Universitat Autònoma de Barcelona

EVALUATION OF NEUROPROTECTIVE EFFECTS OF SIGMA 1 RECEPTOR LIGANDS ON MOTONEURON DEGENERATION MODELS

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It’s all about attitude.

Never give up.

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I. SUMMARY

Motor neuron diseases (MND) include a wide type of sporadic and hereditary neurological disorders characterized by degeneration of motor neurons (MNs), and are highly debilitating and severe conditions for which only limited therapeutic options are available. Amyotrophic lateral sclerosis (ALS), the most frequent MND in adults, is a fatal neurodegenerative disease with degeneration of MNs in the spinal cord and the brain. Currently, available treatments have modest effects on patient survival. Thus, there is an urgent need to understand the molecular mechanisms involved in the MN degeneration to discover new therapeutic targets and develop effective therapies protecting and restoring neuronal integrity. Among the putative targets for neuroprotection, recently the Sigma-1 receptor (Sig-1R) has gained attention.

The aim of the present thesis was to assess the therapeutic potential of several Sig-1R ligands for promoting MN survival and maintenance of neuromuscular function. For this purpose, we first used an *in vitro* model of spinal cord organotypic culture subjected to chronic excitotoxicity, and then, two murine models, the spinal nerve injury and the transgenic $SOD1^{G93A}$ mice.

Our *in vitro* results revealed that the Sig-1R ligands tested (PRE-084, BD1063, SA4503, EST79232 and EST79376) increased MN survival under chronic excitotoxicity. On the other hand, *in vivo* studies showed that the neuroprotective effects observed differed between the Sig-1R ligands tested. In the $SOD1^{G93A}$ mouse treatment with several Sig-1R ligands was effective to preserve neuromuscular function and junctions the hindlimbs, to increase the number of surviving MNs and to modulate glial reactivity in the spinal cord. These Sig-1R ligands were also able to increase MN survival and modulate glial reactivity after a spinal nerve injury. Altogether, the results of this thesis provide clear evidence that Sig-1R is an interesting target for the treatment of MND.

II. INTRODUCTION

1. Motoneuron diseases

Motoneuron diseases (MND) are a set of progressive neurodegenerative disorders characterized by the degeneration of lower and/or upper motoneurons (MN). Based on MN cell body location in the central nervous system (CNS) two main categories could be distinguished: upper or lower MNs. Upper MNs are located in the motor cortex and give rise to the corticospinal and corticobulbar tracts and are involved in the planning and initiation of movement. Lower MNs are located in the motor nuclei of cranial nerves in the brainstem and in the ventral horn of spinal cord, their axons innervate and control the skeletal muscles with a somatotopic distribution (Ragagnin et al., 2019) (Figure 1). Lower MNs are classified as α-MNs (innervate extrafusal muscle fibers in the muscle), γ-MNs (innervate intrafusal muscle fibres of muscle spindles) and β-MNs (innervate both intrafusal and extrafusal muscle fibers) according to the type of muscle fibers innervated. Each MN forms a motor unit with all the muscle fibers that it innervates.

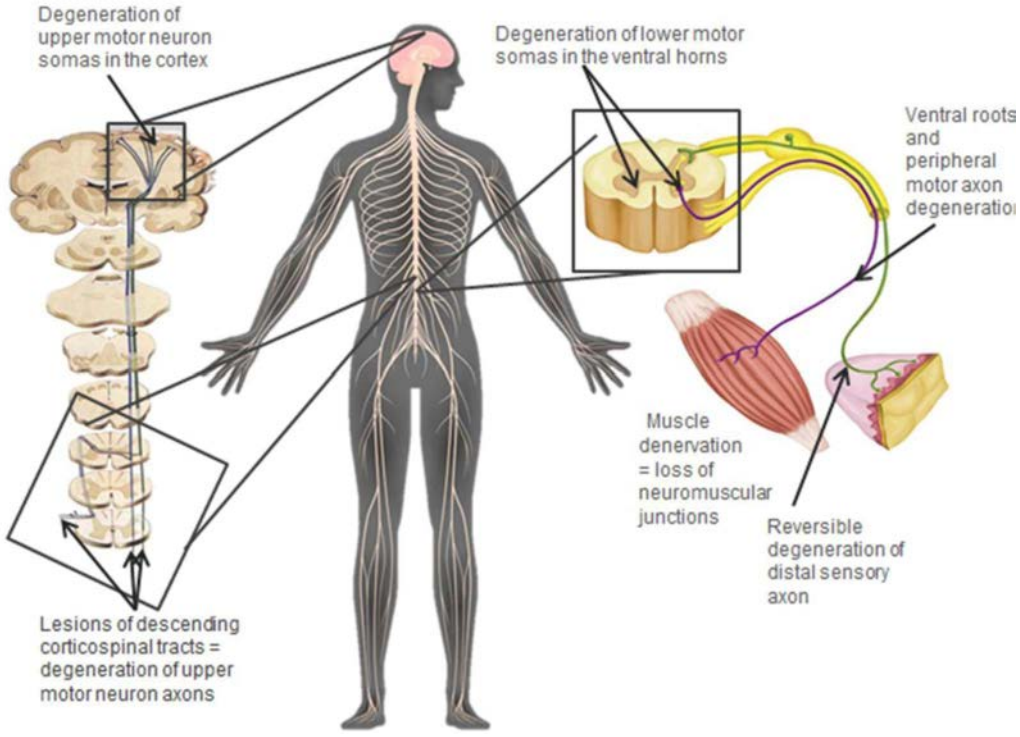


Figure 1. Organitzation of motor system composed by upper and lower MNs, which innervates skeletal muscle. A progressive degneration of upper and lower MNs occurs in ALS. Extracted from Merwin et al. (2017).

There are different types of MND well characterized, either hereditary or sporadic (acquired) and with variable etiology and clinical presentation (Tiryaki & Horak, 2014). Nevertheless, all of them have common manifestations, such as muscle weakness and atrophy, breathing, swallowing and speech difficulties and spasticity on their extremities. The most common

forms of MND are amyotrophic lateral sclerosis (ALS) affecting adults and the monogenic disorder called spinal muscular atrophy (SMA) affecting children (Mancuso & Navarro, 2017). Another type of rare genetic disorders characterized by degeneration of the lower MNs is distal hereditary motor neuropathies (dHMN) also named distal SMA.

Traumatic injuries in the spinal cord or in the nerves are also a type of pathology in which MN dysfunction and degeneration is prevalent, including spinal root injuries and nerve plexus injuries which are considered as peripheral nerve injury (PNI). The disconnection of the nerve-muscle from the spinal cord causes MN degeneration. This is another kind of pathology where the study of MN death is highly relevant.

2. Amyotrophic lateral sclerosis

The first description of ALS was made in 1869 by Jean-Martin Charcot, who observed a decreased number of MNs in the ventral horn of the spinal cord and atrophy (sclerosis) of the lateral funiculi in patients who had muscle atrophy and spasms. ALS is a progressive and fatal neurodegenerative disease which can affect both upper and lower MNs. Depending on the geographic location the ALS incidence varies, in Europe the estimated incidence is 2.1 to 3.8 cases per 100,000 person- years for the general population and its prevalence is between 4.1 and 8.4 per 100,000 persons. Males are more affected than females and onset commonly begins in adulthood, over 50 years of age (Longinetti & Fang, 2019).

The neuropathological hallmarks of this disease include the degeneration of MNs in the spinal cord and in the brainstem, the loss of pyramidal neurons of motor cortex following by degeneration of corticospinal tracts and reactive gliosis. The presence of insoluble protein inclusions in the soma of MNs is another histopathological feature, specifically accumulations of TDP-43 (TAR DNA- binding protein 43) (Mancuso & Navarro, 2017; Neumann et al., 2006). ALS patients have variable phenotypes, considering the origin of ALS several clinical subsets can be distinguished. The majority of ALS presents a spinal onset causing initially muscle weakness and fasciculations in the limbs, and then, progressive paralysis of nearly all muscles leading to death. Patients usually die by respiratory failure within 2-6 years after diagnosis. A less common type is the bulbar onset, in which patients have symptoms related with muscles that control speech (dysarthria), swallowing (dysphagia) and mastication, having worse prognosis (Ragagnin et al., 2019). ALS is a heterogeneous disorder in up to 50% of patients, it affects more than the motor system, they have also impairment of frontotemporal functions (non-motor features) such as cognition and/or behaviour. In ALS with

frontotemporal dementia (FTD) the loss of cortical MNs is accompanied by loss of neurons in the frontal and temporal cortex (Strong et al., 2003).

No definitive test for the diagnosis of ALS is available, but it is usually based on the El Escorial and Airlie House criteria. The diagnosis is fundamentally based on clinical examination supported by nerve conduction tests and electromyography because these tests allow to confirm the muscular denervation (Brown & Al-Chalabi, 2017; Carvalho et al., 2008). Furthermore, diagnostic has to exclude other MNDs that may have similar symptomatology (Hardiman et al., 2017). Usually there is a problematic delay between the first symptom and ALS diagnosis, approximately 12 months.

Nowadays, it is unknown the exact origin of ALS. Approximately 90% of ALS cases are sporadic forms (sALS) which occur without an identified cause, and 10% are familial cases (fALS) linked to genetic mutations, both types are clinically and pathologically similar. In 1993, *Cu/Zn superoxide dismutase 1* (*SOD1*) was identified as the first mutated gene responsible for fALS (Rosen et al., 1993); since then over 150 different mutations have been found in this gene. In the last years, the improvement of genetics technics and analyses (genome-wide association studies, whole genome studies and exome sequencing technologies) allowed the identification of numerous genes related with ALS (Figure 2) such as *TDP-43* (*TARDBP*), *Fused in sarcoma* (*FUS*), *Ataxin2* (*ATXN2*), *Profilin1* (*PFN1*), *Sigma non-opioid intracellular receptor 1* (*SIGMAR1*) or *C9orf72*, among others (Table 1) (Ragagnin et al., 2019).

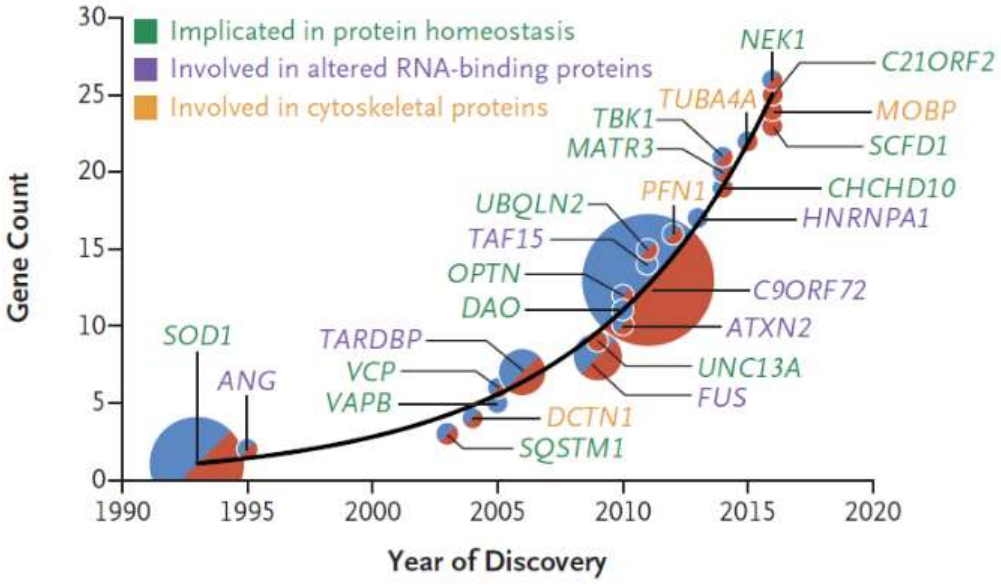


Figure 2. Genetic landscape of ALS since 1993. The identification of multiple novel genes associated with ALS have been enlarging in the last years. The size of each circle indicates the proportion of all fALS cases linked with that gene, and the colour indicates fALS (blue) and sALS (red) cases. Extracted from Brown & Al-Chalabi (2017).

The most commonly mutated gene is *C9orf72*, with an aberrant repeat expansion of a non-coding GGGGCC hexanucleotide sequence localized in the intronic region of human chromosome 9 open reading frame 7, leading to the formation of nuclear RNA foci (DeJesus-Hernandez et al., 2011). This mutation has been found in 40% of fALS and 8% of sALS (Chia et al., 2018). Several genetic abnormalities found in fALS forms are also identified in sALS and in FTD, showing an overlap among FTD and ALS. Interestingly, although mutant proteins are ubiquitously expressed, it remains unknown why MNs are selectively affected.

Table 1. Genes implicated in fALS and sALS forms and their associated protein functions.

Gene	Protein	Locus	Frequency in fALS (%)	Protein function
ALS2	Alsin2	2q33.2	<1	Vesicle trafficking
ANG	Angiogenin	14q11.1	1.5	Angiogenic factor
ATXN2	Ataxin 2	12q24	5	RNA translation, exocytosis
C21orf2	Chromosome 21 open reading frame 2	21q22.3	1.3-1.7	Ciliogenesis, DNA damage repair
C9orf72	Chromosome 9 open reading frame 72	9p21.2	30-50 (Europe, North America)	Endosomal trafficking, autophagy
CCNF	Cyclin F	16p13.3	0.6-3.3	UPS
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain containing 10	22q11.23	3.6	Mitochondrial function
CHMP2B	Charged multivesicular body protein 2B	3p11	-	Vesicle trafficking, proteostasis
DCTN1	Dynactin 1	2p13.1	-	Axonal transport
FUS	Fused in sarcoma	16p11.2	5	DNA/RNA metabolism, stress granule function
hnRNPA1	Human heterogeneous nuclear ribonucleoprotein A1	12q13.13	0.5	RNA metabolism
hnRNPA2B1	Human heterogeneous nuclear ribonucleoprotein A2B1	7p15.2	-	RNA metabolism
MATR3	Matrin 3	5q31.2	1.8	RNA and DNA metabolism mRNA nuclear export
NEFH	Neurofilament, heavy polypeptide	22q12.1	1	Cytoskeleton
NEK1	Never in mitosis gene A (NIMA)-related kinase 1	4q33	-	Cell cycle control and cilia regulation, DNA damage repair
OPTN	Optineurin	10p13	2.6	Autophagy
PFN1	Profilin-1	17p13	2.6	Actin dynamics
SCFD1	Sec1 family domain containing 1	14q12	-	Vesicle transport
SETX	Sentaxin	9q34	-	Ribostasis
SIGMAR1	Sigma non-opioid intracellular receptor 1	9p13.3	-	UPS (ubiquitin-proteasome system) autophagy
SOD1	Superoxide dismutase 1	21q22.11	12-23.5	Scavenger enzyme, oxidative stress, UPS, autophagy
SPG11	Spatacsin	15q14	-	DNA damage
SQSTM1/p62	Sequestosome 1/p62	5q35	1.8	Autophagy
TARDBP	TDP-43	1p36.22	5	DNA/RNA metabolism
TBK1	TANK-binding kinase 1	12q14.2	1-5.2	Autophagy, inflammation
TUBA4A	Tubulin alpha-4A chain	2q35	1.1	Cytoskeleton
UBQLN2	Ubiquilin 2	Xp11.21	0.5-2.1	Autophagy, UPS
VAPB	Vesicle-associated membrane protein (VAMP)-associated protein B	20q13.33	0.6	Vesicle trafficking, UPR
VCP	Valosin-containing protein	9p13.3	1-2.4	Autophagy

Four genes account for up to 70% of all cases of fALS: *C9orf72*, *TARDBP*, *SOD1* and *FUS*. Data extracted and adapted from Chia et al. (2018) and Ragagnin et al. (2019).

Unfortunately, there is no effective treatment for ALS. Some palliative care including assisted ventilation and nutrition (feeding tube) are the only support available. Although symptomatic therapies may improve the quality and prolong the lifespan of patients few months, they do not cure the illness and halt the neurodegenerative process. For ALS treatment only two drugs are approved by the FDA (Food and Drug Administration): Riluzole and Edaravone. Riluzole is an anti-glutamatergic drug that blocks voltage-dependent Na^+ channels inhibiting the presynaptic release of glutamate. It prolongs the lifespans of patients a few months (Bensimon et al., 1994). Edaravone is a free radical scavenger and acts reducing oxidative stress with beneficial effects only in a subgroup of patients at early stages of the disease (Edaravone (MCI-186) ALS 19 Study Group, 2017). However, the European Medicines Agency has not approved this drug yet.

The limited benefits of the available treatments indicate that the molecular and cellular mechanisms implicated in the neurodegenerative cascade must be more investigated to develop new effective therapies to cure the disease.

2.1 Pathophysiology of MN degeneration in ALS

The main feature of ALS is MN dysfunction. Despite knowledge of pathophysiology of MN death has enlarged in the last years, several unanswered questions of molecular mechanisms remain. Similar to other neurodegenerative disease, in ALS pathogenesis a wide variety of cellular pathways have been reported to contribute to MN degeneration, including glutamate excitotoxicity, oxidative stress, mitochondrial dysfunction, protein misfolding and impaired protein homeostasis, endoplasmic reticulum (ER) stress, neuroinflammation, among others (Figure 3) (Mancuso & Navarro, 2015, 2017; Ragagnin et al., 2019). These mechanisms may not be mutually exclusive, hence, its multifactorial pathology but specific mechanism that causes all these processes is still unknown.

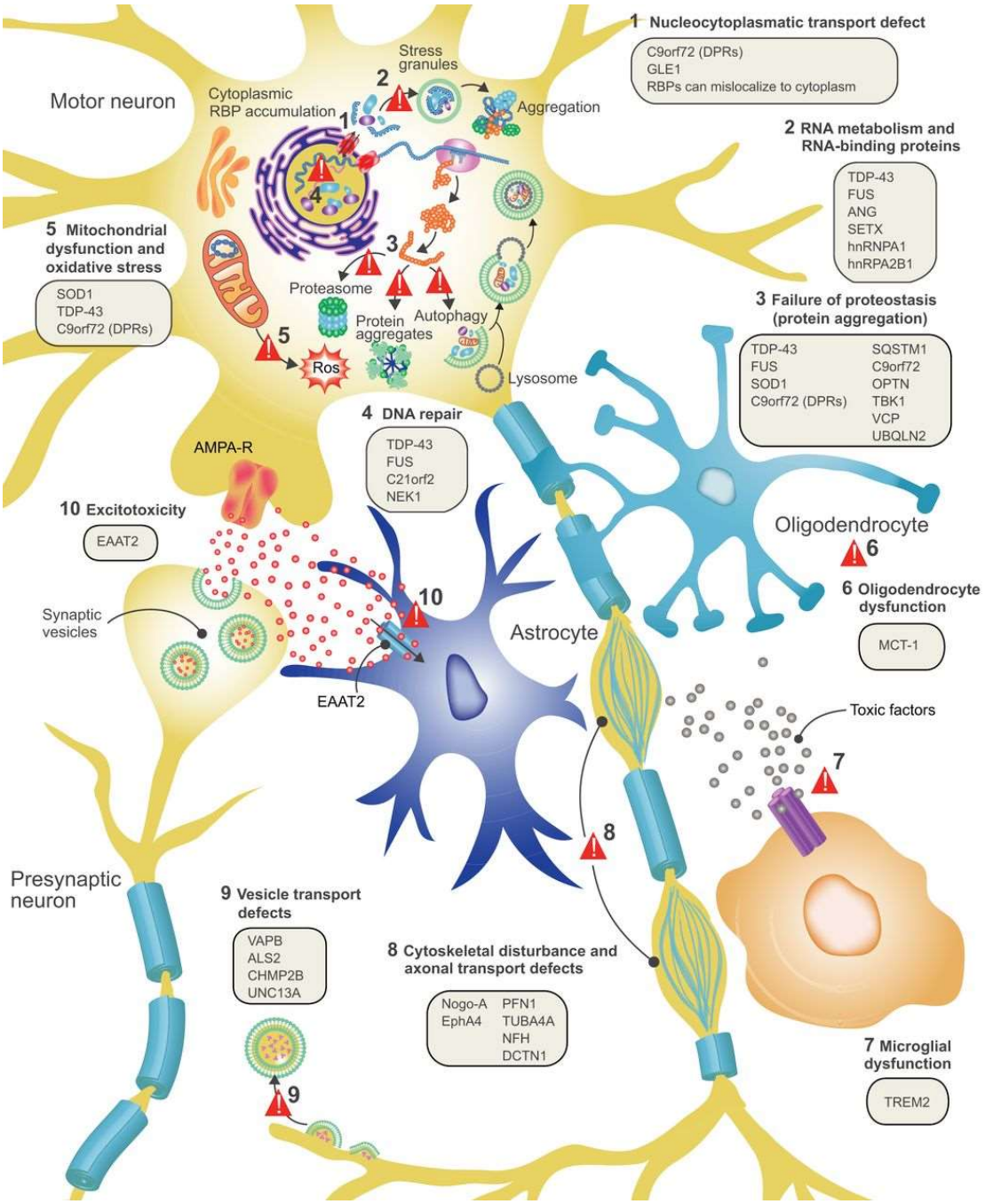


Figure 3. Main pathophysiological mechanisms of MN degeneration. (1) Alterations in nucleocytoplasmic transport of RNA molecules and RNA-binding proteins. (2) Altered RNA metabolism and altered dynamics of stress granule formation or disassembly can propagate cytoplasmic aggregate formation. (3) Impaired proteostasis with accumulation of aggregating proteins. (4) Impaired DNA repair. (5) Mitochondrial dysfunction and oxidative stress: several ALS-related proteins can enter mitochondria and disrupt normal functioning, with increased formation of ROS. (6) Oligodendrocyte dysfunction and degeneration, leading to reduced support for MNs. (7) Neuroinflammation: activated astrocytes and microglia secrete fewer neuroprotective factors and more toxic factors. (8) Defective axonal transport: several ALS-related mutations cause disorganization of the cytoskeletal proteins and disrupt axonal transport. (9) Defective vesicular transport: several ALS-related proteins are involved in vesicular transport. (10) Excitotoxicity: loss of the astroglial glutamate transporter EAAT2 causes accumulation of extracellular glutamate, which causes excessive stimulation of glutamate receptors and excessive Ca^{2+} influx. Extracted from Van Damme et al. (2017).

2.1.1 Excitotoxicity

The process that triggers neuronal death by excessive or prolonged glutamatergic stimulation is called glutamate excitotoxicity. Glutamate is the main excitatory neurotransmitter in the mammalian CNS and acts through iontropic (NMDA, AMPA and kainate) and metabotropic ($mGluR_{1-8}$) receptors. The increased excitatory input leads to a massive Ca^{2+} influx into the cytoplasm that damage the neuron by the activation of Ca^{2+}-dependent proteases, lipases and nucleases. More than twenty years ago, it was reported that glutamate levels are elevated in the cerebrospinal fluid (CSF) of MND patients (Shaw, et al., 1995), finding an important corroboration of the role of this neurotransmitter in disease.

Astrocytes are the principal regulators of extracellular glutamate levels and excitatory amino acid transporter 2 (EAAT2) is the main astroglial synaptic glutamate reuptake transporter protein. The loss of EAAT2 has been reported in both ALS patients (Bristol & Rothstein, 1996) and in rodent models (Howland et al., 2002), the impaired glutamate clearance in the synaptic cleft causes the accumulation of the neurotransmitter and excessive stimulation of receptors. Several lines of evidence, on *in vitro* and *in vivo* studies, suggest that MNs are particularly vulnerable to AMPA receptor stimulation. AMPA receptor Ca^{2+} permeability is largely determined by the GluR2 subunit. The unique AMPA receptor expression profile in MNs may render them selectively vulnerable because MNs express proportionately less GluR2 subunits comparing with other cell types, giving more Ca^{2+} permeability and, thus, more vulnerability to AMPA receptor-mediated excitotoxicity (Kawahara et al., 2003; Sun et al., 2005). Oculomotor neurons express higher levels of GluR2, which may give them less susceptible to excitotoxic injury (Brockington et al., 2013). Also, the importance of AMPA receptors and GluR2 in ALS was clearly observed when transgenic mice expressing mutant *SOD1* (*mSOD1*) and overexpressing the GluR2 subunit in cholinergic neurons (reducing Ca^{2+} permeability) had a marked delay of disease onset and prolonged survival compared to $SOD1^{G93A}$ mice (Tateno et al., 2004).

There is accumulating evidence that MNs may be vulnerable to excitotoxic insults because they possess a lower capacity to buffer Ca^{2+} rises in the cytoplasm than other neurons. Spinal MNs express low levels of parvalbumin and calbindin D28K proteins, bringing less Ca^{2+} buffering capacity. In contrast, less vulnerable MNs such as those in the oculomotor, trochlear and Onuf's nucleus clearly express these binding proteins (Alexianu et al., 1994; Ince et al., 1993). Thus, excitotoxicity damaging effects are primary mediated through Ca^{2+}-dependent pathways and this elevated intracellular Ca^{2+} results in triggering other secondary

degenerative cascades, including mitochondrial dysfunction and induction of proapoptotic pathways.

It is important to remark that one of two approved drugs for ALS treatment, Riluzole is acting by the inhibition of glutamate release, a mechanism that seems to be in common in all the ALS forms.

2.1.2 Oxidative stress

The state of oxidative stress is a condition arising from the imbalance between the removal and production of reactive oxygen species (ROS), which results in a homeostasis break. Mitochondria is the main site of ROS production and their accumulation leads to permanent oxidative damage to cell components, such as lipids (Simpson et al., 2004), proteins (Shaw, et al., 1995b) and nucleic acids (Bogdanov et al., 2000; Chang et al., 2008) promoting MN degeneration. Such damage to cell components was reported in tissues of ALS patients, showing an increase of lipid peroxidation products as HNE (4-hydroxy-2,3-nonenal) (Simpson et al., 2004) or elevated markers of DNA damage (Bogdanov et al., 2000) in the CSF and serum compared to healthy subjects.

Since mutations in *SOD1* gene, which encodes an antioxidant enzyme, were the first identified cause of fALS (Rosen et al., 1993) the role of oxidative stress highlighted interest in ALS. Mutations in SOD1 gene are present in 20% of fALS cases and less than 2% of all ALS (Ragagnin et al., 2019). *In vivo* and *in vitro* models of ALS based on *mSOD1* recapitulate the oxidative damage to protein, lipid and DNA observed in the human disease (Barber & Shaw, 2010; Liu et al., 1999). The mechanism thorough SOD1 causes toxicity is controversial, now it is widely accepted that mSOD1-mediated toxicity is caused by a gain of function rather than the loss of the detoxifying activity of SOD1. This points to a toxic gain of function, potentially mediated through SOD1 misfolding and aggregation (Bruijn et al., 1998).

As a result of ROS accumulation RNA oxidation is observed. A common feature of RNA species (mRNA oxidation) was documented in the SOD1 mouse model and in ALS patients as well (Chang et al., 2008). In models with other ALS-linked proteins (non-SOD1 ALS) oxidative stress was also described. For example, cellular models of *mutated TDP-43* (*mTDP-43*) -related ALS showed that the presence of this mutant protein produces oxidative stress in MN cell lines (Duan et al., 2010).

Finally, it is known that several ALS related-proteins such as SOD1, TDP-43 (Wang et al., 2016) and C9orf72 (Lopez-Gonzalez et al., 2016) can enter mitochondria and disrupt normal functioning, resulting in an increased formation of ROS. Manifesting a cross-link between oxidative stress and other pathogenic mechanisms.

2.1.3 Mitochondrial dysfunction

Numerous studies have highlighted the common role of mitochondria in the pathogenesis of neurodegenerative diseases. The main functions of mitochondria are production of energy, Ca^{2+} homeostasis and control of apoptosis (Ferraiuolo et al., 2011). Particularly, MNs have high energetic demands, even compared to other neurons, and may therefore could be more vulnerable to accumulative oxidative stress over time.

Mitochondrial function is damaged in ALS, reduced amount of mitochondrial DNA associated with increased mutations of mitochondrial DNA, and decreased activity of mitochondrial respiratory chain complexes have been described in the spinal cord of ALS patients compared to controls, suggesting a loss of mitochondria (Wiedemann et al., 2002). Mitochondrial abnormalities are present in ALS patient tissues, like morphological changes in mitochondria in the spinal MNs (Sasaki & Iwata, 2007) and mitochondrial function impairments in the skeletal muscle (Wiedemann et al., 1998). Moreover, these mitochondrial abnormalities such as swelling and vacuolization (morphological changes), alterations in function and/or distribution have been observed in models of fALS associated with SOD1 (Kong & Xu, 1998; Magrané et al., 2014; Vande Velde et al., 2011) and TDP-43 (Magrané et al., 2014) mutations. In $SOD1^{G93A}$ mice, mitochondrial morphological abnormalities were apparent early, prior to onset, whereas in $TDP43^{A315T}$ mice appeared later (Magrané et al., 2014).

Maintaining Ca^{2+} homeostasis is crucial for mitochondria function, dysfunctional Ca^{2+} uptake may therefore result in elevated intracellular levels, thus contributing to neurodegeneration. It has been found that mitochondrial Ca^{2+} buffering capacity is decreased in very early stages of the disease in the brain and spinal cord of $SOD1^{G93A}$ mice (Damiano et al., 2006).

Mitochondria control apoptosis activating the caspase cascade when mitochondrial permeability transition pore is open and cytochrome C releases from mitochondrial intermembrane space into the cytoplasm. Increased expression of the pro-apoptotic proteins Bax and Bad, and decreased expression of the anti-apoptotic Bcl-2 have been found in the spinal cord of transgenic SOD1 mice (Vukosavic et al., 1999). Furthermore, Guégan et al.

(2001) shown the translocation of cytochrome C to the cytosol and protein Bax to the mitochondria during the progression of disease in mice. This translocation also occurs in the spinal cord of sALS patients. Interestingly, inhibition of the mitochondrial permeability transition-mediated cytochrome C release, using minocycline, lengthens the lifespan of $SOD1^{G93A}$ mice (Zhu et al., 2002).

As mentioned before, TDP-43 accumulates in the mitochondria in neurons of ALS subjects and it preferentially binds to mRNAs encoding respiratory chain complex I, causing its disassembly (Wang et al., 2016). In models of *C9orf72*-associated ALS the dipeptide repeat protein poly(GR) preferentially bound to mitochondrial ribosomal proteins and caused mitochondrial dysfunction with DNA damage in part by increasing oxidative stress in induced pluripotent stem cells (iPSCs) derived MNs (Lopez-Gonzalez et al., 2016).

2.1.4 Impaired protein homeostasis (protein misfolding and aggregation)

Analogous with other neurodegenerative disease, ALS is often considered a proteinopathy because of one of the main pathological hallmarks is the insoluble protein inclusions and the aggregates in the soma of MNs. Specifically, inclusions of TDP-43 are the major constituent of that ubiquitinated protein inclusions found in surviving MNs in most forms of ALS. Nevertheless, whether inclusions formation exert cellular toxicity, if they are innocuous neurodegeneration-derived products or if they may represent a protective reaction of the cell to reduce intracellular concentrations of toxic proteins it remains unknown (Mancuso & Navarro, 2015).

Mutations in several ALS-related genes lead to aberrant translation of proteins causing misfolding and/or abnormal cellular localization and accumulation which impairs protein degradation machinery of MNs (Hardiman et al., 2017; Ruegsegger & Saxena, 2016). These intracellular inclusions include protein products from mutated genes mainly *SOD1*, *TDP-43*, *FUS*, *UBQLN2*, *OPTN* and *C9orf72*. Recently, some ALS-associated mutations have been identified to directly target proteostasis network components such as autophagy, ER stress, stress granules, ubiquitin proteasome pathway (UPS), unfolded protein response (UPR) and ER-associated protein degradation (ERAD) (Medinas et al., 2017; Ruegsegger & Saxena, 2016).

The majority of ALS patients present intracellular inclusions in degenerating neurons and glial cells. Cytoplasmic SOD1 inclusions are present in both fALS and sALS cases (Shibata et al., 1994) and in mouse models of *mSOD1* (Bruijn et al., 1998). It has been reported that

ubiquinated SOD1 aggregates overload and inhibit UPS pathways (Bendotti et al., 2012; Urushitani et al., 2002), caused by a decrease of constitutive proteasome subunits in the ventral horn during disease progression in SOD1 mice (Cheroni et al., 2005). At least two types of *mSOD1* cause MN death (activation of ASK-1) through interaction with Derlin-1, a component of ERAD machinery, and triggered ER stress by dysfunction of the proteasome misfold protein degradation in the cytosol (Nishitoh et al., 2008). Also, SOD1 transgenic mice extended the lifespan and had less MN death when Derlin-1 could not bind to SOD1 and in ASK-1 deletion. Thus, ER stress is another pathogenic mechanism with a tight connection with protein aggregation and UPR.

Usually, TDP-43 is concentrated within the nucleus, but in pathologic conditions it is localized in ubiquitinated and hyperphosphorylated cytoplasmatic aggregates (Figure 4), redistribution of TDP-43 suggested the loss of normal function of it. Analysis of post-mortem tissue from sALS and fALS cases demonstrated pathological forms of TDP-43 in neuronal and glial cells that were absent in non-SOD1 fALS cases (Mackenzie et al., 2007; Neumann et al., 2006). FUS is another RNA-binding protein found in neuronal and/or glial cytoplasmic inclusions in ALS patients (Groen et al., 2010; Hewitt et al., 2010). Mutations in genes which encode RNA-binding proteins lead to binding propensity, to excessive accumulation of protein aggregates and the aberrant formation of stress granules in the cytoplasm, this consists a mechanistic link between proteostasis disturbance and aberrant RNA metabolism (Medinas et al., 2017). Altered dynamics of stress granule formation or disassembly can propagate cytoplasmic toxic inclusions formation (Zhang et al., 2019).

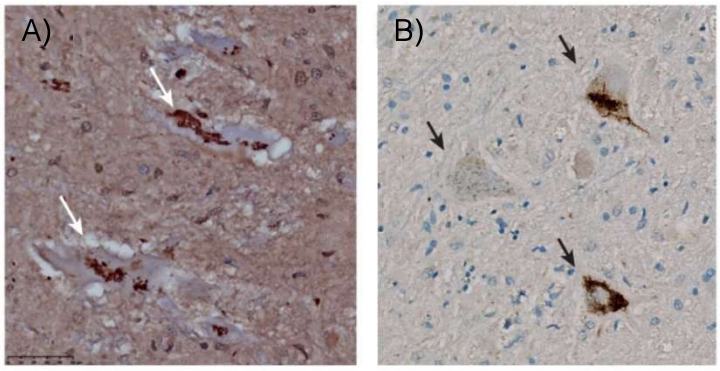


Figure 4. Histopathology of different ALS subtypes. A) SOD1 aggregates in spinal MNs in SOD1-related fALS. B) Aberrant localization of TDP-43 to cytoplasmic inclusions in spinal MNs in sALS. Extracted from Taylor et al. (2016).

C9orf72 ALS/FTD cases are distinguished from other ALS and FTD cases by specific ubiquitin and p62-positive but TDP-43-negative neuronal cytoplasmic inclusions, indicative of impaired autophagy (Mackenzie et al., 2014). How the repeat expansion in *C9orf72* causes ALS is not yet definitively known, one possible mechanism is that the expression of RAN (Repeat-associated non-ATG translated) peptides of C9orf72 repeat expansion has been associated

with proteasome impairment and ER stress (Zhang et al., 2014). Moreover, C9orf72 controls the initiation of autophagy though regulating Rab1a-dependent trafficking of the ULK1 autophagy initiation complex to the phagophore. Basal levels of autophagy were markedly reduced in C9-ALS patients and caused the accumulation of p62-positive inclusions (Webster et al., 2016). All these results suggest that altered regulation of the proteostasis by the accumulated misfolding proteins may affect other related pathogenic mechanisms.

2.1.5 Impaired axonal transport

MNs have very long axons which have length of up to 1 m long in human and connect the soma with distant synaptic sites. An efficient intracellular axonal transport is required to maintain their structure and function such movement of cargo: proteins, mRNA, lipids, membrane-bound vesicles and organelles (De Vos & Hafezparast, 2017). Since 80s, neuropathological studies of post-mortem ALS cases shown axonal cytoskeletal disorganization, specially the accumulation of neurofilaments in MNs cell bodies, implying disruption of axonal transport in these cells (Hirano et al., 1984; Julien et al., 1998). In SOD1 transgenic mice, neuronal cytoskeletal pathology develops resembling human ALS (Tu et al., 1996, Williamson et al., 1998).

Several ALS-related mutations cause defects in motor proteins, disorganization of the cytoskeletal proteins and disrupt axonal transport machinery, such as dynactin, PFN1, tubulin α-4A, neurofilament heavy chain, kinesin-5A, cofilin, among others. Also, SOD1, FUS and TDP-43 mutants impair function of microtubule-associated proteins, tubulin, actin, resulting in defects in anterograde and retrograde axonal traffic (reviewed in (Burk & Pasterkamp, 2019)). For example, it has been demonstrated that mSOD1 interacts with dynein–dynactin complex impairing their functions, and forms aggregates in the spinal cord and sciatic nerve prior to the symptoms and increased over the disease progression in transgenic mice (Zhang et al., 2007). Moreover, because of axonal transport is impair, abnormal redistribution of mitochondria was observed in ALS- related $SOD1^{G93A}$ patients and in $TDP\text{-}43^{A315T}$ transgenic mice at early stages of the disease with pathological clusters along the axon (Magrané et al., 2014). The correct signal transmission between the MN and the muscle is compromised when axonal transport is impaired. The loss of viable mitochondria at sites of high energy demand, such as in the neuromuscular junctions (NMJ), may proceed to devastating consequences leading to muscle denervation. Mitochondrial transport abnormalities could significantly contribute to dying-back hypothesis (Granatiero & Manfredi, 2019).

2.1.6 Altered RNA metabolism

Alterations of RNA processing was proposed as an important pathological mechanism in the disease due to the identification of TDP-43 as a main component of protein inclusion in ALS patients (Neumann et al., 2006). Also, the discovery of mutations in gene *FUS*. Both are DNA-RNA binding proteins with functional homology binding to several RNA target molecules and are implicated in RNA processing-metabolism such as transcriptional regulation, RNA splicing and stability, microRNA processing and RNA transport (Butti & Patten, 2019). Usually, RNA-binding proteins are enriched in the nucleus, but in ALS, proteins such as TDP-43 or FUS become mislocalized with cytosolic accumulation and nuclear depletion (Mackenzie et al., 2007), resulting in the loss of normal processing of their target RNAs and dysregulation of gene expression (Amlie-Wolf et al., 2015; Zhou et al., 2014). For instance, TDP-43 binds to mRNA and regulates the expression of other proteins implicated in ALS. RNA-sequence analyses showed that TDP-43 regulates hundreds of mRNAs, many of them encoding synaptic proteins, neurotransmitters processes, neuronal morphology and neuronal plasticity. mTDP-43 impairs mRNA transport and alters the transcriptional process of crucial genes for MN homeostasis leading MN degeneration process (reviewed in (Butti & Patten, 2019)).

Although C9orf72 protein has a role in autophagy mediating clearance of proteins, in nuclear and endosomal membrane trafficking and it is not an RNA-binding protein, C9orf72 is implicated in RNA toxicity. The pathogenesis of non-coding repeat expansion might be explained by three non-mutually exclusive pathways (Swinnen et al., 2020). First, the repeat expansion might interfere with the normal transcription of the *C9orf72* gene, leading to loss of function of the respective protein reducing the total levels of it (DeJesus-Hernandez et al., 2011). Second, repeat-containing mRNAs are deposited in the nucleus, forming RNA foci that directly sequester various RNA-binding proteins, hence abolishing their normal function. This is called “RNA toxicity”. RNA foci are not restricted to neurons and are also found in astrocytes, microglia and oligodendrocytes, but with smaller amount (Mizielinska et al., 2013). Third, the repeat RNA itself might unconventionally be translated (noncanonical translation process) into peculiar toxic RAN peptides forming repeat dipeptides which result in proteasome impairment (Zhang et al., 2014). This is called “RAN toxicity”. The last two mechanisms are considered a gain of function of the protein, causing chronic cell stress and leading to stress granules formation. In addition, repeat expansions RNA could lead to R-loops formation, which are DNA–RNA hybrid structures, and increase genome instability

through double-strand DNA breaks (DNA damage) and defective kinase ATM-mediated DNA repair (Walker et al., 2017).

There is evidence that disturbance in RNA metabolism is implicated in ALS pathogenesis such as the identification of mRNA oxidation in ALS patients and in transgenic SOD1 mice (Chang et al., 2008), and the description of mutated genes related with RNA metabolism such as *angiogenin*, *DNA-RNA helicase senataxin*, *matrin 3*, *heterogeneous nuclear ribonucleoprotein A2 B1*, among others.

2.1.7 Contribution of non-neuronal cells

Despite ALS is a disease that primary affect MNs, several studies demonstrated the key role of neighbouring non-neuronal cells including microglia, astrocytes, oligodendrocytes and Schwann cells in the disease. Under normal conditions, each glial cell subpopulation is physiologically empowered to perform particularly tasks to warrant an optimal environment for MN survival and activity, but under pathological circumstances, they appear to exacerbate neurodegeneration. Some studies provide clear evidences, transgenic mice limiting the expression of *mSOD1* only in MNs did not cause MN neurodegeneration and motor impairment (Lino et al., 2002; Pramatarova et al., 2001), whereas expressing *mSOD1* only in glial cells induced MN loss and degeneration (Clement et al., 2003). Moreover, to identify differential contribution of mSOD1 in MNs or microglia in disease onset and progression, a double transgenic mouse was generated to express *mSOD1* and Cre-Lox recombination system supressing *mSOD1* expression in each cellular type. Specific deletion of *mSOD1* in MNs delayed the onset of the disease but not the progression. In contrast, the selective removal of *mSOD1* from CD11b+ cells (microglia and macrophages) did not alter disease initiation but delayed the progression and extended the lifespan of mice (Boillée, et al., 2006a, 2006b), indicating that microglia enhance the progression. Fburthermore, transplantation of wild type (WT) microglia into PU.1-SOD1^{G93A} knockout (KO) mice, which lacks microglial cells, extended the lifespan of these animals (Beers et al., 2006). Contradictory findings were obtained in the TDP-43 rNLS8 model, where reactive microglia exerted neuroprotective function, highlighting the beneficial role of this cells activation (Spiller et al., 2018).

On the other hand, transgenic mice expressing *mSOD1* restricted to astrocytes showed astrocytosis but failed inducing MN degeneration (Gong et al., 2000). Nevertheless, other studies evidenced the role of astrocytes in MN degeneration. *In vitro* co-cultures experiments of MNs and astrocytes (or astrocyte-conditioned media) derived from sALS or fALS patients (Haidet-Phillips et al., 2011; Madill et al., 2017; Marchetto et al., 2008) or from mice models

expressing ALS-linked mutations (*mSOD1* or *mTDP-43*) (Díaz-Amarilla et al., 2011; Nagai et al., 2007; Rojas et al., 2014) have reported toxicity to MN by secreted soluble factors. Interestingly, Re et al. (2014) demonstrated that astrocytes from ALS patients triggered MN death through necroptosis, a form of programmed necrosis involving receptor-interacting protein 1 (RIP-1) and the mixed lineage kinase domain-like protein (MLKL-1), and caspase independent death pathway. Several *in vivo* studies, reveal the role of astrocytes in MN degeneration. Thus, genetic excision of *mSOD1* in GFAP+ cells (astrocytes) delayed microglial activation and extended survival in transgenic mice (Wang et al., 2011; Yamanaka et al., 2008). Chronic infusion of conditioned medium derived from *mSOD1*^{G93A} astrocytes induced spinal MN degeneration and neuromuscular dysfunction in healthy rats (Ramírez-Jarquín et al., 2017). Finally, it is important to remember the role of astrocytes in glutamate excitotoxicity (Bristol & Rothstein, 1996), as discussed above in the section on Excitotoxicity.

Oligodendrocytes are the myelinating cells in the CNS and provide metabolic support to neurons; these glial cells also participate in ALS pathology. In the motor cortex and spinal cord of ALS patients, oligodendrocyte dysfunction has been observed as gray matter demyelination and reactive changes in NG2+ cells, oligodendrocytes precursors (Kang et al., 2013). Moreover, inclusions of TDP-43 in oligodendrocyte has been detected in the spinal cord grey matter of sALS (Philips et al., 2013). In ALS animal models myelin abnormalities were also found in transgenic SOD1 rats (Niebroj-Dobosz et al., 2007) and mice (Kang et al., 2013; Philips et al., 2013) at early stages before symptoms onset. In mice, the behaviour of NG2+ glial cells was markedly altered, being enhanced by the end of the disease in the spinal cord, suggesting the progressive dysfunction of oligodendrocytes. Kang et al. (2013) demonstrated that restoring oligodendrocytes function by selective genetic deletion of *mSOD1* from NG2+ cells delayed disease onset and prolonged the lifespan of ALS. Furthermore, oligodendrocytes support MNs function by direct supply to the axon of the energy metabolite lactate by monocarboxylate transporter 1, and this transporter is downregulated in patients and mouse models od ALS, producing axon damage (Lee et al., 2012).

Schwann cells myelinate individual axons in the peripheral nervous system (PNS) and are required for synapse formation, maintenance and repair of the NMJ, specifically the perisynaptic Schwann cells. An initial indication of the involvement of these cells in the pathogenesis of ALS was provided by observation that myelin is altered along femoral nerves from post-mortem patients (Perrie et al., 1993), but little is known about their contribution in ALS. Investigations using trans-gene-driven expression of mSOD1 mice only in protein zero

(P0) positive Schwann cells showed that these mice maintained motor function and normal survival without any evidence of neuronal loss or axonal degeneration (Turner et al., 2010). On the other hand, removal of *mSOD1* from around 70% of Schwann cells by gene-excision experiments showed unexpected results, with significantly accelerated disease progression and reduction of insulin-like growth factor 1 in $SOD1^{G37R}$ mice nerves (Lobsiger et al., 2009). Therefore, results of expression or ablation of *mSOD1* provided contradictory outcomes. Another important role of perisynaptic Schwann cells is guiding motor axonal sprouts to reinnervate NMJs. During ALS progression end-plates are gradually denervated and the sprouting competence mechanism is reduced during ALS course (Fischer et al., 2004; Frey et al., 2000; Mancuso et al., 2011).

Because of NMJ denervation was observed prior to MN degeneration, the question of where MND dysfunction begins arises: two main opposing hypotheses have been proposed. Some studies support the dying-forward hypothesis which suggests that the earliest degenerative process starts from the upper MNs with hyperexcitability and descends to lower MNs and the NMJs (Menon et al., 2015; Thomsen et al., 2014); opposite studies postulate the dying-back hypothesis that the neurodegeneration starts at the level of the NMJs or muscle cells and spreads back to affect MNs (Dadon-Nachum et al., 2011; Fischer et al., 2004; Hegedus et al., 2007; Moloney et al., 2014; Pun et al., 2006).

2.1.8 Neuroinflammation

Neuroinflammation is a common pathological feature in neurodegenerative disorders, particularly, in ALS MN degeneration leads to the activation of microglia, astrocytes and complement enhancing the progression of the disease. This is illustrated by the detection of elevated levels of pro-inflammatory mediators (cytokines/chemokines) and lymphocytes in the CSF of ALS patients (Henkel et al., 2004). Presence of activated microglia and dendritic cells, presence of T cells infiltration (lymphocytes) and reactive astrocytes has been manifested at sites of MN degeneration in spinal cord and brain of ALS patients (Henkel et al., 2004; Kawamata et al., 1992; Turner et al., 2004), and in samples of rodent models of ALS (Alexianu et al., 2001; Nikodemova et al., 2014).

Microglia represents the primary innate immune cells of the CNS. It is known that microglia exists in two states, resting and activated, and activated microglia has a dual phenotype: M1-like which is toxic, and M2-like which is neuroprotective. Nevertheless, this microglial state categorization is considered as an oversimplification as it is clear that microglia elicits graded and context-dependent responses when it is activated. In resting conditions “surveillant”,

microglia shows ramified shape, unlike the activated microglia has amoeboid-shape (Geloso et al., 2017). Activated microglia release ROS, cytokines and growth factors, enhancing inflammation. Several studies with *mSOD1* transgenic mice have been revealed that the number of microglia increased during the progression of the disease, suggesting that the phenotype of microglia evolves from a neuroprotective phenotype at pre-symptomatic stage of the disease to a cytotoxic phenotype at end-stage disease, as the disease progresses microglia undergoes phenotypic transformation. It is very likely that such transition into microglia activation state will occur asynchronously (Beers et al., 2011; Liao et al., 2012; Ohgomori et al., 2016; Spiller et al., 2018). In keeping with this, a dichotomy between whether microglial cells contribute to neuroprotection and neurodegeneration is persisting and it seems that the modulation of microglia function depends on the different stages of microglia polarization during the disease progression (Clarke & Patani, 2020). Several lines of evidence indicate that while microglia do not significantly contribute to disease onset, they accelerate disease progression. For example, ALS progression was slowed down when the *mSOD1* was conditionally deleted in microglia (Boillée, et al., 2006b), and treatment with the antibiotic minocycline, which has anti-inflammatory properties, increased the longevity of $SOD1^{G37R}$ mice (Kriz et al., 2002). Whereas most evidence on glial involvement in ALS pathogenesis came from mSOD1 models, there is accumulating statements for glial contribution in other subtypes of ALS as well (Filipi et al., 2020; O'Rourke et al., 2016; Rostalski et al., 2019). Lack of *C9orf72* in a loss-of-function model of the disease produced no signs of MN degeneration, but led to lysosomal accumulation, altered immune responses in macrophages and microglial cells, and strongly increased expression of inflammatory cytokines, contributing to the pathogenesis in ALS C9orf72 expansion carriers (O'Rourke et al., 2016).

Astrocytes play multiple homeostatic functions, such as metabolic support for neurons, ion and neurotransmitter homeostasis and in the maintenance of blood brain barrier integrity. Therefore, when their properties are altered, astrocytes have a crucial role in the propagation of MN degeneration. Like microglia, astrocytes can exist in two states during the neurodegenerative process, in activated stage, they lose neuroprotective functions and become toxic. It has been described two subsets of reactive astrocytes, A1 and A2. A1 reactive astrocytes are abundant in neurodegenerative diseases and may contribute to the death of neurons and oligodendrocytes (Liddelow et al., 2017). There is an increasing evidence that astrocytes mediate MN degeneration via the release of astrocyte-specific soluble toxic factors which trigger the selective loss of spinal MNs. These factors include inflammatory cytokines, prostaglandins, ROS, TNF-α, TGF-β1 (Tripathi et al., 2017), among

others (Haidet-Phillips et al., 2011; Liddelow et al., 2017; Nagai et al., 2007). Furthermore, astrocytes are involved in the progression rather than onset of ALS. Deletion of *mSOD1* from astrocytes slowed disease progression in $SOD1^{G93A}$ mice (Wang et al., 2011; Yamanaka et al., 2008). Analogous to *mSOD1* astrocytes, human and murine-derived astrocytes with C9orf72 or TDP-43 pathology also display changes in physiological properties and alter function of neighboring MNs (Filipi et al., 2020). Thus, therapeutic approaches targeting astrocytes functionality or transplanting healthy astrocytes are new therapies which might slow down or even halt the ALS progression (Izrael et al., 2020).

2.1.9 MN vulnerability

In ALS pathogenesis all MNs subtypes are not equally affected, some are more resistant to neurodegeneration than others. Upper and lower MNs which innervate voluntary muscles are generally more susceptible than certain lower MN subpopulations (such as MNs of oculomotor nuclei and Onuf's nucleus), reflecting the consistent clinical manifestation of ALS with the loss of the ability to speak, breath or move, and the preservation of eye movements and the function of external sphincters (Mancuso & Navarro, 2015; Ragagnin et al., 2019). Like ALS patients, the oculomotor MNs are spared in the $SOD1^{G86R}$ (Nimchinsky et al., 2000) and in the TDP-43 mouse model (Spiller et al., 2016). The exact reason for this differential vulnerability is not known.

There is a gradient of vulnerability among spinal MNs, where fast motor units are more susceptible to denervate before than slow units, a characteristic detected by electromyographic analysis (Dengler et al., 1990). In two models of *mSOD1* mice it was found that first fast-fatigable α-MNs, and then fast-resistant, are selectively affected at early times in the disease, whereas slow motor units are resistant up to the time when the mice die (Hadzipasic et al., 2014; Pun et al., 2006). This affection results in fast-fatigable muscles (type IIb fibers) such as extensor digitorum longus muscle become paralyzed before slow muscles (type I fibers) as soleus muscle in the disease progression (Hegedus et al., 2007, 2008). Recently, it has been uncovered that inhibitory interneurons contribute to MN vulnerability in ALS. In physiological conditions MNs innervating fast fast-fatigable fibers received stronger inhibitory glycinergic synaptic inputs than slow MNs, but during the ALS progression there was a specific loss of inhibitory synapses onto fast MNs in the $SOD1^{G93A}$ mouse model (Allodi et al., 2021).

Intrinsic MNs factors might therefore underlie their relative vulnerability or resistance to neurodegeneration in ALS. Studies of microarrays analyses and laser capture

microdissection of isolated MNs from different subpopulations reported that resistant subtypes display distinct transcriptional profile compare to susceptible MNs (Brockington et al., 2013; Hedlund et al., 2010). Importantly, the majority of the genes that were differentially expressed encode proteins which act in cellular pathways implicated in ALS pathogenesis, such as ER function, Ca^{2+} regulation, mitochondrial function, among others. Indeed, an up-regulation of subunit GluR2 of AMPA receptor in resistant oculomotor MNs compared to lumbar MNs has been described, resulting in less Ca^{2+} influx into MNs (Brockington et al., 2013). In fact, linked with Ca^{2+} homeostasis, more vulnerable spinal MNs express lower Ca^{2+}-buffering proteins than resistant oculomotor MNs, making them more susceptible to excitotoxic insults (Alexianu et al., 1994).

In addition, the differential susceptibility of MN populations might be related to differential expression of some genes such as semaphorin A3 (Sema3A) or Matrix Metalloproteinase 9 (MMP9). Sema3A is differentially expressed in the terminal Schwann cells based on muscle fiber type, specifically it is up-regulated in Schwann cells near MNJs of vulnerable fast-fatigable muscle fibers (De Winter et al., 2006). The administration of an antibody that binds to the Sema3A receptor, called neuropilin 1, delayed the decline of motor functions while prolonging the lifespan of $SOD1^{G93A}$ mice (Venkova et al., 2014). MMP-9 was identified as another determinant of selective vulnerability in $SOD1^{G93A}$ mice (Kaplan et al., 2014); it is strongly expressed in vulnerable fast-resistant spinal MNs, enhancing activation of ER stress, but in the oculomotor, Onuf's nuclei or slow MNs it is not expressed. Besides, the reduction of MMP-9 expression attenuated muscle denervation in transgenic SOD1 and TDP-43 mice (Kaplan et al., 2014; Spiller et al., 2019).

Further pathogenic mechanisms may explain the selective MN vulnerability, such as excitatory-inhibitory synaptic imbalance, MN excitability properties, aspects related with metabolic specialization, and motor unit size (Mancuso & Navarro, 2015; Ragagnin et al., 2019). Novel evidence supports the potential contribution of C-boutons in ALS pathogenesis, as described below in the *MND pathogenesis in which Sig-1R is involved* section.

3. Spinal root injuries

MN degeneration also occurs in traumatic injuries of the PNS. PNI is a heterogeneous group of lesions that cause partial or total loss of motor, sensory and autonomic functions in the affected limb due to the disconnection between the neuronal soma and its target organ. Patients suffer a reduction in the quality of life as a result of paralysis, sensory disturbance, anaesthesia and lack of autonomic control on the affected body areas, and appearance of positive symptoms as neuropathic pain and ulcerations (Navarro et al., 2007; Romeo-Guitart & Casas, 2019). Injuries affecting the spinal roots and nerve plexus constitute a frequent pathology, mostly due to trauma caused by traffic, work and sport accidents, or as a consequence of birth complications.

After a nerve injury a neuronal retrograde response is initiated and MNs undergo changes in gene expression which has been considered to switch them to a regenerative phenotype (Allodi et al., 2012; Gordon, 2016). The evolution and the severity of that response depends on several factors including distance between the axon injury to neuronal body, age, type of injury and the species of animal (Koliatsos et al., 1994; Ma et al., 2001). A different outcome in MN reaction or degeneration occurs depending on the proximity of the lesion to the neuronal body. It is known that the percentage of spinal MNs loss after a lesion of the sciatic nerve quite distal to the spinal cord is negligible (Karalija et al., 2016; Vanden Noven et al., 1993), whereas, a lumbar root avulsion causes a massive MN death within a few weeks post-injury in adult animals (Koliatsos et al., 1994; Penas et al., 2009). Thus, neuronal death is more severe if the axotomy is produced closer to the cell body, as a root or plexus injury, than after distal and post-ganglionic lesions in adulthood (Gu et al., 1997). Moreover, the age is also another relevant parameter to consider; contrarily to what happens in adult animals, neonatal sciatic nerve injury causes massive death of motor and sensory neurons within the first week of life. Peripheral axotomy (nerve crush) in neonatal (P3-30) rats leads to MN loss and poor regeneration after one month compared with adult rats. Also, nerve crush at P30 or earlier results in significantly less sensory neuron counts in the dorsal root ganglia (DRG) than in older controls (Kemp et al., 2015). In newborn animals almost any type of PNI induces cell death among the axotomized motor and sensory neurons because immature neurons are more dependent of trophic support from their target organ (Ma et al., 2001), while survival of adult neurons is less compromised although they may die if the injury is too close to the cell body.

Despite the fact that regeneration of injured peripheral nerves occurs to some extent, functional recovery is often slow and incomplete. It can be achieved if axotomized neurons survive and injured axons regrow to reinnervate the target organ. In severe injuries with complete nerve transection, the degree of recovery is null, surgical intervention through nerve grafting or root re-implantation is the way to promote axonal regeneration and sensorimotor functional recovery. Nevertheless, these repair techniques produce usually limited and inadequate functional recovery, specially in proximal injuries (Gordon, 2016; Navarro, 2016). A key point to achieve reinnervation is that a suitable number of axotomized MNs remain alive and then can regenerate their axons. Therefore, early application of an effective strategy to promote lesioned MNs survival is required to extend the time-window for surgical repair of nerve or root injuries. In summary, PNI is another kind of pathology where the study of MN death is relevant to understand molecular mechanisms implicated in the neuronal degeneration process.

3.1 Molecular mechanisms in MN neurodegeneration after proximal PNI

Root lesions represent a proximal axotomy that results in a progressive retrograde death of a significant proportion of spinal MNs and sensory neurons of the DRG, and produce changes in the spinal nerve circuits, in gene and protein expression, while inducing a neuroinflammatory response (Navarro et al., 2007). To face up with the injury, MNs shift to a pro-regenerative phenotype activating molecular pathways to promote neuronal survival and axonal regeneration with electrophysiological, morphological and molecular changes (González-Forero & Moreno-López, 2014; Navarro, 2009). The soma of axotomized MNs undergoes several morphological and phenotypic changes, known as neuronal reaction and chromatolysis. These changes are the dispersion of Nissl bodies that comprise the rough ER, the nuclear hypertrophy and eccentricity, the cell swelling and the retraction of dendritic arborization.

The axotomy causes a cascade of events in two distinct temporal phases. First, the rupture of the plasma membrane leads a disruption of the ion balance, a Ca^{2+} wave is rapidly propagated into the cell soma by voltage- gated Ca^{2+} channels and the release of Ca^{2+} from internal stores in the ER. This intracellular Ca^{2+} reaches millimolar concentrations and plays several key roles including, disassembly of the cytoskeleton, growth cone assembly, membrane resealing, activation of endogenous molecular programs such epigenetic modifications (Cho et al., 2013), changes the expression of hundreds of genes and increased the expression of immediate early of genes named as regeneration associated genes (RAGs)

(Ma & Willis, 2015; Mahar & Cavalli, 2018). RAGs include those genes related with neurotrophic factors and their receptors (BDNF, GDNF), cytoskeletal proteins (actin and tubulin) and growth-associated proteins (GAP-43). In this phase injured MNs shift from transmitting to regenerative state with a downregulation of genes that transcribe transmitter-related proteins such as choline acetyltransferase (ChAT) (Gordon, 2016). Then, late events are characterized by retrograde transport of several injury-responsive signalling proteins which include the molecular axis of ERKs, DLK, c-JUNK, the transcription factor signal transducer and STAT3 (Mahar & Cavalli, 2018).

PNI may produce the disconnection and subsequent degeneration of damaged neurons. Despite recent advances, the molecular process leading the retrograde MN death after proximal axotomy is still a matter of controversy. It has been described that in neonates axotomized MNs undergo apoptosis and the death is characterized by apoptotic hallmark cleaved caspase 3 (Romeo-Guitart et al., 2020; Sun & Oppenheim, 2003; Vanderluit et al., 2000). In contrast, adult MNs do not seem to die by apoptosis after root avulsion (Penas et al., 2011a). Nevertheless, some studies showed that MN degeneration is mainly by apoptotic (Martin et al., 1999; Wiberg et al., 2017) or necrotic (Li et al., 1998) process. Therefore, the survival or death of MNs after nerve injury is determined by the type of injury and their developmental stage. Recently it has been reported that several MN endogenous mechanisms of pro-death or pro-survival pathways are activated during early days post root avulsion. Their imbalance leads MNs towards its degeneration. The pro-death mechanisms are apoptosis, necrosis, anoikis, ER stress, nucleolar stress, cytoskeletal rearrangements, selective autophagy and mitochondrial dysfunction. While, the endogenous mechanisms of neuroprotection engaged are anti-apoptosis, anti-anoikis, anti-necrosis, the heat-shock response, autophagy, UPR, ROS hormesis and mitochondrial well function (Casas et al., 2015). In summary, root avulsion induces apoptotic pathways but also anti-apoptotic ones, and their balance leads the neurons to an alternative and unknown death that is not the classical apoptosis.

The ER is an organelle responsible for protein synthesis and Ca^{2+} homeostasis, and after a PNI there is a Ca^{2+} disturbance, which may lead to ER stress (Li et al., 2013; Ohtake et al., 2018; Oñate et al., 2016; Penas et al., 2011a). ER stress and accumulation of unfolded proteins also occur in several neurodegenerative diseases such as ALS or Parkinson disease (Hetz & Saxena, 2017; Medinas et al., 2017). ER stress induces the activation of endogenous mechanism including ER-overload response, ERAD pathways or UPR, which are highly conserved cellular responses. The UPR is triggered by BiP, an ER-resident chaperone that is

the main sensor, when unbound from three major effectors: RNA-activated protein kinase-like ER kinase (PERK), inositol-requiring protein-1 alpha (IRE1α) and activating transcription factor-6 alpha (ATF6) (Valenzuela et al., 2016). Those signaling pathways, englobed in these three branches, will lead to changes in the gene expression profiles of specific proteins (i.e. chaperones, transcriptional factors) and diverse cellular responses aiming to increase the capacity of the cell to restore homeostasis (Hetz & Saxena, 2017). Nevertheless, it is not clear how the activation of ER stress and the relevance of each specific UPR signaling branch is globally coordinated and may differentially contribute to the pathological process depending on the cell type affected and type of injury (Valenzuela et al., 2016). Recent studies have been demonstrated that target ER stress and UPR by pharmacological and genetic approaches may alleviate neurodegeneration improving motor recovery, axonal regeneration or MN survival after axonal injury (Oñate et al., 2016; Penas et al., 2011a).

PNI triggers a neuroinflammatory response in the vicinity of the affected MNs and in the dorsal horn projection areas of injured sensory afferents, the injury stimulates an intense glial reaction marked by the activation and proliferation of microglia and astrocytes. Astrocytes show hypertrophy and within a few days most neurons are extensively enwrapped by their processes. The microglia reaction around facial MNs following nerve injury was first reported by Blinzinger & Kreutzberg (1968) more than 50 years ago. Activated microglia undergoes changes in morphology and gene-protein expression, and surrounds the cell bodies, interposing themselves between the MNs and synaptic boutons participating in the synaptic stripping (Alvarez et al., 2020). Microglia function is also related with neuroprotection because the removal of synapses may protect from excitatory synapsis and releasing cytokines and growth factors. However, the significance of activated microglia surrounding axotomized MNs is not yet settled, it seems that the interactions between microglia polarization and MNs changed with time after axotomy (Rotterman & Alvarez, 2020). Furthermore, proinflammatory response after PNI is required for clearance of tissue debris (Wallerian degeneration) in the distal nerve segment and effective regeneration.

Overall, several mechanisms that are activated in MN degeneration after an axotomy are very similar to pathways activated in MN degeneration in ALS.

4. Models of motoneuron degeneration

Considering the importance of MND, experimental models are useful to elucidate the pathogenic mechanisms related to MN degeneration since they mimic these diseases, and to test new therapeutic strategies to improve MN survival and functional improvement. Notably, the identification of genetic mutations in ALS cases has facilitated the development of a wide range of MND models: *in vitro* and *in vivo* models in mice, rats, zebra fish and drosophila. Each model has its advantages and limitations, providing a translational value to the disease (Gois et al., 2020).

4.1 *In vitro* models

Studies in cell culture models allow to understand specific cellular mechanisms of neurotoxicity. The most simple models are the cell lines, such as NSC-34, which is a murine hybrid cell line obtained by fusion of neuroblastoma cells with motoneuron-enriched embryonic spinal cord cells (Cashman et al., 1992). Differentiated NSC-34 cells are commonly used in ALS research because they share several morphological and physiological characteristics with MNs, including generation of action potentials and respond to agents that affect voltage-gated ion channels, cytoskeletal organization and axonal transport. Although NSC-34 cells share many features with MNs like expressing glutamate receptor subunits, these cells are not suitable for glutamate-induced excitotoxicity studies because no specific effect of glutamate was observed on survival in these MN-like cells (Madji Hounoum et al., 2016). Additionally, NSC-34 cells allow their manipulation by molecular techniques to study ALS-associated gene function, or the toxicity related to the overexpression of WT or mutant proteins. NSC-34 cells transfected with *mSOD1* showed abnormalities in mitochondria morphology and function (Menzies et al., 2002), cytoplasmic inclusions and protein insolubility that correlated with toxicity (Turner et al., 2005), reproducing pathogenic mechanisms that take place in ALS.

Other culture model used to study the mechanisms underlying MND is the MN primary cell culture obtained from spinal cord of rat and mouse embryos. Although, the main limitation is that cells may be maintained only few weeks in culture, this kind of cultures are also very useful to evaluate neuroprotection or toxicity of different compounds to MNs and to investigate the role of mutant protein related with MN degeneration (Nagai et al., 2007; Petrozziello et al., 2017; Vincent et al., 2004). A primary co-culture of MNs and glial cells provides information

of the interaction between different cell types and the contribution of glial cells in the disease pathogenesis (Díaz-Amarilla et al., 2011).

One cellular model that is particularly suited for modelling glutamate neurotoxicity is the spinal cord organotypic culture (SCOC). Opposite to dissociated cell cultures, SCOC preserves the cytoarchitecture and cell interactions of the tissue, providing a more complex model. There is a well stablish rat model based on glutamate excitotoxicity by expose to glutamate (Guzmán-Lenis et al., 2009) or selective inhibitor of glutamate transport, DL-threo-β-hydroxyaspartic acid (THA) (Rothstein et al., 1993) which cause degeneration of spinal MNs over few hours or several weeks, respectively. It is a good model to assess MN degeneration and to evaluate the neuroprotective effects of novel therapeutic targets for MNs (Guzmán-Lenis et al., 2009; Herrando-Grabulosa et al., 2016).

A new model approach is the iPSCs allowing to study both fALS and sALS (Burkhardt et al., 2013; Dimos et al., 2008). iPSCs derived from reprogramming somatic cells of patients, usually fibroblast, could be differentiated into any cell type including MNs and glial cells (Meyer et al., 2014; Zhao et al., 2020). Further, fibroblast from transgenic mice has been reprogrammed into iPSCs- derived MNs (Yao et al., 2013). iPSC-derived lines from patients harbouring distinct ALS mutation are really helpful to define disease mechanisms recapitulating key aspects of the disease pathology and MN dysfunction such as the aggregation and/or cytoplasmic mislocalization of TDP-43, SOD1, FUS, and the formation of RNA foci and dipeptide repeat proteins in the case of C9orf72 repeat expansions (Burkhardt et al., 2013; Hawrot et al., 2020; Zhao et al., 2020). However, each line has a variable phenotype. iPSCs are a tool for finding therapeutic strategies (drug screening) and identification of biomarkers, highlighting that this model provides a powerful method to modelling the sALS forms.

4.2 *In vivo* rodent models

The discovery of mutations in genes that causes ALS has led to develop transgenic mouse models of the main ALS-related genetic mutations as *SOD1*, *C9orf72*, *TARDBP* and *h*(Gois et al., 2020; Lutz, 2018; McGoldrick et al., 2013). Since the first mutation identified in fALS was in the *SOD1* gene (Rosen et al., 1993), SOD1-based mice is the most explored model in ALS research. The first mouse model extensively characterized was $hSOD1^{G93A}$, which ubiquitously overexpresses the mutant human SOD1 protein carrying a substitution of glycine for alanine at position 93 (Gurney et al., 1994). These mice develop progressive MN loss and muscle denervation manifested as hindlimb weakness and deficits of locomotion by 3 months

of age which ultimately progress to muscle paralysis and the death at 4-5 months of age. Deficits in the function of spinal and cortical MNs are detected early using electrophysiological tests (Mancuso et al., 2011). Other mSOD1 mouse models have been studied, all developing progressive MN degeneration but varying in the onset of symptoms as well as the progression of the disease depending on the genetic mutation and level of transgene expression, the genetic background and the gender. This mouse recapitulates the most molecular and clinical features of the human disease, contributing to understand excitotoxicity, glia involvement, axonal transport deficits, mitochondrial abnormalities, among other alterations of the disease (Lutz, 2018).

Various mouse models based on transgenic expression of WT or mutant TDP-43 have also been created with variable phenotypes and grades of MN degeneration, indicating the importance of the level of transgene expression and the promoter used. The first mouse models used transgenic overexpressing strategies to drive mutant (A315T, M337V and Q3311K) TDP-43 cDNAs using the prion protein (Prnp) gene promoter. Prp-TDP-43^{A315T} transgenic mice accumulated pathologic aggregates of ubiquitinated proteins with selective vulnerability of cortical projection neurons and spinal MNs, leading to a severe motor phenotype characterized by neuronal morphological abnormalities, but without cytoplasmic TDP-43 aggregates (Wegorzewska et al., 2009). Recently, transgenic strategies such as specific promoter to drive doxycycline regulated expression or incorporating mutations by bacterial artificial chromosome (BAC) are used to generate TDP-43 transgenic mice which mimics motor impairments and progressive neurodegenerative ALS-like phenotype (Lutz, 2018). Besides mice, transgenic rats ALS models were developed with mSOD1 (Nagai et al., 2001) and mTDP-43 (Zhou et al., 2010), which develop MN degeneration and display paralysis as well.

The high prevalence of C9orf72 mutation causing ALS forms and ALS/FTD has led to a significant interest in pursuing pathogenic mechanisms associated with the G4C2-repeat expansion and several recent studies have been reported different approaches to produce C9orf72 models (Batra & Lee, 2017). Nevertheless, there are discrepancies in neuropathological and disease manifestation between developed mouse models. Some investigators used BAC to generate transgenic mice carrying the G4C2 expansion, which develop widespread RNA foci and RAN proteins aggregates but lack neurodegeneration and clinical features (O'Rourke et al., 2015; Peters et al., 2015). After, Liu et al. (2016) reported the generation of the first FAV-C9orf72 BAC mouse model that recapitulates molecular, pathological and phenotypic hallmarks of ALS/ FTD such as paralysis, muscle denervation,

MN loss, reduced lifespan, TDP-43 inclusions and cortical and hippocampal neurodegeneration, providing an important tool to study this pathology.

As a means of clarifying the mechanisms through *FUS*, *UBQLN2* and *PFN1* mutation cause ALS, animal models with these mutations have been generated. They have a heterogeneous phenotype but most of them have several clinical and pathological features alike human ALS (Lutz, 2018; Mancuso & Navarro, 2015).

The wobbler mouse has been proposed as an alternative experimental model of MND due to its clinical symptoms and prevalent neuropathology. In this mouse, first manifestations of the disease appear at 3 weeks of age, since then, it exhibits progressive locomotion impairment. The *wobbler* mutation causes cellular transport defects, ER and mitochondrial stress, TDP-43 accumulation, neuronal hyperexcitability and neuroinflammation closely resemble to human ALS (Moser et al., 2013). The wobbler mouse offers a model of sALS for testing therapeutic approaches, even though there was no identified a comparable Vsp54 gene mutation in ALS patients.

As referenced earlier, preclinical models of PNI have been used to study molecular mechanisms involved in MNs degeneration due to the disconnection between MNs and their target organ promote changes and MN death. Diverse rodent models have been proposed to mimic proximal PNI, differences between experimental models are observed because many factors involving the severity of the injury, the distance from the soma and the age of the animal modulate the consequences of the lesion (Koliatsos et al., 1994). Moreover, the divergence includes the methodology of root injury, the localization and number of nerve roots affected. One model commonly used is the mechanical traction (avulsion) or traumatic axotomy (transection) of brachial and lumbar plexus or roots, which represent complete nerve transection called neurotmesis. After this injury, surgical repair is needed to re-establish epineural continuity and MN preservation (Eggers et al., 2016). Spinal root injuries without re-implantation are a suitable tool for studying cell death mechanisms and novel therapies which promote MN survival. Therefore, data obtained from these investigations might be used to reveal process that underline degeneration of MNs and therefore could be translational to MND.

5. Sigma-1 receptor

Sigma-1 receptor (Sig-1R) was originally classified as a member of the opioid receptor family because it has high-affinity to opioid ligand SKF-10047 (Martin et al., 1976). Later, molecular and pharmacological studies suggest that Sig-1R is not a traditional receptor and is considered a non-G-protein coupled, non-ionotropic intracellular chaperone with multiple biological functions, including Ca^{2+} homeostasis and neuronal excitability, many of which are relevant to MND. In 1996, Sig-1R was first cloned from liver of guinea pig as 223 amino acid transmembrane protein without homology with any mammalian protein (Hanner et al., 1996). Recently by X-ray crystallography, the structure of the human Sig-1R has been reported in a complex with two different ligands as a membrane-bound trimeric assembly with a single transmembrane region (Schmidt et al., 2016). Nevertheless, the structure of Sig-1R provokes debate because sequence-based prediction structure studies determine two transmembrane domains (Penke et al., 2018).

Many tissues ubiquitously express Sig-1R, including the nervous system, liver and kidney (Alonso et al., 2000; Hanner et al., 1996). In the CNS, Sig-1R is expressed in neurons and in glial cells, particularly it is highly enriched in the ER of MNs in the brain stem and in the spinal cord. At the subcellular level, Sig-1R is located at ER in close contact with cholinergic post-synaptic sides (Mavlyutov et al., 2010) and in the specialized subcomponent called mitochondrial-associated ER membrane (MAM) (Hayashi & Su, 2007). Upon stimulation, Sig-1R dynamically translocate inside cells from ER to plasma or nucleus membrane where it interacts with other receptors and ion channels or with nucleus factors regulating gene transcription, respectively (Mavlyutov et al., 2017; Su et al., 2016). This enigmatic chaperone protein has been involved in various pathological conditions ranging from neurodegenerative disease to drug addiction and cancer.

The generation of homozygous Sig-1R KO mouse, which is viable, fertile and without any apparent phenotype changes compared with their WT littermates, contributed to better understand the role of this receptor (Langa et al., 2003). During the last years, analysis of Sig-1R KO mice revealed that absence of Sig-1R induces various behavioural changes which are consequences of numerous cell type alterations. Sig-1R KO mice studies showed that these mice have alterations in hippocampal neurogenesis and exhibit a depressive-like phenotype (Sha et al., 2015), retinal dysfunction (Ha et al., 2011; Mavlyutov et al., 2011), diminished response to pain (Cendán et al., 2005) and present differences in motor behaviour associated with muscle weakness and MN loss (Bernard-Marissal et al., 2015; Mavlyutov et al., 2010),

among others. Changes and alterations are summarized in Figure 5. It is proposed that those cell type alterations share a common origin, the misregulation of cell stress (Couly et al., 2020).

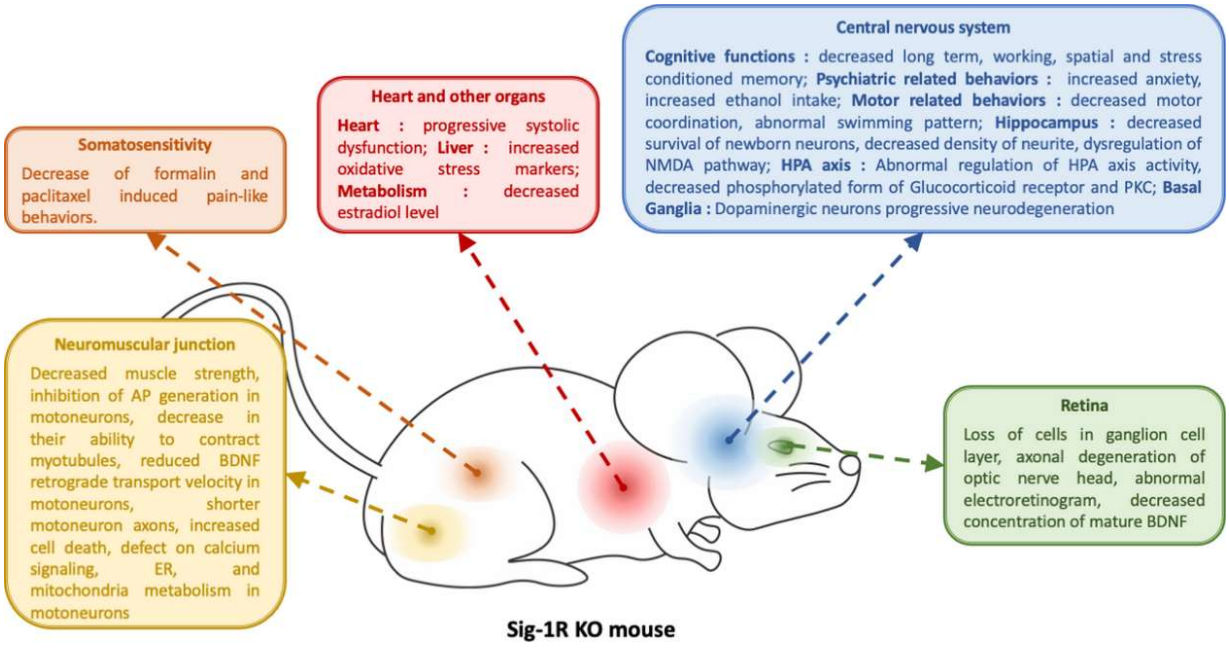


Figure 5. The lack of Sig-1R in mice induce these defects in the body. Several studies reveled that the absence of Sig-1R cause diverse health problems. AP action potential, HPA axis hypothalamic–pituitary–adrenal axis. Extracted from Couly et al. (2020).

Numerous synthetic compounds have been characterized as selective modulators of Sig-1R activities (Table 2), including antidepressants (fluoxetine), antipsychotics (haloperidol)+ and drugs of abuse (cocaine). There is currently no known endogenous ligands exclusive to the Sig-1R, but a variety of endogenous molecules may function as Sig-1R ligands with varying affinities and functionalities, such as steroids (progesterone, dehydroepiandosterone (DHEA) and pregnenolone sulfate), lipid sphingosine derivates, N,N-dimethyl tryptamine (DMT) and choline (Brailoiu et al., 2019; Fontanilla et al., 2009; Mancuso & Navarro, 2017). Interestingly, Indole-N-methyl transferase (INMT), enzyme that converts the amino acid tryptophan into DMT, co-localizes with Sig-1R at C-terminals of MNs (Mavlyutov et al., 2012), implying the local synthesis of this endogenous ligand that regulates the receptor. Many laboratories have been working in understanding the molecular basis of Sig-1R and how is endogenously modulated at physiological or pathological conditions. Based on their biological activity, Sig-1R ligands are classified into agonists and antagonists (Maurice & Su, 2009), however this classification has a lot of controversy.

Remarkably, two subtypes of sigma receptor have been described from pharmacological radioligand-binding studies: Sig-1R and Sigma-2 receptor (Sig-2R) (Hellewell et al., 1994). Both receptors differ in ligand selectivity and function, the activity of Sig-2R is unaffected in Sig-1R KO mice, thereby validating the existence of a second receptor (Langa et al., 2003). However, Sig-2R subtype is less studied than Sig-1R because its gene was recently identified from calf liver and distinguished as transmembrane protein 97 (TMEM97) located in ER (Alon et al., 2017). Sig-2R is involved in cell proliferation, overexpressed in several cancers, and appeared to be linked to neurodegenerative diseases such as Alzheimer disease.

Table 2. Summary of main agonist and antagonist of Sig-1R synthetic and endogenous ligands.

	Sig- 1R ligands	
	Agonist	Antagonist
Synthetic	PRE-084 (2-(4-Morpholinethyl) 1-phenylcyclohexanecarboxylate hydrochloride) SA4503 (Cutamesine) Pridopidine Pentazocine SK-10047 Fluvoxamine Propranolol Cocaine Methamphetamine Memantine Fluoxetine ANVEX-41 (mixed mAChR/σ1 receptor) ANAVEX-71 (mixed mAChR/σ1 receptor) ANAVEX$_2$-73 (blarcamesine) DTG (1,3-di-o-tolyguanidine) Donepezil	BD1063 BD1047 S1RA or MR309 (E-52862) Haloperidol NE-100 PD144,418
Endogenous	Pregnenolone sulfate Dehydroepiandosterone (DHEA) Choline DMT (N,N-Dimethyltryptamine)	Progesterone

Data extracted and adapted from Penke et al. (2018).

5.1 Sig-1R and biological functions in MNs

Sig-1R is a pluri-functional protein implicated in the regulation of multiple cellular processes like Ca^{2+} homeostasis, ER-mitochondria communication, neuritogenesis, modulation of neuronal excitability, regulation protein degradation and reducing oxidative stress like, among others (Mancuso & Navarro, 2017). According to subcellular localization, Sig-1R is present at MAM, but when activated, Sig-1R could be translocated to different subcellular

compartments interacting with other proteins from ion channels to transcriptional factors increasing Sig-1R spectrum of functions (Benarroch, 2018; Penke et al., 2018; Weng et al., 2017).

5.1.1 Sig-1R and the MAM

Sig-1R is mainly located at MAM (Hayashi & Su, 2007), which is a specific area that allow the crosstalk between ER and mitochondria because both organelles physically interact. This structure plays an important role controlling lipid synthesis, Ca^{2+} homeostasis, bioenergetics, protein folding, autophagy initiation and cell survival (Hayashi et al., 2009). At MAM, ER proteins interact with proteins of outer mitochondrial membrane (OMM), there are five ER-mitochondria tethering protein complexes: IP_3R3 (inositol 1,3,5-triphosphate receptor 3)/VDAC, MFN2/MFN1-2, VAPB/PTPIP51, ORP5-8/PTPIP51 and BAP31/FIS1 (Paillusson et al., 2016)(Figure 6A).

Physiologically at MAM, Sig-1R binds to BiP (also known as GRP78) preventing Sig-1R translocation. Nevertheless, several stimuli of ER stress or activation by ligand binding triggers the dissociation of both proteins and allows Sig-1R to act as a chaperone participating in multiple downstream pathways. Sig-1R binds to and stabilizes IP_3R3 which is involved in regulating Ca^{2+} influx from the ER into the mitochondria, thereby promoting normal mitochondrial function such as ATP production and cell survival (Hayashi & Su, 2007). Upon ER Ca^{2+} depletion and Sig-1R activation, it interacts with IP_3R3 and Ca^{2+} releases from the ER to the cytosol through this channel and is immediately captured by VDAC1 at the OMM and mitochondrial Ca^{2+} importer (MCU) at the inner mitochondrial membrane (IMM) (Figure 6B).

Furthermore, Sig-1R as a chaperon modulates UPR induced by ER stress. The three major sensors of the UPR are PERK, IRE1α and ATF6. IRE1α is one of three ER stress sensors specifically located at the MAM to respond to stress caused by mitochondria or ER-derived ROS. Under ER stress or ligand stimulation, Sig-1R binds to IRE1α regulating the stability of it and enhancing the survival pathway IRE1-XBP1 to restore the ER homeostasis (Mori et al., 2013) (Figure 6C).

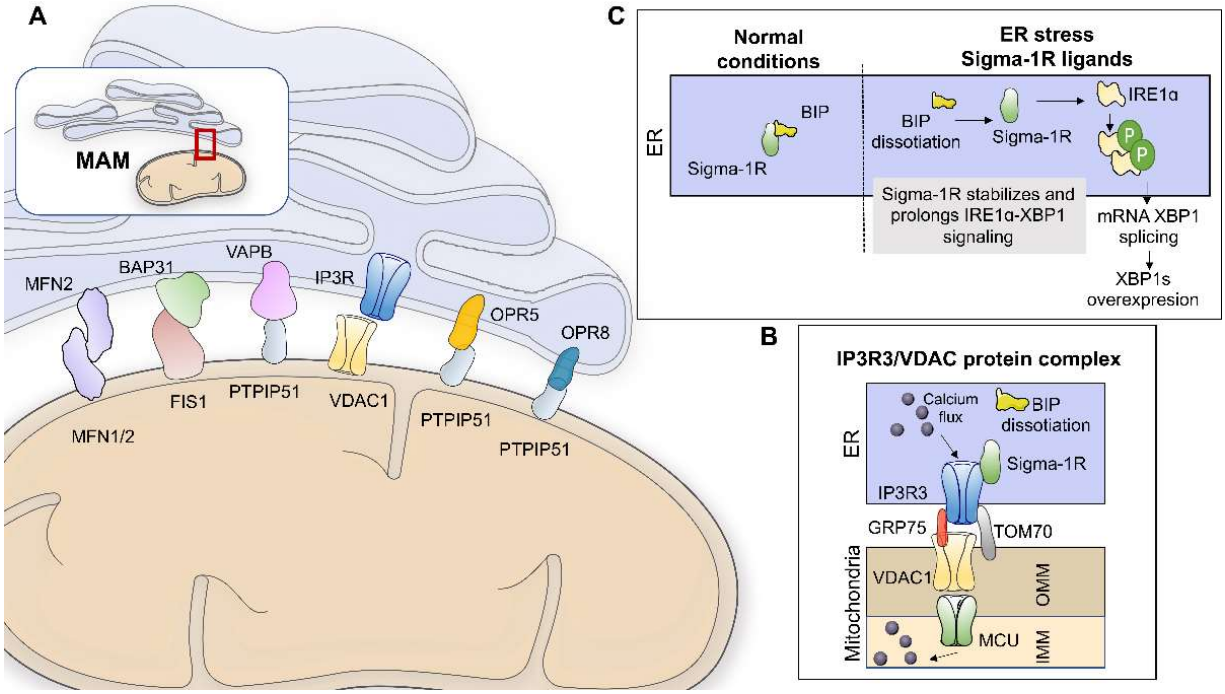


Figure 6. ER-mitochondria tethering protein complexes involved in the MAM signalling. A) The main proteins involved in each complex: MFN2/MFN1-2, BAP31/FIS1, VAPB/PTPIP51, ORP5-8/PTPIP51 and IP_3R/VDAC. B) Detail of the proteins participating in the IP_3R3/VDAC complex in which Sig-1R plays a key role for regulating Ca^{2+} influx into the mitochondria. C) Sig-1R under normal conditions is recruited by BiP, however under ER stress or upon ligand binding, Sig-1R stabilizes and prolongs the survival pathway IRE1-XBP1. IMM inner mitochondrial membrane, OMM outer mitochondrial membrane. Extracted from Herrando-Grabulosa et al. (2021).

5.1.2 Sig-1R interaction with plasma membrane

Sig-1R is highly enrich in the subsurface cisternae of ER adjacent to the plasma membrane in MNs (Mavlyutov et al., 2012) and its activation can also disassociate from BiP and translocate the receptor to the plasma membrane. This allows the interaction of Sig-1R with several proteins that resides in the plasma membrane such as ion channels, ion channel receptors, G protein–coupled receptors, and tyrosine kinase receptors. It has been described that Sig-1R interacts with acid-sensitive cation channels (ASIC), voltage-gated and ligand-gated ion (Na^+, Ca^{2+} and K^+) channels (Kourrich, 2017) and modulates the activity of GluN1 subunit NMDA receptor, muscarinic type-2 acetylcholine receptors (M2AChR)(Hellström et al., 2003), dopamine D1-2 receptors (Navarro et al., 2010), opioid receptor and tyrosine kinase receptors such as TrkB, regulating neuronal activity from synaptic transmission to intrinsic excitability. Further studies are needed to understand how Sig-1R regulates these complex effects because it may be involved by direct or indirect interactions and depends on each cell type.

5.1.3 Sig-1R interaction with nuclear envelope

The Sig-1R is also translocated to the nuclear membrane, specifically it resided in the nucleoplasmic reticulum which is a specialized invagination of the nuclear envelope into the nucleoplasm (Mavlyutov et al., 2017). Upon stimulation with ligands of Sig-1R like cocaine, it translocates and interacts with Emerin, an inner nuclear envelope-resident protein. Then, their recruit various chromatin-remodelling proteins resulting in Sig-1R/Emerin/BAF/HDAC complex which regulates gene transcription (Tsai et al., 2015). Recent studies also suggested that Sig-1R exists in the nuclear pore chaperoning RanGTP-activating protein (RanGAP) and certain nucleoporins (Lee et al., 2020).

5.2 Sig-1R contribution to MND

5.2.1 Mutations

SIGMAR1 mutations have been identified in different MNDs, the first mutation was described in the 3'-untranslated region (UTR) of the gene in patients of ALS-MND pedigree (Luty et al., 2010). Authors suggest that these variants affected the stability of Sig-1R transcripts increasing its gene expression. Brains of these patients displayed unique neuropathological features with cytoplasmic inclusions for either TDP-43 or FUS, but not for Sig-1R. Other genetic variant responsible for the disorder has been reported in the highly conserved transmembrane domain of *SIGMAR1* in juvenile-onset ALS family (Al-Saif et al., 2011), classified as ALS-16. *In vitro* studies with NSC-34 cells testing this E102Q autosomal recessive mutation showed an aberrant membrane distribution of Sig-1R and enhanced apoptosis induced by ER stress with this variant. In kind, the overexpression of Sig-$1R^{E102Q}$ in neuroA2 cells induced mitochondrial impairment impairing ATP production and compromised proteasome activity (Tagashira et al., 2014). A mutation of ALS-16 linked was reported as a loss of function of Sig-1R which induced collapse of MAM because Sig-1R was unable to bind with IP_3R3 (Watanabe et al., 2016).

Distal hereditary motor neuropathies (dHMN) are another phenotype of MND in which several mutations *SIGMAR1* have been found using whole-exome sequencing and homozygosity mapping techniques. To date, these mutations were reported in some families or case report patients with different ethnic populations (Almendra et al., 2018; Gregianin et al., 2016; Li et al., 2015; Ververis et al., 2019). To further investigate the pathophysiological role of Sig-1R, these mutations where evaluated *in vitro* showing that variant mutations affected cell viability, induced RE stress (Li et al., 2015), caused protein mislocalitzation out of MAM and boosted

autophagy (Gregianin et al., 2016). All these findings provide evidence for the involvement of Sig-1R in MN maintenance and its role in MND.

5.2.2 MND pathogenesis in which Sig-1R is involved

Many studies reveal that Sig-1R alterations trigger MN dysfunction and degeneration. The levels of Sig-1R protein were found reduced in the lumbar spinal cord of ALS patients (Prause et al., 2013). In addition, abnormal accumulation of Sig-1R in ER structures and enlarged C-terminals was found in α-MNs during ALS pathogenesis. Remarkably, these alterations were found in distinct ALS models: SOD1 mice, cultured ALS-8 fibroblast's patients with VAPB mutation and NSC-34 cells. Prause et al. (2013) described that shRNA knockdown of Sig-1R in NSC-34 cells led UPR induction and UPS inhibition, causing cell death and accumulation of ER-derived vacuoles associated with autophagy. In the same line, other studies identified changes in C-boutons morphology in MND models (Casas et al., 2013; Pullen & Athanasiou, 2009). In addition, it was found a reduction in protein level and abnormal distribution of Sig-1R at C-boutons in $SOD1^{G93A}$ mice even during early pre-symptomatic stages (Casas et al., 2013), SMA mice model (Cerveró et al., 2018) and wobbler mice where Sig-1R had a diffuse pattern (Peviani et al., 2014). Contrary, other studies found that Sig-1R protein levels were not changing during the disease progression in the spinal cord (Mancuso et al., 2012). Dukkipati et al. (2017) showed that there is no change in C-boutons size during the ALS progression, although the number and density of them are reduced at end stage in SOD1 mice. The possible reason for these failure replication results may be due to variability in analyses and experimental design.

The cholinergic C-boutons are large terminals that arise from local interneurons and specifically contact with soma and proximal dendrites of α-MNs (Deardorff et al., 2014; Hellström et al., 2003). The postsynaptic sites, C-terminalis, have a unique ultrastructure seen at the electronic microscopy level consisting of subsurface cisternae of smooth ER adjacent to the plasma membrane where Sig-1R is highly enrich (Mavlyutov et al., 2012). The postsynaptic membrane contains a high number of proteins, including M2AChR (Hellström et al., 2003), VAMP-2, Ca^{2+} activated K^+ (SK) channels (Deardorff et al., 2013), SK K^+ channels (Miles et al., 2007), voltage-gated Kv2.1, Kv1.4, Kv1.5, connexin 32 and neuregulin-1 (NRG-1) (Gallart-Palau et al., 2014), while in the presynaptic membrane are NRG-1 receptors, ErbB2 and ErbB-4. Sig-1R interact with all these postsynaptic proteins, modulating several events.

Excitability alterations play an important role in MND and C-terminals are implicated in regulating MN excitability. Cholinergic activation of M2AChR leads to increases the

excitability of MNs through reducing a SK-type channels conductance, decreasing after hyperpolarization amplitude (Miles et al., 2007) which results in a higher frequency of MNs firing. Sig-1R can modulate SK channels and a variety of Kv type channels activity (Kourrich, 2017). For instance, under control conditions Sig-1R interacts with Kv1.2, whereas ligand activation of the Sig-1R or presence of Sig-1R-^{E102Q} mutation inhibits its K^+ channel current (Abraham et al., 2019). As mention earlier, Sig-1R KO mice had light motor deficits but did not itself result in ALS phenotype (Mavlyutov et al., 2010). Afterward, same authors demonstrated that MNs firing frequency of Sig-1R KO mice was significantly higher than WT mice, increasing neuronal excitability. Moreover, crossing KO mice with SOD1^{G93A} mouse model exacerbated the motor phenotype and decreased lifespan (Mavlyutov et al., 2013). Furthermore, MNs from Sig-1R KO mice induced less contractions of myotubules than MNs from WT mice (Ionescu et al., 2019).

Deregulation of MAM function and ER-mitochondrial connectivity have been proposed as key mechanisms in neurodegenerative disorders. An *in vivo* and *in vitro* models of loss-of-function of Sig-1R, pharmacological by antagonist NE-100 or KO mice, demonstrated ER-mitochondria disconnection resulting in Ca^{2+} homeostasis impairment, ER stress activation and mitochondrial dynamics and transport alteration. All these cellular changes may lead to axonal and MN degeneration, which are restored by Ca^{2+} scavenging and ER stress inhibition (Bernard-Marissal et al., 2015). In the same line, *in vitro* studies found that Sig-1R disfunction provokes aggregates of its proteins, ER stress activation and Ca^{2+} homeostasis defects (Dreser et al., 2017; Hayashi & Su, 2007; Prause et al., 2013). The collapse of MAM has been reported as a common pathomechanism in *SIGMAR1*- and *SOD1*-linked ALS models, either deficiency of Sig-1R or accumulation of mSOD1 induced IP_3R3 mislocalitzation and MAM disruption. Nevertheless, PRE-084, a Sig-1R agonist, administration restored the Sig-1R and IP_3R3 interaction in SOD1^{G93A} mice (Watanabe et al., 2016). The disruption by deletion either mutation of MAM proteins such VAPB, PTPIP51 and MFN2 is sufficient to cause Ca^{2+} signalling defects (De Brito & Scorrano, 2008; De Vos et al., 2012). Highlighting the importance of Ca^{2+} homeostasis and ER and mitochondrial function regulation for maintenance of MN integrity.

In MNDs, there is an increase of microglia and astroglia reactivity with a disruption in the neuroprotective/toxic phenotype in the spinal cord. Sig-1R is also expressed in microglia and astrocytes and may modulate their activation. *In vivo*, Sig-1R modulation by treatment with agonist ligands has been shown to regulate multiple aspects of glial activity. In wobbler mouse model, the activation of Sig-1R receptor by PRE-084 decreased reactive astrocytosis

and modulated microglial activity by enhancing its anti-inflammatory M2 phenotype and reducing its inflammatory response (M1) (Peviani et al., 2014). Similarly in the mSOD1-ALS model, PRE-084 administration reduced microglial but not astroglial reactivity (Mancuso et al., 2012). Also, this agonist decrease astrogliosis after spinal root avulsion (Penas, et al., 2011b). Of interest, PRE-084 treatment in SMA mice attenuated reactive gliosis and restored the altered M1/M2 phenotype balance, promoting anti-inflammatory action through M2 polarization (Cerveró et al., 2018). The effects of the Sig-1R modulation by its ligands and in Sig-1R KO mice need to be further evaluated to clarify the role of this receptor in astrocytes and microglia in pathological states.

Overall, based on these evidence Sig-1R is involved in several MND pathophysiology. The loss or the gain of function, or both, of Sig-1R may alter chaperon role causing alterations in Ca^{2+} signalling, ER stress, imparted proteostasis, etc. Revelling, that Sig-1R may confer therapeutic effects by modulating mechanisms that are common across a wide array of neurodegenerative conditions.

6. Relevant therapies for MND

One of the main concerns for developing novel therapies is the poor direct translation from favourable results in preclinical models of MND to successful clinical results in humans. Although, multiple cellular processes have been well characterized to be involved in ALS pathophysiology, the concrete mechanism that cause the disease remain unknown. The main achievement is to develop therapeutic strategies to prevent disease progression or to extend survival longer than achieved by Riluzole and Edaravone, the only two drugs approved by the FDA for ALS patients. These both small molecule drugs are non-curative treatment and modestly change the clinical course of the disease improving the quality of life of patients few months. The mechanism of action of Riluzole and Edaravone is based on anti-glutamatergic and antioxidant action, respectively, acting on a single pathway while ALS is a multifactorial disease. For this reason, therapeutic approaches should be addressed to several molecular targets that underlie the MN degeneration. Currently, a wide range of therapies covering pharmacological, gene therapy and cell therapies strategies have been assayed or developed for ALS patients. In addition, drug repurposing is also being investigated to rapidly identify effective therapies in ALS.

Several compounds are being investigated as a potential treatment in clinical trials with ALS patients, described in Filipi et al. (2020) and Mancuso & Navarro (2015) reviews. Despite the

fact that most of the tested drugs have been demonstrated positive results in pre-clinical models and have been proven to be clinically safe and tolerable by humans, the vast majority of the trials failed to demonstrate clinical efficacy. Compounds tested were divided into categories based on their effect: anti-glutamatergic (Ceftriaxone and Talampanel) or antioxidative properties (Coenzyme Q10 and Creatine), the capacity to regulate immune response (Celecoxib, Glatiramer Acetate, minocycline), among other groups (Figure 7).

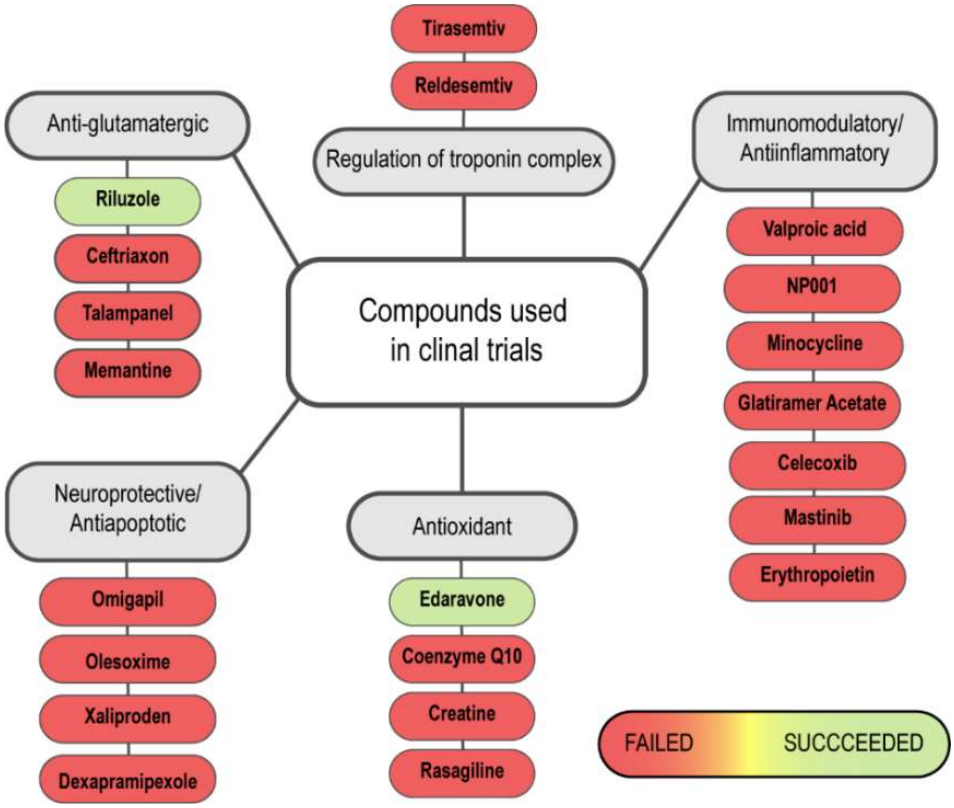


Figure 7. Overview of some drugs used in clinal trials for ALS. Compounds are classified according to their effect or pathway implicated. Picture adapted from Filipi et al. (2020).

Recently, it has described that immune cells like mast cells and macrophages play a role in degenerating motor nerve endings and motor endplates in SOD1^{G93A} rat model skeletal muscle (Trias et al., 2018). Masitinib, a highly specific tyrosine kinase inhibitor, has been demonstrated to slow the progression of the disease and reduce neuroinflammation by targeting microglia, macrophages and mast cell activity. Masitinib underwent a Phase II/III trial with 394 participants, which compared Masitinib in combination with Riluzole versus a Riluzole-placebo and showed that Masitinib at 4.5 mg/kg/d can benefit patients with ALS slowing the disease progression (Mora et al., 2020).

Considering the failure of neuroprotective agents over the past two decades to provide a cure, gene therapies approaches have emerged for the treatment of ALS, involving the delivery of antisense oligonucleotides. This kind of therapy had favourable results in SMA treatment (Mercuri et al., 2018). Antisense oligonucleotides have been tested in clinical trials for patients with *SOD1* or *C9orf72* gene mutations (Cappella et al., 2019).

The failure of possible treatment for ALS can be linked to late diagnosis and the complexity of the disease. New challenges involve the development of reliable biomarkers to early diagnose the pathology and then, sooner therapies will produce better results. Various CSF biomarker candidates have been suggested, but only few have successful. Growing evidence has demonstrated that the levels of neurofilament light chain and phosphorylated neurofilament heavy are increased in the CSF and blood of ALS patients. Neurofilaments, which are neuron- specific cytoskeletal proteins, are promising biomarkers because their increased levels allow to discriminate ALS form other diseases mimics. Levels correlates with disease progression and different phenotypes of ALS, which might permit diagnosis and prognosis (Gagliardi et al., 2019; Gaiani et al., 2017; Steinacker et al., 2016). Although, CSF neurofilament levels are not used to standard clinical practice, in clinal trials neurofilaments are sometimes used to assess individual prognosis and response to experimental drugs. RNA dysregulation has been shown to be involved in the pathogenesis, and extracellular proteins and RNAs, including mRNA, micro RNA, and circular RNA are considered potential biomarkers in ALS (Hosaka et al., 2019). Nowadays, a wide range of molecules such neuroinflammatory molecules have been proposed as novel biomarkers, but further investigations are needed.

6.1 Therapeutic implications targeting Sig-1R in MND: Sig-1R ligands

Sig-1R has been recognized increasingly as a novel target for treatment of neurodegenerative diseases due the fact that Sig-1R ligands have been demonstrated therapeutic potential in a variety of situations. Highlighting, neuroprotective effects of Sig-1R ligands have been found in animal models of pathologies such stroke, Huntington disease (Hyrskyluoto et al., 2013), Parkinson disease (Haga et al., 2019), glaucoma (Mysona et al., 2017), cardiac pathologies (Lewis et al., 2020), Alzheimer disease, ALS, etc. Right now, Sig-1R ligands are in clinical trials for the treatment of ischemic stroke (Urfer et al., 2014), chemotherapy-induced neuropathic pain (Bruna et al., 2018), Huntington disease (Reilmann et al., 2019) and ALS (Ionescu et al., 2019), and Alzheimer's disease (Hampel et al., 2020) with different ligands SA4503, S1RA (MR309), pridopidine and ANVEX$_2$-73, respectively. In June 2020, the FDA

approved the first Sig-1R targeted drug, Fintepla (fenfluramine, ZX008), to treat seizures associated with Dravet syndrome which is a rare, drug-resistant epilepsy and cannot be effectively treated with existing anti-epilepsy medicines. Fenfluramine demonstrated modulatory activity at Sig-1R (Strzelczyk & Schubert-Bast, 2020). For these reasons, ligands of Sig-1R, both agonists and antagonists, are of great interest as potential therapeutic agents against CNS disorders.

Sig-1R ligands have been classified as either agonists or antagonists based on BiP binding assay (Hayashi & Su, 2007) and their effects on animal models in the ability to recapitulate the effects of genetic KO or knockdown of Sig-1R. However, it has been actively questioned the precise molecular definition of Sig-1R agonists or antagonists. Generally, main studies shown that Sig-1R activation is associated with neuroprotection whereas deficits in Sig-1R level or activity are associated with neurodegeneration. In concordance, data with Sig-1R agonist report beneficial effects in several models while there are less studies showing these effects with antagonists. However, there is no clear consensus on the type of Sig-1R ligands that may be more effective to prevent neurodegeneration. Different Sig-1R ligands may act differently, and even contrarily, on these protective mechanisms. A recent work reported that the SA4503 ligand accelerated the cytosolic Ca^{2+} clearance after AMPA receptor activation and IP_3R-mediated ER Ca^{2+} release in G93A mice cultured MNs, whereas the agonist PRE-084 did not modify the cytosolic Ca^{2+} levels (Tadić et al., 2017). These observations suggest that different Sig-1R ligands may act differently on the Ca2+ homeostasis.

6.1.1 Neuroprotective effects of Sig-1R ligands in MND models

Sig-1R ligand treatment has been reported to exert positive effects in several experimental models of MN degeneration. *In vitro*, PRE-084 exerted neuroprotection and neurite elongation activating protein kinase C (PKC) in SCOC excitotoxic damage (Guzmán-Lenis et al., 2009). P56S point mutation causes severe misfolding of the peptide and leads to the formation of cytoplasmic inclusion bodies in fALS, the activation of Sig1-R by PRE-084 in P56S-VABP NSC34 cells ameliorated mutant VAPB aggregation and restored mitochondrial activity alleviating the ER stress exerted by mutant aggregates (Prause et al., 2013). SA4503, another agonist, protected NSC34 cells against $SOD1^{G93A}$-induced cell death activating Akt and ERK1/2 (Ono et al., 2014).

In vivo after a root avulsion in adult rats, PRE-084 administration prevented MN death by increasing GDNF expression in astrocytes (Penas, et al., 2011b). In a murine model of ALS, Sig-1R agonists enhanced preservation of spinal MNs and extended the lifespan of $SOD1^{G93A}$

mice: PRE-084 via modulation NMDA Ca^{2+} influx (Mancuso et al., 2012), pridopidine and SA4503 through the activation of the ERK pathway (Ionescu et al., 2019; Ono et al., 2014). Furthermore, in a model of spontaneous MN degeneration, the wobbler mice, PRE-084 improved MN survival and significantly increased BDNF levels in the gray matter in wobbler mouse (Peviani et al., 2014). All these results illustrate the connection between Sig-1R and neuronal survival. Highlighting that in 2021, Sig1-R ligand pridopidine was added as a one of the four therapies included in the HEALEY ALS platform trial, registered as NCT04297683. In this clinical trial multiple investigational products for ALS will be tested simultaneously as a way to speed the development of those that appear most promising and as new investigational products become available, additional regimens will be added to the trial. Each investigational product will be tested in a regimen and each regimen consists of a placebo-controlled trial, meaning that the active investigational product and matching placebo will be tested in each regimen.

Remarkably, as mentioned above, Sig-1R ligands could modulate the immune response changing glia activation and phenotype in MND models (Cerveró et al., 2018; Mancuso et al., 2012; Penas et al., 2011b). In summary, Sig-1R is a multifunctional protein involved in several cellular processes and its modulation may participate in two interconnected neuroprotective mechanisms, the regulation of neuronal excitability and of Ca^{2+} homeostasis, with an additional contribution changing phenotype of glial cells in pathologic conditions.

III. HYPOTHESIS & OBJECTIVES

In this thesis we hypothesized that Sig-1R ligands target several events involved in the pathophysiology of MN death and may be effective preventing the MN degeneration process.

The main objective of this thesis was to assess whether Sig-1R ligands can significantly increase the survival and function of spinal MNs on the MND models used. To address this general aim, the thesis has been divided in three chapters with the following specific objectives:

Chapter I: Sigma-1 receptor is a pharmacological target to promote neuroprotection in the SOD1^{G93A} ALS mice.

- To assess the neuroprotective effects of three Sig-1R ligands, PRE-084, BD1063 and SA4503, in spinal cord organotypic culture under (SCOC) chronic excitotoxicity.
- To evaluate the contribution of PRE-084, BD1063 and SA4503 treatments to improve motor function and MN survival in SOD1^{G93A} mice.
- To elucidate molecular pathways implicated in the neuroprotective effects of Sig-1R ligands in SOD1^{G93A} mice.

Chapter II: Neuroprotective effects of sigma-1 receptor ligands on motoneuron death after spinal root injury in mice.

- To characterize the progressive MNs death following L4-L5 rhizotomy in mice.
- To test the potential therapeutic effect of Sig-1R modulation on MN preservation after rhizotomy.
- To elucidate the molecular mechanisms implicated in the neuroprotective effects observed in the spinal root injury model.

Chapter III: EST79232 and EST79376, two novel sigma-1 receptor ligands, exert neuroprotection on models of motoneuron degeneration.

- To assess the neuroprotective effects of new synthetized Sig-1R ligands, EST79232 and EST79376, in the SCOC subject to chronic excitotoxicity.
- To test the potential therapeutic effect of Sig-1R ligands, EST79232 and EST79376, on MN preservation following rhizotomy.
- To evaluate the therapeutic potential of the administration of EST79232 and EST79376 in SOD1^{G93A} mice.

IV. STUDY DESING & METHODOLOGIES

In this section there is an overview of the study design and main methodologies performed in each of the three chapters.

Chapter I: Sigma-1 receptor ligands is a pharmacological target to promote neuroprotection in the SOD1^{G93A} ALS mice.

The first chapter of this thesis was focused on evaluating and comparing the treatment with three different Sig1-R ligands, BD1063, SA4503 and PRE-084, in experimental models of MN degeneration.

1. EXPERIMENTAL MODEL

An *in vitro* and *in vivo* model of MN degeneration were used:

A) *In vitro* model: SCOC. SCOCs subject to chronic excitotoxicity caused by THA was used to compare the effect the Sig-1R agonists PRE-084 and SA4503, and the antagonist BD1063 on MN survival. At 28 DIV (days *in vitro*) SCOCs were fixed with paraformaldehyde (PFA) to obtain the slice samples.

B) *In vivo* model: SOD1^{G93A} mice. The ALS mouse model used was the transgenic SOD1^{G93A} mice (B6SJL-Tg [SOD1-G93A]1Gur), that replicates the most relevant phenotypical and histopathological features of the human disease. Different groups of SOD1^{G93A} female mice were administered intraperitoneally (i.p.) daily with saline, PRE-084 (0.25 mg/kg), BD1063 (5 mg/kg) and SA4503 (0.25 mg/kg and 1 mg/kg) from the 8th until the 16th week of age. A group of WT mice, age-matched, was used as negative control of disease.

2. FUNCTIONAL ANALYSES

The SOD1^{G93A} mouse develops a rapidly progressive MN degeneration, which leads to locomotor deficits that were evaluated with:

A) Electrophysiological tests. In the nerve conduction tests the compound muscle action potential (CMAP) was recorded from plantar interossei, tibialis anterior (TA) and gastrocnemius (GM) muscles at 8, 11, 13 and 16 weeks of age. The amplitude, from baseline to the maximal negative peak, and the latency, time from stimulus to the onset of the first negative deflection, of the M wave were measured.

B) Rotarod test. It was performed weekly from 8 to 16 weeks of age to detect abnormalities in coordination, balance and strength. It was also used to determine the onset of signs of disease.

3. HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES

At the end of the follow-up (16 weeks of age), animals were transcardially perfused with PFA in PBS. Tissue samples of lumbar spinal cord and muscles of the hindlimb were harvested, post-fixed and cryopreserved from all the experimental mice groups.

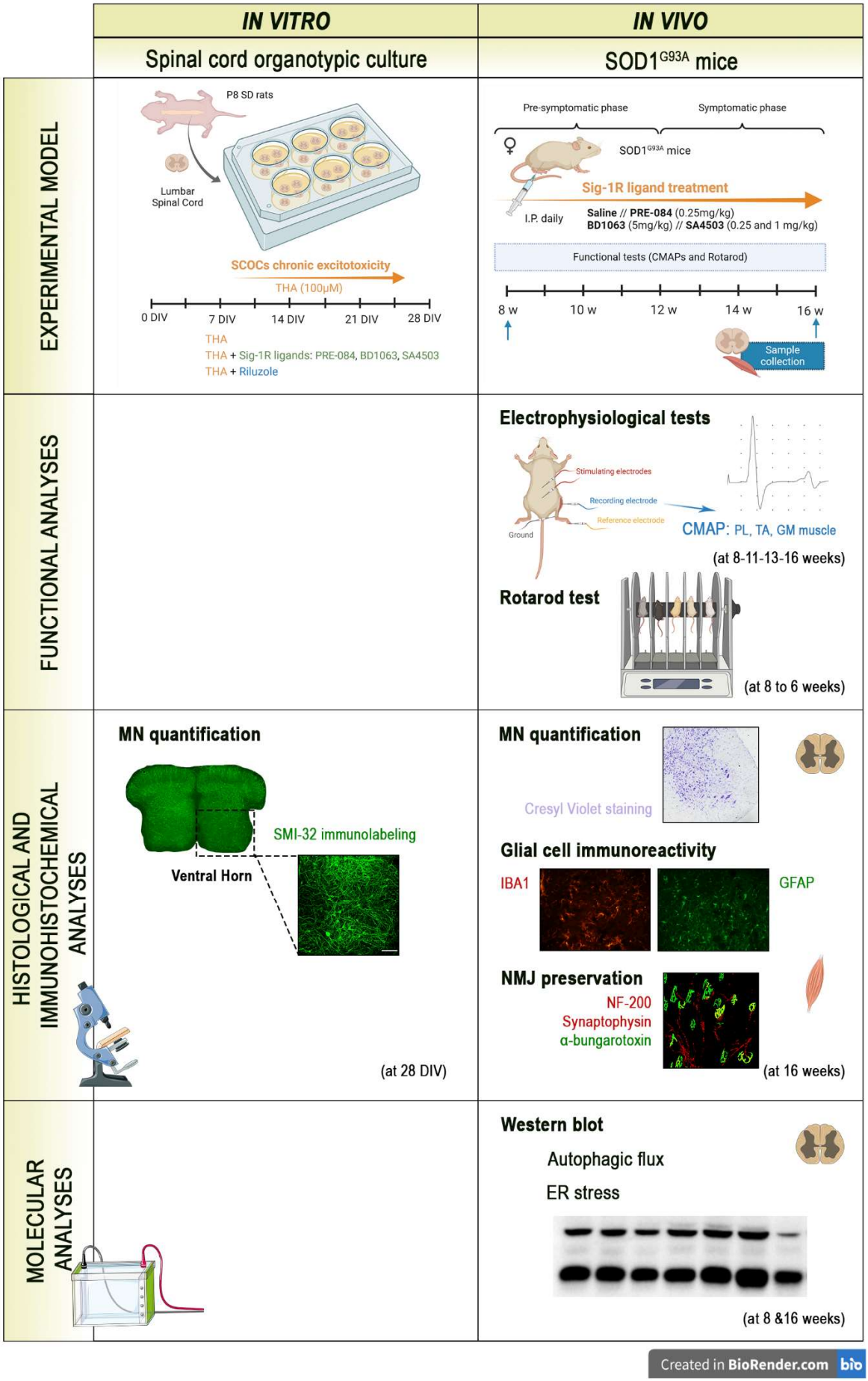
A) MN quantification. To determine the number of MN surviving we used:
   - SMI-32 immunolabeling: Lumbar SCOC slices were immunolabeled with SMI-32 (anti-neurofilament heavy). MN survival was assessed by counting all SMI-32 positive neurons in the ventral horn of each spinal cord hemisection.
   - Cresyl violet staining: L4-L5 lumbar segments of spinal cord of mice were stained with cresyl violet. The number of MNs were quantified in the ventral horns, counting only those MNs with soma larger than 20 μm, polygonal shape and prominent nucleolus.

B) Glial cell immunoreactivity. To label glial cells in the spinal ventral horn Iba1 was used as a marker for microglia cells and GFAP as a marker of astrocytes. Either GFAP or Iba1 immunoreactivity was measured by quantifying the integrated density of the immunostaining using Image J software.

C) NMJ preservation. Transversal sections of GM muscle were immunolabeled with synaptophysin, neurofilament 200 and α-bungarotoxin. The proportion of fully occupied endplates was determined by classifying each endplate as either full or vacant.

4. MOLECULAR ANALYSES

At 8 and 16 weeks of age, samples of lumbar spinal cord were harvested from all the experimental mice groups for protein analyses. By Western Blot (WB) the main protein markers of autophagic flux and ER stress were analyzed to elucidate the mechanisms by which Sig-1R might modulate the MN degenerative process in the $SOD1^{G93A}$ mice.


IN VITRO
IN VIVO
Spinal cord organotypic culture
SOD1G93A mice
EXPERIMENTAL MODEL
P8 SD rats
Lumbar Spinal Cord
SCOCs chronic excitotoxicity
THA (100µM)
0 DIV
7 DIV
14 DIV
21 DIV
28 DIV
THA
THA + Sig-1R ligands: PRE-084, BD1063, SA4503
THA + Riluzole
Pre-symptomatic phase
Symptomatic phase
SOD1G93A mice
Sig-1R ligand treatment
I.P. daily
Saline // PRE-084 (0.25mg/kg)
BD1063 (5mg/kg) // SA4503 (0.25 and 1 mg/kg)
Functional tests (CMAPs and Rotarod)
8 w
10 w
12 w
14 w
16 w
Sample collection
FUNCTIONAL ANALYSES
Electrophysiological tests
Stimulating electrodes
Recording electrode
Reference electrode
Ground
CMAP: PL, TA, GM muscle
(at 8-11-13-16 weeks)
Rotarod test
(at 8 to 6 weeks)
HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES
MN quantification
SMI-32 immunolabeling
Ventral Horn
(at 28 DIV)
MN quantification
Cresyl Violet staining
Glial cell immunoreactivity
IBA1
GFAP
NMJ preservation
NF-200
Synaptophysin
α-bungarotoxin
(at 16 weeks)
MOLECULAR ANALYSES
Western blot
Autophagic flux
ER stress
(at 8 &16 weeks)
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Chapter II: Neuroprotective effects of sigma 1 receptor ligands on motoneuron death after spinal root injury in mice.

The second chapter of this thesis characterizes the model of spinal root injury in mice and evaluates the effects of treatment with Sig1-R ligands, BD1063, SA4503 and PRE-084, in this model.

1. EXPERIMENTAL MODEL

An *in vivo* model of MN degeneration was used:

A) *In vivo* model: Spinal root injury (Rhizotomy). Surgery was performed in adult female and male mice of B6SJL and C57BL6 background and in transgenic Sig-1R KO mice. The right L4-L5 spinal roots were cut at postganglionic level and the stumps of the roots were separated to avoid axon regeneration. Different groups of rhizotomized mice were administered i.p. daily with saline, PRE-084 (0.25 mg/kg), BD1063 (5 mg/kg) and SA4503 (1 mg/kg) starting 30 min after surgery until the mice were euthanized.

2. FUNCTIONAL ANALYSES

In order to confirm the complete denervation of the model, nerve conductions tests were performed at 5 days post-injury (dpi) and at the end of follow-up in both hindlimbs.

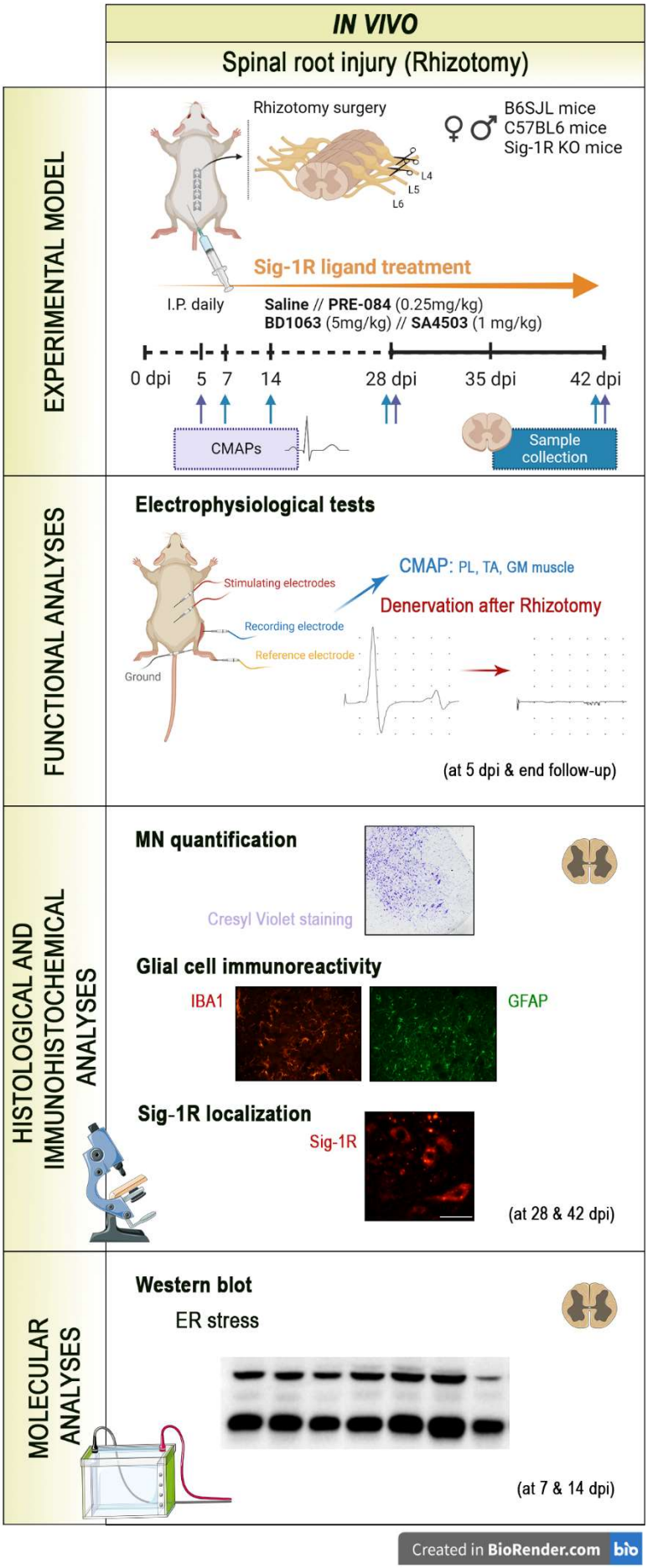
3. HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES

At the end of the follow-up (28 and 42 dpi), animals were transcardially perfused with PFA in PBS. Samples of lumbar spinal cord were harvested, post-fixed and cryopreserved from all the experimental mice groups.

A) MN quantification. To determine the number of MN surviving cresyl violet staining was performed. The number of MNs were quantified in ventral horns, counting only MNs with soma larger than 20 µm, polygonal shape and a prominent nucleolus.
B) Glial cell immunoreactivity. To label glial cells in the spinal ventral horn Iba1 was used as a marker for microglia cells and GFAP as a marker of astrocytes. The integrated density of the immunostainings was quantified using Image J software.
C) Sig-1R immunolabelling. To identify Sig-1R localization in the spinal cord Sig-1R antibody was amplified by Tyramide Signal Amplification (TSA) method with the TSA Biotin Systems.

4. MOLECULAR ANALYSES

At 7 and 14 dpi, samples of ipsi-lateral injured lumbar segment were harvested from all the experimental mice groups. By WB the main protein markers of ER stress were analyzed to elucidate the mechanisms by which Sig-1R affects the MN degenerative process in the rhizotomy model.


IN VIVO
Spinal root injury (Rhizotomy)
EXPERIMENTAL MODEL
Rhizotomy surgery
L4
L5
L6
B6SJL mice
C57BL6 mice
Sig-1R KO mice
Sig-1R ligand treatment
I.P. daily
Saline // PRE-084 (0.25mg/kg)
BD1063 (5mg/kg) // SA4503 (1 mg/kg)
0 dpi
5
7
14
28 dpi
35 dpi
42 dpi
CMAPs
Sample collection
FUNCTIONAL ANALYSES
Electrophysiological tests
Stimulating electrodes
Recording electrode
Reference electrode
Ground
CMAP: PL, TA, GM muscle
Denervation after Rhizotomy
(at 5 dpi & end follow-up)
HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES
MN quantification
Cresyl Violet staining
Glial cell immunoreactivity
IBA1
GFAP
Sig-1R localization
Sig-1R
(at 28 & 42 dpi)
MOLECULAR ANALYSES
Western blot
ER stress
(at 7 & 14 dpi)
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Chapter III: EST79232 and EST79376, two novel sigma-1 receptor ligands, exert neuroprotection on models of motoneuron degeneration.

The third chapter of this thesis was focused on evaluating the therapeutic potential of two new synthetized Sig-1R ligands, EST79232 and EST79376, for MN preservation on three models of MN degeneration. Firstly, the ESTEVE pharmaceutical company synthetized two novel Sig-1R ligands from the same chemical series. Then, using *in vitro* assays the binding affinities for proteins including Sig-1R and functional pharmacological profile (interaction with BiP) of these compounds was determined.

1. EXPERIMENTAL MODEL

An *in vitro* and two *in vivo* models of MN degeneration were used:

A) *In vitro* model: SCOC. SCOC subject to chronic excitotoxicity was used to compare the neuroprotective effect of the Sig-1R ligands EST79232 and EST79376 on MN survival. At 28 DIV SCOCs were fixed with PFA to obtain the samples.

B) *In vivo* model: Spinal root injury (Rhizotomy). Surgery was performed in adult female mice of B6SJL background by sectioning the L4-L5 spinal roots at postganglionic level. Different groups of rhizotomized mice were administered i.p. twice per day (b.i.d) with vehicle, PRE-084 (0.25 mg/kg), EST79232 (0.5 and 5 mg/kg) and EST79376 (0.5 and 5 mg/kg) starting 30 min after surgery until the mice were euthanized at 42 dpi.

C) *In vivo* model: $SOD1^{G93A}$ mice. Different groups of $SOD1^{G93A}$ male mice were administered i.p. twice per day (b.i.d) with vehicle, PRE-084 (0.25 mg/kg), EST79232 (5 mg/kg) and EST79376 (5 mg/kg) from the 8^{th} until the 16^{th} week of age. A group of WT mice, age-matched, was used as negative control of disease.

2. FUNCTIONAL ANALYSES

A) Electrophysiological tests. Nerve conductions tests were performed from plantar interossei, TA and GM muscles at the end of the follow up in the rhizotomy model to confirm the complete denervation, whereas the $SOD1^{G93A}$ mice were evaluated at 8, 11, 13 and 16 weeks of age to assess disease progression.

B) Rotarod test. It was performed weekly from 8 to 16 weeks of age to detect abnormalities in coordination, balance and strength in the $SOD1^{G93A}$ mice.

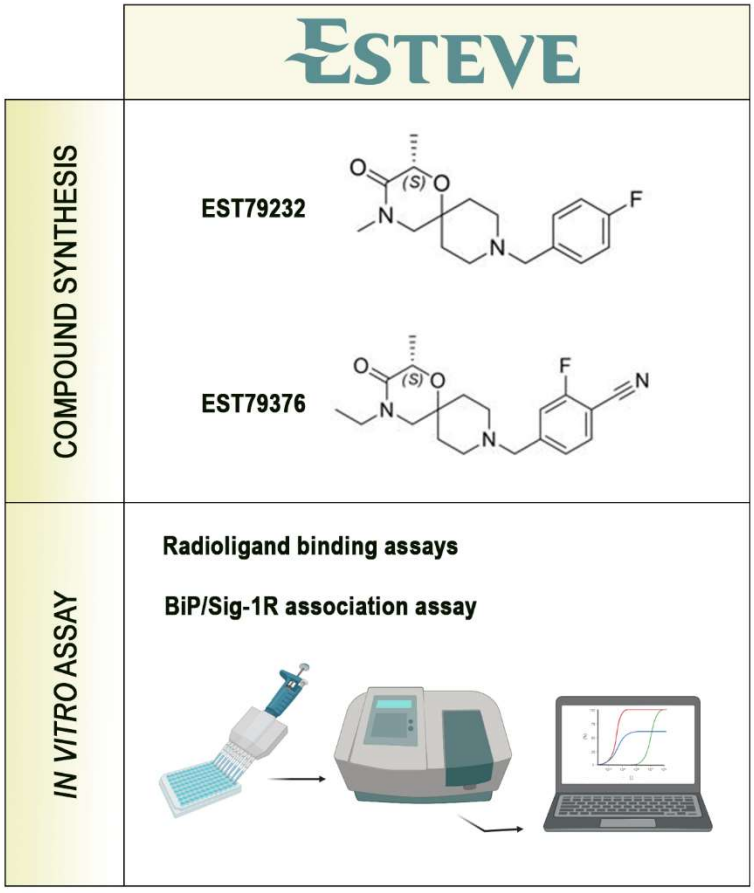
3. HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES

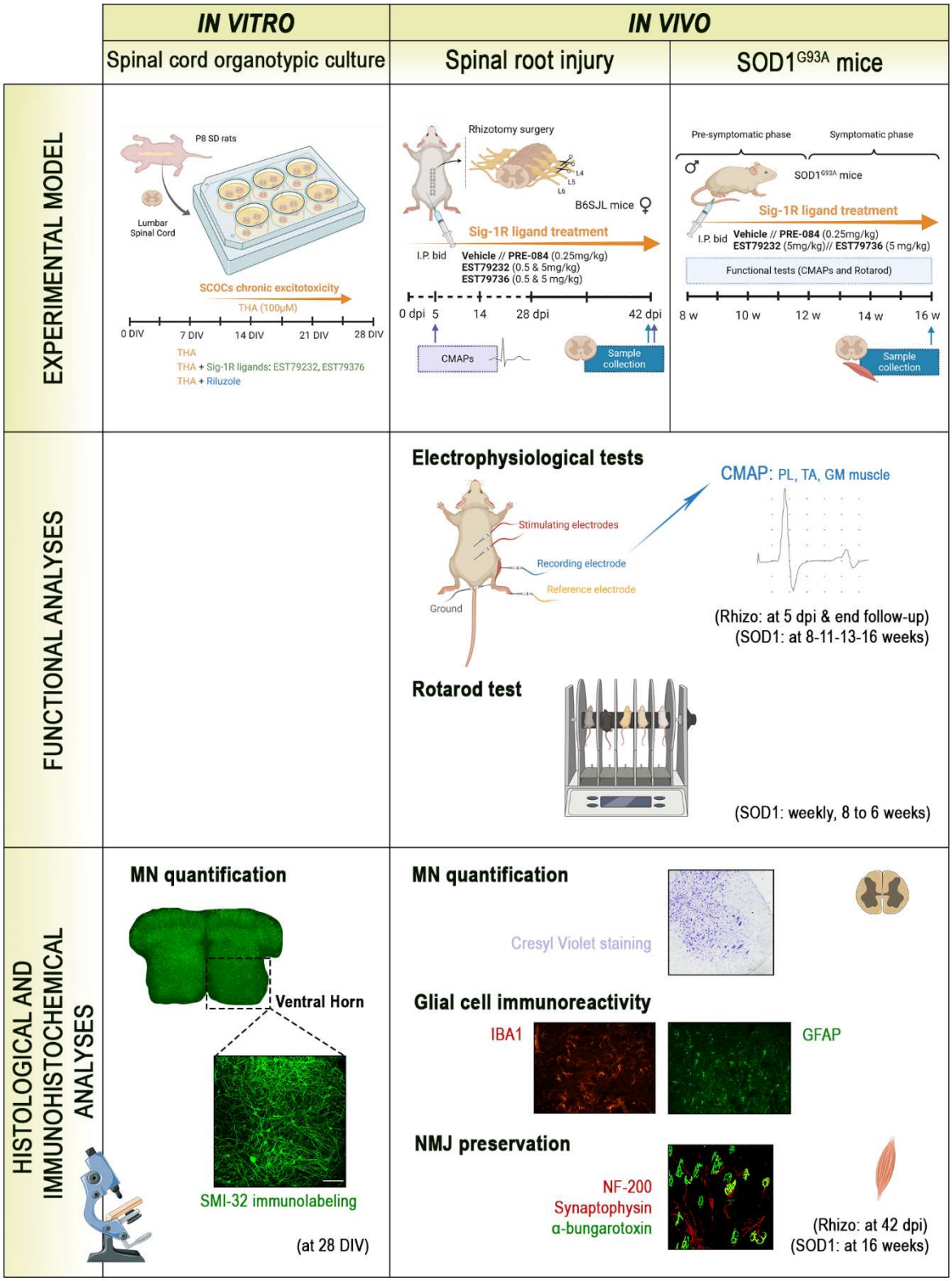
At the end of the follow-up (42 dpi and 16 weeks of age), animals were transcardially perfused with PFA in PBS. Samples of lumbar spinal cord and hindlimb muscles were harvested, post-fixed and cryopreserved from each experimental group.

A) **MN quantification.** To determine the number of MN surviving we used:
   - SMI-32 immunolabeling: Lumbar SCOC slices were immunolabeled with SMI-32. MN survival was assessed by counting all SMI-32 positive neurons in the ventral horn of each spinal cord hemisection.
   - Cresyl violet staining: L4-L5 lumbar segments of spinal cord of mice were stained with cresyl violet. The number of MNs were quantified in the ventral horns, counting only those MNs with soma larger than 20 µm, polygonal shape and prominent nucleolus.

B) **Glial cell immunoreactivity** in the spinal ventral horn by quantifying GFAP and Iba1 immunoreactivity.

C) **NMJ preservation.** Sections of TA muscle were immunolabeled with synaptophysin, neurofilament 200 and α-bungarotoxin. The proportion of fully occupied endplates was determined by classifying each endplate as either full or vacant.





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V. RESULTS

Chapter I:

Sigma-1 receptor is a pharmacological target to promote neuroprotection in the SOD1^{G93A} ALS mice.

Sigma-1 receptor is a pharmacological target to promote neuroprotection in the SOD1^{G93A} ALS mice

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the death of motoneurons (MNs) with a poor prognosis. There is no available cure, thus, novel therapeutic targets are urgently needed. Sigma-1 receptor (Sig-1R) has been reported as a target to treat experimental models of degenerative diseases and, importantly, mutations in the Sig-1R gene cause several types of motoneuron disease (MND). In this study we compared the potential therapeutic effect of three Sig-1R ligands, the agonists PRE-084 and SA4503 and the antagonist BD1063, in the SOD1^{G93A} mouse model of ALS. Pharmacological administration was from 8 to 16 weeks of age, and the neuromuscular function and disease progression were evaluated using nerve conduction and rotarod tests. At the end of follow up (16 weeks), samples were harvested for histological and molecular analyses. The results showed that PRE-084, as well as BD1063 treatment was able to preserve neuromuscular function of the hindlimbs and increased the number of surviving MNs in the treated female SOD1^{G93A} mice. SA4503 tended to improve motor function and preserved neuromuscular junctions (NMJ), but did not improve MN survival. Western blot analyses revealed that the autophagic flux and the endoplasmic reticulum stress, two pathways implicated in the physiopathology of ALS, were not modified with Sig-1R treatments in SOD1^{G93A} mice. In conclusion, Sig-1R ligands are promising tools for ALS treatment, although more research is needed to ascertain their mechanisms of action.

Key words: sigma-1 receptor, amyotrophic lateral sclerosis, motoneuron, SOD1^{G93A} transgenic.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the progressive loss of upper and lower motoneurons (MNs), causing muscle paralysis and early death. Despite persistent efforts to develop treatments for this disease, no effective cure is available for ALS patients. Riluzole and edaravone are the only drugs approved by the FDA, but they have limited therapeutic benefits increasing the lifespan a few months (Edaravone (MCI-186) ALS 19 Study Group, 2017; Ludolph and Jesse, 2009). The exact pathophysiological mechanisms contributing to MN degeneration in ALS remain unclear. Nevertheless, the generation of transgenic animal models carrying ALS-related mutations has accelerated the research on physiopathology and preclinical therapeutic assays for ALS. The $SOD1^{G93A}$ mouse is the most widely used ALS model, which develops the main clinical, electrophysiological and histopathological features of both familial and sporadic forms of the disease (Mancuso et al., 2011b; Turner & Talbot, 2008).

Sigma-1 receptor (Sig-1R) is a protein ubiquitously expressed in the central nervous system (CNS) (Langa et al., 2003) and particularly enriched in the MNs. It is located in the endoplasmic reticulum (ER) cisternae at postsynaptic sites of C-terminals and at the mitochondria associated-endoplasmic reticulum membrane (MAM), an active and dynamic site in which there is crosstalk between mitochondria and ER (Hayashi & Su, 2007; Mavlyutov et al., 2012). Sig-1R is involved in numerous cellular processes, such as ion channel modulation, protein and lipid transport, ER stress response and mitochondria dysfunction (Herrando-Grabulosa et al., 2021; Penke et al., 2018). In recent years, genetic analyses revealed Sig-1R gene mutations involved in a juvenile form of ALS (Al-Saif et al., 2011; Watanabe et al., 2016) and in forms of motor neuropathies (Almendra et al., 2018; Ververis et al., 2019). Moreover, either accumulation of mutant SOD1 or absence of Sig-1R induced MAM disruption and mitochondrial dysfunction (Bernard-Marissal et al., 2015; Watanabe et al., 2016).

Nowadays, several Sig-1R ligands have interest as potential therapeutic agents against CNS disorders, including chronic neurological conditions such as pain, stroke, Huntington disease, among others (Bruna et al., 2018; Reilmann et al., 2019; Urfer et al., 2014). Regarding motoneuron diseases (MND), the Sig-1R agonist PRE-084 has shown positive effects reducing the MN death *in vitro* in organotypic culture of spinal cord subjected to excitotoxic damage (Guzmán-Lenis et al., 2009) and *in vivo* in the $SOD1^{G93A}$ murine model of ALS (Mancuso et al., 2012), in the wobbler mouse model of spontaneous MN degeneration

(Peviani et al., 2014), and after spinal nerve injury in adult mice and rats (Gaja-Capdevila et al., 2021; Penas et al., 2011). Studies testing two other Sig-1R agonists, SA4503 and pridopidine, also showed that treatment ameliorates ALS pathology (Ionescu et al., 2019; Ono et al., 2014), and pridopidine is being tested in a clinical trial for ALS (ClinicalTrials.gov NCT04615923). However, Sig-1R ligands may act differently, and even contrarily, on neuroprotective mechanisms by modulating calcium homeostasis (Tadić et al., 2017). Thus, the Sig1-R appears as a promising target to promote MN protection, but more studies are needed to stablish the role of Sig-1R ligands in MND models, and the type of ligand that may be most effective. Considering the recent data, the aim of the work reported here was to comparatively evaluate the therapeutic efficacy of three Sig-1R ligands in an *in vitro* model of MN degeneration and in the $SOD1^{G93A}$ mouse. Moreover, it was investigated whether the administration of Sig-1R ligands could promote the modulation of glial reactivity. Finally, regarding the importance of ER stress and autophagy in the ALS pathogenesis (Hetz & Saxena, 2017; Medinas et al., 2017; Nguyen et al., 2019), we investigated whether these Sig-1R ligands modulate these molecular pathways.

MATERIAL AND METHODS

Spinal cord organotypic cultures

Spinal cord organotypic cultures (SCOCs) were prepared from lumbar sections of Sprague–Dawley pups (8-9 days-old) as previously described (Mòdol-Caballero et al., 2017). After harvesting, the spinal cord was cut in 350 µm thick transverse sections, that were transferred on Millicell-CM nets (0.4 µm, PICM03050, Millipore) and then into a six-well plate with the incubation medium [50% (v/v) minimal essential medium (MEM, M5775, Sigma), 2 mM glutamine, 25 mM HEPES, 25% (v/v) Hank's Balanced Salt Solution (HBSS-/-, 14175, Gibco) supplemented with 25.6 mg/ml glucose and 25% (v/v) heat-inactivated horse serum (26050-088, Gibco), pH=7.2). After 7 days *in vitro* (DIV), chronic excitotoxicity was induced by adding DL-threo-β-hydroxyaspartic acid (THA; 100 µM) (Rothstein et al., 1993). The Sig-1R ligands were simultaneously co-added in the culture medium and renewed at each medium change twice per week. Sig-1R ligands PRE-084, BD1063 and SA4503 (Tocris) were tested at three different concentrations (30, 3 and 0.3 µM). Riluzole (5 µM) was also assayed as positive control. Slices were maintained for 28 DIV and then fixed with 4% PFA in phosphate-buffered saline (PBS). The *in vitro* experiments have been performed in three independent cultures and resulting in 12 slices for each experimental condition.

Animals and experimental design

Transgenic SOD1^{G93A} mice (B6SJL-Tg[SOD1-G93A]1Gur) and non-transgenic wild type (WT) littermates were used. The transgenic offspring was identified by polymerase chain reaction (PCR) amplification of DNA extracted from the tail. Mice were maintained under standard conditions with access to food and water ad libitum. The experimental procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona and followed the European Communities Council Directive 2010/63/EU.

The study included B6SJL female WT and SOD1^{G93A} mice divided in different experimental groups, either receiving vehicle or a Sig-1R ligand. We first performed a complete study in female mice, and after analyses, the study was also performed in male mice with two compounds, considering the differences in disease progression between sexes in this transgenic mouse. For the functional studies the following experimental groups of SOD1^{G93A} female mice were used: SOD1 + saline (n=15), SOD1 + PRE-084 (n=14), SOD1 + BD1063 (n=12), SOD1 + SA4503 0.25 mg/kg (n=7), SOD1 + SA4503 1 mg/kg (n=7), in addition to untreated WT littermates (n=15). Samples from these animals (n=7-10) were collected for histological analysis at 16 weeks. Subgroups (n=4-5) of WT, vehicle, PRE-084 and BD1063 groups were used for Western blot (WB) analyses at 8 and 16 weeks of age. For the functional studies in male the following groups of SOD1^{G93A} mice were used: SOD1 + saline (n=10), SOD1 + PRE-084 (n=10), SOD1 + BD1063 (n=5), and untreated WT littermates (n=10).

Pharmacological treatment

The Sig-1R ligands were given once a day from 8 to 16 weeks of age by intraperitoneal (i.p) administration of agonists PRE-084 (0.25 mg/kg, TOCRIS) and SA4503 (0.25 mg/kg and 1 mg/kg, TOCRIS) and the antagonist BD1063 (5 mg/Kg, TOCRIS). The compounds were dissolved in saline solution, that was administered in the same volume to the untreated control group. The Sig-1R ligands were administered in a volume of 10 ml/kg.

Electrophysiological tests

Motor nerve conduction tests were performed at 8 weeks of age to obtain baseline values (prior to drug administration) to distribute them between the experimental groups, and then at 11, 13 and 16 weeks of age. Briefly, the sciatic nerve was stimulated by single pulses of 20 μs duration delivered 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 (Mancuso et al., 2011b). The recorded potentials were amplified and displayed on a digital oscilloscope to measure the latency to the onset and the

amplitude of the CMAP. Pentobarbital (50 mg/kg i.p.) was used to anesthetise the mice during the tests and mice body temperature was maintained by means of a thermostated heating pad.

Locomotion tests

The rotarod test was performed to evaluate motor coordination and balance of the animals, weekly from 8 to 16 weeks of age in SOD1^{G93A} and WT mice. Mice were placed onto the rod turning at 14 rpm, each mouse was given five trials and the longest latency without falling was recorded, with an arbitrary cut-off time of 180 s. The symptomatic disease onset for each mouse was determined as the first week when the mouse did not sustain 180 s on the rod.

Histological and immunohistochemical analyses

SCOC were fixed, blocked with 5% normal horse serum in 0.3% Triton X-100 PBS solution (PBS-Tx), and incubated with primary antibody mouse anti-neurofilament H non-phosphorylated (SMI-32, 1:500; 801701, BioLegend) for 48 h at 4°C. Then, after several washes with 0.1% Tween-20 in PBS (PBS-Tw), slices were incubated with secondary antibody Alexa Fluor 488 donkey anti-mouse (1:500; A-21202, Invitrogen) for 2 h at RT. Cell nuclei were labeled with DAPI (1:5000; D9563, Sigma) and the sections were mounted with Fluoromount-G medium (SouthernBiotech). Images of the ventral horns were captured with a confocal microscope (LSM 700 Axio Observer, Carl Zeiss 20x/z0.5). The Cell Counter plugin of ImageJ software was used for quantifying SMI-32 positive neurons (MN survival) in each spinal cord.

At 16 weeks of age mice were sacrificed with an overdose of pentobarbital sodium and transcardially perfused with 4% PFA in PBS. The lumbar segment of spinal cord was post-fixed for 2 h and cryopreserved in 30% sucrose solution in PB, whereas the hindlimb muscles were directly cryopreserved. For assessing MN survival, the spinal cord was serially cut in 20 µm thick transverse sections using a cryostat (Leica) and collected sequentially on series of 10 slides. Slices corresponding to L4-L5 spinal cord sections separated 100 µm were stained for 3 h with an acidified solution of 3.1 mM cresyl violet. Then, the slides were washed, dehydrated and mounted with DPX. MNs were identified by localization in the lateral ventral horn and strict morphological and size criteria: polygonal shape, prominent nucleoli and diameter larger than 20 µm.

For immunofluorescence analysis, lumbar spinal cord sections were blocked with blocking solution (10% normal donkey serum (NDS) and 0.2 mM glycine in PBS-Tx) for 1 h at RT, and then incubated overnight at 4°C with primary antibodies: anti-Iba1 (1:500; 019–19,741, Wako), anti-GFAP (1:500; 13-0300, Invitrogen), SQSTM1/p62 (1:150; 155686, Abcam). After several

washes, sections were incubated for 2 h at RT with the corresponding secondary antibody: Alexa Fluor 488-conjugated secondary antibody (1:500; A-21206, Invitrogen), Cy3-conjugated secondary antibody (1:500; AP182C, Millipore), Alexa Fluor 594-conjugated secondary antibody (1:300; A-21207, Invitrogen) or Alexa Fluor 647-conjugated secondary antibody (1:300; Ab150155, Abcam). NeuroTraceTM 500/525 Green Fluorescent Nissl (1:200; N21480, Invitrogen) and DAPI (1:2000; D9563, Sigma) were used to stain MNs and nuclei, respectively. Finally, samples were washed in PB and mounted with Fluoromount-G. To quantify the glial cell reactivity images of the ventral horn were captured at x40 under the same conditions (sensitivity and exposure time) for each analyzed marker, using fluorescence microscopy (Olympus BX51, Japan). Fluorescence signal intensity (Integrated density) was analyzed using ImageJ software after defining a threshold for background correction. To quantify p62/SQSTM1 immunolabeling, photographs of the ventral horn were taken with confocal microscopy (LSM 700 Axio Observer, Carl Zeiss, 40xOil/z0.5). Integrated density of p62/SQSTM1 was quantified in a total of more than 50 MNs for each animal using a ROI manage tool from Image J software. The p62/SQSTM1 integrated density was analyzed in the glia by quantifying the whole slide image and subtracting the intensity of the MNs.

For neuromuscular junctions (NMJ) labeling, GM muscle was serially cut in 50 µm thick longitudinal sections and collected in sequential series. After blocking, the sections were incubated for 48h at 4°C with the primary antibodies anti-neurofilament 200 (NF200, 1:1000; AB5539, Millipore) and anti-synaptophysin (1:500; AB32127, Abcam). After washes sections were incubated overnight with Alexa Fluor 594-conjugated secondary antibody (1:200; A11042-A21207, Invitrogen) and Alexa Fluor 488-conjugated alfa-bungarotoxin (1:500; B13422, Life Technologies). Slides were mounted in Fluoromount-G with DAPI. Images were captured by confocal microscopy (LSM 700 Axio Observer, Carl Zeiss, 40xOil/z0.5). The proportion of innervated NMJs was calculated by classifying each endplate as occupied or vacant. Four fields with a total of more than 60 endplates were analyzed per each mouse.

Protein extraction and Western blot analysis

At 8 or 16 weeks of age, mice were euthanized with an overdose of pentobarbital sodium. The lumbar spinal cord from WT and $SOD1^{G93A}$ of each experimental group was harvested and frozen in liquid nitrogen for storage. To lysate samples the RIPA lysis buffer with protease inhibitor cocktail (Sigma) and phosphatase inhibitors (PhosphoSTOP Roche) was used. Then, samples were sonicated and centrifuged at 12000 rpm for 10 min at 4°C. Finally, total protein concentration was determined by the BCA Protein Assay Kit (Biorad). 20-30 µg of protein per

sample were loaded into 7.5-15% SDS-polyacrylamide gels and transferred into a PVDF membrane. After blocking, primary antibodies were incubated at 4ºC overnight (Table 1). After incubation with appropriate Horseradish peroxidase (HRP)-coupled secondary antibody (1:3000; Bio-rad) for 1h at RT, proteins were visualized using the Clarity Western ECL Substrate (Cat#1705061, Bio-Rad Laboratories). Images were collected using a transilluminator (Chemidoc MP Imaging System, BioRad) and blots were analyzed using the Lane and band plugin of Image Lab software (Bio Rad). Data were normalized first by the loading control (actin or tubulin) and afterwards by the mean of the control (WT) samples (n=3-6 samples were analyzed per each condition and time-point of the study).

Table 1. Primary antibodies used for WB experiments

Antibody name	Dilution	Description	Reference number
LC3B	1:500	Rabbit polyclonal	#ab51520; Abcam
Beclin 1	1:1000	Rabbit polyclonal	#3738; CST
XBP-1	1:300	Rabbit polyclonal	#37152; Abcam
GADD153/CHOP	1:500	Mouse monoclonal	#sc-7351; SCBT
Sigma-1 receptor	1:250	Rabbit polyclonal	# 223702; Abcam
GRP78/BiP	1:500	Rabbit polyclonal	#G8918; Sigma-Aldrich
IRE1 (phospho S724)	1:250	Rabbit polyclonal	# ab48187; Abcam
IRE1α	1:500	Rabbit polyclonal	#3294; CST
β-Actin	1:10000	Mouse monoclonal	#A5316; Sigma-Aldrich
α-tubulin	1:10000	Mouse monoclonal	#T9026; Sigma-Aldrich

Statistical analysis

GraphPad Prism 8 software was used to perform data analyses, all data is expressed as mean ±SEM. Electrophysiological and functional measurements were analyzed with repeated measurements Two-Way ANOVA and histological and molecular data were analyzed using One-way ANOVA. Bonferroni test was used as the post hoc test for multiple comparisons. Differences were considered significant at $p \leq 0.05$.

RESULTS

Sig-1R ligands exert neuroprotection in SCOCs under chronic excitotoxicity

Addition of THA to the SCOC induced a significant reduction of about 40% in the number of SMI-32 labeled MNs in the ventral horn after 21 DIV, compared to control slices (Figure 1). Slices treated with THA and PRE-084, BD1063 or SA4503 showed significant preservation of MNs at the two doses tested. The Sig-1R agonists PRE-084 and SA4503 significantly reduced MN death in a dose-dependent manner, with maximal protective effect at 30 μM (Figure 1A, B). The antagonist BD1063 also prevented MN death, with a highest effect at 3 μM (Figure 1C). Furthermore, the positive control against excitotoxicity Riluzole presented significant MN protection at similar levels than the Sig-1R ligands tested.

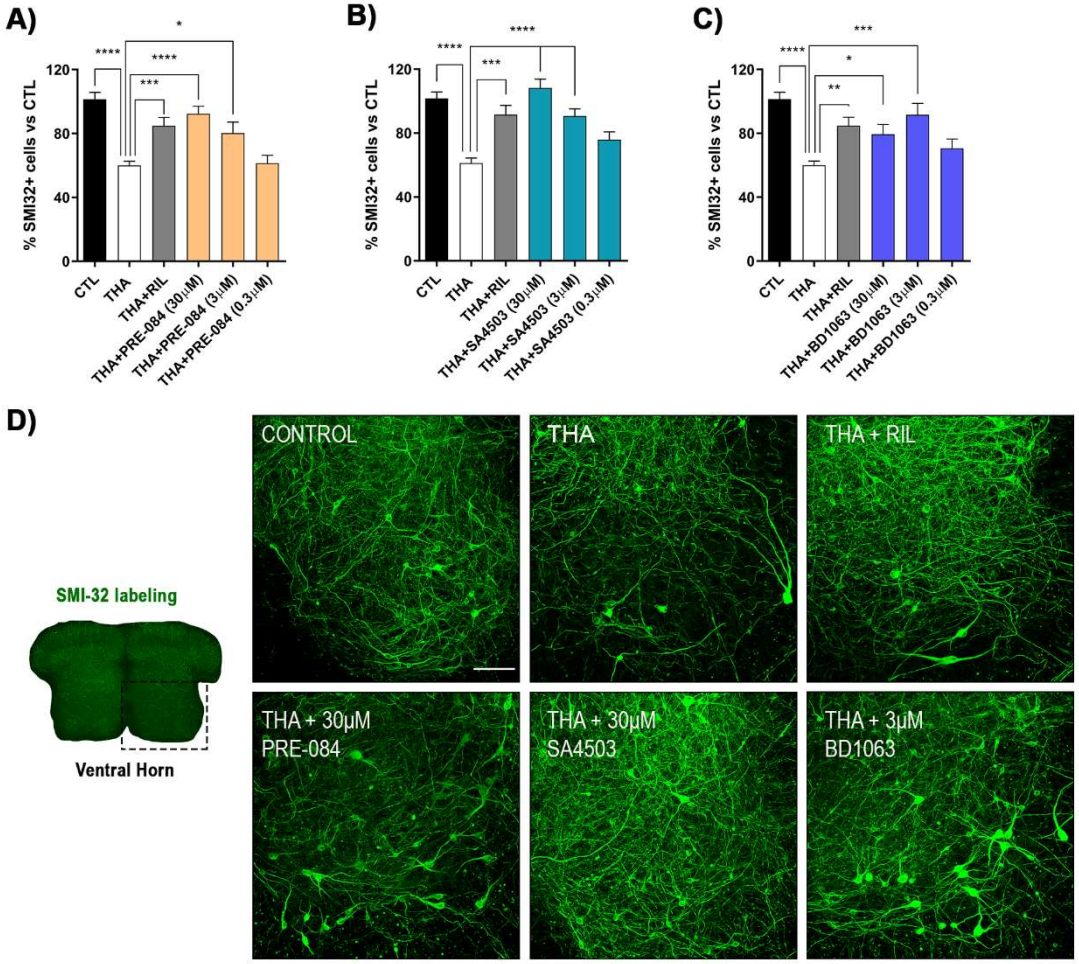

Figure 1. PRE-084, BD1063 and SA4503 prevent MN death under chronic excitotoxicity *in vitro*. A-C) Plots showing the percentage of SMI-32 positive cells in the ventral horn of spinal cord hemislices under excitotoxicity by THA and the addition of Sig-1R ligands (mean ± SEM; n = 16-24 hemisections per treatment). One-way ANOVA followed with Bonferroni's post hoc test: ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 vs. THA alone condition. **D)** Microphotograph of a SCOC slice labeled with SMI-32 antibody, and representative images of the ventral horn at 28 DIV of all tested conditions. Scale bar 100 μm.

Treatment with Sig-1R ligands preserves neuromuscular function in SOD1^{G93A} mice

SOD1^{G93A} mice treated with Sig-1R ligands for 8 weeks maintained a gain of body weight throughout the study, and did not present any secondary effect, indicating lack of general toxicity of these ligands (Figure 2A).

In other to assess the effect of Sig-1R ligands on neuromuscular function of the SOD1^{G93A} mice, we performed motor nerve conduction tests during the follow-up. Results showed that SOD1^{G93A} mice treated with PRE-084 and with BD1063 had a significantly higher TA and GM CMAP amplitude compared to saline administered SOD1^{G93A} mice at 13-16 weeks of age (Figure 2B and Supplementary Figure 1A). SA4503 treated group had a higher CMAP amplitude than untreated mice at the end of the follow up, although differences did not reach statistical significance. Male SOD1^{G93A} treated with PRE-084 had a significant preservation of TA and GM CMAP amplitude throughout the follow-up compared to the saline group, while BD1063 treatment has lower effect (Supplementary Figure 1B, C). The PL CMAP amplitude showed mild preservation for groups treated with PRE-084 and SA4503 at both doses, although it was only significant for PRE-084 (Figure 2C).

The rotarod test revealed that SOD1^{G93A} mice treated with SA4503 at dose of 0.25 mg/kg and PRE-084 significantly improved the functional outcome compared with the untreated group, but animals treated with BD1063 and SA4503 1 mg/kg did not have any improvement (Figure 2D). Furthermore, the disease onset was delayed by one week (14 weeks of age) in mice treated with SA4503 (0.25 and 1 mg/kg) and PRE-084 versus the saline group (13 weeks) (Figure 2E).

The innervation of NMJ of the GM muscle was evaluated at 16 weeks of age. All the SOD1^{G93A} mice treated with Sig-1R ligands had a significantly higher number of innervated endplates compared with the saline group, supporting the preservation of CMAP amplitude observed in the nerve conduction tests (Figure 2F-E).

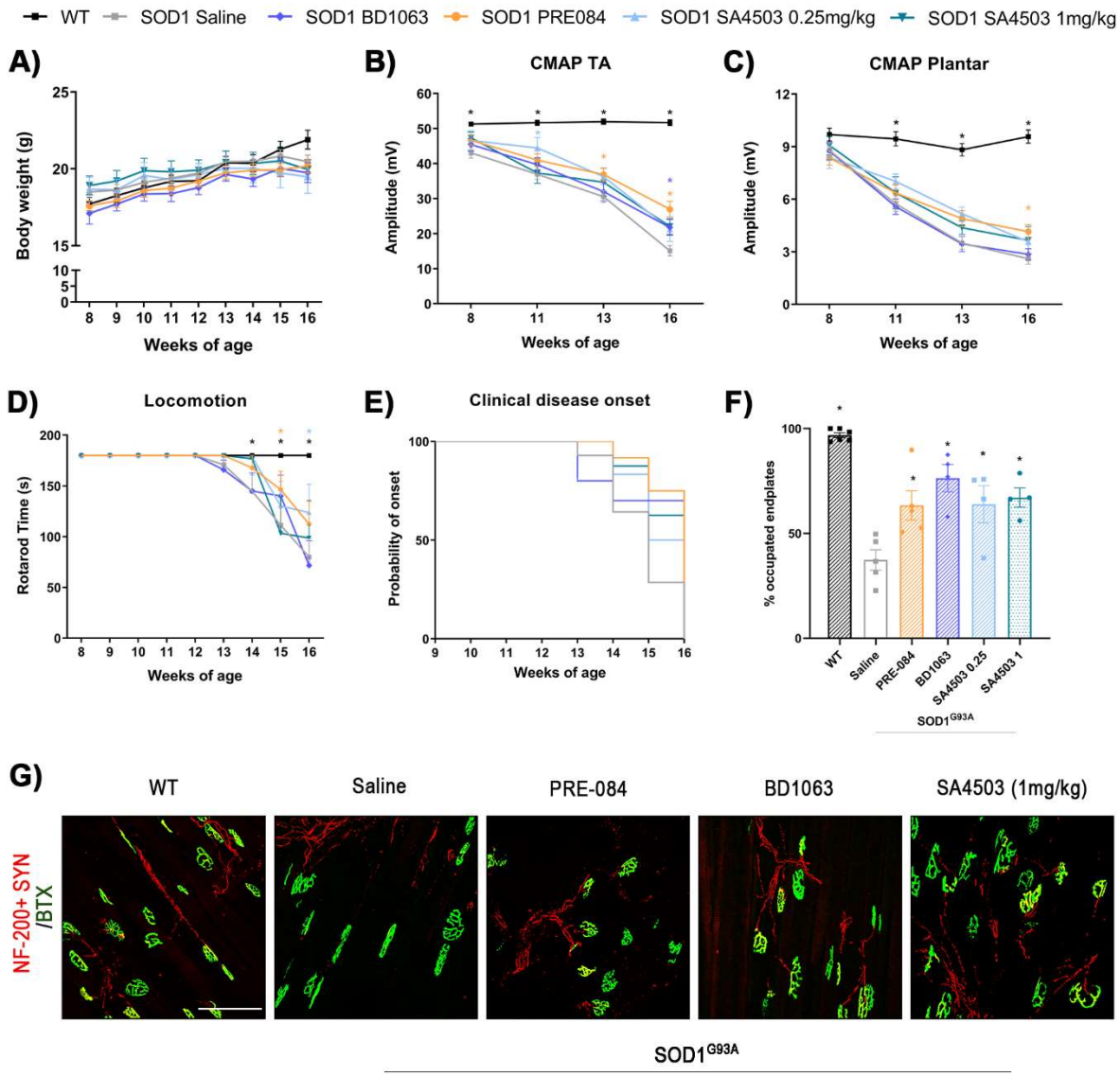

Figure 2. Treatment with PRE-084, BD1063 and SA4503 enhances neuromuscular function in SOD1^{G93A} mice. A) Body weight of mice was monitored weekly during the 8 weeks of the follow-up. B-C) Values of the amplitude of compound muscle action potentials (CMAP) of tibialis anterior (TA) and plantar interosseus muscles. D) Graph showing the effect of the different Sig-1R treatment on functional outcome assessed with the rotarod test. (n=15 WT; n=15 SOD1 saline; n=12 SOD1 BD1063; n=7 SOD1 SA4503 0.25mg/kg; n=7 SOD1 SA4503 1mg/kg, and n=14 SOD1 PRE-084 mice). E) Probability of clinical onset of disease evaluated by the fall in the rotarod test. Some Sig-1R ligands delayed the onset of locomotion deficits but without significant differences. F) Plot of the percentage of innervated NMJ (overlap of signals) in the different experimental groups (n=4-6 mice per group). G) Representative confocal images of GA NMJs at 16 weeks of age. The maximum projection images shown were generated from 1.3 µm z projections. Scale bar 100 µm. Data are mean ± SEM, analyzed by One-way (F) and Two-way (A-D) ANOVA with Bonferroni's multiple comparisons test. *p< 0.05 vs. SOD1^{G93A} saline mice.

Sig-1R ligands promote MN survival and reduce microglial reactivity in SOD1 ^{G93A} mice

The quantification of α-MNs in the ventral horn of lumbar spinal cord sections stained with cresyl violet at 16 weeks of age, revealed that treatment with PRE-084 and BD1063 mildly but significantly prevented the death of spinal MNs at the end-stage of the disease in comparison with untreated SOD1^{G93A} mice, whereas SA4503 at both doses assessed did not have a noticeable effect (Figure 3A-B).

Microglial and astroglial response was found markedly increased in SOD1^{G93A} compared to WT mice. Treatment with Sig-1R ligands reduced microglial reactivity, although only PRE-084 and SA4503 at 0.25 mg/kg caused a significant decrease, whereas the other treatments did not reach statistical significance (Figure 3C-D). Regarding astroglial immunoreactivity in the spinal cord ventral horn, we found that it was not modified by administration of the Sig-1R ligands in female SOD1^{G93A} mice (Figure 3E-F).

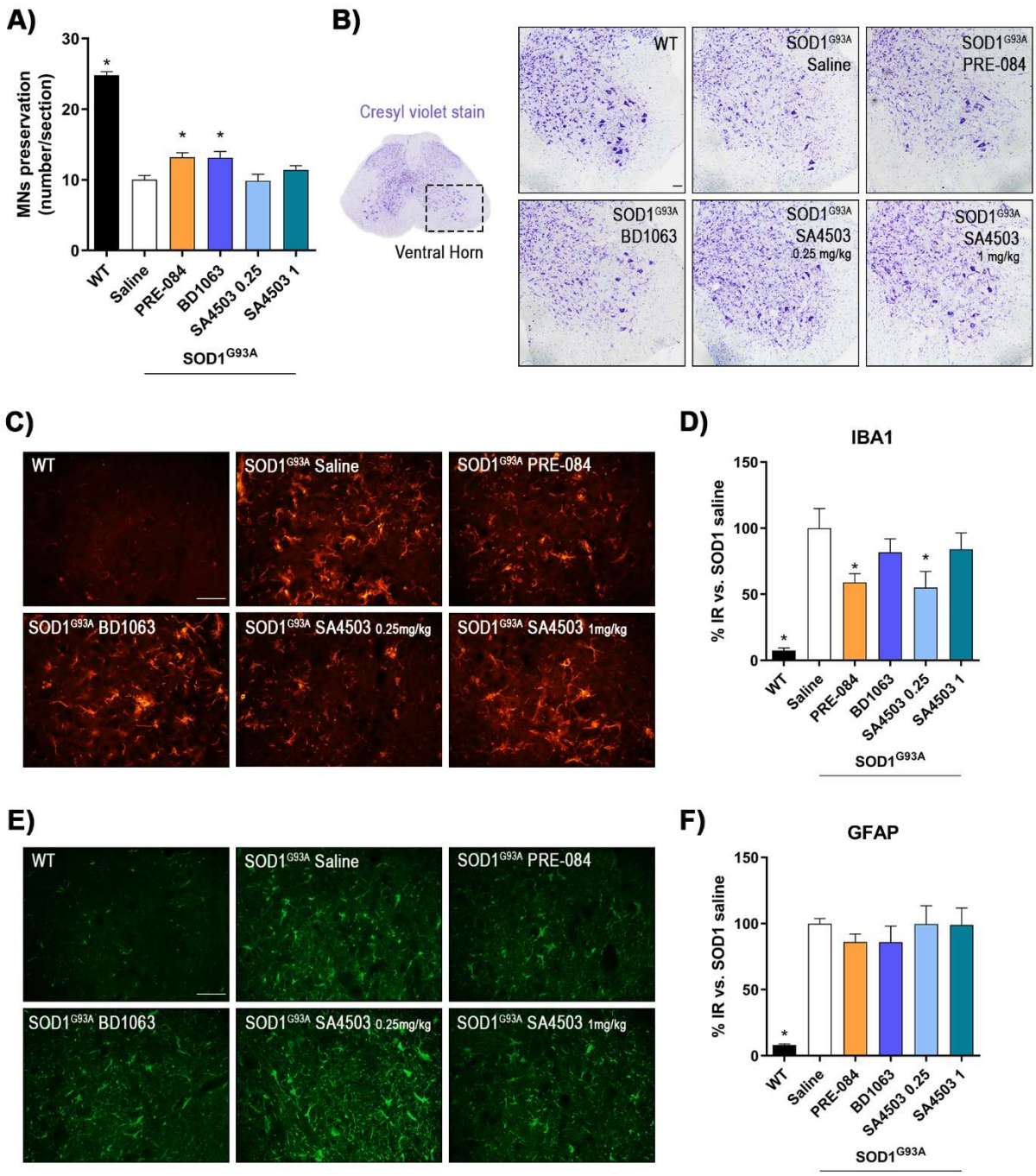

Figure 3. PRE-084 and BD1063 reduce spinal MN degeneration and decrease microgliosis in SOD1^{G93A} mice at 16 weeks of age. A) Quantification of surviving MNs (mean number of MNs per section ± SEM) in the lumbar spinal cord, showing improved MN preservation with Sig-1R ligands PRE-084 and BD1063. B) Representative spinal cord images of MNs stained with cresyl violet. Scale bar 100 µm. C, E) Representative images of immunoreactivity for Iba1 (C), a marker for microglia, and GFAP (E), a marker of astrocytes, in the ventral horn of WT and SOD1^{G93A} mice with or without Sig-1R treatments. Scale bar 50 µm. D, F) Graphs showing the quantification of percentage of Iba-1 (D) and GFAP (F) immunolabeling in ventral horn of spinal cord. (n=10 WT, n=10 SOD1 saline, n=8 SOD1 BD1063, n=7 SOD1 SA4503 0.25mg/kg, n=7 SOD1 SA4503 1mg/kg and n=10 SOD1 PRE-084 mice). *p< 0.05 vs. SOD1^{G93A} saline mice.

Analyses of autophagic flux and ER stress during the progression of ALS with Sig-1R ligands

Considering that PRE-084 and BD1603 significantly preserved spinal MNs, we performed WB analyses of lumbar spinal cord lysates from WT and SOD1^{G93A} saline, PRE-084 and BD1063 treated mice at 8 and 16 weeks of age to evaluate two main molecular pathways implicated in ALS pathogenesis. Three markers of autophagy flux (Figure 4) were analyzed and we found that there was no differences in Beclin-1 protein levels between WT and SOD1^{G93A} mice groups at the two time points evaluated. A progressive increase of LC3-II levels was observed in SOD1^{G93A} mice at 8 weeks and further at 16 weeks compared to WT littermates; treatment with PRE-084 and BD1063 mildly reduced that increase at 16 weeks (Figure 4A-D). Immunofluorescence analyses of lumbar spinal cord sections revealed a significant accumulation of p62/SQSTM1 immunoreactive dots in the MNs and glial cells, mainly astroglia, of SOD1^{G93A} animals at 16 weeks of age (Figure 4E- G). In summary, treatment with Sig-1R ligands PRE-084 and BD1063 did not markedly modify the protein levels of autophagic markers in comparison with saline SOD1^{G93A} mice.

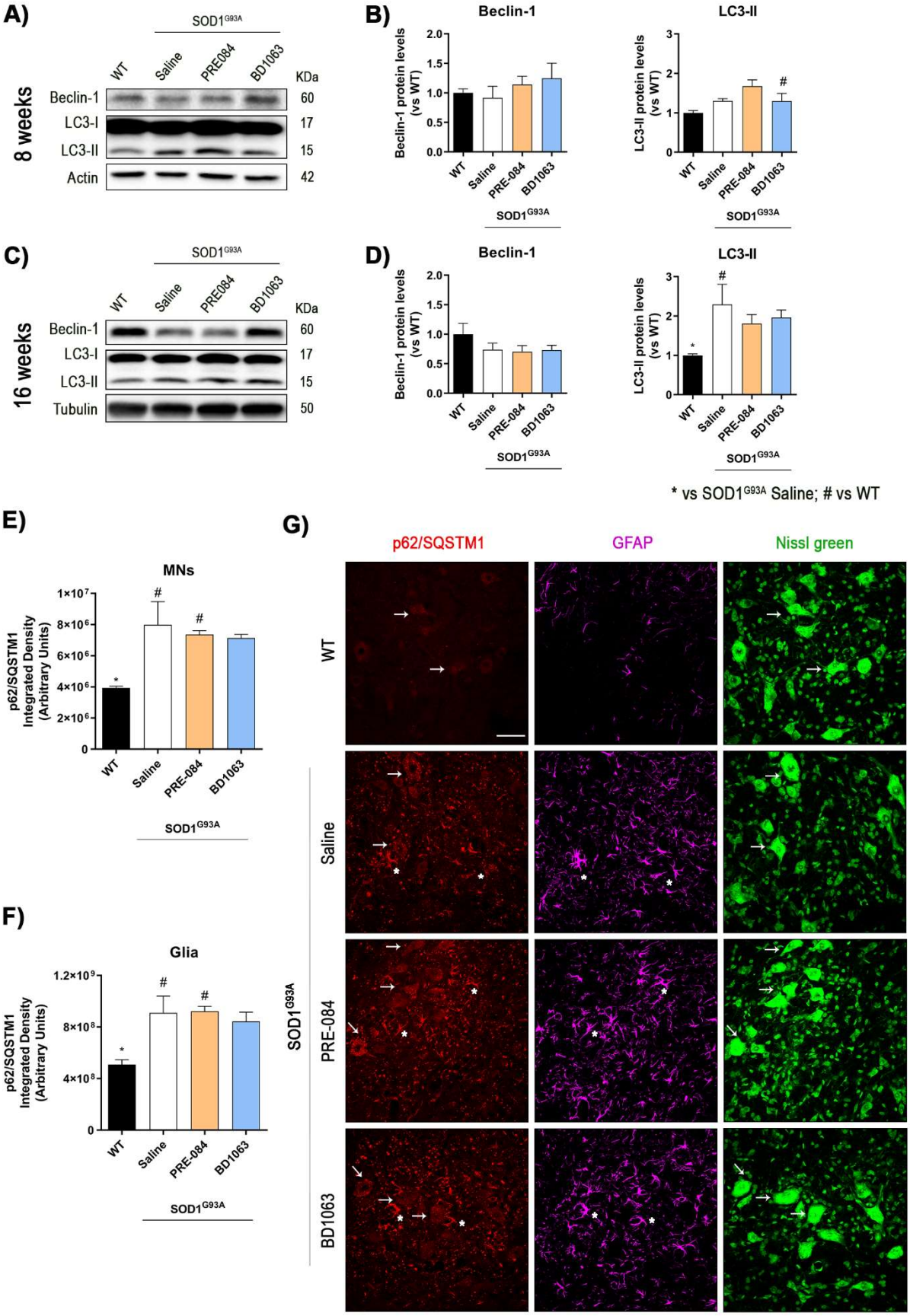

Figure 4. (See legend on next page)

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Figure 4. Protein levels of autophagic flux markers in SOD1^{G93A} mice. Representative blots and protein level quantification of the autophagic markers Beclin-1 and LC3-II at 8 **(A, B)** and 16 **(C, D)** weeks of age. Quantification of the p62/SQSTM1 immunolabeling in the MNs **(E)** and in the glia (whole image without MNs) **(F). G)** Representative images of p62/SQSTM1 (red), GFAP (cyan) and FluoroNissl (green) immunofluorescence in the ventral horn of lumbar spinal cord of WT and SOD1^{G93A} mice at 16 weeks. White arrows show examples of MNs with cytosolic p62 accumulation and asteriscs show p62 immunolabeling in the astroglia. Scale bar 50 µm. Data is mean ± SEM; n = 3-5 mice per group. One-way ANOVA followed with Bonferroni's post hoc test for multiple comparison. *p<0.05 vs SOD1^{G93A} saline mice, #p<0.05 vs WT mice.

We also monitored the levels of ER stress markers in the spinal cord (Figure 5A, B). The protein levels of Sig-1R did not change at 8 and 16 weeks in SOD1^{G93A} mice, as previously described (Mancuso et al., 2012), and administration of Sig-1R ligands PRE-084 and BD1063 did not modify the levels of this receptor (Figure 5C, D). During disease progression, WB analyses revealed a marked increase in the chaperone BiP at 16 weeks of age in SOD^{G93A} mice (Figure 5D). There were no differences in the ratio p-IRE1/IRE between experimental groups at both time points evaluated, whereas there was a significant reduction of the ratio XBP1s/XBP1unspliced at 8 weeks, that was reverted to increased levels at 16 weeks in SOD1^{G93A} mice, with a significant difference for the BD1063 treated compared to WT mice. We also observed a significant increase of CHOP levels in SOD1^{G93A} mice at 8 weeks of age, though no differences were found at 16 weeks of age.

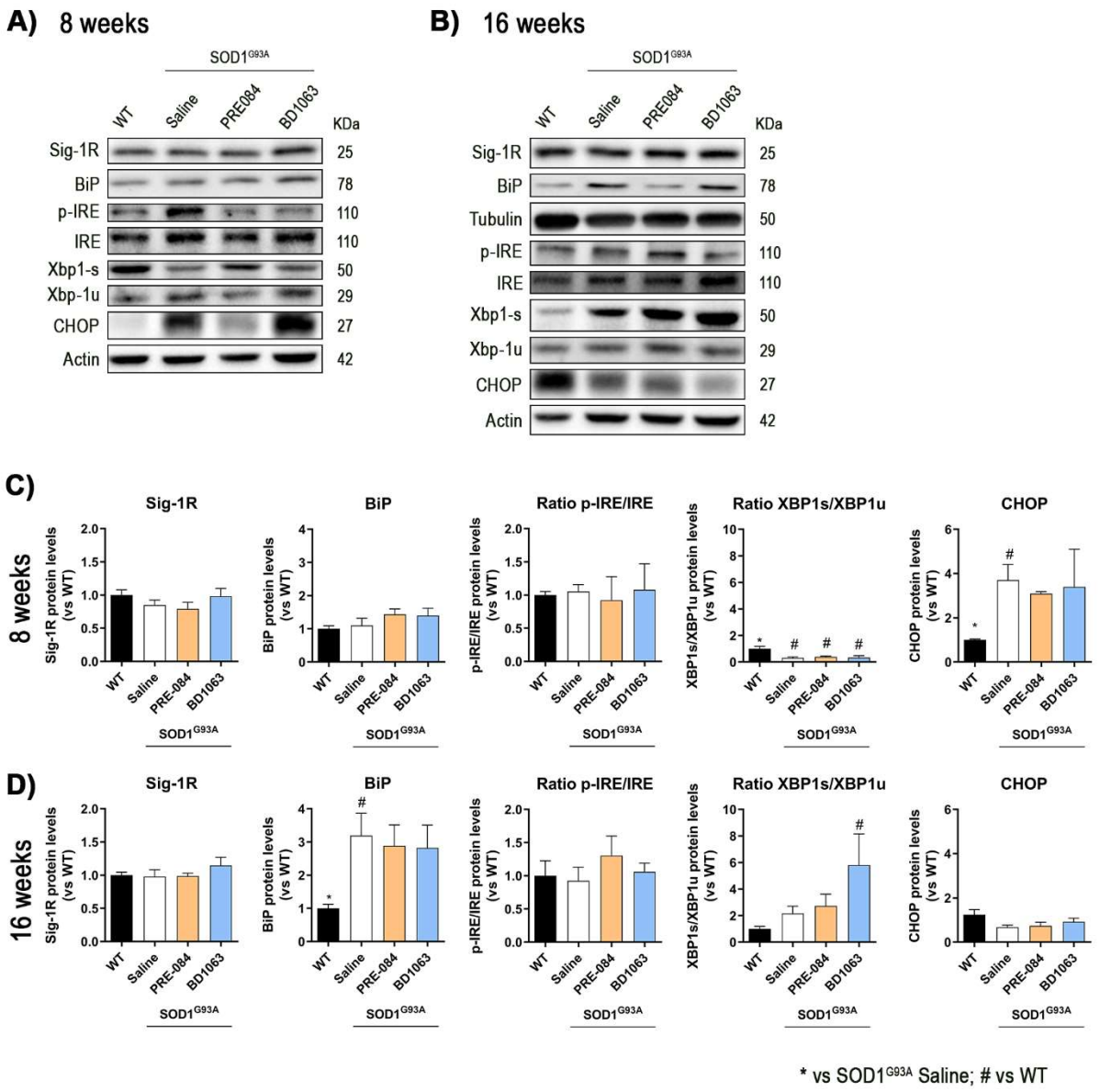


Figure 5. Protein levels of ER stress markers in SOD1^{G93A} mice. Representative blots of ER stress markers at 8 (A) and 16 (B) weeks of age. Quantification of protein levels of Sig-1R, BiP, ratio p-IRE1/IRE1, ratio XBP1s/XBP1unspliced and CHOP at 8 (C) and 16 (D) weeks of age. Data is mean ± SEM; n = 3-5 mice per group. One-way ANOVA followed with Bonferroni's post hoc test for multiple comparison. *p<0.05 vs SOD1^{G93A} saline mice, #p<0.05 vs WT mice.

DISCUSSION

The results of this study demonstrate the beneficial effects of the Sig-1R agonist PRE-084 as well as the Sig-1R antagonist BD1063, on preserving neuromuscular function and attenuating MNs loss in the SOD1^{G93A} mouse model of ALS. The compound SA4503 showed lower effects in ameliorating the progression of the disease. Whereas previous studies have reported that PRE-084 (Mancuso et al., 2012) and SA4503 (Ono et al., 2014) treatment ameliorated the progression and survival of spinal MNs, we report for the first time that a Sig-1R antagonist, such as BD1063, has also neuroprotective effects in preclinical models of ALS. We have recently reported that all PRE-084, SA4503 and BD1063 also prevent MN death in a model of spinal root injury in mice (Gaja-Capdevila et al., 2021), emphasizing the interesting effects of Sig-1R ligands for improving motor neurodegenerative conditions.

Although similar outcomes were obtained in terms of MN preservation *in vitro* in the SCOC with the three Sig-1R ligands tested, there were noticeable differences in the *in vivo* model of ALS. Our *in vitro* results showed that two considered Sig-1R agonists, PRE-084 and SA4503, and the antagonist BD1063 induced protection against chronic excitotoxicity, at the doses of 3 to 30 μM. Indeed, some reports reveal that binding to Sig-1R prevented neuronal death in *in vitro* studies. Our group described that PRE-084 (at 10 μM) protected the SCOC against acute glutamate toxicity (Guzmán-Lenis et al., 2009). Ono and collaborators found that SA4503 (at 10 μM) protected the NSC34 cell line against SOD1^{G93A} and serum free neurotoxicity (Ono et al., 2014). However, those protective effects were inhibited by the co-addition of a Sig-1R antagonist, BD1063 or BD1047, respectively. In our study we assessed each compound alone, without combining them, demonstrating that BD1063 had also similar neuroprotective effects.

Several reports have focused on Sig-1R ligands to modulate ALS progression. PRE-084 (0.25 mg/kg) daily administrated in SOD1^{G93A} from 8 to 16 weeks of age showed neuroprotection, improving MN function and survival and extending the lifespan of SOD1^{G93A} mice (Mancuso et al., 2012). SA4503 (1 mg/kg) treatment from 5 weeks of age extended survival time, but did not affect the onset time in the SOD1^{G93A} mice (Ono et al., 2014). However, we found a delay of one week of disease onset compared to untreated mice. In addition, in our study we evaluate the number of MN survival to compare with the other two Sig-1R ligands. Even though SA4503 did not significantly preserve spinal MNs as PRE-084 and BD1063 treatment did, all the pharmacological treatments significantly preserved NMJ innervation in the hindlimb muscles at 16 weeks of age. This is a remarkable result, since NMJ disruption is an

early event in ALS pathogenesis (Fischer et al., 2004; Mancuso et al., 2011a). Since SA4503 and PRE-084 had been shown to prolong the lifespan of $SOD1^{G93A}$ mice, a further study is needed to elucidate whether BD1063 has similar effect. Recently, pridopidine, a small molecule that modulates axonal transport deficit and causes a reduction in mutant SOD1 aggregates in the spinal cord of $SOD1^{G93A}$ mice though Sig-1R, was reported to attenuate also NMJ disruption (Ionescu et al., 2019). Taken together, it seems that Sig-1R ligands improve several cellular and histological hallmark pathologies related to ALS.

Despite the three Sig-1R compounds assessed, PRE-084, SA4503 and BD1063, have demonstrated potential to bind the Sig-1R, the neuroprotective effects observed in the $SOD1^{G93A}$ mouse model significantly differed between the three ligands. In the same line, Wang and colleagues compared the Sig-1R ligands SA4503, PRE-084 and pentazocine (PTZ) in a model of severe retinopathy (Wang et al., 2020). *In vitro* results yielded similar outcomes, whereas neither PRE-084 or SA4503 afforded in vivo protection comparable to PTZ. A wide range of evidence is now available to support the role of Sig-1R in the treatment of several CNS disorders, such Parkinson disease or ischemia (Haga et al., 2019; Nguyen et al., 2017; Zhao et al., 2019). However, a Sig-1R ligand may act differently in each pathology/degenerative disease, even in opposite way (Tadić et al., 2017). For example, it has been shown that the agonist SA4503 normalized cytosolic Ca^{2+} levels following activation by kainate and by bradykinin in embryonic MNs, whereas PRE-084 (also Sig-1R agonist) did not exert any significant effect (Tadić et al., 2017). Furthermore, it is important to consider that the traditional concept of agonist or antagonist is controversial for Sig-1R ligands, that may act as modulators of this receptor promoting activity in different pathways with a delicate balance of effects (for review see (Herrando-Grabulosa et al., 2021)).

A body of evidence has demonstrated the contribution of neuroinflammation, the role of non-neuronal cells including microglia and astrocytes in ALS pathogenesis. Genetic deletion of mutant SOD1 selectively in microglia increased the lifespan of ALS mice, despite the mutant protein was expressed in MNs and all other cell types (Boillée et al., 2006). We observed a reduction in microglia activation following treatment with PRE-084 and SA4503 at a dose of 0.25 mg/kg (see Figure 3D), but not with BD1063. The effect of PRE-084 on reducing the microglial reactivity and improving the MN environment was already found in the $SOD1^{G93A}$ ALS model (Mancuso et al., 2012), as well as in a mouse model of spinal muscular atrophy ($SMA^{2B/-}$), in which PRE-084 treatment mitigated reactive gliosis restoring the altered M1/M2 balance (Cerveró et al., 2018). On the other hand, we did not observe any effect with any of the three Sig-1R ligands assessed on reactive astrogliosis. Contrarily, after spinal root injury,

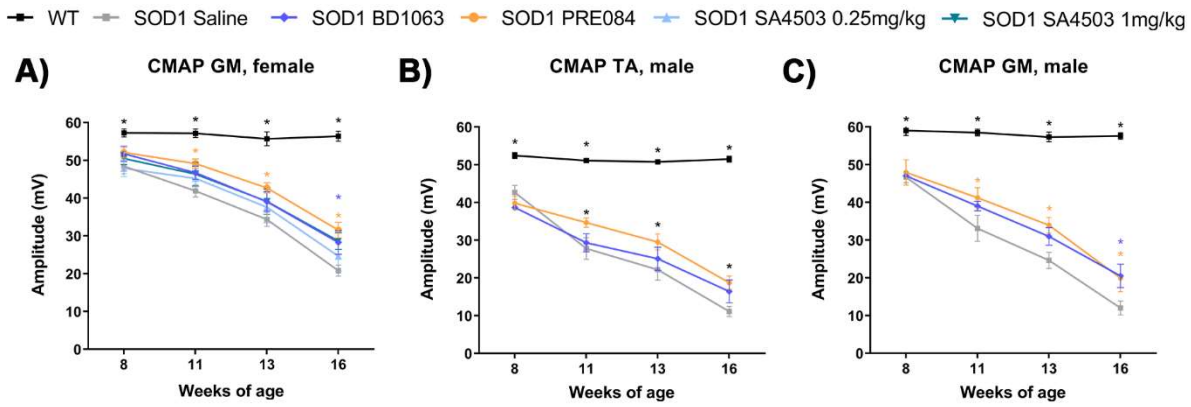
where MN death also occurs, PRE-084 reduced astroglia immunoreactivity (Gaja-Capdevila et al., 2021; Penas et al., 2011). All these studies highlight the role of Sig-1R ligands modulating the neuroinflammatory response.

The etiology underlying the development of ALS remains poorly understood, but abnormal protein aggregation and altered proteostasis are common features of sporadic and familial ALS forms (Medinas et al., 2017). To elucidate the mechanisms through which BD1063 and PRE-084 promote MN preservation we analyzed autophagic flux and ER stress. Autophagy is an intracellular lysosome degradation system responsible for the clearance of cytoplasmic components and organelles. Enhancement of autophagy has been reported in ALS with alterations in several steps. Thus, the level of LC3-II, which is correlated with the extent of autophagosome formation, was found increased in SOD1^{G93A} transgenic mice at symptomatic stage (Morimoto et al., 2007; Tian et al., 2011), as we also found in this study. The marker of the late stage autophagosome, the autophagy adaptor p62, interacts with polyubiquitinated misfolded mutant SOD1 (mSOD1), sequestering mSOD1 into protein inclusions, so fusion of the autophagosome to the lysosome becomes insufficient at the end stage (Tian et al., 2011). Furthermore, autophagy is activated in the ventral spinal cord MNs in sporadic ALS patients, observed by immunostaining for LC3 and p62 (Mizuno et al., 2006; Sasaki, 2011). In our results, we also observed that there was an accumulation of SQSTM1/p62 in the MNs of spinal cord and in the glia cells in SOD1^{G93A} mice. However, there was no difference between untreated group and animals treated with the Sig-1R ligands. It is worth to mention that in ALS the role of autophagy is confusing and it is still unknown whether activation or inhibition of autophagy may influence in the disease progression (Nguyen et al., 2019), and depending on the model used (Medinas et al., 2017).

On the other hand, increased expression of ER stress markers was observed in post-mortem tissues from ALS patients (Hetz et al., 2009), and correspondingly, in this work we observed up-regulation of BiP and XBP1s in the spinal cord of symptomatic SOD1^{G93A} mice. The unfolded protein response (UPR) and the autophagy pathway have been linked (Hetz et al., 2009); thus, XBP1 conditional deletion in the nervous system ameliorated SOD1 mice pathogenesis, through up-regulation of the autophagy pathway boosting the degradation of mSOD1 aggregates. In the context of ALS, the functional significance of ER stress is still unclear, because activating UPR is a protective response to increase protein folding and quality control mechanisms, whereas chronic stress may represent a deleterious signaling due to irreversible disturbance of ER homeostasis. Although we found some differences in the markers analyzed by WB between WT and SOD1^{G93A} mice, we did not observe changes

induced by treatment with the Sig-1R ligands, indicating that autophagy and ER stress pathways are not significantly modified through Sig-1R modulation. However, specific immunolabeling analyses of the spinal cord may reveal changes in specific populations, either MNs or glial cells, that may be obscured in protein analyses of the whole spinal cord tissue. Thus, further experiments are needed to elucidate the mechanisms of action by which Sig-1R ligands yield neuroprotective effects.

SUPPLEMENTARY FIGURES



Supplementary Figure 1. Motor nerve conduction test of SOD1^{G93A} female and male mice treated with Sig-1R ligands. A) Plot showing the amplitude of the compound muscle action potential (CMAP) in gastrocnemius (GM) muscle in females during the follow-up. **B-C)** Plots showing the amplitude of CMAP in the tibialis anterior (TA) and GM muscle in males during the follow-up. (n=10 WT, n=10 SOD1 saline, n=5 SOD1 BD1063 and n=10 SOD1 PRE-084 mice). Data are mean ± SEM, analysed by Two-way ANOVA with Bonferroni's multiple comparisons test. *p< 0.05 vs. SOD1^{G93A} saline mice.

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Chapter II:

Neuroprotective effects of sigma 1 receptor ligands on motoneuron death after spinal root injury in mice.

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Neuroprotective Effects of Sigma 1 Receptor Ligands on Motoneuron Death after Spinal Root Injury in Mice

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Abstract: Loss of motor neurons (MNs) after spinal root injury is a drawback limiting the recovery after palliative surgery by nerve or muscle transfers. Research based on preventing MN death is a hallmark to improve the perspectives of recovery following severe nerve injuries. Sigma-1 receptor (Sig-1R) is a protein highly expressed in MNs, proposed as neuroprotective target for ameliorating MN degenerative conditions. Here, we used a model of L4–L5 rhizotomy in adult mice to induce MN degeneration and to evaluate the neuroprotective role of Sig-1R ligands (PRE-084, SA4503 and BD1063). Lumbar spinal cord was collected at 7, 14, 28 and 42 days post-injury (dpi) for immunohistochemistry, immunofluorescence and Western blot analyses. This proximal axotomy at the immediate postganglionic level resulted in significant death, up to 40% of spinal MNs at 42 days after injury and showed markedly increased glial reactivity. Sig-1R ligands PRE-084, SA4503 and BD1063 reduced MN loss by about 20%, associated to modulation of endoplasmic reticulum stress markers IRE1α and XBP1. These pathways are Sig-1R specific since they were not produced in Sig-1R knockout mice. These findings suggest that Sig-1R is a promising target for the treatment of MN cell death after neural injuries.

Keywords: sigma-1 receptor; spinal root injury; motoneuron death; endoplasmic reticulum stress

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1. Introduction

Injuries that affect the spinal roots and nerve plexuses constitute a frequent and severe pathology, mostly due to trauma caused by traffic, sport and working accidents or birth complications, resulting in partial or total loss of sensory, motor and autonomic functions in the affected limb. After axotomy, a neuronal retrograde response is rapidly initiated due to the disconnection between the neuron soma and its target, and neurons undergo changes in gene expression switching to a regenerative phenotype [1,2]. The course of this response depends on several factors such as the distance of the lesion to cell body, the type of injury, the neuron type, the age and the animal species [3–5]. Regarding the site of lesion, injuries of spinal roots or spinal nerves represent a proximal axotomy that results in progressive death of a significant proportion of spinal motoneurons (MNs) [3,6], and produces changes in the spinal circuits, while inducing a neuroinflammatory response [7]. Surgical intervention through nerve grafting or root reimplantation has been proposed as the only repair solution for spinal root and nerve lesions [8], but they are performed with delay from the injury. Strategies to prevent postinjury neuronal loss and to extend the time-window for surgical repair are needed to allow for subsequent axonal regeneration and eventual functional recovery.

During recent years, attention has focused on several neurotrophic factors offering benefits on neuronal survival after root injuries [9–12], but their clinical use is uncertain. Less studies have reported favorable results in root avulsed animals with pharmacological treatment such as riluzole [13,14], lithium [15] or Neuroheal [16], although none has reached clinical application yet.

Sigma-1 Receptor (Sig-1R) is a transmembrane protein expressed in the central nervous system, particularly highly enriched in the endoplasmic reticulum (ER) of MNs [17]. Alterations in this receptor have been related with MN degeneration and several mutations identified on Sig-1R gene lead to different type of MN diseases, such as amyotrophy lateral sclerosis (ALS) [18,19] or distal hereditary motor neuropathy (dHMN) [20,21]. It has been reported that Sig-1R ligands (such as PRE-084, SA4503, pridopidine) have positive effects reducing MN degeneration in several experimental models. *In vitro*, PRE-084 exerted neu- roprotection and neurite elongation activating protein kinase C in an organotypic culture of spinal cord excitotoxic damage [22], whereas SA4503 protected NSC34 cells against $SOD1^{G93A}$-induced cell death [23]. After root avulsion in adult rats, PRE-084 administration prevented MN death by increasing GDNF expression in astrocytes [24]. Also, in a murine model of ALS, Sig-1R agonists enhanced preservation of spinal MNs and extended the lifespan of $SOD1^{G93A}$ mice, by acting on different pathways: PRE-084 treatment via modulation of NMDA calcium influx [25], pridopidine and SA4503 through activation of the ERK pathway [23,26].

After an injury there is a calcium imbalance and misfolded proteins triggering ER stress as a self-protection mechanism for cell survival. The ER stress induces an adaptive reaction known as unfolded protein response (UPR), in which the ER-resident chaperone BiP is the main sensor. When BiP unbinds from three major effectors, inositol-requiring protein-1 alpha (IRE1α), RNA-activated protein kinase-like ER kinase (PERK) and activating transcription factor-6 alpha (ATF6), there is an intracellular signaling cascade from the ER to the nucleus. The activation of these pathways leads to changes in the gene expression profile of specific proteins and diverse cellular responses aiming to increase the capacity of the cell to restore homeostasis or trigger cell death [27]. Under ER stress or ligand stimulation, Sig-1R binds to IRE1α enhancing the survival pathway IRE1-XBP1 to restore the ER homeostasis [28]. The promotion of the IRE1/XBP1s axis by Sig-1R overexpression also reduces the levels of pro-apoptotic CHOP protein and prevents cell death [29]. Some studies have demonstrated that strategies targeting ER stress and UPR can alleviate neurodegeneration after axonal injury [30,31].

The aim of this study was to evaluate the progressive MN death caused by L4–L5 rhizotomy which mimics a postganglionic spinal root injury in adult wild-type (WT) mice. We investigated whether the administration of Sig-1R ligands (agonist and antagonist) could promote MN survival and modulation of glial reactivity after this injury, and if absence of the Sig-1R protein affects the outcome after rhizotomy in Sig-1R knockout (KO) mice. Our goal was to gain more insight about the molecular mechanisms behind injury induced MN degeneration, such as ER stress, and underlying the neuroprotective effects observed by Sig-1R ligands treatment.

2. Results

2.1 Functional Characterization of L4–L5 Rhizotomized Mice

Nerve conduction tests showed that the compound muscle action potential (CMAP) was totally absent at 5-days post-injury (dpi) in the tibialis anterior (TA) and gastrocnemius (GM) muscles, indicating complete denervation of both muscles after L4–L5 roots section (Figure 1A,B). A reduction of the CMAP amplitude was observed in the plantar muscles in comparison with basal values since they are partially innervated by L5 and L6 ventral roots. Complete denervation of the TA and GM muscles was maintained until the end of the follow-up, confirming correct and selective lesion of L4–L5 lumbar roots. Muscles of the contralateral limb were also tested and showed CMAPs of normal amplitude (data not shown).

Injured animals presented an altered walking pattern, dragging the lesioned right hindlimb [6]. The denervated muscles suffered severe atrophy. At 28 dpi a significant reduction to 50% and 57% of the TA and GM muscles weight compared to the contralateral uninjured muscles was observed. At 42 dpi muscle atrophy progressed, with 45% (TA) and 40% (GM) weight versus the contralateral muscles (Figure 1D–G).

To identify the localization of the MN pools from L4–L5 segments, retrotracers True Blue (TB) in the left and FluoroGold (FG) in the right sciatic nerves were applied after L4–L5 rhizotomy. Spinal lumbar segments, L3 to L6, were identified by the characteristic morphology of the gray matter and comparing with the Atlas of the spinal cord [32]. TB+ MNs were observed at all the segments whereas FG+ MNs were found only at L3 and L6 segments, confirming that L4–L5 roots had been sectioned (Figure 1C).

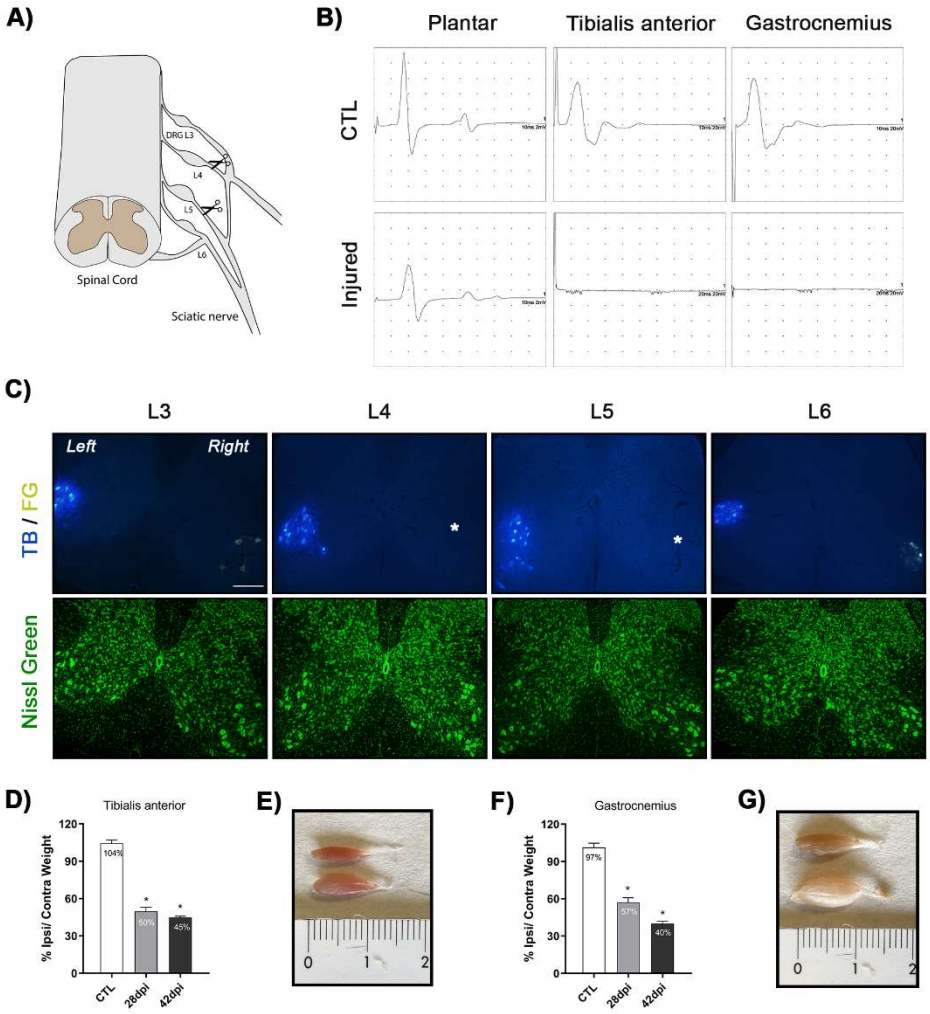

Figure 1. Validation of the L4–L5 rhizotomy model. (**A**) Schematic representation of the surgical procedure. After a small laminectomy, right L4–L5 roots were exposed, and a postganglionic section was made. (**B**) Motor nerve conduction tests recorded the compound muscle action potential (CMAP) in the contralateral side (CTL) and after rhizotomy (Injured). CMAPs were completely abolished in tibialis anterior (TA) and gastrocnemius (GM) muscles at 5 days post injury (dpi) whereas the plantar CMAP was reduced by two thirds. (**C**) Identification of motoneurons (MNs) by sciatic nerve retrolabeling. Representative images of lumbar spinal cord; neurons labelled with True Blue (TB, left) and FluoroGold (FG, right) counterstained with Fluoro Nissl Green. * no FG labelling. Scale bar 200 μm. (**D**–**F**) Graphs of TA and GM muscles weight ratio between ipsilateral and contralateral side at 28 and 42 dpi. Data are mean ± SEM and analyzed with ANOVA and Bonferroni post-hoc test; * $p < 0.05$ vs. CTL. $n = 5–8$ mice per group. (**E**–**G**) Representative images of TA and GM muscles in CTL and injured mice at 42 dpi, showing severe atrophy in the denervated muscles.

2.2 MN Degeneration and Glial Reactivity Following L4–L5 Rhizotomy

The section of L4–L5 roots led to progressive atrophy and loss of MNs. MNs in the ventral horn of spinal cord sections stained with cresyl violet became progressively smaller and rounder, losing their polygonal shape, and had evidence of chromatolysis. The number of MNs in the hemispinal cord of control mice averaged 11.7 ± 0.3 (mean of number of MNs per section ± SEM), whereas after L4–L5 rhizotomy it decreased to 7.6 ± 0.2 (35% loss) at 28 dpi and 6.8 ± 0.3 (42% loss) at 42 dpi. The number of MNs in the contralateral side did not change compared to control intact cords (Figure 2 A,B).

Glial reactivity was assessed by Iba1 and GFAP immunostaining in the ventral horn of lumbar spinal cords. Microglial reactivity was significantly increased in the injured side compared to control mice, being more pronounced at 28 dpi than at 42 dpi (Figure 2C,D). Similarly, a significant increase of astroglia reactivity was observed after rhizotomy similar at 28 and 42 dpi (Figure 2C–E). The contralateral side of the spinal cord did not show significant differences of glial markers in comparison with the intact control group.

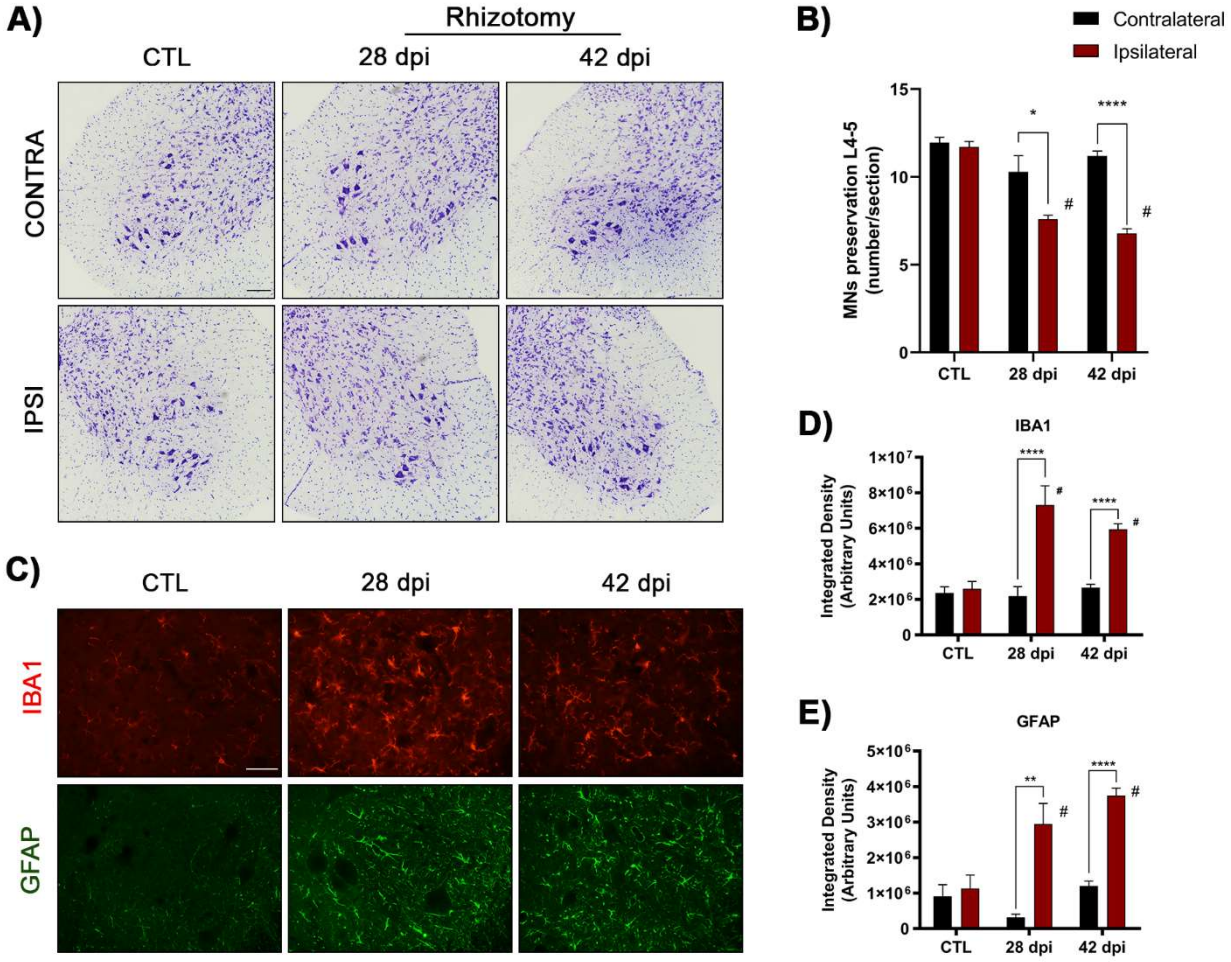

Figure 2. MN death and glial cell activation after rhizotomy. (**A**) Representative images of cresyl violet staining corresponding to ventral horns of L4–L5 spinal segments of the contra- and ipsilateral side to L4–L5 rhizotomy in control and in rhizotomized mice at 28 and 42 dpi. Scale bar 100 µm. (**B**) Plot of number of surviving α-MNs in L4–L5 segments, showing a decrease of MNs in the ipsilateral side to the injury; $n = 4$ female mice at 28 dpi and $n = 14$–16 animals at 42 dpi. (**C**) Images of immunolabeled microglia (Iba1) and astrocytes (GFAP) in the ipsilateral ventral horn of lumbar spinal cord. Scale bar 50 µm. (**D**,**E**) Plots of the integrated density of Iba1 (**D**) and GFAP (**E**) immunoreactivity in the ventral horn of the spinal cord. Rhizotomy caused a significant increase of astrocyte and microglial reactivity. $n = 3$–6 mice per day and group. Data are mean ± SEM. Two-way ANOVA and Bonferroni post-hoc test; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ vs. the contralateral side; # $p < 0.05$ vs. control intact mice.

2.3 Sig-1R Ligands Enhance Preservation of MNs after Rhizotomy

The potential neuroprotective effects of three Sig-1R ligands were evaluated in the rhizotomy model, two of them classified as agonists (PRE-084 and SA4503) and another as antagonist (BD1063) of the Sig-1R. Histological analysis showed that after 42 dpi Sig-1R ligands PRE-084 (8.9 ± 0.4 MNs per section), BD1063 (9.5 ± 0.3) and SA4503 (8.7 ± 0.9) significantly increased the number of MNs preserved between 15 to 22% more in comparison with untreated rhizotomized mice (6.8 ± 0.3) (Figure 3A,B).

Regarding glial reactivity surrounding the axotomized MNs, PRE-084 reduced by 30% microgliosis (69 ± 6% vs. saline mice) after 42 dpi although this difference was not significant (Figure 3C,E). The other two compounds did not reduce microglial reactivity compared with the saline rhyzotomy group. Astrogliosis was increased in the ipsilateral side, and it was significantly reduced in mice treated with PRE-084 (40 ± 6%), BD1063 (54 ± 12%) and SA4503 (46 ± 3%) compared with the saline group at 42 dpi. These results indicate that Sig-1R ligands can prevent MN degeneration and reduce astroglia activation after spinal root injury.

2.4 Influence of Sig-1R Absence in Rhizotomized Mice

Considering that the administration of Sig-1R ligands modulates MN survival, we investigated the consequences of the absence of this protein using transgenic KO mice with Sig-1R gene deletion. Because Sig-1R KO mice have C57BL6 background, the time course of MN death in WT mice of B6SJL and C57BL6 strains was first compared. Results showed no significant differences in the evolution of MN degeneration after L4–L5 rhizotomy between strains; the percentage of MNs loss after rhizotomy in C57BL6 WT mice was 30% and 40% at 28- and 42 dpi, respectively (Figure 4A).

In the spinal cord of WT mice, as previously described, Sig-1R was detected in MNs, specifically in clusters at the post-synaptic sites of C-terminals subsurface cistern (Figure 4B) [33,34]. This labeling was not observed in the Sig-1R KO mice. Western blot also confirmed the lack of Sig-1R protein in the KO mice (Figure 4C).

The number of spinal α -MNs in the ventral horn of L4–L5 spinal segments was the same in WT (11.3 ±0.3 MNs per section) and Sig-1R KO (11.2 ± 0.4) adult mice (Figure 4D–F). After rhizotomy, the number of surviving MNs declined significantly, but there were no significant differences between WT and Sig-1R KO mice at the two time-points analyzed neither for female (Figure 4E) nor for male mice (Figure 4F). Sig-1R KO rhizotomized female mice had about 10% more MN preservation in comparison with injured WT mice, but this difference was not significant. In contrast, astrogliosis and microgliosis were markedly reduced in rhizotomized Sig-1R KO mice compared with injured WT mice at 28 dpi although differences did not reach significance (Figure 4G–I).

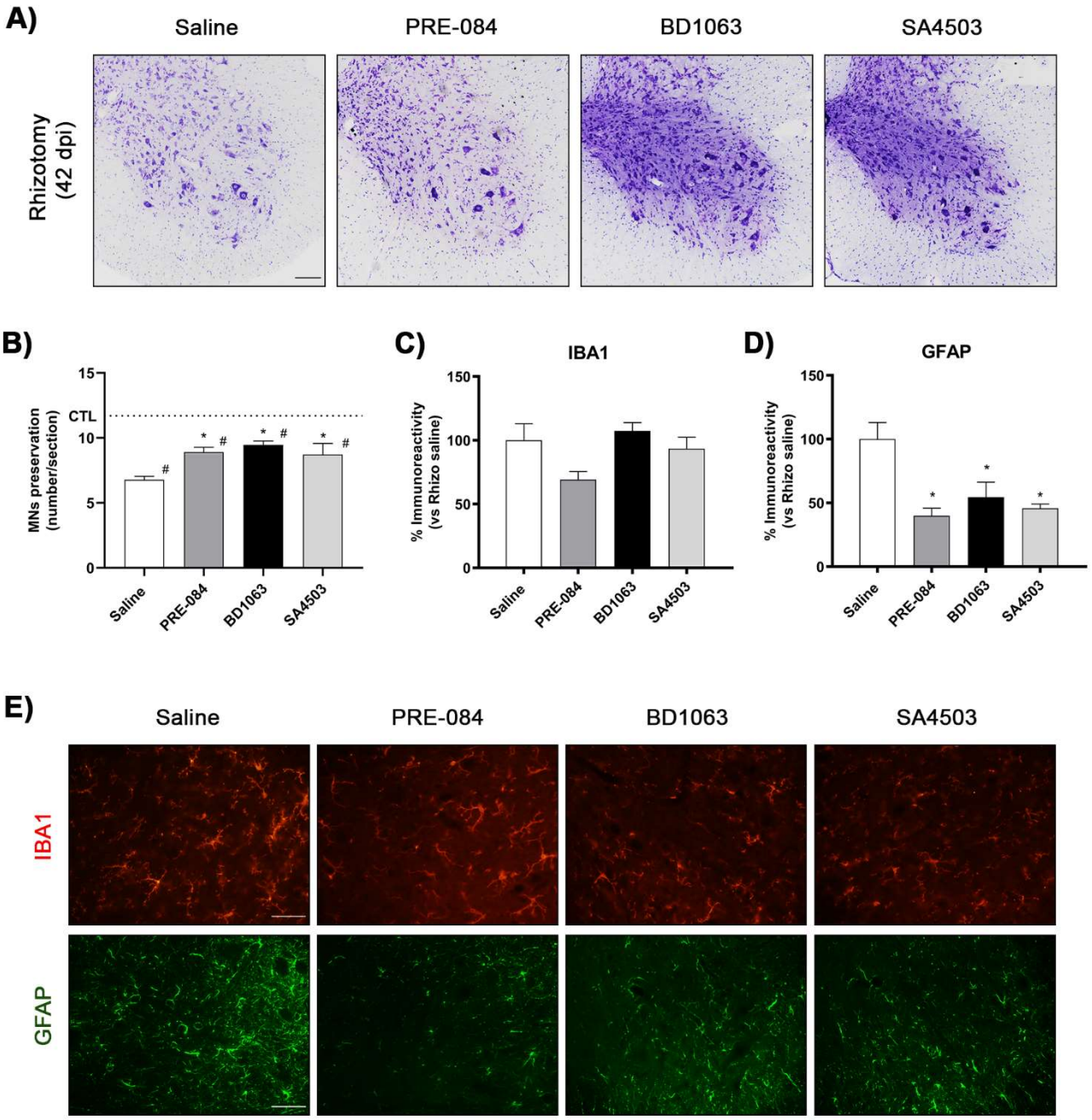

Figure 3. Administration of Sig-1R ligands promotes MN preservation after rhizotomy. (**A**) Representative images of ventral horns of L4–L5 spinal segments ipsilateral to rhizotomy in female mice with or without Sig-1R ligand treatment at 42 dpi. Cresyl violet stain. Scale bar 100 µm. (**B**) Plot of numbers of surviving α-MNs in L4–L5 segments, showing higher numbers of MNs in mice treated with Sig-1R ligands PRE-084, BD1063 and SA4503. Animals per group at 42 dpi: saline $n = 11$, PRE-084 $n = 4$, BD1063 = 4 and SA4503 $n = 4$. Data are mean ± SEM and analyzed with One-way ANOVA and Bonferroni multiple comparisons test. * $p < 0.05$ vs. saline rhizotomy mice at same time-point; # $p < 0.05$ vs. control mice. (**C**,**D**) Plots showing the percentage of immunoreactivity of Iba1 (**C**) and GFAP (**D**) in the ipsilateral ventral horn of the spinal cord after rhizotomy and treatment with Sig-1R ligands. Data are mean ± SEM and analyzed with One-way ANOVA and Bonferroni multiple comparisons test. * $p < 0.05$ vs. saline rhizotomy mice. (**E**) Representative images of immunolabeling of microglia (Iba1) and astrocytes (GFAP) in the ipsilateral ventral horn of rhizotomized mice at 42 dpi. Scale bar 50 µm.

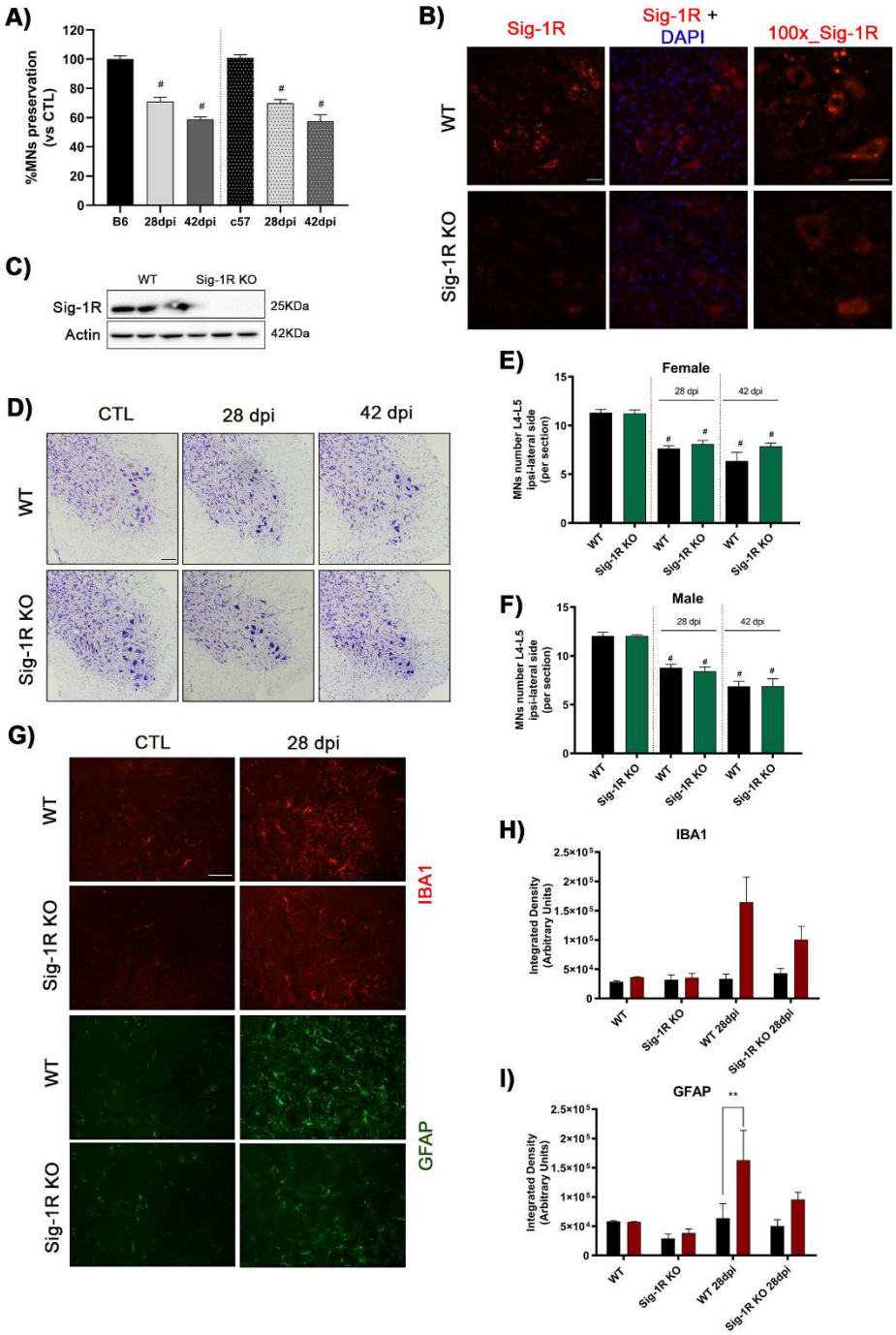

Figure 4. MN degeneration in Sig-1R KO mice after rhizotomy. (**A**) Plot of the percentage of surviving spinal MNs in male and female mice of the B6SJL (B6) and C57BL6 (c57) strains at 28 and 42 dpi. The same time-course of MNs loss occurred in WT mice with the two genetic backgrounds. Animals per group: B6 control n = 18, rhizotomy n = 6 at 28 dpi and n = 18 at 42 dpi; c57 control n = 16, rhizotomy n = 9 at 28 dpi and n = 9 at 42 dpi. Data are mean ± SEM with One-way ANOVA and Bonferroni multiple comparisons test. # $p < 0.05$ vs. control mice. (**B**) Representative images corresponding to Sig-1R immunolabeling in the ventral horn of spinal cord in WT and Sig-1R KO mice. There was absence of labeling in the KO mice whereas in the WT it was mainly localized in MNs. Scale bar 50 µm. (**C**) Western blot confirms the absence of Sig-1R in the spinal cord of KO mice. (**D**) Representative images of ventral horns of L4–L5 spinal segments ipsilateral to the rhizotomy in WT and Sig-1R KO mice at 28 and 42 dpi. Scale bar 100 µm. (**E**,**F**) Plots of number of surviving α-MNs in the ipsilateral side of L4–L5 segments in female (**E**) and male (**F**) mice, showing no differences between WT and Sig-1R KO mice. # $p < 0.05$ vs. uninjured mice. (**G**) Representative images of glial reactivity assessed by Iba-1 and GFAP immunolabeling. Scale bar 50 µm. (**H**,**I**) Bar graph showing the integrated density of Iba1 (**H**) and GFAP (**I**) immunolabeling in the ventral horn of spinal cord (red: ipsilateral side; black: contralateral side). ** $p < 0.01$ vs. contralateral side.

2.5 Activation of ER Stress after Rhizotomy

We then investigated whether rhizotomy induces activation of ER stress responses, similar to what was previously reported in the models of root avulsion in rats [31] and axotomy of the hypoglossal nerve in mice [35]. We found that Sig-1R protein levels in the spinal cord remained lower, although not significantly, than in uninjured control mice at 7 and 14 days after rhizotomy. Pharmacological treatments with Sig-1R ligands PRE-084 and BD1063 did not modify the protein levels of Sig-1R (Figure 5A,B). In physiological conditions Sig-1R is recruited by BiP (Grp78), which also represses the activity of the three main stress sensors, while under ER stress it is dissociated from BiP promoting the activation of downstream ER stress factors. BiP protein levels follow the pattern of Sig-1R. We did not find significant changes of BiP levels in mice after the injury, neither after the pharmacological treatments (Figure 5A). At 7 dpi after rhizotomy, in the saline group ER self-protection mechanisms were promoted through the activation of IRE1α, however at 14 dpi the ratio of activated form of IRE1α /total IRE1α declined to control levels. In contrast, Sig-1R ligands PRE-084 and BD1063 significantly increased the protective levels of activated IRE1α at 14 dpi (Figure 5A,B). IRE1α promotes the alternative spliced form of XBP1 (XBP1s), which induces the translocation of this transcriptional factor into the nucleus and the upregulation of several genes to solve ER stress. The ratio of the spliced versus unspliced forms of XBP-1 was found significantly raised at 7 days after rhizotomy in saline mice in comparison with CTL but declined at 14 days. In contrast, XBP-1s/XBP1u ratio was enhanced significantly at 14 dpi in injured mice treated with PRE-084 and BD1063. In addition, XBP-1 immunoreactivity was observed in the MNs of the ventral horn at 7 dpi compared with control samples (Figure 5C). Protein levels of CHOP were significantly increased at 14 dpi in saline injured mice. PRE-084 treatment reduced the CHOP levels at 14 dpi more efficiently than BD1063 treatment.

The ER stress markers were also analyzed in Sig-1R KO mice. There were no differences in BiP and phosphorylated IRE1α protein levels between WT and Sig-1R KO mice. Moreover, spinal root lesion did not modify the signaling axis IRE1α /XBP1s in Sig-1R KO mice, but the effects on the self-protection mechanisms by PRE-084 and BD1063 treatments were abolished (Figure 5D,E).

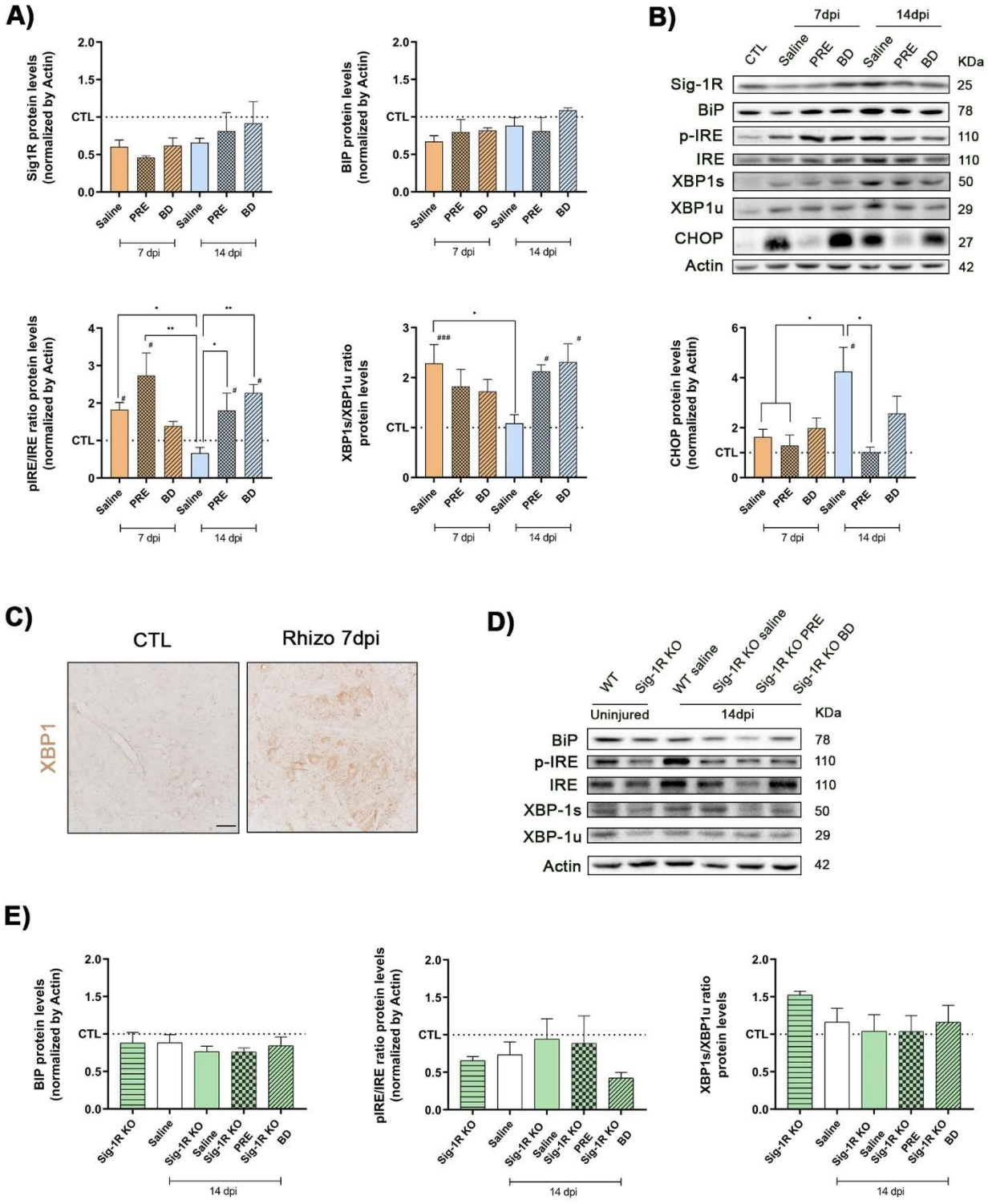

Figure 5. Changes in ER stress markers after rhizotomy. (**A**) Quantitative bar graphs and (**B**) immunoblots and of Sig-1R, BiP, phosphorylated IREα/IRE ratio, XBP-1s/XBP-1unspliced ratio and CHOP proteins in control (CTL) and in injured mice with or without treatment with PRE-084 and BD1063 at 7 and 14 dpi. *n* = 3–4 per group and time point. Data are mean ± SEM and analyzed with One-way ANOVA and Dunnett's multiple comparisons test. ### $p < 0.001$ vs. CTL mice; ** $p < 0.005$, * $p < 0.05$ vs. saline 14 dpi. (**C**) Microphotographs of XBP-1 labeling in the ventral horn of spinal cord confirms the upregulation of this ER stress marker in MNs at 7 dpi. Scale bar 50 µm. (**D**) Immunoblots and (**E**) bar graphs of BiP, phosphorylated IREα/IRE ratio and XBP-1s/XBP-1unspliced ratio, in WT and Sig-1R KO mice groups at 14 dpi. *n* = 3–5 per group. One-way ANOVA and Dunnett's post-hoc test: # $p < 0.05$ vs. CTL mice.

3. Discussion

Presently, there is no effective treatment for proximal spinal root or plexus injuries, other than palliative surgery through nerve or muscle transfers [8,36]. However, the death of a significant number of MNs and DRG sensory neurons after the injury presents an important drawback limiting the possibilities for recovery even after the most advanced surgical procedures [8]. Therefore, research on treatments that prevent neuronal death is necessary to improve the perspectives of recovery following severe nerve injuries. In this study we have used a model of L4–L5 rhizotomy in adult mice to induce MN degeneration and to evaluate the role of Sig-1R ligands as neuroprotective agents. This model of proximal axotomy at the immediate postganglionic level resulted in considerable death, up to 40% of spinal MNs at 42 days after the injury.

In adult animals the proximity of axotomy to the cell body significantly increases the consequences of the lesion, causing retrograde death of both spinal and cranial affected MNs. The majority of spinal MNs survive after a more distal injury, such as a sciatic nerve axotomy [37,38], but 60–80% of MNs dye following a root avulsion that traumatically detaches the axons from its entrance into the spinal cord [3,6,39]. In a model of milder lesion, a lumbar ventral root crush, it was found 25% neuronal loss at 7 days after injury, which increased to 50% at 28 days [40–42]. Our model of postganglionic rhizotomy is produced more distally but it avoids intraspinal surgery, and induces slower degeneration affecting 40% of MN at 42 days. Similarly, the model of hypoglossal neurotomy causes 30 to 40% MN death at 3–4 weeks post injury [35,43]. It is important to highlight that the discordance in the percentage of MN death after root injuries between some studies should also take into account differences in timing and in methods of labeling and quantifying the outcome of MNs [11].

Since survival of MNs is key to allow recovery of the motor functions lost after a neural injury and adequate repair, early and effective approaches for promoting neuroprotection after lesion are needed. We examined whether pharmacological therapy with different Sig-1R ligands could minimize the loss of spinal MNs. We found that daily treatment with PRE-084, BD1063 and SA4503 significantly increased the number of surviving MNs after L4–L5 rhizotomy. The beneficial role of Sig1-R agonist compounds in terms of neuroprotection have been previously reported on in vitro and in vivo models. In the $SOD1^{G93A}$ mouse model of ALS neuroprotective effects with agonists PRE-084 and SA4503 were observed, mildly extending the lifespan and enhancing preservation of MNs via modulation of NMDA calcium influx and through activation of the ERK pathway [23,25]. Recently, Ionescu et al. (2019) found that pridopidine, another Sig-1R agonist, improved several cellular and histological hallmark signs of ALS [26]. PRE-084 also prevented MN death in a model of spinal root avulsion [24] and in the wobbler mouse, a spontaneous model of MN degeneration [44]. These data provide clear evidence of the interesting effects of Sig-1R agonist ligands for ameliorating neurodegenerative conditions.

Here, we demonstrated for the first time that a Sig-1R antagonist BD1063 exerted neuroprotection and reduced astroglial activation. In previous studies BD1063 was tested in coadministration with PRE-084 to demonstrate that the therapeutic action could be attributed to the activation of the Sig-1R by an agonistic compound [24,25]. In fact, in the context of Sig-1R there is controversy between the classical classification as agonists and antagonists of diverse ligand classes showing high affinity for Sig-1R [45]. Indeed, different ligands may provoke various effects, even acting opposite in the regulation of calcium homeostasis and neuronal excitability. A recent work reported that intracellular calcium shuttling can be manipulated by Sig-1R activation; the SA4503 ligand accelerated cytosolic calcium clearance after AMPA receptor activation and IP3R-mediated ER calcium release, whereas PRE-084 did not exert any significant effect on cytosolic calcium levels in $SOD1^{G93A}$ mice cultured MNs [46]. Thus, we found promising results with different types of compounds that have high affinity to Sig-1R.

After rhizotomy, we found that Sig-1R and chaperone BiP protein levels were maintained at 7 and 14 dpi even under treatment with the Sig-1R ligands. This is in line

with previous findings in which PRE-084 administration did not modify Sig-1R protein levels at 3 days after preganglionic root avulsion in rats [24]. Molecular analyses showed that L4–L5 rhizotomy induced ER stress, activating IRE1α /XBP1 pathway, as reported in other models of proximal injuries, such as root avulsion [31] and hypoglossal axotomy [35]. Upon ER stress, IRE1 undergoes dimerization and phosphorylation leading to its active endonuclease form and inducing the unconventional splicing of the mRNA encoding XBP1 with production of the XBP-1s. XBP-1s is essential for the expression of genes involved in protein folding, secretion, phospholipid biosynthesis and ER-associated protein degradation. It has been described that under ER stress or ligand stimulation, Sig-1R binds to IRE1α regulating its stability and enhancing the survival pathway IRE1-XBP1 to restore the ER homeostasis [28]. After rhizotomy we found that the IRE1/XBP1s axis is enhanced by PRE-084 and BD1063 administration, suggesting that Sig-1R ligands exert a pro-survival state by restoring ER function. Here, we also found that the activation of the IRE1/XBP1s axis is due to the modulation of Sig-1R by the ligands, since it did not occur in the Sig-1R KO mice. XBP-1s can bind to gene promoters to regulate gene expression, among others of CHOP, to restore protein homeostasis. Indeed, the ER stress induced after rhizotomy resulted in an increase of pro-apoptotic CHOP levels that would contribute to MN loss. The reduction of CHOP levels by treatment with Sig-1R ligands appeared associated to increased preservation of the spinal MNs. Similarly, in cardiomyocytes, the overexpression of Sig-1R under ER stress enhanced IRE1/XBP1s axis and reduced CHOP levels, preventing cell death [29]. Alternatively, under stress conditions such as neural injuries, Sig-1R could form cholesterol-enriched microdomains in the ER as proposed recently in vitro [47]. These changes in the ER membrane may modulate recruitment of ER proteins, such as IRE1, by forming big clusters enriched with Sig-1R and IRE1. Sig-1R modulation by administration of ligands might be influenced by this formed microdomains. However, further experiments are needed to confirm cholesterol-microdomains formation in the ER after axonal injury and possible changes upon Sig-1R stimulation.

Spinal root injury is associated with a neuroinflammatory response involving local microglia and astrocytes in the spinal cord [3]. Several reports demonstrated the important role of Sig-1R in non-neuronal cells, since in different neurodegenerative disorders the resulting glial activation was modified after treatment with a Sig-1R ligand, such as in stroke [48], pain [49] and MN diseases [24,25,44,50]. We have found in samples at 6 weeks post rhizotomy that PRE-084 treatment reduced by 30% microgliosis, whereas PRE-084, BD1063 and SA4503 administration significantly reduced astrogliosis to around 50% of untreated cords. Such more pre-eminent action on astrocytes was also described for PRE-084 treatment in the Wobbler mouse [44]. Thus, modulation of the glial response is a targeting mechanism of Sig-1R ligands, although the effects may vary depending on the compound and the type of disease. Furthermore, it is well known that non-neuronal cells express Sig-1R in the brain but only a few studies have described its expression in the spinal cord. Choi et al. (2016) found that Sig-1R immunolabeling increased in dorsal horn astrocytes following thoracic spinal cord hemisection, but not in neurons and microglia. Sig-1R protein levels also increased at day 7 post-surgery compared with control mice in the dorsal horn and normalized at 28 dpi [51]. In our study of rhizotomy, Sig-1R protein levels in spinal cord samples remained reduced at 7 and 14 dpi, although not significantly. However, it is important to note that some commercially available antibodies have poor specificity for Sig-1R, generating controversy in data reported in the literature [52].

4. Materials and Methods

4.1. Animals and Experimental Design

Adult mice (10 weeks of age) of B6SJL and C57BL6 background were maintained at the Animal Research Facility of Universitat Autònoma de Barcelona. Adult transgenic Sig-1R knockout (σ1R $^{-/-}$; σ1R KO) mice (10 weeks old) with C57BL6 background, generated and characterized as previously described [53], were provided by Esteve Pharmaceuticals from the colony maintained at Envigo. Mice were kept under

standard conditions of temperature (22± 2 ◦ C) and 12:12 light:dark cycle with access to food and water ad libitum. Mice were maintained 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 were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

WT B6SJL female mice were submitted to L4–L5 rhizotomy and evaluated at 7, 14, 28 and 42 dpi, divided in subgroups with or without Sig1-R ligands treatment. For histological studies, male mice were also used to evaluate if there were differences in MN degeneration between sexes. The following number of animals (*n* = ♀/ ♂) were used per group: uninjured (CTL) (*n* = 13/5), 28 dpi saline (*n* = 3/3), 42 dpi saline (*n* = 11/7), 42 dpi PRE-084 (*n* = 4), 42 dpi SA4503 (*n* = 4) and 42 dpi BD1063 (*n* = 4). For the Sig-1R ablation study, C57BL6 male and female mice were used with the following number (*n* = ♀/ ♂) per group: WT uninjured (*n* = 11/6), Sig1-R KO uninjured (*n* = 5/7), WT 28 dpi (*n* = 6/3) and 42 dpi (*n* = 5/4), Sig1-R KO 28 dpi (*n* = 5/5) and 42 dpi (*n* = 8/6). For molecular analyses groups of female mice were used for obtaining samples at 7 and 14 dpi (*n* = 3–5 per treatment and day).

Intraperitoneal administration of three Sig-1R ligands, two classically considered as agonists, PRE-084 (0.25 mg/kg, TOCRIS, Bristol, UK) and SA4503 (1 mg/kg, TOCRIS, Bristol, UK) and an antagonist BD1063 (5 mg/kg, TOCRIS, Bristol, UK) was given once a day starting 30 min after surgery until the mice were euthanized. All the compounds were dissolved in 0.9% saline solution and administered in a volume of 10 mL/kg. The dosage of Sig-1R ligands was chosen based on previous studies showing benefit in MN degeneration, ATR-X syndrome, retinopathy and pain models [24,25,54,55], and preliminary assays in our laboratory. Control animals were administered with saline.

4.2 Rhizotomy Surgery

Surgery was performed under anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally. An incision was made in the skin of the dorsum above the iliac crest and a small laminectomy was performed to expose the right L4–L5 spinal roots, and the roots were cut at postganglionic level [56] (Figure 1A). Then, the stumps of the roots were separated in opposites sides to avoid axon regeneration. The incision was sutured and disinfected with povidone iodine, and the mice cared until recovery in warm environment. Analgesia was provided with buprenorphine (0.1 mg/kg s.c.) for the next 2 days. During all the follow up, the mice were checked for assessment of general health and mobility daily during the first week and later once per week.

4.3 Retrograde Labelling

Retrograde tracing was applied to the sciatic nerve of mice to identify the MN pools sending axons through lumbar L4–L5 spinal roots after surgery. Under anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg) rhizotomy was performed on the right L4–L5 roots. Thereafter, the sciatic nerve was exposed at the midthigh and sectioned. The proximal stump of the nerve was soaked in retrotracer solution (1 µL) True Blue Chloride (TB, Setareh Biotech, Eugene, OR, USA) in the left side and FluoroGold (FG, Fluorochrome, Denver, CO, USA) in the right side for 45 min to allow dye internalization. Afterwards, the wound was sutured in layers and disinfected. Animals were sacrificed 7 days post tracing.

4.4 Electrophysiological Tests

Motor nerve conduction tests were performed at 5 dpi and at the end of follow-up in both hindlimbs. Briefly, the sciatic nerve was stimulated with small needle electrodes placed at the sciatic notch, and the CMAP of TA, GM and plantar interossei muscles recorded with microneedle electrodes [57]. All the potentials were amplified and displayed on a digital oscilloscope at settings appropriate to measure the latency to the onset and the amplitude to the negative peak. Mice were tested under anesthesia with pentobarbital (50 mg/kg i.p.) and maintained warm by means of a thermostatic heating pad.

4.5 Histological and Immunohistochemical Analyses

At 7, 28 and 42 dpi subgroups of mice were deeply anesthetized with pentobarbital (200 mg/kg) and transcardially perfused with 4% paraformaldehyde in phosphate buffer (PB). The lumbar spinal cord and the muscles TA and GM were harvested, post-fixed, and cryopreserved in 30% sucrose solution in PB at 4°C. Hindlimb muscles of both sides were weighed before cryopreservation.

For assessing MN preservation, 20 µm transverse thick sections were serially cut on a cryostat (Leica, Wetzlar, Germany). Each section of a series of 10 was collected sequentially on separate slides. L4–L5 lumbar spinal cord sections of each animal separated 100 µm were rehydrated and stained for 3 h with an acidified solution of cresyl violet 3.1 mM. The slides were dehydrated with ethanol, cleared with xylol, and mounted with DPX. Images of L4–L5 lateral ventral horn stained with cresyl violet were captured at 20x (Nikon Eclipse Ni, Tokyo, Japan). Then, the number of MN per each section was counted. MNs were identified if they had diameter larger than 20 µm, polygonal shape and prominentnucleoli.

For immunohistochemistry of XBP-1, the endogenous peroxidase activity was inhibited with 70% methanol, 30% Tris-buffered saline (TBS) and 2% H2O2. Lumbar spinal cord sections were blocked with TBS with 3% Triton-X-100 and 1.5% normal donkey serum. Slides were incubated overnight at 4 °C with primary antibody against rabbit anti-XBP-1 (1:200, ab37152, Abcam, Cambridge, UK). Slides were washed with TBS with 0.1% tween 20 and incubated with a secondary antibody horse anti-rabbit biotinylated (Vector Lab- oratories, Burlingame, CA, USA) overnight at 4°C. Afterwards, slides were incubated using VECTASTAIN® Elite ABC complex for 1 h and DAB solution (Vector Laboratories, Burlingame, CA, USA) for brown color development. Finally, after dehydration with a series of ethanol gradients and xylol clearing, slides were mounted with DPX. Images were captured at x20 (Nikon Eclipse Ni, Tokyo, Japan).

To identify Sig-1R localization in spinal cord samples, antigen retrieval was performed by incubating samples with pre-boiled citrate buffer for 30 min at room temperature. The endogenous peroxidase and biotin activity were inhibited with Invitrogen Kit (E21390, B40933, Invitrogen, Waltham, MA, USA). Lumbar spinal cord sections were incubated with blocking solution for 1 h, then incubated overnight at 4°C with primary antibody rabbit anti-Sigma-1 Receptor (1:100; 223702, Abcam, Cambridge, UK). Slides were washed with phosphate buffer saline (PBS) with 0.3% Triton and incubated with a secondary antibody horse anti-rabbit biotinylated (1:200, Vector Laboratories, Burlingame, CA, USA) for 1 h. After washes, samples were incubated for 1 h with streptavidin-HRP linked antibody. TSA kit was applied for 3 min for displaying AlexaFluor 555 to Sig-1R. Finally, slides were mounted with Fluoromount G (Southern Biotech, Birmingham, AL, USA). Images of the ventral horn were captured at x40 and x100 (Nikon Eclipse Ni, Tokyo, Japan).

For immunolabeling glial cells, lumbar spinal cord sections were blocked with PBS with 0.3% Triton-X-100, 10% donkey serum and 0.2 mM glycine 1 h at room temperature. Slices were then incubated overnight at 4 °C with primary antibodies rabbit anti-Iba1 (1:500; 019-19,741, Wako, Osaka, Japan), and rat anti-GFAP (1:500; 13-0300, Invitrogen, Waltham, MA, USA). After washes with PBS with 0.1% tween 20, sections were incubated for 2 h with the respective secondary antibodies: Alexa 488-conjugated secondary antibody (1:500) or Cy3-conjugated secondary antibody (1:500). FluoroNissl (1:200, 990210, Invitrogen, Waltham, MA, USA) and DAPI (1:2000; D9563, Merck-Sigma, Kenilworth, NJ, USA) added to stain MNs and nuclei, respectively, and the slides were mounted with Fluoromount G. Microphotographs of the spinal cord ventral horn were captured under the same exposure time, sensitivity, and resolution (x40) for each analyzed marker by using a fluorescence microscope (Olympus BX51, Tokyo, Japan). A total of 8 images were taken per animal and side, then analyzed. The integrated density of GFAP and Iba1 labeling was measured after defining a threshold for background correction using ImageJ software. The integrated density is the product of area and mean grey value, which is the sum of the grey values of all the pixels in the selection divided by the number of pixels.

4.6 Protein Extraction and Western Blot Analysis

Mice were deeply anesthetized at 7 and14 dpi (n = 3–5) to obtain L4–L5 spinal cord segments of control and rhizotomized mice with or without Sig1-R ligands treatment for Western blot analyses. Samples were divided in ipsi- and contralateral halves and frozen in liquid nitrogen for storage. Tissue samples were homogenized in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% triton- X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing a mixture of protease inhibitor cocktail (Merck- Sigma, Kenilworth, NJ, USA) and phosphatase inhibitors (PhosphoSTOP Roche, Basilea, Switzerland). Lysates were homogenized on ice using a Potter homogenizer, sonicated, and centrifuged at 12,000 rpm during 10 min at 4 ˚C. Protein concentration was determined by BCA Protein Assay (Biorad, Hercules, CA, USA). For Western blotting, equal amount of protein per each sample (20–30 µg) were loaded onto 7.5–15% SDS-polyacrylamide gels and transferred in a PDVF membrane for 1.30 h at 90 V and room temperature. Membranes were blocked with 6% milk or 5% BSA (for phosphorylated antibodies) in TBS 0.1% Tween- 20 for 1 h and then, incubated with primary antibodies at 4 ºC overnight. The following antibodies were used: rabbit anti-Sigma-1 Receptor (1:250; 223702, Abcam, Cambridge, UK), rabbit anti-GRP78/BiP (1:500, G8918, Merck-Sigma, Kenilworth, NJ, USA), rabbit anti- IRE (phosphor S724) (1:200, 48187, Abcam, Cambridge, UK), rabbit anti-IRE1α (1:500, 3294, Cell Signaling, Danvers, MA, USA), rabbit anti-XBP-1 (1:250; 37152, Abcam, Cambridge, UK), mouse anti-GADD153/CHOP (1:500; 3751, Santa Cruz Biotech., Dallas, TX, USA), mouse anti-actin (1:50,000; A5316, Merck-Sigma, Kenilworth, NJ, USA). The following day, after several washes, the membrane was incubated with secondary antibody HRP-conjugated (1:5000; Biorad, Hercules, CA, USA) for 1 h at room temperature. Proteins were visualized using enhanced chemiluminescence method (ECL Clarity kit, BioRad Laboratories, Hercules, CA, USA) and images were captured using Chemidoc apparatus. Image Lab software (BioRad, Hercules, CA, USA) was used for image density quantification. Levels of each protein were normalized by the housekeeping protein (ß-actin) and by each control sample.

4.7 Statistical Analyses

All data are expressed as mean ± SEM. Statistics were performed using GraphPad Prism 6 software. Data were analyzed using ANOVA followed by Bonferroni's post-hoc test. For molecular analysis data were analyzed by one-way ANOVA followed by Dunnett's post-hoc test. Differences were considered significant at p-value ≤ 0.05.

5. Conclusions

In summary, the survival of spinal MNs in adult mice following postganglionic rhizotomy is dependent on the time after the lesion, inducing a loss of about 40% of the spinal MNs at 42 days. Treatment with different Sig-1R ligands exerted neuroprotective effects, preventing cell death by maintaining the IRE1/XBP1s axis, increasing the number of surviving MNs and reducing glial reactivity in the ventral horn after rhizotomy. These and previous findings suggest that Sig-1R is a promising target for the treatment of MN degeneration.

Author Contributions: Conceptualization, N.G.-C., X.N. and M.H.-G.; methodology, validation and formal analysis, N.G.-C., N.H. and M.H.-G.; resources, D.Z., J.M.V. and X.N.; data curation, N.G.-C., N.H., X.N. and M.H.-G.; writing—original draft preparation, N.G.-C. and M.H.-G.; writing—review and editing, J.M.V., M.M., M.H.-G. and X.N.; funding acquisition, X.N. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the guidelines of the European Union Council (Directive 2010/63/EU) and Spanish regulations (RD 53/2013) on the use of laboratory animals. The experimental procedures were approved by the Committee of Ethics in Experimentation in Animals and Humans (CEEAH, procedures #10121 and #10306) of the Universitat Autònoma de Barcelona.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is available from the authors under reasonable request.

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Conflicts of Interest: The authors declare that the work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. JMV, MM and DZ were employed by the company ESTEVE Pharmaceuticals at the time this study was performed. All other authors declare no competing interests.

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Chapter III:

EST79232 and EST79376, two novel sigma-1 receptor ligands, exert neuroprotection on models of motoneuron degeneration.

EST79232 and EST79376, two novel sigma-1 receptor ligands, exert neuroprotection on models of motoneuron degeneration

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ABSTRACT

Motor neuron diseases (MND) include a broad group of sporadic and hereditary neurological disorders characterized by progressive degeneration of motor neurons (MNs). Sigma-1 receptor (Sig-1R) is a protein enriched in MNs, and mutations on its gene lead to various types of MND. Previous studies in experimental models suggested that Sig-1R is a target to avoid MN degeneration. In this study two novel synthesized Sig-1R ligands coded EST79232 and EST79376, from the same chemical series, with the same scaffold and similar physicochemical properties but opposite functionality on Sig-1R, were evaluated as neuroprotective compounds to prevent MN degeneration in three experimental models. Pharmacokinetic analysis confirmed *in vivo* drug exposure. We used an *in vitro* model of spinal cord organotypic cultures under chronic excitotoxicity, and two *in vivo* models, the spinal nerve injury and the $SOD1^{G93A}$ mice, to characterize the effects of these Sig-1R ligands on MN survival and modulation of glial reactivity. Results show that the antagonist EST79376 preserved MNs *in vitro* and after spinal nerve injury but was not able to improve MN death in $SOD1^{G93A}$ mice. In contrast, the agonist EST79232 significantly increased MN survival in the three models of MN degeneration evaluated and had a mild beneficial effect on motor function of the hindlimb muscles in $SOD1^{G93A}$ mice. These data further support the interest of Sig-1R as a therapeutic target for neurodegeneration.

Key words: amyotrophic lateral sclerosis, motoneuron degeneration, sigma-1 receptor, spinal cord, $SOD1^{G93A}$ mice, spinal nerve injury.

INTRODUCTION

Motor neuron diseases (MND) represent a heterogeneous group of chronic sporadic and hereditary neurological disorders involving the upper or lower motor neurons (MNs), mainly represented by amyotrophic lateral sclerosis (ALS) in adults and spinal muscular atrophy (SMA) in children. There is no effective treatment available yet for most of these diseases; three drugs based on gene therapy are approved for SMA and only two drugs are approved for ALS, riluzole and edaravone, which slightly prolong the lifespan of the patients (Edaravone (MCI-186) ALS 19 Study Group, 2017; Ludolph and Jesse, 2009). This is in good part due to the multiple etiopathogenetic mechanisms, with a proposed complex interplay between excitotoxicity, neuroinflammation, oxidative stress, protein aggregation, mitochondrial dysfunction, and axonal transport defects, contributing to MN degeneration (Mancuso and Navarro, 2015).

Sigma-1 receptor (Sig-1R) is a protein enriched in the endoplasmic reticulum of MNs. Although, it has no direct downstream signaling, Sig-1R acts as a chaperone modulating plenty of essential cellular processes (Langa et al., 2003; Mavlyutov et al., 2010). Several mutations on the *Sig-1R* gene have been identified that lead to different types of MND, such as a severe, juvenile-onset form of ALS (ALS16) (Al-Saif et al., 2011), development of FTD-ALS (Luty et al., 2010), and some familial cases of distal hereditary motor neuropathies (dHMN) (Almendra et al., 2018; Ververis et al., 2019). In addition, the administration of Sig-1R ligands was shown to exert neuroprotection in various experimental models of MN degeneration, including *in vitro* excitotoxicity (Guzmán-Lenis et al., 2009; Ono et al., 2014) and in vivo models of ALS (Ionescu et al., 2019; Mancuso et al., 2012a; Ono et al., 2014), spinal root injury (Penas et al., 2011, Gaja-Capdevila et al., 2021) and the wobbler mouse, a model of spontaneous MN degeneration (Peviani et al., 2014). Taken together, all these data illustrate the connection between Sig-1R and MN survival.

The aim of this study was to evaluate the therapeutic effect of novel selective Sig-1R ligands, coded as EST79232 and EST79376, on well characterized in *vitro* and *in vivo* models of MN degeneration. First, the compounds were tested *in vitro* to estimate the affinity to the Sig-1R and to define the pharmacological profile of both new compounds. Then, spinal cord organotypic culture (SCOC) under chronic excitotoxicity was used instrumentally for initial screening of a library of compounds and to assess the concentration profile. Then, the two compounds were moved to assays in a non-genetic model of MN death induced by spinal nerve injury and in the transgenic $SOD1^{G93A}$ mouse model of ALS.

MATERIAL AND METHODS

Human Sig-1R Radioligand Assay

Plates were incubated with binding buffer contained Tris-HCl (50 mM, pH 8), at 37°C for 120 min. After the incubation period, the reaction mix was transferred to MultiScreen HTS, FC plates (Millipore) presoaked in 0.1% polyethyleneimine and filtered. Then, plates were washed with ice-cold Tris-HCl (10 mM, pH 7.4). Filters were dried and counted at approximately 40% efficiency in a MicroBeta scintillation counter (PerkinElmer) using an EcoScint liquid scintillation cocktail (Díaz et al., 2021).

Human Sigma-2 receptor Radioligand Assay

The binding properties of the compounds to human Sigma-2 receptor (Sig-2R) were similarly studied in transfected HEK-293 Sig-1R knockout membranes using [^{3}H]-1,3-di-o-tolylguanidine (DTG) (PerkinElmer, NET-986) as the radioligand. The assay was carried out with 15 µg of membrane suspension, [^{3}H]-1,3-di-o-tolylguanidine (DTG) (10 nM, 100 µL), in either absence or presence of either buffer or 10 µM haloperidol for total and nonspecific binding, respectively. Binding buffer contained Tris-HCl (50 mM, pH 7.4). Plates were incubated at 25°C for 120 min. After incubation, the reaction mix was transferred to MultiScreen HTS, FC plates (Millipore) presoaked in 0.5% polyethyleneimine and filtered. Then, plates were washed with ice-cold Tris-HCl (10 mM, pH 8). Filters were dried and counted as above (Díaz et al., 2021).

Selectivity profile

Binding affinities of EST79232 and EST79376 for proteins other than Sig-1R and Sig-2R were determined by commercial radioligand binding assays by EurofinsPanlabs. A selectivity profile including a panel of more than 180 radioligand binding assays for different receptors, ion channels, enzymes and transporters was performed according to their standard *in vitro* screening assay protocols (http://www.eurofinspanlabs.com). Assays were done with concentrations tested in duplicate.

BiP/Sig-1R association assay

The interaction between Sig-1R and the chaperone BiP was used to identify the functional nature (agonistic or antagonistic) of compounds. The assay was conducted by Amylgen according to their standard assay protocol (https://www.amylgen.fr/). Each assay condition consisted of six determinations.

Spinal cord organotypic cultures (SCOCs)

SCOCs from lumbar sections of 8 day-old Sprague–Dawley rats were prepared as previously described (Mòdol-Caballero et al., 2017). Briefly, the spinal cord was collected and cut in 350 µm thick transverse sections with a McIlwain Tissue Chopper. Four lumbar sections were transferred onto Millicell-CM nets (0.4 µm, PICM03050, Millipore) in medium [50% (v/v) minimal essential medium (MEM, M5775, Sigma), 2 mM glutamine, 25 mM HEPES, 25% (v/v) Hank's Balanced Salt Solution ($HBSS^{-/-}$, 14175, Gibco) supplemented with 25.6 mg/ml glucose and 25% (v/v) heat-inactivated horse serum (26050-088, Gibco), pH=7.2). Cultures were maintained at 37°C in a 5% CO_2 humidified cabin and let to stabilize. After 7 days *in vitro* (DIV), DL-threo-β-hydroxyaspartic acid (THA; 100 µM) was added to induce chronic excitotoxicity (Rothstein et al., 1993). The neuroprotective effect of each Sig-1R ligand was assessed by its coaddition to the culture with THA and renewing at each medium change. EST79232 (in DMSO 100 mM) and EST79376 (in PBS 1 mM) were tested at four concentrations (30, 3, 0.3 and 0.03 µM). Comparisons were performed against slices with vehicle as negative control, and with addition of Riluzole (5 µM) as positive control (Guzmán-Lenis et al., 2009; Herrando-Grabulosa et al., 2016). *In vitro* experiments were performed in three independent cultures, with at least 12 different SCOCs for each experimental condition.

Slices were maintained for 28 DIV and then fixed with 4% PFA. Fixed SCOC slices were immunolabeled with primary antibody mouse anti-neurofilament H non-phosphorylated (SMI-32; 1:500; 801701, BioLegend), washed and incubated with secondary antibody Alexa Fluor 488 donkey anti-mouse (1:500; A-21202, Invitrogen) (Mòdol-Caballero et al., 2017). Cell nuclei were labeled with DAPI (1:2000) and the sections mounted with Fluoromount-G medium (SouthernBiotech). Images of the ventral horn were captured with a confocal microscope (LSM 700 Axio Observer, Carl Zeiss 20x/z0.5). MN survival was assessed by counting all SMI-32 positive neurons in each spinal cord using the Cell Counter plugin of ImageJ software.

Animals

For spinal nerve injury studies, adult female mice with B6SJL background were used, whereas for the ALS murine model, transgenic male mice carrying the G93A human mutation in SOD1 gene (B6SJL-Tg[SOD1-G93A]1Gur) and non-transgenic wild type (WT) littermates as controls were used (Mancuso et al., 2012b). The transgenic offspring was identified by polymerase chain reaction (PCR) amplification of DNA extracted from the tail. Mice were kept under standard conditions 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. All experimental procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Rhizotomy procedure

Surgeries were performed in 3 months old mice under anesthesia with ketamine-xylazine (100-10 mg/kg i.p.) as previously described (Gaja-Capdevila et al., 2021). Briefly, the L4-L5 spinal roots were exposed by a small laminectomy on the right side. Then, roots were cut at the exit from the intervertebral foramina and the root stumps were separated. Mice were cared until recovery in warm environment. Analgesia was provided with buprenorphine (0.1 mg/kg) for the next 48 h post-surgery.

Drug administration

The following selective Sig-1R ligands were used: PRE-084 at a dose of 0.25 mg/Kg (TOCRIS), EST79232 and EST79376 at doses of 0.5 and 5 mg/kg (synthesized and supplied by Esteve Pharmaceuticals). The compounds were dissolved in 0.5% hydroxypropyl-methylcellulose (HPMC; Sigma-Aldrich) in distilled water. The compounds were administered by intraperitoneal (i.p.) route twice daily (bid) in a volume of 10 ml/kg. Administrations were given from 30 min after rhizotomy surgery until 42 days post-injury (dpi), the end of the study; in the $SOD1^{G93A}$ study, treatments were given from 8 to 16 weeks of age.

For the spinal nerve injury (rhizotomy, rhizo), female WT mice were distributed in the following experimental groups: uninjured control (CTL) (n=16), rhizo + vehicle 0.5% HPMC (n=14), rhizo + PRE-084 0.25 mg/kg (n=4), rhizo + EST79232 0.5 mg/kg (n=5), rhizo + EST79232 5 mg/kg (n=6), rhizo + EST79376 0.5 mg/kg (n=4), rhizo + EST79376 5 mg/kg (n=5). For the $SOD1^{G93A}$ studies, male transgenic mice were divided in 4 groups: SOD1+ vehicle 0.5% HPMC (n=13), SOD1 + PRE-084 0.25 mg/kg (n=5), SOD1 + EST79232 5 mg/kg (n=7), SOD1+ EST79376 5 mg/kg (n=6), with B6SJL male WT age-matched mice used as negative control of the disease (n=13).

Pharmacokinetic studies of EST79232 and EST79376 in plasma and brain

Adult female C57-BL6 mice (Charles River) were given a single i.p. dose of 10 mg/kg of EST79232 or EST79376 in 0.5% HPMC (10 ml/kg). Blood and brain samples were extracted at selected time points (15 min, 1, 3 and 6 h) from two animals per sampling point. Blood samples were collected by intracardiac puncture into heparinized tubes. Plasma was obtained by blood centrifugation at 4°C and 2280g for 10 min. Plasma and brain samples

were kept at −80°C until analysis. Brains were homogenized with Dulbecco´s phosphate saline buffer pH 7.4 (4 ml/g tissue). Plasma and brain samples were assayed by ultra-performance liquid chromatography–triple quadrupole mass spectrometry (UPLC-MS/MS) after protein precipitation. Standard pharmacokinetic parameters, such as the area under the curve (AUC), peak plasma concentration (C_{max}), time to peak concentration (t_{max}), and terminal half-life ($t_{1/2}$) were determined by noncompartmental analysis of the plasma and brain concentration–time curves (Phoenix v.6.2.1.51, Pharsight, CA).

Plasma levels associated with pharmacological activity

Plasma levels were determined in the two *in vivo* models evaluated to be able to associate the pharmacological activity with the levels present in plasma. In the rhizotomy model, the plasma levels were evaluated at day 42 after surgery and at 15 min after compound administration (0.5 and 5 mg/kg i.p.). In the $SOD1^{G93A}$ model, the plasma levels were evaluated at 16 weeks of age (after 8 weeks of administration) and 15 min after compound administration (5 mg/kg i.p.). Plasma samples were assayed by ultra-performance liquid chromatography–triple quadrupole mass spectrometry (UPLC-MS/MS) after plasma protein precipitation. Results are expressed as mean ± standard deviation (n=2-6).

Electrophysiological tests

Motor nerve conduction tests were performed before the surgery to obtain baseline values and at the end of the follow up in the rhizotomy model, whereas the $SOD1^{G93A}$ mice were evaluated at 8 weeks (prior to starting drug administration) and then every 2-3 weeks until the end point at 16 weeks of age. The sciatic nerve was stimulated by means of single pulses delivered through needle electrodes placed at the sciatic notch. The compound muscle action potential (CMAP) was recorded from tibialis anterior (TA), gastrocnemius (GM) and plantar interossei muscles with microneedle electrodes (Mancuso et al., 2011). The CMAPs were amplified to measure the latency to the onset and the amplitude from baseline to the maximal negative peak. The mice were anesthetized with pentobarbital (50 mg/kg i.p.) and their body temperature was maintained by means of a thermostated warming pad.

Locomotion tests

Rotarod test was performed to evaluate motor coordination and strength. The time that each animal remained on the rotating rod at a speed of 14 rpm was measured for five trials per mouse, and the longest time until falling was recorded; 180 sec was chosen as the cut-off time. The test was performed weekly from 8 to 16 weeks of age in $SOD1^{G93A}$ and WT mice,

and disease onset was determined as the first week when each mouse was unable to keep walking for 180 sec on the rod.

Histological and immunofluorescence analyses

At the end of follow up (42 dpi for rhizotomy and 16 weeks of age for ALS mice) the animals were deeply anesthetized and transcardially perfused with 4% PFA in PBS. The lumbar spinal cord and the TA muscle were harvested and cryopreserved in 30% sucrose solution in PB. The spinal cords were serially cut in 20 µm-thick transverse sections with a cryostat (Leica). For MN counting, L4-L5 spinal cord sections separated 100 µm were stained for 3 h with an acidified solution of 3.1 mM cresyl violet. MNs were identified by their localization in the lateral ventral horn of the spinal cord and were counted following strict size and morphological criteria: only MNs with diameter larger than 20 µm, polygonal shape and prominent nucleoli were counted (Gaja-Capdevila et al., 2021; Mancuso et al., 2012a).

For glial cells immunofluorescence, other lumbar spinal cord sections were blocked with 10% normal donkey serum and incubated overnight with primary antibodies rabbit anti-Iba1 (1:500; 019–19,741, Wako) and rat anti-GFAP (1:500; 13-0300, Invitrogen) to label microglia and astroglia, respectively. After several washes, sections were incubated with secondary antibodies Alexa Fluor 488-donkey anti-rat (1:500; A-21208, Invitrogen) or Alexa Fluor 594 donkey anti-rabbit (1:500; A21207, Invitrogen). Finally, sides were mounted with Fluoromount G. To quantify glial cell reactivity, images of the ventral horn were acquired with an epifluorescence microscope (Nikon Eclipse Ni, Japan) using the same conditions for each analyzed marker. After defining a threshold for background correction, the integrated density of GFAP or Iba1 labeling was measured using ImageJ software.

For neuromuscular junctions (NMJ) labeling, 50 µm-thick longitudinal sections of TA muscle were serially cut. Sections were blocked with 5% normal donkey serum and incubated with primary antibodies rabbit anti-synaptophysin (1:500; AB32127, Abcam) and chicken anti-neurofilament 200 (NF200, 1:1000; AB5539, Millipore, USA) 48h at 4ºC. After washes, sections were incubated overnight with secondary antibody Alexa Fluor 594-donkey anti-rabbit and anti-chicken (1:200; A11042-A21207, Invitrogen, USA) and Alexa 488 conjugated α-bungarotoxin (1:500; B13422, Life Technologies, USA). Confocal images were captured (LSM 700 Axio Observer, Carl Zeiss, 40xOil/z0.5) and the maximum projection images generated from 1.3 µm z projections. The proportion of innervated NMJs was calculated by classifying each endplate as occupied (when presynaptic terminals overly the endplate) or

vacant (no presynaptic label in contact with the endplate). At least 60 endplates were analyzed per muscle.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistics were performed using GraphPad Prism 6 software. Histological data were analyzed using One-way ANOVA followed by Bonferroni post hoc test for multiple comparisons, and for *in vitro* pharmacological profile Dunnett's post-hoc multiple comparisons was used. Electrophysiological and functional measurements were statistically analyzed using One-way or Two-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons. Statistical significance was set at $p<0.05$.

RESULTS

In vitro pharmacological profile of EST79232 and EST79376

The two new compounds synthesized, EST79232 and EST79376 (Figure 1A), were highly selective Sig-1R ligands. They showed high affinity for the human Sig-1R (K_i=32 nM and K_i=36 nM, respectively) while they did not show affinity for the Sig-2R (IC_{50}>10000 nM for both of them). A selectivity profile (Spectrum Screen) including a panel with more than 180 binding assays for different receptors, ion channels, transporters and enzymes was performed for EST79232 and EST79376 at EurofinsPanlabs according to their specifications. Both compounds showed no significant affinity (% inhibition < 50% at 10 μM) for any of the targets included in the selectivity panel, apart from the Sig-1R. Similarly, PRE-084 has good affinity for the human Sig-1R (K_i=72 nM) and no affinity for human Sig-2R (IC_{50}>10000 nM).

To determine the Sig-1R functional profile of EST79232 and EST79376, an *in vitro* assay consisting of the modulation of the interaction between Sig-1R and BiP was performed at Amylgen. As described Hayashi and Su (2007), the activation of Sig-1R results in its dissociation from BiP, whereas the antagonists stabilize or do not affect this interaction. EST79232 produced a concentration-dependent dissociation of Sig-1R from BiP, as observed with the prototypical Sig-1R agonist PRE-084, while EST79376 did not produce any change in the interaction between the two proteins (Figure 1B). Moreover, the agonist effect of EST79232 on Sig-1R could be reverted using the prototypical Sig-1R antagonist NE100 (Figure 1C) and the effect of PRE-084 could be reverted by EST79376 (Figure 1D). These results confirmed the differential functional profile of EST79232 and EST79376 on Sig-1R, with EST79232 showing an agonist behavior and EST79376 an antagonist one.

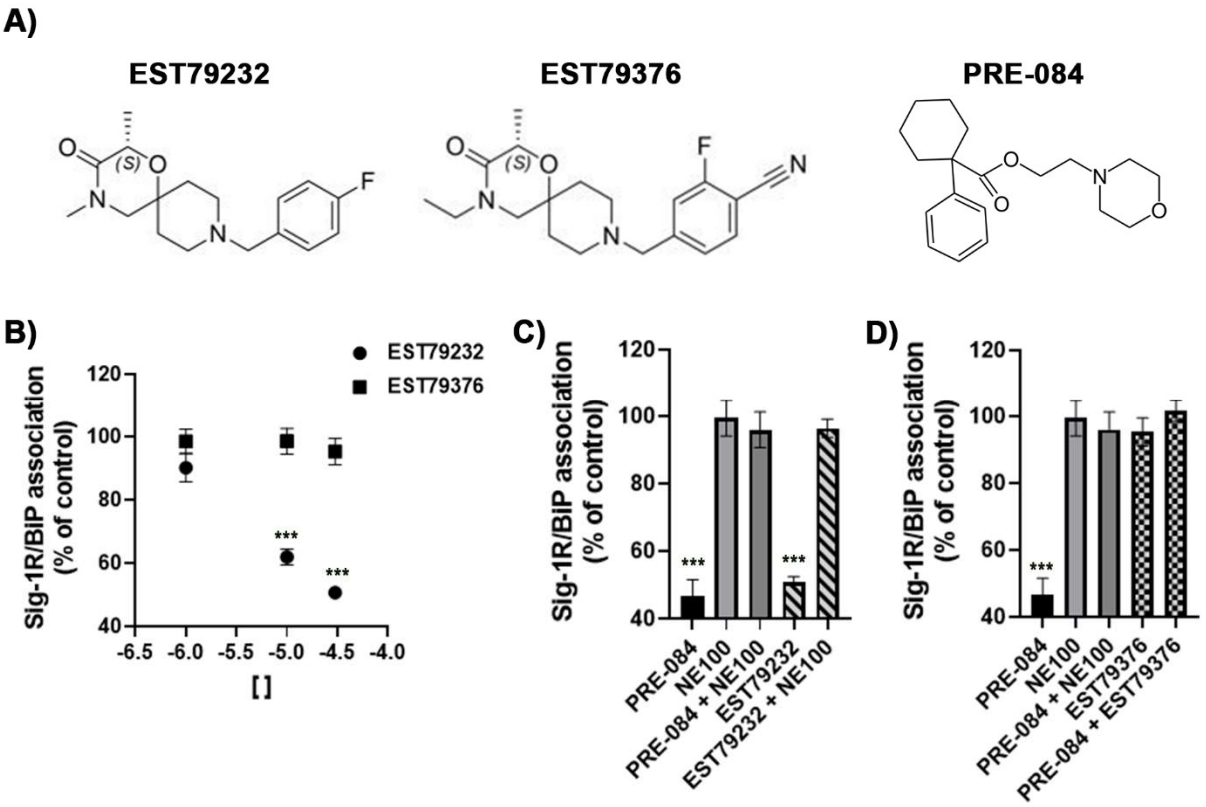

Figure 1. *In vitro* pharmacology of EST79232 and EST79376 at Sig-1R: Sig-1R and BiP interaction assay. A) Chemical structure of the two new compounds synthesized, EST79232 and EST79376 from the same chemical series and PRE-084. B) Concentration-response curves of EST79232 and EST79376 on the Sig-1R/BiP interaction. C) and D) show the agonist effect of 10 μM of PRE-084 and the antagonist effect of 10 μM of NE100. NE100 was able to revert the effect of PRE-084. While EST79232 at 30 μM was able to reduce the association between Sig-1R and BiP and this effect was reverted by NE100 (C), EST79376 at 30 μM had no effect on its own but it was able to revert the effect of PRE-084 (D). Values are expressed as mean ± SEM of 6 determinations. *** $p < 0.001$ vs control. One-way ANOVA followed by the Dunnett's post-hoc multiple comparison test.

EST79232 and EST79376 prevent MN death in SCOC under chronic excitotoxic stress

In the SCOC, the number of SMI-32 positive MNs was significantly reduced in the ventral horn of slices treated with THA compared to the control slices (Figure 2). Riluzole, used as a positive control against excitotoxicity, preserved MNs at levels of control cultures. As shown in Figure 2B, slices treated with EST79232 had significantly more SMI-32 positive neurons at three concentrations tested with the highest effect at 0.3 to 3 μM. The two highest concentrations of EST79376 assessed (3 and 30 μM) also significantly preserved MNs (Figure 2C).

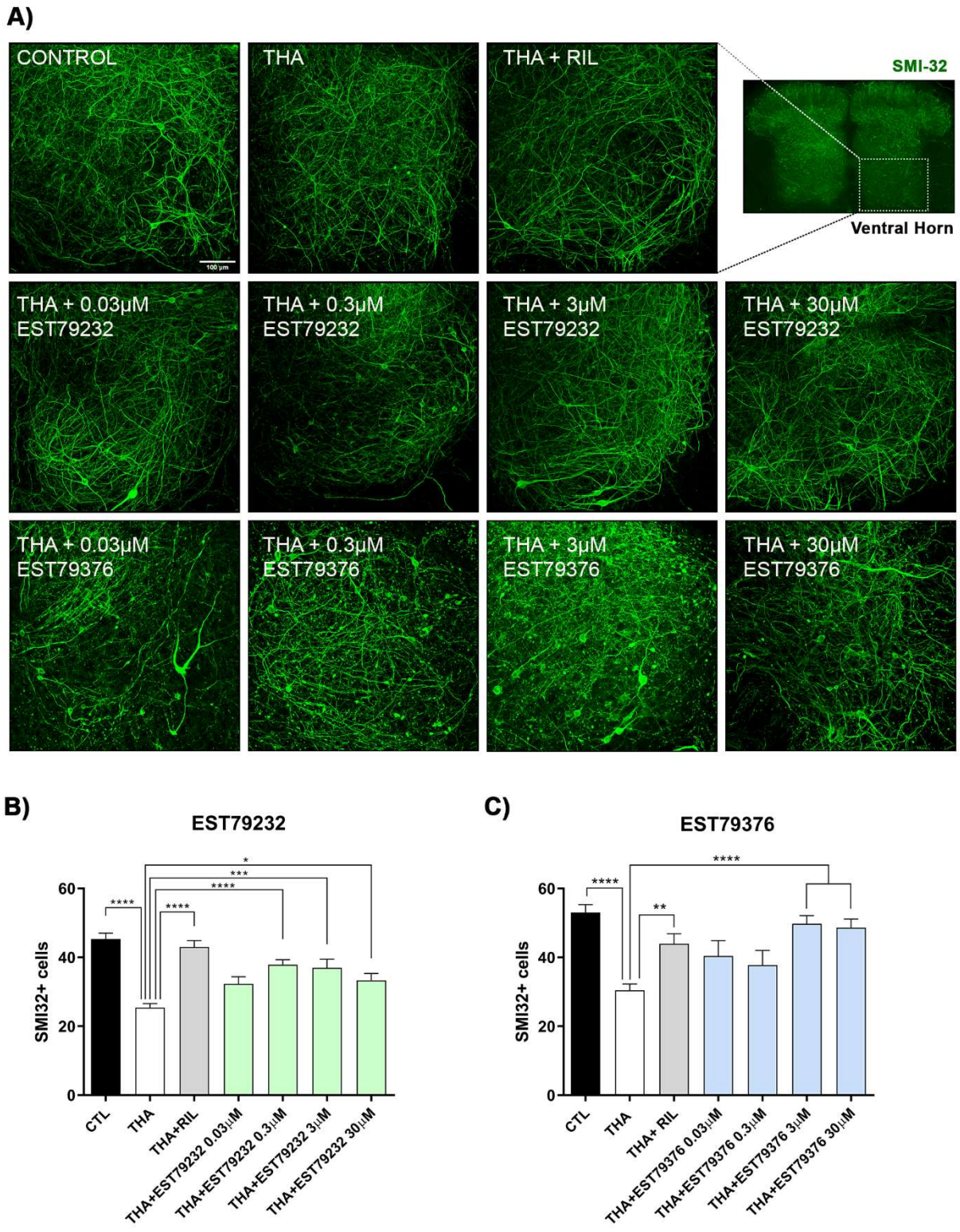

Figure 2. EST79232 and EST79376 ligands prevent MN death under chronic excitotoxicity. A) Representative confocal images of spinal cord ventral horns labeled with SMI32 antibody at 28 DIV for control, THA and THA plus EST79232 or EST79376 treated at four concentrations (0.03, 0.3, 3 and 30 μM). Scale bar 100 μm. B-C) Bar graphs showing the number (mean±SEM; n = 20-24 hemisections per treatment) of SMI-32 positive cells of each spinal cord hemislice. One-way ANOVA followed by Bonferroni's post hoc test; ****p<0.0001, ***p<0.001; **p<0.01, *p<0.05 vs THA condition.

Pharmacokinetic analyses of Sig-1R ligands in plasma and brain

The pharmacokinetic profile of Sig-1R ligands EST79232 and EST79376 was determined in female mice after i.p administration of a single dose of 10 mg/kg (Table 1, Figure 3). Brain and plasma kinetics were parallel for both compounds. Brain concentrations were higher than the corresponding plasma concentrations as shown by a brain-to-plasma ([brain]/[plasma]) ratio based on AUC of 4.4 and 2.1 for EST79232 and EST79376, respectively. These results indicate that these compounds have a good penetration into the brain, necessary to act in the central nervous system. PRE-084 is also known to penetrate the brain ([brain]/[plasma] ratio about 1.2 at T_{max} (5 min) following single i.p. administration at 10 mg/kg to male CD1 mice) (Marra et al., 2016).

Table 1. Pharmacokinetic parameters of EST79232 and EST79376 compounds and PRE-084 in plasma and brain after single intraperitoneal administration of 10 mg/kg to female mice.

Compound	Sample type	$t_{1/2}$ (h)	C_{max} (ng/ml or ng/g)	t_{max} (h)	AUC (ng·h/ml or ng·h/g)	Brain-to-plasma AUC ratio
EST79232	Plasma	>5	1348	0.25	1116	4.4
	Brain	>5	5405	0.25	4873	
EST79376	Plasma	2.2	1343	0.25	802	2.1
	Brain	3.4	2652	0.25	1698	
PRE-084[a,b]	Plasma	-	659	-	-	1.2
	Brain	-	774	-	-	

$t_{1/2}$: terminal half-life, C_{max}: peak plasma concentration, t_{max}: time to peak concentration, AUC: area under the curve. [a] From literature (Marra et al., 2016).[b] Concentrations determined at 5 min post-administration.

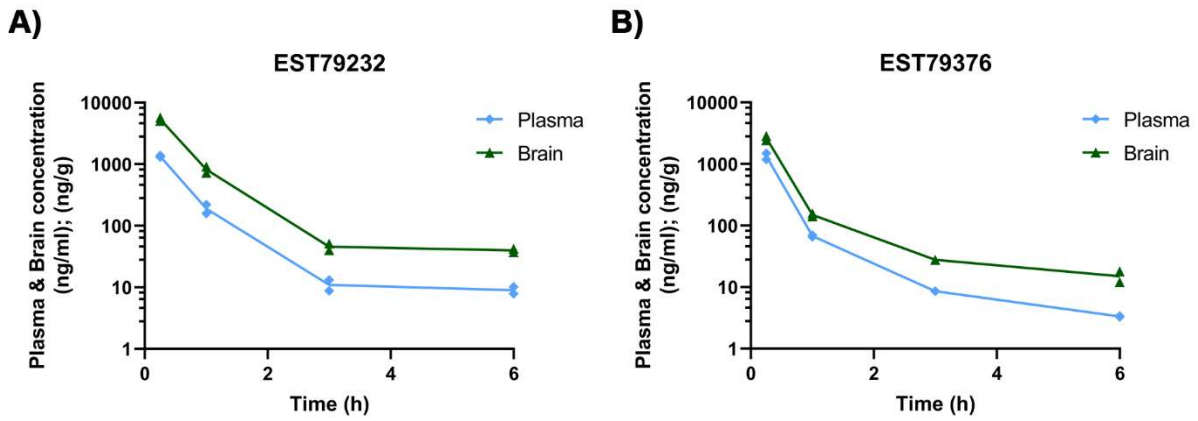

Figure 3. Plasma and brain concentration-time curves of EST79232 (A) and EST79376 (B) after single i.p. administration of 10 mg/kg to mice. At 15 min, 1, 3 and 6 h samples of blood and brain were extracted to determine the concentrations of both compounds (n=2 mice per sampling point). Curves in semilogarithmic scale.

EST79232 and EST79376 enhance MN survival after rhizotomy

After rhizotomy surgery, nerve conduction tests were done to confirm the complete denervation of both TA and GM muscles, thus ensuring a complete L4 and L5 root section. There were no recordable CMAPs from TA and GM muscles, and only partial preservation of the plantar muscle CMAP in all the mice included in the study after surgery and at the end of the follow-up, as in previous studies (Gaja-Capdevila et al., 2021).

In the rhizotomy mice, a similar plasma concentration, about 20 ng/ml, was found for EST79232 and EST79376 15 min after the last 0.5 mg/kg i.p. administration (day 42 after surgery, end of the study). At 5 mg/kg i.p. the levels obtained in plasma for EST79232 were lower than those obtained for EST79376, 265 and 592 ng/ml, respectively (Table 2). However, taking into account the brain-to-plasma ratio (4.4 for EST79232 and 2.1 for EST79376), estimated brain concentrations at 5 mg/kg i.p. are similar for both compounds.

Histological analyses revealed that rhizotomy (L4-L5 spinal nerve section) caused severe MN loss (43%) in the affected lumbar segments and that the three Sig-1R ligands reduced MN degeneration at 42 days after the injury. Rhizotomized mice treated during 6 weeks with EST79232 and EST79376 at a dose of 5 mg/kg/bid i.p. had a significantly higher number of spinal MNs (8.6 ± 0.6 and 8.7 ± 0.5, respectively) compared to the untreated injured group (6.8 ± 0.3) in the ipsilateral ventral horn of spinal cord sections stained with cresyl violet (Figure 4A-B). The administration of these new Sig-1R ligands increased the number of surviving MNs at the same level as PRE-084 (8.9 ± 0.4), used as a positive control of MN

preservation (Gaja-Capdevila et al., 2021; Mancuso et al., 2012a; Penas et al., 2011). Administration of EST79232 and EST79376 at the low dose (0.5 mg/kg) did not exert significant MN protective effects (7.6 ± 0.9 and 7.4 ± 0.7, respectively) after rhizotomy.

As a consequence of the rhizotomy, microglia and astrocytes were activated surrounding lumbar MNs. IBA1 and GFAP immunostaining showed a marked increase in the untreated rhizotomized group (26×10^4 ± 3×10^4 and 19×10^4 ± 5×10^4, respectively; mean of integrated density ± SEM) compared with control mice (8×10^4 ± 1.5×10^4 and 2.5×10^4 ± 0.6×10^4) (Figure 4C-E). Regarding pharmacologic treatments, EST79232 at the highest dose significantly diminished microglial (13×10^4 ± 2×10^4) and astroglial (4×10^4 ± 0.4×10^4) reactivity at levels similar to the positive control PRE-084 (15×10^4 ± 1.4×10^4 and 6×10^4 ± 0.9×10^4). In contrast, EST79376 at 5 mg/kg did not modulate glial reactivity (25×10^4 ± 4×10^4 and 15×10^4 ± 2.7×10^4). The low doses tested of both Sig-1R ligands showed a non-significant tendency to decrease microgliosis and astrogliosis.

Finally, it is important to note that twice daily administration for 6 weeks of these new compounds did not result in noticeable side effects on mice, with no difference in body weight compared with the vehicle group, thus suggesting that chronic administration of EST79232 and EST79376 did not cause toxicity in the animals (Figure 4F).

Table 2. Plasma levels of EST79232, EST79376 and PRE-084 after chronic intraperitoneal administration.

Model	Compound	Dose (mg/kg)	Plasma concentration (ng/ml) 15 min post-administration	
			Mean	SD
Spinal Nerve Injury female mouse	EST79232	0.5	20	8
		5	265	53
	EST79376	0.5	21	7
		5	592	176
	PRE-084[a]	0.25	3.6	0.8
Transgenic $SOD1^{G93A}$ male mouse	EST79232	5	425	129
	EST79376	5	692	206
	PRE-084[a]	0.25	2.6	1.3

[a]: Concentrations determined at 30 min post-administration

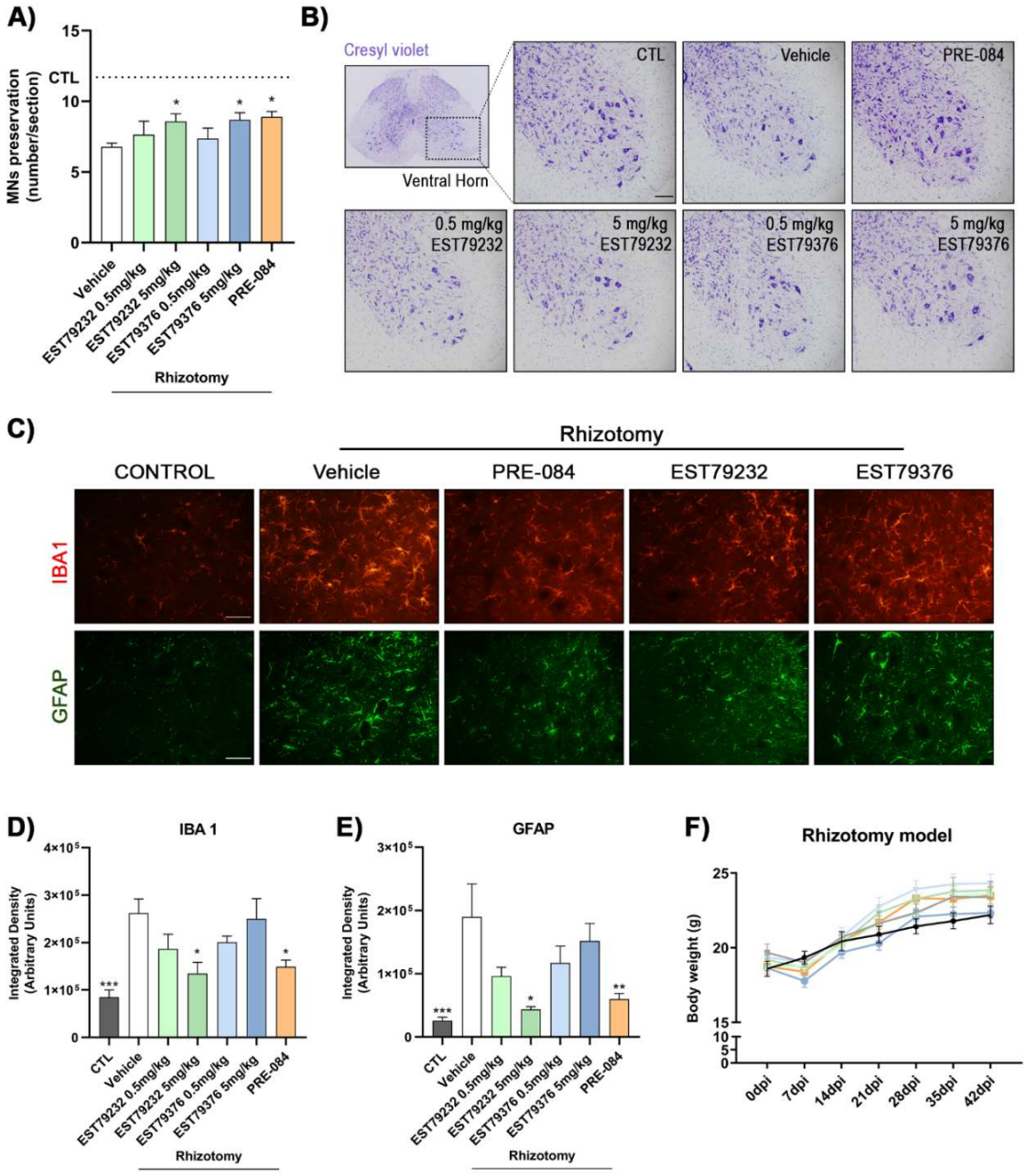

Figure 4. Administration of EST79232 and EST79376 increased MN survival and modulated glia activation after L4-L5 spinal roots injury. A) Plot of number of α-MNs in L4-L5 segments, showing higher number of MNs in mice treated with Sig-1R ligands than in untreated ones. Animals per group: control (CTL) n=14, vehicle n=14, PRE-084 n=4, EST79232 0.5 mg/kg n=5 and 5 mg/kg n=6, EST79376 0.5 mg/kg n=4 and 5 mg/kg n=5. B) Representative images corresponding to ventral horns of L4-L5 cord segments ipsilateral to rhizotomy in female mice with or without Sig-1R ligands treatment at 42 dpi. Scale bar 100 µm. C) Representative images of glial reactivity assessed by microglia (IBA1) and astrocytes (GFAP) immunolabeling in the ventral horn of control and rhizotomized mice with vehicle or Sig-1R treatment (PRE-084, EST79232 and EST79376 at dose of 5 mg/kg) at 42 dpi. Scale bar 50 µm. D-E) Bar graph showing the integrated density of Iba-1 and GFAP immunolabeling in the ipsilateral ventral horn of spinal cord. n=3-6 mice per group. F) Plot of the body weight of rhizotomized mice during the study. All the mice groups presented a mild reduction of the body weight the first week after surgery, then gained weight normally during the follow-up. Data are mean ± SEM, analyzed with One-way (A,D,E) or Two-way (F) ANOVA and Bonferroni's multiple comparisons test. *p<0.05, **p<0.001,***p<0.0001 vs vehicle rhizotomized mice.

EST79232 treatment slows disease progression in SOD1^{G93A} mice

The neuroprotective effect of the new Sig-1R ligands were also assessed in the SOD1^{G93A} mice model of ALS. In this study only the dose of 5 mg/kg of each compound was tested based on previous results in the rhizotomy model. After administration of 5 mg/kg i.p. for 8 weeks (from 8 to 16 weeks), plasma concentrations 15 min after last dose administration were around 500 ng/ml, similar for both Sig-1R ligands, EST79232 and EST79376 (Table 2). Considering the brain-to-plasma ratio of compounds (4.4 for EST79232 and 2.1 for EST79376), the estimated brain concentration of EST79232 is about two-fold the concentration of EST79376.

At early (11 weeks) and mid (13 weeks) stages of the disease, motor nerve conduction tests showed that administration of EST79232 significantly prevented the decline in amplitude of the CMAP of TA and plantar muscles, similar to PRE-084. However, at 16 weeks, the effect was reduced to levels found in the vehicle group (Figure 5A-B). Mice treated with EST79376 only showed significant preservation of the CMAP amplitude in the plantar muscle at 13 weeks of age compared to the vehicle group. Results of the rotarod test showed that animals treated with both new Sig-1R ligands had a delay of one week (15 weeks) in the onset of the motor impairment in comparison with untreated animals (14 weeks). When compared, EST79232 produced a significant improvement of SOD1^{G93A} mice in the rotarod performance at 15 weeks of age, but PRE-084 treatment significantly improved the functional outcome at 15 and 16 weeks (Figure 5C).

The histological analyses of NMJ of the hindlimb muscle showed that PRE-084 treatment significantly increased the proportion of innervated endplates compared to untreated SOD1^{G93A} mice at 16 weeks, consistent with the electrophysiological results of higher CMAP amplitude values. EST79232 and EST79376 caused a tendency to preserve NMJ innervation, although differences did not reach statistical significance when compared with the vehicle group (p=0.081 and p=0.057, respectively) (Figure 5D-F).

Similar to findings in the rhizotomy study, the daily i.p. administration of the Sig-1R ligands EST79232, EST79376 and PRE-084 to SOD1^{G93A} mice did not cause differences in body weight compared with the vehicle group (Figure 5E).

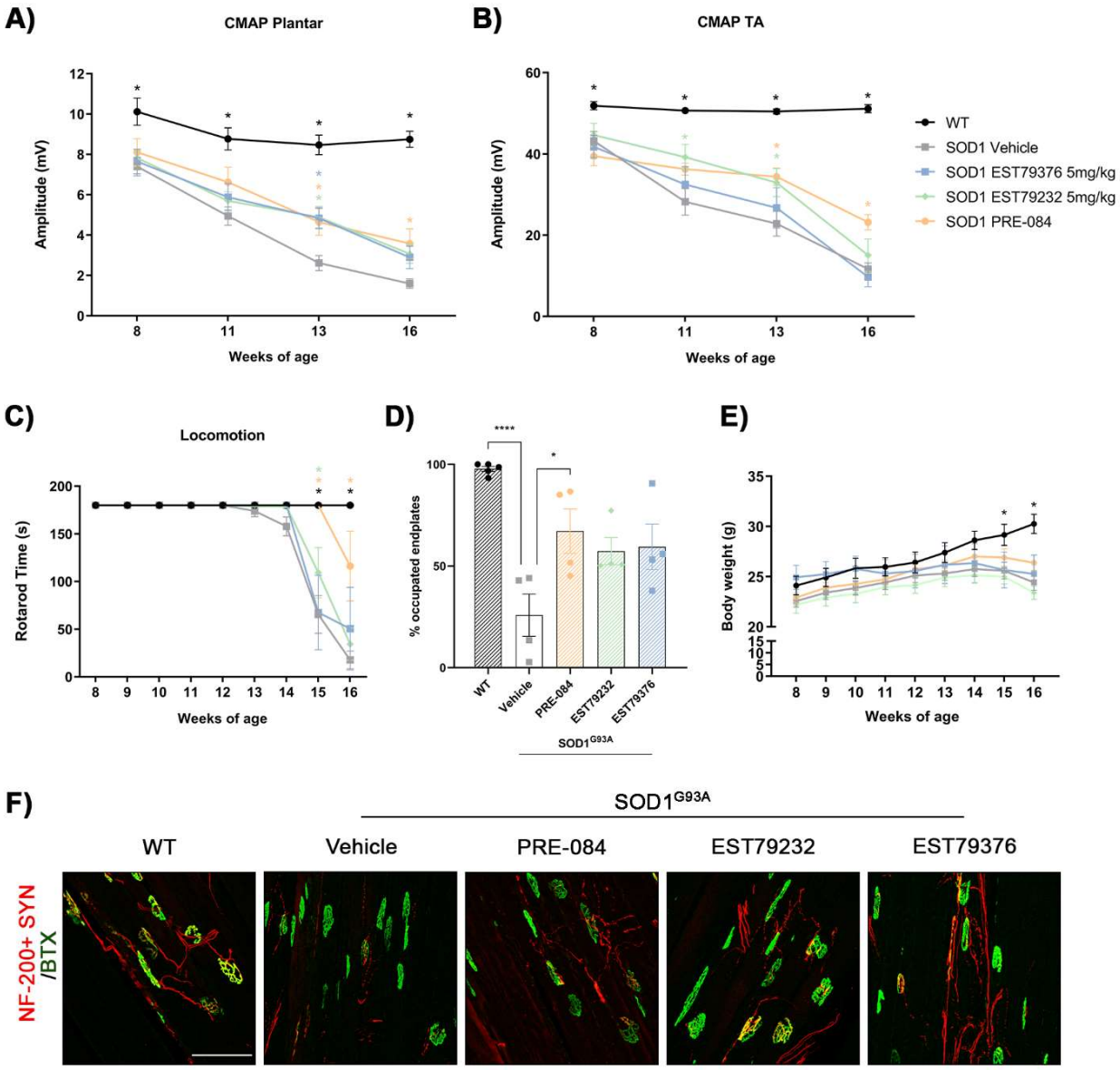

Figure 5. Effects of administration of EST79232 and EST79376 on disease progression of SOD1^{G93A} mice. A-B) Plots of the CMAP amplitude of plantar and tibialis anterior (TA) muscles. C) EST79232 treatment produced a slight improvement in the rotarod performance in treated SOD1^{G93A} mice (n=9 SOD1 vehicle, n=7 SOD1 EST79232, n= 4 SOD1 EST79376 and n=5 SOD1 PRE-084 mice). D) Histological analyses of NMJ showed a tendency to maintain the proportion of occupied endplates in the GM muscle of treated SOD1^{G93A} with the two novel Sig-1R ligands compared to vehicle group (n= 4-5 per group). F) Plot of the body weight of the different groups of mice during the follow-up. At 15 and 16 weeks there was a significant difference between SOD1^{G93A} mice and WT mice. E) Representative confocal images of NMJ in the GM muscle of WT mice and SOD1^{G93A} male mice with or without Sig-1R ligands treatment at 16 weeks of age. Scale bar 100 µm. Data are mean ± SEM, analyzed with One-way (D) or Two- way (A, B, C, E) ANOVA with Bonferroni's multiple comparisons test. ****p<0.0001,*p< 0.05 vs. SOD1^{G93A} vehicle.

EST79232 treatment protects spinal MNs and reduces astroglial activation in SOD1^{G93A} mice

Counts of α-MNs in ventral horns of spinal cord sections revealed that untreated SOD1^{G93A} mice had a loss of around 60% MNs (6.9 ± 0.5 MNs per section) compared to WT mice (25.3 ± 0.5) at 16 weeks of age. SOD1^{G93A} mice treated with EST79232 (5 mg/kg) had a significantly higher number of surviving spinal MNs (10.8 ± 1.1), like animals administered with PRE-084 (12.4 ± 0.9), whereas EST79376 did not produce a significant effect (8.5 ± 1.0) (Figure 6A, B). Glial reactivity showed a huge increase in SOD1^{G93A} with respect to WT mice. Treatment with the new Sig-1R ligands EST79232 and EST79376 produced a significant decrease of astrocyte activation as did PRE-084, whereas they did not reduce microglial activation (Figure 6C-E).

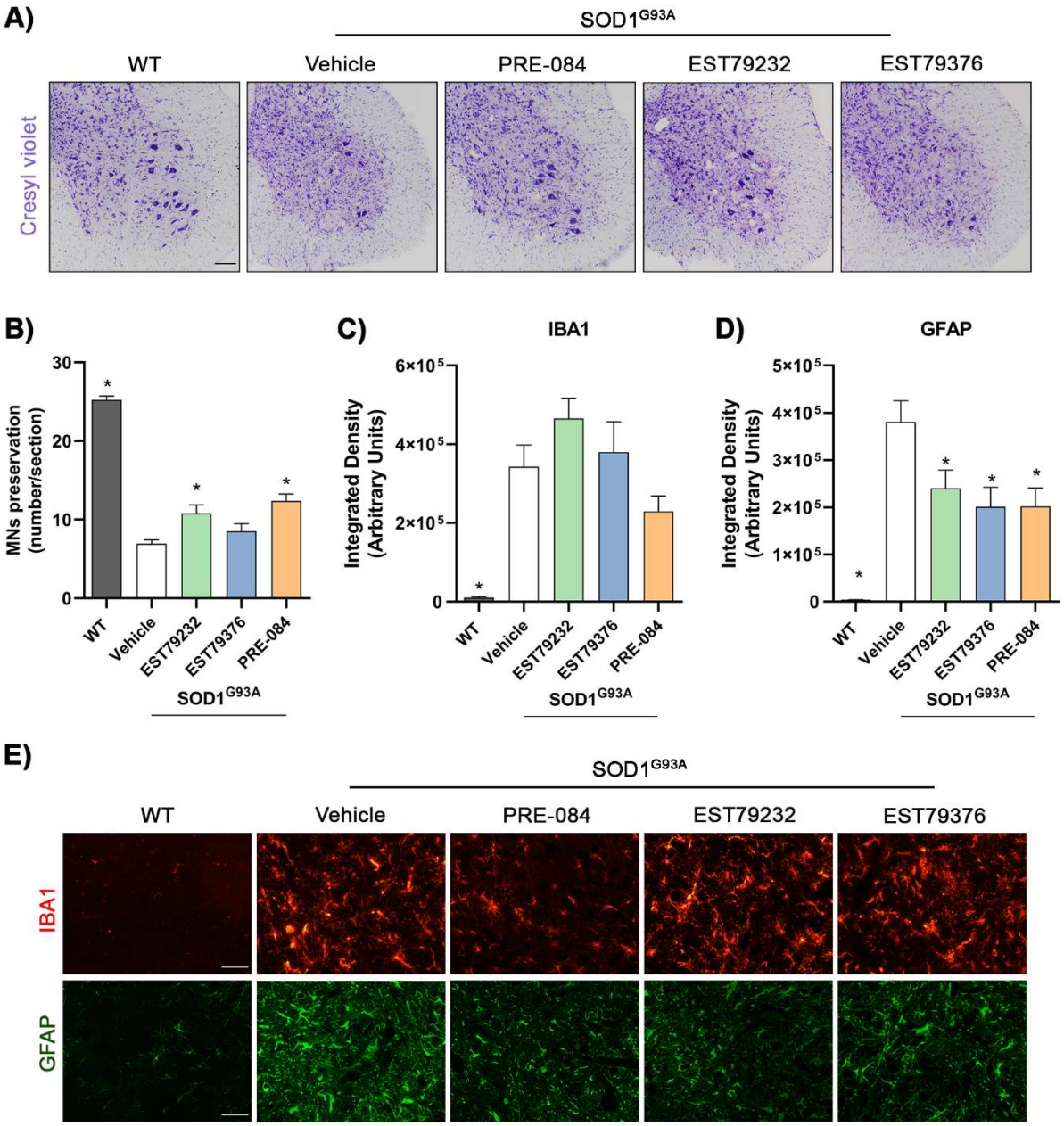

Figure 6. Neuroprotective effects of Sig-1R ligands administration on MNs and glial reactivity in SOD1^{G93A} mice at 16 weeks. A) Representative images of the ventral horns stained with cresyl violet of WT and SOD1^{G93A} male mice with or without Sig-1R ligands treatment. Scale bar 100 µm. B) Histological analyses showed an increase of number of MNs in mice treated with EST79232 and PRE-084. Animals per group WT n= 13, SOD vehicle n=16, SOD PRE-084 n=5, SOD EST79232 n=7, SOD EST79376 n=5. C-D) Bar graph showing the integrated density of Iba-1 and GFAP immunolabeling in the ventral horn of spinal cord. n=5-10 mice per group. Data are expressed as mean ± SEM, and analyzed by One-way ANOVA and Bonferroni's multiple comparisons test. *p<0.05 vs SOD1^{G93A} vehicle mice. D) Representative images of glial reactivity assessed by IBA and GFAP to label microglia and astrocytes in the ventral horn. Scale bar 50 µm.

DISCUSSION

The results of this study show that two novel Sig-1R ligands, coded as EST79232 and EST79376, exert neuroprotective effects in three different experimental models of MN degeneration, thus adding to previous studies highlighting Sig-1R as a promising target to treat MND. These two new synthesized compounds have high affinity for the human Sig-1R and high selectivity against a panel of 180 targets, including no affinity for Sig-2R. These compounds may thus be advantageous to avoid interfering and/or adverse effects due to the binding to other receptors (off-target effects). Neuroprotective activity found in this study may be thus attributable to Sig-1R binding.

Sig-1R is implicated in multiple cellular processes, such as calcium release modulation through inositol triphosphate receptor type 3 (IP_3R) stabilization, endoplasmic stress regulation with the formation of a complex with BiP protein and cell survival (Herrando-Grabulosa et al., 2021; Weng et al., 2017). Several studies have supported a neuroprotective role of Sig-1R activation, by administering an agonist ligand, on neurodegenerative disorders, including Alzheimer disease, Parkinson disease, Huntington disease, ALS, stoke, retinal degeneration and depression (Haga et al., 2019; Hampel et al., 2020; Ionescu et al., 2019; Mancuso et al., 2012a; Reilmann et al., 2019; Zhao et al., 2019). On the other hand, blocking Sig-1R by administration of antagonists was shown useful for the amelioration of psychosis, pain, drug abuse and cancer (Bruna et al., 2018).

In the present study, we used the *in vitro* model of SCOC to assess the potential to protect against chronic excitotoxicity as a drug screening before testing promising compounds on *in vivo* models (Herrando-Grabulosa et al., 2016; Rothstein et al., 1993). Both novel Sig-1R compounds tested exerted neuroprotective effects by reducing MN death, at more than one concentration (EST79232 at 0.3 to 30 µM and EST79376 at 3 to 30 µM). It has been previously reported that the agonist PRE-084 exerted a bell-shape dose-dependent protective response of MNs against in a similar model of glutamate excitotoxicity in organotypic cultures of spinal cord (Guzmán-Lenis et al., 2009). SA4503, another Sig-1R agonist, reduced NSC34 SOD1G93A-induced cell death in a concentration-dependent manner, with more significant effect at 10 µM (Ono et al., 2014). On the contrary, the Sig-1R antagonists BD1063 and BD1047 abolished the protective effect obtained with PRE-084 and SA4503, respectively (Guzmán-Lenis et al., 2009; Ono et al., 2014). In our study, neuroprotective effects were observed with the compounds EST79232 and EST79376, classified as Sig-1R agonist and antagonist, respectively, based on BIP binding assay. These results highlight the importance

of Sig-1R modulation by ligands independently on their classification as agonists or antagonists, especially because ligand functionality classification may depend on the assay and, importantly, on the dose (bi-phasic/bell-shaped dose-response effect, with antagonism profile at higher doses is a common feature in numerous preclinical models *in vitro*, *in vivo* and in clinical trials) (Maurice, 2021). Furthermore, Tadić et al. (2017) demonstrated that Sig-1R agonists, SA4503 and PRE-084, act differently enhancing the cytosolic calcium exchange in SOD1^{G93A} mice cultured MNs. Thus, there is no clear consensus on the type of Sig-1R ligands that may be more effective to prevent neurodegeneration, highlighting the need to evaluate their effects in reliable models.

Pharmacokinetic analyses revealed that EST79232 and EST79376 were present at high concentration in the brain after a single i.p. administration, confirming that they cross the blood brain barrier. Thus, we proceeded to test them in two *in vivo* experimental models of MN degeneration. A proximal nerve injury, at the root or spinal nerve level results in death of a significant number of MNs (Gaja-Capdevila et al., 2021; Koliatsos et al., 1994; Penas et al., 2011), as in our L4-L5 rhizotomy model mice. We reported that the Sig-1R agonist PRE-084 and SA4503 and the antagonist BD1063 enhanced MN preservation in this model, through IRE1-XBP1 activation and modulation of glia reactivity (Gaja-Capdevila et al., 2021). Similarly, administration of EST79376 and EST79232 at the dose of 5 mg/kg produced an increase of the number of surviving lumbar spinal MNs, similar to PRE-084, indicating that modulating the Sig-1R represents a good strategy to prevent MN death and extend the time-window for surgical repair after spinal root and plexus injuries.

In the SOD1^{G93A} transgenic mice, the most common ALS model, we found that daily administration of EST79232 from 8 to 16 weeks of age improved functional outcomes, and delayed the onset of disease. EST79232 treatment also increased the number of surviving MNs and showed a tendency to preserve innervated NMJ in treated SOD1^{G93A} mice. The results of EST79232 treatment were similar to those obtained with the prototypic agonist PRE-084. Accordingly, PRE-084 is known to exert an enhancing effect on the preservation of spinal MNs and extend the lifespan in the SOD1^{G93A} mouse (Mancuso et al., 2012a). Beneficial effects on SOD1^{G93A} mice were also observed with the Sig-1R agonist SA4503 that extended the survival time compared with untreated animals (Ono et al., 2014). Interestingly, we found that treatment with EST79376 also produced positive effects, although less marked than with EST79232 and PRE-084, despite its putative consideration as a Sig-1R antagonist. Curiously, the best results overall were obtained with the ligand PRE-084, which has less affinity for the Sig1R than the two new developed compounds. This comparison points out to the complexity

of the Sig-1R modulation. Further studies are needed to better understand the function of this receptor and the ligand particularities driving the actual action of its ligands.

In addition to MNs, microglia and astrocytes also express Sig-1R (Jia et al., 2018). Glial cell reactivity is associated and has been proposed to play a causative role in MN death following root lesions (Koliatsos et al., 1994), and also in MND models (Brites and Vaz, 2014; Filipi et al., 2020; Pehar et al., 2017). Thus, reducing glial activation may help to ameliorate the deleterious spinal MN environment in these conditions. Our results show that treatment with the EST79232 reduced glial reactivity after rhizotomy, similarly to PRE-084. Similarly, other Sig-1R ligands, including PRE-084, BD1063 and SA4503 reduced astrogliosis after spinal root injuries (Gaja-Capdevila et al., 2021; Penas et al., 2011). PRE-084 treatment was reported to reduce microglia activation in SOD1^{G93A} mice (Mancuso et al., 2012a). In contrast, we found that EST79232 and EST79376 had an effect reducing astroglia activation in the ALS model, but no effect on microglial cells. In the spinal muscular atrophy Smn$^{2B/-}$ mice, PRE-084 attenuated reactive astrogliosis and restored the imbalance of M1/M2 microglia, despite no improvement in clinical outcome was observed (Cerveró et al., 2018). Thus, treatment with Sig-1R ligands may contribute to regulate the deleterious role of activated glial cells in MN degenerative conditions, although the precise outcome of such phenotypic modulation merits further investigation.

CONCLUSIONS

The pharmacological activity and neuroprotective effect on MN degeneration of two new potent and selective Sig-1R ligands is reported. *In vitro* treatment with the agonist EST79232 and antagonist EST79376 significantly reduced MN death caused by chronic excitotoxicity. *In vivo,* Sig-1R ligand EST79232 had more potent effect on preventing MN degeneration than EST79376. In addition, administration of EST79232 had effects on reducing astroglial reactivity in both models, and EST79376 only in the SOD1^{G93A} mice model. The observation that Sig-1R ligands exert protective/preventive effects on different experimental models of MN degeneration opens promising perspectives for targeting Sig-1R for MND.

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Conflicts of Interest: The authors declare that the work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. SY, RFR, JB, AM, MM and JMV were employed by the company ESTEVE Pharmaceuticals at the time this study was performed. All other authors declare no competing interests.

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VI. GENERAL DISCUSSION

Despite MN alterations and diseases have been extensively studied over the years, the exact pathogenetic mechanisms of MN degeneration have not been completely elucidated yet. In ALS, the process of neurodegeneration involves a complex array of molecular and genetic pathways and, although a combination of mechanisms may contribute to the aggressive development of motor dysfunction, the primary factors underlying this global disturbance in ALS are not fully known. In fact, the treatment options in ALS management are still limited. For these reasons, in the present thesis we performed a detailed investigation in the role of Sig-1R ligands on the MN survival using experimental models of MND.

Sig-1R is a poorly understood membrane protein expressed throughout the body (Alonso et al., 2000; Hanner et al., 1996). Despite Sig-1R had been misclassified in the mid-1970s as a subtype of opioid receptor (Martin et al., 1976), it is nowadays classified as a unique ligand-regulated chaperone membrane receptor. Extensive studies have shown that Sig-1R interacts with and modulates the activity of multiple proteins with important biological functions (Herrando-Grabulosa et al., 2021; Weng et al., 2017). Sig-1R has been shown to be localized, at the subcellular level in MNs, in the MAM (Hayashi & Su, 2007) and in the ER cisternae at postsynaptic sites of C-terminals (Mavlyutov et al., 2012). It has been described to re-localize to the nuclear membrane and to the plasma membrane upon activation or ligand binding (Mavlyutov et al., 2017). The endogenous ligands exclusive of Sig-1R remain unknown, however several molecules such as progesterone, lipid sphingosine derivates, DMT and choline (Brailoiu et al., 2019; Fontanilla et al., 2009; Mancuso & Navarro, 2017) are identified to bind and regulate this receptor. Nevertheless, due to the chemical diversity and therapeutic potential of the exogenous ligands, the enigmatic Sig-1R has attracted attention during the last decades.

It has been reported that the modulation of Sig-1R through ligands ameliorates pathological phenotypes in CNS disorders such as Alzheimer disease, Parkinson disease, Huntington disease, ALS, stroke, retinal degeneration, pain and depression (Bruna et al., 2018; Haga et al., 2019; Hampel et al., 2020; Ionescu et al., 2019; Mancuso et al., 2012; Reilmann et al., 2019; Zhao et al., 2019). The importance of Sig-1R in vital cellular roles including the regulation of mitochondria and ER functions made Sig-1R as a novel therapeutic target for several diseases or pathological conditions where those organelles are dysregulated. The ability of Sig-1R to be involved in several diseases is explained by its ubiquitous expression of its gene *SIGMAR1*. Focusing on MND, several compounds revealed the neuroprotective effects of Sig-1R activation *in vivo*, for instance positive effects were described in the

SOD1^{G93A} murine model of ALS (Ionescu et al., 2019; Mancuso et al., 2012; Ono et al., 2014), in the wobbler mouse which is a model of spontaneous MN degeneration (Peviani et al., 2014), and after spinal nerve injury in adult mice and rats (Gaja-Capdevila et al., 2021; Penas, Pascual-Font, et al., 2011), illustrating the relation between Sig-1R and MN survival. In this thesis we have compared the potential therapeutic effects of three well known Sig-1R ligands, the considered agonists PRE-084 and SA4503 and the antagonist BD1063, and also two newly synthetized Sig-1R compounds, the agonist EST79232 and the antagonist EST79376, in three preclinical models of MN degeneration.

Firstly, we used the *in vitro* SCOC model, which is a primary culture in which the anatomical organization of the spinal cord and neuronal and glial interactions are preserved. The well stablished model of chronic excitotoxicity induced by THA is based on glutamate toxicity and allows neuroprotective drug screening before testing the promising compounds on *in vivo* models (Herrando-Grabulosa et al., 2016; Rothstein et al., 1993). We found that all the five Sig-1R ligands tested conferred MN protection at least at two concentrations evaluated, displaying that both agonists and antagonists of Sig-1R exerted neuroprotection under chronic excitotoxicity. The *in vitro* studies also revealed the importance of the bell shape dose effect of Sig-1R ligands to reverse pathologic phenotypes with specific concentrations to avoid the death of MN. This model mimics one of the main mechanisms inducing MN degeneration in ALS, glutamate excitotoxicity; for that reason we added Riluzole, drug approved to treat this disease, as a positive control as an anti-glutamatergic drug. Regarding the mechanisms of action, it has been described that PRE-084 promotes neuroprotection through PKC signalling (Guzmán-Lenis et al., 2009), while SA4503 through activating the Akt-Erk1/2 pro-survival pathway. However, further molecular studies are needed to elucidate the mechanisms through which Sig-1R binding exerted neuroprotection. We hypothesize that due to the important role of Sig-1R in Ca^{2+} regulation, these ligands may act in this pathway modulating Ca^{2+} homeostasis. In this line, Tadić et al. (2017) described that intracellular Ca^{2+} shuttling could be modified by Sig-1R activation. In embryonic SOD1^{G93A} mice cultured MNs, SA4503 accelerated the cytosolic Ca^{2+} clearance after AMPA receptor activation and IP3R-mediated ER Ca^{2+} release, whereas PRE-084 did not modify Ca^{2+} stores. These observations suggest that different Sig-1R ligands may act differently on the Ca^{2+} homeostasis in the same pathological condition.

In the following *in vivo* studies, we demonstrated that Sig-1R ligands exerted neuroprotection in the SOD1^{G93A} mouse model of ALS. In agreement with previous studies (Mancuso et al., 2012), here, we showed that administration of PRE-084 improved motor function until late stages of the disease in SOD1^{G93A} female and male mice. Furthermore, we describe for the first time that daily administration from 8 to 16 weeks of age of the Sig-1R antagonist BD1063 had also neuroprotective effects in this animal model, preserving neuromuscular function of the hindlimbs and increasing the number of surviving MNs in the SOD1^{G93A} mice. On the previous study, BD1063 was co-administered with PRE-084 and the beneficial effects observed with the agonist were blocked (Mancuso et al., 2012). It is important to consider that in the previous study BD1063 was assessed at a low dose of 0.25 mg/kg and we used it at 5 mg/kg, and we gave the compound alone. Whereas SA4503 treatment tended to improve motor function maintaining the amplitude of TA CMAP during the disease and NMJ at 16 weeks of age, it did not improve MN survival. Interestingly, a previous study found that SA4503 1 mg/kg treatment from 5 weeks old extended survival time, but did not affect the onset of motor signs in the SOD1^{G93A} mice (Ono et al., 2014). However, we found a delay of one week of disease onset compared to untreated mice. Concerning the treatment with the two new Sig-1R compounds, daily administration of EST79232 from 8 to 16 weeks of age improved functional outcomes and delayed the onset of disease. In line with these functional data, EST79232 treatment also attenuated MNs loss and showed a tendency to preserve innervated NMJ in treated SOD1^{G93A} male mice. The results of EST79232 treatment were similar to those obtained with the agonist PRE-084 as a positive control. In contrast, EST79376 preserved MNs *in vitro* and after spinal nerve injury but was not able to improve MN death in SOD1^{G93A} mice. Finally, when we compared the outcomes of several Sig-1R ligands in SOD1^{G93A} mice the neuroprotective effects significantly differed between them. In the same line, Wang et al. (2020) compared the Sig-1R ligands SA4503, PRE-084 and pentazocine (PTZ) in a model of severe retinopathy, and reported that *in vitro* the three drugs yielded similar outcomes, whereas neither PRE-084 nor SA4503 afforded *in vivo* protection comparable to PTZ. Regarding the differences in disease progression between genders in the SOD1^{G93A} mouse model, PRE-084 was assessed in both studies in both sexes. The beneficial effects with the prototypic agonist in the functional and histological results were similar in female and in male SOD1^{G93A} mice, as previous observed (Mancuso et al., 2012). For this reason, we did not assess all the ligands in both sexes.

In the present thesis, we also characterized a spinal nerve injury, or rhizotomy, model in mice, in which MN degeneration does not depend on genetic alterations thus being relevant due to

the majority of sporadic ALS cases and other MND. The section of L4–L5 roots leads to a progressive loss of MNs, causing the death of 35% and 42% MNs at 28 and 42 dpi, respectively, in the affected ventral horn of the spinal cord. Moreover, no differences were found in the degenerative MN pattern comparing sexes. In the past, it has been reported a potent neuroprotective effect of PRE-084 treatment on spinal MNs after spinal root avulsion injury in adult rats (Penas, et al., 2011b). Thus, we questioned whether the administration of Sig-1R agonist or antagonist ligands could promote MN survival after this injury in mice. Our data showed that PRE-084, SA4503 and BD1063 reduced MN loss by about 20%. In addition, the two new synthesized compounds with high affinity and specificity for the human Sig-1R, EST79376 and EST79232 at the dose of 5 mg/kg produced an increase of the number of surviving lumbar spinal MNs, similar to the other compounds. Taking into consideration that similar neuroprotective effects were obtained with both Sig-1R agonist and antagonist ligands, a better understanding of the essential functions of Sig-1R will help to guess in which conditions the use of a specific Sig-1R compound is beneficial or detrimental (Jia et al., 2018; Ryskamp et al., 2017; Ye et al., 2020). However, these obtained results showed that modulating the Sig-1R represents a good strategy to prevent MN death and extend the time-window for surgical repair after spinal root and plexus injuries.

Since 2003, when the first model of Sig-1R KO mice was generated (Langa et al., 2003), the knowledge of the biological functions of the Sig-1R has considerably increased. Broadly, Sig-1R alterations may worsen the progression of neurodegenerative diseases, whereas its activation prompts potent neuroprotective effects, promoting neuronal survival, and restoring neuronal plasticity to slow disease progression via mechanisms like modulation of Ca^{2+} homeostasis, attenuation of oxidative stress and inflammation, maintenance of ER and mitochondrial integrity (Ye et al., 2020). Here, we also investigated whether absence of the Sig-1R protein affects the outcome after rhizotomy, using Sig-1R KO mice. Our data showed that the percentage of MN death after a spinal root injury did not change between WT and Sig-1R KO adult mice. In concordance, several studies showed that spontaneous locomotion is not affected by the absence of Sig-1R, indicating no motor dysfunction in the animals (see the review by Couly et al. (2020)). Contrarily, other studies revealed that Sig-1R KO mice at 10 and 20 weeks of age displayed locomotor deficits, having lower scores in the rotarod test compared to age-matched WT and had muscle weakness, axonal degeneration and MN loss at 20 weeks compared to WT mice (Bernard-Marissal et al., 2015). Similarly, Mavlyutov et al. (2010) showed that Sig-1R KO mice remained less time on the rotarod, a shorter latency period, compared to WT and with larger differences at 14 weeks, especially at constant

speed. Later, this research group also described that the double transgenic mice SOD1^{G93A} and Sig-1R KO mice had a reduction in lifespan (Mavlyutov et al., 2013). These results were also confirmed by Hong et al. (2017), who observed an age-related decline of motor coordination with an increase in aggregation and phosphorylation of α-synuclein in dopaminergic neurons of substantia nigra in Sig-1R KO mice. In that study, however, no difference was observed in rotarod scores at 12 weeks of age, but the rotarod score was lower than the WT with a decrease in muscle strength at 48 weeks (12 months of age). To conclude, these observations from different experiments uncover that with aging the absence of Sig-1R induces cell stress dysregulation and alterations that may be compensated in the young stage, but the pathological phenotypes appear when Sig-1R KO mice get old.

Since mutations in *SIGMAR1* have been found to cause forms of ALS (Al-Saif et al., 2011; Luty et al., 2010) and dHMN (Almendra et al., 2018; Li et al., 2015; Ververis et al., 2019), in several studies the levels of Sig-1R were analysed in ALS samples. Firstly, it was described that the levels of Sig-1R protein were reduced in the lumbar spinal cord of ALS patients, and it was abnormally accumulated in enlarged C-terminals and ER structures of MNs. These Sig-1R accumulations were also observed in SOD1 transgenic mice (Prause et al., 2013). Here, we observed that Sig-1R protein levels did not change along the pathology progression in SOD1^{G93A} mice, and that treatment with PRE-084 or BD1063 did not modify this protein levels in the whole lumbar spinal cord. These results are in agreement with previous studies in the laboratory (Mancuso et al., 2012). Furthermore, Sig-1R levels were maintained at 7 and 14 dpi after rhizotomy, even under treatment with the Sig-1R ligands, in line with previous findings in which PRE-084 administration did not modify Sig-1R protein levels after preganglionic root avulsion in rats (Penas, et al., 2011b).

A common feature that occurs in ALS, after CNS injury or in other neurodegenerative diseases is the neuroinflammatory response. Then, another controversial issue about Sig-1R modulation is whether Sig-1R ligands play a role in the regulation of glial cells which also express this receptor (Jia et al., 2018). After rhizotomy, a huge increase of glial activation occurs; we found that PRE-084 and EST79232 (5 mg/kg) treatment reduced microgliosis, whereas PRE-084, BD1063, SA4503 and EST79232 (5 mg/kg) administration significantly reduced astrogliosis to around 50% of untreated cords. In agreement with our data, PRE-084 also reduced astrogliosis after root avulsion in rat (Penas et al., 2011b) and in the wobbler mouse (Peviani et al., 2014). In the ALS model we had controversial results regarding glia immunoreactivity in the lumbar spinal cord. On one hand, in SOD1^{G93A} female mice, we observed a reduction in microglia immunoreactivity with PRE-084 and SA4503 treatment but

there were no changes in astroglia activation comparing to untreated mice. On the other hand, in SOD1^{G93A} male mice, a marked reduction of astroglia response was observed with all the compounds assessed (PRE-084, EST79232 and EST79376), and only PRE-084 treated group presented a tendency to reduce microglia reactivity. Mancuso et al. (2012) also showed that PRE-084 administration significantly reduced microglial, but not astroglial immunoreactivity in both male and female SOD1 animals. Similarly, in the mouse model of SMA, PRE-084 attenuated reactive gliosis mitigating the M1/M2 imbalance (Cerveró et al., 2018). With these results it is difficult to delineate the specific contribution of microglia and astroglia to disease; indeed, contradictory results about the impact of the Sig-1R modulation on glial cells are present in the literature. Nowadays, therapeutic approaches trying to modulate the function of endogenous astrocytes are recognized as promising for treating neurodegenerative disorders. Thus, it is important to characterize the mechanisms which regulate astrocyte activation. Wang et al. (2019) elucidated the role of Sig-1R in the activation of astrocytes; pharmacological activation of Sig-1R with the agonist SKF-10047 directly promoted the activation of primary cultured astrocytes via the ERK1/2 and glycogen synthase glycogen synthase kinase 3β (GSK3β) signaling pathway. All these studies highlighted the role of Sig-1R ligands modulating the glial response.

A complex neurodegenerative process occurs after rhizotomy with Ca^{2+} imbalance and misfolded proteins triggering ER stress as a self-protection mechanism for cell survival. We investigated whether rhizotomy induced activation of ER stress responses, similar to what was previously reported in the models of root avulsion in rats (Penas, et al., 2011a) and axotomy of the hypoglossal nerve in mice (Romeo-Guitart et al., 2018). Indirect regulation of transcriptional activity by Sig-1R contributes to its neuroprotective properties. For example, Sig-1R facilitates dimerization of the ER stress sensor and endonuclease IRE1 at the MAM domain, leading to splicing-dependent activation of the transcription factor XBP1, XBP1s which goes on to upregulate several ER chaperones (Mori et al., 2013). We found that the IRE1/XBP1s axis is enhanced by PRE-084 and BD1063 administration at 14 dpi, suggesting that Sig-1R ligands exert a pro-survival state by restoring ER function after rhizotomy. Also, the activation of the IRE1/XBP1s axis is due to the modulation of Sig-1R by the ligands, since it did not occur in the Sig-1R KO mice. Therefore, Sig-1R ligands activate these endogenous neuroprotective mechanisms to sustain MN survival.

Dysfunction or loss of Sig-1R has been correlated to disturbed ER-mitochondria contacts, intracellular Ca^{2+} signaling, ER stress activation and an increase in UPR (Bernard-Marissal et al., 2015; Hong et al., 2017; Prause et al., 2013). For this reason, we analysed autophagic flux

and the ER stress, two pathways implicated in the physiopathology of ALS, in the spinal cord samples of SOD1^{G93A} mice treated with Sig-1R ligands. WB revealed that the autophagic flux and the endoplasmic reticulum stress were not notably modified with Sig-1R treatments in SOD1^{G93A} mice. However, we found an increase of autophagic markers at 16 weeks of age in SOD1^{G93A} mice compared with the WT littermates.

Despite significant research efforts during the last decades, there has been a continuous failure in the translation of preclinical data into the clinics. This lack of success could be due to the fact that most of these clinical approaches targeted exclusively one of the multiple pathologic mechanisms involved in ALS, and unfortunately, the vast majority of clinical trials failed to show clinical efficacy (Petrov et al., 2017). ALS is a heterogeneous disease and is likely to require a combined therapy targeting several events involved in the pathology. Another possible reason for the lack of translation of preclinical data is the experimental models used. Since the first causative mutation for ALS was described in the *SOD1* gene (Rosen et al., 1993), SOD1-based mice are the most extensively investigated models of the disease (Gurney et al., 1994). These mice closely mimic human ALS pathology developing progressive MN loss and muscle denervation manifested as hind limb weakness and deficits of locomotion. However, mutations in the *SOD1* gene account for approximately 20% of fALS cases, representing 1–2.5% of all ALS cases, reaffirming the importance of replicating therapeutical studies in other animal models of MND because the SOD1 mutations represent a small proportion of ALS patients.

It is interesting to note that this year the Sig-1R ligand pridopidine is one of the four therapies added in the HEALEY ALS platform trial (NCT04297683) helping to accelerate the development of effective treatments for ALS. Preclinical models of ALS have shown that pridopidine prevents neuronal cell death, boosts the connections between neurons, and preserves motor function (Ionescu et al., 2019). Platform trials are designed to evaluate several treatments at the same time to speed the development of those most promising drugs, while significantly reducing costs. So, the importance of Sig-1R in ALS pathology has been growing to reach the stage of clinical trials.

Overall, the results presented in this thesis provide evidence of the essential role of Sig-1R on MN survival. Specifically, we found that Sig-1R ligands improved motor function, maintained the number of surviving MNs and modulated the glia reactivity in the SOD1^{G93A} mice at the end-stage of the disease. However, it is worth to note that each compound tested had specific effects ameliorating the pathology. Moreover, treatment with different Sig-1R

ligands exerted neuroprotective effects after spinal root injury, preventing MN cell death by maintaining the IRE1/XBP1s axis. All these data suggest that this topic still needs deeper investigation and demonstrated the ongoing interest for the Sig-1R ligands therapy in the MN degeneration process.

VII. CONCLUSIONS

Chapter I: Sigma-1 receptor is a pharmacological target to promote neuroprotection in the SOD1^{G93A} ALS mice.

1. The Sig-1R ligands PRE-084, SA4503 and BD1063 promote MNs survival under chronic excitotoxicity in SCOCs.
2. Administration of PRE-084 (0.25 mg/kg) and BD1063 (5 mg/kg) preserved neuromuscular function of the hindlimbs and increased the number of surviving spinal MNs in the SOD1^{G93A} female mice. SA4503 had lower effects but did not improve MN survival.
3. PRE-084 and SA4503 (0.25 mg/kg) treatments reduced microglia reactivity in the spinal cord of SOD1^{G93A} female mice.
4. The autophagic flux and the ER stress were not notably modified with Sig-1R treatments in SOD1^{G93A} mice.

Chapter II: Neuroprotective effects of sigma 1 receptor ligands on motoneuron death after spinal root injury in mice.

1. The section of L4–L5 spinal roots led to progressive loss of MNs, causing death of 35% and 42% MN at 28 and 42 dpi, respectively.
2. The absence of Sig-1R protein (Sig-1R KO mice) did not change MN degeneration after spinal root injury in both sexes.
3. Treatment with Sig-1R ligands, PRE-084, SA4503 and BD1063, exerted neuroprotective effects, preserving spinal MNs and reducing astroglia reactivity after spinal root injury.
4. PRE-084 and BD1063 administration maintained the IRE1/XBP1s axis activation to sustain MN survival.

Chapter III: EST79232 and EST79376, two novel sigma-1 receptor ligands, exert neuroprotection on models of motoneuron degeneration.

1. The newly synthesized compounds, EST79232 and EST79376, are highly selective Sig-1R ligands with high affinity for the human Sig-1R. EST79232 has an agonist while EST79376 an antagonist profile.
2. *In vitro*, addition of the agonist EST79232 and the antagonist EST79376 significantly reduced MN death caused by chronic excitotoxicity in SCOCs.
3. EST79232 and EST79376 at a dose of 5 mg/kg/bid enhanced MN survival following rhizotomy. Only EST79232 significantly reduced microglial and astroglial reactivity.

4. The agonist EST79232 significantly increased MN survival and had a mild beneficial effect on motor function of the hindlimb muscles in SOD1^{G93A} male mice.
5. In the ALS mouse model, treatment with EST79232 and EST79376 significantly reduced the astroglia activation in the lumbar spinal cord at 16 weeks of age.
6. *In vivo,* Sig-1R ligand EST79232 had more potent effect on preventing MN degeneration than EST79376.

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IX. ABBREVIATIONS

ALS: amyotrophic lateral sclerosis
ATF6: activating transcription factor-6 α
BAC: bacterial artificial chromosome
Ca^{2+}: calcium ion
ChAt: choline acetyltransferase
CMAP: compound muscle action potential
CNS: central nervous system
CSF: cerebrospinal fluid
DHEA: dehydroepiandosterone
dHMN: distal hereditary motor neuropathies
DIV: days *in vitro*
DLK: dual leucine zipper bearing kinase
DMT: N,N-dimethyl tryptamine
dpi: days post-injury
DRG: dorsal root ganglia
EAAT2: excitatory amino acid transporter 2
ER: endoplasmic reticulum
ERAD: ER-associated protein degradation
fALS: familiar ALS
FDA: Food and Drug Administration
FTD: frontotemporal dementia
FUS: fused in sarcoma
GM: gastrocnemius
IMM: inner mitochondrial membrane
INMT: indole-N-methyl transferase
I.P.: intraperitoneal
IP_3R3: inositol 1,4,5-triphosphate receptor type 3
iPSCs: induced pluripotent stem cells
IRE1α: inositol-requiring protein-1 α
KO: knockout
MAM: mitochondrial-associated ER membrane
MMP9: matrix metalloproteinase 9
MN: motoneuron
MND: motoneuron disease
mSOD1: mutant superoxide dismutase
mTDP-43: mutated TDP-43
NMJ: neuromuscular junction
OMM: outer mitochondrial membrane
PBS: phosphate-buffered saline
PERK: RNA-activated protein kinase-like ER kinase
PFA: paraformaldehyde
PFN1: profilin 1
PKC: protein kinase C
PNI: peripheral nerve injury
PNS: peripheral nervous system
RAG: regeneration associated genes
RAN: repeat-associated non-ATG translated
RIP-1: receptor-interacting protein 1
ROS: reactive oxygen species
sALS: sporadic ALS
SCOC: spinal cord organotypic culture
Sema3A: semaphorin A3
Sig-1R: sigma-1 receptor
Sig-2R: sigma-2 receptor
SMA: spinal muscular atrophy
SOD1: superoxide dismutase
TA: tibialis anterior
TDP-43: TAR DNA-binding protein 43
THA: DL-threo-β-hydroxyaspartic acid
TSA: tyramide signal amplification
UPR: unfolded protein response
UPS: ubiquitin proteasome system
WB: western blot
WT: wild type

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