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Combined Epigenetic Drugs Elicit Neuroprotective Effects on Sex Dimorphic Features in ALS Mice

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disorder. There is no cure and current treatments fail to slow the progression of the disease. Epigenetic modulation in the acetylation state of NF-kB RelA and histone 3 (H3) protein, involved in the development of neurodegenerative disease, is a drugable target for HDAC inhibitors and Resveratrol. In this study, we demonstrated that the combination of two epigenetic drugs, Valproate and Resveratrol, can restore the normal acetylation state of RelA in the SOD1(G93A) murine model of ALS, to obtain the neuroprotective form of NF-kB. We also investigated the dimorphic development of the disease, as well as the sex-sensibility to the treatment administered. We showed that the combined drugs, which rescued AMPK activation, RelA and histone 3 acetylation state, reduced the motor deficit and the disease pathology associated with motor neuron loss and microglial reactivity, BDNF and Bcl-xL reduction. Specifically, vehicle-administered males showed earlier onset and slower progression of the disease when compared to females. The treatment, administered at 50 days of life, postponed the time of onset in males by 22 days, but by 7 days in females. Nevertheless, in females, the drugs significantly reduced symptoms severity of the later phase of the disease and prolonged the mice survival. Only minor beneficial effects were produced in the later stage in males. Overall, this study shows a beneficial and dimorphic response to Valproate and Resveratrol treatment in ALS mice.

TABLE OF CONTENT

1. AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is classified as a fatal neurodegenerative disorder with no specific cure and is the most common form of motor neuron disease (Zucchi et al., 2020). It is also known as Charcot's disease, named after Jean-Martin Charcot, a neurologist who first diagnosed the disease in 1869 (Ma & Li, 2019), or Lou Gehrig's disease, named after the famous baseball player, who was diagnosed with the disease in 1939 (Cardoso et al., 2002). Being a motor neuron disorder, ALS mainly affects motor cells, which are specialized cells located in the brain and spinal cord (Morris, 2015). These motor neurons (MNs) come together to form a network known as motor circuits and are responsible for sending information between the brain and voluntary muscles, thereby initiating complex behaviours such as locomotion. There are two classes of MNs: The upper motor neurons (UMNs), which originate in the cerebral cortex and passes down to the brain stem and spinal cord, while the lower motor neurons (LMNs) travel from the spinal cord to innervate muscles and glands throughout the entire body (Zayia & Tadi, 2021). Both UMNs, as well as LMNs, are involved in the onset and progression of ALS. The degeneration of these cells results in muscle weakness, atrophy, and progressive loss of voluntary movement, eventually leading to premature death usually due to respiratory failure (Hardiman et al., 2017). The term amyotrophic lateral sclerosis has its etymology from several Greek words. "Amyotrophic" can be divided into three parts – "a" which means absent, "myo" which refers to the muscles and "trophic" which means nourishment or nutrition. Taken together, Amyotrophic can be interpreted as "a lack of muscle nourishment". The term "lateral" refers to the areas of the spinal cord which contain the nerve cells that are affected by the disease. While the term "sclerosis" is derived from the progressive hardening of the myelinated pathway that occurs as a result of astroglia activity and response to neuronal death (Mandal., 2019) The clinical onset of ALS is associated with symptoms such as muscle weakness, muscle cramps, difficulty walking, breathing, swallowing and speech (Siddique & Siddique, 1993). However, the molecular onset of this disease precedes the physical clinical onset and involves mitochondrial and motor neuron dysfunction which begins to occur in UMNs and LMNs before the manifestation of clinical symptoms (Dresselhaus & Meffert,

2019). Furthermore, compensatory mechanisms in the motor pathway help to maintain motor function until about 50% of motor neurons die, after which the disease progresses rapidly (Majoor-Krakauer et al., 2003).

Although ALS is a disease that primarily affects motor neurons, recent findings have implicated other areas in the frontal and temporal lobe to be associated with the development and progression of the disease (Hardiman et al., 2017). The molecular onset of ALS can either be spinal or bulbar, with about 70% of diagnosed patients initially experiencing the spinal onset of the disease. However, as the disease progresses, most of these patients begin to experience bulbar changes (Hardiman et. al. 2017). Fewer patients develop bulbar onset, which is most likely the most devastating form of the disease as it is characterized by a rapid decline, a shorter survival rate (<2 years after diagnosis), and significantly reduces the quality of life (Shellikeri et al., 2017). The bulbar onset is linked to the development of lesions in the motor neurons of the brain and bulbar trunk, which eventually leads to motor speech disorder (dysarthria) and difficulty in swallowing food (dysphagia) (Hardiman et al., 2017). In Europe and North America, the incidence rate of ALS (number of cases diagnosed per year) varies between 1.5 and 2.7 per 100,000 people/year. While, the prevalence rate (number of patients living with ALS), varies between 2.7 and 7.4 per 100,000 people/year (Nakken et al., 2016). ALS is more prevalent in males, with a ratio of 1:2, and is responsible for about 1 in 1000 deaths (Jun et al., 2019; Palese et al., 2019). The onset of the disease is often associated with people of advanced age (between 55 and 65 years of age). However, the disorder can also be found in younger individuals between the age of 20 and above 30 years old (Nakken et al., 2016). About 90.95% of ALS cases are idiopathic i.e., of unknown origin, and are referred to as sporadic ALS (sALS). The remaining 5-10% are associated with several genetic mutations are known as familial ALS (fALS) (Hardiman et al., 2017).

1.1   FAMILIAL AND SPORADIC ALS

ALS can either be sporadic (sALS) meaning it occurs at random without being associated with any known cause or family history and accounts for about 90-95%

of all cases, or genetic, also known as familial ALS (fALS), which only accounts for 5-10% of ALS cases. Most cases of ALS are sporadic. Although the cause of sALS is generally unknown, studies have suggested that a combination of environmental and genetic factors could play a key role in its development (Ajroud-Driss & Siddique, 2015).

1.1.1 Superoxide dismutase 1 (SOD1)

Several genetic mutations are linked with ALS pathogenesis. However, the Superoxide dismutase 1 (SOD1) gene is the first to be implicated in the pathogenesis and progression of the disease. Genetic mutation in the SOD1 gene accounts for 12% of the fALS and about 1% of the sALS (Renton et al., 2014). Three distinct superoxide dismutase enzymes exist in humans: Cu/Zn/SOD1(SOD1), Mn SOD2(SOD2), and extracellular SOD3 (SOD3). The SOD1 binds to copper and zinc ions and can be found in the cytoplasm as a dimer, SOD2 is a magnesium-dependent enzyme located in the mitochondria, while SOD3 is an extracellular superoxide dismutase. Both SOD2 and SOD3 are tetramers (Fetherolf et al., 2017). These enzymes can degrade the toxicity of reactive oxygen species (ROS) produced by mitochondrial respiration and protect cells from oxidative stress. About 160 amino acid mutations of the SOD1 gene have been associated with the development of ALS (http://alsod.iop.kcl.ac.uk/), with most of them being missense mutations. In humans, the SOD1(A4V) mutation is the most common type of amino acid substitution in ALS. This mutation is responsible for the most virulent form of ALS and patients with this form of mutation usually have a short life span after the disease onset (Deng et al., 1993). Another type of mutation is the H48Q mutation which is located within the copper-binding domain and results in a rapid progression of the disease (Enayat et al., 1995), while the SOD1(H46R) mutation is associated with a slower progression of the disease and prolonged survival. Furthermore, in this form of mutation, the presentation of clinical onset and respiratory failure usually exceeds 10 years, making it a less severe form of ALS (Holmoy et al., 2007).

1.1.2   Chromosome 9 open reading frame 72 (C9orf72)

The C9orf72 mutation in ALS accounts for about 40-50% of fALS and about 7% of sporadic cases, and as such, is the most common mutation found in ALS patients (Renton., 2014). This genetic mutation results from a hexanucleotide (GGGGCC) repeat expansion in the intron 1 position of the C9orF72 gene. The pathogenic mechanisms involved in C9orF72 mutation include RNA toxicity, as well as loss-of-function, sense and anti-sense foci (Haeusler et al., 2016; Hayes & Rothstein, 2016). Furthermore, patients with C9orF72 mutation also have ubiquitinated p62-positive TDP-43 inclusions in the cytoplasm of neurons and glial cells (Cooper-Knock et al., 2012). Under normal conditions, C9orF72 is highly abundant in the cortex, cerebellum, and spinal cord, however, its exact role remains unclear. Studies have linked C9orf72 protein to endosomal trafficking and autophagy in cells (Farg et al., 2014), and has also been implicated in nucleocytoplasmic transport in neurons (Xi et al., 2015).

1.1.3   TAR   DNA-binding   protein   (TDP-43)   and   Fused   in sarcoma/translocated in liposarcoma (FUS/TLS)

TDP-43, a protein encoded in the TARDBP gene, is involved in multiple processes such as transcription, translational control, RNA splicing and transport. It also has a major role in stress granule response and microRNA biogenesis (Baralle et al., 2013). TARDBP gene mutations account for 4% of fALS, and 1% of sALS (Corcia et al., 2017). Mutations in the TARDBP gene cause neurodegeneration due to the formation of protein aggregates in the cytoplasm of neurons (Neumann et al., 2006). Most TDP-43 mutations are located in the exon 6 region of the glycine-rich C-terminal of the protein (Pesiridis et al., 2009). TARDBP genetic mutations have been associated with frontotemporal dementia (FTD) (Sreedharan et al., 2008). However, most patients are most likely to develop ALS than FTD. TDP-43 inclusions are found in approximately 97% of ALS patients [Mackenzie et al 2007], and 50% of FTD victims (Ling et al., 2013). It is still unclear what determines a patient's predisposition to either ALS or FTD, while some patients even develop both diseases (Stephenson & Amor, 2017). Furthermore, TDP-43 inclusions can also be found in neurodegenerative diseases such as Alzheimer's, Parkinson's and

Huntington's (Chen-Plotkin et al., 2010), as well as in older patients, suggesting a possible role of TDP43 aggregation during aging (Nakashima-Yasuda et al., 2007).

Another gene implicated in ALS is Fused in sarcoma (FUS). It is a DNA/RNA-binding protein that is involved in cellular processes such as transcription, RNA splicing, and transport (Vance et al., 2009). It shares similar characteristics to TARDBP and is primarily located in the nucleus. However, in ALS patients, mutations cause a reduction of FUS in the nucleus and lead to the formation of protein aggregates within the cytoplasm (Kwiatkowski et al., 2009; Vance et al., 2009). FUS is responsible for approximately 4% of familial ALS cases and 1% in sporadic cases (Renton et al., 2014).
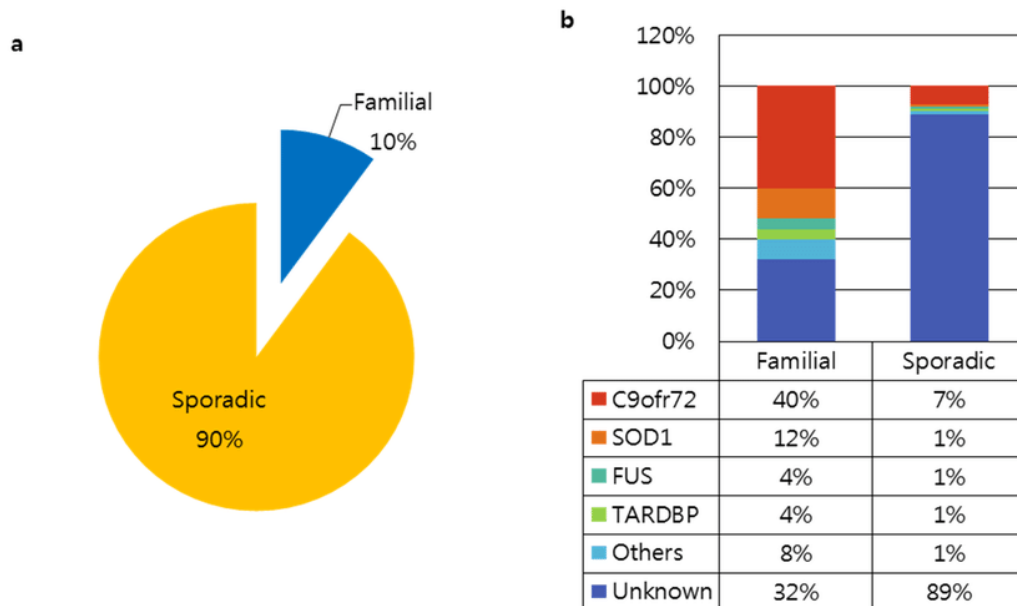
Figure 1.1 The diagram shows the genetic forms of ALS. (a) Prevalence of fALS and sALS. (b) Rate of occurrence of the causative gene in fALS and sALS (Yun & Ha, 2020).

1.2   PATHOGENIC MECHANISMS INVOLVED IN ALS

The cause of MN degeneration in ALS is still unknown. Several processes that involve a combination of environmental and genetic factors have been implicated in the pathology of ALS. Some of these factors include mitochondrial dysfunction, oxidative stress, glutamate excitotoxicity and protein aggregation. It is for this

reason that ALS is regarded as a multifactorial disease (Bonafede & Mariotti, 2017).

1.2.1 Mitochondrial dysfunction

Mitochondria are generally referred to as the powerhouse of the cell. It is responsible for energy production and cellular respiration. MN undergo mitochondria alterations to their morphology both in ALS patients and in the SOD1(G93A) mice model (Muyderman & Chen, 2014). This structural change could present itself as a swollen and vacuolated appearance, usually due to the accumulation of mutant SOD1 protein aggregates in the space of the internal mitochondrial membrane (Pollari et al., 2014). The aggregation of the SOD1 enzyme causes oxidative damage to mitochondrial proteins and induces a defect in respiratory function and axonal transport of mitochondria (Pickles et al., 2016). The oxidative damage causes an increase in the production of reactive oxygen species (ROS), which may trigger another pathogenic mechanism of ALS known as oxidative stress (Smith et al., 2017). Mitochondria also play a key role in calcium homeostasis. Ca^{2+} transport in neurons is regulated by ion channels located in the internal and external mitochondrial membrane and modulates the release of neurotransmitters. Therefore, maintaining the morphological integrity of the mitochondria is necessary for neuronal function and survival (Pollari et al., 2014).

1.2.2 Oxidative stress

Reactive oxygen species (ROS) are a natural metabolic by-product of enzymatic and non-enzymatic reactions (Pizzino et al., 2017). In a healthy system, there is a balance between the production and removal of ROS (Birben et al., 2012). However, oxidative stress occurs when the ability of an organism to maintain this balance is compromised due to excessive production of ROS (Bodega et al., 2019). When these oxidants are produced in excess, they lead to a reduction of necessary antioxidants. This limits the ability of the cell to remove or repair the damage caused by the ROS (Ferraiuolo et al., 2011). As a result, significant dysfunction is observed in macromolecules such as DNA, proteins and lipids, eventually leading to necrosis and cell death (Calingasan et al., 2005). An excessive production of ROS associated with insufficient antioxidant defence mechanism has been linked to ALS pathology (Tam et al., 2019). Mitochondria is an important organelle that is

involved in intracellular ROS production. This is mainly due to their role in the production of oxidative ATP (Kausar et al., 2018). Furthermore, the activity of proteins such as dihydroorotate dehydrogenase (Hey-Mogensen et al., 2014), monoamine oxidases (Kaludercic et al., 2014), cytochrome P450 (CYP) enzymes (Omura, 2006) and complex II (Quinlan et al., 2012), also contribute to ROS production in the mitochondria. However, mitochondria are not the only site of ROS production. Outside the mitochondria, enzymes such as cycloxygenases, CYP450, nicotinamide adenine dinucleotide phosphate oxidase (NOX), lipoxygenases, and xanthine oxidase, are all involved in ROS production (Rani et al., 2016). Several enzymatic and non-enzymatic antioxidants are involved in the clearance of excess ROS from the cell (Phaniendra et al., 2015). Enzymatic antioxidants such as glutathione peroxidase (GPx), Superoxide dismutases, catalase (CAT), thioredoxin (Trx) and glutathione reductase (GR), play an important role in the catalytic removal of these ROS. Non-enzymatic antioxidants include flavonoids, glutathione (GSH), vitamins A, C, and E, as well as specific proteins (e.g., metallothionein, albumin, and ceruloplasmin) also assist with the clearance of excess ROS (Niedzielska et al., 2016).

1.2.3   Glutamate excitotoxicity

Glutamate is the main excitatory neurotransmitter in the central nervous system and is involved in several cellular processes such as energy metabolism, synaptogenesis, and synaptic plasticity (Sundaram et al., 2012). Glutamate binds to either α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) or N-methyl-D-aspartate (NMDA) receptors and causes depolarization of postsynaptic neurons. This, in turn, results in the opening of the voltage-dependent calcium gated channels, allowing an influx of Ca^{2+} into the cytoplasm of the cell. A group of transmembrane proteins known as excitatory amino acid transporters (EAATs) is produced by glial cells, and are responsible for the clearance and recycling of glutamate in the synaptic cleft (Sundaram et al., 2012). Accumulation of glutamate neurotransmitters in the synaptic cleft of neurons causes an increase in the influx of Ca^{2+} into the cytoplasm of the cell which leads to neuronal degeneration. Ca^{2+} overload can also lead to excessive production of ROS, resulting in damage to the mitochondria and endoplasmic reticulum (ER) (Lanznaster et al., 2018).

Several studies have linked glutamate excitotoxicity to the development of ALS. According to Lin et al., 1998, the astroglial glutamate transporter EAAT2, was reported to be downregulated in the motor cortex and spinal cord of ALS patients (Lin et al., 1998). An increase in glutamate present in the CSF has also been observed both in ALS patients and the SOD1(G93A) mice model. This increase has been associated with a decrease in the production of the astrocytic glutamate transporter (GLT) -1 in the motor cortex and spinal cord (Lanznaster et al., 2018; Spreux-Varoquaux et al., 2002; Trotti et al., 1999).

1.2.4 Protein aggregate

Protein aggregation in the cytoplasm of MN is a major factor in the pathology of ALS. This occurs due to mutation of specific genes such as SOD1 and FUS, which lead to spontaneous misfolding and aggregation of proteins that form inclusions and become toxic (Benkler et al., 2018). Excessive cytoplasmic inclusions could affect the normal functioning of chaperon proteins and ubiquitin-proteasome, thereby inducing MN degeneration (Julien, 2001). Recent studies have identified prion-like propagation of protein aggregation to be a primary pathogenic mechanism in ALS (McAlary & Yerbury, 2019). ALS is characterized by the formation of cytoplasmic protein inclusions such as TDP-43 (Neumann et al., 2006), SOD1 (Boillee et al., 2006), or FUS (Kwiatkowski et al., 2009), in both UMN and LMN. These studies provide evidence that the aggregation and cytoplasmic inclusions of these proteins can originate in one MN and be transferred from one neuron to another in a prion-like manner. This prion-like propagation supports the well-known fact that clinical progression of ALS begins with the loss of a subgroup of MN in one region (either bulbar or spinal onset) and then progresses to other regions of the CNS (Sangwan & Eisenberg, 2016). Spontaneous aggregation of neurofilament (NF) proteins have also been implicated in MN degeneration. NF are structural proteins and are an integral part of the neuronal cytoskeleton. They are abundant in the axons of the MN and serve transportation functions between the soma and the synaptic terminals of MN. Therefore, abnormal aggregation of NF can lead to dysfunction in axonal transport and induce MN degeneration (Xiao et al., 2008).

1.2.5 Autophagy dysfunction

Autophagy is a natural clearance process whereby the cell removes dysfunctional or toxic cellular components through a lysosome-dependent regulated mechanism (Klionsky, 2008). Since ALS is a disease that is caused by the many factors already described above, it should come as no surprise that there is possible damage in the clearance system which is needed to eliminate the excess build-up of these factors such as ROS, protein aggregates, glutamate excitotoxicity, etc. Numerous studies have implicated autophagy dysfunction in the pathogenesis of several neurodegenerative diseases including ALS (Wong et al., 2015). Specific autophagy proteins such as LC3, Beclin 1, p62, and Atg5–Atg12 complex, are increased in spinal MN of sALS and fALS patients, as well as in animal models (Hetz et al., 2009; Tokuda et al., 2016). During disease progression, the accumulation of these complex spreads to glial cells such as astrocytes and microglia (Tian et al., 2011). Studies using electron microscopy have demonstrated the accumulation of autophagosomes in spinal cord MN of late-symptomatic ALS animal models and post-mortem spinal tissue from ALS patients (Ito et al., 2011; Li et al., 2008). Furthermore, in ALS mice models, in vivo imaging of the spinal cord has shown an increase in the level of green fluorescent protein (GFP)-LC3 signal in spinal MN, which indicates the accumulation of autophagosome, throughout the course of the disease (Tian et al., 2011). The accumulation of protein aggregates and morphologically altered mitochondria in the brain and spinal MN, leads to a reduction in autophagy degradation which has been observed in ALS mice models (Watanabe et al., 2001), and ALS patients (Arai et al., 2006; Neumann et al., 2006).
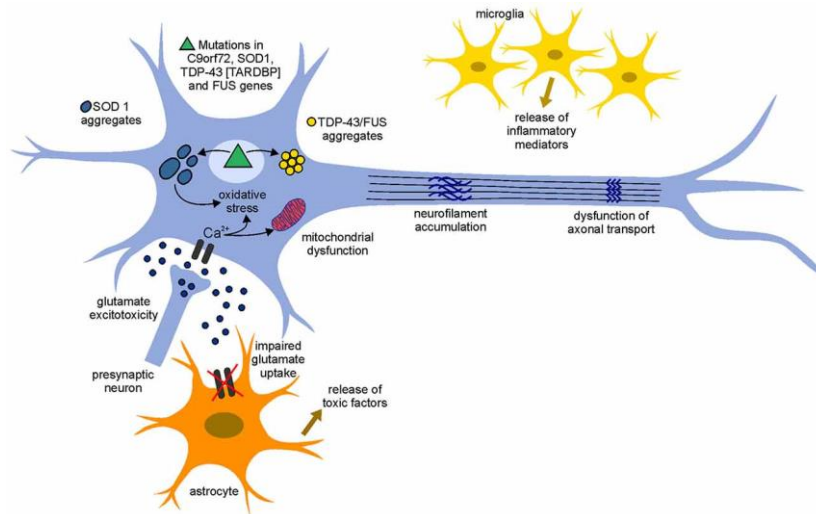
Figure 1.2. The diagram shows the pathogenic mechanisms involved in ALS pathogenesis (Bonafede & Mariotti, 2017)

2.   IN VIVO, IN VITRO, AND ENVIRONMENTAL MODELS OF ALS

Several in vitro and in vivo models have been created with different ALS-linked gene mutations. This was after the discovery of ALS phenotypes in mice that were caused by the overexpression of mutated SOD1 and presented similarities to the human pathology (Gurney et al., 1994). In recent years, environmental models of ALS have been developed by inducing motor neuron degeneration through exposure to environmental factors commonly believed to be involved in the development of ALS (Morrice et al., 2018). These novel environmental models play a key role in understanding the development and progression of the disease, particularly the sporadic ALS which are thought to be linked to environmental factors. Understanding these models will help develop therapeutic interventions.

2.1   In vivo Models of ALS

2.1.1   SOD1

Several SOD1 mouse models that express one of the human SOD1 mutant genes (G37R, G85R, D90A, G93A, missense mutations, or truncated SOD1) have been created to mimic the common ALS characteristics found in humans such as misfolded SOD1, motor dysfunction, and motor neuron loss, gliosis activation, among others (Wang et al., 2009). The murine mice model overexpressing the SOD1(G93A) mutation is the most widely used model of study for ALS because it causes motor neuron degeneration and best elicits ALS traits. The SOD1(G93A) mice begin to develop motor deficits symptoms between 80-90 days after birth and reach the end stage of the disease at around 120-130 days (Gurney et al., 1994). Despite the SOD1(G93A) being the most widely used model of study for ALS, its positive attributes are not without their limitations. Studies have shown that the mSOD1 model mouse has the propensity to randomly delete copy numbers, which can directly impact the clinical presentation of the disease (Lutz, 2018; Zwiegers et al., 2014). Another study reported that the overexpression of human SOD1 leads to axonopathy in mice, leaving many uncertainties about the actual role of the mutation in the development and progression of the disease (Joyce et al., 2011). Furthermore, UMN deficits are not presented in the SOD1(G93A) mouse model (Morrice et al., 2018). Regardless of these limitations, the SOD1(G93A) mice still prove to be the most suitable model of ALS study.

Another popular SOD1 model is found in zebrafish. This model shows evidence of adult-onset, LMN loss, progressive reduction in motor function, reduced survival, and exhibits similarities to many aspects of the disease in patients (Morrice et al., 2018). This model also presents some advantages over the transgenic mice mSOD1 model because it relies on only a slight overexpression of the mutant SOD1. Furthermore, the expression of wild-type SOD1 in zebrafish does not produce the motor neuron defects which is observed in mice (Lemmens et al., 2007). The major disadvantage of this model is that it does not show evidence of muscle denervation and cannot be used as an appropriate model for muscle dysfunction. Also, similar to the SOD1(G93A) mice model, the zebrafish cannot be used to model UMN defect (Joyce et al., 2011).

2.1.2 TARDBP

Many TARDBP mouse models have been developed, some with causal mutations while others overexpress the wild-type TARDBP also known to present ALS phenotype. The TDP-43 mice model displays symptoms such as the TDP-43 proteinopathy, gliosis, metabolic deficiencies and motor neuron degeneration, and signs of FTD (Stephenson & Amor, 2017). In theory, TDP-43 mutation models have the best chance of adequately representing the ALS pathology. However, developing the best TDP-43 mice model has come with many challenges (Morrice et al., 2018). Several attempts are still being made to develop the best model for the TDP-43 pathology. In 2018, White et al used CRISPR/Cas9 to create a new type of TDP-43 mouse model which eliminated the common problem of overexpression of TDP-43, making it a good genotypic model. Unfortunately, it does not present the typical TDP-43 pathology or enough phenotypic characteristics associated with the disease (White et al., 2018). Another mice model that overcomes the problem of overexpression of the mutant TDP-43 is the TDP43-Q331K mice model (Arnold et al., 2013). This model displays many ALS-like symptoms such as muscle atrophy, progressive motor dysfunction, degeneration of motor neurons and decreased NMJ integrity. The disadvantage of this model is that it does not experience UMN phenotype, has a limited period of progressive degeneration lasting only 20 months and the ALS features which are produced are mild and do not cause the death of the mice. Additionally, no TDP-43 cytosolic aggregation is produced in this model

(Arnold et al., 2013; Lutz, 2018). About 20 different TDP-43 mice models have been created (Lutz, 2018). Presently, The TDP-43(A315T) model is the most ideal TDP-43 mouse model due to its ability to elucidate pathological and phenotypic characteristics of the disease (Wegorzewska et al., 2009). However, this model overexpresses TDP-43 and presents gastrointestinal issues in the mice model, but a gel-based diet has been used to overcome this problem (Coughlan et al., 2016; Wegorzewska et al., 2009).

Several TDP-43 models also exist in zebrafish. For example, the TDP43-A315T zebrafish model exhibit defects in motor function, motor neuron axons as well as axonal branching. However, similar to the TDP43- Q331K mice model, the TDP43-A315T zebrafish does not present the TDP-43 cytosolic protein aggregations (Joyce et al., 2011; Laird et al., 2010).

2.1.3 FUS

FUS mutation accounts for about 4% of fALS and sALS cases (Nashabi et al., 2000). Many FUS mouse models have already been developed, either by knocking out the FUS gene or through the insertion of human wild-type FUS. Similar to the TDP-43 mice model, the FUS mouse model presents symptoms such as gliosis, motor neuron degeneration, muscle weakness, metabolic deficiencies as well as FUS proteinopathy (Stephenson & Amor, 2017). In neonatal mice, deletion of the FUS gene drastically reduced survival, and resulted in rapid death and chromosomal instability of the neonates (Hicks et al., 2000). However, the overexpression of the human wild-type FUS gene induced motor dysfunction and paralysis in 56 days and death in about 70 days after birth, making the wild-type FUS overexpressing model the best model of study (Mitchell et al., 2013).

2.1.4 C9orF72

Many C9orF72 mouse models have been created, however, most of these models do not present the necessary ALS phenotypes. Typical C9ORF72 mouse models of ALS would show signs of TDP-43 proteinopathy, motor neuron degeneration, sense and anti-sense foci, DPRs, gliosis, metabolic deficits, muscle weakness and signs of FTD (Stephenson & Amor, 2017). Studies carried out by Koppers et al showed that the knockout C9orf72 mice model did not exhibit any ALS features (Koppers

et al., 2015). Chew et al demonstrated that the transfection of ALS mice with mutated C9orF72 gene, using a viral vector produced some pathological features of ALS such as the motor deficit, TDP-43 inclusions, and intranuclear RNA foci (Chew et al., 2015). Other studies have also obtained similar results to Chew et al with the use of artificial chromosomes carrying the mutated C9orF72 gene (O'Rourke et al., 2015; Peters et al., 2015). Another recent murine model of C9orF72 developed similar ALS pathology. The mice model displays signs of anxiety, NMJ impairment, hippocampus neurodegeneration, paralysis, motor neuron loss, and a decreased survival rate (Liu et al., 2016). However, the $h(G_4C_2)_{37-500}$ model is still the most accepted model of study for the C9orF72 mutation as it exhibits all the expected phenotypes and pathology (Chew et al., 2015).

It is important to know that the C9orf72 knockdown zebrafish model is one of the few ALS models that supports the loss of function hypothesis in disease pathogenesis. In this model, a knockdown of the zebrafish homolog to the human C9orF72 gene produced a reduction in motor function. However, aggregation of TDP-43 in the cytoplasm was not evident in this model (Ciura et al., 2013).

2.1.5   Cerebrospinal Fluid (CSF)

In recent years, animal models based on the cerebrospinal fluid of ALS patients have been developed as a promising model of study for the disease. CSF obtained for ALS patients are infused into mice models via intracerebroventricular and intrathecal routes. These animal models with CSF infusion develop transferrin and TDP-43 proteinopathy in the cytoplasm, decreased motor performance, cystatin C overexpression, oxidative stress, mitochondrial dysfunction and neuronal apoptosis (Gomez-Pinedo et al., 2018; Sankaranarayani et al., 2014; Sharma et al., 2016).

2.2   In vitro Models of ALS

In vitro experiments using cell cultures models have also been developed to better understand the pathogenic mechanisms associated with the development of ALS. Primary neuronal cells from ALS rodents and motor neurons derived from human cells are the most widely used cell cultures (Veyrat-Durebex et al., 2014). Primary neuronal cells are mostly obtained from the cortex or hippocampus of embryos of

ALS mice between E14-E17 (Boutahar et al., 2011; Caioli et al., 2011). These cell cultures are easy to isolate and are harvested in a high population number of cells. The primary culture of motor neurons is obtained from the spinal cord of embryos obtained from the ALS mice model (Camu & Henderson, 1992). Common neural cell lines used in the study of ALS are the Neuro-2a, SH-SY5Y and NSC-34 (Cashman et al., 1992; Olmsted et al., 1970). Induced pluripotent stem cells (iPSCs) and human embryonic stem cells (hESC) are also used to study both fALS and sALS. These cells provide promising models of study as well as therapeutic interventions because of their ability to be differentiated to motor neurons (Cashman et al., 1992). Cell culture models are often used to study the common genetic mutations already linked to ALS. For example, motor neuron cultures associated with SOD1 expression display pathogenic mechanisms such as Ca^{2+} dysregulation associated with excitotoxicity, presence of reactive oxygen species (ROS), mitochondrial dysfunction, proteinopathies, axonal transport dysfunction, gliosis, and cell death (Chang & Martin, 2016; Tran et al., 2014). Similarly, spinal motor neuron cultures from ALS patients have demonstrated an increase of TARDBP mRNA in the cytoplasm and splicing of multiple alternative introns in TARDBP mRNA induced by TDP-43 (Koyama et al., 2016). Vascular endothelial growth factor (VEGF) has also been used as a therapeutic intervention to mitigate the cytoplasmic aggregation of TDP-43 and FUS using NSC-34 cells (Shantanu et al., 2017). Furthermore, human spinal motor neurons, neuronal cell lines, and primary cortical neurons were used to study the C9orF72 gene and the role it plays in endosomal trafficking. It was observed that C9orF72 colocalizes the Rab1, Rab5, Rab7 and Rab11 proteins that have been linked to autophagy and endocytic transport (Farg et al., 2014).

Despite the promising nature of cell lines as a model of study for ALS, it is important to note that they still present major disadvantages. In vitro analyses are done in a highly controlled environment and as such, cell lines are not exposed to the internal environmental conditions of an ALS patient or animal model. Such limitations must be considered when choosing cell lines as a model of study and when concluding on the results obtained from in vitro studies (Gois et al., 2020).

2.3   Environmental Models of ALS

2.3.1   Bisphenol A (BPA) exposure in zebrafish

Bisphenol A (BPA) is a toxic chemical primarily found in polycarbonate plastics and epoxy resins. Polycarbonate plastics are used in the packaging of food, water and carbonated drinks. They can also be found in compact discs and some medical devices. Epoxy resins are used to coat some metal products such as food and drink cans, bottle tops, and water supply pipes. Some dental sealants and composites may also contribute to BPA exposure (Nowicki et al., 2016). Humans are constantly exposed to this chemical in manufacturing companies, but mostly in our diet as BPA tends to combine with our foods and drinks when they are stored in these plastic materials for too long. In 2003/2004, the Centres for Disease Control and Prevention (CDC) conducted the National Health and Nutrition Examination Survey (NHANES III) and found high levels of BPA in 93% of 2,517 urine samples that were examined from people six years and older (NIEHS., 2021) Recent studies have linked BPA exposure to motor neuron degeneration (Morrice et al., 2018). Based on this finding, Morrice et al developed a novel model of study by environmentally inducing motor neuron degeneration in zebrafish after exposure to BPA. This motor neuron degeneration was associated with increased microglia activity and ultimately cell death similar to that observed in ALS. Unfortunately, this model presents several limitations, one of which is that exposure to BPA does not cause selective motor neuron death, but also causes the death of other types of neurons. Furthermore, other features of ALS such as TDP-43 cytoplasmic aggregation are yet to be examined in this model (Morrice et al., 2018). While BPA exposure has not been linked to the development of ALS, this model may be a useful way to study specific features of the disease, and possibly provide more insight into sALS, which are believed to be caused by a combination of environmental factors.

2.3.2   Amino beta-N-methylamino-L-alanine (BMAA)

The High prevalence of neurodegenerative diseases such as Parkinson's, Alzheimer's and ALS on the island of Guam has been a major point of study for decades, resulting in the terminology "Guam disease" (Steele, 2005). The development of these neurodegenerative diseases was later linked to the consumption of cycads seeds with the neurotoxin amino beta-N-methylamino-L-

alanine (BMAA), by individuals living on the island of Guam (Gois et al., 2020). Other studies also reported that the consumption of bats that had previously fed on the toxic cycads seed contributed to the development of the disease (Holtcamp, 2012). Animal models of ALS have been created by injecting pregnant mice (gestational day 14) and new-born (post-natal day 10) with BMAA. This treatment reproduced similar features of ALS and the animal models displayed motor deficits, motor neuron degeneration, protein aggregation and gliosis (Karlsson et al., 2009; Yin et al., 2014), making this a promising model of study for ALS and possibly other neurodegenerative diseases.

2.3.3   b-Sitosterol-b-d-glucoside (BSSG) exposure in mice

b-Sitosterol-b-d-glucoside (BSSG) is another toxic compound that has been found in cycad seed and has been linked to ALS epidemiology (Wilson et al., 2002). Based on this, an environmental mice model was created by feeding animals with cycad flour, or the synthesized form of BSSG. These animals showed signs of progressive motor dysfunction, motor neuron degeneration, astrocytosis and microgliosis, reduced NMJ integrity, and ultimately neuronal cell death (Tabata et al., 2008; Wilson et al., 2002). Surprisingly, similar exposure to BSSG in rats resulted in progressive motor neuron degeneration in the substantia nigra pars compacta (Snpc) and was associated with cognitive deficits (van Kampen et al., 2015). This suggests that BSSG exposure may prove to be a valuable environmental model of study for Parkinson's disease in rats, as it is for ALS in mice models.

3. SEX DIMORPHISM IN ALS

Several animal models of ALS have been created and studied to determine which best elucidates both the genotypic and phenotypic features of the disease. However, there is still a lot of controversy about the role which the sex and genetic background of these animal models, play in how experiments are conducted, and how results are interpreted. In general, it has been reported that male patients are at higher risk of developing the disease than female patients, with a male-to-female ratio of 1:2 (Jun et al., 2019; Palese et al., 2019). Some studies have also reported a male-to-female ratio of 1:3 (Manjaly et al., 2010), but these variances depend on the geographical region. It is still unclear what predisposition factors determine this higher prevalence rate reported in males. The importance to therefore take into consideration the sex differences involved in both clinical ALS patients and animal models of the disease cannot be overemphasized.

3.1 Dimorphism in ALS Patients

It is widely known that men have a higher risk of developing ALS than women. However, the age at disease onset as well as the rate of progression of ALS is highly variable in male and female patients. Several studies have reported that male patients develop an earlier onset of the disease compared to females. In Ireland, the mean age at onset is said to be 64.2 years of while females have a mean onset of 67.8 years (Traynor et al., 1999). In southeast England, men have a disease onset at the mean age of 58.9 years, but women experience later onset at about 63.0 years of age (Manjaly et al., 2010). Japan has recorded the mean age at disease onset in male patients to be 64.6 years while in the females it is 66.4 years old (Atsuta et al., 2009). Similarly, in Italy, the average age at onset in male patients is 64.4 years old, and 65.3 years old in female patients (Chio et al., 2009). Similar reports of earlier disease onset in men have also been reported in France (Marin et al., 2009), Sweden (Fang et al., 2009) and India (Nalini et al., 2008). Furthermore, studies have reported that the molecular onset of ALS begins in the lumbar spinal cord of male patients, while in females, it begins in the bulbar region (Blasco et al., 2012). The type of phenotypic features presented in the disease also varies between male and female patients. An example of this is the flail arm syndrome (FAS), which is an atypical symptom of ALS characterized by progressive and predominantly

proximal upper limbs weakness, without any dysfunction observed in the lower limbs, respiratory muscles and bulbar (Yoon et al., 2014). Although FAS is relatively rare, it is 4 to 9 times more common in male patients than in females(Czaplinski et al., 2004; Hu et al., 1998; Wijesekera et al., 2009). Studies have identified the mutant SOD1 as a possible biological marker for these observed sex differences. SOD1 concentration is said to be dysregulated in the cerebrospinal fluid (CSF) and is generally higher in male than female ALS patients. However, this difference was not observed in patients presenting other forms of genetic mutations associated with ALS, suggesting a specific dysregulation of SOD1 metabolism in male and female patients (Frutiger et al., 2008). Sex also plays an important role in the C9orF72-linked ALS. Studies have reported that C9orF72 expansion reduces the rate of survival in men but has little impact in women (Rooney et al., 2017), implicating a possible role of adverse androgen receptor (AR) action in male patients (Trojsi et al., 2020).

There are several biomarkers for ALS that have been studied to find the best diagnostic measure for the disease. Studies have shown that some of these biomarkers are expressed differently depending on the sex of the ALS patient. Phosphorylated axonal neurofilament H is a potential blood biomarker for ALS (Boylan et al., 2009). It is produced from axons in neurodegenerative diseases and is used to determine the rate of axonal breakdown (McCombe & Henderson, 2010). Studies showed that the mean concentration of this marker was higher in male ALS patients compared to females (Boylan et al., 2009). Other biomarkers of ALS include the vascular endothelial growth factor (VEGF)-β (Gomes-Trolin et al., 2002), monocyte chemotactic protein (MCP)-175 (Turner et al., 2010), transforming growth factor (TGF)-β (Houi et al., 2002), and human neutrophil elastase (HNE) (Kuhle et al., 2009). These markers are key indicators of oxidative stress, making them potential biomarkers of the pathologic processes that lead to neuron degeneration in ALS (Turner et al., 2010). The mean value of VEGF-β was examined in ALS patients and was found to be slightly higher in females (439 ng/L) compared to males (332 ng/L), which could be an indicator of a higher inflammatory response in women than men (Gomes-Trolin et al., 2002). However, studies on (MCP)-175, (TGF)-β, and HNE did not report any influences of sex

differences on the expression of these biomarkers (Houi et al., 2002; Kuhle et al., 2009).

In general, the type of ALS (either sALS or fALS) is rarely impacted by sex. However, few cases of fALS have been reported where sex plays a role in modifying the disease. A Phe20Cys mutation in the SOD1 (Kim et al., 2007)gene which had autosomal dominant inheritance was reported to be present in all members of a Korean family, however, it had low penetrance in the females . This suggests that the clinical course of the disease within a family can vary depending on the sex (McCombe & Henderson, 2010). Studies have also shown that ALS females have a higher risk of developing impaired executive function, indicating that females are more vulnerable to cognitive dysfunction compared to male patients (Palmieri et al., 2015). Several studies have implicated sex hormones in the onset and progression of ALS. Estrogen has been shown to play a protective role against some ALS mechanisms, while androgens might be involved in the earlier disease onset and higher predisposition to the disease observed in male patients (Miller et al., 1998). De Jong et al demonstrated a link between a longer reproductive cycle, susceptibility to ALS, and the rate of survival in female patients. This study showed that longer exposure to estrogen results in neuroprotective effects on motor neurons in ALS patients (de Jong et al., 2013).

3.2 Dimorphism in ALS Models

Sex differences have also been implicated in the onset and course of ALS animal models. The SOD1(G93A) is the most widely used model of study for ALS. In this model, male animals are known to develop an earlier disease onset compared to females, both in rats (Suzuki et al., 2007) and mice (Veldink et al., 2003). Furthermore, the genetic background of the SOD1(G93A) mice is significant in determining the effect of sex on the phenotypic features presented by the mice model. One study showed that SOD1(G93A) mice with C57BL/6 background exhibited a delay in clinical onset of symptoms, an increase in survival, and an overall prolonged disease duration compared to their sex-matched SOD1(G93A) counterparts with B6SJL background (Pfohl et al., 2015). It was also observed that the female SOD1(G93A) mice with B6SJL background generally experience extended survival and delayed disease onset compared to their male counterparts,

while female SOD1(G93A) mice with the C57BL/6 background show a delay in disease onset but no significant difference in the survival time compared to their male counterparts (Pfohl et al., 2015). However, sex did not have any noticeable impact on the loss of muscle force in the SOD1(G93A) mice model (McCombe & Henderson, 2010). The chromogranin B (CHGB) gene has also been linked to sex-dependent differences in ALS mice models (Gros-Louis et al., 2009; Ohta et al., 2016). Chromogranin is a vital component of secretory vesicles that acts as a chaperone by binding to mutant SOD1 protein and promoting their secretions from neuronal cells. Studies have demonstrated that the co-expression of CHGB[L413] allelic variant in SOD1(G37R) mice lead to pathological changes associated with earlier disease onset in female mice (Ohta et al., 2016). This may be due to the presence of an SRY silencer element of the CHGB promoter, which results in higher expression of CHGB by neuronal cells in female mice compared to males (Vegeto et al., 2020). Similar to clinical ALS patients, estrogen has also been linked to a neuroprotective effect in SOD1(G93A) female mice compared to males, as estrogen therapy was shown to improve motor function and extend survival in SOD1(G93A) mice (Choi et al., 2008). Estrogen is not only found in females but is also present in males although to a lesser degree. In both male and female mice, cytoplasmic aromatase, and nuclear estrogen receptors (ERs; ERα and ERβ) both colocalize with SMI-32 and GPR30, which are motoneuron specific marker in the ventral horn of the lumbar spinal tract of adult mice (Ji et al., 2017). Based on this, it is believed that phytoestrogens and estradiol may directly have neuroprotective effects on spinal cord motoneurons (Trieu & Uckun, 1999). Another study showed that exposing SOD1(G93A) mice to increased environmental activity accelerated disease progression in female mice, but no difference was observed in males (Stam et al., 2008).

4.   ROLE OF EPIGENETICS AND TRANSCRIPTIONAL ACTIVITY IN ALS

As described before, ALS is a multifactorial disease, and the exact cause of MN degeneration is still unknown. In recent years, epigenetic factors such as transcription dysregulation have been implicated in the pathology of several neurodegenerative diseases, including ALS (Bennett & La Spada, 2018). Epigenetics generally refers to the study of heritable phenotypic changes that can occur without any change to the DNA sequence (Dupont et al., 2009). Epigenetic mechanisms influence gene expression by activating or deactivating specific parts of the genome. These mechanisms include DNA methylation, post-translational histone modification and chromatin remodeling (Ptashne, 2007; Zhang et al., 2019). Studies have suggested that these mechanisms could play a unique role in the expression of phenotypic traits observed in ALS (Wong et al., 2013). Covalent modifications that include alterations to histone proteins (acetylation of lysine, serine and threonine phosphorylation, lysine and arginine methylation), as well as DNA methylation are often related to each other. They act through enzyme-catalyzed reactions that involve the addition or removal of chemical compounds from DNA and histone proteins, leading to the activation or suppression of the target gene (Lo & Weksberg, 2014).

4.1   DNA Methylation

DNA methyltransferase (Dnmt) is the main enzyme involved in DNA methylation. It is a biological process in eukaryotic cells that involves the addition of a methyl group to the DNA molecule. This results in an alteration of the activity of the targeted DNA segment without any modification to the DNA sequence (Jones, 2012). Of the four nucleotide bases that exist, two, adenine and cytosine, are involved in DNA methylation. The addition of a methyl group to these bases results in the formation of N^6-methyladenine, 5-methylcytosine and N^4-methylcytosine (Dunn & Smith, 1958; Vanyushin et al., 1970). These changes can affect DNA-protein interactions, causing alterations in chromatin structure and the rate of gene transcription (Jones, 2012). Studies have shown that the activities of Dnmts that lead to either hyper or hypomethylation of DNA can cause MN degeneration in ALS. High levels of Dnmt1 and Dnmt3 enzymes have been observed in the brain

and spinal cord MN of mice and ALS patients. Inhibition of Dnmt enzymatic activity with the use of a small inhibitory molecule, RG108, and procainamide prevented motor neuron degeneration caused by excessive DNA methylation in cell-cultured NSC34 cells and a mouse model of ALS(Wong et al., 2013).

4.2 Histone Acetylation

The acetylation of histone proteins is another epigenetic mechanism that regulates gene expression. These proteins are found in the nucleosome and associated with DNA which wraps around the protein to form the chromatin structure (Ma et al., 2015). There arrange themselves into octamers, composed of two copies of the four histone types H2A, H2B, H3, and H4 (Lazo-Gomez et al., 2013). Chromatin can basically exist in two states. In the euchromatin state, the chromatin unwinds and becomes uncondensed in a transcriptionally active state. This allows easy access for external transcriptional factors to reach the promoter region of the DNA and initiate transcription. The second stage is the heterochromatin state which is an inactive condensed state. Here, the DNA is inaccessible, and the gene is inactive. This is due to negatively charged DNA being closely packed with the positively charged histone proteins, thereby inducing a closed conformation of the nucleosome and preventing the binding of external transcriptional factors (Berger, 2007; Cuvier & Fierz, 2017). Histone proteins can undergo several post-translational modifications (PTM) necessary for cellular homeostases, such as acetylation, phosphorylation, methylation, among others. Histone acetylation is the most studied PTM because dysregulation in the acetylation state of histone homeostasis has been associated with MN degeneration in several neurodegenerative diseases including ALS (Lazo-Gomez et al., 2013). Acetylation in histone is regulated by two enzymes: histone acetyltransferase (HATs), which catalyze the acetylation of histone proteins, and the histone deacetylases (HDACs) which are responsible for histone deacetylation (de Ruijter et al., 2003; Kazantsev & Thompson, 2008). There are eighteen HDACs which are divided into four groups depending on their structural composition, localization, and function: class I (HDAC 1-3, 8) class II (HDAC 4-7,9,10), class III (SIRT 1-7) and class IV (HDAC 11). These enzymes interact with the lysine residue present at the N-terminal tail of the histone to catalyze either the acetylation or deacetylation of the protein.

Increased acetylation state of histone improves transcriptional activity. This is due to the neutralization of the positive charge of the histone protein by the acetyl group, causing an unwinding and relaxation of the chromatin structure. This makes the promoter region of the DNA more accessible to transcriptional factors. Contrary to this, a decrease in the acetylation state of histone causes suppression of transcriptional activities. Positive charges of the poorly acetylated histones interact with the negatively charged phosphate group of the DNA, preventing the unwinding of the chromatin structure (Cuvier & Fierz, 2017; Lazo-Gomez et al., 2013). Studies have shown that pharmacological inhibition of HDAC6 induces cell death in primary motor neurons (Boutillier et al., 2003). Furthermore, loss of (cAMP)-response element-binding protein (CPB)/p300 in neuronal cell cultures induced neurodegeneration (Rouaux et al., 2003). Another study showed that inducing oxidative stress through the administration of H_2O_2 to NSC34 cell culture reduced the expression of HAT CPB/p300 and reduced the acetylation state of H3 (Rouaux et al., 2007), while another study demonstrated that the genetic knockdown of HDAC6 in SOD1(G93A) mice had protective effects (Taes et al., 2013). These studies support the idea that an imbalance of HATs and HDACs enzymatic activity, dysregulates cellular homeostasis and triggers cell apoptosis in neurodegenerative diseases.

4.3 Neuroinflammation Caused by Nuclear Transcription Factor, NF-κB

As described earlier, HATs and HDACs are responsible for the acetylation and deacetylation of histone proteins respectively. However, these enzymes are also involved in the modification of other non-histone proteins such as the *nuclear factor kappa-light-chain-enhancer of activated B cells* (NF-κB) (Spange et al., 2009). NF-κB is a transcriptional factor that is made up of two of the five DNA binding proteins (p50, p52, p65 RelA, c-Rel, RelB). NF-κB is composed of the p50/RelA subunits and plays a critical role in the transcriptional regulation of key neuronal genes, proliferation and differentiation of cells, and monitoring the physiological and pathological mechanism involved in the progression of neurodegenerative (Lanzillotta et al., 2015). It is also thought to be the major regulator of inflammation and immunity activities. NF-κB is activated when various stimuli such as DNA damage, activate IkB kinases (IKKs) which cause phosphorylation of IkB inhibitory

protein (IkBα). This leads to the production of NF-κB which is transported into the nucleus and binds to the DNA, promoting the transcription of anti-apoptotic genes, such as Bcl-xL protein, and Bcl-2 family members (Chen et al., 2000; Lu et al., 2015). RelA/p65 acetylation and phosphorylation play important role in the regulation of NF-κB, and the transcriptional action of NF-κB is mediated by nuclear PTM (Chen & Chen, 2015). The acetylation state of p50/RelA dimer is catalysed by HATs and HDACs. The addition of an acetyl group (mediated by HATs) or removal (mediated by HDACs) from the lysine residues of the histone protein can induce either neuroprotective or neurotoxic effects. Studies have associated the acetylation of RelA at the level of lysine 310 to an improvement in the transcriptional activity of NF-kB, its ability to regulate inflammatory response. However, deacetylation of lysine 310 by HDAC3 promotes binding to IκBα and inhibits the transcriptional activity of RelA, causing cell apoptosis in response to inflammatory stimuli (Chen & Chen, 2015). In brain ischemia, studies have demonstrated that RelA elicits aberrant acetylation of its lysine residues, associated with overall deacetylation of the lysine except lysine 310 in murine models, inducing the transcription of the pro-apoptotic factor known as Bim (Lanzillotta et al., 2013). Schiaffino et al also made similar observations in the global deacetylation state of the lysine residue of RelA excluding the specific lysine 310 but in ALS mice model SOD1(G93A) (Schiaffino et al., 2018). Both studies used a combination of two epigenetic drugs (MS-275 and Resveratrol) to restore the normal acetylation state of RelA and increase the transcription of the anti-apoptotic gene (Bcl-xL) which promoted a neuroprotective effect in both brain ischemia and ALS mice models (Lanzillotta et al., 2013; Schiaffino et al., 2018). Furthermore, studies have linked an upregulation of NF-κB to ALS in vitro (Ikiz et al., 2015) and in vivo (Apolloni et al., 2014) models of the disease, as well as in ALS patients (Jiang et al., 2005), suggesting a possible correlation between RelA subunit and the degeneration of MNs.
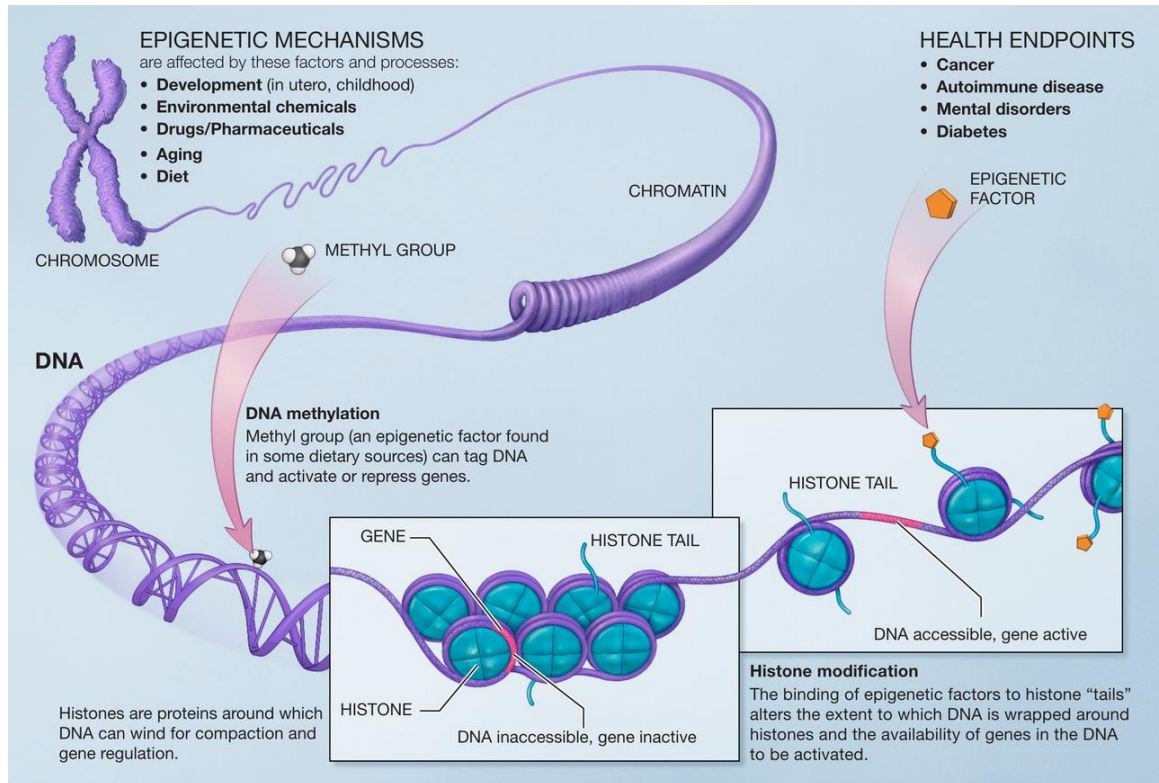
Figure 2. The diagram shows the processes involved in different epigenetic mechanisms (http://commonfund.nih.gov/epigenomics/figure.aspx).

5.   THERAPEUTIC INTERVENTIONS IN ALS

As has been stated earlier, many factors have been implicated in the pathogenic mechanism of ALS. As a result of this, several therapeutic approaches have been made to target these different pathogenic mechanisms. Riluzole, and more recently, Edaravone, are the only drugs approved by the Food and Drug Administration in the management of ALS (Bensimon et al., 1994; Bhandari et al., 2018). But even these drugs still have their limitations and a possible cure for ALS is yet to be discovered. Based on this, the need to find promising therapies for the treatment of this neurodegenerative disease cannot be overemphasized. Below are several therapeutic interventions that are being explored every day in the race to find the most suitable approach to managing ALS.

5.1   HDACs Inhibitors.

In recent years, transcriptional dysregulation has been implicated in the development and progression of ALS (Bennett & La Spada, 2018). HDACs enzymes, which are involved in transcriptional activities through the catalyzation of histone deacetylation, are very likely the most widely studied transcriptional enzyme involved in the course of ALS. Studies have demonstrated hypoacetylation of histone in SOD1(G86R) (Rouaux et al., 2007) and SOD1(G93A) (Schiaffino et al., 2018) mice models of ALS. Furthermore, HDAC2 and HDAC11 mRNA expression levels are altered on the post-mortem spinal cord and brain tissue of ALS patients (Janssen et al., 2010). Based on these finding, several drugs are known as HDACs inhibitors (HDACi) have been developed that act by inhibiting the activities of HDACs enzymes, with the hope of restoring the normal acetylation state of histone proteins in ALS (Table 1).

Table1. Effects of HDACi in pre-clinical and clinical trials of ALS

HDAC inhibitors	Model	Target	Pre-clinical outcome	Reference	Clinical Outcome	Reference
MC1568	SOD1(G93A)	HDAC class II	Preserved motor neuron, improved motor performance, restored glutamate	(Buonvicino et al., 2018), (Lapucci et al., 2017)	-	-

			upregulation in the spinal cord.			
RGFP966	FUS(R521H)	HDAC3	Preserved nuclear mutant FUS	(Kuta et al., 2020)	-	-
Trichostatin A	SOD1(G93A)	Pan-HDAC	Improved life span and delayed disease progression	(Yoo & Ko, 2011)	-	-
Tubastatin A	FUS(P525L)	HDAC6	Improved α-tubulin acetylation, restored axonal transport function	(W. Guo et al., 2017)	-	-
Valproate	SOD1(G93A)	PAN-HDAC	Reduced motor neuron death, no improvement in survival	(Crochemore et al., 2009)	No difference compared to placebo (phase II trial)	(Piepers et al., 2009)
Resveratrol	SOD1(G93A)	Sirt1 activator	Improved motor function and survival	(Lee et al., 2012; Mancuso et al., 2014)	-	-
Sodium phenylbutyrate	SOD1(G93A)	Pan-HDAC (classes I and II)	Increase in lifespan	(S. J. Del Signore et al., 2009; Ryu et al., 2005)	No effect was observed (phase II trial)	(Cudkowicz et al., 2009)

5.2 Mitochondria Protectants

It has been previously established that mitochondrial dysfunction plays a significant role in ALS pathology. Previous studies have reported alterations to mitochondrial morphology and activity both in the SOD1(G93A) mice model, and in post-mortem tissue of ALS patients (Pollari et al., 2014). Table 2 shows the several drugs which target mitochondrial dysfunction and are currently being examined.

Table 2. Effects of mitochondrial protectants in pre-clinical and clinical trials of ALS

Drug	Model	Pre-clinical outcome	Reference	Clinical outcome	Reference
Dexpramipexole	SOD1(G93A)	Reduced motor neuron degeneration	(Gribkoff & Bozik, 2008)	No improvement was observed	(Bozik et al., 2011; Cudkowicz et al., 2011)
Creatine	SOD1(G93A)	Improved motor function and survival	(Klivenyi et al., 1999)	No improvement was observed	(Groeneveld et al., 2003; Rosenfeld et al., 2008)
Olesoxime	SOD1(G93A)	Delayed onset, preserved motor neurons, increased survival	(Sunyach et al., 2012)	No improvement was observed	(Lenglet et al., 2014)
Cyclosporine	SOD1(G93A)	Increased life span and protected motor neurons	(Karlsson et al., 2004)	No improvement was observed	(Appel et al., 1988)
Nortriptyline	SOD1(G93A)	Delayed onset and increased survival	(Wang et al., 2007)	-	-
P7C3A20	SOD1(G93A)	Protects motor neurons, improves motor function	(Tesla et al., 2012)	-	-

5.3   Anti-excitatory Drugs

Excitotoxicity is another pathogenic mechanism that has well been documented in murine models and patients with ALS. Riluzole acts as an anti-excitatory drug and is currently one of the only two drugs that have been approved for the management of ALS. The mechanism of action of riluzole involves the inhibition of glutamatergic transportation which causes a reduction in glutamate concentration, thereby reducing excitotoxicity and protecting MNs from death (Cheah et al., 2010). In clinical trials, riluzole significantly slowed down the rate of disease progression and improved survival in ALS patients (Bensimon et al., 1994; Lacomblez et al., 1996). However, no improvement was observed in the phenotypic features such as tremor and debilitating motor function typical of ALS pathology (Borrás-Blasco et al., 1998). Due to the positive but yet minimal effect of riluzole in the management of ALS, several anti-excitatory drugs are still being tested with the hope of a better outcome for ALS therapy (Table 3).

Table 3. Effects of anti-excitatory drugs in pre-clinical and clinical trials of ALS

Drug	Model	Pre-clinical outcome	Reference	Clinical outcome	Reference
Riluzole	SOD1(G93A)	Improves motor function and survival	(Gurney et al., 1996)	Improves survival, no effect on motor function	(Bensimon et al., 1994; Lacomblez et al., 1996)
L-Arginine	SOD1(G93A)	Improves motor function and survival	(Lee et al., 2009)	-	-
Ceftriaxone	SOD1(G93A)	Improves motor function and survival	(Rothstein et al., 2005)	No improvement was observed	(Berry et al., 2013; Cudkowicz et al., 2014; Zhao et al., 2014)

Drug	Model	Pre-clinical outcome	Reference	Clinical outcome	Reference
Gabapentine	SOD1(G93A)	Increased survival	(Gurney et al., 1996)	No improvement was observed	(Miller et al., 2001)
Gacyclidine	SOD1(G93A)	Increased survival and motor function	(Gerber et al., 2013)	-	-
Memantine	SOD1(G93A)	Increased survival and motor function	(Joo et al., 2007; Wang & Zhang, 2005)	No improvement was observed	(de Carvalho et al., 2010)
Vitamin D	SOD1(G93A)	Improves motor function	(Gianforcaro et al., 2013)	No improvement was observed	(Blasco et al., 2015; Karam et al., 2013)
N-Acetylated α-linked acidic dipeptidase	SOD1(G93A)	Improves motor function and survival	(Ghadge et al., 2003)	-	-

5.4 Pro-autophagy Drugs

Autophagy dysfunction has recently been implicated in the pathogenesis of neurodegenerative diseases, including ALS. Based on this, several studies have investigated autophagy as a possible therapeutic target, using autophagy-modulating molecules, in the treatment of ALS (Corti et al., 2020).

Table 4. Effects of pro-autophagy drugs in pre-clinical and clinical trials of ALS

Drug	Model	Pre-clinical outcome	Reference	Clinical outcome	Reference
Spermidine	FTLD-U mice	Increased autophagy, increased motor neuron, improved motor function	(I. F. Wang et al., 2012)	-	-

Rapamycin	FTLD-U mice	Increased autophagy, improved motor function	(I. F. Wang et al., 2012; Zhang et al., 2011)	-	-
Metformin	SOD1(G93A)	Increased motor neuron	(Kaneb et al., 2011)	-	-
Trehalose	SOD1(G93A)	Sex dimorphism was observed. Male: Increased autophagy, increased motor neuron, increased survival, Female: Increased autophagy, increased motor function, no improvement in survival	(Y. Li et al., 2015; Zhang et al., 2014)	-	-
Tamoxifen	FTLD-U	Increased autophagy, increased motor neuron, improved motor function	(I. F. Wang et al., 2012)	Slowed disease progression minimally	(Chen et al., 2020)
Lithium	SOD1(G93A)	Increased motor neuron, improved motor function, prolonged survival	(Shin et al., 2007)	No improvement observed	(Chio et al., 2010; Miller et al., 2011; Verstraete et al., 2012)
Valproate	SOD1(G93A)	Improved survival	(Sugai et al., 2004)	No improvement was observed	(Piepers et al., 2009)
Resveratrol	SOD1(G93A)	Sex dimorphism was observed. Male: increased motor function, increased survival	(Han et al., 2012; Markert et al., 2010)	-	-

		Female: No improvement was observed			

5.5   Anti-oxidative Drugs

Inhibition of ROS accumulation prevents MN degeneration due to oxidative stress. Studies have shown that the administration of Bromocriptine in cultured rat MNs rescued the cells from neuronal death by inhibiting oxidative damage (Iwasaki et al., 1997). In SOD1 transgenic mice Bromocriptine delayed the motor function impairment and increased lifespan (Tanaka et al., 2011). Resveratrol reduces oxidative stress by activating sirt1 and AMPK in the cells (Han et al., 2015). Edaravone is one of the only two drugs that has been approved for the treatment of ALS (Bhandari et al., 2018). Edaravone has been previously used in the treatment of acute stroke. In 2017, a Japanese team demonstrated that edaravone, a free radical eliminator, slowed the progression of ALS in patients who were still in the early stage of the disease, and in 2018 this drug was approved for clinical use in Canada (Breiner et al., 2020).

Table 5. Effects of anti-oxidative drugs in pre-clinical and clinical trials of ALS

Drug	Model	Pre-clinical outcome	References	Clinical outcome	References
Bromocriptine	SOD1(G93A)	Delayed onset and improved survival	(Tanaka et al., 2011)	-	-
M30-HLA20	SOD1 (G93A)	Protected motor neuron, delayed onset, increased survival	(Wang et al., 2011)	-	-

AEOL-10150	SOD1(G93A)	Increased survival	(Crow et al., 2005)	-	-
Coenzyme Q	SOD1(G93A)	Delayed onset, increased survival	(Matthews et al., 1998)	No improvement was observed	(Ferrante et al., 2005; Kaufmann et al., 2009)
Hydrogen-rich Saline	SOD1(G93A)	Delayed onset, improved survival, increased motor neuron	(Zhang et al., 2016)	-	-
EGCG	SOD1(G93A)	Delayed onset, improved survival	(Koh et al., 2006)	-	-
DP-109 and DP-460	SOD1(G93A)	Delayed onset, improved survival, increased motor neuron	(Petri et al., 2007)	-	-
FeTCPP	SOD1(G93A)	Delayed onset, improved survival, increased motor neuron	(Wu et al., 2003)	-	-
N-Acetylcysteine	SOD1(G93A)	Delayed onset, improved survival, increased	(Andreassen et al., 2000)	No improvement was observed	(Louwerse et al., 1995; Vyth et al., 1996)

		motor neuron			
Resveratrol	SOD1(G93A)	Delayed onset, improved survival, increased motor neuron	(Mancuso et al., 2014; Song et al., 2014)	-	-
Rasagiline	SOD1(G93A)	Improved survival, improved motor function	(Waibel et al., 2004)	No improvement was observed	(Macchi et al., 2015)
Vitamin E	SOD1(G93A)	Delayed onset, improved survival	(Gurney et al., 1996)	No improvement was observed	(Desnuelle et al., 2001)

5.6 Anti-inflammatory Drugs

Inflammatory responses caused by glial activities have been associated with the degeneration of MN in ALS. Several drugs that target the overactivation of microglia in the CNS of ALS patients have been tested (Boillee et al., 2006). Thalidomide is an anti-inflammatory drug that inhibits the release of TNF-α, a cytokine that causes inflammatory activation and triggers MN neurodegenerative processes in ALS (Olmos & Llado, 2014). The administration of thalidomide in SOD1(G93A) mice caused a reduction in MN loss by inhibiting the production of TNF-α. This led to an improvement in motor function and increased survival (Kiaei et al., 2006). However, thalidomide did not show any improvement in the phase II clinal trial but rather had adverse effects (Stommel et al., 2009).

Minocycline is another drug that has been examined in the treatment of ALS (Kriz et al., 2002). Studies have shown that the drug slowed down the progression of the disease and improved survival of ALS murine models (Kriz et al., 2002; Van Den Bosch et al., 2002; Zhu et al., 2002). Unfortunately, no positive effects were

observed when used as a treatment in ALS patients (Gordon et al., 2004). Other anti-inflammatory drugs that have been used in the treatment of ALS can be found in table 6.

Table 6. Effects of anti-inflammatory drugs in pre-clinical and clinical trials of ALS

Drugs	Model	Pre-clinical outcome	Reference	Clinical outcome	Reference
Thalidomide	SOD1(G93A) mouse	Increased survival, Delayed, onset Improved motor function, increased motor neuron	(Kiaei et al., 2006)	No improvement was observed	(Stommel et al., 2009)
Glatiramer Acetate	SOD1(G93A) mouse and SOD1(G37R) mouse	Limited effects	(Habisch et al., 2007; Haenggeli et al., 2007)	No improvement was observed	(Meininger et al., 2009; Mosley et al., 2007)
AM-1241	SOD1(G93A) mouse	Increased survival	(Kim et al., 2006; Shoemaker et al., 2007)	-	-
Δ9-THC	SOD1(G93A) mouse	Increased survival	(Raman et al., 2004)	No improvement was observed	(Joerger et al., 2012; Weber et al., 2010)
Anakinra	SOD1(G93A) mouse	Increased survival	(Meissner et al., 2010)	No improvement was observed	(Maier et al., 2015)

| Minocycline | SOD1(G93A) mouse and SOD1(G37R) mouse | Increased survival, delayed onset, Improved motor function | (Kriz et al., 2002; Van Den Bosch et al., 2002; Zhu et al., 2002) | No improvement was observed | Gordon et al., 2004) |
| Celastrol | SOD1(G93A) mouse | Increased lifespan, protects motor neurons | (Kiaei et al., 2006) | - | - |

5.7   Anti-Apoptotic Drugs

Several anti-apoptotic drugs have been studied as possible therapies for the treatment of ALS, both in murine models and in ALS patients. A reduction in hematopoietic growth factor erythropoietin (EPO) has been observed in the CSF of ALS patients (Brettschneider et al., 2007). Therapeutic administration of EPO led to an inhibition of apoptosis in the motor neurons of in vitro ALS models (Naganska et al., 2010). In SOD1(G93A) mice, EPO resulted in the delay of disease onset, but no improvement was observed in survival (Grunfeld et al., 2007). However, treatment with recombinant human erythropoietin (rhEPO) did not have any positive effects in phase III clinical trials (Lauria et al., 2015). Other anti-apoptotic drugs that have been tested in the treatment of ALS include the following.

Table 7. Effects of anti-apoptotic drugs in a pre-clinical and clinical trial of ALS

Drug	Model	Pre-clinical outcome	Reference	Clinical outcome	Reference
EPO	SOD1(G93A) mouse	Delayed onset, protected motor neuron	(Grunfeld et al., 2007)	No improvement was observed	(Kim et al., 2014; Lauria et al., 2015)
Dasitinib	SOD1(G93A) mouse	Increases survival	(Katsumata et al., 2012)	-	-

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Drug	Model	Pre-clinical outcome	Reference	Clinical outcome	Reference
TCH346	SOD1(G93A) mouse PMN mouse	No effect was observed in SOD1(G93A), Increased survival, Improved motor function in PMN mouse	(Groeneveld et al., 2004; Sagot et al., 2000)	No improvement was observed	(Miller et al., 2007)
zVAD-fmk	SOD1(G93A) mouse	Increased survival, Improved motor function	(Li et al., 2000)	-	-

5.8 Protein Clearance Drugs

Accumulation of aggregated misfolded proteins such as SOD1 and FUS, in the cytoplasm of cells, caused MN degeneration. Several drugs that target the removal of protein aggregates from MN have been studied (Webster et al., 2017). Heat shock proteins play important roles in cell recycling. Arimoclomol is a drug that increases the expression of these heat shock proteins in the MN (Kalmar et al., 2014). Studies have observed that the administration of Arimoclomol in ALS mice improved motor function and increased survival of the animals (Kieran et al., 2004). Edaravone is the most recently approved drug for the treatment of ALS (Breiner et al., 2020). This drug can also be classified as a protein clearance drug as studies have shown that it decreases the aggregation of mutated SOD1 and slowed down disease progression in SOD1(G93A) mice (Ito et al., 2008). In clinical trials, the administration of the drug caused a reduction in plasma biomarkers of oxidative stress (Nagase et al., 2016).

Table 8. Effect of protein clearance drugs in pre-clinical and clinical trials of ALS

Drug	Model	Pre-clinical outcome	Reference	Clinical outcome	Reference

Arimoclomol	SOD1(G93A) mouse	Increased survival, Improves motor function	(Kieran et al., 2004)	No improvement was observed	https://clinicaltrials.gov/ct2/show/NCT03491462
Edaravone	SOD1(G93A) mouse	Improves motor function, Protects motor neurons	(Ito et al., 2008)	Reduce oxidative damage	(Nagase et al., 2016)
Pyrimethamine	SOD1(G93A) mouse	Increased survival, Delayed onset Improved motor function	(Lange et al., 2013; Lange et al., 2017)	-	-

5.9 Stem Cell Therapy

Stem cells have been investigated as a promising therapy in the treatment of several diseases, including ALS. The major aim of stem cell therapy in ALS is to help increase the MN population that has been lost during the progression of the disease (Goutman et al., 2019). They are unique in their ability because they can differentiate into other cell types. Embryonic stem cells (ESCs) are totipotent cells and can differentiate into any cell type. Pluripotent stem cells (PSCs) can differentiate into a specific type of cells, while neural progenitor cells (NPCs) are PSCs and have the ability to differentiate into neuronal or glial cells (Gage, 2000). Stem cells obtained from human umbilical cord blood (hUCB) resulted in a delay of disease onset and increased lifespan in SOD1(G93A) mice (Chen & Ende, 2000). However, in humans, bone marrow-derived mesenchymal stem cells (MSCs) treatment in a phase I/II clinical trial did not result in any improvement compared to the placebo group (Karussis et al., 2010).

Table 9. Effects of stem cell therapy in pre-clinical and clinical trials of ALS

Drug	Model	Pre-clinical outcome	Reference	Clinical outcome	Reference

Bone marrow-derived MSCs	Muscle deficient (MDF) mice	Improved motor function, no effect on survival	(Pastor et al., 2012)	No safety concerns, no improvement was observed	(Karussis et al., 2010)
Olfactory Ensheathing Cell (OECs)	SOD1(G93A)	Improved motor function, increased motor neuron count, increased lifespan	(Li et al., 2011)	Slowed down disease progression, no adverse effects	(Huang et al., 2008)
Neural Progenitor Cell (NPCs)	SOD1(G93A)	Increased motor neuron survival, decrease in survival, sex dimorphism were observed (female mice showed a rapid decline compared to males)	(Park et al., 2009)	Slowed down disease progression in patients without bulbar onset, no adverse effect was observed	(Glass et al., 2012; Riley et al., 2012; Riley et al., 2014)

5.10   Combined Therapy

Combined therapy involves the use of two or more drugs to achieve a combined beneficial effect. This can be done to either improve the effectiveness of the primary drug with the secondary drug, activate or inhibit different molecular targets at the same time, or enhance the effect of the treatment if one of the drugs only produces positive effects in the presence of the other. Several drugs have been used in combination to produce synergic and beneficial effects in the treatment of ALS. Schiaffino et al., 2018 demonstrated that the combined administration of Entinostat, an inhibitor of HDAC class 1-3, and resveratrol an activator of Sirt1 and AMPK,

significantly delayed disease onset and improved survival in SOD1(G93A) mice. This was associated with a restoration of RelA and histone 3 acetylation to its normal state (Schiaffino et al., 2018).

Table 10. Effects of combined therapy in pre-clinical and clinical trials of ALS

Drug	Model	Pre-clinical outcome	References	Clinical outcome	References
Entinostat + Resveratrol	SOD1(G93A)	Increased RelA acetylation, Increased survival, delayed onset	(Schiaffino et al., 2018)	-	-
RGFP109 + Arimoclomol	FUS(R521H)	Increased nuclear FUS relocalization, enhanced DNA repair	(Kuta et al., 2020)	-	-
Valproate + Lithium	SOD1(G93A)	Increased survival, delayed onset	(Feng et al., 2008)	-	-
Sodium phenylbutyrate + AEOL10150	SOD1(G93A)	Increased survival	(Petri et al., 2006)	-	-
Sodium phenylbutyrate + Riluzole	SOD1(G93A)	Increased survival	(Steven J Del Signore et al., 2009)	-	-
Acamprosate + Baclofen	Primary neuromuscular cell culture	Preserved neuromuscular junction (NMJ), protected motor neurons	(Boussicault et al., 2020)	-	-

PXT864 + Riluzole	Primary neuromuscular cell culture	Preserved neuromuscular junction (NMJ), protected motor neurons	(Boussicault et al., 2020)	-	-

6. AIM OF STUDY

ALS pathogenesis has been linked to the activation of the NF-κB complex, coupled with a reduction in the acetylation state of histone 3 protein. Previous studies carried out by Schiaffino et al., 2018 demonstrated that the combined therapy of MS-275 (Entinostat) and Resveratrol, was able to restore the acetylation state of RelA and histone 3 to normal levels in SOD1(G93A) mice, which was associated with an increase in motor performance and survival. However, MS-275 is still yet to be approved for clinical trials. Furthermore, ALS is a disease that is prevalent in male patients compared to females, and differences have been observed in the phenotypic and genotypic features of the disease, presented in male and female patients, and animal models of the disease. The overall aim of this project is to:

- Demonstrate the efficacy of replacing MS-275 with valproate, another epigenetic drug that acts in a similar way to MS-275 and has already been approved for clinical trials in ALS patients. Together with resveratrol, valproate will be used in combination, as a possible treatment for ALS. This project will analyze for the first time in the ALS mice model, the effect of valproate and resveratrol in improving motor function and prolonging the lifespan of ALS mice.

- Explore the molecular mechanism underlying the MN degeneration in ALS and determine alterations in RelA mRNA expression and RelA acetylation rate about cell survival or non-survival-promoting molecules.

- Determine the capability of the epigenetic drugs to directly protect MNs from neurodegeneration in SOD1(G93A) mice, through the induction of the production of pro-survival factors.

- Examine the effect of the drug on the different sex dimorphic features observed in ALS mice.

7. BENEFICIAL AND DIMORPHIC RESPONSE TO COMBINED EPIGENETIC DRUGS VALPROATE AND RESVERATROL IN THE TREATMENT OF ALS MICE.

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7.1 Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a fatal progressive neurodegenerative disease that affects nerve cells, responsible for controlling voluntary muscle movement, in the brain (upper motor neurons) and spinal cord (lower motor neurons) leading to weakness, loss of motor function, muscle atrophy and eventually death due to respiratory failure (Hardiman et al., 2017). ALS is the most common form of motor neuron disease (Zucchi et al., 2020), with about 90% of all ALS cases being sporadic, while the other 10% are familial, and about 15-20% of all familial cases are caused by a mutation in the superoxide dismutase-1 glycine 93 to alanine gene (SOD1 G93A) (Alsultan et al., 2016). Furthermore, studies have reported genetic and clinical differences in the onset and progression of the disease in male and female ALS mice models, as well as in clinical patients (Vegeto et al., 2020). SOD1(G93A) female mice with B6SJL

background generally experience a delay in disease onset and an increase in survival time compared to their male counterparts (Pfohl et al., 2015). In terms of genetic differences, SOD1(G93A) female mice often show a delay in mitochondrial dysfunction due to decreased mitochondria oxidative phosphorylation associated with protein oxidative damage, compared to their male SOD1(G93A) mice. This delayed mitochondrial dysfunction has been associated with higher estrogen levels in females (Cacabelos et al., 2016). In clinical patients, it is generally reported that men are at higher risk of developing ALS with a male-to-female ratio of 1:2 (Jun et al., 2019; Palese et al., 2019). Moreover, the ALS symptomatology is different in men and women and depends on which neurological region or motor level is affected: there is a predominance of earlier limb onset in males and later bulbar onset in females (Blasco et al., 2012). However, the survival is similar in the two sexes (Blasco et al., 2012). Many factors have been implicated in the degeneration of motor neurons (MN), such as glutamate excitotoxicity, mitochondrial dysfunction, inflammatory response, and oxidative stress (Bonafede & Mariotti, 2017). More recently, transcriptional dysfunction, a mechanism that involves aberrations of the molecular machinery that regulates gene expression, has been implicated in MN degeneration (Bennett & La Spada, 2018). The level at which these aberrations in gene expression occur is known as epigenetic marks. Among these marks, acetylation of histone, the protein that makes up the chromatin component, has been characterized as an important mechanism involved in increased DNA transcriptional activity (Ibeagha-Awemu & Zhao, 2015). Defect in this histone homeostasis (acetylation and deacetylation) has been implicated in several neurodegenerative diseases, including ALS (Belzil et al., 2016; Bennett & La Spada, 2018; Paez-Colasante et al., 2015). The status of histone homeostasis strongly depends on the activity of a family of enzymes known as histone deacetylases (HDACs), which catalyze histone deacetylation in the chromatin structure and have been implicated in several cellular processes such as cell death and stress response (Yang & Seto, 2007). HDACs also play an important role in regulating the acetylation of non-histones proteins such as the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB). NF-κB is a transcription factor involved in a variety of physiological processes in the nervous system (Dresselhaus

& Meffert, 2019), but also in pathological mechanisms associated with neurodegeneration (Bellucci et al., 2020; Srinivasan & Lahiri, 2015). Five subunits compose the NF-κB family of proteins: RelA (p65), RelB, c-Rel, p50, p52. NF-κB neuroprotective or neurotoxic role depends on the subunits forming the transcriptional active dimer (Gilmore & Wolenski, 2012; Lanzillotta et al., 2015). The acetylation state of the RelA subunit can promote both neuroprotective and neurotoxic actions (Lanzillotta et al., 2010; Mincheva-Tasheva & Soler, 2013). In particular, we previously showed that a specific aberrant acetylation pattern of RelA, characterized by a reduced level of total acetylation, but site-specific acetylation at the lysine 310, RelA Ac(K310) triggers the expression of pro-apoptotic genes in brain ischemia (Lanzillotta et al., 2010).

Several studies suggested the role of RelA in ALS pathogenesis (Moges et al., 2009). RelA subunit is upregulated in cellular (Ikiz et al., 2015; Prell et al., 2014; Yin et al., 2018) and animal models of ALS (Frakes et al., 2014; Swarup et al., 2011). Of note, RelA levels were elevated also in the spinal cord of ALS patients (Jiang et al., 2005; Yamamoto et al., 2007). Importantly, we recently demonstrated that the pro-apoptotic acetylation state of RelA, consisting of global lysine deacetylation and increased lys 310 acetylations, was evident in the lumbar spinal cord of SOD1(G93A) mice (Schiaffino et al., 2018).

Several studies have indicated that pharmacological inhibition of HDACs with drugs such as butyrate and vorinostat help to improve cell survival by promoting acetylation of histone, gene transcription and protein synthesis ischemic stroke and a murine model of ALS (G93A) (Lazo-Gomez et al., 2013). Combined drug treatment of lithium and Valproate (VPA) have been shown to improve motor function, delay disease onset, and prolong the life span of SOD1(G93A) mice (Feng et al., 2008). Furthermore, pharmacological stimulation of the class-III HDAC sirtuin 1 with Resveratrol (RESV) has been shown to protect against motor neuron death and improve survival in the SOD1(G93A) ALS mice model (Mancuso et al., 2014).

We reported that the combined administration of low doses of the HDAC inhibitor MS-275 (entinostat) and RESV, an activator of the AMP-activated kinase (AMPK)-

sirtuin 1 pathway, promote the normalization of RelA acetylation state and revert the pathological histone H3 deacetylation in brain ischemia and ALS animal models (Schiaffino et al., 2018). The combination MS-275 and RESV corrected the pathological acetylation state of RelA by, respectively, enhancing the RelA general acetylation and reducing the acetylation at the lys 310 via SIRT1 activation (Lanzillotta et al., 2013; Schiaffino et al., 2018). Moreover, RESV activated the AMPK pathway, leading to an increase of acetyl-CoA and the subsequent activation of histone acetyltransferases (HATs), the enzymes responsible for the acetylation of both RelA and H3 histones. By induction of NAD+, the fundamental cofactor for class III HDACs (Ruderman et al., 2010), AMPK could also sustain SIRT1 activation. In SOD1(G93A) mice the correction of RelA acetylation by the epigenetic drugs protected MNs from neurodegeneration, activated the anti-apoptotic Bcl-xL and neurotrophic BDNF, delayed symptoms' onset and extended lifespan (Schiaffino et al., 2018).

MS-275 is currently in clinical trials for the treatment of different types of cancer but has not yet been approved for clinical use (Trapani et al., 2017). For translational purposes, the present study investigated the efficacy of MS-275 replacement with valproate (VPA), an antiepileptic drug also reported to be a class I HDAC blocker and is already in clinical use. VPA has major inhibitory activity on class I HDACs 1,2,3 and 8, but no effects on HDACs 6,7, and 9 (Khan et al., 2008). As a class I HDACs inhibitor, VPA could represent an alternative to MS-275, with the advantage of being suitable for translational application in clinical trials for ALS, as it is already being routinely used to treat epileptic and bipolar patients (Loscher, 2002). Furthermore, VPA has been reported to be neuroprotective in both animal and cellular models of brain ischemia (Hunsberger et al., 2012; Z. Wang et al., 2012). Resveratrol (trans-3,4',5-trihydroxystilbene) is a natural polyphenol endowed with multiple beneficial properties including neuroprotective effects (Rauf et al., 2017). Of note, we recently showed that VPA and RESV can synergize in promoting neuroprotection in cellular and animal models of brain ischemia by re-storing the RelA pathological acetylation state and reverting the histone H3 hypoacetylation (Faggi et al., 2018).

Based on these previous findings, we investigate, for the first time, the effect of the combined treatment of VPA with RESV in ALS SOD1(G93A) male and female mice. The combined administration of RESV and VPA postponed the time of onset in males, while in the female counterpart reduced symptoms severity, especially in the late phase of the disease, and prolonged the survival. Furthermore, the combined drugs were able to restore the correct RelA and histone 3 acetylation state in the lumbar spinal cord of mice in both sexes. This effect was associated with an overall neuroprotective effect on motor neurons and a reduced microglial immunoreactivity in the lumbar spinal cord.

7.2 Materials and Methods

7.2.1 Animals

The experiments were conducted using 44 transgenic mice (n = 22 males and n = 22 females) overexpressing human Cu/Zn superoxide dismutase 1 carrying a Gly93 - Ala mutation (SOD1(G93A)) (strain designation: B6SJL - TgN [SOD1 - G93A] 1Gur, stock number 002726), which were originally obtained from Jackson Laboratories (Ben Harbor, ME, USA). Transgenic SOD1(G93A) mice and wild-type (WT, n = 12) non-transgenic littermates were maintained in a B6SJL genetic background and housed under controlled environmental conditions with 12 hours of light and dark cycle and received food and water ad libitum. The research complies with the commonly accepted "3Rs". The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the University of Verona committee on animal research (Centro Interdipartimentale di Servizi per la Ricerca she utilizza Animali da Laboratorio – C.I.R.S.A.L.) and by the Italian Ministry of Health (ministerial authorization number 56DC9.72, obtained on 13 August 2021). The transgenic mice were identified by polymerase chain reaction specific for human Sod1 with G93A mutation, as outlined by the Jackson Laboratories (Henriques et al., 2010).

7.2.2 Disease progression assessment

The efficacy of the drug treatment on SOD1(G93A) mice was evaluated every week, starting from postnatal day 50 to the end-stage of the disease. To carry out this evaluation, the animals were subjected to the Neurological score test, Paw Grip

Endurance (PaGE) and Rotarod test, followed by body weight evaluation every week. Each animal was assessed using a neurological scoring system as follows: 4 indicated that there is no sign of motor dysfunction; 3 indicated tremors in the hind limbs when mice were suspended from the tail; 2 when gait anomalies were present; 1 when the animal dragged at least one hind limb; 0 when the animal was unable to right itself within 30 seconds when it was placed in a supine position (Schiaffino et al., 2018). This method provided a rapid, non-invasive measure of disease onset and progression in SOD1(G93A) mice, in a combination with motor tests. The end-stage of the disease was determined when the motor score was equal to 0 (humane endpoint).

7.2.3 Paw Grip Endurance (PaGE)

The PaGE test was used to evaluate the grip strength of the animals by positioning them in the center of a metal grid and quickly rotating. The animals attempted this test twice for 120 secs each which was set as the cut-off time, with an interval of 15 mins between each trial. The latency time was noted and estimated as the period spent up until the mice detached the hind limbs from the grid. The animals failed the PaGE test when they were unable to reach the cut-off time, and the onset of the disease was established from the first day the test was failed.

7.2.4 Rotarod test

The Rotarod test was used to analyze the motor coordination of the animals. The mice were placed in a rotor tube (Acceler Rota-Rod 7650, UGO BASILE, Italy) at a constant speed of 16rpm. The temporal cut-off was set at 180 secs and in case of failure, three attempts were allowed with 5 mins resting phase between each attempt. The longest latency time was recorded.

7.2.5 Drugs treatment

Resveratrol (RESV) (Merck) was dissolved in dimethyl sulfoxide (DMSO), while Sodium Valproate (VPA) (Sigma-Aldrich) was diluted in saline solution (0,9% NaCl in water). The final DMSO concentration used was approximately 0.1%. The drugs were combined daily from the already prepared stock solutions and injected intraperitoneally into SOD1(G93A) mice. Groups were balanced concerning gender: the Vehicle (VEH) group (n = 20, 10 males and 10 females) received saline

+ 0.1% DMSO, at the same concentration used in the TREATED group, while the TREATED group (VPA and RESV) (n = 24, 12 males and 12 females) received 136 μg/Kg of RESV and 40 μg/Kg of VPA in combination. Treatment started at postnatal day 50 and continued until the animals reached the end stage of the disease. In line with the results we previously obtained treating SOD1(G93A) mice with the combination MS-275 and RESV (Schiaffino et al., 2018), RESV was used at 136 μg/Kg. Keeping this RESV concentration, a VPA dose of 40 μg/Kg was selected based on the ratio VPA: RESV we found effective in an animal model of brain ischemia (Schiaffino et al., 2018).

7.2.6 Tissue preparation, staining procedures and stereological count of lumbar spinal cord

The end-stage SOD1(G93A) mice of both VEH (n=10) and TREATED group (n=12) were anesthetized with Ketamine + Xilazina + Altadol® (130 + 12 + 7 mg/kg) and transcardially perfused using 0.1 M PBS followed by 4% paraformaldehyde (PFA). The spinal cord was dissected out and was further post-fixed in 4% PFA and stored overnight at 4°C. The lumbar tract was soaked in a solution containing 30% sucrose and included in the OCT. Using a cryostat, the section was cut into 15 μm transverse slices, collected and mounted on Surgipath® Apex ™ upper adhesive slides (3800080E, Leica Biosystems).

For the stereological count of MN, the Nissl staining method was used. The slides were air-dried and hydrated with water for 30 seconds. Staining was performed by incubating the slides in a 37 °C oven for 15 minutes in a solution containing 0.1% cresyl violet, 92% glacial acetic acid and 8% sodium acetate. The sections were then gradually placed in increasing concentrations of ethanol (70%, 95% and 100%), cleaned with xylene, mounted with Entelan (Merck, Darmstadt, Germany) and covered with a coverslip. The MN of the lumbar tract (L1-L5) were counted using a computer-assisted microscope (Olympus BX6 with Retiga 2000R camera) with the Stereo investigator software (MicroBrightField, Williston, VT, USA) at 40x magnification. Cells with nucleoli on the plane of focus, size and shape typical of MN were counted. The values from the sections were computed for the summation, the mean number was then computed from the average number derived from each animal.

To investigate gliosis activation to detect microglia cells in the lumbar tract of the spinal cord, immunohistochemistry for light microscopy was performed. The sections were incubated for 15 minutes in 1% H_2O_2 to quench endogenous peroxidase and preincubated for 1h in 5% of Normal Goat Serum (NGS) and 0.3% Triton X-100 in PBS. The slides were then incubated overnight in mouse anti-mouse Iba1 antibody (1:500, SKN4887, Fujifilm) in 1% NGS and 0.3% Triton X-100 in PBS. The sections were rinsed in water 3 times for 5 mins each and incubated for 1h in biotinylated goat anti-mouse IgG (1:200, BA-9500, Vector Laboratories) in 1% NGS and 0.3% Triton X-100 in PBS. The reaction was developed with the avidin-biotin-peroxidase kit (ABC kit; Vector) using 3‑3′ -diaminobenzidine as the chromogen. The sections were dehydrated through increasing grades of ethanol (70%, 95% and 100%), cleared in xylene, and cover-slipped with Entellan. The microglial cells of the lumbar tract (L1-L5) were analyzed using a computer-assisted microscope (Olympus BX6 with Retiga 2000R camera) with the Stereo investigator software (MicroBrightField, Williston, VT, USA).

7.2.7 Immunofluorescence procedures

Immunofluorescence procedure was performed on lumbar spinal cord MN of end-stage SOD1(G93A) of both VEH (n=11) and TREATED (n=10) groups, and age and sex-matched WT (120-140 days, n=4) to investigate the acetylation state of the Lys9 of H3 and SMI-32. The animals were deeply anesthetized and transcardially perfused as described above. First, the slides were incubated for 1h in 2.5% NGS and 0.3% Triton X-100 diluted in PBS. Then the slides were incubated overnight in H3AcK9 (1:250, GeneTex, GTX88007) and SMI-32 (1:1000, Biolegend, #SMI-32P) antibodies in 1.25% NGS and 0.3% Triton X-100 diluted PBS. The slides were rinsed and the sections were incubated for 1h in goat anti-rabbit 594 IgG (1:1000, A11012, TermoFisher Scientific) and goat anti-mouse IgG 488 secondary (1:1000, A32723, TermoFisher Scientific) antibodies in 1% GS and 0.3% Triton X-100 in PBS. The nuclei were counterstained with DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride) (1:2000, D1306 TermoFisher Scientific) for 5 minutes and washed in PBS. The sections were then coverslipped with Fluorescent Mounting Medium (S3025, Dako), and immunofluorescence was analyzed with a

TCS-SP5 confocal microscope (Leica-Microsystems, Wetzlar, Germany), in a dual-channel acquisition setup, using UV, 488nm, and 543nm excitation beams.

7.2.8 Immunoprecipitation and western blot

Fresh lumbar spinal cords were collected from SOD1(G93A) (n=21) and WT (age-matched, n=6) mice after cervical dislocation. To investigate the RelA acetylation state was performed immunoprecipitation assay, while the expression of an enzyme involved in RelA acetylation (AMPK), anti-apoptotic (Bcl-xL) and neurotrophic factor (BDNF) was achieved by western blot assays. The proteins of the nuclear and cytoplasmic fraction were obtained using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific) according to the manufacturer's instructions. Protein concentration was determined using the Pierce™ Detergent Compatible Bradford Assay (23236, Thermo Fisher Scientific). Fifty micrograms of nuclear protein fraction were incubated with goat anti-RelA (10 µg/mg lysate, LS-C290611-100) overnight at 4 °C on a rotor plate. The RelA protein associated with the antibodies was precipitated with Protein A-Sepharose (CL-4B, 10233478, GE Healthcare) according to the user's manual. After washing, the immunoprecipitated proteins were resolved by 4–20% SDS-polyacrylamide gel (Biorad) and transferred to polyvinylidene difluoride (PVDF) membrane. Following antibodies were used to detect the immunoprecipitated proteins: rabbit anti-NF-kB p65 (1:1000, GTX107678, GeneTex), rabbit anti-AcetylLysine (1:500, AB3879, Merck Millipore) and rabbit anti-NF-kB p65 (acetyl lys 310) (2.5 µg/ml, ab19870, Abcam). Horseradish peroxidase (HRP) polyclonal goat anti-rabbit immunoglobulins (1:2000, #P0448, Dako) was used to detect primary antibodies.

Thirty micrograms of cytoplasmic protein fraction were separated on 4–20% SDS-polyacrylamide gel and transferred onto the PVDF membrane. Primary antibodies were used at 1:1000 dilution and appropriate HRP-conjugated secondary antibodies were used at 1:2000 dilution in 5% BSA and PBS-T 0.1% blocking solution. The primary antibodies against murine AMPKα rabbit mAb (#5831, Cell Signaling) and p-AMPKα (Tr172) rabbit mAb (#2535, Cell Signaling), rabbit anti- Bcl-xL (#2762, Cell Signaling), mouse anti-BDNF (ab203573, Abcam) and a mouse monoclonal anti-GAPDH (AM4300, TermoFisher Scientific) were used. All membranes were

then incubated with a chemiluminescent HRP substrate (WBKLS0500, Merck Millipore) and detected with G: BOX F3 GeneSys (Syngene, UK).

7.2.9   Data analysis and statistics

The data obtained from the motor tests were analyzed using two-way ANOVA followed by Bonferroni multiple comparison tests. Data are expressed as mean ± standard error of the mean (SEM). Regarding data of the disease onset and the survival, the Log-rank test (Mantel-Cox) was used. Data are expressed as a mean ± SEM. Concerning stereological count of MN two-tailed Student's T was used. Data are reported as mean ± SEM. GraphPad Prism 8 software was used for statistical analysis and graphs and significance was accepted at p <0.05. Densitometric analysis of Western blot performed with Fiji (ImageJ) software open source (Schindelin et al., 2012) and the stereological MN count data were analyzed using one-way ANOVA followed by Tukey's multiple for comparisons tests. Data are reported as mean±SEM. For all statistical analysis and graphs, GraphPad Prism 8 software was used, and significance was accepted at p<0.05.

7.3   Results

7.3.1   Untreated SOD1(G93A) female mice show delayed decline in motor performance compared to male counterparts.

To determine the difference in the clinical onset and rate of disease progression in both male and female SOD1(G93A) mice, the animals were divided into two groups based on their sex. Both male and female animals were subjected to two motor tests. Paw grip endurance test (PaGE), which was used to examine the grip strength of the animals, and the rotarod test which examines motor coordination in the animals. In the PaGE test, the female animals exhibited a delay in the decline of motor performance compared to male mice. Female animals began to show a decrease in motor performance at week 14 compared to male mice that experienced a decline in motor performance as early as week 9. A significant difference was observed between the two sexes at week 12 (p= 0.0038), week 13 (p= 0.0003) and week 14 (p= 0.0006), with female animals showing better performance as opposed to male mice (Figure 7.1A). Similar results were obtained in the rotarod test, where the

female animals performed significantly better than their male counterparts at week 17 (p= 0.0025), indicating that female SOD1(G93A) mice with B6SJL background, generally show a delay in clinical onset compared to male mice (Figure 7.1B).
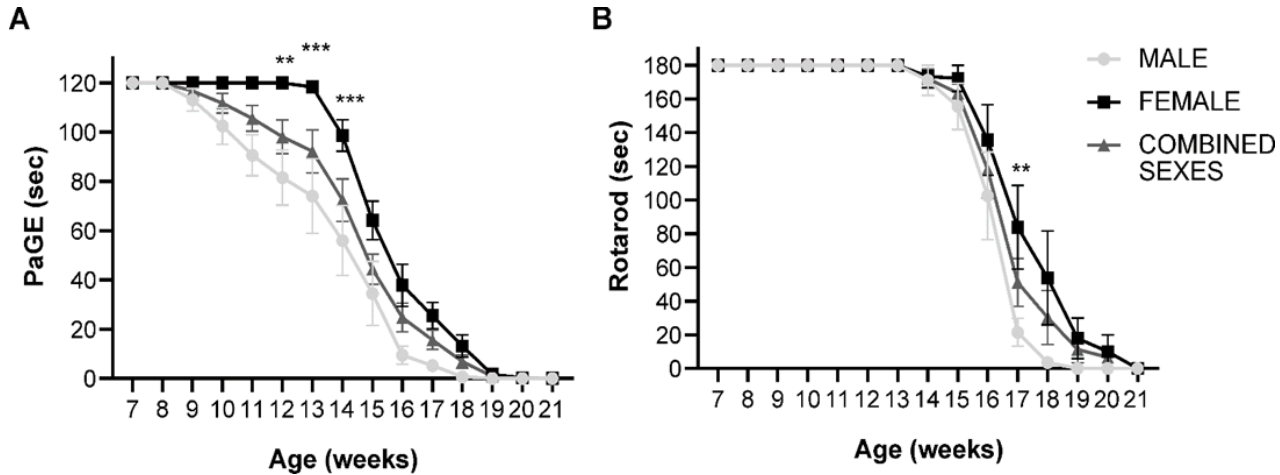

Figure 7.1. PaGE and Rotarod test of untreated male, female and combined sexes: (a) PaGE test showing grip endurance of un-treated SOD1(G93A) animals. There was a significant difference between male and female untreated groups at week 12 (p= 0.0038), week 13 (p= 0.0003) and week 14 (p= 0.0006), where the female animals displayed significantly better performance compared to the male mice, (n= 10 male, 10 female); (b) Rotarod test showing motor coordination of untreated SOD1(G93A) animals. A significant difference between male and female mice was observed, with the female untreated mice showing significantly better performance at week 17 (p= 0.0025) compared to male animals. Results were analyzed by two-way ANOVA followed by Bonferroni multiple comparisons test.   *p<0.05; **p<0.005; ***p<0.0005; and ****p<0.0001. Data in all graphs are expressed as mean ± SEM.

7.3.2   VPA and RESV improve motor performance in SOD1(G93A) mice.

To evaluate the effect of the combined drugs (RESV and VPA) on the motor performance of the SOD1(G93A) mice in combined sexes, male and female mice, the animals were divided into the treated group (TREATED), which were administered a combined treatment of RESV (136 µg/kg) and VPA (40 µg/kg), and the vehicle group (VEH). Both groups of animals began treatment at post-natal week 7, close the onset stage, as detected through the PaGE test, and continued the treatment until they were sacrificed, at the end stage of the disease. The dose of the drug to be administered was regulated every week based on the body weight of the animals. Bodyweight as well as motor tests, PaGE and rotarod, were assessed every week. Regarding the grip persistence of the animals, results revealed an overall

improvement in motor performance of animals in the TREATED group compared to the VEH group. Specifically, in the combined sexes, the drug treatment significantly delayed the loss of motor function at week 12 (p= 0.0382), week 13 (p= 0.0272), week 14 (p<0.0001), week 15 (p<0.0001) and week 16 (p= 0.010) (Figure 2A). Interestingly, when divided into different sexes, the male mice treated with the drugs showed a higher significant improvement in motor performance at week 12 (p= 0.0430), week 14 (p= 0.007), week 15 (p=0.0480) and week 16 (p= 0.0327) compared to the VEH group (Figure 7.2B), while the female animals only showed a significant improvement at week 15 (p= 0.0009) compared to VEH group (Figure 7.2C). The rotarod test was also performed to examine motor coordination in the TREATED and VEH animals. In the combined sexes, it was observed that animals in the TREATED group showed significant improvement in motor coordination at week 17 (p<0.0001) and week 19 (p= 0.0021) compared to the VEH group (Figure 7.2D). Surprisingly, the male animals only showed a trend in the improvement of motor function after drug treatment without any statistical significance (Figure 7.2E). The female animals in the TREATED group showed better performance in the rotarod test with statistical significance at week 17 (p= 0.0040), week 18 (p= 0.0262), week 19 (p= 0.0077) (Figure 7.2F). An overall significant improvement in motor performance was recorded in the drug-treated groups compared to the control.
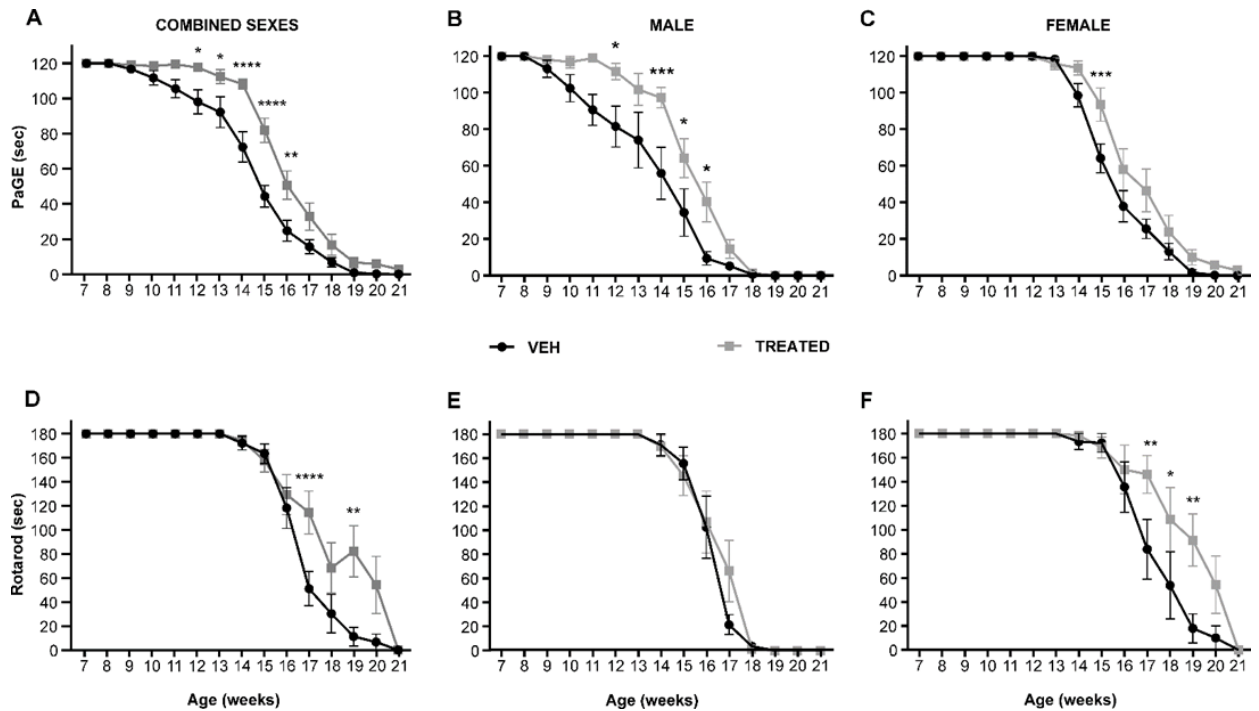
Figure 7.2. PaGE and Rotarod tests in combined sexes, male and female SOD1(G93A) mice treated with pharmacological combination of VPA and RESV (TREATED), or untreated (VEH): (a) PaGE test for combined sex animals showed that in the TREATED animals, a significant improvement in motor performance was observed at week 12 (p= 0.0382), week 13 (p= 0.0272), week 14 (p<0.0001), week 15 (p<0.0001) and week 16 (p= 0.010) compared to the VEH (n= 20 VEH, 24 TREATED); (b) Male SOD1(G93A) mice showed a significant improvement in the treated group compared with VEH at week 12 (p= 0.0430), week 14 (p= 0.007), week 15 (p= 0.0480) and week 16 (p= 0.0327; n= 10 VEH, 12 TREATED); (c) The female mice displayed a significant improvement at week 15 in the TREATED versus the VEH group (p= 0.0009; n= 10 VEH, 12 TREATED); (d) The motor coordination test of the combined sexes showed a significant improvement in motor performance of the TREATED mice compared with the VEH animals at week 17 (p<0.0001) and week 19 (p=0.0021; n= 20 VEH, 24 TREATED); (e) Rotarod test in the SOD1(G93A) male mice showed no statistical significance in the treated versus the VEH animals (n= 10 VEH, 12 TREATED); (f) A significant improvement was observed in the TREATED female SOD1(G93A) mice at week 17 (P=0.0040), week 18 (p=0.0262), week 19 (p=0.0077) with respect to the VEH group, (n= 10 VEH, 12 TREATED). Results were analyzed by two-way ANOVA followed by Bonferroni multiple comparisons test. *p<0.05; **p<0.005; ***p<0.0005; and ****p<0.0001. Data in all graphs are expressed as mean ± SEM.

7.3.3 Combined administration of VPA and RESV showed a postponement of disease onset and survival in a sex-depending manner in SOD1(G93A) mice.

To examine the effect of the combined drug treatment, in the ALS murine model SOD1(G93A), the clinical onset of the disease, as well as the life span were

monitored in both the TREATED and VEH groups of the combined sexes, male and female mice. We first evaluated the disease onset in the combined sexes. Here, we observed that there was a significant delay in the disease onset of TREATED animals (p= 0.0055), concerning the VEH mice. The TREATED mice experienced disease onset at approximately 110 days after birth, while animals in the VEH group displayed earlier disease onset at 94 days. The mean difference between the two groups was recorded as 16±5.5 days (Figure 7.3A). Surprisingly, the further sex-related analysis revealed that the male animals treated with the drugs exhibited a significant delay in the onset of the disease (107 days) compared to the VEH animals (84 days). A delay of 22±7.8 days was observed between the two groups (p= 0.0107) (Figure 7.3B). The female animals only showed 7 days delay in clinical onset when treated with the drugs, without any statistical significance (p= 0.3294) (Figure 7.3C). The survival rate of the animals was also taken into consideration. In the combined sexes (Figure 7.3D), a significant increase in survival time was observed in the TREATED group (145 days) concerning the VEH mice (136 days). The mean difference between the two groups was 8.3±2.9 days (p= 0.0074). Specifically, the male animals in the TREATED group did not show any significant improvement in the rate of survival compared to the VEH group, although a trend was observed (p= 0.0702) (Figure 7.3E). Interestingly, treated females showed a statistically significant improvement in survival rate (148 days) (p= 0.00497), as opposed to the VEH female mice (139 days). A mean difference of 8.5±4 days in the survival rate was observed (Figure 7.3F).
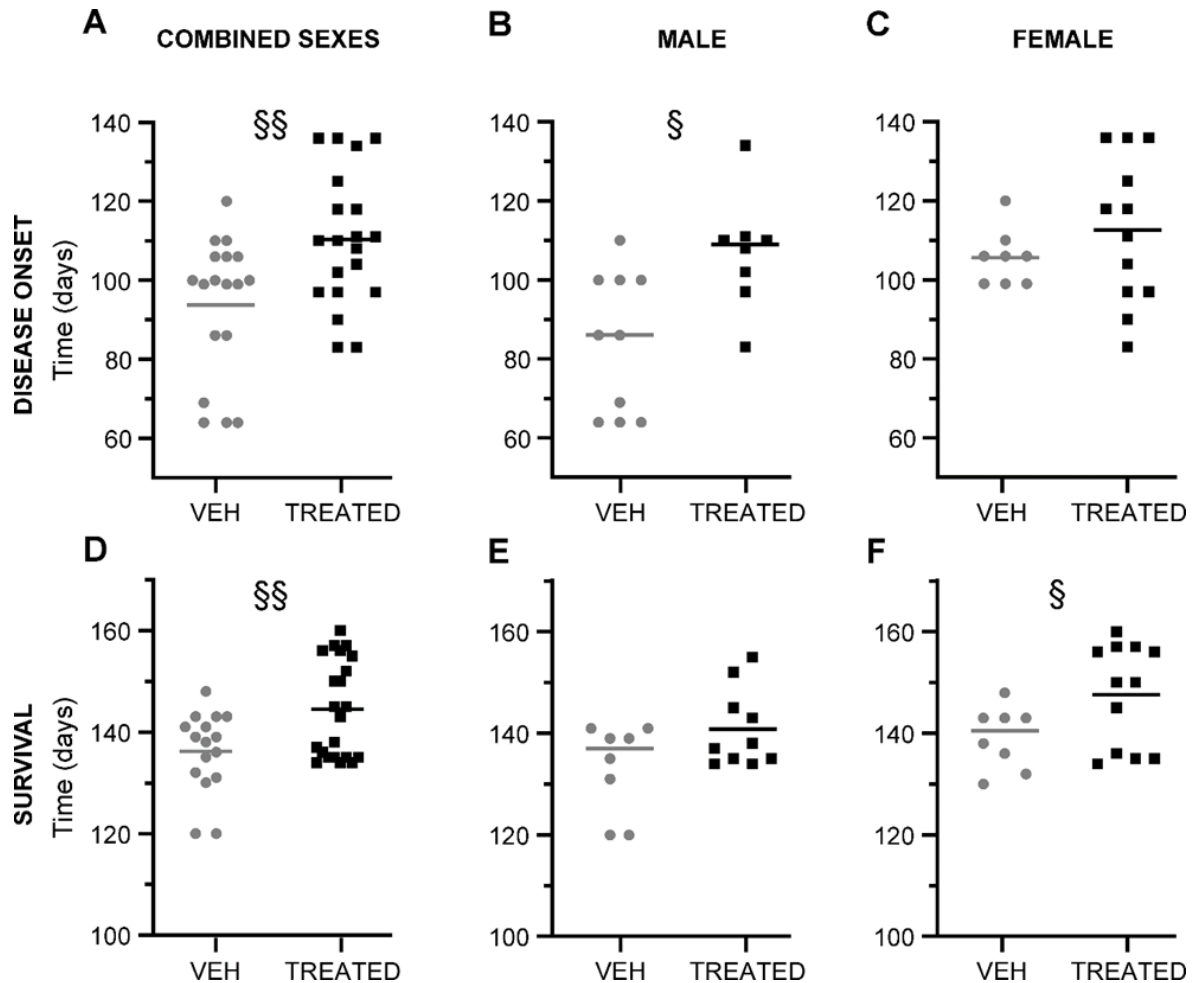
Figure 7.3. Disease onset and survival of combined sexes, male and female SOD1(G93A) mice in TREATED and VEH group: (a) The graph shows the disease onset of combined sex mice in the TREATED and VEH group. A significant delay in disease onset was observed in the TREATED animals compared to the VEH mice, (p= 0.0055; n= 18 VEH, 20 TREATED); (b) The male mice in the TREATED group showed a statistically significant delay of disease onset compared to the VEH, (p= 0.0107; n= 10 VEH, 8 TREATED); (c) In the female animals, a trend was observed in the delay of disease onset of animals treated with the drugs, but was without statistical significance (p= 0.3294; n= 8 VEH, 12 TREATED); (d) The graph shows the rate of survival of combined sexes in the TREATED group compared to the VEH. The treated mice showed a significant improvement in survival rate compared to the VEH (p=0.0074; n= 16 VEH, 22 TREATED); (e) Male animals in the TREATED group displayed no significance in survival rate (p= 0.0702; n= 8 VEH, 10 TREATED); (f) In the female mice, an improvement in the lifespan of treated animals was observed with respect the VEH mice (p = 0.0497; n= 8 VEH, 12 TREATED). Statistical analysis was performed with the Log-rank (Mantel-Cox) test. Data in all graphs are expressed as mean ± SEM. § p<0.05; §§ p<0.005.

7.3.4 Drug treatment protects the lumbar spinal cord from neurodegeneration.

To evaluate the neuroprotective effect of RESV and VPA on SOD1(G93A) mice, Nissl staining and stereological count of motoneurons (MN) in the lumbar spinal cord was performed at the end stage of the disease. The MN population per area in the ventral horn of the lumbar tract (L1-L5) was analyzed in both the TREATED and VEH groups. In the combined sexes, a 40% increase in MN number was observed in TREATED mice compared to animals in the VEH group (P=0.0002) (Figure 4b). Furthermore, we examined the MN population in both male and female SOD1(G93A) mice and obtained similar results. Both male and female mice in the TREATED group showed a significant increase in the MN population compared to their VEH counterparts (57% and 60% respectively, see supplementary data, Figure S7.1). The increase in the MN population was also associated with a significant decrease in microglia activation in the TREATED group of male, female and combined sexes (33%, 38% and 31% respectively) (see supplementary data, Figure S7.2). These results revealed a protective effect of the combined drug treatment on lumbar MN (Figure 7.4).
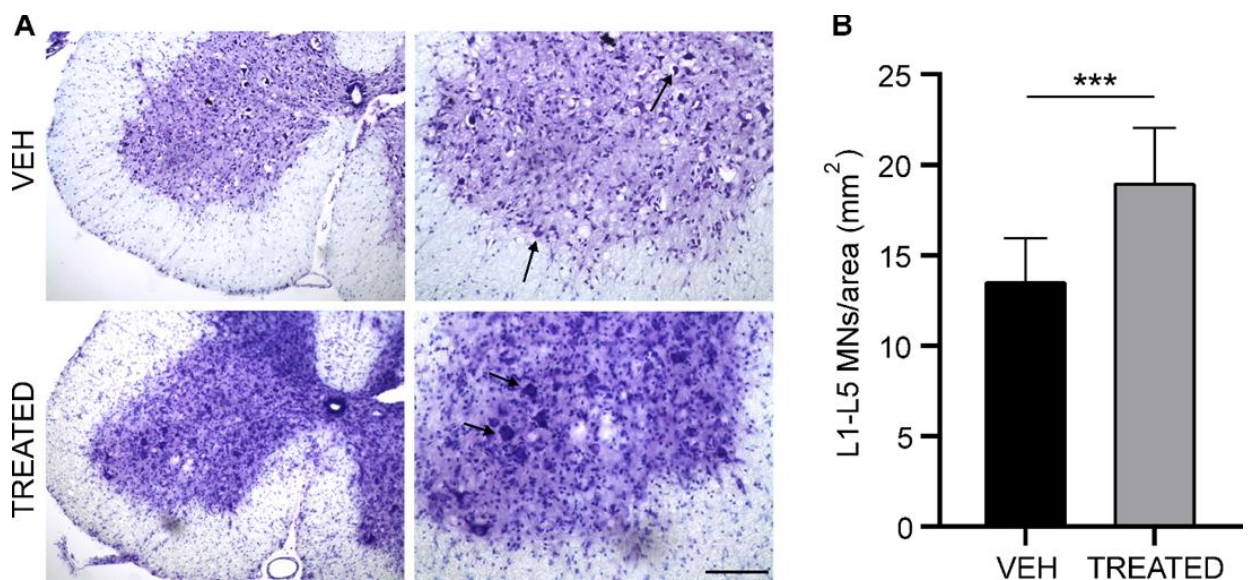

Figure 7.4. MN count in the lumbar tract of the spinal cord (L1- L5): (a) Nissl staining on the L3 lumbar segment of the spinal cord at the end stage of the disease in VEH (VEH) and TREATED groups. The arrow shows stained MN; (b) The graph shows the MN number/area in the L1-L5 tract of both the TREATED and VEH groups. In the TREATED group, there was a significant increase in MN survival compared to the VEH group (p= 0.0002; n= 10 VEH, 12 TREATED). Images on

the left panel have a magnification of 10x, which images on the right panel have a magnification of 20x (scale bar= 100μm). Data were analyzed by student t-test, expressed as mean ± SEM.

7.3.5   VPA and RESV restore RelA acetylation state in the lumbar spinal cord of SOD1(G93A) mice.

Immunoprecipitation and western blot were performed to assess the acetylation state of RelA protein from the nuclear fraction of the lumbar spinal cord of WT, VEH and TREATED SOD1(G93A) male and female mice at the end-stage of the disease. There was an increase of the RelA expression in the VEH group relative to the WT but without statistical significance. Animals in the TREATED group experienced a reduction in RelA expression (Figure 7.5B). Furthermore, mice in the VEH group showed a significant reduction in acetylation of RelA immunoprecipitated (Ac-RelA, tot lys), compared to the WT (p= 0.0035). However, the treatment led to a significant increase in the acetylation state (p= 0.0461) (Figure 7.5C). Immunoprecipitation was also performed to examine the acetylation of specific lysine 310. Results obtained showed a significant increase in acetylation in the VEH group compared to WT (p= 0.0216), followed by a significant restoration of the acetylation state in animals that received the treatment (p= 0.0439) (Figure 7.5D). The animals were also specifically divided into male and female groups and the RelA acetylation was examined. Similar results were obtained, and no difference was observed between the acetylation state of male and female animals (see supplementary data, Figure S7.3). As reported in previous studies (Schiaffino et al., 2018), we demonstrated a general alteration of the acetylation state of RelA in SOD1(G93A) mice, and the combined drug treatment resulted in a restoration of RelA acetylation state similar to WT animals.
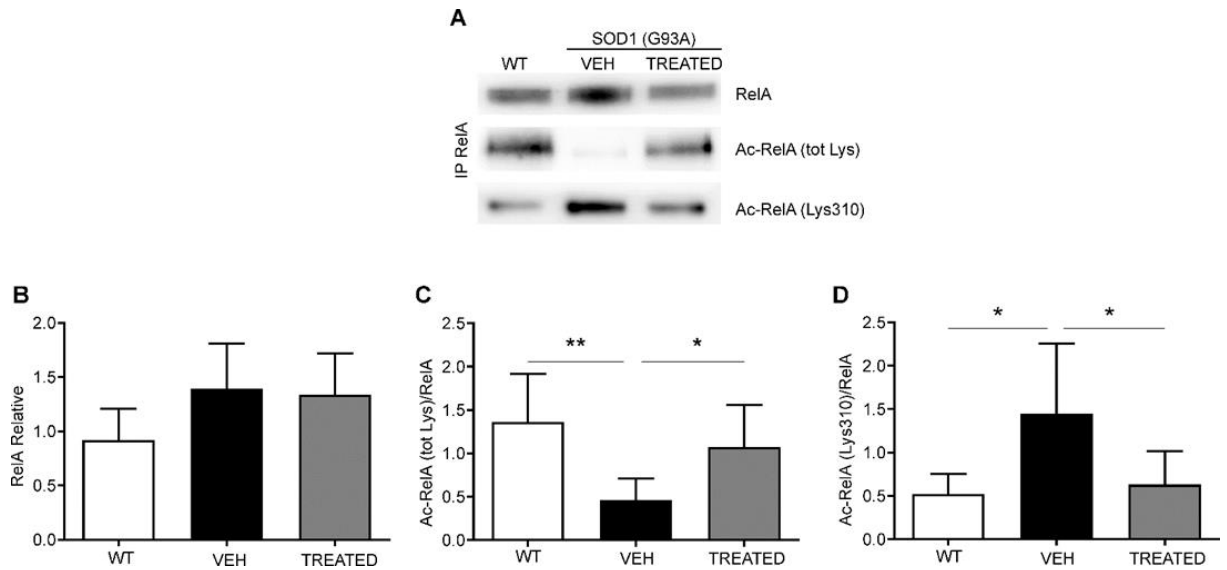
Figure 7.5. Immunoprecipitation and densitometric analysis of RelA subunits in combined sex mice: (a) Representative image of immunoprecipitation and western blot of RelA (65 kDa), total lysine acetylation (Ac-RelA (tot lys)) and acetylation of lysine in the 310 positions in RelA (Ac-RelA (lys310)); (b) Densitometric analysis shows an increase in RelA expression in the VEH compared to the WT and TREATED group without any significance; (c) Ac-RelA (tot lys) acetylation was statistically reduced in the VEH group compared to the WT (p= 0.0035), and TREATED group (p= 0.0461); (d) Ac-RelA (lys 310) was significantly increased in the VEH group compared to the WT (p= 0.0216), and to the TREATED group (p= 0.0439). (n= 6 WT, 10 VEH, 12 TREATED) Results were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Graphs are shown as mean ± SEM. *p<0.05; **p<0.005.

7.3.6 RESV Increases phosphorylation of AMPK in the lumbar spinal cord of SOD1(G93A) mice.

To further examine the effect of RESV on the protein target AMPK, the phosphorylated state of AMPK (pAMPK) was analyzed using the cytoplasmic protein fraction of the lumbar spinal cord of WT, VEH, and TREATED mice, at the end stage of the disease. A significant decrease in the phosphorylation state of Tr172 of pAMPK was observed in VEH animals compared to WT (p<0.0001) (Figure 7.6B). However, the drug treatment resulted in a subsequent significant increase in phosphorylation of AMPK compared to the VEH (p<0.0001), as well as the WT (p<0.0001). Regarding the sex-specific study, similar results were obtained in both male and female SOD1(G93A) mice, indicating no sex-specific differences in AMPK phosphorylation at the end stage of the disease (see supplementary data, Figure S7.4).
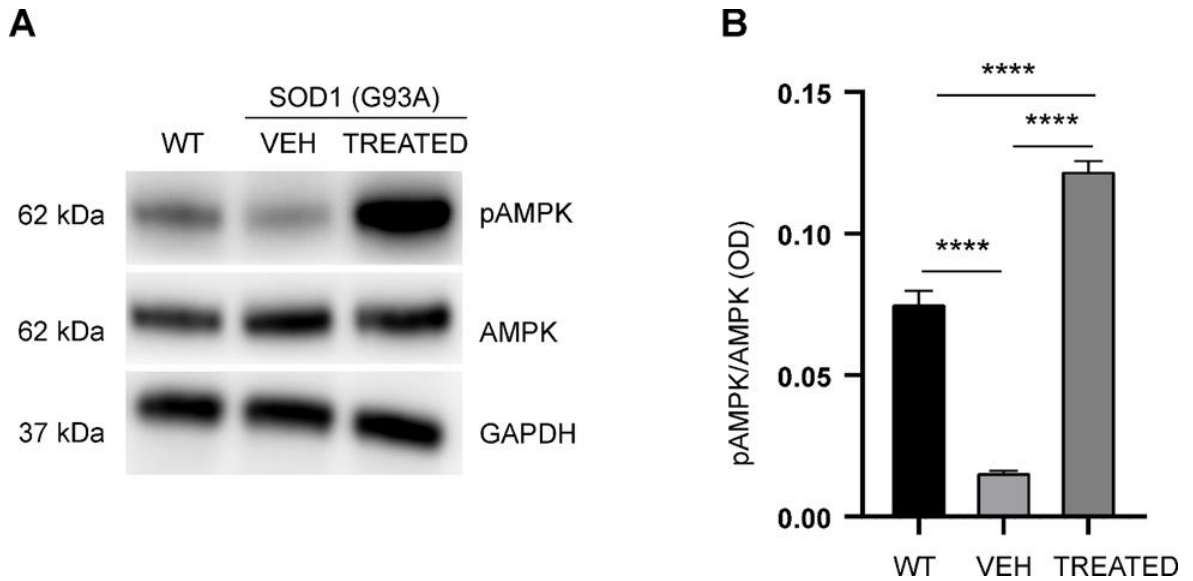
Figure 7.6. Analysis of phosphorylation state of AMPK: (a) Representative western blot showing the phosphorylation of AMPK; (b) Phosphorylation state of AMPK was reduced in the VEH group compared to the WT with a significance of $p<0.0001$. in the TREATED group, the phosphorylation state was increased with a significance of $p<0.0001$. There was also a significant difference between WT and TREATED groups ($p<0.0001$). (n= 6 WT, 10 VEH, 12 TREATED). Results were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Data are shown as mean ± SEM. *$p<0.05$; **$p<0.005$; ***$p<0.0005$; and ****$p<0.0001$.

7.3.7 Increased neurotrophic factor BDNF and anti-apoptotic Bcl-xL protein levels in the lumbar spinal cord of SOD1(G93A) mice.

The expression level of brain-derived neurotrophic factor (BDNF) and Bcl-xL protein was examined in the cytoplasmic protein fraction of the lumbar spinal cord of WT, VEH and TREATED SOD1(G93A) mice at the end stage of the disease. It was observed that the neurotrophic factor BDNF was significantly reduced in the VEH group compared to the WT ($p= 0.0019$). The drug treatment resulted in a significant increase in the BDNF levels in the TREATED group concerning the VEH ($p= 0.0001$) and the WT ($p= 0.0049$), (Figure 7.7B). Moreover, a reduction in the anti-apoptotic factor the Bcl-xL protein was observed in the VEH animals compared to the WT ($p= 0.0176$), while the combined drug treatment significantly improved the expression of Bcl-xL protein concerning the VEH ($p<0.0001$), and WT ($p= 0.0001$) (Figure 7.7D). Similar results were obtained both in male and female animals (see supplementary data, Figure S7.5).
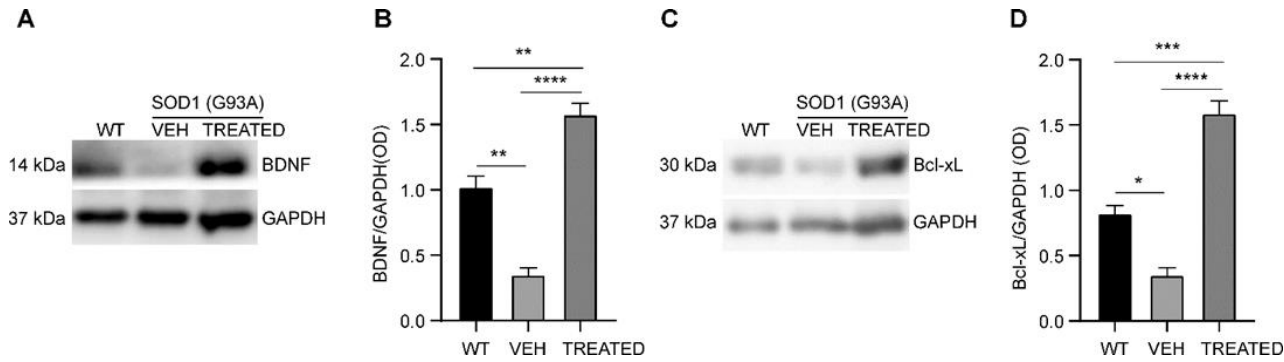
Figure 7.7. Densitometric analysis of the expression of neurotrophic factor, BDNF and anti-apoptotic factor Bcl-xL normalized to GAPDH: (a) Representative image of western blot assay showing the expression of BDNF (14 kDa) and GAPDH (37 kDa); (b) BDNF level was reduced in the VEH animals compared to the WT with a significance of ($p= 0.0019$). The treatment resulted in an increased expression of BDNF compared to the VEH group with a significance of ($p<0.0001$). There was also a significant increase of the TREATED group compared to the WT ($p= 0.0049$); (c) A representative image of Bcl-xL (30 kDa) and GAPDH (37 kDa) western blot; (d) The anti-apoptotic factor Bcl-xL was reduced in the VEH group compared to the WT with a significance of ($p= 0.0176$). Pharmacological treatment resulted in an increase in the expression level of the protein with a significance of ($p<0.0001$) concerning the VEH group. Furthermore, Bcl-xL was significantly increased in the TREATED animals compared to the WT ($p= 0.0001$). The same number of animals ($n= 6$ WT, 10 VEH, 12 TREATED) used for AMPK analysis was also used for the BDNF and Bc-xL studies. Results were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Data are shown as mean $\pm$ SEM. $*p<0.05$; $**p<0.005$; $***p<0.0005$; and $****p<0.0001$.

7.3.8 Restoration of H3 acetylation state in the lumbar spinal cord of SOD1(G93A) mice.

Immunofluorescence staining was performed to examine the effect of epigenetic drugs on the acetylation state of Lysine 9 of histone 3 (H3AcK9) (Figure 7.8 b, g, l), colocalized with the nuclei (DAPI) (Figure 8 a, f, k) in the lumbar MN (SMI-32) (Fig 7.8 c, h, m) of WT, VEH and TREATED SOD1(G93A) male mice. Qualitative analysis of immunofluorescence images revealed a decrease in the acetylation of H3 in the VEH group (Figure 8 g) compared to matched WT (Figure 7.8 b). Furthermore, we observed that the treatment led to a restoration of H3 acetylation (Figure 7.8 l), compared to animals that did not receive any treatment (VEH) (Figure 7.8 g). The level of H3 restoration was similar to what was observed in WT animals (Figure 7.8 b). It is important to note that the same results were observed also in the female SOD1(G93A) mice, proving that VPA was able to regulate the

activity of HDACs class I and restore the H3 acetylation state in both sexes (see supplementary data, Figure S7.6).
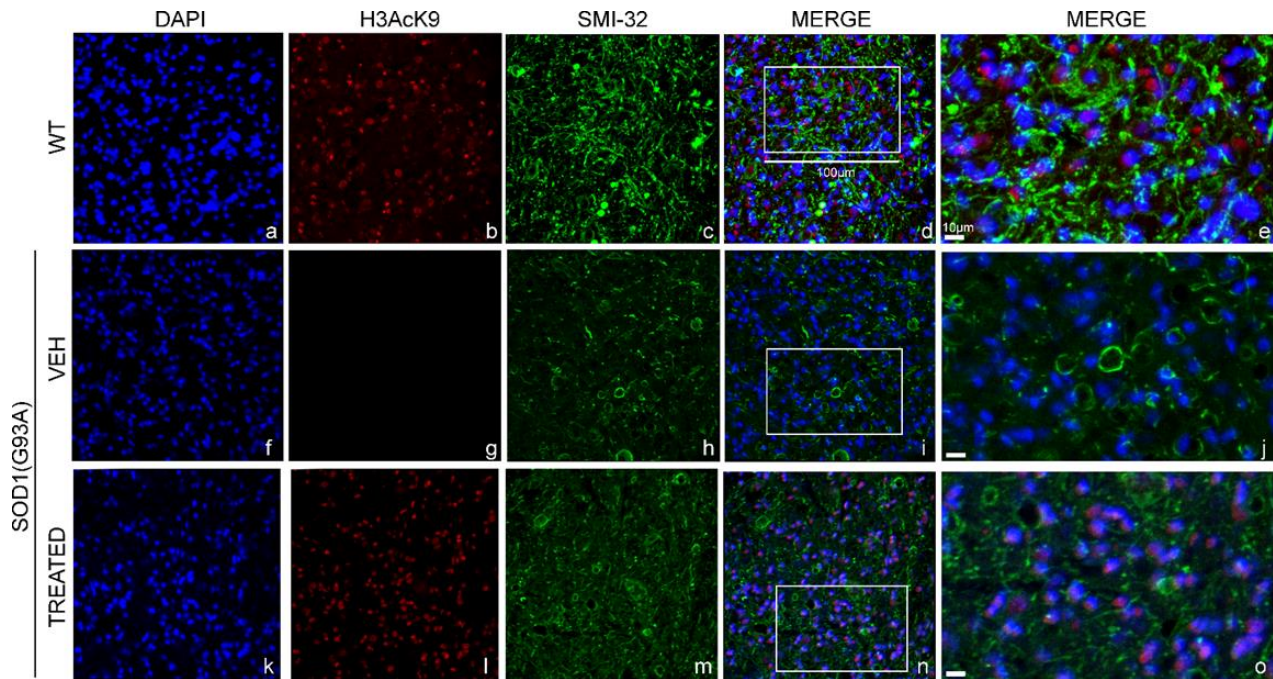

Figure 7.8. Histone 3 acetylation in the lumbar spinal cord of male WT and SOD1(G93A) mice: The figure panel shows the different acetylation states of lysine 9 of histone 3 in the lumbar spinal cord of wild type (WT) mice, VEH and TREATED SOD1(G93A) groups. The nuclei were stained in blue with DAPI (a, f, k). The acetylation state of histone 3 was identified by the H3AcK9 antibody in red (b, g, i), while the motor neurons (MN) were detected by identifying the antibody neurofilament H with the SMI-32 antibody in green (c, h, m). The acetylation state of histone 3 was drastically reduced in the VEH group (g) compared to WT animals (b). The treatment with the epigenetic drugs led to a restoration of the acetylation of histone 3 in the TREATED group (i), (n= 3 WT, 5 VEH, 6 TREATED). Figure d, i, n shows the superimposed images of DAPI, SMI-32 and H3AcK9 in the WT, VEH, and TREATED groups respectively. Magnification 20x, scale bar = 100μm (a-d, f-i and k-n). The figure panel e, j, o shows the highlighted area in d, i, n at higher magnification 40x, scale bar = 10μm.

7.3.9 Supplementary Data
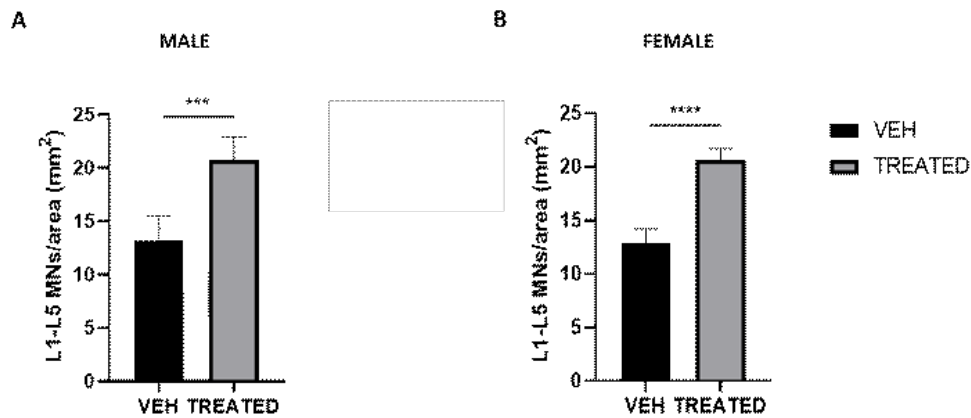
Figure S7.1: Motor neuron (MN) count in the lumbar tract of the spinal cord (L1- L5) of male and female SOD1(G93A) mice: (a) In the male mice, a significant increase in MN count was observed in the TREATED group concerning the VEH (p= 0.0004; n= 5 VEH, 5 TREATED); (b) Similar to the male SOD1(G93A) mice, the female mice showed a significant increase in MN count in the L1-L5 segment of the TREATED group concerning the VEH (P<0.0001; n= 5 VEH, 6 TREATED). Data were analyzed by student t-test, expressed as mean ± SEM. *p<0.05; **p<0.005; ***p<0.0005; and ****p<0.0001.
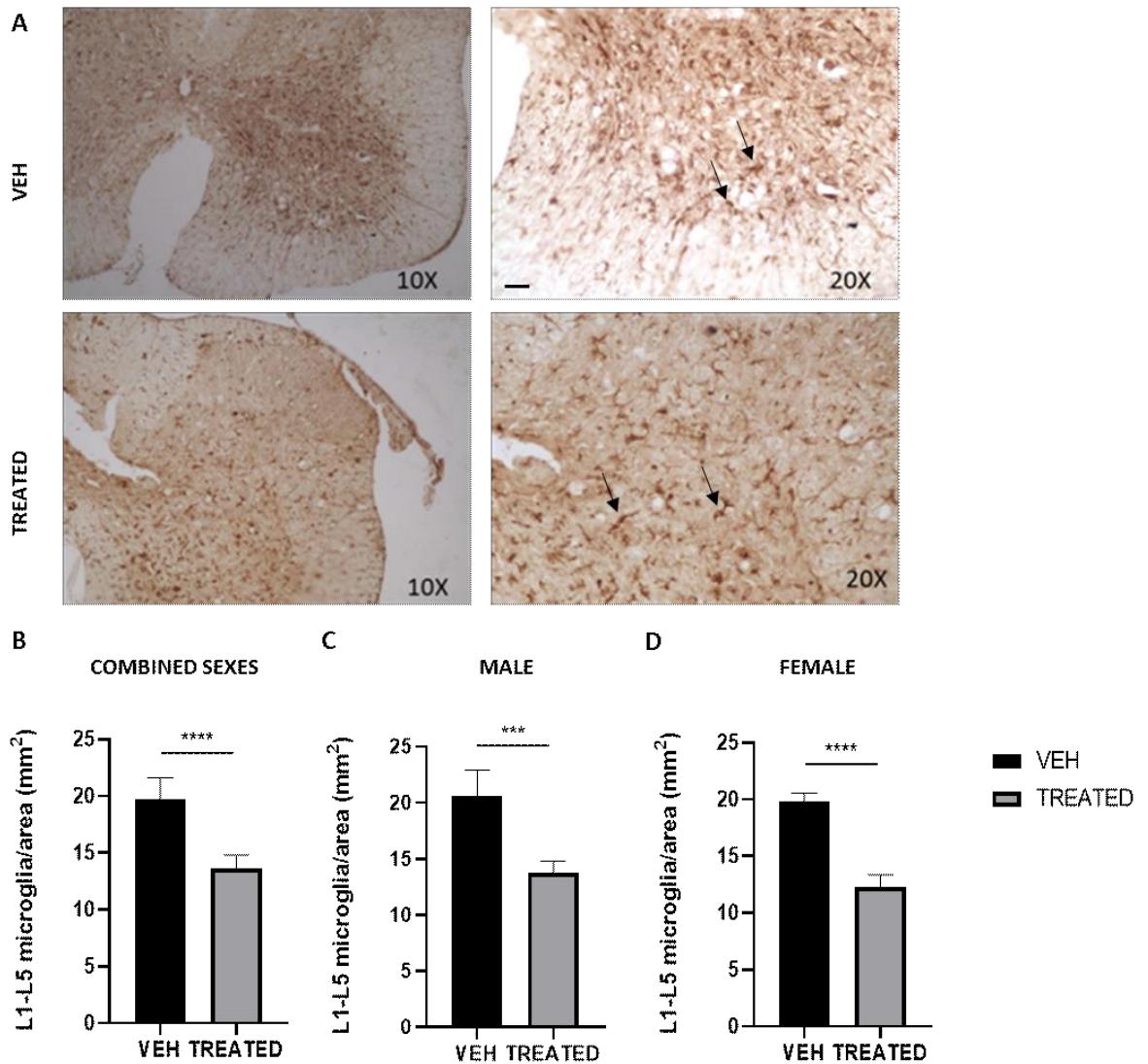
Figure S7.2. Microglia count in the lumbar tract of the spinal cord (L1- L5) of combined sex, male and female SOD1(G93A) mice: (a) Image showing Iba1 positive cells in the L5 segment of VEH and TREATED SOD1(G93A) mice. The arrows indicate visible microglia; (b) The graph shows the number of Iba1 positive cells in the lumbar tract of combined sex. A significant decrease (31%) in microglia count was observed in the TREATED group compared to the VEH (P<0.0001; n= 10 VEH, 12 TREATED); (c) In the male mice, there was a significant decrease in microglia count (33%) in TREATED compared to the VEH (p= 0.0001; n= 5 VEH, 6 TREATED); (d) Female mice also showed a significant decrease (38%) in the number of Iba1 positive cells in the L1-L5 tract in the TREATED concerning the VEH, (p<0.0001; n= 5 VEH, 6 TREATED). Images on the left panel have magnification of 10x, while images on the right panel have a magnification of 20x, scale bar 100μm. Data were analyzed by student t-test, expressed as mean ± SEM. ***P<0.0005; and ****P<0.0001.

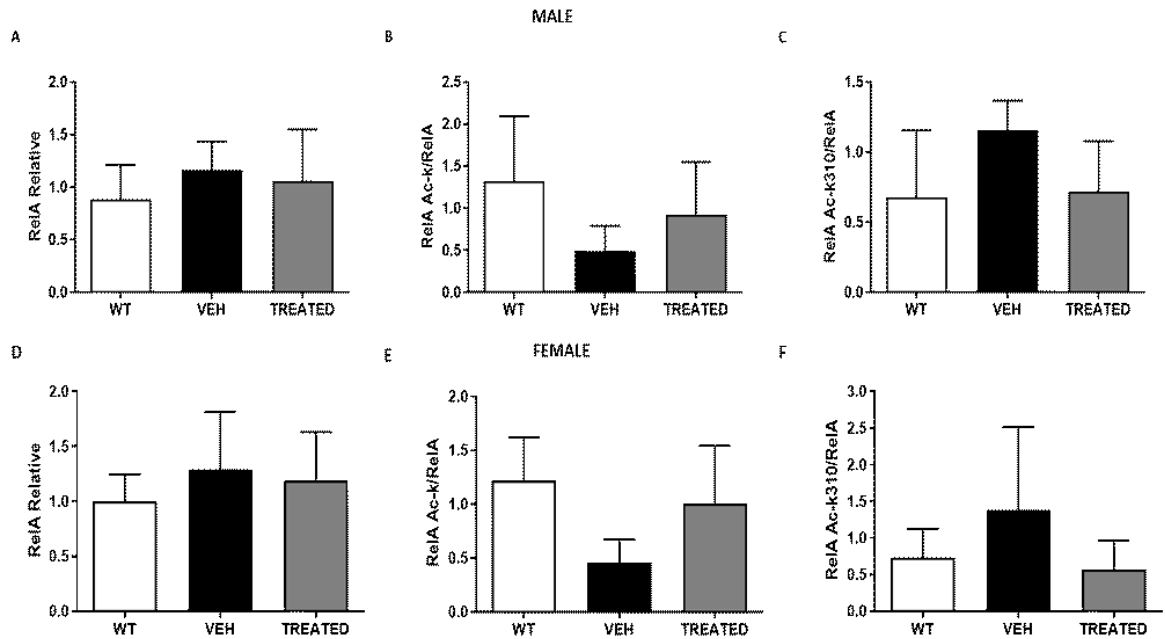**Figure S7.3: Immunoprecipitation of RelA subunits in male and female SOD1(G93A) mice**: (a) An increase in RelA expression in the VEH group of male mice was observed compared to the WT and TREATED animals without statistically significance; (b) A reduction was observed in total lysine (RelA Ac-k) acetylation in the VEH group compared to the WT. This was accompanied by an increase in RelA Ac-k acetylation of male animals in the TREATED group without statistical significance; (c) Furthermore, acetylation of the specific RelA Ac-k310 was increased in the VEH group compared to the WT, while the treatment led to a decrease in acetylation concerning the VEH without significance. (n= 3 WT, 5 VEH, 6 TREATED); (d) In female mice, densitometric analysis shows an increase in RelA expression in the VEH group compared to the WT and TREATED group without statistical significance; (e) RelA Ac-k acetylation was reduced in the VEH group compared to the WT while the acetylation was increased in the TREATED group but without any significance; (f) An increase was observed in the acetylation at K310 in the VEH group of female mice compared to the WT, while the treatment decreased the RelA K310 acetylation compared to the VEH. This was, however, not statistically significant (n= 3 WT, 5 VEH, 6 TREATED). Results were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Graphs are shown as mean ± SEM.
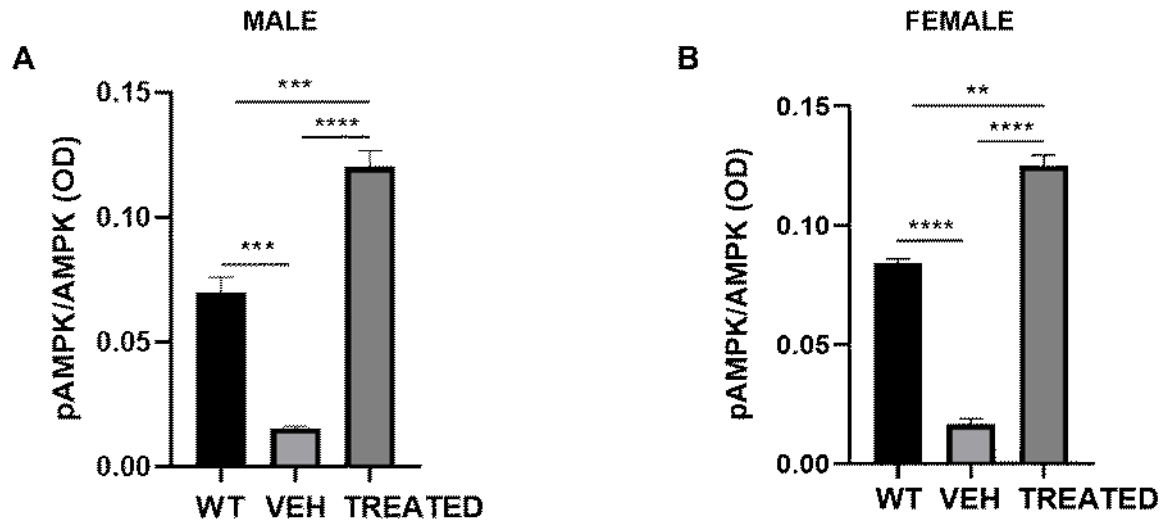
Figure S7.4: Analysis of phosphorylation state of APMK in male and female SOD1(G93A) mice: (a) Phosphorylation state of AMPK male mice was reduced in the VEH compared to the WT with a significance of (p= 0.0002). Animals in the TREATED group showed an increased phosphorylation state with a significance of (p<0.0001) concerning the VEH. A significant increase was also observed in the TREATED mice compared to the WT (p= 0.0003; n= 3 WT, 5 VEH, 6 TREATED); (b) In female mice, a significant reduction (p<0.0001) was observed in the phosphorylation state of AMPK in the VEH group compared to the WT. TREATED mice also displayed increased phosphorylation compared to the VEH with a significance of (p<0.0001). Furthermore, there was a significant increase in the phosphorylation state of TREATED animals compared to the WT (p= 0.0010; n= 3 WT, 4 VEH, 7 TREATED). Results were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Graphs are shown as mean ± SEM. *p<0.05; **p<0.005; ***p<0.0005; and ****p<0.0001.
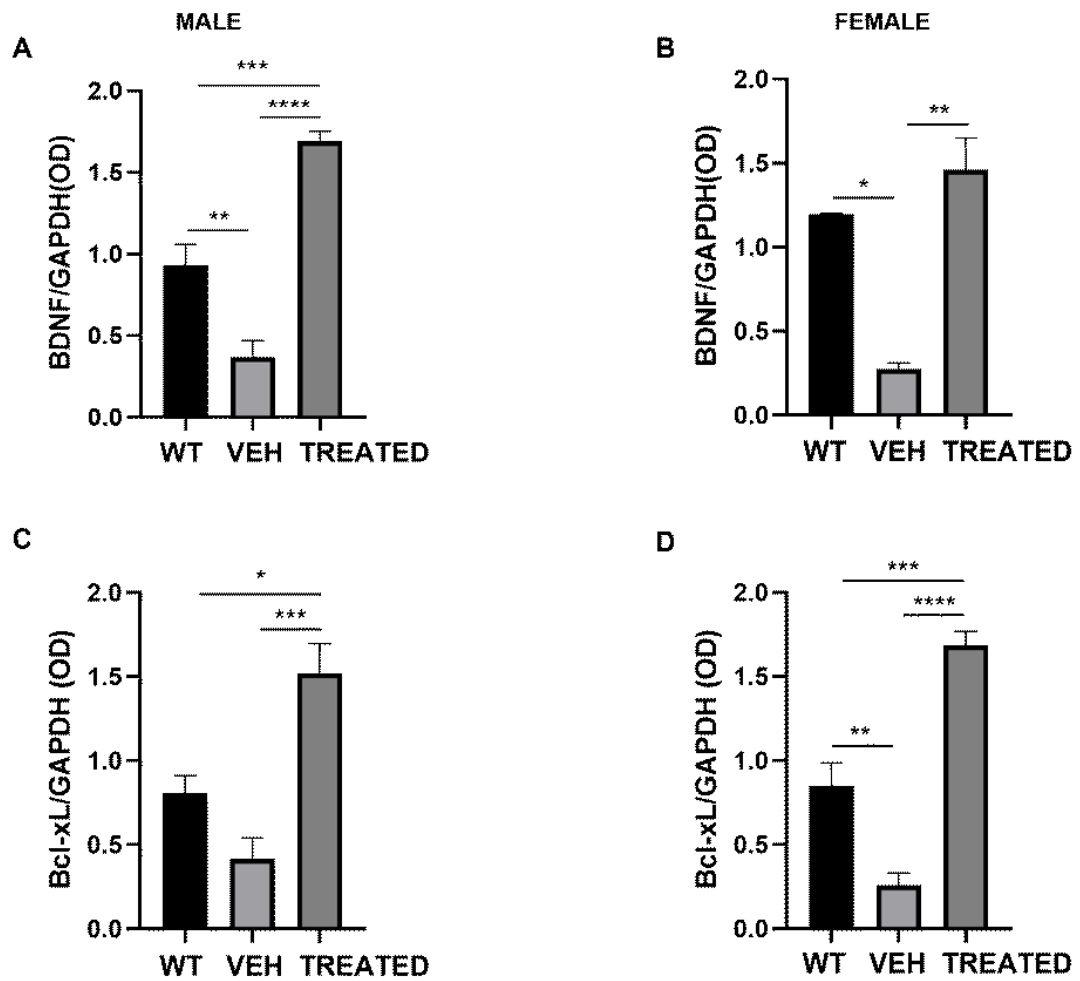
Figure S7.5: Analysis of neurotrophic factor BDNF and anti-apoptotic factor Bcl-xL male and female SOD1(G93A) mice: (a) Expression level of BDNF in males mice was significantly reduced ($p= 0.0049$) in the VEH compared to the WT. TREATED mice displayed an increase in BDNF concerning VEH with a significance of ($p<0.0001$). Similarly, a significant increase was observed in the VEH group compared to the WT, ($p= 0.0003$; n= 3 WT, 5 VEH, 7 TREATED); (b) The neurotrophic factor BDNF in female animals was reduced in VEH mice compared to the WT with the significance of ($p= 0.0365$). The treatment led to an increased expression of BDNF with a statistical significance of ($p= 0.0055$). No significance was observed between WT and TREATED group ($p= 0.6878$); (c) In male animals, the anti-apoptotic protein was reduced in VEH animals compared to the WT without any significance ($p= 0.3497$). Treatment with the epigenetic drugs resulted in an increased expression of Bcl-xL with a significance of ($p= 0.0007$). Likewise, a significant increase was observed in the Bcl-xL expression of the treated animals compared to the WT ($p=0.0400$; n= 3 WT, 5 VEH, 7 TREATED; (d) The anti-apoptotic factor Bcl-xL was significantly reduced in the VEH female animals compared to the WT ($p=0.0097$). In the TREATED

mice, a drastic increase (p<0.0001) in the expression of the protein was observed concerning the VEH Lastly, the treated animals also displayed a significant increase compared to the WT (p= 0.0009; n= 3 WT, 4 VEH, 7 TREATED). Results were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Graphs are shown as mean ± SEM. *p<0.05; **p<0.005; ***p<0.0005; and ****p<0.0001.
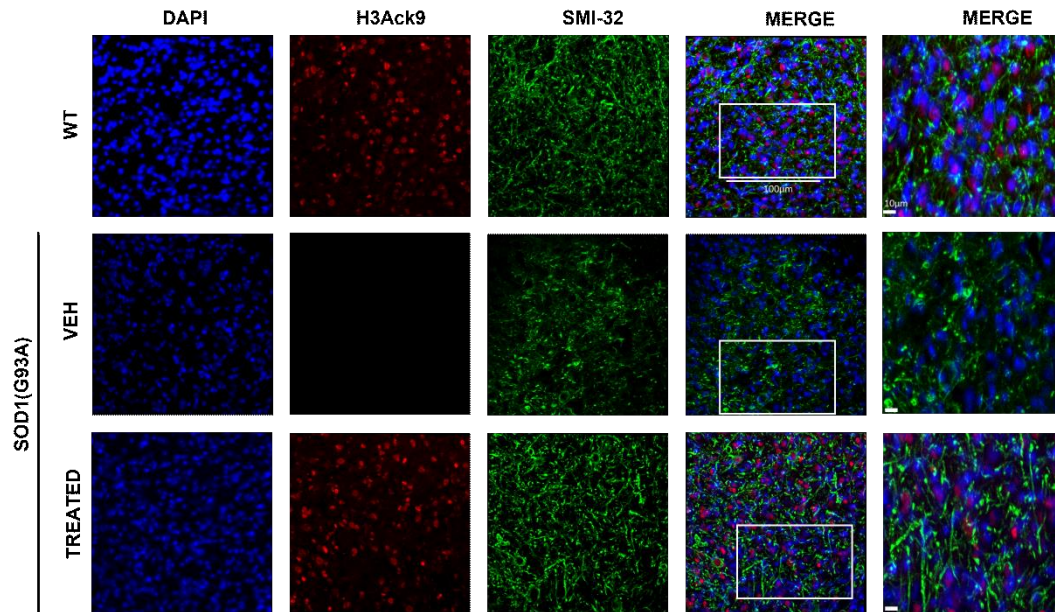
**Figure S7.6: Histone 3 acetylation in the lumbar spinal cord of female WT and SOD1(G93A) mice:** The figure panel shows the different acetylation state of lysine 9 of histone 3 in the lumbar spinal cord of wild type (WT) mice, untreated (VEH) and treated SOD1(G93A) groups. The nuclei were stained in blue with DAPI (a,f,k). The acetylation state of histone 3 was identified by the H3AcK9 antibody in red (b,g, i), while the motor neurons (MN) were detected by identifying the antibody neurofilament H with the SMI-32 antibody in green (c,h,m). The acetylation state of histone 3 was drastically reduced in the VEH group (g) compared to WT animals (b). The treatment with the epigenetic drugs led to a restoration of the acetylation of histone 3 in the treated group (i), (n= 3 WT 5 VEH, 6 TREATED). Figure d, i, n shows the superimposed images of DAPI, SMI-32 and H3AcK9 in the WT, VEH, and treated groups respectively. Magnification 20x, scale bar 100μm (a-d, f-I, and k-n). The figure panel e, j, o shows the highlighted area in d, i, n at higher magnification 40x, scale bar 10μm.

7.4   Discussion

The treatment of SOD1(G93A) mice with the combination VPA and RESV promoted a significant improvement of motor performances, the delay of disease onset, and longer survival.

We observed a dimorphic behaviour of the ALS mouse model in response to the treatment. In males, the combination VPA and RESV exerted a beneficial action in the early phase of the disease, resulting in an onset delay, but not in the later phase. Conversely, in females, the effect of the drug appeared more evident in the later phase of the disease when we detected a reduction of motor deficits and a significant extension of survival.

The beneficial effect of the treatment on motor performances was coupled to a protective effect on MN and a reduced microglial immunoreactivity in the lumbar spinal cord. Moreover, by our previous study employing the combination MS-275 and RESV in SOD1(G93A) mice, we observed an increased expression of the anti-apoptotic protein Bcl-xL and the neurotrophic factor BDNF in the lumbar spinal cord of TREATED animals (Schiaffino et al., 2018). Previous studies reported that the enhancement of Bcl-xL levels protected MN, quenched microglial activation, delayed disease onset, extended survival, and ameliorated motor deficits in SOD1(G93A) mice (W. Li et al., 2015). Interestingly, we previously showed that MS-275 and RESV drive RelA recruitment and H3 acetylation to the Bcl-xL promoter, allowing the expression of the anti-apoptotic factor (Lanzillotta et al., 2013).

Similarly, BDNF promoted MN differentiation and survival (Yuen, 2001). A protective role of BDNF has been observed in a cellular model of ALS (Shruthi et al., 2017). Furthermore, the use of recombinant human BDNF has been shown to improve motor functions in the Wobbler mouse model of ALS (Ikeda et al., 1995). Of note, BDNF was able to promote the anti-apoptotic effect by upregulating Bcl-xL expression *in vivo* (Chao et al., 2011), indicating crosstalk between the two pathways.

By our initial hypothesis, the combination of VPA and RESV reverted the mismatch of RelA acetylation state in the spinal cord of SOD1(G93A) mice by improving the

RelA general acetylation and reducing the acetylation at lys 310. The drugs combination also dramatically enhanced the activation of AMPK, therefore supporting the HATs activity necessary to restore the proper RelA acetylation (Lanzillotta et al., 2013). Moreover, the treatment reverted the histone H3 deacetylation observed in the lumbar spinal cord of SOD1(G93A) mice. Taken together, these results indicate that in the SOD1(G93A) mouse model VPA synergizes with RESV in a similar fashion and with a similar mechanism as was seen with the combination MS-275 and RESV (Schiaffino et al., 2018).

We did not observe any differences among SOD1(G93A) males and females in terms of the effect of the drug on the disease pathology. It is important to notice that the pathology analysis was performed on tissue samples collected from animals at the disease endpoint. It is plausible that possible differences among sexes, occurring earlier in the disease progression and that we missed, could have contributed to the observed dimorphic response to the drugs.

 The reasons underlying the dimorphic effect of the drugs on motor performance and survival remain unknown. One explanation may rely on the different progression of the disease observed in males and females. This study confirmed a sexual dimorphism in the SOD1(G93A) mouse model of ALS (Vegeto et al., 2020). By previous reports, SOD1(G93A) male mice showed earlier motor deficit symptoms and disease onset, as well as slower disease progression than females (Cervetto et al., 2013; Pfohl et al., 2015). Hypothesizing a beneficial effect of the treatment in mild and moderate stages of the pathology, it is conceivable that the drugs were more active on males only in the first phase of the disease, when symptoms were milder.

Another intriguing hypothesis is that the drugs, besides the investigated effect on NF-κB/RelA and histone acetylation, could specifically act on sex-related molecular targets. The molecular bases of sex-dimorphism in ALS are not yet fully understood (Vegeto et al., 2020). Several factors have been suggested to be involved in ALS sex differences, including *C9ORF72* genetic factor (Rooney et al., 2017), mitochondrial function (Cacabelos et al., 2016), and sex hormones. In particular, female sex steroids have been proposed to play an important beneficial

role in ALS, exerting both neuroprotective and anti-inflammatory actions (Vegeto et al., 2020). Interestingly, estrogen receptors (ERs) and aromatase, the enzyme responsible for estrogen synthesis, are expressed in the lumbar spinal cord of adult mice, suggesting that circulating and locally synthesized estrogens could mediate neuroprotection in spinal cord MN. Estrogen efficiently improved locomotor function when administered after spinal cord injury or in ALS (Ji et al., 2017). We speculate that VPA and RESV could potentiate female sex steroids in the SOD1(G93A) mouse model resulting in a sustained function of degenerating MN. RESV is structurally similar to natural and synthetic estrogens and is endowed with estrogenic activity (Qasem, 2020). This occurs *via* multiple mechanisms, including a direct action on ERs, modulation of steroidogenesis, and inhibition of estrogens metabolism that potentially strengthens estrogen action (Qasem, 2020). Moreover, studies on different experimental models indicated that SIRT1 and AMPK, two proteins activated by RESV, are necessary for modulating ER-signaling pathways (J. M. Guo et al., 2017; Hajializadeh & Khaksari, 2021; Yao et al., 2010). Likewise, VPA has been shown to upregulate ERs in cancer and heart tissues (Fortunati et al., 2010; Rabadiya et al., 2018).

Both VPA and RESV have been individually tested against ALS with mixed results (Moges et al., 2009). Oral treatment of SOD1(G93A) male mice with VPA at the antiepileptic dose of 500 mg/kg/day increased lifespan without postponing disease onset (Sugai et al., 2004). In another study, VPA orally administered at antiepileptic dose slowed down MN death but failed in improving lifespan in SOD1(G93A) mice of both sexes (Crochemore et al., 2009). Intraperitoneal administration of VPA twice a day at the dose of 300 mg/kg delayed disease onset, ameliorated motor deficits, and prolonged lifespan in SOD1(G93A) male and female mice (Feng et al., 2008). VPA injected to SOD1(G86R) male mice (250 mg/kg/day, i.p.) postponed disease onset without increasing lifespan (Rouaux et al., 2007). VPA has also been tested in phase II clinical trials at a dose used in epilepsy (1500 mg daily), but it did not provide any beneficial effect on survival or disease progression in ALS patients (Piepers et al., 2009).

Oral administration of RESV (160 mg/kg/day) to SOD1(G93A) male and female mice delayed disease onset, extended life span, and reduced MN neurodegeneration

(Lanzillotta et al., 2013). A lower dose of RESV (25 mg/kg/day) administered with the diet did not promote any beneficial effect in SOD1(G93A) female mice (Markert et al., 2010). On the other hand, intraperitoneal injections of RESV twice a week at the dose of 20 mg/kg delayed disease onset, extended survival, and preserved MN survival in SOD1(G93A) male mice (Han et al., 2012).

Although some of the studies listed above provided beneficial results sometimes comparable to the ones reported in this research, the doses of VPA and RESV administrated individually were several folds higher than the dosage of the molecules in combination. Therefore, the combination of VPA and RESV at very low doses may minimize the dose-related side effects of the drugs. These include the known VPA adverse reactions during long-term treatment, such as tremors, gastrointestinal disturbances, liver toxicity, pancreatitis, and neurological disorders (Loscher, 2002). Furthermore, the fact that VPA and RESV impact on multiple molecular targets, including class I HDACs, SIRT1, AMPK, and possibly sex-related pathways, could reduce off-target effects.

7.5 Conclusion

In conclusion, several factors, including the low doses of the epigenetic drugs, their multi-target activity, and the well-known pharmacology of VPA, make the combination of VPA and RESV worthy of further investigations. Future studies on different ALS models are needed to validate this promising pharmacological treatment and to clarify its mechanism of action. A particular effort is requested to elucidate the effect of these epigenetic drugs on sex-related molecular targets in ALS. Only a better knowledge of these aspects will allow the design of personalized preventive strategies and therapies for the treatment of ALS.

7.6   Bibliography

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