget disease and frontotemporal dementia (Forman et al., 2006; Farpour et al., 2012).

Recent studies have reported a novel mechanism in the crosstalk between ER and mitochondria underlying the sigma-1 receptor in MN death (Mancuso & Navarro., 2015). Sigma-1 receptor is a chaperone protein specially expressed in MNs and located at the interface between the ER and mitochondria, called mitochondria-associated membranes (MAMs), and at the sub-synaptic cisternae of cholinergic boutons (Mavlyutov et al., 2012). Sigma-1 receptor mutations were established in FTD-ALS (Luty et al., 2010). Also, impairment of sigma-1 receptor function in MNs

disturbed the ER-mitochondrial contacts, affecting intracellular calcium signalling, inducing ER stress and changing the mitochondrial transport (Bernard-Marissal et al., 2015).

In conclusion, there is a body of evidence that the impaired protein homeostasis induced by the disruption of the proteasomal and autophagic degradation machinery contributes to the cellular failure that characterizes MNs in certain forms of ALS. This dysfunction seems to be multifactorial and involves both gain-of-function and loss-of-function mechanisms (Robberecht & Philips., 2013).

Neuroinflammation

Neuroinflammation is a essential pathogenic feature for neuronal injury and ALS progression since MNs damage promotes the activation of glial cells, further enhancing neurodegeneration (Troost et al., 1990; Zhao et al., 2013). Glial cells become increasingly activated as the disease progresses in both animal models and patients (Corcia et al., 2012; Brites et al., 2014). Studies of mutant SOD1 mice have shown that MN cell death is non-cell autonomous (Robberecht & Philips., 2013).

Microglial cells, which are the innate immune cells of the nervous system become activated in ALS (Taylor et al., 2016). These immune cells possess a dual activation phenotype, which can be neuroprotective (known as the M2 phenotype) or toxic (M1 phenotype) (Hardiman et al., 2017). Studies from transgenic SOD1 mice show that the phenotype of microglia changes from neuroprotective to neurotoxic as disease progresses, with a transformed cytokine release profile at the end-stage of the disease (Liao et al., 2012). Also, the synthesis of mutant SOD1 by microglia is an important determinant of rapid disease progression, as determined by selectively silencing of the mutant gene SOD1 in microglia (Boillée et al., 2006a) or by using cell grafts to replace microglia expressing mutant SOD1 with normal microglia (Beers et al., 2006). Moreover, the downregulation of CX3CL1 modulates the microglial activity in ALS (Cardona et al., 2006). An altered microglial function also contributes in the pathogenesis of ALS patients with C9ORF72 mutations (Taylor et al., 2016). Indeed, the inactivation of this protein in mice results in a dysfunctional microglia and neuroinflammation (Burberry et al., 2016; Jiang et al., 2016; O'Rourke et al., 2016).

Astrocytes provide nutrients for MNs, ion buffering and recycle the neurotransmitter glutamate. The selective reduction or loss of mutant SOD1 synthesis by astrocytes in mice delays the onset and progression of the disease (Lepore et al., 2008; Wang et al., 2011). This delay was accompanied by a late activation of microglia, showing that there is a functional crosstalk between mutant-SOD1-expressing astrocytes and microglia. Moreover, co-cultures of astrocytes from familial and sporadic ALS are toxic to MNs since there is an upregulation of inflammatory gene expression (Haidet-Philips et al., 2011).

Abnormal RNA processing

Impairment of the RNA processing proteins is an important pathogenical mechanism of the disease (Pasquali et al., 2014). mRNA bears a complex processing as it is transported from the nucleus to the cytoplasm, where it is translated (Hardiman et al., 2017). In neurons, mRNA can also be translated in the axonal compartment. Recent findings regarding mutations in TARDBP and FUS as rare causes of ALS has identified an essential pathogenic role for RNA-binding proteins that comprise low complexity domains (Hardiman et al., 2017). TDP-43 is mostly localized in the nucleus where it has a role in several mechanisms for RNA processing. A mislocalization of TDP-43 from nucleus to cytoplasm was found in affected areas of the brain and the spinal cord of ALS patients excluding those based on mSOD1 and FUS mutations (Mackenzie et al., 2007; 2010). It is important to say that TDP-43 mislocalization has been described in both sporadic ALS and most familial forms of ALS. Indeed, at least one-third of the transcriptome is changed in models of TARDBP-related ALS and impairment of gene expression has also been shown about mutations in C9ORF72, SOD1 and FUS genes, including changes in transcription, alternative splicing of mRNA, axonal transport of mRNA and biogenesis of micro RNAs (Chen-Plotkin et al., 2010; Ratti et al., 2016).

As reported, an abnormal expansion of the GGGGCC hexanucleotide (G4C2) sequence in the C9ORF72 gene leads to the formation of stable parallel unimeric and multimeric G-quadruplex structures, which rapidly interact with RNA processing factors (Reddy et al., 2013; Haeusler et al., 2014). This interaction leads to the production of aberrant RNA species that can be identified as nuclear RNA foci, and

might produce direct RNA toxicity, for instance sequestering RNA-binding proteins (Lee et al., 2013; Donnelly et al., 2013; Cooper-Knock et al., 2014). Furthermore, this repeat expansion can lead to the formation of R-loops, meaning DNA-RNA hybrid structures, that enhances susceptibility to DNA damage and genome instability (Haeusler et al., 2016; Santos-Pereira et al., 2015).

Other mutations have provided more evidence of RNA metabolism disturbance. Angiogenin (ANG) which is an angiogenesis promoter during hypoxia, can also act as a transfer RNA-specific ribonuclease, regulating ribosomal RNA transcription (Kieran et al., 2008). Mutations in ANG have a deleterious role through loss of function since ANG overexpression extends mSOD1 mice lifespan (Kieran et al., 2008). Also, mutations in SETX gene which regulates the transcription of ribosomal RNA (Saxena et al., 1992; Pizzo et al., 2013) can cause juvenile-onset FALS (Chen et al., 2004).

Role of muscles and neuromuscular junction

Numerous studies have shown that skeletal muscle pathology and early functional impairments occur in human sALS (Vielhaber et al., 1999; Krasnianski et al., 2005; Echaniz-Laguna et al., 2006), fALS (Comi et al., 1998; Corti et al., 2009) and also in transgenic mouse models (Derave et al., 2003; Dupuis et al., 2004; Mahoney et al., 2006) before the start of MN degeneration. Indeed, Wong and Martin (2010) developed a transgenic animal with muscle-restricted mSOD1 overexpression and proved that muscular mSOD1 is enough to cause MN degeneration.

In addition, semaphorin 3a (Sema3A) is an axonal chemorepellent molecule that was described as a possible contributor to MN degeneration. This protein is produced in terminal Schwann cells corresponding to the most vulnerable fast-fatigable motor units (De Winter et al., 2006). Treatment with an antibody that binds to the Sema3a receptor, neuropilin, improved motor function and prolonged survival in transgenic mice (Venkova et al., 2014). Another candidate protein to contribute to the ALS pathogenesis in the skeletal muscle is Nogo-A, which is highly expressed in ALS rodents and ALS patients (Dupuis et al., 2002). Indeed, the administration of antibodies against Nogo-A promoted an improvement of

neuromuscular function at least during the early stages of the disease (Bros-Facer et al., 2014).

1.4 Motoneuron vulnerability and final common pathway

One of the particularities of ALS is the selective vulnerability of certain groups of MNs, and the resistance of others. A consistent clinical feature of ALS is the preservation of eye movements and the external sphincters function. In fact, in mSOD1 mice, oculomotor nuclei are also relatively spared (Nimchinsky et al., 2000).

The specific pathogenic mechanisms that may predispose MNs to degeneration are various. First, a possible explanation of MN vulnerability is based on the structure and energy metabolism. MNs, as previously explained, are large neurons, with cell bodies of approximately 50-60 μm and axons of up to 1m long in humans. Therefore these cells demand a high metabolic input, and are more exposed to oxidative stress. Therefore, this could produce an increased ROS generation and a decrease in the mitochondrial efficiency (Barber & Shaw., 2010).

Second, several studies reinforce the theory that large phasic MNs have a special susceptibility in the degenerative process of ALS. Larger and stronger motor units are certainly more affected by the disease compared to the smaller ones, as it was shown by electromyographic analysis (Dengler et al., 1990), and histopathological studies have indicated more degeneration of large MNs in ALS (Sobue et al., 1983). Strikingly in mSOD1 mice, Pun and collaborators (2006) described a selective vulnerability of large fast-fatigable hindlimb motor units before the clinical onset, followed by degeneration of fast fatigue-resistant motor units at clinical onset, but not of slow motor units. Furthermore, other studies reported that muscles with fast fatigable motor units such as the extensor digitorum longus have a rapid denervation whereas muscles such as soleus mainly formed by slow motor units are resistant as described for $SOD1^{G93A}$ mice (Hegedus et al., 2007; 2008).

Third, this vulnerability can be further enhanced by the high susceptibility of MNs to glutamate-induced excitotoxicity. Permeability of a subtype of glutamate receptors, the GluR2 subunit, to calcium is a major determinant of this susceptibility.

In the absence of GluR2, the AMPA receptor-ion-channel complex becomes permeable to calcium. Indeed, gene array results revealed an up-regulation of the GluR2 subunit in resistant oculomotor MNs compared to the vulnerable lumbar MN population, thus reducing calcium influx into the cells (Brockington et al., 2013). MNs also express low levels of cytosol calcium-binding proteins compared to other neuronal populations, indicating that reduced cytosol calcium buffering supports the selective vulnerability of MNs (Reiner et al., 1995; Appel et al., 2001).

Fourth, some studies have shown an excitatory-inhibitory imbalance, affecting synaptic inputs into spinal MNs (Sunico et al., 2011). GABA is the most important inhibitory neurotransmitter in the CNS. Brockington and collaborators (2013) showed that in oculomotor MNs, there is an upregulation of six GABA-A receptor subunits and of GABA-B receptor subunit 2 in comparison with spinal MNs, contributing to an increased inhibition. Moreover, loss of parvalbumin-positive inhibitory interneurons in the motor cortex of ALS patients may also act reinforcing the development of cortical hyperexcitability (Nihei et al., 1993). However, it is not clear whether an increased or decreased excitation of the MNs in ALS is positive or not. In fact, vulnerable fast-fatigable spinal MNs are those with larger cell bodies and more phasic activity pattern (Mancuso & Navarro et al., 2015). Interestingly, an early administration of an AMPA receptor agonist preserved spinal MNs whereas an AMPA receptor antagonist increased the MNs degeneration in SOD1^{G93A} mice (Saxena et al., 2013). Another protein that is involved in the excitatory-inhibitory imbalance is the potassium-chloride co-transporter 2 (KCC2), which maintains low intracellular chloride levels (Bos et al., 2013; Boulenguez et al., 2010). Strikingly, recent studies reveal that in late stages of the disease KCC2 is dysregulated in the spinal MNs of SOD1^{G93A} mice (Fuchs et al., 2010; Mòdol et al., 2014). Furthermore, other studies demonstrated that the functional overload is able to rescue motor units in SOD1^{G93A} mice (Gordon et al., 2010), supporting the hypothesis of hypoexcitability as one potential factor underlying selective MN degeneration.

Fifth, there is emerging evidence showing the potential contribution of C-boutons participating in ALS pathophysiology (Casas et al., 2013; Gallart-Palau et al., 2014; Casanovas et al., 2017; Salvany et al., 2019). C-boutons are large, cholinergic synaptic terminals that arise from local interneurons and specifically contact spinal

α-MNs (Gallart-Palau et al., 2014). The C-boutons postsynaptic membrane contains numerous proteins, including sigma-1 receptor (Mavlyutov et al., 2012), and Neuregulin-1 (NRG1) whereas the presynaptic membrane contains neuregulin-1 receptors, ErbB2 and ErbB4 (Gallart-Palau et al., 2014). Interestingly, it has been shown that mutations in Sigma-1 receptor cause juvenile ALS (Al-saif et al., 2011; Prause et al., 2013). Indeed, knocking down the sigma-1 receptor in the SOD1 mice caused a reduced lifespan (Mavlyutov et al., 2013), whereas treatment with a Sigma-1 receptor agonist is neuroprotective (Mancuso et al., 2012). Moreover, loss-of-function mutations in the ErbB4 receptor are reported to produce late-onset, autosomal dominant ALS in patients (Takahashi et al., 2013), and Neuregulin/ErbB expression alterations have been shown in $SOD1^{G93A}$ mice (Song et al., 2012).

And six another prospective marker of the selective vulnerability of MNs is the matrix metalloproteinase-9 (MMP-9) (Kaplan et al., 2014). Indeed, while this protein is present in most of cranial and spinal MNs, in the oculomotor and Onuf's nuclei MMP-9 is not expressed. Also, its expression before the disease onset defines the subset of MNs that are predetermined to die and plays an essential role activating ER stress. Strikingly, partial reduction of MMP-9 levels in mSOD1 mice caused a delay in the muscle denervation and an extension of lifespan (Kaplan et al., 2014).

Furthermore, there is a controversy regarding the MN involvement in the disease. Some studies postulate that the MN death occurs due a dying back process, in which the lower MN degenerates earlier (Kiernan & Hudson., 1991, Pamphlett et al., 1995). Others hypothesize that the death is produced dying forward, in which the upper MN death takes places before the development of clinical features of lower MN dysfunction (Eisen et al., 1993; Zanette et al., 2002; Vucic & Kiernan et al., 2006; Vucic et al., 2008; Menon et al., 2015). However, in mSOD1 animal models results showed that lower MN degeneration occurs earlier than upper MN death (Mancuso et al., 2011; Ozdinler et al., 2011; Zang & Cheema., 2002).

Finally, multiple pathogenic mechanisms seem to be implicated in each mutation. However, the initial cause of the MN degeneration that triggers all these features remains unsolved. For the familial mutations, the mechanisms underlying

the MN dysfunctions begin to be clear but the question about how sALS starts is still not elucidated.

1.5 ALS models

ALS in vitro models

A commonly used in vitro model is the mouse cell line NSC-34 (Gomes et al., 2010). These cells result from the fusion of neuroblastoma cells and MNs from spinal cord (Cashman et al., 1992). Upon differentiation, NSC-34 cells display numerous MN properties including generation of action potentials, expression of neurofilament proteins and acetylcholine synthesis (Cashman et al., 1992). Furthermore, when NSC-34 cells were transfected with mSOD1 cytoplasmic inclusions and protein insolubility were observed (Turner et al., 2005), validating this model to evaluate ALS pathogenic mechanisms. Interestingly, treatment with glycoursodeoxycholic acid (GUDCA) prevents and repairs caspase-9 and MMP-9-associated MN dysfunction in the NSC-34 cells expressing $SOD1^{G93A}$. However, there is some controversy related to the physiological response of NSC-34 cells to glutamate, which as explained, is a major contributor to MN degeneration. Recent data have shown that this model is not suitable to explore the pathogenesis of glutamate-mediated toxicity (Madji Hounoum et al., 2016). Indeed, no specific effect of glutamate was observed on cultured NSC-34 cell survival and morphology. Moreover, these cultures do not to contain only differentiated cells (Madji Hounoum et al., 2016).

During the past years, the in vitro models have undergone impressive development, and have been shown as useful tools to study the pathogenic mechanisms underlying ALS (Myszczynska & Ferraiuolo., 2016). Indeed, the finding that adult human fibroblasts could be reprogrammed to induce pluripotent stem cells (iPSCs) with the use of selected transcription factors (Takahashi et al., 2007) paved the way to study not only the familial but especially the sporadic disease in vitro. The use of stem cells started with the mouse embryonic stem cells (mESC) which were established from the $SOD1^{G93A}$ mice (Di Giorgio et al., 2007). The expression of mutated-SOD1 did not affect the differentiation of these cells to MN, but after some weeks in culture, they showed SOD1 inclusions and accumulation of

ubiquitinated proteins (Di Giorgio et al., 2007). SOD1 mouse fibroblasts have been also reprogrammed into iPSCs cells and differentiated into MNs, which were proved to be electrophysiologically active (Yao et al., 2013). Human ESCs were also transfected with SOD1 mutations and have been a useful tool to study the cell-autonomous mechanisms involved in MN degeneration (Karumbayaram et al., 2009). Indeed, the authors observed a reduction in neurite length and reduced cell survival in this ALS in vitro model.

Furthermore, Egawa and collaborators (2012) found that ALS patient-specific iPSC-derived MNs had TDP-43 cytosolic inclusions similar to those seen in postmortem tissue from ALS patients. Moreover, treatment with anacardic acid, a histone acetyltransferase inhibitor, successfully rescued the abnormal ALS MN phenotype, thus indicating that iPSC-derived cells can be effectively used to identify new therapeutic compounds (Egawa et al., 2012).

Also, numerous studies have shown that iPSCs, and iPSC-derived human neurons from patients carrying C9ORF72 repeat expansion display some of the neuropathological hallmarks of ALS/FTD, like the presence of RNA foci containing GGGGCC repeats (Almeida et al., 2013). In fact, iPSC-derived MN harbouring SOD1, C9ORF72 and FUS mutations also showed hyperexcitability determined by electrophysiology methods (Wainger et al., 2014). Interestingly, a report identified deficits in the nucleocytoplasmic transport in Drosophila cells expressing hexanucleotide repeats and in iPSC-derived neurons from ALS patients, which were rescued with ASOs or small molecules targeting the G-quadruplexes (Zhang et al., 2015).

Despite these findings, most of the studies performed using iPSCs have focused on samples from patients with fALS (Myszczynska & Ferraiuolo., 2016). Only very few studies have focused on the sporadic cases. In this line, TDP-43 aggregates were found in iPSC-derived MN from sALS patients (Burkhardt et al., 2013). Also, a transcriptome analysis from MN differentiated iPSCs derived from sporadic ALS patients identified mitochondrial dysfunction as one of the strongest dysregulated mechanisms in sporadic ALS MNs compared with controls (Alves et al., 2015).

Amyotrophic lateral sclerosis, as explained, is a very complex and comprehensive disease. To study particular pathogenic mechanisms, such as

excitotoxicity, the spinal cord organotypic cultures (SCOCs) have emerged as a candidate in vitro model. This model is based on the hypothesis of glutamate toxicity in ALS, which may be induced in vitro by exposure to glutamate (Guzmán-Lenis et al., 2009) or more long lasting by DL-*threo*-β-hydroxyaspartic acid (THA) (Rothstein et al., 1993), although it may be used also to test other damaging pathways. SCOCs have some advantages over dissociated cell cultures; first, the preservation of the synaptic and anatomical organization of the neural circuitry. Second, the functional characteristics similar to those found *in vivo*, and third, the cellular stoichiometry is preserved in the spinal cord. Whereas SCOCs are not well characterized in mice yet (Kosuge et al., 2009), in rats it has provided a useful model of ALS that can be used for preclinical screening of potentially therapeutic drugs (Guzmán-Lenis et al., 2009; Herrando-Grabulosa et al., 2016; Pandamooz et al., 2016) and also trophic factors that might be involved in the disease (Mòdol-Caballero et al., 2018). SCOC represents a good model to study MN degeneration that does not depend on known genetic alterations, thus being relevant for the majority of sporadic ALS cases and other MN diseases.

ALS in vivo models

During the last 20 years, the study of ALS genetic mutations has led to the development of different transgenic animals models. Mutations in the gene coding for SOD1 on the chromosome 9 were the first identified genetic causes of ALS (Rosen et al., 1993), and the research on the abnormal function of mSOD1 has been essential for the understanding of ALS pathogenesis. However, nowadays the exact mechanisms underlying mSOD1 pathology remain unknown. The first hypothesis was based on the loss of function of the SOD1 dismutase activity. However, it was shown that in the SOD1 mice the degeneration of the MNs is likely to result from a toxic gain-of-function (Gurney et al., 1994). The first animal model carried the human mutation of a glycine to alanine conversion at the 93rd codon of SOD1 gene in a high copy number (Ripps et al., 1995). These mice develop progressive MN degeneration, which leads to hindlimb paralysis and death around 4-5 months of age and outline numerous clinical and histopathological features of both familial and sporadic forms of the human disease (Ripps et al., 1995). Apart of the development

of this model, other mSOD1 models have been created carrying other mutations such as G85R or G37R, and this led to different phenotypes in terms of disease onset, progression and survival.

Recently some publications have reported controversial results about the possible role of wtSOD1 in ALS pathogenesis. Jaarsma and collaborators (2008) showed that overexpressing wtSOD1 produced a mild axonopathy without motor dysfunction. However, another study demonstrated that overexpression of human wtSOD1 in mice contributes to the progressive MN degeneration, and results in abnormal hindlimb reflexes and reduced lifespan (Graffmo et al., 2013). It was also described that an altered conformation of wtSOD1 could play a role in sALS patients (Bosco et al., 2010). Therefore, further experiments are required to elucidate the contribution of SOD1 in ALS pathology.

Other animal models carrying different mutations have been developed. Several groups have generated TDP-43-ALS mouse models, but different phenotypes have been observed, with none of these models completely replicating an ALS phenotype (Mancuso & Navarro., 2015). Furthermore, the development of the ALS pathogenesis in the TDP-43-ALS transgenic mice is highly reliant upon the level of transgene expression, as it happens in the SOD1 mice models. The most relevant model that presents MN degeneration and phenotypical alterations may be the Prp-hTDP-43^{A315T} transgenic mouse, that shows pathologic aggregates of ubiquitinated proteins in specific neuronal populations, including pyramidal neurons in frontal cortex and spinal MNs (Wegorzewska et al., 2009; Espejo-Porras et al., 2019). These mice show motor deficits from 10 weeks of age and progressive neurodegeneration reminiscent of both ALS and FTLD. Other models carrying a FUS mutation have also been developed. These transgenic mice show different phenotypic manifestations; from motor function involvement with muscle denervation, axonopathy and spinal MN degeneration, to cognitive deficits with memory deterioration and hippocampal neurons death (Mancuso & Navarro., 2015).

Finally, C9ORF72 transgenic mice have been created using an adeno-associated virus (AAV) expressing the aberrant hexanucleotide expansion (G4C2)66, in the nervous system. This mice present neuronal loss, and also show

behavioral changes resembling C9FTD/ALS patients, including hyperactivity, anxiety, antisocial behavior and motor deficits (Chew et al., 2015). However, deletion of C9ORF72 in neurons in a conditional C9ORF72 KO mouse did not promote MN degeneration, defects in motor function activity or an altered lifespan (Koppers et al., 2015). The most interesting achievement is a BAC mouse model of C9ORF72 that shows decreased survival, paralysis, muscle denervation, motor neuron loss, anxiety-like behavior, and cortical neurodegeneration. These mice express C9ORF72 sense transcripts and upregulated antisense transcripts. RAN protein accumulation and TDP-43 inclusions were found in degenerating brain regions in end-stage animals, thus resulting in a promising ALS/FTD phenotype (Liu et al., 2016).

1.6 Relevant therapeutic strategies

Many clinical trials have been performed to assay new therapies for ALS. Unfortunately, most of them have failed. The most used treatment for ALS patients is Riluzole. The beneficial effect of this drug is achieved because of its antiglutamatergic actions, blocking the NMDA receptors, and the voltage dependent sodium channels (Doble et al., 1996). As indicated above, despite its wide use, in most cases riluzole provides a very limited benefit, prolonging survival for an average of 3-4 months. Recently, a new drug named edaravone (also known as MCI-186) showed positive effects in a phase III clinical trial with ALS patients (Edaravone ALS Study Group., 2017). It is described as a free-radical scavenger of peroxyl radicals and peroxynitrite, therefore the main effect that it produces is reducing the oxidative stress. Indeed, clinical trial with this drug showed efficacy in a small subset of people with ALS who met criteria in a post-hoc analysis. However, there is no indication that edaravone could be useful in a larger population of patients with ALS who do not meet these criteria (Edaravone ALS Study Group., 2017).

Apart of these two clinically approved drugs, other treatments that produced positive experimental results but were not successful upon translation in clinical trials, include numerous antioxidants, such as vitamin E, N-acetyl-L-cysteine (NAC), catalase creatine or coenzyme Q10 (Groeneveld et al., 2003; Shefner et al., 2004; Orrell et al., 2005; Rosenfeld et al., 2008; Kaufmann et al., 2009). The antibiotic

minocycline was especially effective expanding the lifespan of mSOD1 mice (Kriz et al., 2002), but it produced harmful effects in a phase III clinical trial (Gordon et al., 2007). A similar case occurred with glatiramire acetate (copaxone) that produced almost 25% increased survival in mSOD1 mice (Angelov et al., 2003) but in a phase III clinical trial a treatment with this drug resulted in no impact on disease progression in human ALS patients (Meininger et al., 2009).

Moreover, Arimoclomol is one of the last novel candidate treatments for ALS. It is a co-induced of the heat shock protein (HSP) response and stimulates natural folding of nascent proteins and refolding of misfolded proteins (Benatar et al., 2018). In the SOD1 mice, treatment with arimoclomol slowed disease progression and increased survival (Kieran et al., 2004). Upon translation in a class II clinical trial, it was proved to be safe and well-tolerated by the patients, although not powered for therapeutic effect (Benatar et al., 2018).

Since one of the particularities of this disease is the loss of MNs, other therapies have been focused on growth factors. Glial-derived neurotrophic factor (GDNF), insulin growth factor (IGF-1), vascular endothelial growth factor (VEGF) and ciliary neurotrophic factor (CNTF) showed promising effects on mSOD1 models but have not reached successful translation to clinical trials (Scarrott et al., 2015; Picher-Martel et al., 2016).

Another therapeutic approach used to treat ALS is stem cell therapy. iPSC as explained, are capable of differentiating into any cell type, including MNs. Therefore, these cells can be used for drug screening or studying ALS cell mechanisms or to perform cell therapies. However, this therapy presents some potential complications after the transplantation; first, the iPSC-derived cells have the ability to grow and form a teratoma, and second, it is unlikely that these cells can replace the lost MNs, its connections to the upper MNs and the muscles and therefore produce a long-term benefit in ALS pathology (Mancuso & Navarro, 2015). There are other three types of cells that have been tested as a therapy for ALS. First, mesenchymal stem cells (MSCs) transplanted in the SOD1 mice delayed the disease onset and increased the lifespan compared to untreated mice (Zhao et al., 2007). The translation into a phase I clinical trial demonstrated that the MSC transplantation into the spinal cord of ALS patients was safe, but ALS disease progression in most of

the patients was not slowed by the treatment (Mazzini et al., 2010; 2012). Second, hematopoietic stem cells have also been used as a therapeutic approach. Injection of a suspension of these cells into the spinal cord parenchyma of adult ALS mice produced functional improvement, as well as an increase in the number of MNs, but no neural differentiation of the transplanted cells was observed (Pastor et al., 2012). And third, a cell line of Neural stem cells (NSCs), called NSI-566RSC, have been transplanted into the spinal cord of SOD1 rats and mice, and the results showed contradictory preclinical results regarding the effect on motor behaviour and lifespan (Xu et al., 2011; Hefferan et al., 2012). Nevertheless, there was a significant increase in the number of spinal MNs at the site of transplantation, suggesting that NSCs might have beneficial effect on ALS pathology. The translation of this therapy to a phase I trial concluded that the transplant was safe and well-tolerated and no obvious deterioration in patient progression was observed (Feldman et al., 2014).

Controversies of preclinical studies with animal models of ALS

Most of the new therapies generated to treat ALS have failed to translate positive experimental data into successful human trials (Rothstein., 2003; Benatar., 2007). There are some evidences that can explain this failure. First of all, in vivo models such as mSOD1 mice only represent a small proportion of familial ALS rather than sporadic ALS cases. Since ALS pathophysiology is not completely understood, probably these models may lack some of the features that can resolve the success of the therapies. Also, the dose and bioability of the treatments are difficult to translate to humans based on the animal experimental data. Related with this, most therapies are usually applied before the clinical onset of the disease in the murine models, which is difficult to replicate in human sALS. In this line, the limitations of the animal models can also be an explanation for the restricted beneficial effects that can be achieved. Another concern raises on methodological issues. It is important to develop and use functional techniques that can provide solid and objective preclinical data and that can be correlated with clinical measures. However, few studies have been directed on creating new tools to interpretate correctly the findings from preclinical studies (Mancuso & Navarro, 2015).

Finally, the failure of previous clinical trials for ALS can be a result from the

disease heterogeneity and the absence of early detection biomarkers (Hardiman et al., 2017). It may be important to stratify patients who have a shared ALS pathology and phenotype, in order to identify potential subgroups of patients who respond to a given therapy. In this regard, in a recent phase III trial for edaravone, a post hoc analysis was performed to identify and recruit the possible responders (Edaravone ALS Study Group., 2017). Therefore, a clinically homogeneous group of ALS patients that were likely to respond to the treatment participated in this clinical trial which was essential for its success (Hardiman et al., 2017).

1.7 Gene therapy

Gene therapy is a promising therapeutic strategy to treat ALS. The main advantage of this therapy is that it permits to specifically deliver a gene of interest to different cells such as MNs, overcoming the difficulty of crossing the blood-brain barrier (Federici & Boulis., 2006). Particularly, adeno-associated vectors (AAV) are one of the most promising gene therapy vectors for clinical application in the CNS. They have some advantages over other viral vectors, such as the ability to provide sustained gene expression, mostly as an episomal form, avoiding the risk for insertional mutagenesis, transducing dividing and nondividing cells, the absence of toxicity associated to wild type virus, and the possibility of easily produce pure high-titer viruses in the laboratory. Viral transgene expression can also be restricted using promoters with selective and defined expression patterns (Kügler., 2016). Combining knowledge of viral tropism with promoter selectivity thus provides a potential method for cell type-specific targeting (Von Jonquieres et al., 2013). However, due to the immune response, repeated AAV dosing is not recommendable (Lorain et al., 2008) and that once an AAV has been delivered, relatively little can be done to regulate transgene expression.

Numerous AAV serotypes and hybrids have been used to date, and some of them are capable to efficiently transduce neurons and glia. These virus serotypes have been screened to demonstrate their neurotropism with age, host species, and route of administration (Hardcastle et al., 2018). Indeed, AAV9 and AAVrh10 serotypes have been shown to be good candidates for ALS (Deverman et al., 2016; Chan et al., 2017). Retrograde AAV delivery systems coding for IGF-1 and GDNF have

shown beneficial effects in the mSOD1 mice (Wang et al., 2002; Kaspar et al., 2003). Additionally, intramuscular injections of plasmids encoding multiple isoforms of hepatocyte growth factor (HGF) or a transcription factor able to increase the expression of vascular endothelial growth factor (VEGF) have been tried in ALS patients (Scarrott et al., 2015).

During the design of a gene therapy it is important to consider what cells and tissues need to be targeted and how exactly this can be most effectively achieved (Hardcastle et al., 2018). Indeed, early therapeutic interventions in mSOD1 mice also often result in greater impact on disease, as it was shown by a pre-symptomatic injection of AAV-mediated IGF-1 delivery, resulting in greater delay in disease onset and progression compared to injection at disease onset (Kaspar et al., 2003). Also, these studies show that even delivering gene therapies after the onset of the symptoms survival of mutant SOD1 rodents can be extended. Therefore, it indicates that there is a therapeutic window, in which disease progression can at least be slowed, if not stopped or even partially reversed.

Routes of administration

The routes of administration can be classified in two groups: remote or direct delivery. Remote delivery includes the minimally invasive routes of administration that rely on the potential of specific AAV serotypes to travel from the periphery to the spinal cord using the cellular axonal transport machinery (Hardcastle et al., 2018). In this group, intramuscular (IM) or intraneural (IN) injections are included. Therefore, ALS was thought to be an ideal candidate for remote gene delivery, since affected MNs could be easily targeted. Indeed, many studies have successfully shown beneficial effects following AAV-mediated remote delivery in the ALS mice models. However, remote delivery injections have revealed some problems. There have been difficulties to reproduce this retrograde axonal transport of the AAV vectors which can occur due to differences in vector production and purification methods (Towne et al., 2010). Furthermore, problems can be related to the amount of vector necessary to transduce large muscles in human patients, as well as the distances that the vector will need to reach the MNs in the spinal cord. Also, both IM

and IN injections confine gene expression to a small area corresponding to the injected motor units (Hardcastle et al., 2018).

Interestingly, since it has been shown that AAV vectors are capable of crossing the blood-brain barrier (BBB), intravenous (IV) administration has been postulated as a therapeutic approach to deliver therapeutic genes to the CNS and the muscles of the whole body. Also, this route of administration shows an important advantage compared to the IM and IN injections: the IV AAV injection can reach many more affected areas and therefore produce a systemic effect. Indeed, following this systemic administration scAAV9 crossed the BBB without any pharmacological help and successfully transduced the whole spinal cord of an SMA mice model (Duque et al., 2009; Foust et al., 2009). Moreover, systemic IV administration of AAV8 and AAV9 vectors can transduce not only all the hepatocytes but also the skeletal muscles throughout the body and the entire myocardium in mice (Nakai et al., 2005; Wang et al., 2005; Inagaki et al., 2006) Nevertheless, long-term safety of this therapy has not yet been demonstrated.

An alternative to the intravenous injection, is the intrathecal administration (IT) since it minimizes the potential for off-targets and transduces the whole spinal cord (Hardcastle et al., 2018). This viral vector delivery method consists of transferring the viral vector solution directly into the CSF, so it can diffuse and penetrate into the spinal cord parenchyma. Interestingly, in animals it may require a surgical procedure, but in humans the IT space can easily be accessed via lumbar puncture in a non-invasive percutaneous procedure. Studies in mouse models showed that following injection of AAVrh10 a striking transduction of the entire spinal cord was achieved (Guo et al., 2016).

Finally, as a direct delivery approach, the intraparenchymal injections (IP) of viral vectors have been successfully achieved in animal models of neurodegenerative diseases (Hardcastle et al., 2018). Like IM and IN, this delivery method is confined to a local area, but it can target specific regions of the spinal cord and needs much lower doses of the vector compared to the remote delivery approaches.

Combinatorial treatment

Considering the multiple ALS disease mechanisms, it is probable that combining gene therapies with different targets could provide additive effects in ALS.

In an ex vivo gene therapy approach targeting skeletal muscle of SOD1 transgenic rats, human MSC were modified to release different neurotrophic factors, with properties to protect MNs (Krakora et al., 2013). Injection of hMSC-GDNF or of hMSC-VEGF, but not of cells releasing BDNF and IGF, prolonged survival and slowed the loss of motor function in the ALS rats. Interestingly, a combined ex vivo delivery of GDNF and VEGF showed a strong synergistic effect in extending survival and protecting neuromuscular junctions and spinal MNs.

In another approach, genetic suppression of the NF-kB pathway in microglia and shRNA-mediated knockdown of SOD1 in MNs and astrocytes via systemic AAV9 administration resulted in an additive amelioration of the treated $SOD1^{G93A}$ mice (Frakes et al., 2017). Moreover, the median lifespan of these mice was expanded to a maximum survival of 204 days. It is thus clear that prognosis can be improved in ALS models by attempting a multifaceted gene therapy approach.

ASOs and RNAi

Currently, as the understanding for the gain and loss-of-function mechanisms of genetic forms of ALS grows, novel gene therapies containing antisense oligonucleotides (ASOs) and interference RNA (RNAi) have emerged (Tosolini et al., 2017). Currently, gene therapy technologies to reduce toxic RNA and/or proteins and to protect motor neurons by modulating gene expression are at the forefront of the field (Scarrott et al., 2015). However, despite broad advances in target gene identification in ALS and advances in gene therapy methodologies, a successful gene therapy for ALS continues to be elusive.

Therapeutic agents have been created to specifically target and reduce levels of toxic, mutant proteins known to cause ALS, such as SOD1, C9ORF72, TDP-43 and FUS. Indeed, results applying these oligonucleotide-mediated therapeutic approach to rodent models have shown promising results. IONIS-SOD1Rx treatment into CSF

of SOD1^{G93A} rats resulted in reduced SOD1 protein in the spinal cord (Winer et al., 2013). The ASO therapy was then translated into a clinical trial, which showed that an intrathecal delivery of 12h was safe and well-tolerated (Miller et al., 2013). Furthermore, since many sALS patients possess mutations in genes linked to fALS, ASOs translation in ALS patients may have a broader applicability.

However, ASOs show some disadvantages. They do not penetrate tissues and their site of action is limited (Tosolini et al., 2017). In fact, it is estimated that <1% of ASOs reach their target, since most of them are distributed in undesirable organs such as the liver (Godfrey et al., 2017). This suggests that to reach a therapeutic effect several injections should be performed, which can cause adverse effects (Haché et al., 2016) and toxic ASO accumulation (Godfrey et al., 2017). Therefore, numerous modifications in the ASOs have been performed to improve its safety and pharmacological properties (Evers et al., 2015).

1.8 Neuregulin 1/ErbB signaling pathway: a promising target for ALS

Neuregulin 1/ErbB pathway

Neuregulins (NRGs) constitute an extensive family of growth factors that have been involved in neural development and the homeostasis of the central and peripheral nervous system (Falls., 2003; Esper et al., 2006; Mei & Nave., 2014). Neuregulin 1 (NRG1) was the first member of the family discovered for very different biological roles, participating at all levels of the nervous system. Five additional NRG genes (NRG2, NRG3, NRG4, NRG5, and NRG6) have been identified later.

NRG1 is a 44-kD glycoprotein that interacts with the ErbB tyrosine kinase receptors. At least 16 different isoforms are produced from the NRG1 gene by alternative splicing (Falls., 2003; Steinthorsdottir et al., 2004). However, there are three major NRG1 types that can be distinguished by structural and functional differences in their N-terminal regions but share an epidermal growth factor (EGF)-like domain, essential to activate the receptors (Esper et al., 2006). NRG1 type I was identified as Heregulin (HRG, Neuregulin 1 type I), which contains an extracellular heparin-binding and an immunoglobulin-like domain (HBD) (Holmes et al., 1992;

Peles et al., 1992). NRG1 type II was identified as glial growth factor (GGF) and also has an heparin-binding domain and a kringle domain (Lemke & Brockes., 1984). The NRG1 type III was identified as the sensory and motor neuron-derived factor isoforms (SMDF, Neuregulin 1 type III) and contains a cysteine rich domain (CRD) (Ho et al., 1995).

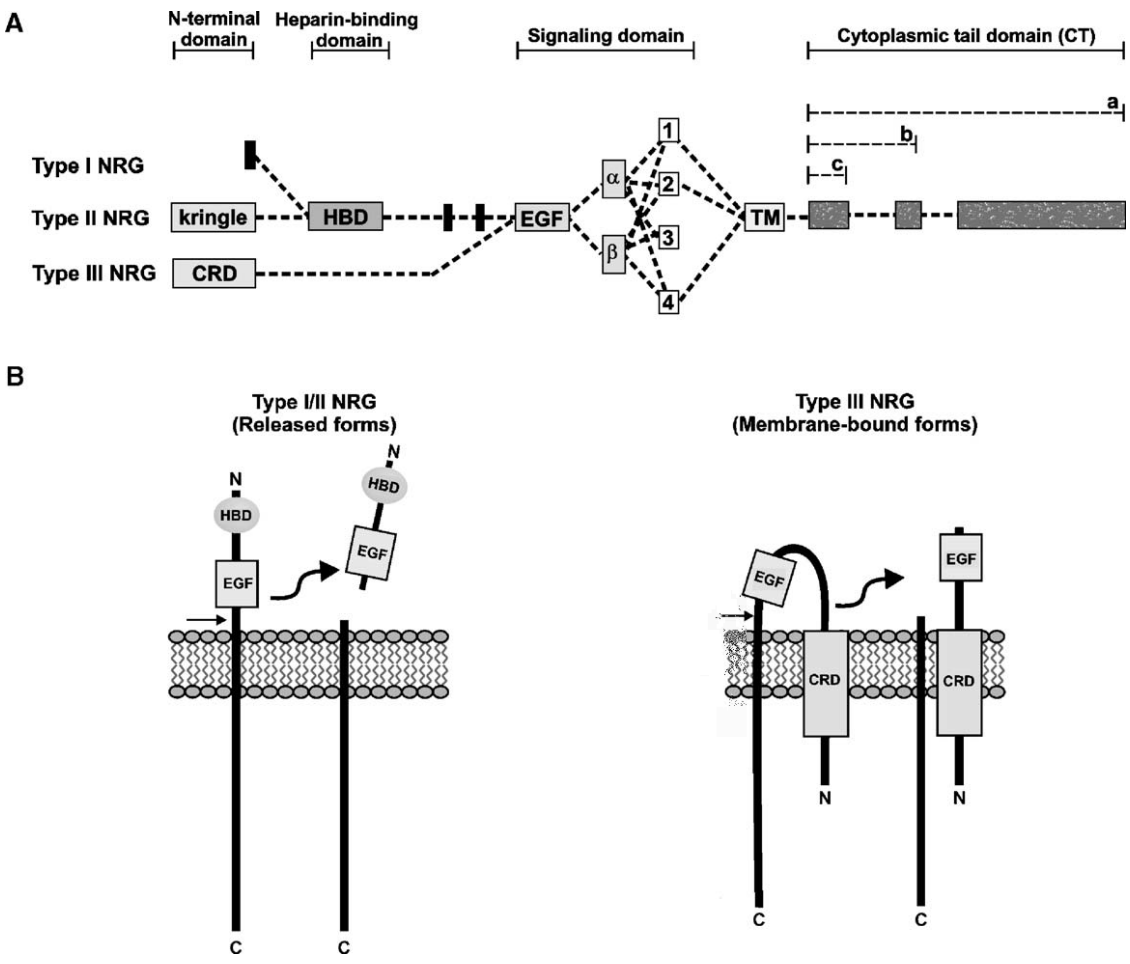

Figure 3. Alternative splicing of the NRG1 gene yields distinct isoforms. (A) The NRG1 gene produces at least 15 different proteins through alternative splicing through the use of multiple promoters. There are three major types of NRG1 proteins (I, II, III) distinguished by the N-terminal domain. All forms of NRG1 share a common EGF-like domain that is necessary and sufficient for signaling. Most forms of NRG1 are synthesized as transmembrane (TM) precursors with one of three possible cytoplasmic tails (CT), a, b, and c. (B) The Type I and II forms of NRG1 contain a heparin-binding domain (HBD) near their N-terminus, which functions to target NRG1 activity through interactions with HSPGs. After

proteolytic cleavage and release of the ectodomain, these forms of NRG1 signal to nearby cells in a paracrine manner. The Type III forms of NRG1 have a cysteine-rich domain (CRD) near their N-terminus, which loops back into the plasma membrane to function as a transmembrane anchor. Following proteolytic cleavage, this form of NRG1 signals to adjacent cells in a juxtracrine manner. (Extracted from Esper et al., 2006).

They are tissue-specific and differ significantly in their structure. Immature NRGs are transmembrane proteins, which can be cleaved by extracellular proteases such as protease β-secretase 1 (BACE1) and disintegrin and metalloproteinase (ADAM) (Hu et al., 2006; Willem et al., 2006; Savonenko et al., 2008; Luo et al., 2011; Velanac et al., 2012) and neuropsin to release soluble, mature NRG1 in the case of type I and type II (Tamura et al., 2012), or remain tethered to the membrane even after proteolytic processing as a membrane-associated form for type III (Schroering & Carey et al., 1998; Wang et al., 2001; Cabedo et al., 2002; Falls et al., 2003). Once released, localization of NRG1 type I and type II forms are restricted through interactions with cell-surface and extracellular matrix heparan-sulfate proteoglycans (HSPGs) (Loeb & Fischbach., 1995; Meyer et al., 1997; Loeb et al., 1999; Li & Loeb., 2001; Loeb., 2003; Li et al., 2004a,b). Through the EGF domain, NRGs activate the receptor tyrosine kinases of the ErbB family, a family of tyrosine kinase transmembrane receptors, each of which initiates intracellular signaling pathways in a specific way, through canonical and noncanonical mechanisms. The ErbB family of proteins contains four receptor tyrosine kinases, structurally related to the epidermal growth factor receptor (EGFR), its first discovered member. In humans, the family includes Her1 (EGFR, ErbB1), Her2 (Neu, ErbB2), Her3 (ErbB3), and Her4 (ErbB4). The gene symbol, ErbB, is derived from the name of a viral oncogene to which these receptors are homologous: erythroblasticleukemia viral oncogene.

Insufficient ErbB signaling in humans is associated with the development of neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease (Bublil & Yarden., 2007), while excessive ErbB signaling is associated with the development of a wide variety of solid tumors (Cho & Leahy., 2002). Each one of the ErbB family members has an exclusive group of ligands except ErbB2, whose ligand

is unknown. Also, the ErbB3 receptor has a disrupted kinase activity. Particularly, NRG1 binds to either ErbB3 and ErbB4 receptors, after which it can produce ErbB3/ErbB2 or ErbB4/ErbB2 heterodimers or ErbB4 homodimers able to promote complex signaling cascades (Lee et al., 1995; Pinkas-Kramarski et al., 1996a,b; Riese et al., 1995). These receptors are able to form 28 different homo and heterodimers in order to activate their downstream targets (Iwakura & Nawa., 2013).

The NRG1/ErbB signaling networks consist of several modules that are interconnected and overlapping; soluble NRG1-I binds and activates ErbB kinases to activate the canonical pathways (Mei & Nave, 2014). This activation promotes a subsequent phosphorylation of the intracellular domains (ICDs) which create docking sites of adaptor proteins including growth factor receptor-bound protein 2 (Grb2) and SH2 domain (Src homology 2 domain) for the extracellular signal-regulated kinases (Erk) activation and for phosphoinositide 3-kinase (PI3K) activation (Loeb & Fischbach., 1995; Gerecke et all, 2001; Holbro & Hynes., 2004; Ferguson., 2003; Bagossi et al., 2005). NRG1/ErbB signaling can also activate noncanonical pathways. NRG1 type III can directly interact with transmembrane ErbB4 in a cell adhesion-dependent manner, that may be kinase-independent (Chen et al., 2008; Krivosheya et al., 2008; Fazzari et al., 2010; Del Pino et al., 2013). Moreover, cleavage of NRG1 or ErbB4-C-terminal fragments release their respective ICDs that are believed to signal into the nucleus (Ni et al., 2001; Lee et al., 2002; Bao et al., 2003; 2004; Sardi et al., 2006). Interestingly, neurotrophic factors such as GDNF, BDNF and NT-3 secreted by muscle and glial cells can stimulate the release of NRG1 by MNs (Loeb and Fischbach., 1997; Loeb et al., 2002). Indeed after blockade by curare at neuromuscular synapses, both BDNF and GDNF are able to recover NRG1 expression (Loeb et al., 2002; Loeb., 2003).

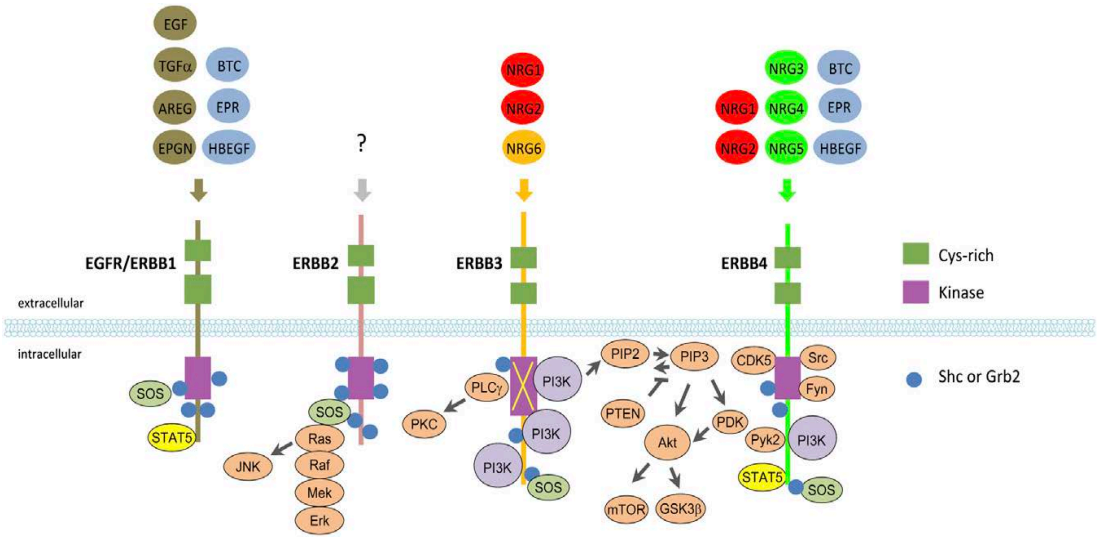

Figure 4. NRG1-ErbB canonical signaling (Extracted from Mei & Nave, 2014).

NRG1/ErbB in the CNS

NRG1 has an essential role during development and in the mature nervous system. NRG1-I and specially NRG1-III are the predominant isoforms expressed in the brain (Law et al., 2004; Liu et al., 2011; Weickert et al., 2012) and also in the spinal cord by MNs (Ho et al., 1995; Meyer et al., 1997; Loeb et al., 1999; Wolpowitz et al., 2000), astrocytes (Pinkas-Kramarski et al., 1994) and oligodendrocytes (Nave & Salzer., 2006). Likewise, ErbB receptors are also expressed in the CNS by MNs, astrocytes, oligodendrocytes and microglial cells (Canoll et al., 1996; Cannella et al., 1999; Deadwyler et al., 2000).

NRG1/ErbB signaling participates at multiple stages of cortical circuit development. NRG1 and ErbB kinases, in particular ErbB4, are essential for the assembly of the GABAergic circuitry, including interneuron migration, axon and dendrite development and synapse formation (Mei & Nave., 2014). Indeed, two models have been postulated to explain the NRG1 role in interneuron migration during cortical development. In the first one, the interneurons that express ErbB4 migrate on a permissive corridor of NRG1 through the developing striatum in response to soluble NRG1 type I in the cortex (Flames et al., 2004). In the second one, NRG1 and NRG3 proteins function as repellents that channel interneurons as they migrate from the medial ganglionic eminence (MGE) to cortical destinations (Li

et al., 2012). Moreover, postsynaptic ErbB4 may regulate presynaptic differentiation by transsynaptic interaction with transmembrane NRG1 or another binding partner (Krivosheya et al., 2008).

Since NRG/ErbB signaling is crucial for the development of neural circuits in the brain and also in the adult brain, it can be expected that NRG and ErbBs may also contribute to neuropsychiatric diseases. Indeed, many NRGs and ErbBs have been suggested to play a role in schizophrenia and other psychiatric disorders (Mei & Nave., 2014). In particular, NRG1 has been identified as a candidate gene for this disease because of a genetic analysis from schizophrenic patients from Iceland (Stefansson et al., 2002). Other studies have corroborated the genetic association between NRG1 and schizophrenia in other populations (Petryshen et al., 2005; Li et al., 2006; Munafò et al., 2006; Norton et al., 2006).

NRG1/ErbB expression has also important functions in neurotransmission and neuroplasticity mechanisms. ErbB4 is found postsynaptically in excitatory synapses where it interacts with PSD-95 (Garcia et al., 2000; Huang et al., 2000). Indeed, neutralization of endogenous NRG1 and mutation of ErbB4 both enhance hippocampal long-term depression (LTP), suggesting that synaptic plasticity is regulated by NRG1 in vivo (Chen et al., 2010; Pitcher et al., 2008; Agarwal et al., 2014).

Moreover, the NRG1/ErbB signaling may have an important role in the dopaminergic circuitry (Mei & Nave., 2014). ErbB4 is expressed in dopaminergic neurons of the midbrain (Steiner et al., 1999; Zheng et al., 2009; Abe et al., 2009; Neddens & Buonanno., 2011). Indeed, injection of NRG1 dorsal to the substantia nigra increased dopamine levels in the midbrain (Yurek et al., 2004). How NRG1 promotes dopamine release remains unclear, but it was proposed that NRG1 may elevate dopamine release through a disinhibitory circuit consisting of ErbB4-positive interneurons (Kwon et al., 2008). In the adult brain, ErbB4 receptors are also localized at excitatory synapses on interneurons (Fazzari et al., 2010; Ting et al., 2011). One function of ErbB4 is to promote GABA release, which can be increased by soluble NRG1 (Woo et al., 2007; Chen et al., 2010). These observations propose that GABAergic activity may be under control of endogenous NRG1 and thus, NRG1 is associated in a wide spectrum of brain functions, from autonomic control of blood

pressure (Matsukawa et al., 2013) to cognition.

NRG1/ErbB in the PNS

Since NRG1 KO mutant mice died from heart failure at E11 (Meyer & Birchmeier., 1995) the first in vivo evidence for the role of NRG1 in the PNS came from mutant mice lacking ErbB3 (Riethmacher et al., 1997). These mice had a complete lack of Schwann cells and also lost most of the sensory DRG neurons and spinal MNs. These results proposed a potential neurotrophic function of Schwann cells for the survival of associated neurons. Indeed, axonal NRG1-III is a key signaling molecule that regulates the behavior of myelinating Schwann cells, which express the ErbB2 and 3 receptors (Syroid et al., 1996). As reported NRG1-III it is the most prominent NRG1 isoform expressed in MNs (Meyer et al., 1997; Loeb et al., 1999; Wolpowitz et al., 2000). Certainly, mutant embryos that lack this membrane-associated isoform have a striking perinatal death, which is associated with a lack of Schwann cells and reduction of DRG cells and MNs (Wolpowitz et al., 2000). When comparing transgenic mice expressing either NRG1 type I or type III cDNA transgenes, only NRG1-III emerged as regulator of myelination. Its overexpression caused Schwann cells to hypermyelinate axons in peripheral nerves. On the contrary, loss of this gene caused significant hypomyelination. Together, these observations identified an important developmental function for NRG1-III (Michailov et al., 2004).

The role of NRG1-I is not fully elucidated. Transgenic mice overexpressing neuronal NRG1-I exhibited hyperactivity, impaired working memory, contextual fear conditioning and social interaction (Deakin et al., 2009; Kato et al., 2010; Deakin et al., 2012; Luo et al., 2013; Yin et al., 2013). Currently, this isoform has been related to axoglial development (Esper & Loeb., 2004) and the remyelination process of the PNS after nerve injury. In vitro, soluble NRG1-I enhances survival and proliferation of cultured Schwann cells (Dong et al., 1995) whereas in intact nerves, axonal NRG-III controls myelination and inhibits NRG1-I expression by Schwann cells. After Wallerian degeneration, Schwann cells are detached and the axonal NRG1-III signal is lost. At this point, NRG1-I expression is upregulated and promotes Schwann cell differentiation and remyelination in a transient autocrine/paracrine signaling loop

(Stassart et al., 2013).

NRG1-I is also critical for the formation of muscle spindles (Andrecheck et al., 2002; Hippenmeyer et al., 2002) and the maintenance of neuromuscular synapses (Sandrock et al., 1997; Loeb et al., 2002; Loeb., 2003). Indeed NRG1-I has an important role in mediating the nerve-dependent accumulation of acetylcholine receptors (AChRs) in the postsynaptic membrane (Sandrock et al., 1997). Moreover NRG1/ErbB signaling in the NMJ helps to maintain the structural integrity of the postsynaptic apparatus and the stability of AChRs (Schmidt et al., 2011). The authors also described that loss of NRG1/ErbB signaling destabilizes the anchoring of acetylcholine receptors in the postsynaptic muscle membrane.

Neuregulin 1 expression is altered in ALS

In brief, alterations of the NRG-1/ErbB system have been recently related to ALS since loss-of-function mutations on NRG1 receptor ErbB4 produce late-onset, autosomal-dominant ALS in human patients (Takahashi et al., 2013). A recent study reported that ErbB4 ectodomain fragments were reduced in the CSF and plasma of ALS patients, as well as in plasma of $SOD1^{G93A}$ mice, suggesting impaired NRG1-ErbB signaling (López-Font et al., 2019). The levels of NRG1 type III expression have been found reduced in the spinal cord of both ALS patients and $SOD1^{G93A}$ mice in parallel with MN loss, but NRG1 type I was increased and associated with glial activation (Song et al., 2012). Remarkably both spliced forms are highly expressed in MNs. However some controversy exists regarding the NRG1-I expression, since Lasiene and collaborators (2016) found that this isoform was decreased in SOD1 transgenic mice. Therefore, it may be hypothesized that the two isoforms, I and III, have different roles in ALS.

Furthermore, increased ErbB2 receptor activation was observed on activated microglia in both ALS patients and in $SOD1^{G93A}$ mice, observed at the time of disease onset, and prior to upregulation of NRG1-I gene expression in the mice (Song et al., 2012). This supports the hypothesis that NRG1 released from injured neurons and other cells in the spinal cord induce microglial activation, which could contribute to progressive MN degeneration in ALS (Song et al., 2012). Indeed, blocking the excessive NRG1 signaling with an antagonist (HBD-S-H4) resulted in reduction of

microglial activation and MN loss. This treatment also resulted in a delay in disease onset and an increase in survival of the transgenic mice (Liu et al., 2018).

NRG1 localization seems also to be altered during ALS progression (Gallart-Palau et al., 2014; Lasiene et al., 2016). In normal conditions NRG1 accumulates in the postsynaptic subsurface cistern of C-boutons, whereas ErbB2 and ErbB4 are located presynaptically, suggesting that NRG1 retrograde signaling occurs in these synapses (Gallart-Palau et al., 2014). However, during the disease progression in the $SOD1^{G93A}$ mice there is a transient increase in NRG1 in C-boutons (Gallart-Palau et al., 2014). Nevertheless, Lasiene et al. (2016) described that there was a loss of NRG1 expression and that ErbB4 and ErbB3 were reduced in MNs of ALS mice around the disease onset. Also, viral-mediated delivery of NRG1-III in the CNS restored the number of C-boutons and produced an increase of the survival of $SOD1^{G93A}$ mice (Lasiene et al., 2016). Since ErbB4 receptor was mainly localized in the MN in the spinal cord, NRG1 may provide neuroprotection to MNs in an autocrine manner. Therefore, maintenance of the NRG1-ErbB4/3 axis might be a relevant strategy for amelioration of MNDs.

Nonetheless, the exact role of NRG1-I in ALS is not fully known. Overexpression of NRG1-I by means of an adeno-associated viral (AAV) vector, produced functional improvement in the gastrocnemius muscle by enhancing the collateral sprouting in the $SOD1^{G93A}$ mice and in wild type mice after partial muscle denervation (Mancuso et al., 2016). Indeed, NRG1 increases terminal Schwann cells survival after denervation and promotes extension of axonal sprouts required for new NMJ formation (Fricker et al., 2011; Mancuso et al., 2016). Thus, NRG1-I plays a relevant role in the muscular collateral reinnervation process. Thereby, the manipulation of NRG1 expression might represent a promising novel therapeutic target on ALS disease since it acts on the key element affected: the motor neurons and the neuromuscular connections.

OBJECTIVES

The general aim of this thesis is to evaluate the therapeutic effect of overexpressing NRG1 in the CNS and the PNS. For this purpose we established the following specific objectives:

1. To characterize and evaluate the role of exogenous NRG1 in the SCOC based on chronic excitotoxicity
2. To overexpress NRG1-III by gene therapy to enhance its expression in the spinal cord of the $SOD1^{G93A}$ mice and exert protection of spinal MNs
3. To overexpress NRG1-I in skeletal muscles by gene therapy under the desmin promoter to maintain muscle innervation in the $SOD1^{G93A}$ mice
4. To perform a combined gene therapy for overexpressing NRG1-I and NRG1-III to preserve the MNs in the spinal cord and the muscle innervation in the $SOD1^{G93A}$ mice.

CHAPTER I

NEUREGULIN 1 REDUCES MOTONEURON CELL DEATH AND PROMOTES NEURITE GROWTH IN AN IN VITRO MODEL OF MOTONEURON DEGENERATION

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
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Neuregulin 1 Reduces Motoneuron Cell Death and Promotes Neurite Growth in an *in Vitro* Model of Motoneuron Degeneration

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Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disorder with no effective treatment currently available. Although the mechanisms of motoneuron (MN) death are still unclear, glutamate excitotoxicity and neuroinflammatory reaction are two main features in the neurodegenerative process of ALS. Neuregulin 1 (NRG1) is a trophic factor highly expressed in MNs and neuromuscular junctions. Several recent evidences suggest that NRG1 and their ErbB receptors are involved in ALS. However, further knowledge is still needed to clarify the role of the NRG1-ErbB pathway on MN survival. In this study we used an *in vitro* model of spinal cord organotypic cultures (SCOCs) subject to chronic excitotoxicity caused by DL-*threo*-β-hydroxyaspartic acid (THA) to characterize the effect of NRG1 on MN survival. Our results show that addition of recombinant human NRG1 (rhNRG1) to the medium significantly increased MN survival through the activation of ErbB receptors which was ablated with lapatinib (LP), an ErbB inhibitor, and reduced microglial reactivity overcoming the excitotoxicity effects. rhNRG1 activated the pro-survival PI3K/AKT pathway and restored the autophagic flux in the spinal cord culture. Moreover, addition of rhNRG1 to the medium promoted motor and sensory neurite outgrowth. These findings indicate that increasing NRG1 at the spinal cord is an interesting approach for promoting MN protection and regeneration.

Keywords: neuregulin 1, ErbB receptors, motoneuron, excitotoxicity, spinal cord, organotypic culture, amyotrophic lateral sclerosis, neurite growth

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common form of motoneuron (MN) disease, characterized by the loss of MNs of primary motor cortex, brainstem and spinal cord (Wijesekera and Leigh, 2009). Most patients with ALS die within 3–5 years after symptoms onset due to respiratory failure (Robberecht and Philips, 2013). Unfortunately, there is no therapy for this disease, and the only drugs approved for use in ALS, riluzole and edaravone, only slightly prolong patients' survival. The etiopathogenesis of ALS remains to be elucidated, but it has been proposed that a complex interplay between excitotoxicity, neuroinflammation, oxidative stress, protein aggregation and mitochondrial dysfunction contribute to MN degeneration (Robberecht and Philips, 2013; Mancuso and Navarro, 2015). Among these pathogenetic mechanisms, excitotoxicity

is considered as a firm mechanism involved in the disease because of data obtained from ALS patients and animal and cellular models as well as inferred by the therapeutic effect of riluzole, an antiglutamatergic drug. The importance of excitotoxicity in MN death has been demonstrated in ALS mouse models in which excessive extracellular glutamate levels contribute to MN loss (Van Damme et al., 2005). Several *in vitro* models have been used to study glutamate neurotoxicity. Among them, the spinal cord organotypic culture (SCOC) offers advantages for assessing MN degeneration and potential therapeutic agents (Herrando-Grabulosa et al., 2016). This model is based on the selective inhibition of glutamate transport which continuously raises the concentration of glutamate in the culture medium, resulting in a slow degeneration of spinal MNs over several weeks (Rothstein et al., 1993).

Neurotrophic factors derived from alternatively spliced forms of the neuregulin 1 (NRG1) gene have been shown to play an important role in peripheral nerve development and regeneration, myelination, maintenance of neuromuscular junctions, and also on microglial activation in peripheral nerve diseases, whereas ablation of NRG1 impairs axonal regeneration (Mei and Nave, 2014). NRG1 acts through the EGF domain of ErbB receptors, a family of tyrosine kinase transmembrane receptors. NRG1 is localized in the MN endomembrane system, destined to be anterogradely transported either for axon-Schwann cell signaling or for delivery to the neuromuscular junction. Interestingly, NRG1 has been found present within the subsurface cistern in postsynaptic sites of cholinergic C terminals apposed to spinal MNs (Issa et al., 2010; Gallart-Palau et al., 2014). ErbB2 and ErbB4 receptors are present in the presynaptic compartment, suggesting that NRG1 acts as a retrograde signaling molecule in MNs synaptic connections. A role of NRG1 expressed in C synaptic boutons was recently suggested for ALS, since spinal MNs showed a transient increase of NRG1 during disease progression in $SOD1^{G93A}$ mice, whereas oculomotor MNs, which are spared in ALS, lack both C boutons and associated NRG1 (Gallart-Palau et al., 2014). In fact, it was recently reported that viral-mediated delivery of type III-NRG1 to the spinal cord restored the number of C-boutons and slightly extend the survival of SOD1 transgenic mice (Lasiene et al., 2016).

Alterations in the NRG1/ErbB system have been related to MN degeneration and ALS. Loss-of-function mutations on the NRG1 receptor ErbB4 produce late-onset, autosomal-dominant ALS in human patients (Takahashi et al., 2013). NRG1 type III expression was found reduced in both ALS patients and $SOD1^{G93A}$ mice in parallel with MN loss, but NRG1 type I was increased and associated with glial activation (Song et al., 2012). We recently reported that increased expression of NRG1 in skeletal muscle promotes collateral reinnervation and neuromuscular junction maintenance in the $SOD1^{G93A}$ mouse model of ALS (Mancuso et al., 2016).

Our goal in this study was to evaluate the role of NRG1 in the SCOC subjected to chronic excitotoxicity, in order to assess the potential effects of exogenous NRG1 on MN survival and regeneration, and its mechanisms of action.

MATERIALS AND METHODS

Spinal Cord Organotypic Cultures for Assessment of Neuroprotection

SCOCs were prepared on the basis of the method previously described (Rothstein et al., 1993). The experimental procedure was approved by the Ethics Committee of Universitat Autònoma de Barcelona and followed the European Communities Council Directive 2010/63/EU. P8 Sprague-Dawley rats were used in this study. After euthanasia, the spinal cord was aseptically harvested and placed in ice-cold high glucose-containing (6.4 mg/mL) Gey's Balanced Salt Solution (GBSS; Sigma-Aldrich, St. Louis, MO, USA), and meninges were removed. The spinal cord was cut transversely in 350 μm thick slices using a McIlwainTissue Chopper (The Mickle Laboratory Engineering Co., Surrey, UK).

To investigate MN survival, glial reactivity and the ErbB and signaling pathways activation SCOCs were obtained from at least three independent cultures performed at different days and resulting in 12 slices for condition. L4-L5 lumbar sections were carefully transferred on Millicell-CM porous membranes (0.4 μm; Millipore, Burlington, MA, USA) into a six-well plate containing 1 mL of incubation medium (50% minimal essential medium (MEM), 25 mM Hepes, 25% heat-inactivated horse serum, 2 mM glutamine, and 25% Hank's Balanced Salt Solution (HBSS) supplemented with 25.6 mg/ml glucose; pH 7.2). Cultures were let to stabilize for 1 week. During the first week in a SCOC, a high number of neurons die naturally and glial cells show strong reactivity due to the axotomy performed during the culture procedure, which later stabilize. Then DL-*threo*-β-hydroxyaspartic acid (THA; 100 μM) was added to induce chronic excitotoxicity (Corse et al., 1999). Concomitantly, some slices were treated with recombinant human NRG1 (rhNRG1; 100 ng/ml) or with lapatinib (LP), an ErbB blocker, at two different concentrations (6 μM and 12 μM). PRE084 (10 μM) a sigma-1 receptor agonist that promotes neuroprotection and neurite elongation was used as a positive control (Guzmán-Lenis et al., 2009). THA and the treating compounds were renewed at each medium exchange, twice per week until 14 days *in vitro* (DIV) for western blot analysis and histological stainings, or to 28 DIV for MN survival and microglial reactivity analyses. In this model of chronic excitotoxicity, THA induces around 40% of MN death after 3 weeks of treatment (28 DIV; Rothstein et al., 1993), whereas microglial activation occurs after 2 weeks of THA treatment (21 DIV) and is maintained at 28 DIV (Lee et al., 2012).

Protein Extraction and Western Blot

SCOC slices were prepared for protein extraction and homogenized in modified RIPA buffer (50 mM Tris–HCl pH 7.5, 1% Triton X-100, 0.5% sodium dodecyl sulfate (SDS), 100 mM NaCl, 1 mM EDTA) adding 10 μl/ml of Protease Inhibitor cocktail (Sigma) and PhosphoSTOP phosphatase inhibitor cocktail (Roche). Twenty to forty microgram of protein of each sample were loaded in SDS-polyacrylamide gels at different percentages (7.5%–15%). For heavy proteins (ErbB receptors) the western blot transfer was applied 3.5 h at room temperature with constant intensity of 360 mA. For the rest of the proteins the transfer was made 1 h at room

temperature with constant voltage of 90 V. The membranes were blocked with 5% BSA in TBS plus 0.1% Tween-20 for 1 h, and then incubated with primary antibodies at 4°C overnight. The primary antibodies used were: anti-NRG1 (1:200, sc-228916, Santa Cruz Biotechnology), anti-ErbB4 (1:500, 4795S, Cell Signaling), anti-ErbB3 (1:500, 12708S, Cell Signaling), anti-ErbB2 (1:500, 4290S, Cell Signaling), anti-pErbB4 (1:500, 4757S, Cell Signaling), anti-pErbB3 (1:500, 2842S, Cell Signaling), anti-pErbB2 (1:500, 2243L, Cell Signaling), anti-Akt (1:1000, 4691S, Cell Signaling), anti-pAkt (1:1000, 4060, Cell Signaling), anti-ERK1/2 (1:500, 4348, Cell Signaling), anti-pERK1/2 (1:500, 9106, Cell Signaling), anti-LC3 (1:500, ab51520, Abcam), anti-p62 (1:500, 610833, BD Biosciences), anti-Beclin 1 (1:1000, ab62557, Abcam), anti-Actin (1:10,000, A5316, Sigma) and anti-GAPDH (1:10,000, MAB374, Millipore). Horseradish peroxidase-coupled secondary antibody (1:3000; Vector Laboratories, Burlingame, CA, USA) incubation was performed for 1 h at room temperature. The membranes were visualized using enhanced chemiluminescence method and the images were collected using a Chemidoc apparatus. Western blots were then analyzed using the Lane and Band plugin from the Image Lab software (BioRad), and normalized first by the loading control (actin and GAPDH) and afterwards by each control sample. Each sample analyzed was extracted from four slices and 3–7 samples were analyzed per each treatment condition.

Immunoflurorescence Analyses

We fixed slices in the different experimental conditions with 4% paraformaldehyde in PBS for 1 h at RT. After blocking with 5% normal horse serum (Vector Laboratories, Burlingame, CA, USA) and 0.3% Triton-X-100 in TBS (TBS-TX), we incubated the sections for 48 h with primary antibodies against anti-neurofilament H non-phosphorylated (SMI-32, 1:1000, BioLegend) or anti-ionized calcium binding adapter molecule 1 (Iba-1, 1:1000; Wako, Japan). Slices were thoroughly washed in TBS with 0.1% Tween-20 (TBS-T) and incubated with the appropriate secondary antibody Alexa Fluor®488 donkey anti-rabbit IgG (1:500) and Alexa Fluor®594 donkey anti-mouse IgG (1:500; Invitrogen, Carlsbad, CA, USA), diluted in TBS-T for 2 h. Finally, cell nuclei were labeled with DAPI (1:2000) for 1 min in TBS and the sections mounted with Fluoromount-G medium (SouthernBiotech, Birmingham, AL, USA). We analyzed the slides under confocal microscopy, and counted the SMI-32 positive cells in each hemislice using the tool Cell Counter from ImageJ software (NIH)[1]. MNs were selected according to the following criteria: localization in ventral horns and polygonal shape, with clear dendrites. To quantify the microglia, we selected the ventral zone using for each image the same area, and then quantified the integrated density (area of the ROI × mean of the ROI) using the ROI manager tool of ImageJ.

Immunohistochemical Analyses

Slices were fixed with 4% paraformaldeyde in PBS for 24 h at 4°C, and then, cryoprotected in 30% sucrose in PBS and stored at 4°C. Cyroprotected slices were then sectioned with a cryostat (Leica) into 6–8 10 μm thick sections from each SCOC. The endogenous peroxidase activity was inhibited (70% Methanol, 30% TBS 1X, 2% H_2O_2) and thereafter a blocking solution (5% normal horse serum and 1% BSA in TBS-T) was added. We incubated the slides overnight at 4°C with primary antibodies against anti-ErbB4 (1:100, 4795S, Cell Signaling) and anti-ErbB2 (1:100, 4290S, Cell Signaling). Slides were washed with TBS-T and incubated with a secondary antibody horse anti rabbit HRP conjugate (Vector Laboratories, USA) overnight at 4°C. Afterwards, we incubated the slides with the VECTASTAIN® Elite® ABC complex (Vector Laboratories, USA) for 1 h at RT and a DAB solution (Vector Laboratories, USA) was used for brown color development. We finally counterstained the slides with cresyl violet to localize the MNs in the ventral horn.

Organotypic Cultures for Assessment of Neurite Growth

For assessing neurite outgrowth, we used spinal cord sacral sections and also dorsal root ganglia (DRG) explant cultures (n = 11/group) embedded in a collagen gel, as previously described (Allodi et al., 2011; Santos et al., 2016). Collagen type I solution (#354236, Corning) at a concentration of 3.83 mg/ml was mixed with basal Eagle's medium (Gibco) and 7.5% of sodium bicarbonate solution. NRG1 at a concentration of 100 ng/ml was added to the collagen gel in the treated slices, whereas the same volume of PBS was used for the control slices. Single 30 μl drops were deposited on poly-D-lysine (1 g/ml, Sigma) coated coverslips, which were placed in 24-well multidishes (Iwaki, Asahi Technoglass, Chiba, Japan) and kept in the incubator for 2 h to induce collagen gel formation. Spinal cord slices and DRG explants were then embedded in the gelled collagen droplets, and placed in the incubator for 45 min before adding Neurobasal medium (NB, Invitrogen), supplemented with B27 (Invitrogen), glutamine and penicillin/streptomycin (Sigma). After 1 day in culture, the medium of spinal cord cultures was removed and changed by a penicillin/streptomycin free medium. DRG explants were cultured for 2 days, and spinal cord slices for 4 days.

Neurite Growth Analysis

Spinal cord and DRG cultures were fixed with 4% paraformaldehyde in PBS for 30 min. Afterwards, the samples were incubated for 48 h with primary antibody mouse RT97 that recognizes phosphorylated epitopes of neurofilaments (1:200, Developmental Studies Hybridoma Bank) at 4°C, washed and incubated with secondary antibody AF594 conjugated donkey anti-mouse (1:200, Jackson IR) overnight at 4°C. After two washes samples were mounted on slides with Mowiol containing DAPI (1:10,000, Sigma) nuclear counterstain.

Cultures were visualized with an Olympus BX51 fluorescence microscope, images of different areas were taken with Cell A software (Olympus) and merged using Adobe Photoshop CS3 (Adobe System). Whole culture images were analyzed with the

[1]http://rsb.info.nih.gov/ij/

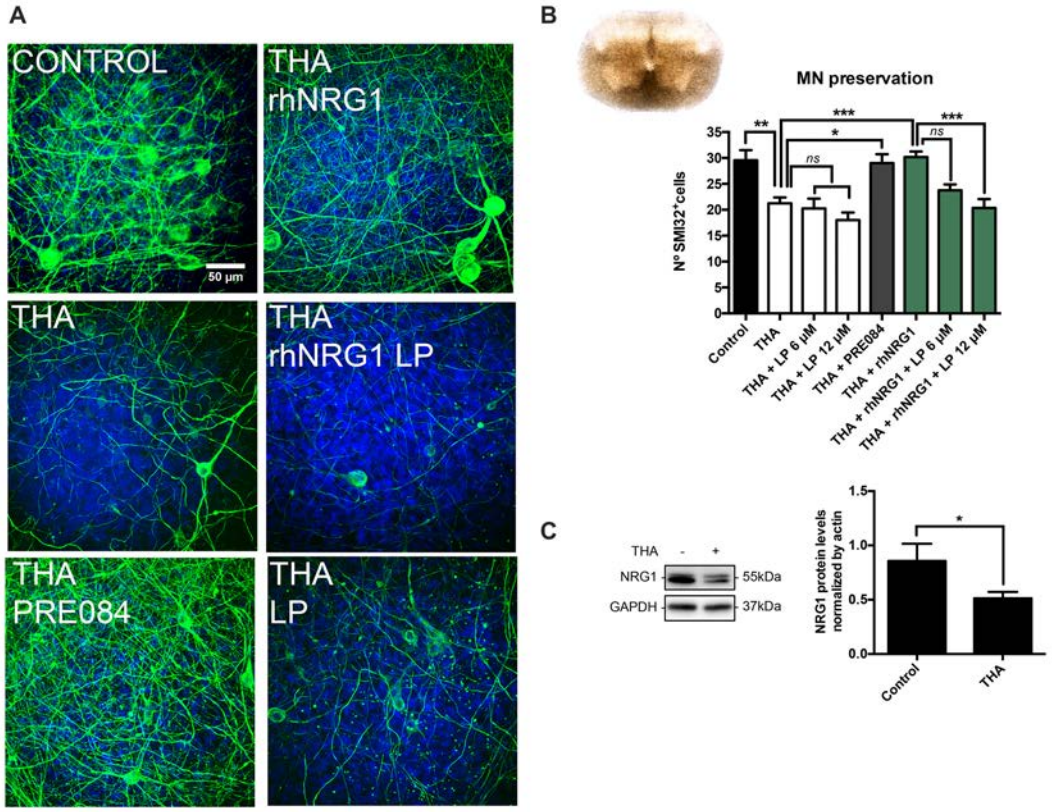

FIGURE 1 | Recombinant human NRG1 (rhNRG1) promotes motoneuron (MN) survival in the spinal cord organotypic culture (SCOC) under excitotoxicity. **(A)** Representative microphotographs of MNs in the ventral horn of the slices labeled with the SMI-32 antibody and DAPI at 28 days in vitro (DIV). Cultures were subjected to excitotoxicity by DL-threo-b-hydroxyaspartic acid (THA) alone or co-treated with rhNRG1 with or without lapatinib (LP) inhibitor, or with PRE084, a sigma 1 receptor agonist used as a positive control. Scale bar = 50 µm. **(B)** Microphotograph of a spinal cord slice in culture at 28 DIV is shown. Histogram graph showing the number (mean ± SEM, n = 24–31 hemisections per treatment) of SMI-32 positive cells in the ventral horn of each spinal cord slice. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$. **(C)** Western blot of the Neuregulin 1 (NRG1) protein in SCOCs under control or THA condition at 14 DIV. Bar graph showing the mean ± SEM protein levels of NRG1 (n = 3 cultures per condition). $^{*}p < 0.05$.

Neurite-J plug-in (Torres-Espín et al., 2014) for ImageJ software and the number of neurites grown at 50 µm intervals from the explant was compared between sets of cultures. The length measured for each neurite was the distance from the end of the neurite straight back to the DRG body radially (Deister and Schmidt, 2006) or to the neuronal body in spinal cord slices (n = 10–15 per slice).

Data Analysis

Data were evaluated using GraphPad Prism 5 software (San Diego, CA, USA). For statistical analysis, immunofluorescence data were analyzed by one-way ANOVA followed by Bonferroni multiple *post hoc* comparisons test. Western blot quantification results were analyzed by two-tail unpaired t-test for two groups comparison and one-way ANOVA followed by Tukey multiple *post hoc* test for multiple group comparison. Data were expressed as mean ± SEM. For the neurite length assessment, results were analyzed by performing a two-tailed unpaired t-test. All differences were considered statistically significant when $p < 0.05$.

RESULTS

rhNRG1 Prevents Motoneuron Cell Death

In SCOCs prepared for the assessment of neuroprotection, addition of THA in the medium significantly reduced the number of SMI32 positive cells in the ventral horn (21 ± 1) compared to the control slices (30 ± 2; **Figures 1A,B**). We also evaluated by western blot the endogenous level of NRG1 in the SCOCs after chronic excitotoxicity, and found that after 21 days of exposure to THA, NRG1 levels were significantly reduced (0.51 ± 0.05) compared to control cultures (0.85 ± 0.15; **Figure 1C**). With the aim to investigate if NRG1 could act as a neuroprotective agent for spinal cord MNs, we added 100 ng/mL of rhNRG1 to the SCOCs exposed to THA, and found significant preservation of MNs (30 ± 1) at similar level than with addition of PRE084 (29 ± 2; **Figures 1A,B**). The neuroprotective effect induced by rhNRG1 alone was abolished by simultaneous treatment with LP, an ErbB blocker, at 12 µM (20 ± 2) and partially at 6 µM (24 ± 1; **Figure 1B**). Addition of LP alone at 6 µM and 12 µM had no influence on the loss of MNs induced by

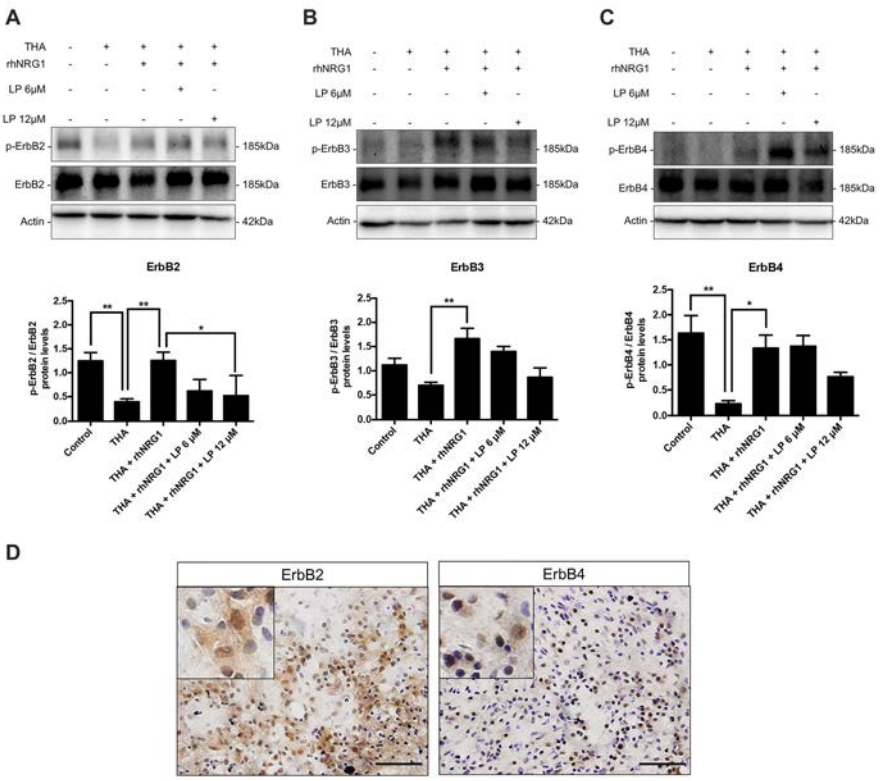

FIGURE 2 | ErbB receptors activation. Western blot of SCOCs revealed an increased phosphorylation ratio of ErbB2 **(A)**, ErbB3 **(B)**, and ErbB4 **(C)** receptors upon rhNRG1 treatment, that was totally or partially blocked by co-addition of LP. Data are shown as mean ± SEM with $n = 3–5$ values per treatment. $^{**}p < 0.01$; $^{*}p < 0.05$. **(D)** Microphotographs of ErbB2 and ErbB4 protein labeling with DAB and cresyl violet in control SCOCs at 14 DIV. Inset show MNs labeled against ErbB receptor at higher magnification. Scale bar = 100 μm.

THA (20 ± 2 and 18 ± 2, respectively). These results indicate that rhNRG1 induces protection to MNs under an excitotoxic chronic insult through ErbB receptors.

rhNRG1 Signaling under Chronic Excitotoxicity

In order to evaluate the signaling pathways underlying the neuroprotective effect of rhNRG1, we performed immunoblot analyses of the ErbB receptors. The total isoform expression of ErbB2, 3 and 4 receptors was not significantly modified in the different SCOC conditions as measured by immunoblot. However, excitotoxicity caused a significant reduction of the proportion of activated ErbB2 and ErbB4 and not significant for ErbB3 in comparison with control cultures (**Figure 2**). The activation of ErbB receptors was determined by calculating the ratio between the phosphorylated vs. the total protein levels. Interestingly, the level of phosphorylated ErbB receptors was restored by addition of rhNRG1 to the culture medium (**Figure 2**). On the other hand, addition of LP at 12 μM significantly inhibited the activation of ErbB2 induced by rhNRG1, and tended to block although not significantly the levels of phosphorylated ErbB3 and ErbB4 receptors (**Figure 2**). In addition, histological staining was performed to localize the ErbB receptors in the ventral horn of control SCOC. We confirm that ErbB2 and ErbB4 are expressed in MNs in the spinal cord slices taken at 14 DIV. The ErbB2 expression pattern was similar to that in intact, non-cultured, spinal cord. ErbB4, which is located in the cytosol of the MNs, appeared partly distributed to the nucleus after the axotomy produced for the culture (**Figure 2D**).

To corroborate the functional activation of the ErbB receptors through NRG1 signaling, we analyzed changes in the phosphorylation levels of AKT and ERK1/2, as downstream targets of ErbB related to neuronal survival. Increased activation of AKT by rhNRG1 treatment was evidenced by the significantly higher ratio of the phosphorylated vs. the total form of the protein compared to addition of THA alone (**Figures 3A,C**). In contrast, we did not find significant changes in the activation of ERK1/2 (**Figures 3B,C**). Because AKT pathway can modulate macroautophagic mechanisms, we examined the expression of different markers. Upon rhNRG1 treatment, we found a reduction of Beclin 1, a phagophore formation marker, and of p62, a protein involved in the proteasomal degradation of ubiquitinated proteins, compared to the increase produced by THA treatment alone, although the differences were not significant (**Figures 4A,B**). Interestingly we found differences in the LC3-II, a marker

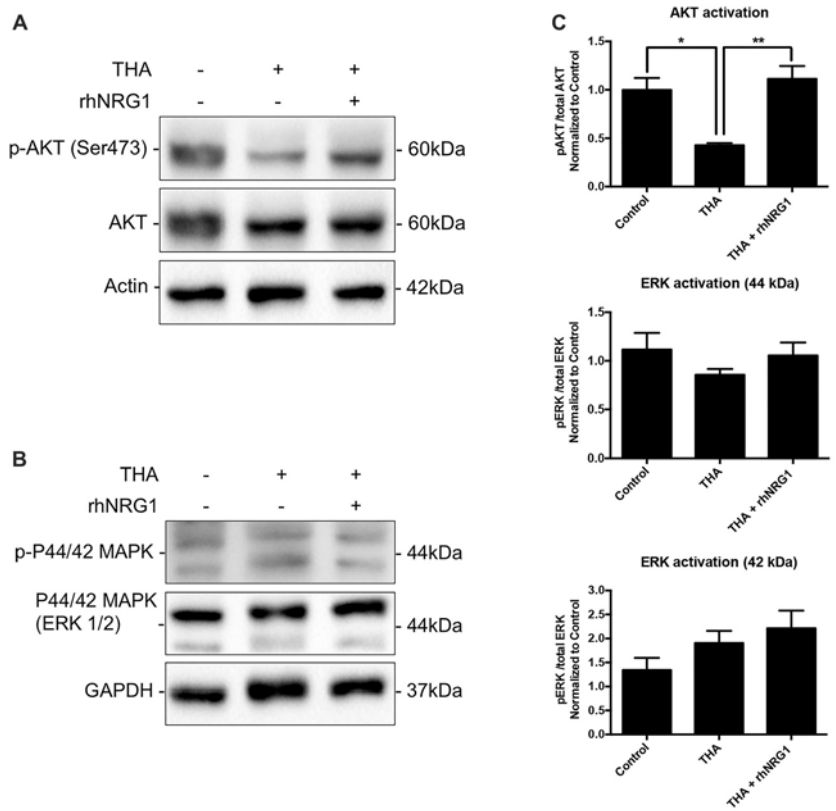

FIGURE 3 | Activation of downstream targets by rhNRG1. **(A,C)** Western blot of SCOCs revealed increased phosphorylation of AKT upon rhNRG1 treatment compared to the excitotoxic treatment alone. **(B,C)** No significant differences for ERK activation. Data are shown as mean ± SEM with n = 3–5 values per treatment. $^{**}p < 0.01$; $^{*}p < 0.05$.

of autophagosome formation. Under chronic excitotoxicity the LC3-II levels were enhanced, whereas addition of rhNRG1 maintained the levels of LC3-II similar to the control condition (**Figure 4C**).

rhNRG1 Reduces Microglial Reactivity

We also analyzed whether rhNRG1 had a role modulating the microglial activation in the SCOC. Microglial reactivity markedly increased with THA treatment, as revealed by the integrated density of Iba-1 labeling ($5.19 \times 10^9 \pm 8.30 \times 10^8$) at 28 DIV (**Figure 5**). Addition of rhNRG1 under chronic excitotoxicity significantly reduced microgliosis ($2.70 \times 10^9 \pm 4.98 \times 10^8$) at the same level than control slices ($2.41 \times 10^9 \pm 4.86 \times 10^8$). PRE084 caused a less marked reduction of microglial reactivity that did not reach significance ($3.41 \times 10^9 \pm 8.40 \times 10^8$).

rhNRG1 Enhances Motor and Sensory Neurite Outgrowth

Because we observed in rhNRG1-treated slices a larger bundle of tangled axons than in control slices, we assessed whether rhNRG1 might influence neurite growth by using modified organotypic culture models (**Figures 6A,B**; Allodi et al., 2011). rhNRG1 treated SCOCs showed significantly higher number of growing motor neurites and increased average length of the longest neurites (694 ± 82 μm) compared to the untreated control cultures (271 ± 16 μm; **Figures 6E,F**). Additionally, we also evaluated the role of rhNRG1 on sensory neurite elongation (**Figures 6C,D**). rhNRG1-treated DRG also showed increased number of growing sensory neurites and length of the longest neurites (945 ± 42 μm) compared to control cultures (677 ± 42 μm; **Figures 6G,H**). However, the effect was less marked for sensory than for motor neurites.

DISCUSSION

The results of this study demonstrate that NRG1 exerts neuroprotective effects on MNs under chronic excitotoxicity, and also enhances neurite growth. These properties point to the NRG1-ErbB system as a potential target of interest for the treatment of MN degenerative diseases.

We used two relevant models of neural organotypic cultures in order to independently assess these two actions *in vitro*. The SCOC has the advantage to preserve the anatomical organization of the spinal cord, neuronal connectivity and glial–neuronal interactions. The SCOC model under chronic excitotoxicity

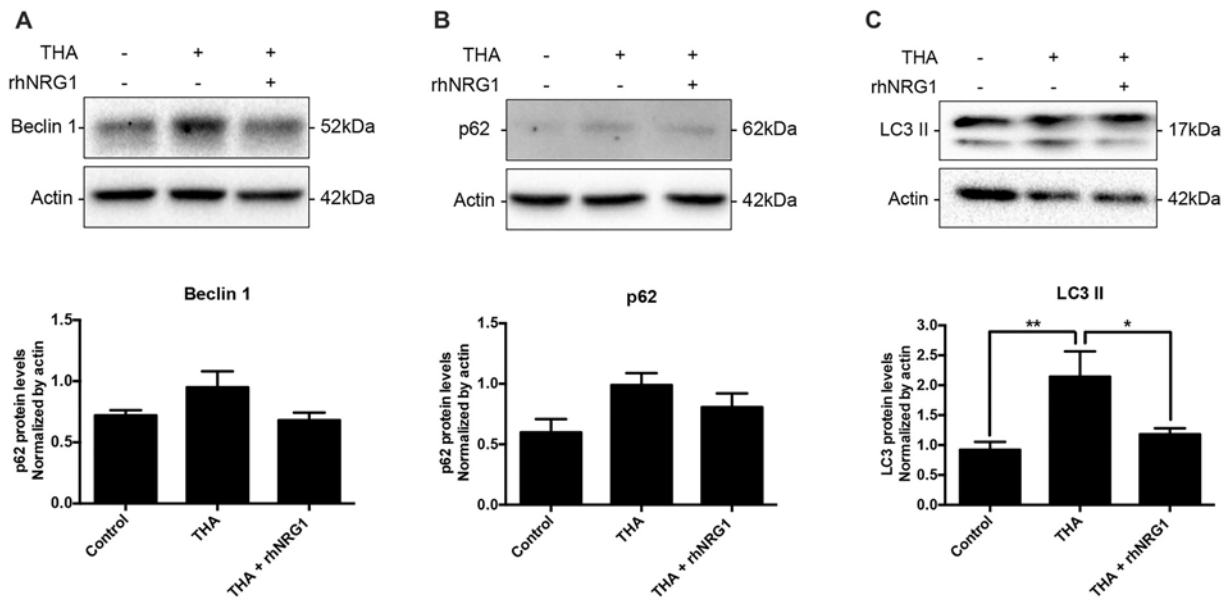

FIGURE 4 | Autophagic flux modulation by rhNRG1. Western blot of SCOCs did not show significant changes for Beclin 1 **(A)** and p62 **(B)**. However, THA induced an increase of autophagosome marker LC3II **(C)**, which was prevented when rhNRG1 was applied to the SCOCs. Data are shown as mean ± SEM with $n = 3–7$ values per treatment. $^{**}p < 0.01$; $^{*}p < 0.05$.

induced by THA is an *in vitro* model based on the role of glutamate toxicity. It has been proved a useful *in vitro* model for screening potential therapeutic drugs (Guzmán-Lenis et al., 2009; Herrando-Grabulosa et al., 2016; Pandamooz et al., 2016) against MN degeneration that does not depend on known genetic alterations, thus being relevant for the majority of sporadic ALS cases and other MN diseases. The role of NRG1 and its isoforms on the ALS pathogenesis is still controversial. The studies investigating the expression of NRG1 and its ErbB receptors in the spinal cord have reported several alterations in samples of mouse models and ALS patients. Regarding the mRNA levels of NRG1, the Type III isoform was found decreased in the ventral horn of the spinal cord from ALS human samples as well as in $SOD1^{G93A}$ transgenic mice in parallel with MN loss, whereas the Type I isoform was reported to increase at advanced stage of the disease in $SOD1^{G93A}$ mice in one study (Song et al., 2012) and to decrease in another (Lasiene et al., 2016). In the SCOCs under chronic excitotoxicity we found low expression of NRG1 compared to control slices, and addition of exogenous NRG1 significantly preserved the number of surviving MNs in the ventral horn. The rhNRG1 corresponds to the EGF-domain of NRG1, which is able to activate ErbB receptors 2, 3 and 4. The EGF-domain of NRG1 can bind to ErbB3 and ErbB4 by inducing their homodimerization or the heterodimerization with ErbB2. As a consequence of the dimerization ErbB receptors phosphorylate. We showed that the neuroprotective effects of rhNRG1 are mediated by the activation of ErbB receptors because the addition of LP, an inhibitor of ErbB receptors, blocks the neuroprotective action of rhNRG1. In addition we showed that the ErbB2 and ErbB4 receptors are expressed in MNs, although we could not confirm the expression of ErbB3 due to limitations of the antibodies available. The ErbB2 pattern was the same as previously shown at *in vivo* samples (Song et al., 2012), while ErbB4 appeared in the nucleus of the MNs after the axotomy produced in the culture. Upon phosphorylation of ErbB receptors, the PI3K-AKT pro-survival pathway was activated as demonstrated by immunoblot. In cultures of embryonic rat MNs, NRG1 was shown to inhibit apoptosis during the period of embryonic programmed cell death by a PI3K-dependent pathway, although in this case it did not increase the relative level of p-AKT (Ricart et al., 2006). Despite the fact that ERK pathway is another cascade induced upon ErbB receptor activation, no differences of ERK1/2 were detected after rhNRG1 treatment.

Autophagy is another common feature in MN diseases that has already been described in the SCOC under chronic excitotoxicity (Matyja et al., 2005; Herrando-Grabulosa et al., 2013). However, the role of autophagy in promoting neuronal cell death or survival is a subject of debate. After THA treatment we found evidence of accumulation of autophagosomes that corresponds to an early stage of autophagy cell death induction. In contrast, addition of rhNRG1 to the culture decreased the levels of the phagopore formation marker Beclin 1 and the autophagosome marker LC3-II. Therefore, rhNRG1 may act restoring the autophagic flux by reducing the number of autophagosomes formed and starting p62 degradation in the lysosome after the fusion, avoiding accumulation in the cytosol.

MNs are preferentially damaged by the prolonged blockade of glutamate uptake produced by THA addition, causing excitotoxicity. In the SCOC this mechanism is mediated by non NMDA receptors, such as AMPA receptor

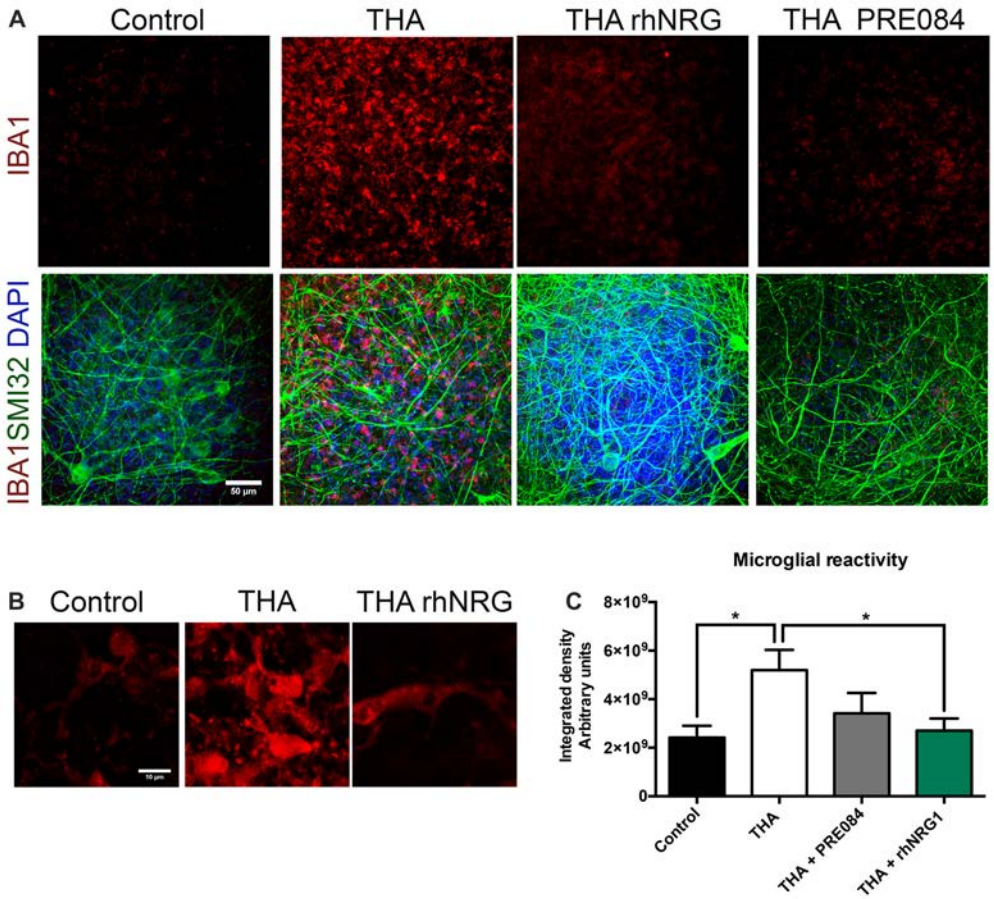

FIGURE 5 | Reduction of microgliosis by rhNRG1. **(A)** Representative microphotographs showing microglia stained with anti-IBA1 (red), non-phosphorylated H neurofilament SMI32 (green) and DAPI (blue) at 21 DIV post-THA treatment alone or co-treated with rhNRG1 (100 ng/ml) or PRE084 (10 μM). Scale bar = 50 μm. **(B)** Higher magnification images of microglia stained with anti-IBA1 showing morphological differences between control, THA added and THA+ rhNRG1 conditions. Scale bar = 10 μm. **(C)** Bar graph showing the microglial reactivity measured by the integrated density in the ventral horn of SCOCs (mean ± SEM, $n = 15$ hemisections per treatment). $^*p < 0.05$.

(Rothstein et al., 1993). While these channels permit rapid Ca^{2+} entry, MNs buffer the consequent cytosolic Ca^{2+} load poorly (Lips and Keller, 1998), with the consequence that much of the Ca^{2+} is readily taken up into mitochondria, resulting in strong reactive oxidative species (ROS) generation (Carriedo et al., 2000; Rao et al., 2003). Furthermore, ROS may be able to exit the MN, disrupting glutamate transport in surrounding astrocytes, resulting in increased extracellular glutamate accumulation, and further propagation of the injury cascade (Rao et al., 2003; Yin and Weiss, 2012). Recently it has been shown that NRG-1β plays an important role in modulating Ca^{2+} homeostasis and preventing apoptosis through activating PI3K/AKT pathway in DRG sensory neurons subjected to excitotoxicity induced by glutamate (Liu et al., 2011). This evidence suggests that in the SCOC, rhNRG1 treatment may modulate Ca^{2+} homeostasis and thus reduce the excitotoxicity through this mechanism.

On the other hand, we used SCOCs and DRG explants embedded in a 3D collagen matrix, which creates a permissive environment for neurite elongation (Allodi et al., 2011; Santos et al., 2016), to assess the effects of NRG1 on neurite growth. Our results showed that rhNRG1 increased the number and length of the neurites emerging from both motor and sensory neurons in the SCOC and DRG cultures respectively. NRG1 plays an important role in myelination during development and also after injury in the peripheral nerve, and promotes the role of Schwann cells supporting axonal regeneration (Fricker and Bennett, 2011; Gambarotta et al., 2013; Stassart et al., 2013). Immediately after injury, the soluble NRG1 transcript is upregulated in the lesioned nerve, mostly released by reactive Schwann cells (Stassart et al., 2013), suggesting that soluble NRG1 plays a role also during the early phases of axonal degeneration and regrowth (Gambarotta et al., 2013). NRG1 receptors ErbB2, ErbB3 and ErbB4 are variably expressed by adult DRG sensory neurons and spinal cord MNs, and their levels increase although in a variable pattern after their axotomy, indicating that NRG1 proteins may act directly on the neurons (Pearson and Carroll, 2004). In our *in vitro* organotypic

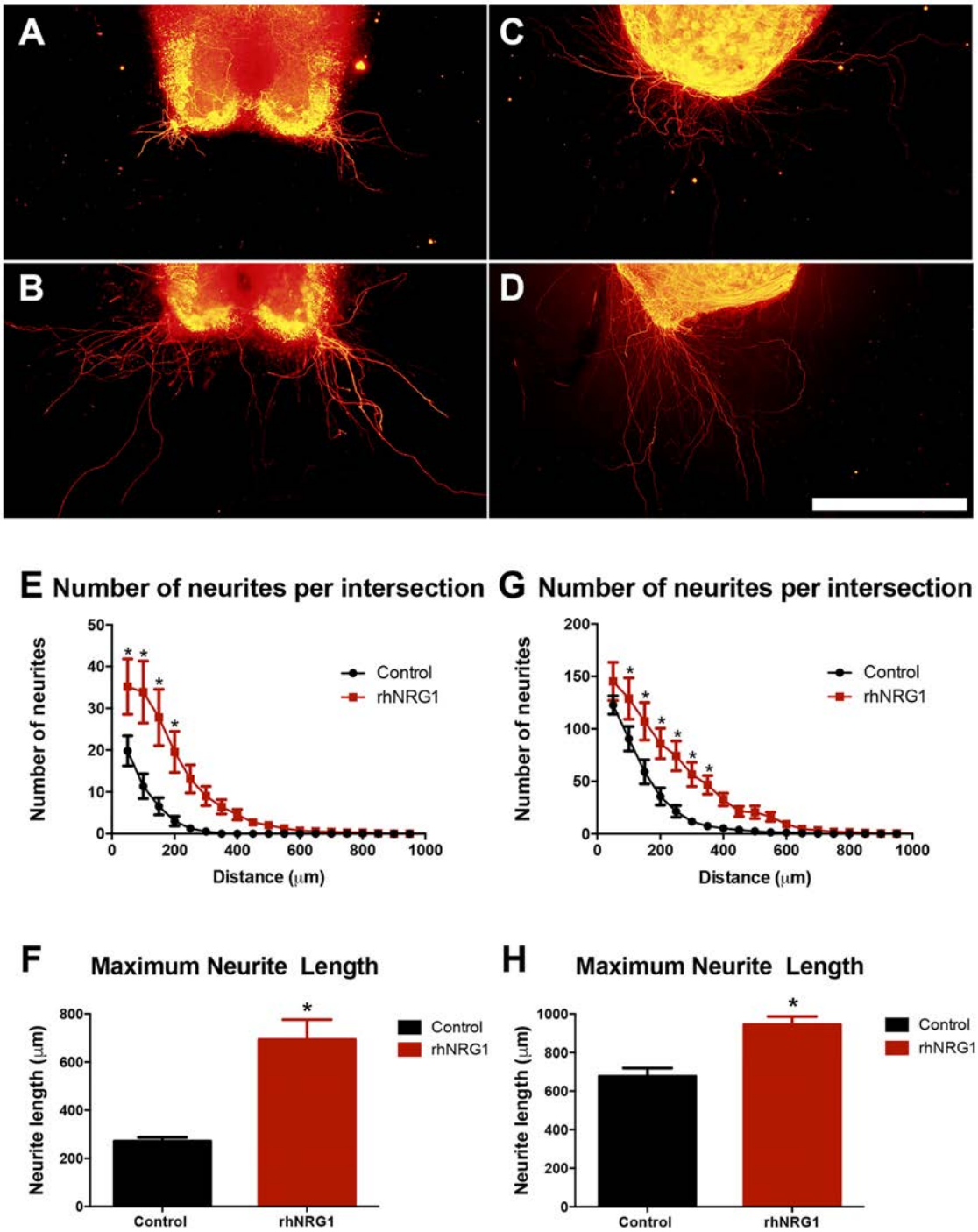

FIGURE 6 | rhNRG1 enhances neurite outgrowth. Representative microphotographs of untreated and rhNRG1-treated SCOC **(A,B)** and dorsal root ganglia (DRG) **(C,D)** cultures embeeded in collagen. Graphs show the number of neurites per intersection and the maximum neurite length in the SCOC **(E,F)** and the DRG **(G,H)** cultures. Scale bar = 500 μm. Data are shown as mean ± SEM with $n = 11$ slices per treatment. $^{*}p < 0.05$.

cultures, addition of exogenous NRG1 promoted the initial phase of neurite growth, likely acting on the neuronal processes. Accordingly, increased supply of exogenous NRG1 was shown to promote nerve regeneration *in vivo* (Chen et al., 1998; Nicolino et al., 2003; Joung et al., 2010). Our group has also recently reported that intramuscular administration of NRG1 Type 1 enhances the emergence of collateral sprouts of motor axons that reinnervate previously denervated muscle fibers in wild type and $SOD1^{G93A}$ mice (Mancuso et al., 2016).

Another relevant aspect in the issue is whether the NRG1-ErbB pathway participates in the regulation of the neuroinflammatory response mediated by microglial cells, a common feature that occurs in human patients as well as in murine models of neurodegeneration. Activated ErbB2 receptors predominantly present on microglia and, to a lesser extent, on astrocytes were found overexpressed as a function of disease progression in the $SOD1^{G93A}$ transgenic mouse, correlating with the pattern of microglial activation (Song et al., 2012). These findings led to suggest that NRG1 isoforms could contribute to disease pathogenesis through glial cell activation. Chronic excitotoxicity also induced a marked microglial response in the SCOC that was markedly reduced by administration of rhNRG1. Furthermore, we observed that microglial cells showed thinner and more ramified processes under rhNRG1 treatment compared to THA alone where microglia had an ameboid morphology and larger size. These results suggest that in this chronic excitotoxic model, rhNRG1 modulates the ErbB2 receptor expressed in microglia. Indeed, NRG1 signaling via the ErbB2 receptor was shown to be involved in microglial proliferation and chemotaxis after peripheral nerve injury (Calvo et al., 2010).

Recently, it has been reported that autophagy might influence inflammation and activation of microglia, as well as inflammation might promote or inhibit the process of autophagy (Su et al., 2016). Interestingly, IGF-I has been described to protect hippocampal neurons against early excitotoxicity via the NR2B/PI3K-AKT-mTOR pathway, suppressing the excess of autophagy (Wang et al., 2014). Considering these links between the different pathogenic mechanisms we hypothesize that rhNRG1 reduces the autophagy caused by excitotoxicity activating the ErbB receptors and the PI3K/AKT pathway in both MNs and microglial cells. This activation promotes a dual effect; the survival of the MNs and the reduction of the neuroinflammatory response mediated by the microglia. Therefore, the modulation of the expression of NRG1 at the central nervous system seems an interesting approach for promoting neuroprotection and maintenance of connectivity of the MNs.

AUTHOR CONTRIBUTIONS

GM-C carried out neuroprotection studies and wrote the manuscript. DS performed neurite growth studies. XN and MH-G made the experimental design, helped to perform the experiments, analyzed the data and wrote the manuscript.

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CHAPTER II

THERAPEUTIC ROLE OF FULL-LENGTH NEUREGULIN 1 TYPE III IN SOD1-LINKED AMYOTROPHIC LATERAL SCLEROSIS

Therapeutic role of full-length Neuregulin 1 Type III in SOD1-linked Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a devastating motoneuron disease without effective cure currently available. Death of motoneurons (MNs) in ALS is preceded by failure of neuromuscular junctions and axonal retraction. Neuregulin 1 (NRG1) is a neurotrophic factor highly expressed in MNs and neuromuscular junctions that supports axonal and neuromuscular development and maintenance. Recent evidence suggests that NRG1 and its ErbB receptors are involved in ALS. Reduced NRG1 expression has been found in ALS patients and in the ALS SOD1^{G93A} mouse model, however the expression of the isoforms of NRG1 and its receptors is still controversial. Moreover, the role of the NRG1-ErbB pathway on MN survival is not fully known. Due to the reduced levels of NRG1 Type III (NRG1-III) in the spinal cord of ALS patients, we used gene therapy based on adeno-associated virus to overexpress NRG1-III in SOD1^{G93A} mice. The mice were evaluated from 9 to 16 weeks of age by means of electrophysiology and rotarod tests. At 16 weeks they were sacrificed for histological and molecular biology analyses. Our results indicate that overexpression of NRG1-III full length (FL) is able to preserve motor function of the hindlimbs, improve the locomotor performance, maintain the number of surviving MNs at 16 weeks, and reduce glial reactivity in the treated female SOD1^{G93A} mice. Furthermore, the NRG1-III/ErbB4 axis appears to regulate MN excitability by modulating the chloride transporter KCC2 and reduces the expression of the MN-vulnerability marker MMP-9. These findings indicate that increasing NRG1-III at the spinal cord is a promising approach for promoting MN protection and functional improvement in ALS.

Key words: Neuregulin 1, ErbB receptors, motoneuron, neuromuscular function, amyotrophic lateral sclerosis, spinal cord.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset motoneuron degenerative disease, characterized by progressive paralysis of skeletal muscles of the limbs, speech, swallowing and breathing (Wijesekera & Leigh., 2009). The most common form of ALS is sporadic, of unknown etiology. Around 10% or more of the cases are inherited, caused by mutations in several genes, the most prevalent mutations involving the superoxide dismutase 1 (SOD1), the TAR-DNA binding protein (TDP-43) and the hexanucleotide repeat expansions in chromosome 9 open reading frame 72 (C9ORF72) (Rosen et al., 1993; Kabashi et al., 2008; Yokoseki et al., 2008; DeJesus-Hernandez et al., 2011; Renton et al., 2011). The pathophysiological mechanisms underlying the development of ALS are multifactorial, with emerging evidence of a complex interaction between genetic and molecular pathways. The precise molecular mechanism that specifically affects the motoneuron (MN) to cause its death is still to be elucidated. In addition, neighboring cells, such as microglia, astrocytes and interneurons, contribute to the disease (Brites et al., 2014; Mancuso & Navarro, 2015). Several animal models carrying ALS-related mutations have been developed during the last decades; the most widely used ALS model is a transgenic mouse over-expressing the human mutated form of the SOD1 gene with a glycine to alanine conversion at the 93rd amino acid (SOD1^{G93A}) (Gurney et al., 1994; Ripps et al., 1995), which recapitulates the most relevant clinical and pathological features of both familial and sporadic ALS (Ripps et al., 1995; Mancuso et al., 2011). Alterations in SOD1 protein have been also found in sporadic ALS patients (Bosco & Landers, 2010), and accumulation of wild-type SOD1 was reported to produce ALS in mice (Graffmo et al., 2013).

Nowadays, no effective cure exists for ALS. One of the promising therapeutical approaches for ALS is gene therapy, since it permits to stably and specifically deliver treatments to cells participating in the disease process, including MNs, overcoming the difficulty of crossing the blood-brain barrier and avoiding non-specific effects (Federici & Boulis., 2006). Particularly, adeno-associated vectors (AAV) are one of the most used gene therapy vectors for human clinical applications. They show advantages over other viruses, such as the capacity of generating long-term expression, mostly as an episomal form, avoiding the risk for insertional

mutagenesis, the absence of toxicity associated to wild type viruses, and the possibility of easily producing pure high-titer viruses in the laboratory. Viral transgene expression can also be restricted depending on the viral tropism and using promoters with selective and defined expression patterns (Kügler, 2016).

Neuregulin 1 (NRG1) is a widely expressed protein of the epidermal-growth factor (EGF) family, involved in several biological functions directed to maintain the homeostasis of the central and peripheral nervous system (Syroid et al., 1996; Wolpowitz et al., 2000; Michailov et al., 2004; Nave & Salzer., 2006; Velanac et al., 2012). NRG1 increases terminal Schwann cells survival after denervation and promotes extension of axonal sprouts required for new neuromuscular junction (NMJ) formation (Fricker et al., 2011; Mancuso et al., 2016). The diversity of the amino-terminal sequences of NRG1 and the alternative splicing processes result in six major isoforms, NRG1 type I-VI (Falls et al., 2003; Steinthorsdottir et al., 2004). It has been recently reported that NRG1-III expression is reduced in the spinal cord of both ALS patients and SOD1^{G93A} mice. However, some controversy exists about the levels of NRG1-I in the spinal cord of SOD1^{G93A} mice (Song et al., 2012; Lasiene et al., 2016). Importantly, loss-of-function mutations of NRG1 receptor ErbB4 were reported to produce late-onset, autosomal-dominant ALS in human patients (Takahashi et al., 2013). Furthermore, we have reported that ErbB4 ectodomain fragments were reduced in cerebrospinal fluid and plasma of ALS patients, indicating an impairment of the NRG1-ErbB signaling (López-Font et al., 2019). One of the hallmarks of ALS, is the contribution of non-neuronal cells to the progression of the pathology. Recent work showed that in SOD1^{G93A} mice spinal cord microglial cells express the activated form of ErbB2 receptor (Song et al., 2012). Moreover, corticospinal tracts from ALS human patients show enhanced levels of NRG1 in the microglial cells (Song et al., 2012). Therefore, more evidence is needed to define the role of NRG1 and ErbB receptors in the MN and non-neuronal cells of the spinal cord in MN degeneration. In the present study, we investigated the distribution of NRG1 and ErbB4 receptor in the spinal cord of ALS patients and SOD1^{G93A} mice. Furthermore, we overexpressed NRG1-III by gene therapy to test its effect on motor function and spinal MN preservation in the SOD1^{G93A} mice ALS model. We have also analyzed its effects on the potassium chloride cotransporter 2 (KCC2) and the metalloproteinase 9 (MMP-9), which are known markers related to MN

vulnerability (Fuchs et al., 2010; Kaplan et al., 2014). The results showed that viral-mediated delivery of NRG1-III promotes motor function improvement of the hindlimb muscles and increases MN survival while reducing glial cell reactivity and regulating the expression of KCC2 and MMP-9 in the female SOD1^{G93A} mice, suggesting that the modulation of the NRG1-III/ErbB4 axis is relevant for MN survival and function.

Materials and Methods

Human samples

Cryopreserved lumbar spinal cord sections from five ALS patients and two healthy controls without evidence of neurological disease were provided by the Tissue Bank of the Hospital de Bellvitge (Table 1). Postmortem time intervals ranged from 2 to 6 hours.

Table 1. Clinical information of the ALS patients from whom samples have been analyzed in this study.

Subject	Gender	Age	Postmortem delay
CTRL 1	Male	66	4h20m
CTRL 2	Male	63	3h50m
ALS1	Male	69	2h
ALS2	Male	57	4h20m
ALS3	Female	75	4h5m
ALS4	Female	79	2h10m
ALS5	Female	57	10h

CTR, healthy control subjects; ALS, patients with Amyotrophic Lateral Sclerosis

Animals

Transgenic mice carrying the mutation G93A in the SOD1 gene and nontransgenic wild-type (WT) littermates as controls were used. $SOD1^{G93A}$ high copy mice (Tg[SOD1-G93A]1Gur) were obtained from the Jackson Laboratory (Bar Harbor, ME), with B6xSJL background. These mice were bred and maintained as hemizygotes by mating transgenic males with F1 hybrid (B6SJLF1/J) females obtained from Janvier Laboratories (France). Transgenic mice were identified by polymerase chain reaction amplification of DNA extracted from the tail. Mice were kept in standard conditions of temperature (22±2 °C) and a 12:12 light:dark cycle with access to food and water *ad libitum*. Animals were maintained at the Animal Service of the Universitat Autònoma de Barcelona and were cared for and handled in accordance with the guidelines of the European Union Council (Directive 2010/63/EU) and Spanish regulations on the use of laboratory animals. The experimental procedures had been approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

The study included B6xSJL female and male mice that were divided in two groups of WT mice and two groups of $SOD1^{G93A}$ mice, that were administered at 8 weeks of age with either AAV coding for NRG1 type III or mock vector, respectively. We first performed a complete study in female mice, and after analyses, the study was also performed in male mice, considering the differences in disease progression between sexes in this mouse model. For the functional studies we used the following number of mice per group: WT Mock mice (n=6 females, n=6 males), WT NRG1-III mice (n=6 females, n=8 males), SOD Mock mice (n=14 females, n=6 males), SOD NRG1-III mice (n=20 females, n=6 males). These animals were then further distributed in two different subgroups at 16 weeks. First, a subgroup to assess the mRNA expression: WT Mock mice (n=3 females), WT NRG1-III mice (n=3 females), SOD Mock mice (n=3 females), SOD NRG1-III mice (n=5 females). Second, a subgroup for histological analysis: WT Mock mice (n=3 females, n=3 males), WT NRG1-III mice (n=3 females, n=3 males), SOD Mock mice (n=4 females, n=3 males), SOD NRG1-III mice (n=8 females, n=3 males). For the survival analysis a subset of female mice was used: SOD Mock mice (n=9), SOD NRG1-III mice (n=9).

Viral vectors production and administration

Full length (FL) NRG1 type III sequence, cloned between AAV2 ITRs under the regulation of the CMV promoter and containing a HA-tag sequence, was used to produce an AAVrh10 pseudotype. AAV viral stock for pseudotype 10 was generated by triple transfection into HEK293-AAV cells of the expression plasmid, RepCap plasmids containing AAV genes for each serotype and pXX6 plasmid containing adenoviral genes needed as helper virus. AAV particles were purified by iodixanol gradient. Titration was evaluated by picogreen quantification. Control serotype-matching AAV vectors coding for mock were also generated.

FL AAVrh10NRG1 type III was administered intrathecally at the lumbar region of 8 weeks old mice under anesthesia with ketamine/xylacine (100/10 mg/kg i.p.). After exposure of the lumbar vertebrae, 10 µl of viral vectors ($1x10^{11}$ vg of AAVrh10Nrg1type III FL vector or mock vector) were delivered into the cerebrospinal fluid (CSF) using a Hamilton syringe and a 30-gauge needle placed between L3 and L4 vertebrae. Adequate injection into the intrathecal space was confirmed by the animal's tail flick. The needle was left in place at the injection site for 1 additional minute to avoid reflux. Then, the wound was sutured by planes.

Electrophysiological tests

For motor nerve conduction tests, the sciatic nerve was stimulated percutaneously by means of single pulses of 20µs duration (Grass S88) delivered by two needle electrodes placed at the sciatic notch. The compound muscle action potential (CMAP) was recorded from tibialis anterior (TA), gastrocnemius (GM) and plantar (PL) interossei muscles with microneedle electrodes (Navarro & Udina., 2009; Mancuso et al., 2011) at 9, 12, 14 and 16 weeks of age. Recorded potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S), measuring the latency and amplitude of the CMAP. During the tests, the mouse body temperature was kept constant by means of a thermostated heating pad.

Motor unit number estimation (MUNE) was performed using the incremental technique (Shefner et al., 2006; Mancuso et al., 2011) with the same setting as for motor nerve conduction tests. Starting from a subthreshold intensity the sciatic

nerve was stimulated with pulses of gradual increasing intensity. Then, quantal increases in the CMAP were recorded. The increments higher than 50 μV were considered as indicative of the recruitment of an additional motor unit. The mean amplitude of individual motor units was calculated as the average of consistent increases. Finally, the estimated number of motor units resulted from the equation: MUNE = CMAP maximal amplitude/mean amplitude of single motor unit action potentials.

For evaluation of the central pathways, motor evoked potentials (MEP) were recorded from the TA muscles after electrical stimulation of the motor cortex with pulses of 0.1 ms duration and supramaximal intensity, delivered through subcutaneous needle electrodes placed over the skull overlaying the sensorimotor cortex (Mancuso et al., 2011).

Locomotor test and Clinical Disease Onset

Rotarod test was performed to evaluate motor coordination, strength and balance of the animals (Miana-Mena et al., 2005). Mice were placed onto the rod rotating at a constant speed of 14 rpm. The time during which each animal remained on the rotating rod was measured. Each mouse was given three trials and the longest time until falling recorded; 180 sec was chosen as the cut-off time. The test was performed weekly from 9 to 16 weeks of age. Clinical disease onset for each animal was determined as the first week that the cut-off time was lower than 180 seconds.

Survival analysis

For survival assessment, 9 $SOD1^{G93A}$ mice per group were followed until the defined end-point, that was considered when the mouse was unable to upright standing in 30 s when placed on its side.

Histological analyses

At 16 weeks of age, after functional follow-up, the mice were transcardially perfused with 4% paraformaldehyde in PBS. The lumbar spinal cord was harvested,

postfixed during 2h, and cryopreserved in 30% sucrose in PBS. For spinal MN evaluation, 20 µm transverse sections were cut using a cryotome (Leica, Germany) and collected in sequential series of 10 slides. Slides corresponding to L4-L5 lumbar spinal cord sections separated 100 µm were stained with cresyl violet. Motoneurons were identified by their localization in the ventral horn and following strict size and morphological criteria: only neurons with diameter larger than 20 µm, polygonal shape and prominent nucleoli were counted.

Slides containing 20 µm thick lumbar spinal cord transverse sections from either ALS patients or $SOD1^{G93A}$ mice were used for immunolabeling of NRG1 and its ErbB receptors. The endogenous peroxidase activity was inhibited (70% Methanol, 30% TBS1X, 2% H_2O_2) and a blocking solution (5% normal horse serum and 1% BSA in TBS-T) was added. Slides were incubated overnight at 4ºC with primary antibodies against anti-panNRG (1:500, sc-28916, Santa Cruz, USA), anti-NRG1 Type III (1:200, AB 5551, Millipore, USA), anti-ErbB4 (1:100, 4795S, Cell Signaling, USA), anti-ionized calcium binding adapter molecule 1 (Iba-1, 1:1000; 019-19741, Wako, Japan), and anti-glial fibrillary acidic protein (GFAP, 130300; Invitrogen, USA). Slides were then washed with TBS-T and incubated with a secondary antibody horse anti rabbit HRP conjugate (Vector Laboratories, USA) overnight at 4ºC. Afterwards, we incubated the slides with the VECTASTAIN® Elite® ABC complex (Vector Laboratories, USA) for 1h at RT and the DAB solution (Vector Laboratories, USA) was used for brown color development. Dehydration with a series of ethanol gradients was performed. Finally, after xylol incubation, slides were mounted with DPX (06522, Sigma, USA) and analyzed under microscope (Nikon Eclipse Ni, Japan).

Immunofluorescence

Spinal cord sections were blocked with PBS-Triton-Donkey serum and incubated 24h at 4°C with primary antibodies: anti-Iba-1 (1:1000; 019-19741, Wako, Japan), and anti-GFAP (1:1000, 130300; Invitrogen, USA), anti-ErbB4 (1:100, 4795S, Cell Signaling, USA), anti-MMP9 (1:200, ab38898, Abcam, UK), anti-KCC2 (1:400, 07-432, Millipore, USA) and anti-ChAT (1:100, AB144P, Millipore, USA). After washes, sections were incubated overnight with the corresponding secondary antibody: Alexa 488-conjugated secondary antibody (1:200; A21206, Invitrogen,

USA) or Cy3-conjugated secondary antibody (1:200; 712-165-150, Jackson IR, USA). Finally, Fluoronissl (1:200, 990210, Invitrogen USA) and DAPI (1:2000; D9563-10MG, Sigma) was used to stain the motoneurons and the nucleus respectively. Slides were mounted in Fluoromount-G (Southern Biotech, USA). GFAP, Iba1, MMP-9, ChAT and KCC2 labelings were viewed using fluorescence microscopes (Olympus BX51, Japan, or Nikon Eclipse Ni, Japan). Alternatively, ErbB4 staining was analyzed under confocal microscopy (Zeiss LSM 700, Germany). Assessement of astroglia and microglia immunoreactivity, photographs of the ventral horn were taken at ×40 and, after defining a threshold for background, the integrated density of GFAP or Iba1 labeling, respectively, was measured using ImageJ software.

RNA extraction and real time PCR

To obtain RNA samples, the mice were sacrificed by decapitation after deep anaesthesia. L4-L5 spinal cord segments were rapidly dissected, maintained in RNA-later solution and processed for mRNA analyses. Total RNA was extracted and 1 µg per sample was reverse-transcribed using 10 µmol/l DTT, 200 U M-MuLV reverse transcriptase, 10 URNase Out Ribonuclease Inhibitor and 1µmol/l oligo(dT), 1µmol/l random hexamers. The reverse transcription cycle conditions were 25°C for 10 min, 42°C for 1 h and 72°C for 10 min. Gene-specific mRNA analyses were performed by SYBRgreen real-time PCR using the MyiQ5 real-time PCR detection system.

Statistical analysis

All experiments were performed by researchers blinded with respect to treatment received by each mouse group, using randomized groups. Sample sizes were selected according to previous observations in our lab. Data were expressed as mean ± SEM. Electrophysiological and locomotion tests results were analyzed using two-way ANOVA, with Bonferroni post-hoc test. For MUNEs and MEPs electrophysiological results t-Student test was applied. For clinical disease onset and survival results Log-rank (Mantel-Cox) test was applied. Histological and

molecular biology data were analyzed using ANOVA and t-Student, applying Bonferroni post-hoc test when necessary.

Results

Neuregulin 1 type III expression in ALS

To determine the localization and level of expression of NRG1 in the spinal cord of SOD1^{G93A} mice and ALS patients, immunohistochemistry and qPCR analyses were performed. For immunohistochemistry, two types of antibodies were used according to its specificity for the N-terminal of NRG1-III or the C-terminal domain of all NRG1 isoforms. In spinal cord samples of ALS patients, the immunoreactivity of the C-terminal domain of NRG1 appeared reduced in preserved MNs and was mostly expressed by neighboring cells (Fig. 1A), likely microglial cells according to their morphology, whereas in healthy controls NRG1 was mainly localized in the spinal MNs. In contrast, NRG1-III was specifically expressed in MNs in healthy controls and also in ALS patients, despite the low expression compared with control samples. These results are in agreement with the mRNA levels of NRG1-I and NRG1-III in spinal cord from SOD1^{G93A} ALS mice. At the advanced symptomatic stage of the disease (16 weeks), NRG1-I was upregulated (1.15±0.02), whereas the NRG1-III isoform was downregulated (0.68±0.05) compared to the WT mice (Fig. 1B).

To assess the role of NRG1-III, we administered the AAVrh10Nrg1-III FL vector to overexpress the full-length form of NRG1-III in SOD1^{G93A} mice. The mRNA levels of NRG1-III indicate that it was overexpressed in the spinal cord of treated WT and SOD1^{G93A} mice (Fig. 1C). These results corresponded with the viral genome counting, that was largely increased in the treated mice (Fig. 1D). Moreover, immunohistochemical labeling showed that NRG1-III was overexpressed in the spinal MNs of the ventral horn in mice administered with the functional vector (Fig. 1E).

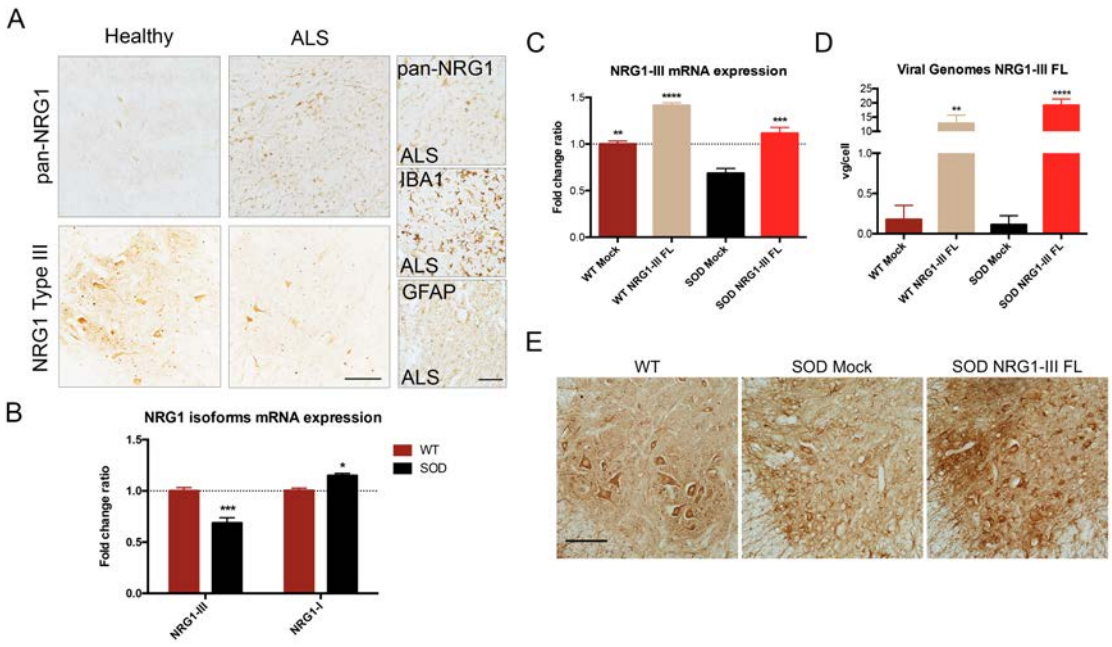

Figure 1. *Neuregulin 1 type III expression in ALS patients and SOD1^{G93A} mice.* A) Microphotographs of pan-NRG1 and NRG1 type III labeling by DAB staining in the spinal cord ventral horn of healthy and ALS patients. Higher magnification images show that pan-NRG1 labeling may colocalize with Iba1 in ALS patients (scale bar = 100 μm). B) mRNA expression of NRG1 isoforms reveals a downregulation of type III whereas type I is increased in the SOD1^{G93A} mice at 16 weeks of age. C) mRNA expression analysis shows that overexpression of NRG1-III FL compensates the loss of this isoform in the SOD1^{G93A} mice. D) Viral genome analysis showing that the intrathecal AAV injections were successful in the treated mice (n=3 WT Mock, 3 WT NRG1-III, 3 SOD Mock, 3-5 SOD NRG1-III mice per group). E) Microphotographs of NRG1-III labeling further confirms downregulation of this isoform in the SOD1^{G93A} mice that is recovered upon viral-mediated overexpression (scale bar = 100 μm, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

NRG1-III overexpression slows the disease progression of SOD1^{G93A} ALS female mice

Next, we assessed the contribution of NRG1-III overexpression to the functional outcome in the female SOD1^{G93A} mice. AAVrh10Nrg1-III FL injected at 8 weeks resulted in an improvement of the motor function. The electrophysiological

results showed that overexpression of the NRG1-III FL significantly reduced the progressive decline of the CMAP amplitude of the PL, TA and GM muscles during the follow-up in comparison with the mice treated with the mock vector (Fig. 2A, B, C). The recording of MEPs of the TA muscle at 16 weeks revealed a significant preservation of the amplitude in the treated mice (Fig. 2D). We then estimated the size and number of motor units of the TA muscle, and found a significant increase of the mean amplitude and of the number of preserved motor units (Fig. 2E) in agreement with the higher CMAP amplitude of the hindlimb muscles. Furthermore, the frequency distribution of the TA motor units demonstrates a shift to the right in the treated group (Fig. 2E). The rotarod test results showed that the treated group had better functional outcome than the mock group (Fig. 2F). In addition, we found that treated mice had a delay in the disease onset, although it did not reach statistical significance compared with the mock group ($p=0.07$) (Fig. 2G). Finally, we used a subset of animals to assess the survival of the treated mice. The results obtained did not show a significant difference between the two groups of $SOD1^{G93A}$ mice (Fig. 2H).

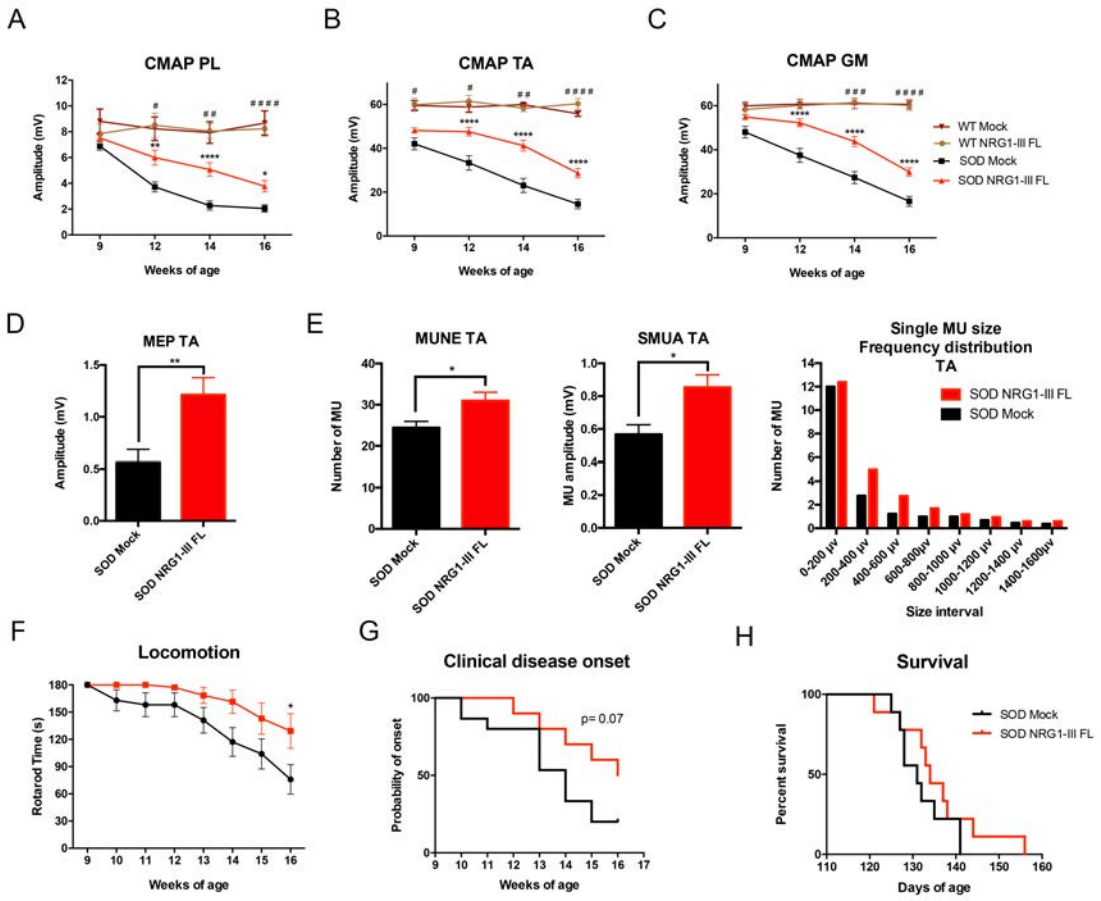

Figure 2. *NRG1-III FL overexpression slows the disease progression of SOD1^{G93A} mice.* Electrophysiological tests show that AAV-NRG1-III FL injection produced significant preservation of the CMAP amplitude of plantar (A), tibialis anterior (B), and gastrocnemius (C) hindlimb muscles in the SOD1^{G93A} mice (n=6 WT Mock, 6 WT NRG1-III, 13-14 SOD Mock, 19-20 SOD NRG1-III mice per group, two-way ANOVA, *p<0.05 vs SOD Mock mice; #p<0.05 vs WT Mock and WT NRG1-III mice). D) The gene therapy increased the amplitude of MEPs in SOD1^{G93A} mice, indicating improved connectivity between upper and lower MNs (**p<0.01, *p<0.05 vs SOD Mock mice). E) Electrophysiological estimation of motor unit number (MUNE) and mean amplitude of single motor unit action potential (SMUA) of the tibialis anterior muscle shows preservation of large motor units, confirmed by the frequency distribution (**p<0.01, *p<0.05 vs SOD Mock mice). F) NRG1-III FL overexpression produced improvement in the Rotarod performance of treated SOD1^{G93A} mice along time (n=6 WT Mock, 6 WT NRG1-III, 13-14 SOD Mock, 19-20 SOD NRG1-III mice per group, two- way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). G) The onset of locomotion dysfunction was

delayed but without significant differences. H) Overexpression of NRG1-III FL did not improve significantly the survival of the treated mice (n=9 SOD Mock, 9 SOD NRG1-III mice per group, Mantel-Cox test, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

NRG1-III overexpression preserves spinal MNs and decreases glial reactivity

Histopathological analysis of the lumbar spinal cord of $SOD1^{G93A}$ mice at 16 weeks of age revealed that NRG1-III FL overexpression significantly increased the number of surviving MNs (12.9±0.4; number of MNs per section) compared to mice treated with mock virus (9.1±0.7) (Fig. 3A, B). In WT mice the overexpression of NRG1-III FL did not modify the number of motoneurons (20.1±0.6) compared with the WT mock group (19.2±1.5) (Fig. 3B). These data provide clear evidence of the beneficial contribution of NRG1 type III in the CNS of ALS mice.

Since ErbB receptors are also expressed in astrocytes and microglial cells, we assessed their immunoreactivity. We found that our gene therapy reduced astrocyte ($2.37x10^8$±$1.70x10^7$ integrated density) and microglial ($1.82x10^8$±$3.36x10^7$) activation compared to the $SOD1^{G93A}$ mock mice ($6.05x10^8$±$2.08x10^8$ and $8.78x10^8$±$4.28x10^8$ respectively), suggesting a positive effect of NRG1-III FL on glial cells in degenerative pathologies (Fig. 3C, D).

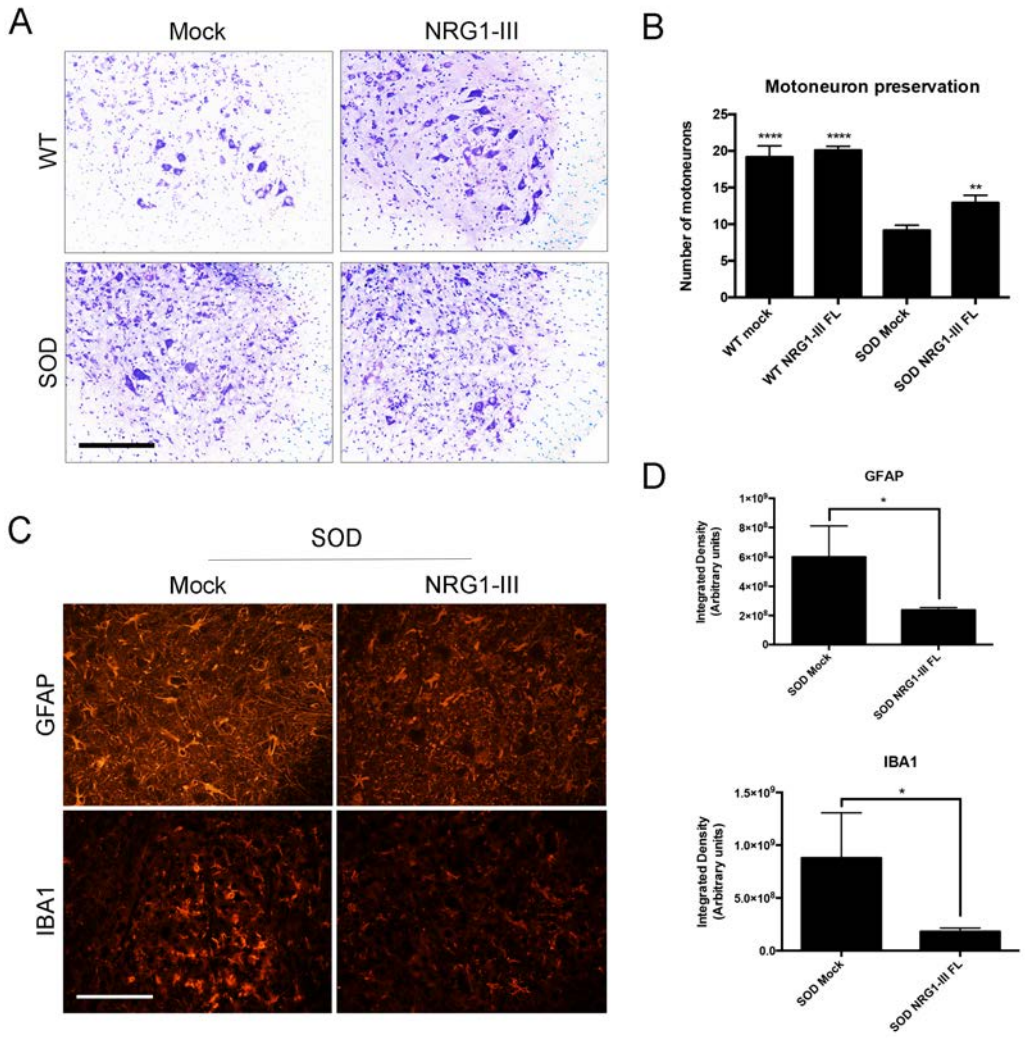

Figure 3. *Effect of NRG1-III FL overexpression on MNs preservation and glial reactivity in SOD1^{G93A} mice.* A) Representative images of L4 spinal cord of wild type and SOD1^{G93A} mice, treated with NRG1-III FL or with mock vector (scale bar = 100 µm). B) Histological analysis showed higher number of MN in the ventral horn of the treated mice compared with mock mice (n=3 WT Mock, 3 WT NRG1-III, 3 SOD Mock, 7 SOD NRG1-III mice per group, one-way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). C) Representative confocal images of astrocytes labeled against GFAP, and microglia labeled against Iba-1, in the spinal cord ventral horn of SOD1^{G93A} mice (scale bar = 100 µm). D) AAV-mediated delivery of NRG1-III FL reduced the astrocyte and microglial reactivity in the spinal cord (n=4 SOD Mock, 8 SOD NRG1-III mice per group, T-student, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

Modulation of NRG1/ErbB4 signaling upon NRG1-III overexpression

We evaluated the expression and distribution of ErbB4 receptor in spinal cord samples of SOD1^{G93A} mice and ALS human patients. The levels of ErbB4 mRNA in transgenic mice at 16 weeks were slightly downregulated (0.81±0.02) compared to the WT mice (1.00±0.01) whereas NRG1-III overexpression restored the receptor levels (0.96±0.01) (Fig. 4A). In addition, immunofluorescence labeling showed intranuclear localization of the C-terminal domain of ErbB4 receptor in spinal MNs of SOD1^{G93A} mice ($1.3x10^7$±$1.5x10^6$), that was not observed in control samples ($2.6x10^6$±$8.1x10^5$) (Fig. 4B). The C-terminal domain of ErbB4 was also localized within the nucleus of the preserved MNs in the spinal cord of ALS patients (Fig. 4C).

We next analyzed whether NRG1-III overexpression influenced two well-known markers related to MN vulnerability, MMP-9 and KCC2. MMP-9 is selectively expressed by the fast MNs, the first affected in ALS, and in the presence of mutant SOD1 it enhances the activation of ER stress which is sufficient to trigger axonal die-back (Kaplan et al., 2014). Our results showed that the SOD1^{G93A} treated mice had more MMP-9 negative MNs (72.2±3.5%) compared to the untreated mice (47.0±4.3%) (Fig. 4D). Furthermore, loss of the inhibitory tone induced by downregulation of KCC2 in motoneurons has been shown to contribute to spasticity (Brocard et al., 2016). Immunohistochemical analysis showed a significant decrease of KCC2 immunofluorescence in SOD1^{G93A} mice injected with mock construct (0.60±0.03), but not in those SOD1^{G93A} mice injected with NRG1-III FL vector (0.84±0.04) compared to WT mice (Fig. 4E).

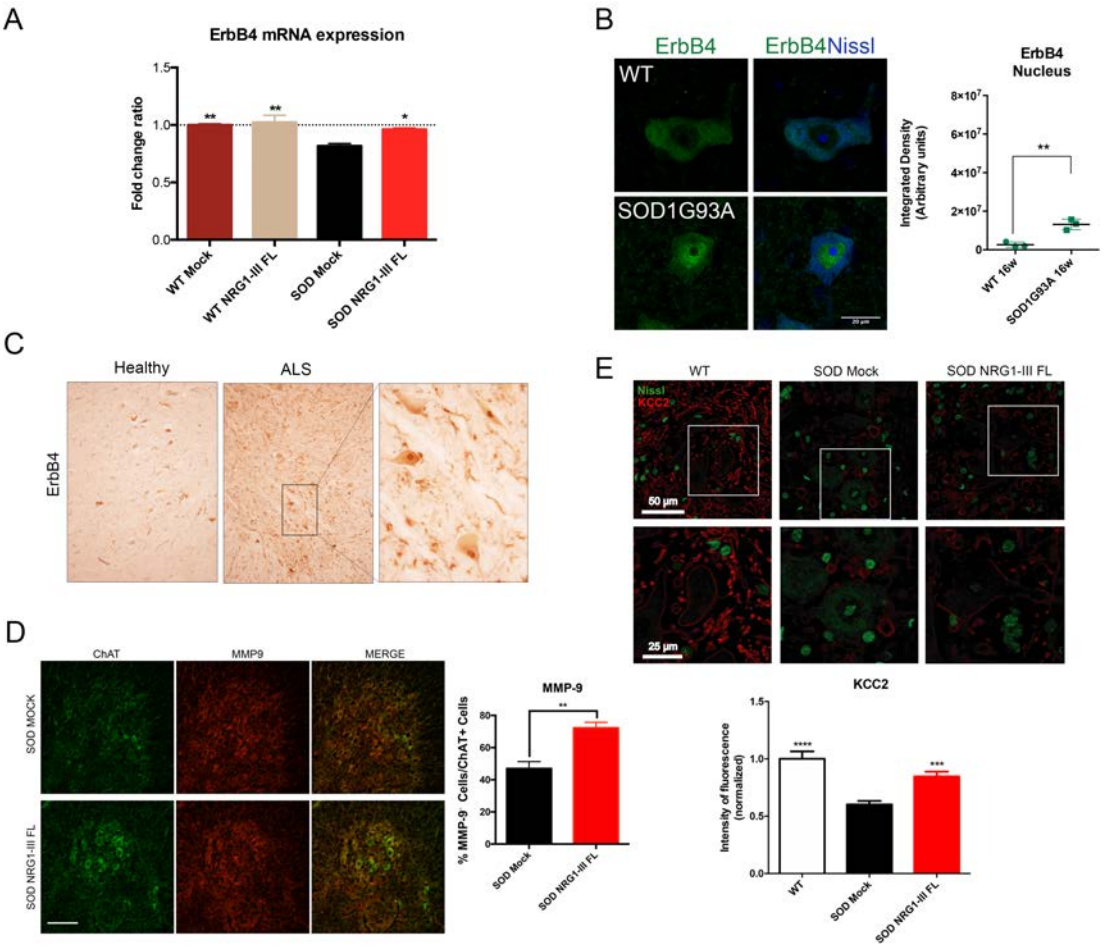

Figure 4. *Effect of NRG1-III FL overexpression on NRG1/ErbB4 signaling and MN excitability markers in SOD1^{G93A} mice.* A) Treatment with NRG1-III FL increased the ErbB4 receptor expression to normal levels in the SOD1^{G93A} mice (n=3 WT Mock, 4 WT NRG1-III, 3 SOD Mock, 4 SOD NRG1-III mice per group, one-way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). B) Confocal images showed ErbB4 translocation to the nucleus of MNs in the SOD1^{G93A} mice (scale bar = 20 µm). The integrated density of ErbB4 immunolabeling was significantly increased in the nucleus in SOD1^{G93A} mice (n= 3 WT, 3 SOD mice per group, T-student, **p<0.01, *p<0.05). C) Microphotographs of spinal cord samples labeled for ErbB4 showed also presence of ErbB4 in the nucleus of MNs in ALS patients (Scale bar = 20 µm). D) Representative images of spinal cord ventral horn MNs immunolabeled for ChAT (green) and MMP-9 (red). NRG1-III overexpression increased the number of MMP-9 negative MNs (scale bar = 100 µm, n=4 SOD Mock, 8 SOD NRG1-III mice per group, T-student, **p<0.01, *p<0.05. E) Representative immunostaining of KCC2 (red) in the membrane of MNs labeled with FluoroNissl (green) (scale bar = 50

μm). Higher magnification images (bottom) showed that KCC2 staining was decreased specially around the MN soma (scale bar = 25 μm). NRG1-III FL treatment rescued the KCC2 downregulation observed in the $SOD1^{G93A}$ mice (n=3 WT, 4 SOD Mock, 8 SOD NRG1-III mice per group, one-way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

NRG1-III overexpression does not alter the disease progression of the male $SOD1^{G93A}$ mice

To further characterize the effect of NRG1-III overexpression we administered the AAVrh10Nrg1-III FL vector again at 8 weeks in male mice. The electrophysiological results revealed that the treatment in the $SOD1^{G93A}$ male mice did not reduce the progressive decline of the CMAP amplitude of the hindlimb muscles in comparison with the mice treated with the mock vector (Fig. 5A,B). The rotarod test results showed that treatment with NRG1-III did not improve the functional outcome of the $SOD1^{G93A}$ mice in comparison with the mock group (Fig. 5C). Moreover, histopathological analysis of the lumbar spinal cord of male $SOD1^{G93A}$ mice at 16 weeks of age revealed a similar number of surviving MNs of the NRG1-III FL treated mice (8.1±0.1) compared to mice treated with mock virus (7.4±0.7) (Fig. 5D,E). In WT male mice the overexpression of NRG1-III FL did not modify the number of motoneurons (18.9±1.4) compared with the WT Mock group (19.5±1.6) (Fig. 5D,E).

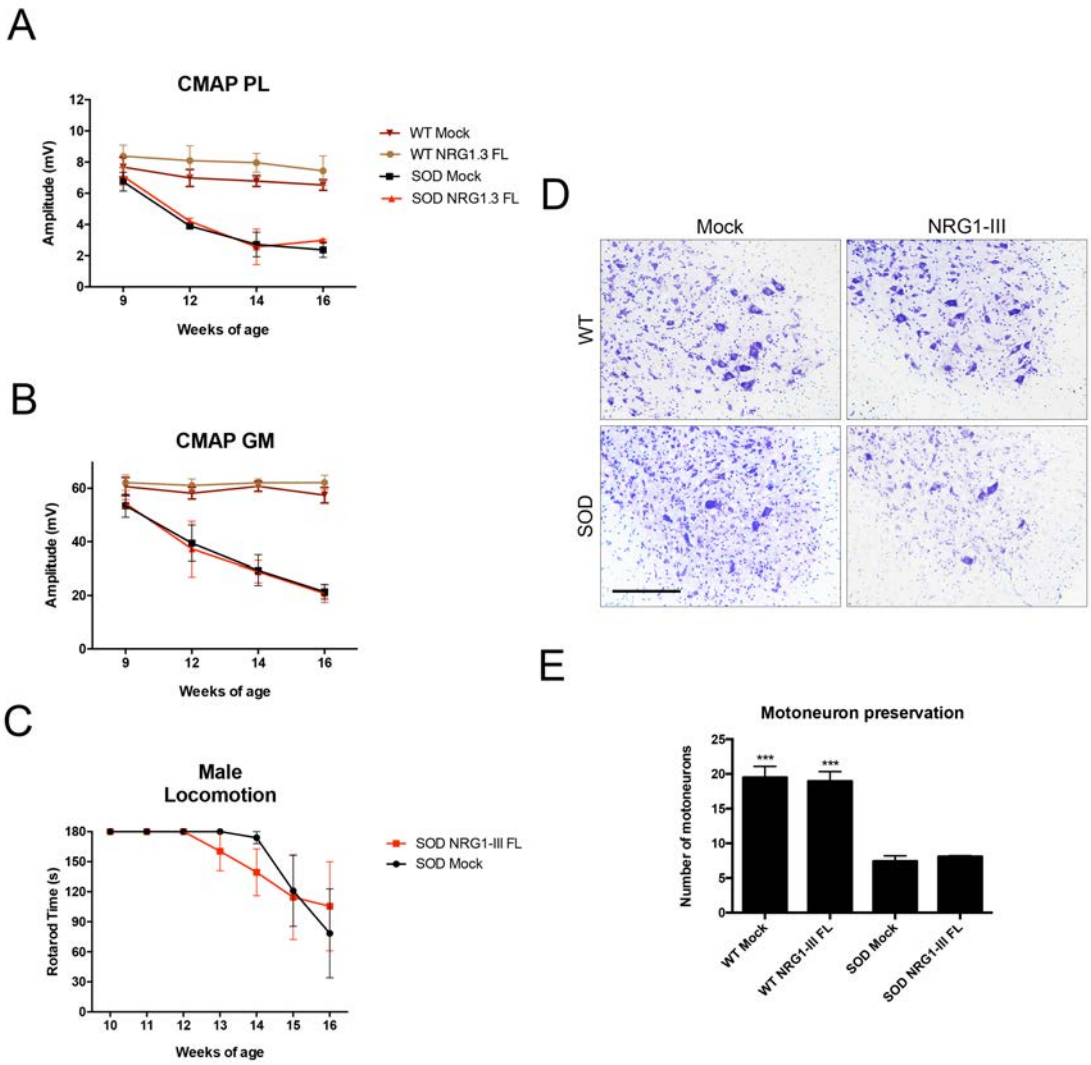

Figure 5. *NRG1-III overexpression does not produce beneficial effects in the male SOD1^{G93A} mice.* Electrophysiological tests show that AAV-NRG1-III FL injection did not preserve the CMAP amplitude of plantar (A) and gastrocnemius (B) hindlimb muscles in the male SOD1^{G93A} mice (n=6 WT Mock, 8 WT NRG1-III, 6 SOD Mock, 6 SOD NRG1-III mice per group, two-way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). C) Treatment with NRG1-III did not improve the Rotarod performance of the male mice (n=6 SOD Mock, 4 SOD NRG1-III mice per group, two-way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). D) Representative images of L4 spinal cord of wild type and SOD1^{G93A} mice, treated with NRG1-III FL or with mock vector (scale bar = 100 μm). E) Histological analysis showed a similar number of MNs in the spinal cord ventral horn of the treated SOD1^{G93A} mice compared to the mock mice (n=3 WT Mock, 3 WT NRG1-III, 3 SOD1^{G93A} mock, 3 SOD1^{G93A}

NRG1-III mice per group, one-way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

Discussion

The results of this study provide novel insights into the mechanisms of NRG1-III/ErbB4 signaling on spinal MN preservation in the pathophysiology of ALS. NRG1-III has been reported as an important isoform for neuronal survival (Wolpowitz et al., 2000; Bao et al., 2003; Xu et al., 2006; Li et al., 2007; Chen et al., 2019), for synaptic plasticity (Loeb & Fischbach, 1997; Buonanno & Fischbach, 2001; Roysommuti et al., 2003; Agarwal et al., 2014), and it is the most prominent NRG1 isoform expressed in spinal cord MNs (Ho et al., 1995; Meyer et al., 1997; Loeb et al., 1999). Interestingly, mutant embryos that lack selectively this isoform suffer perinatal death, associated with a striking lack of Schwann cells and a severe reduction of MNs and DRG cells (Wolpowitz et al., 2000). NRG1-III is mostly localized at the endoplasmic reticulum-related subsurface cistern (SSC) adjacent to the postsynaptic membrane to C-boutons (Gallart-Palau et al., 2014), where it has been hypothesized to play a role in the regulation of MN excitability.

While recent studies suggested that type I and type III NRG1 transcript and protein levels were decreased in the lumbar spinal cord of symptomatic SOD1^{G93A} mice (Lasiene et al., 2016), we found that NRG1-I was slightly increased in the spinal cord of ALS patients and of SOD1^{G93A} mice, due to increased expression in glial cells, whereas NRG1-III appeared decreased in the spinal MNs, in agreement with previous observations by Song et al. (2012). Enhancement of NRG1-I in ALS patients and SOD1^{G93A} mice may exert a detrimental effect by promoting glial reactivity upon the activation of ErbB receptors. In contrast, NRG1-III seems to play a critical role on preservation of MN activity. These results suggest that early changes of the expression of NRG1 isoforms in spinal cord may be involved in the pathogenesis of the disease. Consequently, the modulation of NRG1/ErbB axis may affect the progression of MN degeneration. Indeed, we recently demonstrated, using organotypic spinal cord cultures, that NRG1 exerts neuroprotective effects on MNs subjected to chronic excitotoxicity, and also enhances neurite outgrowth (Mòdol-

Caballero et al., 2018). Lasiene and collaborators (2016) also suggested that overexpression of NRG1-III by gene therapy extended the survival of the ALS mice via restoration of C-boutons of spinal MNs, however more detailed studies were needed to reveal the possible beneficial role of the enhancement of NRG1-III in ALS. Since a previous paper reported that the ICD of the NRG1-III protein could translocate to the nucleus and promote the survival of neurons (Bao et al., 2003) we decided to overexpress the full-length form of NRG1-III in the spinal cord of $SOD1^{G93A}$ mice. Our results show that this gene therapy approach produced significant preservation of motor function during the symptomatic stage of the disease of the $SOD1^{G93A}$ female mice. The histological analysis corroborates the beneficial role of NRG1-III overexpression, since we found a higher preservation of spinal MNs. Indeed, the spinal MNs expressed higher level of NRG1-III, which may cause the protective effect by an autocrine fashion (Lasiene et al., 2016). However, despite this viral-mediated therapy was able to delay the disease onset it did not increase survival of the mice. This suggests that the treatment ameliorates the disease in the early stages but is not able to induce a long-term positive effect.

In addition, here we show that NRG1-III overexpression in the spinal cord does not produce a similar positive effect on the male $SOD1^{G93A}$ mice. In a previous study similar results were obtained, since the expression of NRG1 antagonist in the spinal cord of an EAE mouse model protected female but not male mice (Allender et al., 2018). Interestingly, in a model of nerve root ligation the authors showed that progesterone specifically facilitated the expression of NRG1 in the spinal cord which in turn modulates nociceptive sensitivity (LaCroix-Fralish et al., 2008). Indeed, it is described that progesterone provides neuroprotection, since it is able to rescue MNs from degeneration in the Wobbler mouse, a genetic model of spinal cord MN disease (Gonzalez Deniselle et al., 2002). Consequently, progesterone might play a key role on the modulation of the NRG1 expression in the spinal cord of the $SOD1^{G93A}$ mice and therefore enhancing the neuroprotective effects observed.

The role of NRG1/ErbB signaling on inflammation is controversial. Resident glial cells and infiltrating immune cells express NRG1 receptors (Tynyakov-Samra et al., 2011; Gauthier et al., 2013). Increased ErbB2 receptor activation is observed on activated microglia in ALS patients and transgenic $SOD1^{G93A}$ mice (Song et al.,

2012), and treatment with a NRG1 antagonist reduces microglial reactivity in the SOD1^{G93A} mice through the reduction of ErbB2 phosphorylation (Liu et al., 2018). However, treatment with NRG1 has been shown to attenuate astrogliosis after spinal cord injury (Alizadeh et al., 2017; 2018). In this regard, our results show that overexpression with NRG1-III isoform decreased both astrocyte and microglia reactivity. Microglial cells revealed thinner and ramified processes under NRG1-III overexpression compared to the control SOD1^{G93A} mice, in which microglia showed larger size and amoeboid morphology. Therefore, while upregulation of NRG1-I might have a deleterious role in the SOD1^{G93A} mice, NRG1-III overexpression has a beneficial role reducing the neuroinflammatory response. Interestingly, we found that our treatment did not only increase the expression of NRG1-III in MNs but also in other type of cells, likely astrocytes. It was previously described that NRG1 is expressed and secreted by astrocytes (Pinkas-Kramarski et al., 1994) and treatment attenuates the upregulation of chondroitin sulfate proteoglycans (CSPGs) which plays an inhibitory role for neural regeneration in spinal cord injury (Bradbury et al., 2002; Dyck & Karimi-Abdolrezaee., 2015; Alizadeh et al., 2017). Therefore, a MN-astrocyte signaling mechanism might be involved, in which astrocytes may be acting via neuronal ErbB receptor signaling to induce synaptic plasticity (LaCroix-Fralish et al., 2008).

The intrathecal AAV-mediated therapy applied induced a physiological expression of NRG1-III FL and its most important receptor ErbB4 in the spinal cord. Interestingly we found that in both ALS patients and SOD1^{G93A} mice the ErbB4 receptor translocated into the nucleus of the MNs whereas wild-type mice only showed labeling in the cytoplasm, suggesting a detrimental effect of the ICD domain. Indeed, for NRG1/ErbB signaling, both NRG1-III and ErbB4 have intracellular domains that can be internalized by the neuron and translocate to the nucleus (Lee et al., 2002; Bao et al., 2003). Presilin-dependent cleavage of ErbB4 generates the soluble B4-ICD that functions in the nucleus presumably regulating gene transcription and cell fate (Lee et al., 2002; Ni et al., 2001; Sardi et al., 2006), or to the mitochondria where it promotes apoptosis of breast cancer cells (Naresh et al., 2006). For the nuclear accumulation, ErbB4 ICD domain requires sumoylation by the sentrin-specific proteases (SENPs) (Knittle et al., 2017). Intriguingly, other data showed that ErbB4-mediated synapse maturation requires the extracellular domain

of ErbB4 rather than the ICD where the kinase domain is located, whereas the tyrosine kinase activity modulates primary neurite formation (Krivosheya et al., 2008). Therefore, while NRG1-III cleavage produces neuroprotection, the ErbB4 ICD signaling may play a role on the neurodegeneration process.

The balance between excitatory and inhibitory synaptic inputs is critical for the physiological control of MNs. Loss of NRG1 from cortical projection neurons resulted in increased inhibitory neurotransmission (Agarwal et al., 2014) and it has been described that NRG1-III has an essential role for cholinergic transmission (Salvany et al., 2019). Indeed, blocking cholinergic neurotransmission through C-boutons results in an increase in neurotoxic misfolded SOD1 in MNs of SOD1 ALS mouse (Saxena et al., 2013). Furthermore, Salvany and collaborators proposed that NRG1-III may serve as postsynaptic SSC organizer, a structure that includes the potassium channel Kv2.1. Therefore NRG1-III overexpression may interact with postsynaptic components of C-type synapses such as Kv2.1 channel regulating the MN excitability. Also, the maintenance of a low-intracellular chloride concentration by the KCC2 transporter is essential for the efficacy of the fast-synaptic inhibition of MNs (Fuchs et al., 2010). Interestingly, it was previously reported that in late stages of the disease KCC2 is dysregulated in the spinal MNs of $SOD1^{G93A}$ mice (Fuchs et al., 2010; Mòdol et al., 2014). We found that KCC2 transporter expression, which also regulates MN excitability, is upregulated following NRG1-III overexpression preventing late hyperreflexia. Therefore, NRG1-III/ErbB4 signaling might also regulate the MN excitability through KCC2. In addition, loss of the inhibitory tone induced by downregulation of KCC2 in MNs has been shown to contribute to the spastic behavior. Our recent investigation (Brocard et al., 2016) demonstrates that spasticity results from an excess of motoneuron excitation due to concomitant upregulation of the persistent sodium current (I_{NaP}) with downregulation of KCC2 after spinal cord injury. We reported the casual relationship between the activation of calpain and the increase of I_{NaP}. In ALS genetic models, motoneuronal hyperexcitability is also characterized by an increase of I_{NaP} (ElBasiouny et al., 2010). It thus appears that the upregulation of the I_{NaP} and the downregulation of KCC2 are common in both spinal cord injury and ALS but the molecular disturbances responsible for this dysfunction remain unknown.

Another prospective marker for ALS-vulnerable MNs is MMP-9. MMP-9 expression prior to disease onset triggers neurodegeneration and enables activation of ER stress (Kaplan et al., 2014). Furthermore, the removal of MMP-9 gene leads to an increase in lifespan (Xia et al., 2006). Intriguingly, treatment with NRG1 remarkably attenuates the production and activity of MMP-9 following spinal cord injury (Alizadeh et al., 2017) and activation of EGFR (ErbB1) enhances nociception in dorsal root ganglia neurons through a mechanism involving the PI3K/AKT pathway and the upregulation of MMP-9 (Martin et al., 2007). Our results showed that the overexpressing NRG1-III decreased the number of MMP-9 positive MNs, therefore conferring neuroprotection to the most vulnerable population of MNs.

Conclusions

In summary, NRG1-III overexpression improves motor function and significantly preserves the spinal MNs, through the activation of the NRG1-III/ErbB signaling in female, but not in male SOD1^{G93A} mice. It also regulates MN excitability and improves MN-vulnerability markers producing a functional improvement at the symptomatic stage of the disease. Our results point out that upregulation of the full-length form of NRG1-III may be important for counteracting MN degeneration, and that further investigation to elucidate differences between sex and the intracellular mechanisms that mediate neuroprotection are required.

Acknowledgments

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CHAPTER III

GENE THERAPY OVEREXPRESSING NEUREGULIN 1 TYPE I ALONE AND IN COMBINATION WITH NEUREGULIN 1 TYPE III PROMOTES FUNCTIONAL IMPROVEMENT IN THE SOD1^{G93A} ALS MICE

Gene therapy overexpressing Neuregulin 1 type I alone and in combination with Neuregulin 1 type III promotes functional improvement in the SOD1^{G93A} ALS mice

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Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder affecting motoneurons, with no effective treatment currently available. The molecular mechanisms that are involved in the motoneuron (MN) death are complex and not fully understood, with partial contributions of surrounding glial cells and skeletal muscle to the disease. Neuregulin 1 (NRG1) is a trophic factor highly expressed in MNs and neuromuscular junctions. Recent studies have suggested a crucial role of the isoform I (NRG1-I) in the collateral reinnervation process in skeletal muscle, and NRG1-III in the preservation of MNs in the spinal cord, opening a new window for developing novel therapies. In this study, we performed two gene therapies, one overexpressing NRG1-I in the skeletal muscles, and a second combining NRG1-I with NRG1-III overexpression in the spinal cord of the $SOD1^{G93A}$ mice. The results show that both NRG1 gene therapy approaches were able to increase the number of MNs and neuromuscular junctions, and reduce the astroglial reactivity in the spinal cord of the treated $SOD1^{G93A}$ mice. Furthermore, NRG1 gene therapies preserved the motor function of the hindlimb muscles and delayed the onset of clinical disease. However, the combinatorial gene therapy did not produce the expected synergic effect. In summary, our data indicates that NRG1 plays an important role on MN survival and muscle innervation in ALS, and that viral-mediated overexpression of NRG1 isoforms may be considered as a promising approach for ALS treatment.

Key words: Neuregulin 1, ErbB receptors, motoneuron, motor function, amyotrophic lateral sclerosis, spinal cord, neuromuscular junction.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by loss of motoneurons (MNs) of primary motor cortex, brainstem and spinal cord (Wijesekera & Leigh., 2009). The vaste majority of the cases are sporadic (sALS) in which the etiology is unknown, and about 10% correspond to familiar forms (fALS), associated with inherited alterations in genes, such as superoxide dismutase 1 (SOD1) (Rosen et al., 1993), TAR-DNA binding protein (TDP-43) (Kabashi et al., 2008; Yokoseki et al., 2008), hexanucleotide repeat expansions in chromosome 9 open reading frame 72 (C9ORF72) (DeJesus-Hernandez et al., 2011; Renton et al., 2011), and others less frequent. In both sALS and fALS the loss of MNs leads to fastly progressive muscle atrophy and weakness, accompanied with fasciculations and spasticity (Robberecht & Philips, 2013). The pathophysiological mechanisms underlying the development of ALS are multifactorial, with rising evidence of a complex interaction between genetic and molecular pathways (Mancuso & Navarro., 2015). The particular molecular mechanisms that specifically affect the MN to cause its death is still to be elucidated. Moreover, neighboring cells, such as microglia, astrocytes and interneurons, contribute to the disease (Brites et al., 2014). MN degeneration is preceded by alterations and disjunction of neuromuscular junctions (NMJs) (Fischer et al., 2004; Mancuso et al., 2011), and a toxic role for skeletal muscle has also been reported (Moloney et al., 2014),

During the last decades several animal models carrying ALS-related mutations have been developed. The most widely used ALS model is a transgenic mouse expressing the human mutated form of the SOD1 gene with a glycine to alanine conversion at the 93rd amino acid (SOD1^{G93A}) (Gurney et al., 1994; Ripps et al., 1995), which outlines the most important clinical and pathological features of ALS (Ripps et al., 1995; Mancuso et al., 2011). Alterations in SOD1 protein have been also found in sporadic ALS patients (Bosco & Landers, 2010), and accumulation of wild-type SOD1 was shown to produce ALS in mice (Graffmo et al., 2013), thus, increasing the potential interest of the SOD1-linked ALS models.

Unfortunately, there is no cure for ALS, and there are only two drugs approved for use in ALS, riluzole, that only slightly prolongs survival for 3-4 months, and

edaravone, a drug that presumably works to mitigate oxidative injury in central neurons. Symptomatic and paliative measures (including feeding and respiratory support) are the mainstay of patients management (Pasinelli & Brown., 2006). One of the many therapeutic strategies that have emerged recently to treat ALS is gene therapy. The main advantage of this approach is that it permits to specifically deliver a gene of interest to MNs overcoming the difficulty of crossing the blood-brain barrier of large proteins (Federici & Boulis., 2006). Particularly, adeno-associated vectors (AAV) have some advantages over other viral vectors, such as the ability to provide sustained gene expression avoiding the risk for insertional mutagenesis. Viral transgene expression can also be restricted depending on the viral tropism and using promoters with selective and defined expression patterns (Kügler, 2016).

Neurotrophic factors derived from the spliced forms of Neuregulin 1 (NRG1) have been shown to be critical for MN survival, supporting axonal and neuromuscular development and maintenance (Syroid et al., 1996; Sandrock et al., 1997; Loeb et al.,1999; Wolpowitz et al., 2000; Michailov et al., 2004; Nave et al., 2006; Velanac et al., 2012). NRG1 has been localized in spinal MNs, focused in the subsurface cisterns underlying postsynaptic sites of cholinergic C boutons (Issa et al., 2010, Gallart-Palau et al., 2014). NRG1 acts through the EGF domain of ErbB receptors, and NRG1-ErbB system alterations have been related to ALS since loss-of-function mutations on NRG1 receptor ErbB4 produce late-onset, autosomal-dominant ALS in human patients (Takahashi et al., 2013). We have recently reported that ErbB4 ectodomain fragments were reduced in cerebrospinal fluid and plasma of ALS patients, suggesting impaired NRG1-ErbB signaling (López-Font et al., 2019). Interestingly, several studies support that NRG1 type III expression is reduced in the spinal cord of both ALS patients and $SOD1^{G93A}$ mice, whereas NRG1 type I appears to be upregulated (Song et al., 2012; Mòdol-Caballero et al., 2019). Furthermore, viral-mediated delivery of NRG1-III in the CNS restored the number of C-boutons and produced an increase of survival of the $SOD1^{G93A}$ mouse (Lasiene et al., 2016). Also, treatment with an AAV coding for the full-length form of NRG1-III improves the progressive decline in motor function and motoneuron number and reduces glial reactivity in $SOD1^{G93A}$ mice (Mòdol-Caballero et al., 2019). However, the exact role of NRG1-I is not fully known. It has been reported that NRG1-I expression by Schwann cells is essential for promoting axonal regeneration and remyelination

(Stassart et al., 2012). Indeed, overexpression of NRG1-I by means of an AAV vector, injected locally in the gastrocnemius muscle produced functional improvement by enhancing motor axons collateral sprouting in SOD1^{G93A} mice (Mancuso et al., 2016). Therefore, NRG-I plays a crucial role in the collateral reinnervation process.

Considering these recent findings, in the present study our aims were, first, to perform a gene therapy to overexpress NRG1-I in all the skeletal muscles to maintain motor innervation in the SOD1^{G93A} mouse. For this reason, we used the promotor of the desmin gene, which is only expressed in muscles, to design the viral vector. Second, we combined two gene therapies to overexpress NRG1-III in the spinal cord to preserve MNs (Mòdol-Caballero et al., 2019) and NRG1-1 in muscles to maintain their innervation, searching for a synergic effect.

Materials and methods

Animals

Transgenic mice carrying the mutation G93A in the SOD1 gene and non transgenic wild-type (WT) littermates as controls were used. SOD1^{G93A} high copy mice (Tg[SOD1-G93A]1Gur) were obtained from the Jackson Laboratory (Bar Harbor, ME). These mice were bred and maintained as hemizygotes by mating transgenic males with F1 hybrid females obtained from Janvier Laboratories (France). Transgenic mice were identified by polymerase chain reaction amplification of DNA extracted from the tail. Mice were maintained in standard conditions with access to food and water *ad libitum* at the Animal Service of the Universitat Autònoma de Barcelona, and were cared for and handled in accordance with the guidelines of the European Union Council (Directive 2010/63/EU) and Spanish regulations on the use of laboratory animals. The experimental procedures had been approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

For a preliminary part of the study we also used C57BL/6 mice, divided in two groups of WT mice and two groups of SOD1^{G93A} mice (n=5 each) that were administered at 10 weeks of age with either AAV8 or AAV9 coding for luciferase under the desmin promoter. To determine the specificity for muscle targeting after

systemic e.v. administration, we performed luciferase in vivo imaging (IVIS) in these mice (see below).

After proving the effectiveness of the viral vector and considering the differences observed in disease progression depending on mouse strain (Mancuso et al., 2012), we performed the therapeutic assay with B6xSJL female and male mice. The animals were divided in a group of WT mice and two groups of $SOD1^{G93A}$ mice, that were administered at 6 weeks of age with either AAV8 coding for NRG1-I or mock vector. For the functional studies we used the following number of mice per group: WT mice (n=6 females, n=5 males), SOD Mock mice (n=14 females, n=12 males), SOD NRG1-I mice (n=12 females, n=5 males). These animals were then further distributed in two different subgroups at 16 weeks. One subgroup to analyze mRNA expression: WT (n=3 females), SOD Mock (n=4 females), SOD NRG1-I (n=4 females); the second subgroup for histological analysis: WT (n=3 females), SOD Mock (n=4 females), SOD NRG1-I (n=7 females).

Finally, we aimed to combine this gene therapy with the overexpression of NRG1-III FL (Mòdol-Caballero et al., 2019). In this study we included B6xSJL female mice, divided in a group of WT mice and two groups of $SOD1^{G93A}$ mice administered at 7 weeks with either AAV coding for NRG1-IIIFL and NRG1-I, or the respective mock vectors. For the functional studies we used the following number of mice per group: WT mice (n=6 females), SOD Mock mice (n=22 females), SOD NRG1-I + NRG1-III FL mice (n=11 females). These animals were then further distributed in two subgroups at 16 weeks. A subgroup for mRNA expression analysis: WT (n=3 females), SOD Mock (n=4 females), SOD NRG1-I + NRG1-III FL (n=4 females), and a second subgroup for histological analysis: WT (n=3 females), SOD Mock (n=5 females), SOD NRG1-I + NRG1-III FL (n=6 females).

Virus production and administration

The cDNA sequence of firefly luciferase or the extracellular domain of NRG1-I isoform containing a HA-tag (provided by G. Corfas, Harvard Medical School, Boston, MA, USA) were cloned between AAV2 ITRs under the regulation of the human desmin promoter (provided by G. Lemke, Salk Institute, La Jolla, CA, USA). The woodchuck hepatitis virus responsive element (WPRE) was added at 3' to stabilize

mRNA expression. AAV8 or AAV9 viral stocks were produced by the Viral Production Unit of UAB (http://sct.uab.cat/upv). Briefly, vectors were generated using the triple transfection system in HEK293-AAV cells of the expression plasmids, Rep1Cap8 or Rep1Cap9 plasmids containing AAV genes (provided by J.M. Wilson, University of Pennsylvania, Philadelphia, USA) and pXX6 plasmid containing adenoviral genes, needed as helper virus (Zolotukhin et al., 1999). After 48 hours, AAV vectors were harvested, treated with benzonase, purified in an iodixanol gradient, and titred using the Picogreen (Invitrogen) system (Piedra et al., 2015) quantification and calculated as viral genomes per milliliter (vg/ml). Control serotype-matching AAV8 or AAV9 vectors coding mock or GFP were used as control. Full-length (FL) NRG1 type III AAVrh10 virus was produced as in a previous study (Mòdol-Caballero et al., 2019).

For the luciferase assay, intravenous administration of $1x10^{12}$ vg of AAV8 or AAV9-Luciferase in a total volume of 250 µl was performed in 10 weeks old mice. AAV8 or AAV9-null virus was used as control. Five weeks later, animals were analyzed in vivo and ex vivo using the IVIS Spectrum (Perkin Elmer).

For the NRG1-I gene therapy, a suspension of $7.6x10^{13}$ vg of AAV8-NRG1-I in 250 µl was injected intravenously in 6 weeks old mice. Intravenous injection was performed in a tail vein. For the combined therapy, intravenous administration of $1x10^{12}$ vg of AAV8-NRG1-I and intrathecal administration of AAVrh10NRG1-IIIFL were performed in 7 weeks old mice. For intrathecal injection, the spine was surgically exposed, under anesthesia with ketamine/xylacine (100/10 mg/kg i.p.), and 10 µl of viral vector ($1x10^{11}$ vg of AAVrh10Nrg1-IIIFL vector or mock vector) were delivered between lumbar vertebrae L3 and L4 into the cerebrospinal fluid (CSF) with a 30G needle attached to a Hamilton syringe. Suitable injection into the intrathecal space was confirmed by the animal's tail flick. The needle was left in place at the injection site for 1 minute to avoid reflux.

Electrophysiological tests

Motor nerve conduction tests were performed by stimulation of the sciatic nerve by means of single pulses of 20µs duration (Grass S88) delivered by two needle electrodes placed at the sciatic notch. The compound muscle action potential

(CMAP) was recorded from tibialis anterior (TA), gastrocnemius (GM) and plantar interossei (PL) muscles with microneedle electrodes (Navarro & Udina., 2009; Mancuso et al., 2011) at 9, 12, 14 and 16 weeks of age. The recorded potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S), measuring the latency and amplitude of the CMAP. The mouse body temperature was maintained constant by means of a thermostated heating pad.

Motor unit number estimation (MUNE) was performed using the incremental technique (Shefner et al., 2006; Mancuso et al., 2011) with the same setting as for motor nerve conduction tests. The sciatic nerve was stimulated with pulses of gradual increasing intensity, starting from a subthreshold intensity. Then, quantal increases in the CMAP were recorded. The increments higher than 50 μV were considered as due to the recruitment of an additional motor unit. The mean amplitude of single motor units was calculated as the average of consistent increases. The MUNE was calculated as the ratio between the CMAP maximal amplitude and the mean amplitude of single motor unit action potentials.

To evaluate the central descending pathways, motor evoked potentials (MEP) were recorded from the TA and GM muscles following electrical stimulation of the motor cortex with pulses of 0.1 ms duration and supramaximal intensity, delivered through subcutaneous needle electrodes placed over the skull overlaying the sensorimotor cortex (Mancuso et al., 2011).

Locomotion tests

The Rotarod test was performed to evaluate motor coordination, strength and balance of the animals. Mice were placed onto the rod rotating at a constant speed of 14 rpm. The time that the animal remained on the rotating rod was measured. Each mouse was given three trials and the longest time until falling recorded; 180 sec was chosen as the arbitrary cut-off time. The test was performed from 9 to 16 weeks of age. Clinical disease onset for each mouse was determined as the first week when the maintenance time was lower than 180 seconds.

RNA extraction and real time PCR

To obtain RNA samples at 16 weeks of age, mice were sacrificed by decapitation after deep anesthesia. L4-L5 spinal cord segments and gastrocnemius muscles were rapidly dissected, maintained in RNA-later solution and processed for mRNA analyses. Total RNA was extracted and 1 µg per sample was reverse-transcribed using 10 µmol/l DTT, 200 U M-MuLV reverse transcriptase, 10 URNase Out Ribonuclease Inhibitor and 1µmol/l oligo(dT), 1µmol/l random hexamers. The reverse transcription cycle conditions were 25°C for 10 min, 42°C for 1 h and 72°C for 10 min. Gene-specific mRNA analyses were performed by SYBR green real-time PCR using the MyiQ5 real-time PCR detection system.

Histological analysis

At 16 weeks of age after functional follow-up, subgroups of mice were transcardially perfused with 4% paraformaldehyde in PBS, and the lumbar spinal cord and the gastrocnemius muscle were harvested. Spinal cords were postfixed during 2h, then cryopreserved in 30% sucrose in PBS. Gastrocnemius muscles were directly cryopreserved in 30% sucrose in PBS.

For spinal MN counting, 20 µm thick transverse sections were cut with a cryotome (Leica, Germany) and serially collected in gelatinized slides. For each animal, slides corresponding to L4-L5 spinal cord sections with a separation of 100 µm were stained with cresyl violet. Motoneurons were identified by their localization in the ventral horn of the spinal cord and counted following strict size and morphological criteria: only neurons with a diameter larger than 20 µm, polygonal shape and prominent nucleolus were counted.

For astrocyte and glial immunofluorescence, slides from L4-L5 segements were chosen. Sections were blocked with PBS-0.3%Triton-10%Normal Donkey serum and 20 mM glycin and incubated 24h at 4°C with primary antibodies anti-ionized calcium binding adapter molecule 1 (Iba-1, 1:1000; 019-19741, Wako, Japan), and anti-glial fibrillary acidic protein (GFAP, 1:1000; 130300, Invitrogen, USA). After washes, sections were incubated overnight with Alexa 488-conjugated secondary antibody (1:200; A21206, Invitrogen, USA) or Cy3-conjugated secondary

antibody (1:200; 712-165-150, Jackson IR, USA). Finally, DAPI (1:2000; D9563-10MG, Sigma, USA) was used to stain the nucleus. Slides were mounted in Fluoromount-G (Southern Biotech, USA), and viewed under a fluorescence microscope (Nikon Eclipse NI, Japan). A region of interest centered in the ventral horn was used to measure the integrated density of Iba1 and GFAP labeling using ImageJ software.

For muscle immunohistochemistry, 60 µm longitudinal sections were serially cut and collected in 24-well plates in sequential series of 4 slices per well in Olmos solution. Sections were blocked with PBS-0.3%Triton-5%Normal Donkey serum and incubated 48h at 4ºC with primary antibodies anti-synaptophysin (1:500; AB130436, Abcam, UK) and anti-neurofilament 200 (NF200, 1:1000; AB5539, Millipore, USA). After washes, sections were incubated overnight with Alexa 594-conjugated secondary antibody (1:200; A11042, Invitrogen, USA) and Alexa 488 conjugated alfa-bungarotoxin (1:200; B13422, Life Technologies, USA). Slides were mounted in Fluoromount-G (Southern Biotech, USA). Confocal images were captured with a scanning confocal microscope (LSM 700 Axio Observer, Carl Zeiss 40x/1.3 Oil DIC M27, Germany). Maximum projections images shown in this study were generated from 1.5 µm z projections. For NMJ analysis, the proportion of fully occupied endplates was calculated by classifying each endplate as fully occupied (when presynaptic terminals overly the endplate), partially occupied (when presynaptic terminals were not clearly within the endplate) or vacant (no presynaptic label in contact with the endplate). At least 4 fields with more than 100 endplates were analyzed per muscle.

Statistical analysis

All experiments were performed by researchers blinded with respect to treatment received by each mouse group, and randomized allocation of animal in groups. Sample sizes were selected according to previous observations in our lab. Data are expressed as mean ± SEM. Electrophysiological and locomotion tests results were statistically analyzed using one-way or repeated measurements ANOVA, with Bonferroni post-hoc test when necessary. For the MUNEs and MEPs electrophysiological results t-Student test was applied. For clinical disease onset

data Log-rank (Mantel-Cox) test was applied. Histological and molecular biology data were analyzed using ANOVA and t-Student, applying Bonferroni post-hoc when necessary.

Results

Viral-delivery of NRG-I in skeletal muscles

Luciferase in vivo imaging (IVIS) showed that both vectors AAV8 and AAV9 coding for luciferase expression under the desmin promoter were widely expressed in the skeletal muscles, especially on hindlimb and cardiac muscles, after systemic administration (Fig. 1A). Moreover, ex vivo analysis of luciferase activity further confirmed the expression in the skeletal muscles, and vector genome showed a similar biodistribution in the analyzed tissues that was independent of genotype and sex (Fig. 1B).

We then aimed to test the effect of the AAV8-desmin-NRG1-I viral vector in the skeletal muscles based on a single intravenous injection of 7.6×10^{13} viral genomes previous to the clinical disease onset (at 6 weeks) in order to allow a higher expression of NRG1-I at the symptomatic stage. At the end stage of the disease (16 weeks) we confirmed that the treated $SOD1^{G93A}$ mice had a higher expression of NRG1-I in the gastrocnemius (GM) muscle (18.64 ± 7.09) compared to the $SOD1^{G93A}$ mock mice (0.76 ± 0.41) (Fig. 1C). This result correlated with the high viral genomes detected in the treated $SOD1^{G93A}$ mice (0.24 ± 0.05) compared to the $SOD1^{G93A}$ mock mice ($0.6 \times 10^{-3} \pm 0.01 \times 6 \times 10^{-3}$)(Fig. 1D).

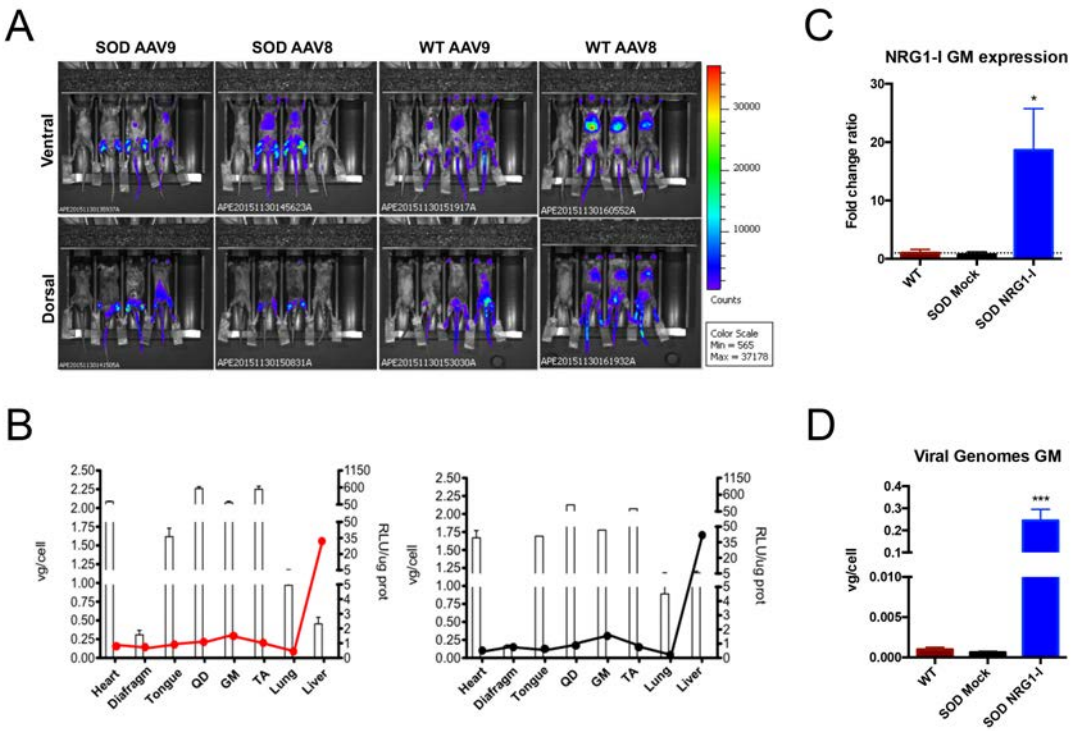

Figure 1. *AAVs under the desmin promoter coding for NRG1-I enhance specifically the NRG1-I expression in the hindlimb muscles of SOD1^{G93A} mice.* A) Luciferase in vivo imaging shows viral distribution of AAV serotypes 8 and 9 specially in the hindlimb muscles and the cardiac muscle. B) Luciferase ex-vivo analysis confirms the distribution of the viral vector in the skeletal muscles. Also, the viral genomes determination reveals that vector biodistribution was independent of genotype and sex. (n=5 WT, n=5 SOD1^{G93A} mice including 2 males and 3 females per group). C) mRNA expression analysis shows that NRG1-I is overexpressed in the GM muscle of the treated mice compared to the control SOD1^{G93A} and WT mice. D) Viral genome analysis reveals that the intravenous AAV injection was effective in the treated mice. (n=5 WT, 4 SOD Mock, 4 SOD NRG1-I mice per group, one-way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

NRG1-I overexpression preserves neuromuscular function of the treated SOD1^{G93A} mice

To study the effect of the NRG1-I viral injection we evaluated by means of electrophysiological tests the hindlimb muscles of SOD1^{G93A} mice. The results showed that there was a significant preservation of the PL, GM and TA CMAP amplitude at 16 weeks in the female (Fig. 2A,B,C) and male treated SOD1^{G93A} mice (Supplementary Fig. 1A,B,C). This higher CMAP amplitudes of hindlimb muscles was explained by an increase of the mean amplitude and the number of surviving motor units in female (Fig. 2D,E,F) and also male mice (Supplementary Fig. 1D,E,F). The distribution of TA motor units grouped by amplitude of the action potential show a higher number in most of the groups of females (Fig. 2F) and males (Supplementary Fig.1F). The amplitude of the MEPs of the GM and TA muscles was significantly higher in treated SOD1^{G93A} mice (Fig. 2G and Supplementary Fig. 1G).

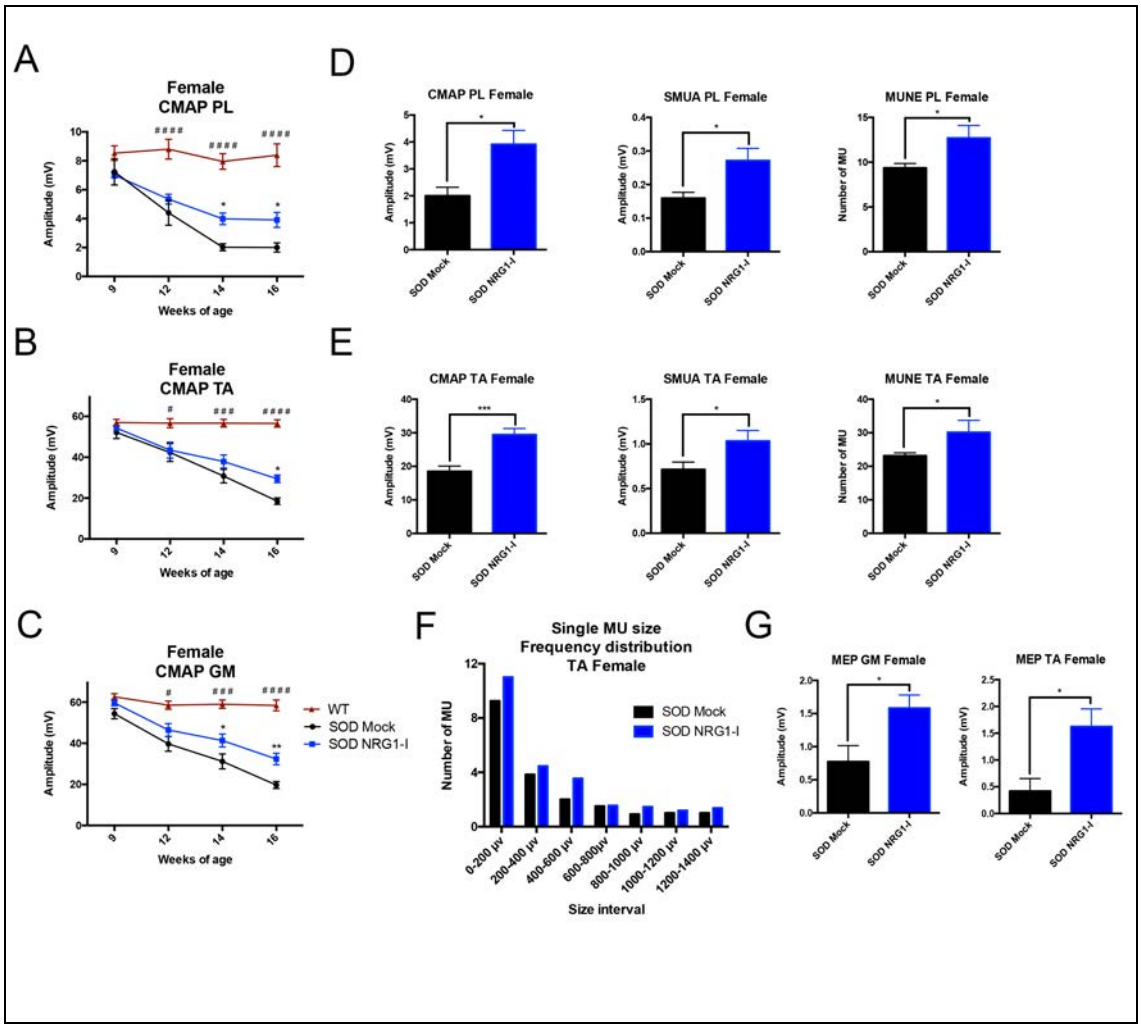

Figure 2. *NRG1-I overexpression promotes motor functional improvement of the SOD1^{G93A} mice.* A) Electrophysiological tests show that AAV-NRG1-I injection produced a significant preservation of the CMAP amplitude of plantar (A), tibialis anterior (B), and gastrocnemius (C) muscles in the SOD1^{G93A} mice along time (n=6 WT, 6-13 SOD Mock, 10-11 SOD NRG1-I mice per group, two-way ANOVA, *p<0.05 vs SOD Mock mice; #p<0.05 vs WT mice). Electrophysiological estimation of motor unit number (MUNE) and mean amplitude of single motor unit potential (SMUA) of the plantar (D) and tibialis anterior (E) muscles show that the increased CMAP amplitude was the result of preservation of more motor units and of larger size, confirmed by the frequency distribution (F). G) NRG1-I treatment increased the amplitude of the motor-evoked potentials (n=6-14 SOD Mock, 10-12 SOD NRG1-I mice per group, T-student, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

NRG1-I overexpression preserves spinal MNs and reduces glial reactivity

We then performed the histological analysis of the SOD1^{G93A} mice at 16 weeks. The results showed that the NRG1-I overexpression preserved the number of MNs (12.08±0.49 mean number of MNs per ventral horn ± SEM) compared to the SOD1^{G93A} mock mice (6.00±0.49) (Fig. 3A,B) in line with our electrophysiological findings. The increased CMAP and motor unit number in the GM muscle at 16 weeks was further confirmed by the NMJ analysis, since the gene therapy treatment significantly preserved the number of occupied motor endplates (68.78±5.06; percentage of occupied endplates ± SEM) compared to the mock SOD1^{G93A} group (35.87±7.17) (Fig. 3A,B). We also assessed the glial immunoreactivity in the spinal cord and we found a significant decrease of astrocyte and microglial activation ($3.89x10^7$±$6.60x10^6$ and $5.52x10^7$±$1.08x10^7$ respectively; integrated density ± SEM) compared to the SOD1^{G93A} mock mice ($1.825x10^8$±$2.67x10^7$ and $2.25x10^8$±$9.07x10^7$ respectively) (Fig. 3C,D).

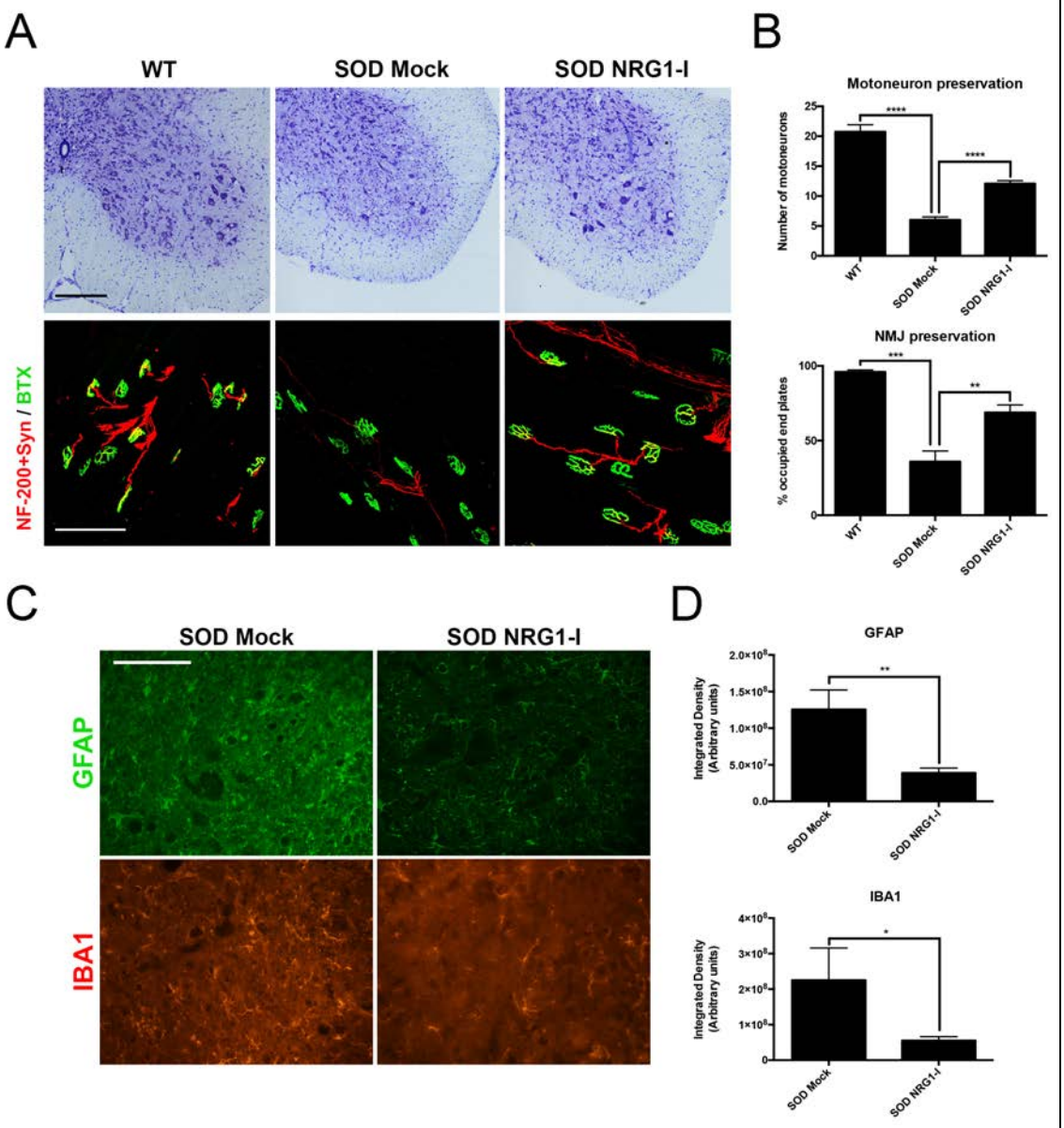

Figure 3. *Viral-delivery of NRG1-I preserves spinal motoneuron number and decreases glial reactivity.* A) Representative images of L4 spinal cord and GM neuromuscular junctions of WT and SOD1^{G93A} mice, treated with NRG1-I or with mock vector (scale bar = 100 μm). B) Histological analysis showed a higher number of spinal MNs number, as well as an increased proportion of innervated NMJs in the gastrocnemius muscle in the treated SOD1^{G93A} mice compared to the mock mice (n=3 WT, 3-4 SOD Mock, 7 SOD NRG1-I mice per group, one-way ANOVA, **p<0.01 *p<0.05 vs SOD Mock mice). C) Representative confocal images of astrocytes labeled against GFAP, and microglia labeled against Iba-1, in the spinal cord ventral horn of SOD1^{G93A} mice (scale bar = 100

μm). D) The viral-mediated delivery of NRG1-I reduced the astrocyte and microglia reactivity in the treated SOD1^{G93A} mice (n=3-4 SOD Mock, 5-6 SOD NRG1-I mice per group, T-student, **p<0.01, *p<0.05). Data are shown as mean ± SEM.

NRG1-I signaling through ErbB2/3 receptors produces motor functional improvement in the treated SOD1^{G93A} mice

We then evaluated the expression of the ErbB 2, 3 and 4 receptors in GM muscle samples of SOD1^{G93A} mice. Upon NRG1-I overexpression, the levels of ErbB2 (1.7±0.42) (Fig. 4A) and ErbB3 (3.36±0.26) (Fig. 4B) were upregulated compared to the SOD1^{G93A} mock mice (0.59±0.12 and 1.57±0.43 respectively) and the WT mice. However, we did not find significant differences for the ErbB4 receptor levels in NRG1-I SOD1^{G93A} treated mice (1.48±0.66) compared to the SOD1^{G93A} mock group (0.79±0.13) and WT mice (Fig. 4C).

This NRG1-I/ErbB signaling in the skeletal muscles was able to produce functional improvement in the rotarod performance in the treated SOD^{G93A} mice (Fig. 4D) and a significant delay of the clinical disease onset (Fig. 4E) compared to the control SOD1^{G93A} mice.

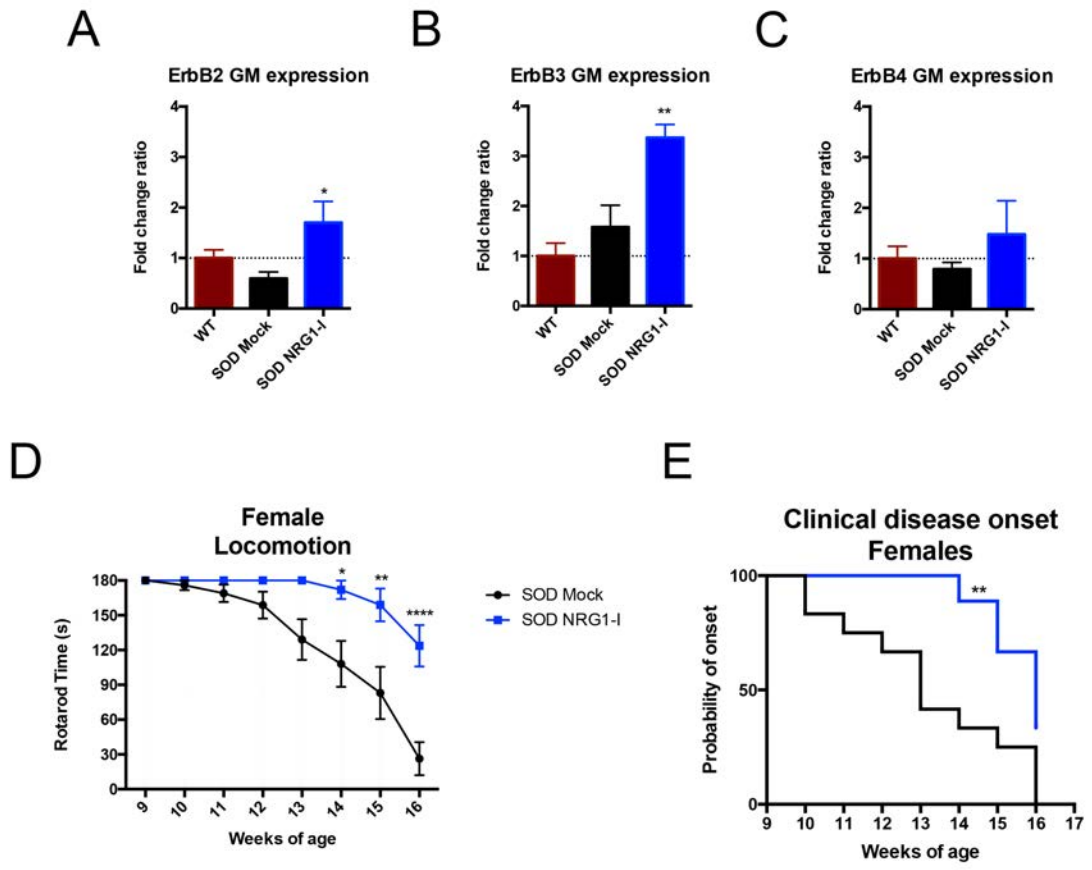

Figure 4. *NRG1-I/ErbB pathway delays the disease onset and produces global functional improvement in the treated mice.* A) mRNA expression analysis of ErbB receptors shows a significant upregulation of ErbB2 (A) and ErbB3 (B) upon NRG1-I overexpression in the treated SOD1^{G93A} mice compared to the mock mice, whereas the levels of ErbB4 receptor showed a non-significant tendency to increase (C) (n=3 WT, 3-4 SOD Mock, 4 SOD NRG1-I mice per group, two-way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). D) NRG1-I overexpression produced an improvement in the rotarod performance of treated SOD1^{G93A} mice from 14 to 16 weeks (n=12 SOD Mock, 7 SOD NRG1-I mice per group, two-way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). E) Clinical onset of disease was significantly delayed (n=12 SOD Mock, 9 SOD NRG1-I mice per group, Mantel-Cox test, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

NRG1-I and NRG1-III overexpression are detected in both skeletal muscles and spinal cord

Taking into account the positive results obtained from the NRG1-I gene therapy and our previous data from the NRG1-III overexpression (Mòdol-Caballero et al., 2019), we then aimed to test whether a combined therapy overexpressing both isoforms at different sites of the motor unit could produce a synergic effect on the SOD1^{G93A} mice. Vector injections were performed at the same time, previous to disease onset (at 7 weeks). mRNA expression at the end-stage of the disease showed that NRG1-I was increased in the GM muscle (38.77±11.18) in comparison with the control SOD1^{G93A} mice (0.76±0.41) (Fig. 5A), and NRG1-III was increased in the spinal cord (1.12±0.04) compared to the respective of SOD1^{G93A} mice control (0.68±0.05) (Fig. 5B).

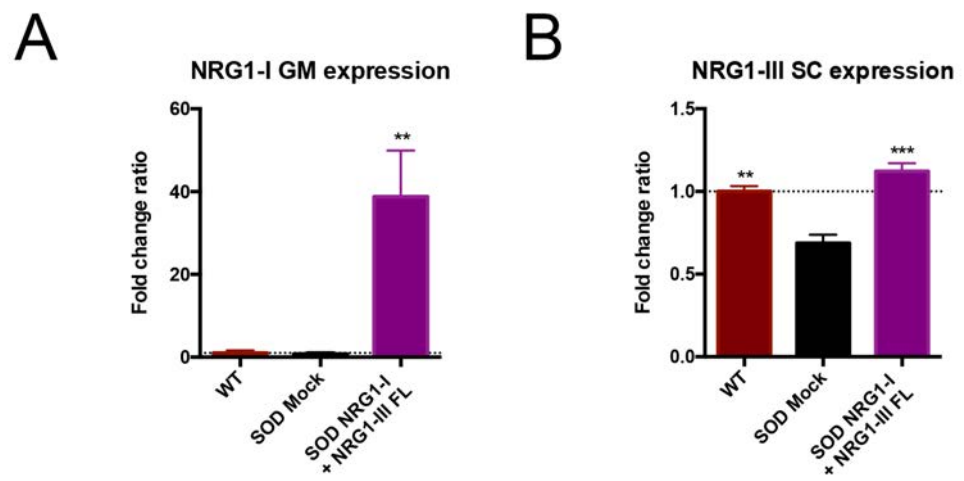

Figure 5. *Combined-gene therapy upregulates NRG1-I in the GM muscle and NRG1-III in the spinal cord.* A) mRNA expression analysis shows that NRG1-I is overexpressed in the GM muscle upon treatment in the SOD1^{G93A} mice. B) In the lumbar spinal cord, NRG1-III FL overexpression rescues NRG1-III mRNA levels similar to WT group, whereas it is downregulated in the SOD1^{G93A} mock group (n=3 WT, 3-4 SOD Mock, 4 SOD NRG1-I + NRG1-III FL mice per group, one-way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

Combined gene therapy does not produce a synergic effect on neuromuscular function in SOD1^{G93A} mice

The electrophysiological results showed that there was a significant preservation of the PL, GM and TA CMAP amplitude (Fig. 6A,B,C). MUNE also showed a higher number and mean amplitude of the motor units for the PL and TA muscles (Fig. 6D,E,F). The amplitude of the GM and TA MEPs also indicated preserved connection with the upper MNs (Fig. 6G). The histological analysis further confirmed the improvement in neuromuscular function. There was a higher MN number (11.59±0.40) compared to SOD1^{G93A} mock mice (6.87±0.49) (Fig. 7A,B) and NMJ counting showed a higher number of occupied MN-end plates (74.66±3.73) compared to SOD1^{G93A} mock mice (42.97±4.67) (Fig. 7A,B). The combined gene therapy also produced a decrease of astrocyte and microglial activation ($6.38x10^7$±$7.18x10^6$ and $3.54x10^7$±$9.42x10^6$ respectively) whereas the untreated mice showed marked neuroinflammation in the ventral horn ($5.05x10^8$±$1.99x10^8$ and $2.27x10^8$±$8.86x10^7$ respectively) (Fig. 7C,D).

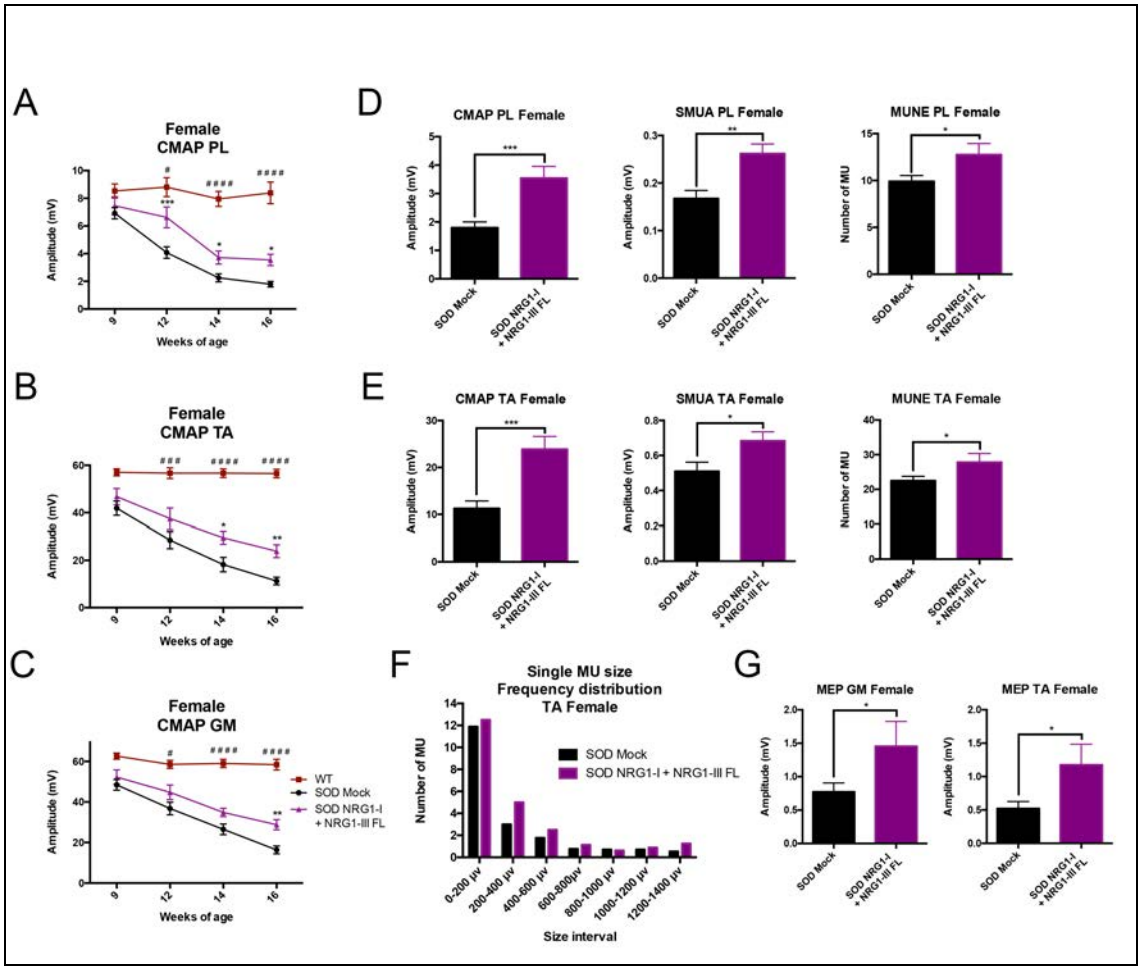

Figure 6. *Combined-gene therapy promotes motor functional improvement of the SOD1^{G93A} mice.* Electrophysiological tests show that AAV-NRG1-I and AAV-NRG1-III FL injections produced a significant preservation of the CMAP amplitude of plantar (A), tibialis anterior (B), and gastrocnemius (C) muscles in the SOD1^{G93A} mice (n=6 WT, 16-18 SOD Mock, 7-9 SOD NRG1-I + NRG1-III FL mice per group, two-way ANOVA, *p<0.05 vs SOD Mock mice; #p<0.05 vs WT mice). Electrophysiological estimation of motor unit number (MUNE) and mean amplitude of single motor unit potential (SMUA) of plantar (D) and tibialis anterior (E) muscles show a general preservation of motor units induced by the combined therapy, confirmed by the frequency distribution (F). G) The combined therapy increased the amplitude of the motor-evoked potentials, indicating enhanced connection between upper and lower MNs. (n=14-21 SOD Mock, 5-9 SOD NRG1-I + NRG1-III FL mice per group , T-student, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

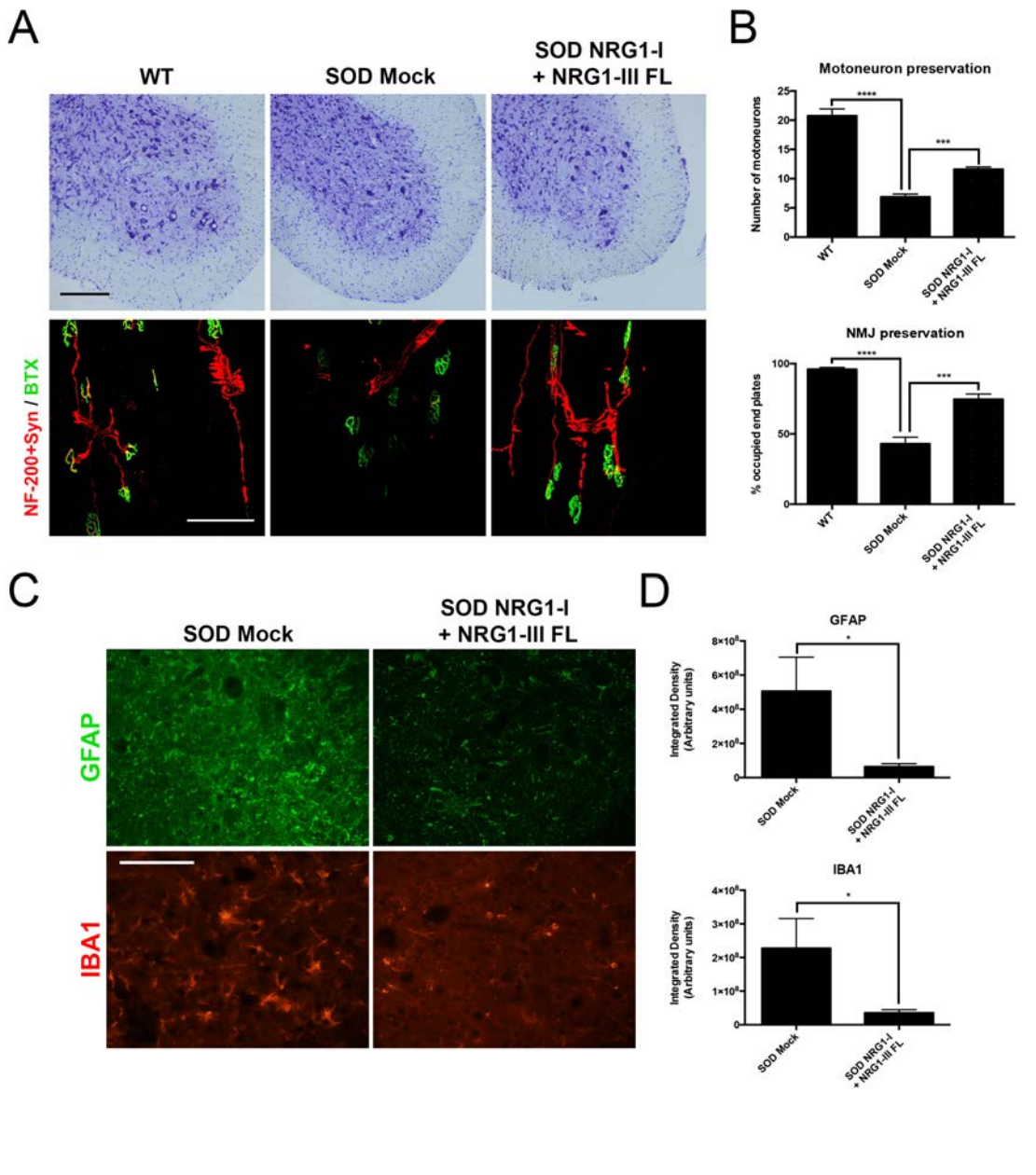

Figure 7. *Overexpression of NRG1-I and NRG1-III preserves spinal motoneurons and reduces glial reactivity.* A) Representative images of L4 spinal cord and GM neuromuscular junctions of wild type and SOD1^{G93A} mice, treated with NRG1-I and NRG1-III FL or with mock vector (scale bar = 100 µm). B) Histological analysis showed a higher preservation of the number of spinal MNs, as well as an increased proportion of innervated NMJs in the gastrocnemius muscle of the treated SOD1^{G93A} mice (n=3 WT, 5 SOD Mock, 5-6 SOD NRG1-I + NRG1-III FL mice per group, one-way ANOVA, **p<0.01 *p<0.05 vs SOD Mock mice). C) Representative confocal images of astrocytes labeled against GFAP, and microglia labeled against Iba-1, in the spinal cord ventral horn of

SOD1^{G93A} mice (scale bar = 100 μm). D) The viral-mediated delivery of both NRG1-I and NRG1-III FL decreased astrocyte and microglia reactivity in the spinal cord of the SOD1^{G93A} mice (n=3-4 SOD Mock, 5-6 SOD NRG1-I + NRG1-III FL mice per group, T-student, **p<0.01, *p<0.05). Data are shown as mean ± SEM

Finally, NRG1-I and NRG1-III overexpression produced a global functional improvement in the rotarod test and delayed the clinical disease onset (Fig. 8A,B). However, when we compared the functional and histological results obtained by the combined gene therapy with the two gene therapies alone, the results showed that it was not able of producing a synergic effect, better than single gene therapies.

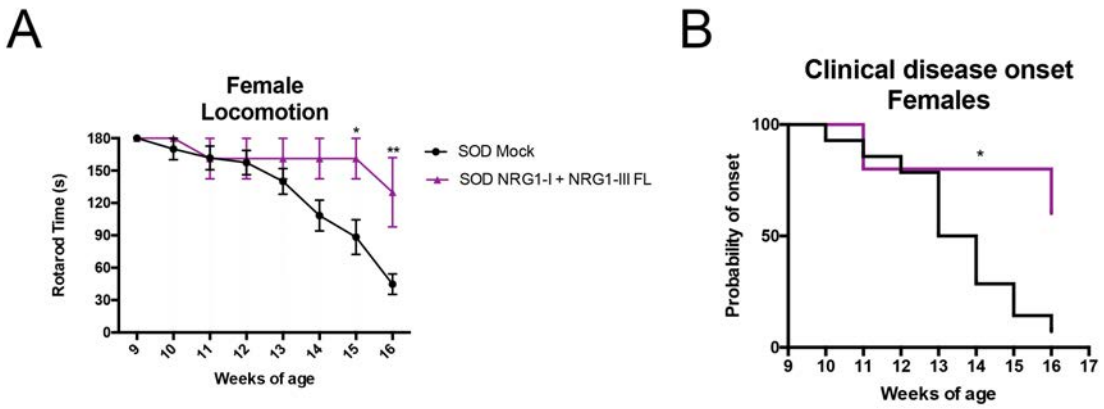

Figure 8. *NRG1 combined expression delays the disease onset and produces global functional improvement in the treated mice.* A) NRG1-I overexpression produced an improvement in the Rotarod performance of the treated SOD1^{G93A} mice from 14 to 16 weeks. (n=14 SOD Mock, 5 SOD NRG1-I + NRG1-III FL mice per group, two-way ANOVA, **p<0.01, *p<0.05 vs SOD Mock mice). B) The clinical onset of disease was significantly delayed by the combined therapy (n=14 SOD Mock, 5 SOD NRG1-I + NRG1-III FL mice per group, Mantel-Cox test, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

Discussion

The results of this study indicate that generalized overexpression of NRG1-I in body muscles was possible with the AAV vector produced. NRG1-I acts through the ErbB2 and 3 receptors in the periphery and promotes neuromuscular functional improvement and increases MN survival while reducing glial cell reactivity around spinal MNs. However, the combined therapy with NRG1-III administered intrathecally despite producing also a significant functional improvement, did not produce a synergic effect in the SOD1^{G93A} mice.

NRG1 isoforms are important trophic factors due to their multiple functions in the nervous system (Esper et al., 2006). NRG1/ErbB system alterations have been related to ALS human patients (Takahashi et al., 2013; Lopez-Font et al., 2019). The NRG1-III expression was found reduced in the spinal cord of both ALS patients and SOD1^{G93A} mice in parallel with MN loss (Song et al., 2012; Lasiene et al., 2016; Mòdol-Caballero et al., 2019). Indeed, viral-mediated delivery of NRG1-III in the CNS restored the number of C-boutons and produced a slight increase on the survival of the SOD1^{G93A} mice (Lasiene et al., 2016), and we have demonstrated that addition of NRG1 exerts neuroprotective effects on MNs under chronic excitotoxicity in an in vitro model (Mòdol-Caballero et al., 2018), and overexpression of NRG1-III in the SOD1^{G93A} mice improves motor function and maintains the number of surviving MNs (Mòdol-Caballero 2019).

Effects of gene therapy for overexpressing NRG1-I

However, the role of NRG1-I isoform has remained unclear. NRG1 is critical for the to axoglial development and intercommunication (Loeb et al., 1999; Esper & Loeb., 2004), the formation of muscle spindles (Andrecheck et al., 2002; Hippenmeyer et al., 2002) and the maintenance of the mature NMJ (Sandrock et al., 1997; Loeb et al., 2002; Loeb., 2003). Indeed, NRG1/ErbB signaling is important for the stability of AChRs at the NMJ and the structural integrity of the postsynaptic site (Schmidt et al., 2011). While Song and collaborators (2012) found that NRG1-I isoform was increased and associated with glial activation in the spinal cord of SOD1^{G93A} mice, Lasiene and collaborators (2016) showed decreased mRNA levels. However, when they applied a virus-mediated delivery to overexpress this isoform

in the lumbar spinal cord, their results showed that it did not extend the survival of the treated mice (Lasiene et al., 2016). Strikingly, our previous data showed that NRG1-I overexpression localized in the GM muscle produced functional compensation by enhancing collateral sprouting in the SOD1^{G93A} mouse and in WT after partial muscle denervation (Mancuso et al., 2016). However, an increased expression of NRG1-I in a single muscle was not able to globally affect ALS disease progression. Therefore, we attempted to promote a general overexpression of NRG1-I in most skeletal muscles to produce a global functional improvement. Our gene therapy delayed the disease onset and produced a significant functional preservation of the hindlimb muscles in both female and male SOD1^{G93A} mice. These results were corroborated by the upregulation of NRG1-I in the muscle and by the preservation of the NMJ innervation.

NRG1-I overexpression produced an upregulation of the ErbB receptors, which in turn, are expressed by the terminal Schwann cells in the NMJs (Syroid et al., 1996). Indeed, a bidirectional communication between Schwann cells and MNs supports their mutual survival and differentiation during development and after nerve injury (Esper & Loeb., 2004) when NRG1-I overexpression promotes axonal regeneration and remyelination in a transient autocrine/paracrine signaling loop with Schwann cells expressing ErbB2 and 3 receptors (Stassart et al., 2012). Moreover, constitutively active ErbB2 in Schwann cells resulted in proliferation and process extension from these cells (Hayworth et al., 2006) and loss of NRG/ErbB signaling destabilizes the anchoring of acetylcholine receptors in the postsynaptic muscle membrane (Schmidt et al., 2011). Therefore, NRG1-I expressed at the NMJ may act on terminal Schwann cells, which play a critical role for maintaining muscle innervation and motor axon survival.

Since ALS pathogenesis is considered to develop in a dying-back process, in which the nerve terminals and NMJ are partially degraded when cell bodies in the spinal cord are still intact, viral-mediated NRG1-I therapy may be a suitable approach to for MN degeneration diseases. However, further experiments should be performed to elucidate the pathway that causes these changes upon NRG1-I overexpression. In this study, we introduced the use of a combined therapeutic strategy to deliver NRG1-I in most of the skeletal muscles of the SOD1^{G93A} mice with

a single systemic injection. Therefore, this viral-mediated therapy can be considered for the translation to treat ALS patients since it is minimally invasive, only requires a single administration, permits the overexpression of the gene of interest in most of the skeletal muscles affected in ALS but not in other cell types, and the effect of the therapy is stable and allows a long-term expression even at the end-stage of the disease.

Combined gene therapy for overexpressing NRG1-I in muscle and NRG1-III in spinal cord

In light of these and our previous results (Mòdol-Caballero 2019), we were encouraged to combine two gene therapies for overexpressing NRG-I and NRG1-III to produce a synergic effect on the SOD1^{G93A} mice. Although data published shows that dual AAV therapies injected in murine models can be successful (Yang et al., 2016; Bengtsson et al., 2017), this issue still presents some concerns. In order to produce the minimal activation of the immune system in mice, we performed both vector injections the same day, avoiding the possibility to activate the immune system with two separate injections. Indeed, the injection of the viral vectors produced an increased mRNA expression of NRG1-III in the spinal cord, and a high expression of NRG1-I in the muscle. Furthermore, the results showed that the combined-gene therapy promoted functional improvement of the SOD1^{G93A} mice, although it did not produce the expected synergic effect. One of the possible explanations could be regarding the limitations of the used model. As Mancuso and collaborators reported, very few studies report synergistic effect after combinatory therapies (Mancuso et al., 2014). This might reflect that there is an endogenous limitation for the beneficial effects that can be achieved using the SOD1^{G93A} mouse model.

It is worth to comment, the significant effect of the NRG1 isoforms on spinal cord neuroinflammatory response. NRG1-ErbB signaling was associated with microglial chemotaxis in vitro and in spinal cord dorsal horn after peripheral nerve injury (Calvo et al., 2010; 2011). Indeed, it was proved that treatment with an NRG1 antagonist reduced the microglial reactivity in the SOD1^{G93A} mice through the reduction of ErbB2 phosphorylation (Liu et al., 2018). Furthermore, C-boutons

degenerate along the course of ALS and in this process the NRG1/ErbB signal-transduction pathway can act as a chemotactic factor for microglia (Casanovas et al., 2017). On the other hand, treatment with NRG1 attenuated astrogliosis in spinal cord injury (Alizadeh et al., 2017; 2018). Interestingly, we found that in both therapies, overexpression of NRG1-I alone and in the combined-NRG1 therapy, there is a notable reduction of astrocyte and microglial reactivity. Therefore, while NRG1-I might have a deleterious role upon upregulation in the spinal cord of $SOD1^{G93A}$ mice (Song et al., 2012), its overexpression in the muscle preserves the NMJs and the spinal MNs, and thus it can reduce the central neuroinflammatory response.

Regarding sex differences, we show here that the beneficial effects of NRG1-I overexpression are similar in female and in male $SOD1^{G93A}$ mice. However, in our previous paper we found that gene therapy targeting NRG1-III in the spinal cord produced positive effects only in female but not in male $SOD1^{G93A}$ mice. We hypothesized that progesterone may participate in the modulation of the NRG1-III expression in the spinal cord of the mice and have a role on the neuroprotective effects observed in females (Gonzalez-Deniselle et al., 2002; LaCroix-Fralish et al., 2008). Moreover, considering that here we overexpressed NRG1-I in the skeletal muscles, it seems that progesterone does not influence NRG1 signaling at the peripheral level.

Finally, in view of the past and recent results, NRG1-I and NRG1-III may differ in their functions in the nervous system but share positive effects in terms of MN survival and inflammation when applied in the peripheral and central nervous system, respectively. Thus, the beneficial effects of these isoforms might overlap somehow in the pathways activated in the MNs, since the combined gene therapy did not show any further positive result compared with both gene therapies alone. Certainly, NRG1-I activates the PI3K/AKT and ERK pathways when overexpressed in the GM muscle of the $SOD1^{G93A}$ mice (Mancuso et al., 2016), whereas NRG1 treatment also activates the same pathways in the CNS to promote MN survival (Mòdol-Caballero et al., 2018; Chen et al., 2019). Consequently, the maximum effect that can be reached by the overexpression of NRG1 isoforms may be limited.

Conclusions

In summary, the NRG1-I overexpression significantly improved the MN functional preservation and promoted neuroprotection, accompanied by a global functional improvement and a delay in the disease onset. However, when we applied the combined gene therapy with the viral-mediated injection of NRG-III we did not see an improved effect compared to the NRG1-I overexpression alone. Together, our study shows that the gene therapies overexpressing NRG1-I, and the combination of this isoform with NRG1-III represent a novel therapeutic approach to treat ALS, despite not producing a synergic effect in the $SOD1^{G93A}$ mice.

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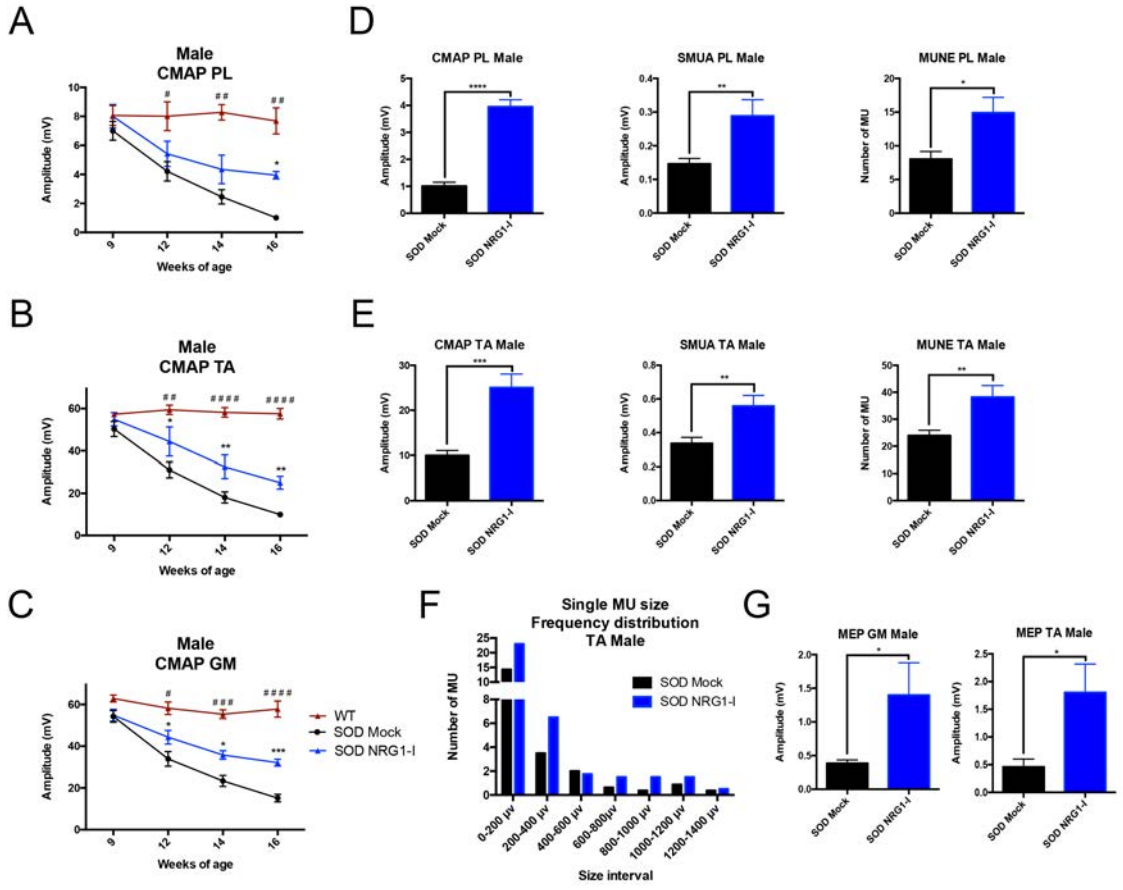
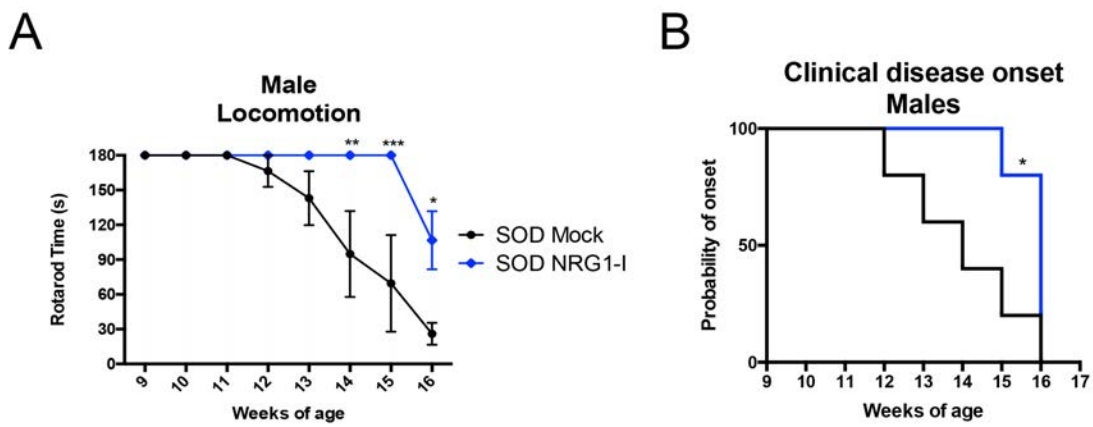
Supplementary Data



Supplementary Figure 1. *NRG1-I overexpression also promotes functional improvement in the male SOD1^{G93A} mice.* Electrophysiological tests reveal that AAV-NRG1-I injection produced a significant preservation of the CMAP amplitude of plantar (A), tibialis anterior (B), and gastrocnemius (C) hindlimb muscles in the male SOD1^{G93A} mice (n=5 WT, 5-12 SOD Mock, 4-5 SOD NRG1-I mice per group, two-way ANOVA, *p<0.05 vs SOD Mock mice; #p<0.05 vs WT mice). Electrophysiological estimation of motor unit number (MUNE) and mean amplitude of single motor unit potential (SMUA) of plantar (D) and tibialis anterior (E) muscles show that the increased CMAP amplitude was the result of preservation of motor units, confirmed by the frequency distribution (F). G) Furthermore, the treatment increased the amplitude of the motor-evoked potentials. (n=4-9 SOD Mock, 4-5 SOD NRG1-I mice per group, T-student, **p<0.01, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.



Supplementary Figure 2. *Viral-delivery of NRG1-I delays the disease onset and produces a global functional improvement in the treated male mice.* A) Effect of NRG1-I overexpression produced an improvement of the Rotarod performance in the treated SOD1^{G93A} mice from 14 to 16 weeks (n=5 SOD Mock, 5 SOD NRG1-I mice per group, two-way ANOVA, *p<0.05 vs SOD Mock mice). B) The clinical onset of disease was significantly delayed compared to the mock mice (n=5 SOD Mock, 5 SOD NRG1-I mice per group, Mantel-Cox test, *p<0.05 vs SOD Mock mice). Data are shown as mean ± SEM.

GENERAL DISCUSSION

Discussion

Overall, the results presented in this thesis provide new insights about the essential role of NRG1 on the MN survival in ALS. We found that the upregulation of NRG1-I and NRG1-III improved motor function, maintained the number of surviving MNs and reduced astrocyte and microglial reactivity in the SOD1^{G93A} mice at the end-stage of the disease. Our studies suggest that gene therapy overexpressing NRG1 isoforms may be a feasible therapeutic approach for ALS.

Neuregulin and motoneurons

NRG1 and its isoforms have emerged as important trophic factors due to its multiple functions in the nervous system (Esper et al., 2006). NRG1/ErbB signaling has been shown to be essential for the excitability and the survival of the MNs (Riethmacher et al., 1997; Wolpowitz et al., 2000; Agarwal et al., 2014). In the CNS, NRG1-III expression was found reduced in the spinal cord of both ALS patients and SOD1^{G93A} mice in parallel with MN loss, but NRG1-I was increased and associated with glial activation (Song et al., 2012). Accordingly, we corroborated that NRG1-I was increased in the spinal cord of ALS patients and of the SOD1^{G93A} model mice, due to increased expression in glial cells, whereas NRG1-III appeared to decrease in the spinal MNs. In the CNS, the increase of NRG1-I in ALS samples patients and SOD1^{G93A} mice suggests that this isoform may exert a detrimental effect, promoting microglial reactivity upon the activation of ErbB receptors (Song et al., 2012). In contrast, NRG1-III might play a beneficial role on the preservation of MN activity. Salvany and collaborators (2019) also suggested that the two isoforms have different role on MN synaptic connections; NRG1-III may serve as a postsynaptic SSC organizer but in contrast, NRG1-I stimulates promotes a substantial increase in the number and size of cholinergic inputs.

The addition of exogenous NRG1 in the spinal cord organotypic cultures (SCOC) exposed to chronic excitotoxicity significantly maintained the number of surviving MNs in the ventral horn, thus providing a solid proof-of-concept of the MN protective effects of NRG1. The rhNRG1 corresponds to the EGF domain of NRG1, which can bind to ErbB3 and ErbB4 by inducing their homodimerization or the heterodimerization with ErbB2. As a consequence of the dimerization ErbB

receptors phosphorylate. We demonstrated that the neuroprotective effects of rhNRG1 are mediated by the activation of ErbB receptors because the addition of lapatinib, an inhibitor of ErbB receptors, blocked the neuroprotective action of rhNRG1. Moreover, ErbB4 is the main receptor for NRG1 signaling, since ErbB2 contains the functional kinase domain but is unable to bind NRG1 (Klapper et al., 1999), and ErbB3 binds to NRG1 but is unable to propagate the signal to the cells because of lack of kinase activity (Guy et al., 1994), suggesting that ErbB4 is the main responsible of the beneficial effects of NRG1 in the CNS. Interestingly we found that in the SCOC, as well as in samples of $SOD1^{G93A}$ mice and ALS patients the ErbB4 receptor translocated into the nucleus of the MNs, whereas control samples did only show labeling in the cytoplasm, suggesting a detrimental effect of the ICD domain. Indeed, for NRG1/ErbB signaling, both NRG1-III and ErbB4 have intracellular domains that can be internalized by the neurons and translocate to the nucleus (Lee et al., 2002; Bao et al., 2003). Presinilin-dependent cleavage of ErbB4 generate the soluble B4-ICD that functions in the nucleus presumably at transcriptional level (Ni et al., 2001; Lee et al., 2002) or to the mitochondria where it promotes apoptosis of breast cancer cells (Naresh et al., 2006). For the nucleus accumulation, ErbB4 ICD domain requires sumoylation by the sentrin-specific proteases (SENPs) (Knittle et al., 2017). Therefore, while NRG1-III cleavage produces neuroprotection, the ErbB4 ICD signaling may play a role on the neurodegeneration process. At the same time the NRG1-III/ErbB4 interactions might modulate MN survival against degenerative insults. Consequently, the modulation of NRG1/ErbB axis may influence the progression of ALS.

Based on the results demonstrating the dysregulation of NRG1/ErbB axis, we studied the signaling pathways underlying the neuroprotection effect. In the SCOC, upon phosphorylation of ErbB receptors, the PI3K-AKT pro-survival pathway was activated as demonstrated by immunoblot. Indeed, in cultures of embryonic rat MNs, NRG1 was shown to inhibit apoptosis during the period of embryonic programmed cell death by a PI3K-dependent pathway, although in this case it did not increase the relative level of p-AKT (Ricart et al., 2006). Furthermore, the ERK pathway is another cascade induced upon ErbB receptor activation, but we did not detect differences of ERK1/2 after rhNRG1 treatment. It has been also reported that autophagy might influence inflammation and activation of microglia, as well as

inflammation might promote or inhibit the process of autophagy (Su et al., 2016). Strikingly, IGF-I has been described to protect hippocampal neurons against early excitotoxicity via the NR2B/PI3K-AKT-mTOR pathway, suppressing the excess of autophagy (Wang et al., 2014). Autophagy is a pathogenic mechanism in ALS that has already been described in the SCOC under chronic excitotoxicity (Matyja et al., 2005; Herrando-Grabulosa et al., 2013). However, the role of autophagy in promoting survival of neurons or apoptosis is not fully clarified. After THA treatment in the SCOC we found an accumulation of autophagosomes that correspond to an early stage of autophagy cell death induction. In contrast, addition of rhNRG1 to the culture decreased the levels of the phagopore formation marker Beclin 1 and the autophagosome marker LC3-II. Therefore, rhNRG1 might act restoring the autophagic flux by reducing the number of autophagosomes formed and starting p62 degradation in the lysosome after the fusion, avoiding accumulation in the cytosol. Considering the links between the different pathogenic mechanisms we hypothesize that rhNRG1 reduces the autophagy in the SCOC caused by excitotoxicity activating the ErbB receptors and the PI3K/AKT pathway in both MNs and microglial cells. This activation promotes a dual effect; the survival of the MNs and the reduction of the neuroinflammatory response mediated by the microglia.

In the SCOC model used MNs are preferentially affected by the prolonged blockade of glutamate uptake produced by THA addition, causing excitotoxicity. This mechanism is mediated by non-NMDA receptors, such as AMPA receptor (Rothstein et al., 1993). While these channels allow rapid calcium entry, MNs buffer the consequent cytosolic calcium load poorly (Lips et al., 1998), with the consequence that much of the calcium is taken up into mitochondria, resulting in strong reactive oxidative species (ROS) generation (Carriedo et al., 2000; Rao et al., 2003). Furthermore, ROS may be able to exit the MN, disrupting glutamate transport in surrounding astrocytes, resulting in increased extracellular glutamate accumulation, and further propagation of the injury cascade (Rao et al., 2003; Yin & Weiss., 2012). Recently it has been shown that NRG-1β plays an important role in modulating calcium homeostasis and preventing apoptosis through activating PI3K/AKT pathway in DRG sensory neurons subjected to excitotoxicity induced by glutamate (Liu et al., 2011). These results suggest that in the SCOC, rhNRG1

treatment may modulate calcium homeostasis and thus reduce the excitotoxicity through this mechanism.

Gene therapy targeting NRG1-III in the spinal cord

In the following studies in vivo, using the SOD1^{G93A} mouse model of ALS, we assayed the effects of NRG1 overexpression at the spinal cord by mean of gene therapy with AAV vectors directly administered by intrathecal route. While the secreted-isoform NRG1-I overexpression in the CNS had no effect on the disease onset and survival of SOD1^{G93A} mice in other studies (Lasiene et al., 2016; Liu et al., 2018), we found that NRG1-III overexpression by gene therapy in the CNS conferred MN protection and preservation of motor function until the end-stage of the disease of the female SOD1^{G93A} mice. Indeed, the spinal MNs expressed an increased amount of NRG1-III upon gene therapy at the late stage; however, in contrast to previous results by Lasiene and collaborators (2016), this viral-mediated therapy was able to delay the disease onset but did not increase the survival of the mice. This suggests that the treatment ameliorates the disease progression in the early stages but is not able to induce a long-term positive effect.

We hypothesize that NRG1-III acts through the ErbB4 receptor by autocrine signaling in the MNs (Lasiene et al., 2016), regulating its excitability, and also modulating microglial reactivity to reduce the inflammatory reaction and ameliorate the damaging MNs environment in the spinal cord. One of the controversial issues about the NRG1-ErbB pathway is whether it participates in the regulation of the neuroinflammatory response mediated by microglial cells, a common feature that occurs in ALS and other neurodegenerative diseases. One the one hand, intrathecal treatment with the EGF domain of NRG1 promoted microglial proliferation and chemotaxis contributing to microgliosis and pain following peripheral nerve injury (Calvo et al., 2010; 2011). Furthermore, activated ErbB2 receptors predominantly present on microglia and, to a lesser extent, on astrocytes were found overexpressed as a function of disease progression in the SOD1^{G93A} transgenic mouse, correlating with the pattern of microglial activation (Song et al., 2012). Indeed, treatment with a NRG1 antagonist reduced microglial reactivity in the spinal cord of the SOD1^{G93A} mice, through the reduction of ErbB2

phosphorylation (Liu et al., 2018). In agreement with those findings, we observed a marked reduction of the neuroinflammatory response in SCOC under chronic excitotoxicity and in SOD1^{G93A} mice upon treatment with NRG1. Furthermore, we observed that microglial cells showed thinner and more ramified processes under NRG1 treatment compared to untreated samples in our studies in vitro and in vivo.

When AAV coding for NRG1-III was applied to the SOD1^{G93A} mice, there was a decrease in microglial as well as in astrocyte reactivity. In SOD1^{G93A} mice, we observed that the gene therapy did not only increase the expression of NRG1-III in MNs but also in other cells, by the shape probably astrocytes. It was previously described that NRG1 is expressed and secreted by astrocytes (Pinkas-Kramarski., 1994). On the other hand, it has been reported that treatment with NRG1 attenuated astrogliosis after spinal cord injury and reduced their production of chondroitin sulfate proteoglycans (CSPGs) which play an inhibitory role in neural regeneration (Bradbury et al., 2002; Alizadeh et al., 2017; 2018). Therefore, a MN-astrocyte signaling mechanism might be involved, in which astrocytes may be acting via neuronal ErbB receptor signaling to potentiate synaptic transmission and induce synaptic plasticity (LaCroix-Fralish et al., 2008).

Gene therapy targeting NRG1-I in the skeletal muscle

In the PNS, NRG1-I, the soluble heparin-binding form of NRG1, is highly expressed early in motor and sensory axons and it is essential for the axoglial development and intercommunication (Loeb et al., 1999; Esper & Loeb, 2004), the formation of muscle spindles (Andrecheck et al., 2002; Hippenmeyer et al., 2002), and the maintenance of the NMJ (Sandrock et al., 1997; Loeb et al., 2002; Loeb., 2003). Indeed, NRG1/ErbB signaling in NMJ helps to maintain the efficacy of synaptic transmission by stabilizing the anchoring of acetylcholine receptors (AChRs) at the postsynaptic site (Schmidt et al., 2011).

As previously reported, the NRG1-I/ErbB system is altered in the SOD1^{G93A} mice and in ALS patients (Song et al., 2012; Lasiene et al., 2016; Mancuso et al., 2016). Interestingly, previous data from our laboratory showed that NRG1-I overexpression localized in one skeletal muscle promoted functional reinnervation by enhancing collateral sprouting of motor axons within the muscle in the SOD1^{G93A}

mouse and also in the wild type mouse after partial muscle denervation, an action that was presumably mediated through NRG1-I acting on ErbB receptors in the terminal Schwann cells (Mancuso et al., 2016). However, sole NRG1-I increased expression in only one muscle was not able to globally affect ALS disease progression. Therefore, we promoted a general overexpression of NRG1-I in the skeletal muscles by a new AAV vector with the desmin promoter in order to transfect a large number of muscles and thus to produce general functional improvement. Indeed, such a gene therapy strategy was able to delay the disease onset and improve the hindlimb muscles function in both female and male SOD1^{G93A} mice along the course of their disease. Furthermore, NRG1-I viral-delivery produced a significant increase on the NMJ innervation of the GM muscle. mRNA analysis of this same muscle showed that our treatment upregulated the ErbB 2 and 3 receptors, and less markedly ErbB4, which in turn are expressed by the terminal Schwann cells in the NMJs (Syroid et al., 1996). Indeed, a bidirectional communication between Schwann cells and MNs supports their mutual survival and differentiation during development and after nerve injury (Esper & Loeb, 2004), when NRG1-I promotes axonal regeneration acting on ErbB2 and 3 receptors of denervated Schwann cells (Stassart et al., 2013). Therefore, NRG1-I expressed at the NMJ may act on terminal Schwann cells, which play a key role in maintaining muscle innervation and motor axon survival.

Interestingly, NRG1-I upregulation in the skeletal muscles was able to decrease the glial reactivity in the lumbar spinal cord of the SOD1^{G93A} mice. We think that it may be a consequence of the reduced MN death, rather than a direct effect of the therapy, considering the local expression of this isoform in the skeletal muscles. However, we cannot discard that the increased levels of NRG1-I may reach the spinal cord by retrograde axonal transport or by entering the blood flow. Thus, NRG1-I might have a deleterious role upon upregulation in the spinal cord of SOD1^{G93A} mice (Song et al., 2012), but upon upregulation in the skeletal muscles its beneficial effects on MNs might produce a signaling with glia in the CNS that consequently reduces neuroinflammation.

Regarding sex differences, we provide evidence here that the beneficial effects of NRG1-I overexpression are similar in female and in male SOD1^{G93A} mice. However,

we also found that gene therapy targeting NRG1-III in the spinal cord produced positive effects only in female but not in male SOD1^{G93A} mice. We hypothesize that progesterone might play a role in the modulation of the NRG1-III expression in the spinal cord of the mice and participate on the neuroprotective effects described in females also in other pathological conditions (Gonzalez Deniselle et al., 2002; LaCroix-Fralish et al., 2008). Considering that here we overexpressed NRG1-I in the skeletal muscles, it seems that progesterone does not alter NRG1 signaling at the peripheral level.

In this study, we introduced the use of an AAV vector with the desmin promoter which allows the delivery of the gene of interest in most of the skeletal muscles of the animal with a single systemic injection (Pacak et al., 2008). Indeed, this therapy shows numerous advantages compared to other approaches since it only requires a single administration, permits the overexpression of the gene of interest, in our case NRG1-I, in most of the skeletal muscles affected in ALS but not in other cell types, and the effect of the therapy is stable and allows a long-term expression even at the end-stage of the disease. Finally, since ALS physiopathology is considered to evolve in a dying-back process in which the nerve terminals and NMJ are partially degraded when cell bodies in the spinal cord are still intact (Fischer et al., 2004; Mancuso et al., 2011), viral-mediated NRG1-I therapy may be a proper approach for MND, as demonstrated with our results, if the NMJ remains functional for longer time.

Furthermore, combined-gene therapy with NRG1-III and NRG1-I delivered in the CNS and PNS respectively delayed the disease progression of the SOD1^{G93A} mice but did not produce the synergic effect that we expected. One of the possible explanations could be regarding the limitations of this model. Indeed, there may be a limited therapeutic opportunity which these therapies cannot overcome. As previously explained, a very few studies have reported a synergistic effect after combinatory approaches (Mancuso et al., 2014). This might reflect that there is an endogenous limitation for the beneficial effects that can be achieved using the SOD1^{G93A} mouse model. However, we must take into account that we did not assess the survival for the gene therapies performed in chapter III, that could elucidate in

the end-stages of the disease whether there is a difference between our therapeutic approaches or not.

Motoneuron vulnerability and excitability

One of the particularities of ALS and other MND is the predominant vulnerability of certain groups of MNs, and the resistance of others. While in the $SOD1^{G93A}$ mice oculomotor MNs are relatively spared (Nimchinsky et al., 2000), dysfunction of large fast-fatigable motor units was reported well before the clinical onset, followed by degeneration of these motor units at clinical onset, but with sustained sparing of slow motor units (Pun et al., 2006). This has been corroborated in the murine model, in which the extensor digitorum longus muscle that is mainly formed by fast fatigable motor units has a rapid denervation, whereas the soleus muscle mainly composed by slow motor units is quite resistant (Hegedus et al., 2007; 2008). Among the possible explanations, MN vulnerability could be explained because of the structure of this cells and the energy metabolism, since they are large cells with long axons, and they require a high metabolic input. Also, some studies have revealed an excitatory-inhibitory imbalance, affecting synaptic inputs into spinal MNs (Sunico et al., 2011). The potential contribution of C-boutons participating in ALS pathophysiology has also received attention, since they regulate spinal MN excitability. Indeed, blocking cholinergic neurotransmission through C-boutons results in an increase in neurotoxic misfolded SOD1 in MNs of the $SOD1^{G93A}$ mouse (Saxena et al., 2013). C-boutons contain numerous proteins, including sigma-1 receptor (Mavlyutov et al., 2012) and NRG1 (Gallart-Palau et al., 2014), and the presynaptic membrane contains NRG1 receptors, ErbB2 and ErbB4 (Gallart-Palau et al., 2014).

The balance between excitatory and inhibitory synaptic inputs is critical for the physiological control of MNs. Loss of NRG1 from cortical projection neurons results in increased inhibitory neurotransmission (Agarwal et al., 2014) and it was recently described that NRG1-III has an essential role for cholinergic transmission (Salvany et al., 2019). Indeed, blocking cholinergic neurotransmission through C-boutons increases the neurotoxic misfolded SOD1 in MNs of SOD1 ALS mouse (Saxena et al., 2013). Moreover, Salvany and collaborators (2019) proposed that

NRG1-III may serve as postsynaptic SSC organizer, a structure that includes the potassium channel Kv2.1. Therefore NRG1-III may interact with post synaptic components of C-type synapses such as Kv2.1 channel regulating the MN excitability. On another pathway, the maintenance of a low-intracellular chloride concentration by the KCC2 is important for the efficacy of the fast-synaptic inhibition of MNs (Fuchs et al., 2010). In fact, it is known that in late stages of the disease, KCC2 is dysregulated in the spinal MNs of $SOD1^{G93A}$ mice (Fuchs et al., 2010; Mòdol et al., 2014). We found that KCC2 transporter expression is upregulated upon NRG1-III overexpression preventing hyperreflexia. Therefore, NRG1-III/ErbB4 signaling may also regulate the MN excitability through KCC2, avoiding the deleterious effect that downregulation of KCC2 in MNs has contributing to spasticity (Brocard et al., 2016).

Another proposed marker for vulnerable MNs is MMP-9. Its expression provokes neurodegeneration and enables activation of ER stress (Kaplan et al., 2014). Moreover, the removal of MMP-9 gene leads to a 25% increase in lifespan of the ALS mice (Xia et al., 2006). Interestingly, treatment with NRG1 attenuates the production and activity of MMP-9 following spinal cord injury (Alizadeh et al., 2017) and activation of EGFR (ErbB1) enhances nociception in DRG neurons through a mechanism involving the PI3K/AKT pathway and the upregulation of MMP-9 (Martin et al., 2017). Our results showed that treatment overexpressing NRG1-III decreases the number of MMP-9 positive MNs in the spinal cord, therefore suggesting that it conferred neuroprotection to vulnerable MNs through the reduction of the MMP-9 expression.

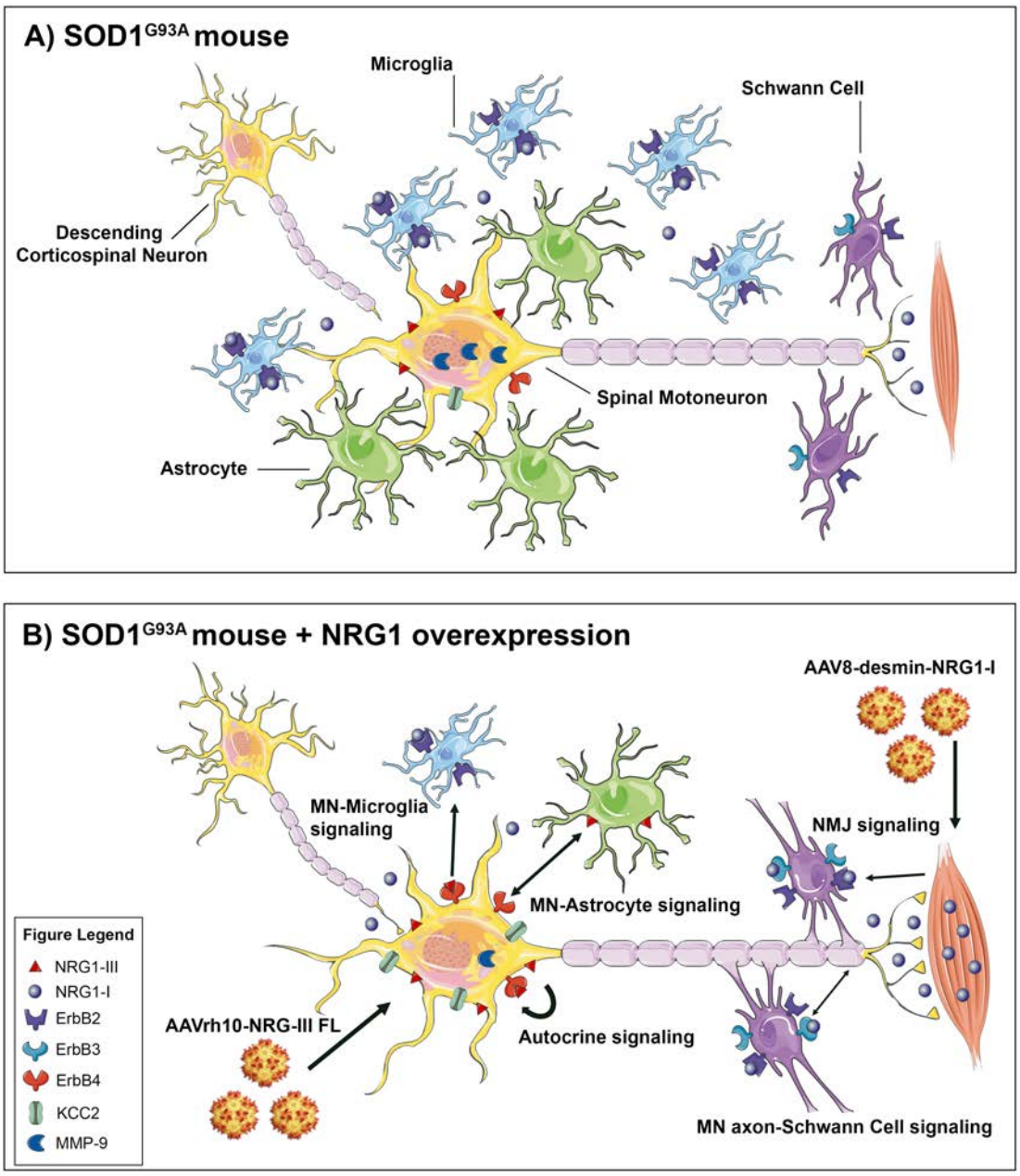


Figure 5. Effect of Neuregulin 1 overexpression in the SOD1^{G93A} mouse. A) Alterations in the NRG1/ErbB system have been reported in the end-stage of the disease of SOD1^{G93A} mouse model. In the CNS, an upregulation of the NRG1-I isoform released from spinal MNs, contributes to the activation and proliferation of the microglial cells expressing the ErbB2 receptor (Song et al., 2012). In contrast, NRG1-III is reduced in parallel with MN loss, as well as the ErbB4 receptor. The MN-vulnerability marker MMP-9 is increased in MNs together with a dysregulation of the KCC2 transporter, increasing the MN excitability (Fuchs et al., 2010; Kaplan et al., 2014; Mòdol et al., 2014). Other pathogenic

features reported include the excitotoxicity caused by astrocytes, and the disconnection and loss of the upper MNs. At the same time in the PNS denervation and muscle atrophy is observed and the low expression of NRG1-I in the NMJ cannot activate the Schwann cells to induce motor axonal reinnervation. B) Treatment with gene therapies overexpressing NRG1-III in the CNS and NRG1-I in the PNS promote MN survival, probably through an autocrine signaling (Lasiene et al., 2016). The upregulation of NRG1-III in the spinal cord also decreases the astroglial reactivity and may provide a NRG1-III/ErbB4 bidirectional signaling with astrocytes to potentiate synaptic transmission and induce synaptic plasticity (LaCroix-Fralish et al., 2008). Also, MMP-9 is reduced in parallel with an increase of KCC2, regulating the MN excitability. Furthermore, in the PNS NRG1-I in the NMJ signals with terminal Schwann cells expressing ErbB2 and 3 receptors to promote axonal reinnervation. Also, a bidirectional signaling including NRG1-I/ErbBs between Schwann cells and motor axons to support their mutual survival is feasible (Esper et al., 2004).

CONCLUSIONS

Conclusions

Chapter I: Neuregulin 1 reduces motoneuron cell death and promotes neurite growth in an in vitro model of motoneuron degeneration

1. In the spinal cord organotypic culture submitted to chronic excitotoxicity by exposure to THA, the levels of NRG1 levels were significantly reduced compared to control cultures
2. Addition of rhNRG1 promotes motoneuron survival in the spinal cord organotypic culture under excitotoxicity
3. NRG1 activates the PI3K/AKT pathway through activation of ErbB receptors
4. The autophagic flux is modulated by NRG1, reducing the expression of LC3-II and beclin autophagosome markers
5. Addition of NRG1 reduces the microglial reactivity, overcoming the excitotoxic effect caused by THA
6. NRG1 promotes motor and sensory neurite outgrowth as shown in spinal cord culture and in dorsal root ganglia culture, respectively.

Chapter II: Role of the full-length Neuregulin 1 type III in SOD1-linked Amyotrophic Lateral Sclerosis

1. In spinal cord samples of ALS patients, the immunoreactivity of NRG1 appeared reduced in preserved motoneurons and was increased in microglial cells, whereas NRG1-III was specifically expressed in motoneurons but at lower levels than in healthy controls
2. The mRNA levels of NRG1-I were upregulated whereas those of NRG1-III were downregulated in the spinal cord of $SOD1^{G93A}$ ALS mice at the symptomatic stage of the disease
3. The intrathecal administration of an AAVrh10NRG1-III produced a marked overexpression of NRG1-III FL in the spinal cord
4. NRG1-III overexpression improves neuromuscular function of the hindlimb muscles and slows the disease progression of the $SOD1^{G93A}$ transgenic mice
5. NRG1-III treatment preserves the motoneuron number and decreases the astrocyte and microglial reactivity

6. NRG1-III activates the ErbB4 receptor signaling pathway, inducing a reduction of the MMP-9 vulnerability marker and modulating the motoneuronal excitability by increasing expression of KCC2
7. The positive effects of gene therapy to overexpress NRG1-III FL in the spinal cord were found in the female but not in the male SOD1^{G93A} mice
8. NRG-III viral-mediated therapy did not increase the survival of the SOD1^{G93A} mice

Chapter III: Gene therapies overexpressing Neuregulin 1 type I and combination with Neuregulin 1 type III promote motor functional improvement in SOD1-linked amyotrophic lateral sclerosis

1. AAV vectors coding for luciferase expression under the desmin promoter were widely affecting the skeletal muscles, especially on the hindlimbs, after systemic endovenous administration
2. General NRG1-I overexpression in skeletal muscles produces significant preservation of the neuromuscular function and of the locomotion performance in the female and in the male treated SOD1^{G93A} mice
3. NRG1-I overexpression in skeletal muscles preserves the number of surviving spinal motoneurons and decreases the astrocyte and microglial reactivity
4. Under NRG1-I overexpression, the levels of ErbB2 and ErbB3 are significantly increased in skeletal muscle of SOD1^{G93A} mice compared to the mice given a mock vector
5. Simultaneous administration of AAV8-desmin-NRG1-I endovenously and AAVrh10-NRG1-IIIFL intrathecally allows for effective overexpression of NRG1-I at the periphery and NRG1-III at the central nervous system, without causing any adverse effect
6. The combined gene therapy with NRG1-I and NRG1-III improves neuromuscular function and locomotor performance and significantly delays the disease onset in SOD1^{G93A} mice
7. The combined gene therapy with NRG1-I and NRG1-III, however, despite producing a significant functional improvement, does not have a synergic

effect over the single therapies.

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