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Overview of impaired BDNF signalling, their coupled downstream serine-threonine kinases and SNARE/SM complex in the neuromuscular junction of the amyotrophic lateral sclerosis model SOD1-G93A mice

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The mice were cared for in accordance with the guidelines of the European Community's Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. All experiments on animals have been reviewed and approved by the Animal Research Committee of the Universitat Rovira i Virgili (Reference number: 0233). Also, animal handling and experimentation were performed in line with approved Institutional Animal Care and Use Committe protocols at the University of Paris Descartes and followed the national authority (Ministère de la Recherche et de la Technologie, France) guidelines for the detention, use and the ethical treatment of laboratory animals based on European Union Directive 2010/63/EU.

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Authors' contributions

Data collection: LJ, EH, VC, MT Quantitative analysis and statistics: LJ, EH Graphical abstract design: LJ Data interpretation: LJ, EH, VC, OB, FC, JT, MAL, NG. Literature search: LJ, EH, VC, JT, MAL, NG. Manuscript preparation: LJ, EH, VC, JT, MAL, NG. Conception and design: JT, MAL, NG. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a chronic neurodegenerative disease characterized by progressive motor weakness. It is accepted that is caused by motoneuron degeneration leading to a decrease in muscle stimulation. However, ALS is being redefined as a distal axonopathy, in that neuromuscular junction dysfunction precedes and may even influence motoneuron loss. In this synapse, several metabotropic receptor-mediated signalling pathways converge on effector kinases that phosphorylate targets crucial for synaptic stability and neurotransmission quality. We have previously shown that, in physiological conditions, nerve-induced muscle contraction regulates the Brain-derived neurotrophic factor/Tropomyosin-related kinase B (BDNF/TrkB) signalling to retrogradely modulate presynaptic protein kinases PKC and PKA, which are directly involved in the modulation of acetylcholine release. In ALS patients, the alteration of this signalling may significantly contribute to functional motor impairment. Here we investigate whether BDNF/TrkB signalling, the downstream PKC (cPKCβI, cPKCα and nPKCε isoforms) and PKA (regulatory and catalytic subunits) and some SNARE/SM exocytotic machinery proteins (Munc18-1 and SNAP-25) are altered in the skeletal muscle of pre- and symptomatic SOD1-G93A mice. We found that this pathway is strongly affected in symptomatic ALS mice muscles including an unbalance between I) BDNF and TrkB isoforms, II) PKC isoforms and PKA subunits and III) Munc18-1 and SNAP-25 phosphorylation ratios. Changes in TrkB.T1 and cPKCβI are precociously observed in presymptomatic mice. Altogether, several of these molecular alterations can be partly associated to the known fast-to-slow motor unit transition during the disease process but others can be related with the initial disease pathogenesis.

KEY WORDS

ALS; TrkB; PKC; PKA, BDNF; Munc18-1; SNAP-25; skeletal muscle; NMJ.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a chronic neurodegenerative disease characterized by progressive motor weakness and loss of muscle innervation originating from selective motoneuron (MN) cell death. Consequently, it causes synaptic dysfunction at the neuromuscular junction (NMJ) as well as in other synapses [1, 2]. Although several cellular processes are altered in ALS patients and mouse models, as glutamatergic excitotoxicity and oxidative stress, the precise pathogenesis of the disease remains unknown. About 90% of ALS cases are sporadic and only the 10% of the cases are familial (FALS) [3]. Genetic studies in FALS patients have identified different mutations in genes including ataxin-2, TDP-43, Fus, and C9ORF72 [4]. However, about the 20% of the cases can be attributed to a mutation-induced misfolding in the Cu^{2+}/Zn^{2+} superoxide dismutase 1 (SOD1), which is a ubiquitously-expressed free-radical defence enzyme [5, 6]. Because of that, the overexpression of human mutant SOD1 in transgenic mice, which mimics the symptoms and progression of the human disease, has been used to try to understand ALS physiopathology.

It is widely accepted that ALS is caused by MN degeneration. However, it has been shown that NMJ degeneration appears before the pathology shows up in animal models [7] and in humans [8]. The loss of a correct nerve-muscle contact contributes to motor impairment (atrophy and paralysis) in different diseases, because of that, the NMJ alteration could be a primary cause for ALS. In this context, many mechanisms explaining the early NMJ alteration have been proposed such as the disruption of anterograde/retrograde axonal transport, genomic and proteomic changes and abnormal cellular metabolism and/or tropism of the cells making synapse [9, 10].

The Brain-derived neurotrophic factor/ Tropomyosin-related kinase B (BDNF/TrkB) neurotrophic signalling is one of the most implicated in the maintenance of synapses and the neuronal survival in the CNS and it is also implicated in NMJ stability and functionality [11–15]. Indeed, several studies have demonstrated that BDNF prevents lesion-induced degeneration of spinal motoneurons or in models of neurodegenerative disease [16–19]. Genetic manipulation of $p75^{NTR}$, a member of the tumor necrosis factor receptor family, and BDNF/TrkB signalling is effective in the treatment of ALS in animal models [20, 21]. Moreover, TrkB.T1 deletion significantly slows the onset of motoneuron degeneration in an ALS mice model [22] while the functional isoform of TrkB receptor

(TrkB.Fl) is much less phosphorylated in ALS spinal cords [23], indicating that it is less functional in the pathology. However, despite these evidences, all preclinical [24] and clinical [25] attempts to module the BDNF/TrkB signalling in the CNS failed to demonstrate beneficial effects on motor neuron survival [26, 27] indicating that further investigation on this signalling pathway is needed. Neuromuscular activity is an essential regulator of BDNF/TrkB signalling in the skeletal muscle, which triggers the activity of presynaptic PKC isoforms (cPKCβI and nPKCε) to adapt neurotransmission through the phosphorylation of key molecules of the synaptic vesicle exocytosis, such as the SM protein Munc18-1 and the SNARE protein SNAP-25 (Besalduch et al., 2010; Hurtado et al., 2017a; Obis et al., 2015a; Simó et al., 2018). However, no information about how the BDNF/TrkB signalling is affected in the skeletal ALS muscle and which role this signalling could play on the primary degeneration of the NMJ has been reported.

Fast-twitching muscles are more affected by ALS than slow-twitching muscles, due their functional properties [32]. Moreover, during ALS progression, fast-twitch muscles undergo a fast-to-slow transition [33, 34] because surviving slow motor neurons sprout over denervated fast-twitch myofibers in an activity-dependent way [34]. Furthermore, extra-ocular muscles, which are known to be the fastest muscles in mammalians, are far less affected than limb and respiratory muscles [35, 36], due to their specific characteristics [32, 37].

All these data evidence that it could be a relation between the activity-dependent BDNF/TrkB/PKC signalling at the muscle level and the ALS phenotype suggesting that its alteration could contribute to a deficient regulation of the presynaptic function. This could decrease the synaptic protection capability and affect the retrograde neuroprotection over the MNs. Because of that, our aim is to investigate how this pathway is (1) affected in the fast-twitch plantaris of pre- and symptomatic ALS mouse model, and (2) differentially expressed in control condition between the ALS-sensitive fast-twitch plantaris and the ALS-resistant slow-twitch soleus. This work will allow us (1) to evaluate how this pathway is altered in an ALS-involved fast-twitch muscle and (2) to link BDNF/TrkB signalling to muscle susceptibility to ALS.

Our results show, firstly, that the full BDNF/TrkB signalling is altered in symptomatic ALS plantaris and some of these changes (i.e. TrkB.T1 isoform) appear precociously altered in presymptomatic muscles. Secondly, BDNF/TrkB signalling molecular pattern is different between control fast-twitch and slow-twitch muscles

reinforcing its potential role in the protection against the ALS-induced neuromuscular denervation. Moreover, we demonstrate that the alterations found in ALS plantaris can be partly explained by the well-known fast-to-slow transition during the disease process, but also by a specific ALS-dependent process, revealed by the increase of mature BDNF and TrkB.T1 levels, which could directly impact the pathogenesis.

MATERIALS AND METHODS

SOD1–G93A mice model

Transgenic male B6/SJL-Tg (SOD1-G93A) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). They were crossed with wild-type B6/SJL females (Janvier, le Genest-Saint-Isle, France) and only littermate males were used in this study. This was done i) to reduce variability in the results and ii) because incidence and prevalence of ALS are greater in men than in women [38]. All mice were kept on the animal facility under standard conditions: constant temperature (22º±2ºC), relative humidity (50±10%) and a 12-hour light/dark schedule. All experimental procedures which included minimizing the number of animals used and their suffering were approved by the policies of the French Agriculture and Forestry Ministry and by the Animal Research Committee of the Universitat Rovira i Virgili following the guidelines of theEU Directive 2010/63/EU for animal experiments.

ALS onset was defined as the time corresponding to the first observation of myotonia symptoms in the mice hind limb (around P90) and the disease progression was assessed by a trained observer that evaluated myotonia symptoms and weighing. Five animals were euthanized at P50 (ALS P50), which represents a presymptomatic stage of the disease and five animals at P115, which represents the end stage of the disease (ALS P115). Five littermates of these animals without the mutation were used as controls (WT P50 and WT P115).

Antibodies

Primary and secondary antibodies used for Western blot and immunohistochemistry (described below) analysis were obtained from different commercial manufacturers and are specified in the Table 1. As a control, primary antibodies were omitted from some samples. These controls never revealed bands of the

appropriate molecular weight nor staining in nonspecific regions. All antibodies specificity has been previously determined [14, 28, 30, 31, 39].

Table 1. List of primary and secondary antibodies used.

Target	*Immunogen*	*Source*	*Reference*	*Dilution*
BDNF	Peptide corresponding to the amino acids 130–247 of the human protein.	Rb pAb	Sc-20981	1/500
NT4	Peptide corresponding to an intern region of the human protein.	Rb pAb	Sc-545	1/500
$p75^{NTR}$	Peptide corresponding to the amino acids 274–425 of the rat protein.	Rb pAb	07-476	1/800
TrkB	Peptide corresponding to the amino acids 37-75 of the human protein.	Ms mAb	Sc-377218	1/1000
pTrkB (Tyr816)	Phosphopeptide corresponding to the sequence containing the Tyr816 near the C-terminus of the rat protein.	Rb pAb	ABN1381	1/1000
PDK1	Peptide corresponding to the amino acids 229-556 of the human protein.	Ms mAb	Sc-17765	1/1000
pDPK1 (Ser241)	Phosphopeptide corresponding to residues around Ser241 of the human protein.	Rb pAb	#3061	1/1000
cPKCα	Peptide corresponding to the C-terminus of the human protein.	Rb pAb	Sc-208	1/800
pcPKCα (Ser657)	Phosphopeptide corresponding to the amino acids 654-663 of the protein.	Rb pAb	06-822	1/1000
cPKCβI	Peptide corresponding to the C-terminus of the human protein.	Rb pAb	Sc-209	1/1000
pcPKCβI (Thr642)	Phosphopeptide corresponding to the residues around the phosphorylation site of Thr642.	Rb pAb	Ab75657	1/1000
nPKCε	Peptide sequence corresponding to the C-terminus of the human protein.	Rb pAb	Sc-214	1/1000
pnPKCε (Ser729)	Phosphopeptide sequence corresponding to the amino acids around the Ser729 of the human protein.	Rb pAb	Sc-12355	1/1000
PKA Cα	Peptide corresponding to the C-terminus of the human protein.	Rb pAb	Sc-903	1/1000
PKA Cβ	Peptide corresponding to the C-terminus of the human protein.	Rb pAb	Sc-904	1/1000
PKA RIα	Peptide corresponding to the amino acids 1-381 (full sequence) of the human protein.	Ms mAb	Sc-136231	1/1000
PKA RIβ	Peptide corresponding to the C-terminus of the human protein.	Rb pAb	Sc-907	1/1000
PKA RIIα	Peptide corresponding to the C-terminus of the mouse protein.	Rb pAb	Sc-909	1/1000
PKA RIIβ	Peptide corresponding to the amino acids 21-110 mapping near the N-terminus of the human protein.	Ms mAb	Sc-376778	1/1000
Munc18-1	Peptide corresponding to residues around Tyr157 of human protein.	Rb mAb	13414	1/1000
pMunc18-1 (Ser313)	Phosphopeptide corresponding to amino acids 307-319 (internal sequence) containing the Ser313 of the human protein.	Rb pAb	Ab138687	1/1000
SNAP-25	Peptide corresponding to residues surrounding Gln116 of human protein.	Rb mAb	#5309	1/1000
pSNAP-25 (Ser187)	Phosphopeptide corresponding to amino acids around Ser187 of the rat protein.	Rb pAb	Ab169871	1/1000
pSNAP-25 (Thr138)	Phosphopeptide corresponding to residues around Thr138 from the human protein.	Rb pAb	Orb163730	1/1000
ChAT	Human placental enzyme (full sequence)	Gt pAb	AB144	1/800
Secondary antibodies	**Anti-rabbit conjugated HRP**	Dk pAb	711-035-152	1/10.000
	Anti-mouse conjugated HRP	Rb pAb	A9044	1/10.000
	Anti-goat conjugated Alexa fluor 568	Dk pAb	A-11057	1/500

Western blotting

Mice muscles were dissected and frozen in liquid nitrogen and homogenized using a manual homogenizer in ice-cold lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, sodium orthovanadate 1; NP-40 1%, Triton X-100 0.1% and protease inhibitor cocktail 1% (Sigma-Aldrich, Saint Louis, MO, USA). Protein lysates were obtained collecting supernatants after removing insoluble materials by

centrifugation at 4ºC and aliquots were stored at -80ºC. Protein concentrations were determined by DC protein assay (Bio-Rad, Hercules, CA, IL).

Protein samples of 30 µg were separated by 8% or 12% SDS-polyacrylamide electrophoresis and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Hybond™-P; Amersham, GE Healthcare) using Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA). For immunodetection, membranes were blocked with Tris-buffered saline 0.1% Tween 20 (TBST) containing 5% (W/V) phosphoblocker or Bovine Serum Albumin (BSA) for phosphorylated proteins and non-fat dry milk for non-phosphorylated proteins for an hour. Then, membranes were incubated in primary antibody (Table 1) overnight and with a corresponding secondary antibody horseradish peroxidase-conjugated for one hour. Membranes were revealed with Bio-Rad ECL kid on the Plus and ChemiDoc XRS+ machine (Bio-Rad, Hercules, CA). The bands optical density was normalized in relation to: (1) the background values; and to (2) the total protein transferred on PVDF membranes, measured by total protein analysis (Sypro Ruby protein blot stain, Bio-Rad [40].

The relative variations between ALS and WT plantaris were calculated from the same membrane image, and the same was done for soleus and tibialis WT muscles. Data was taken from densitometry measurements made in at least three separate western blots for each of the five animals in each group. To simplify data expression, the normalized value of the bands representing the control (both P115 and P50) animals were adjusted to 1 and the bands representing ALS animals were calculated in relation with the respective control ones.

Immunohistochemistry

The spinal cord of P115 mice (n=5 in each group) was dissected, post-fixed in 4% PFA and rinsed 2 times in PBS azide 0.01% buffer. The L1 to L5 lumbar region of the spinal cord was sectioned with a vibrating blade microtome (VT-1000S, Leica Microsystems SAS, Nanterre, France) at 50 µm thickness. One out of every six sections was subsequently processed for immunostaining on free-floating sections (an average of 7 sections per animal were studied). The immunohistochemical analysis was based on detection of choline acetyltransferase (ChAT) to stain motoneurons (Table 1). Moreover, DAPI was also used to stain cell nuclei. Sections were mounted in Vectashield mounting

medium (Vector Laboratories, Burlingame, CA, USA) and collected with a CMOS camera (ORCA Flash 2.8, Hamamatsu Photonics France, Massy, France) mounted on a Zeiss AxioObserver microscope (Z1, Carl Zeiss SAS, Le Pecq, France) using the ZEN 2012 software (Carl Zeiss SAS). The staining specificity was checked by performing the incubation in the absence of the primary antibodies. All counts were performed using the ZEN 2012 software (Carl Zeiss SAS).

Statistical analysis

All values are expressed as means ± standard deviation (SD) within each group. Statistical significance of the differences between WT and ALS groups was evaluated under a non-parametric Kruskal-Wallis test followed by Dunn's post hoc test. On the other hand, statistical significance of the differences between WT muscles were evaluated using the Friedman test and Bonferroni correction (Graph Pad Prism Software, San Diego, USA). All the data presented in this study were considered as statistically different when the statistical power exceed 95%. The criterion for statistical significance was: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

RESULTS

Firstly, we analysed total and phosphorylated protein levels of representative molecules of the BDNF-operated transmitter release regulation in the fast plantaris muscle (one of the most affected in ALS) of presymptomatic (50 days-old) and symptomatic (115 days-old) ALS mice. The entire neurotrophic pathway has been studied to its three molecular levels: i) the neurotrophins BDNF and NT4 and their receptors TrkB and $p75^{NTR}$ ii) the coupled serine-threonine kinases (PKC isoforms) and priming kinase -PDK1- and the different subunits of the cAMP-dependent kinase (PKA) and iii) PKC and PKA targets related with neurotransmitter release. In addition to the figures showing quantitative data (Figures 1-4), Figure 6A (changes in P50) and 6B.1 (P115) shows a schematic representation of the main results.

Neurotrophins and receptors

To investigate how the BDNF signalling is affected in ALS mice, we compared mature BDNF (mBDNF) and proBDNF protein levels between WT and ALS mice in plantaris muscles from symptomatic (P115) and presymptomatic (P50) mice. We used an anti-BDNF antibody raised against a region present in both proBDNF (32 kDa) and mBDNF (14 kDa) [41]. Results showed that mBDNF is significantly increased in P115 ALS mice (3-fold) without changes in proBDNF levels (Figure 1A). In presymptomatic mice, both forms of BDNF are unchanged. Moreover, neurotrophin-4 (NT4) is also increased ~ 3-fold in P115 ALS mice and, in this case, a significant increase is observed since P50 (Figure 1A).

Then, we analysed the BDNF/NT4 receptors, $p75^{NTR}$ and TrkB, both expressed in WT skeletal muscle. Alternative splicing of TrkB generates full-length receptors (TrkB.Fl) with strong survival effects for nervous cells and truncated receptors (TrkB.T1) without intracellular tyrosine kinase domain. We used an anti-TrkB antibody raised against a peptide sequence shared by both TrkB.Fl (145-150 kDa) and TrkB.T1 (95-100 kDa). Our results show that $p75^{NTR}$ and TrkB.Fl are significantly decreased in about a half in ALS P115 while TrkB.T1 is significantly increased. As a result, the Fl/T1 ratio decreases about 2/3 in ALS mice at P115. Interestingly, TrkB.T1 is already increased in ALS P50, without changes in $p75^{NTR}$ nor TrkB.Fl, therefore also the Fl/T1 ratio is similarly decreased at P50 (Figure 1B; Table 2A – first line).

Since TrkB signalling starts with the TrkB.Fl phosphorylation [42], we next analysed it and found that pTrkB.Fl (Y816) is decreased in about a half in P115. However, the ratio pTrkB.Fl/TrkB.Fl is not affected due to the similar decrease of TrkB.Fl. A moderate but significant decrease of pTrkB.Fl is yet observed at P50 (Figure 1B; Table 2A – first line). Taken together, these results confirm that the neurotrophic pathway is altered in ALS plantaris muscles, with an increase in mBDNF and NT4 expression and a decrease in TrkB-F1 activation, probably since the presymptomatic stage, due to the precocious increase of TrkB.T1. The alteration of neurotrophic receptors and neurotrophins could decrease their intracellular signalling.

Serine-threonine kinases

TrkB.Fl (Y816) phosphorylation triggers the gamma phospholipase C (PLCγ) signalling pathway, which sequentially activates PKCs [43]. Because of that, we next analysed the protein levels of representative serine-threonine kinases: the ubiquitous cPKCα and the presynaptic cPKCβI and nPKCε and also the PKA subunits. All of them have been functionally related to the BDNF/TrkB receptor complex in the presynaptic component of the NMJ [28, 30, 31, 39, 44, 45].

PKC

Total cPKCα protein levels increase without affecting the phosphorylated form at P115 (Figure 2A). Moreover, cPKCβI decreases while pcPKCβI increases in P115 ALS muscles. In contrast, at P50 only pcPKCβI significantly decreases (Figure 2B). Finally, nPKCε and pnPKCε decrease both at P115 and P50 (Figure 2C). This indicates that ALS differently affects the three PKC isoforms in plantaris muscles. When considering the ratio "phosphorylated/total protein" of these kinases, we observed that at P50, pcPKCα/cPKCα, pcPKCβI/cPKCβI and pnPKCε/nPKCε do not change, suggesting that at the presymptomatic stage the potential efficacy of the kinase activity is barely affected. On the other hand, at P115, pcPKCβI/cPKCβI increases more than four-fold while pnPKCε/nPKCε and pcPKCα/cPKCα show a moderate decrease (Table 2B – first line).

PKC maturation includes three phosphorylation steps and the first one is mediated by phosphoinositide-dependent kinase-1 (PDK1). Previous results showed that PDK1 is exclusively located in the nerve terminal of the NMJ and that synaptic activity enhances pPDK1 (S241) in the membrane, where it realizes its function [30]. Here, we found that PDK1 increases about 2.5-fold and pPDK1 decreases about 1/3 at P115 while an increase of 0.5-fold is observed in both molecules at P50 (Figure 2D). Thus, this result points to an imbalance of the pPDK1 and its substrate pcPKCβI that highlights the affectation of cPKCβI in symptomatic ALS and suggest PDK1-independent modulation of the cPKCβI phosphorylation.

PKA

Regarding the PKA, the regulatory subunits do not change at P50 whereas the catalytic subunits Cα and Cβ increase moderately. However, at P115, Cα significantly increase (two-fold, Figure 3A). Moreover, RIα significantly decreases (about 2/3) accompanied by a significant increase of the RIIβ subunit (of two-fold) (Figure 3B).

These results suggest a change in the pattern of activation of the PKA pathway, which could modulate downstream phosphorylation targets implicated in synaptic function.

SNARE/SM proteins

We next analysed different representative targets of PKC and PKA closely related with the exocytotic machinery: the regulatory SM family protein, Munc18-1 and the SNARE protein, SNAP-25.

Munc18-1

Munc18-1, is a neuron-specific member of the Sec1/Munc18 protein family (SM family) that binds to syntaxin-1 and regulates the formation of the SNARE/SM complex [46–52]. Moreover, it has two targets for PKCs (Serine 306 and 313) that change its conformation to enhance exocytosis [53–57].

Results show a significant increase of pMunc18-1 (of more than two-fold) in P115 ALS plantaris, whereas total Munc18-1 does not change. Therefore, pMunc18-1/Munc18-1 ratio increases. These results indicate that in symptomatic ALS mice, Munc18-1 phosphorylation is enhanced, resulting in the upregulation of the synaptic exocytotic machinery. This points out the complex dysregulation of this molecule at the end stage of the disease, as they do not change at P50 (Figure 4A).

SNAP-25

SNAP-25 is one of the three SNARE proteins of the core fusion machine implicated in vesicle exocytosis. PKA-mediated phosphorylation of SNAP-25 at T138 controls the size of the releasable vesicle pools and PKC-mediated phosphorylation at S187 regulates pool refilling after they have been emptied [58, 59].

SNAP-25 levels decrease at P50 but it recovers at P115. pSNAP-25 S187 and T138 increase (almost two-fold in both cases) at P115 while they are unaffected at P50 (Figure 4B). When considering the ratio "phosphorylated/total protein", the data show an increase both at P50 (the ratios pSNAP-25 S187/SNAP-25 and pSNAP-25 T138/SNAP-25 are increased: 2.28 ± 0.43 and 2.38 ± 0.26 respectively, $P<0.05$ in both cases) and at P115 (due to pSNAP-25 S187 and T138 increase) (Table 2D - first line). This indicates a good phosphorylating efficacy of both PKC and PKA on SNAP-25 along the disease progression which suggests that the changes in the kinases modulate SNAP-25

phosphorylation to influence neurotransmission in ALS plantaris muscles at the end stage of the disease.

Differential expression of the BDNF/TrkB/PKC signalling in slow and fast-twitching muscles

Considering the preferential degeneration of the fast motor units in ALS progression and the fast-to-slow transition of the muscle phenotype, we wondered whether the above exposed molecular changes at the end stage of the disease could be related to this transition, or, by the contrary, be specific of ALS. To assess this, we compared slow-twitching muscles with fast-twitching ones in WT animals. This allowed us to relate the values of slow WT muscles with the fast ALS ones.

Table 2A sums up the differential pattern of the neurotrophic pathway. The WT slow soleus muscle has lower levels of mBDNF, TrkB.Fl and pTrkB.Fl than the WT fast plantaris muscle. Also, in comparison, soleus has less cPKCβI and pcPKCβI and more nPKCε (Table 2B). PKA catalytic and regulatory subunits Cα, RIα and RIIβ levels are higher in WT soleus (Table 2C). Finally, there is less Munc18-1, pMunc18-1, SNAP-25 and pSNAP-25 (T138) but more pSNAP-25 (S187) in the soleus muscle (Table 2D). In addition to the WT plantaris, we included WT tibialis as a control in the comparisons. Interestingly, the value of some molecules in this muscle are different from the plantaris (see Table 2A-D) which could be attributed to specific muscle fiber composition and should be further investigated.

Thus, there are evident differences in the molecular pattern of the studied signalling pathway between WT fast and slow muscles. In the Table 2, the protein values in the ALS plantaris that become similar to the values in the WT soleus muscles are indicated in blue. This similarity may be related to the fast-to-slow transition caused by ALS and may represent the part of the pathway that works to prevent the complete loss of NMJ. On the contrary, the protein values that are different in ALS plantaris and WT soleus muscles or that go in another direction when compared with the plantaris WT are highlighted in yellow. This dissimilarity may be related not with the fast-to-slow transition but with another ALS mechanism closely related with the cause of the disease. Figure 6B.2 shows a diagrammatic representation of these results.

Table 2. Protein levels in different muscles in relation with WT Plantaris. (A) Differential pattern of the TrkB signalling molecules. **(B)** PDK1 and PKC isoforms. **(C)** PKA catalytic and regulatory subunits. **(D)** SNARE/SM proteins Munc18-1 and SNAP-25. The numerical data show the mean ± SD of the considered molecules in ALS plantaris, WT soleus and WT tibialis in relation with WT plantaris muscle, whose value has been normalized to 1. In blue, the molecules that in the ALS plantaris muscles become similar to the values in the WT soleus. In yellow, the molecules that in the ALS plantaris muscles are different from WT soleus. The molecules that do not change are in white. Significant differences between the muscles and WT plantaris are indicated with *; significant differences between WT soleus and WT tibialis are indicated with #; significant differences between ALS plantaris and WT soleus are indicated with +. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant). Statistical significance was evaluated with the non-parametric Kruskal-Wallis test followed by Dunn's post hoc test and with the Friedman test followed by a Bonferroni correction.

A

	Neurotrophins and neurotrophin receptors							Ratios			
	proBDNF	**mBDNF**	**NT4**	**p75^{NTR}**	**TrkB.T1**	**TrkB.FL**	**pTrkB.FI (Y816)**	**mBDNF/ proBDNF**	**mBDNF/ NT4**	**pTrkB.FI (Y816)/ TrkB.FI**	**TrkB.FI/ TrkB.T1**
ALS Plantaris	**1,06** ± 0,20 ns	**3,01** ± 0,29 ***	**3,22** ± 0,04 ***	**0,53** ± 0,13 ***	**2,16** ± 0,24 ***	**0,58** ± 0,19 ***	**0,52** ± 0,14 ***	**2,84** ± 0,59 ***	**0,94** ± 0,09 ns	**0,89** ± 0,37 ns	**0,27** ± 0,09 **
WT Soleus	**1,05** ± 0,31 ns ns ns	**0,57** ± 0,27 * # +++	**1,89** ± 0,03 *** ns +++	**1,11** ± 0,03 ns ns +++	**0,92** ± 0,06 ns ns +++	**0,63** ± 0,04 ** ## ns	**0,19** ± 0,01 *** ### ns	**0,55** ± 0,28 * # ++	**0,30** ± 0,15 *** ns +++	**0,30** ± 0,03 *** ## ++	**0,68** ± 0,06 ** ns ++
WT Tibialis	**0,97** ± 0,18 ns	**1,08** ± 0,01 ns	**2,37** ± 0,23 ***	**0,99** ± 0,27 ns	**1,11** ± 0,10 ns	**1,06** ± 0,20 ns	**0,99** ± 0,31 ns	**1,11** ± 0,20 ns	**0,46** ± 0,04 **	**0,93** ± 0,34 ns	**0,95** ± 0,20 ns

B

	Downstream Protein Kinase C (PKC) signaling								Ratios			
	PDK	**pPDK (S241)**	**cPKCα**	**pcPKCα (S657)**	**cPKCβI**	**pcPKCβI (T641)**	**nPKCε**	**pnPKCε (S729)**	**pPDK (S241)/ PDK**	**pcPKCα (S657)/ cPKCα**	**pcPKCβI (T641)/ cPKCβI**	**pnPKCε (S729)/ nPKCε**
ALS Plantaris	**2,38** ± 0,13 ***	**0,62** ± 0,11 **	**1,33** ± 0,08 **	**0,87** ± 0,15 ns	**0,36** ± 0,29 ***	**1,57** ± 0,09 **	**0,88** ± 0,14 *	**0,62** ± 0,14 **	**0,26** ± 0,05 ***	**0,65** ± 0,12 *	**4,37** ± 3,60 **	**0,71** ± 0,17 *
WT Soleus	**1,35** ± 0,09 ns ns +++	**0,99** 0,26 ns ns ns	**1,19** ± 0,01 ns ns ns	**0,61** ± 0,03 ** ns ns	**0,15** ± 0,11 *** ## ns	**0,51** ± 0,22 *** ns +++	**1,65** ± 0,32 *** ns +++	**0,77** ± 0,06 ns ns ns	**0,74** ± 0,20 ns ns +	**0,51** ± 0,03 *** ns ns	**3,43** ± 2,96 * # ns	**0,46** ± 0,10 ** ns ns
WT Tibialis	**1,19** ± 0,02	**0,91** ± 0,21	**1,08** ± 0,38	**0,83** ± 0,18	**0,60** ± 0,31	**0,77** ± 0,03	**1,48** ± 0,06	**1,01** ± 0,28	**0,77** ± 0,18	**0,77** ± 0,32	**1,28** ± 0,65	**0,69** ± 0,19

	ns	ns	ns	ns	**	ns	***	ns	ns	ns	ns	ns	*

C

	Protein Kinase A (PKA) Subunits					
	PKA Cα	**PKA Cβ**	**PKA RIα**	**PKA RIβ**	**PKA RIIα**	**PKA RIIβ**
ALS Plantaris	**2,06** ± 0,47 ***	**0,94** ± 0,17 ns	**0,20** ± 0,01 ***	**0,74** ± 0,12 ns	**1,28** ± 0,17 ns	**1,58** ± 0,25 ***
WT Soleus	**2,04** ± 0,07 *** ### ns	**1,13** ± 0,43 ns # ns	**2,40** ± 0,56 *** ### +++	**1,15** ± 0,01 ns ns +	**0,79** ± 0,09 ns ns ns	**1,98** ± 0,07 ** ns ns
WT Tibialis	**0,85** ± 0,00 ns	**0,68** ± 0,14 *	**1,13** ± 0,58 ns	**1,64** ± 0,28 *	**0,76** ± 0,19 ns	**1,44** ± 0,13 *

D

	SNARE/SM proteins							
						Ratios		
	Munc18-1	**pMunc18-1 (S313)**	**SNAP-25**	**pSNAP-25 (S187)**	**pSNAP-25 (T138)**	**pMunc18-1 (S313)/ Munc18-1**	**pSNAP-25 (S187)/ SNAP-25**	**pSNAP-25 (T138)/ SNAP-25**
ALS Plantaris	**1,07** ± 0,13 ns	**2,33** ± 0,49 ***	**1,07** ± 0,24 ns	**1,84** ± 0,06 ***	**1,95** ± 0,35 ***	**2,18** ± 0,53 *	**1,73** ± 0,39 *	**1,82** ± 0,52 *
WT Soleus	**0,75** ± 0,12 ns ns ns	**0,54** ± 0,11 *** ### +++	**0,75** ± 0,17 ns ns ns	**2,31** ± 0,06 *** ### ns	**0,35** ± 0,21 *** ### +++	**0,72** ± 0,18 * ns ++	**3,08** ± 0,69 *** ### +++	**0,46** ± 0,29 ** ### +++
WT Tibialis	**1,02** ± 0,13 ns	**1,07** ± 0,14 ns	**0,95** ± 0,10 ns	**1,13** ± 0,01 ns	**1,68** ± 0,32 ***	**1,05** ± 0,19 ns	**1,19** ± 0,13 ns	**1,77** ± 0,39 *

Note: Table 2 needs to be in colour.

Motoneuron loss in P115 ALS mice spinal cord

It is described that ALS involves loss of MNs, preferentially the larger ones innervating faster muscle fibers that are the ones that change their phenotype [33, 34]. In the context of the present study, we confirmed this selective loss of larger-fast MN. We analysed the changes in the percentage of fast (larger) and slow (smaller) MN in the spinal cord of the symptomatic ALS mice (115 days-old) compared with WT animals (Figure 5). When analysing soma's area, we observed an increase of the 300-900 μm^2 MN (mainly slow) from 25% to 40% whereas the percentage of higher of 900 μm^2 MN (mainly fast) diminishes from 25% to 15%. Thus, in the studied spinal cord of ALS mice, the proportion of small (and therefore mainly slow) MN increasees.

DISCUSSION

Neurotrophic dysfunction at the NMJ influences its stability and may contribute to motor impairment in ALS muscles. The present results show that ALS disease strongly alters in the plantaris muscle, even in presymptomatic but mainly in symptomatic animals, protein and phosphorylation levels of many molecules of the putative BDNF-NT4/TrkB-$p75^{NTR}$/PKC-PKA/SNARE-SM pathway, which is essential to modulate NMJ maintenance and promote neurotransmission. These complex changes are represented in the intuitive graphic of the Figure 6 and, despite that the relations between molecular changes may not be evident, we discuss their meaning that I) may be specific of ALS pathogenesis or II) can be explained by the fast-to-slow fiber transition.

The TrkB signalling

The functional isoform of TrkB receptor is TrkB.Fl. However, the truncated TrkB.T1 is the predominant form in mammalian skeletal muscle [14]. It affects cellular viability when it is over-expressed in artificial or pathological situations [60] and regulates negatively TrkB.Fl, consequently affecting its signalling.

ALS patients have increased BDNF and NT4 in skeletal muscle and spinal cord and decreased pTrkB.Fl [23, 61]. Our results confirm the neurotrophin increase (NT4 even in presymptomatic mice), the decrease of pTrkB.Fl (since P50 and due to the loss of total TrkB.Fl protein levels in symptomatic mice) and additionally, the great TrkB.T1

increase in the plantaris skeletal muscle in both stages. The early deregulation of TrkB alternative splicing -yet in presymptomatic animals- may lead to an impaired neuromuscular function which could underlie motoneuron loss. This suggestion is supported by experiments in which TrkB.T1 deletion in mutant SOD1 mice delays the onset of the disease, slows down the motoneuron loss and improves mobility tests results at the end stage of the disease compared with normal mutant SOD1 mice [22]. In addition, the deletion of TrkB.T1 increases neuromuscular function and nerve-evoked muscle contraction [60]. Altogether, it can be suggested that the over-presence of TrkB.T1 limits BDNF and NT4 effect by hijacking it and prevents its action through TrkB.Fl, with a direct impact on the signal transduction, despite being overproduced. Because of that, it seems that the increase of BDNF and NT4 in ALS is an insufficient compensatory mechanism to promote neuronal survival of injured motoneurons because of the lack of TrkB.Fl available, as it has been already proposed in other studies [22]. Also, $p75^{NTR}$ is strongly related with cell death and neurodegeneration in the adult nervous system. In fact, different results have found that under high doses of neurotrophines, $p75^{NTR}$ acquires a proapoptotic role which goes through the activation of caspase 3 [20, 62]. Therefore, it seems to be also directly related with ALS process despite of its total levels being decreased.

Enhancement of BDNF signalling may have a great potential in therapy for neurological disorders like ALS, due to its strong pro-survival effects through TrkB and $p75^{NTR}$ in developing and injured MN [25, 63, 64]. However, intrathecally administered BDNF did not show significant effects on motor function and survival in ALS patients [65] or autonomic nervous system function [66]. We show here that ALS not only shows a timely dysregulation of the ligands but also of the receptors which suggest an early alteration of the alternative splicing of the TrkB.

In summary, the neurotrophic signalling that under normal conditions guarantees the stability and functionality of the NMJ through synaptic activity [14] is highly and precociously affected in ALS. As a result, the long term compensatory increase of neurotrophins is not sufficient due to the TrkB.T1 dominance since the presymptomatic stage of the disease.

PKC and PKA in ALS muscle

TrkB.Fl stimulates the PLCγ, which activates PKC [14, 42, 43, 67]. Therefore, when TrkB.Fl is downregulated (as it happens in ALS), the PKC activation may be decreased or lost. Here, we analysed three PKC isoforms (α, βI and ε; the two last are exclusive of the presynaptic site) which are upregulated by synaptic activity and muscle contraction through BDNF/TrkB signalling [14, 28, 31] to control the neuromuscular function [14, 29].

However, in ALS symptomatic muscles, we show (see Figure 5A) that the phosphorylation of these isoforms do not change (cPKCα), increase (cPKCβI) or even decrease (nPKCε). Thus, a relevant modification in the normal balance of the βI and ε isoform activity can be intuited. Interestingly, the pcPKCβI increase is accompanied with a reduction in total cPKCβI protein and the pnPKCε decrease occurs in parallel with a reduction in nPKCε, thus, increasing the evidences of the dysregulation between PKC isoforms. Moreover, cPKCα increases and, therefore, the ratio pcPKCα/cPKCα is reduced. Therefore, whereas the ratio phosphorylated/non-phosphorylated form of α and ε isoforms are reduced and not modified respectively in the ALS symptomatic muscle, this ratio strongly increases for βI isoform. The normally constitutive upstream kinase for PKCs (PDK1) is highly altered in ALS muscles (Figure 5A) and may influence in the changes of phosphorylation of the PKC isoforms. All these results suggest an increased activity and consumption of cPKCβI whereas nPKCε activity decreases. Both kinases regulate the neuromuscular transmission (Obis et al., 2015; Hurtado et al., 2017) and, therefore, their imbalance could directly affect it. In accordance, our results in the ALS plantaris muscle show that the imbalance of the levels of PKC isoforms coincide with a significative increase in the protein levels of PKC-mediated phosphorylations of Munc18-1 and SNAP-25 (in the Ser-313 and Ser-187 respectively; see later), indicating a dysregulation of the exocytotic synaptic machinery. The modification of the normal balance of PKC isoforms and PDK1 have already been observed at the presymptomatic ALS muscle. Changes at P115 for nPKCε are already found at P50, while pPDK1 and pPKCβI modifications are opposed to the ones occurred at P50 suggesting their profound and complex alteration in the progression of the disease.

The changes in the balance of the presynaptic PKC isoforms may be related with the imbalance of the TrkB isoforms. However, it could be suggested that other presynaptic metabotropic receptors related with PLCγ or PLCβ (such as adenosine receptor A_1 and

muscarinic receptor M_1) may contribute to the selective modulation of different PKC isoforms in the muscle, and this must be investigated because their activity is related with TrkB and PKC [12, 68, 69].

Neurons affected by ALS have high Ca^{2+} concentrations, which have been related with apoptosis induction due to ion imbalance [70, 71] and sustained calcium-dependent PKC activation [72]. Also, immunohistochemical analyses have reported a decrease of PKCs in spinal cord motoneurons affected by ALS, which has been associated with a selective degeneration of the largest motoneurons [73]. These changes are in accordance with the decrease of cPKCβI and nPKCε that we found in the symptomatic skeletal muscle.

On the other hand, the increase of the catalytic PKA isoforms (both at P50 and P115) together with maintenance of the total count of RI and RII regulatory isoforms could be the reason why its target pSNAP-25 T138 is increased in symptomatic ALS mice. This could be due to the impossibility of RIα to modulate PKA activity in this situation, as it is known that it works as a buffer to modulate it in normal conditions [74, 75]. These results coincide with previous studies done in central nervous system, where total PKA was increased not only in mice and rats but also in human patients [76, 77] therefore contributing to the changes in neurotransmission that occur in ALS muscles.

Exocytotic synaptic proteins in ALS muscle

cPKCβI, nPKCε and PKA subunits regulate the neuromuscular synapse [14, 39]. Therefore, their changes may influence neurotransmission in ALS. In the presymptomatic phase, endplate potentials (EPP) amplitude and their quantal content is increased, suggesting an abnormal upregulation in Ca^{2+} levels in the nerve terminals [78]. In spite of the important changes in neurotrophin and kinases signalling described, only SNAP-25 expression is reduced to the half at P50. However, the ratios pSNAP-25 S187/SNAP-25 and pSNAP-25 T138/SNAP-25 are high, indicating a good phosphorylating efficacy of both PKC and PKA on SNAP-25. These data, in concordance with unchanged levels of pMunc18-1 in this presymptomatic stage indicate a good operation of vesicle release to support the high quantal content in presymptomatic stage.

At the same time that the disease progresses and the large MN die, the EPPs amplitude and quantal release is reduced [79, 80]. This phenomenon could be in part due

to the fast-to-slow transition because the small and slow MN generate EPP with smaller quantal content than big and fast MN [81]. In fact, the ALS plantaris muscle at P115 show a significative increase in the protein levels of pSNAP-25 S187 in a similar way to the slow WT soleus. However, pSNAP-25 T138 and pMunc18-1 S313 are also really increased contrarily to soleus. Therefore, the ratio phosphorylated/total protein of the three molecules is very high in ALS P115, maybe because the remaining motoneurons generate bigger amounts of pMunc18-1 and pSNAP-25 to maintain neurotransmission but they accumulate and do not do their function.

Fast-to-slow transition in the ALS fast-twitching muscles

The molecular changes observed in end-phase ALS mice may be related either with the cause of the disease, the consequence or a combination of both. Motoneuron loss occurs in the ventral horns of the symptomatic ALS animals mainly affecting medium and large somas and muscle phenotype changes in parallel with it. In the fast-twitch muscles (like the plantaris), there is a significant fast-to-slow transition from type II fibers to type I fibers and, within the type II fiber population, from type IIb/IIx to IIa fibers [33, 34]. In accordance, a fat mass reduction and weight loss, together with altered energy metabolism has been observed both in animal models and human patients [82]. These changes occur before the first motor symptoms in mice [83] and have been related with a switch of the source of energy of the cells from carbohydrates to lipids [84]. Some of the changes we observed at P115 resemble the molecular pattern of slow muscles like the wild soleus (Table 2 and Figure 6B.2, in blue), which could be a side effect of the fast-to-slow transition. Some molecular differences between WT soleus (slow) and WT plantaris (fast) observed in this study can be related with the differences in quantal content of transmitter release, that is approximately a 30-40% higher in fast-twitch muscles than in slow-twitch muscles contributing to the higher safety factor in the fast NMJ [81, 85]. In the slow WT soleus, low levels of pSNAP-25 (T138) may be related with a small releasable pool of vesicles whereas the high level of pSNAP-25 (S187) may be related with constant refilling after the pools have been emptied to sustain tonic stimulation of the slow muscle during extended day use [58]. As stated, a high level of pSNAP-25 S187 similar to the soleus is observed in the P115 ALS plantaris.

On the other hand, there are several molecules in the ALS plantaris muscles with a very different value not only from the WT plantaris muscles but also from the WT

soleus muscles (Table 2 and Figure 6B.2, in yellow). These molecular changes may represent an adaptative effort to go beyond the fast-to-slow transition trying to ameliorate the increasing impairment of the neuromuscular function. Because the described molecular pattern in the P115 ALS plantaris muscles belongs to the more resistant motor units, which have survived the course of the disease process, it can be speculated that the survival capacity may be linked with these independent changes. Decreased levels of $p75^{NTR}$ could indicate less proapoptotic activity, whereas elevated pcPKCβI, low PKA RIα (inhibitory) and elevated values of pMunc18-1 and pSNAP-25 (T138), may be adapted to improve function and favour transmitter release and neurotransmission.

Finally, some of the molecular changes observed during the disease progression could be directly related with the etiology of ALS. Between many possible causes, a genetic (or epigenetic, in sporadic ALS) change in the free-radical defense enzyme SOD1 could be detrimental for $p75^{NTR}$ and TrkB expression and turnover in MNs or NMJs (as shown here) and impair neuromuscular activity.

CONCLUSIONS

In ALS disease, NMJ degeneration appears before MN death, suggesting that the loss of a correct nerve-muscle contact could be a primary cause for ALS. One of the most important mechanisms involved in NMJ stability is the BDNF-NT4/TrkB neurotrophic signalling, which suffers several changes in the pre- (P50) and symptomatic (P115) SOD1-G93A mice model's plantaris muscle. The main changes are the misbalance between i) neurotrophins (BDNF and NT4), the different TrkB receptor isoforms and the $p75^{NTR}$ receptor, ii) their coupled PKC isoforms themselves (presynaptic cPKCβI and nPKCε) and their upstream priming kinase PDK1, iii) the PKA catalytic and regulatory subunits and iv) the targets Munc18-1 and SNAP-25 phosphorylation. The increase of NT4 and TrkB.T1 and the decrease of the PKC isoforms already occurs in presymptomatic mice. The molecular pattern observed in symptomatic ALS plantaris muscles may be partially explained by the fast-to-slow fiber transition, which affects the motor units of the fast-twitching muscles during the progression of the disease (like pSNAP-25 S187 upregulation, which is normal in slow WT muscles). However, other

molecular changes such as elevated pMunc18-1 and pSNAP-25 (T138), may represent an adaptative effort to ameliorate the increasing impairment of the neuromuscular function.

The precocious and sustained increase of TrkB.T1 along with the decrease of the functional receptor TrkB.FL, and the unbalance of pcPKCβI and pnPKCε, seems to be specific of the ALS physiopathology and may be involved in the initial course of the disease. These molecular changes may deregulate the presynaptic function and decrease the retrograde neurotrophic protection over MNs. Further characterization of over-expressing wild-type SOD1 mice would help to discern possible changes induced by increased SOD1 activity. Moreover, imposed physical training could help to identify those molecular changes that could be prevented by activity and evaluate their therapeutic potential.

LIST OF ABBREVIATIONS

ALS, amyotrophic lateral sclerosis; BDNF, brain derived neurotrophic factor; MN, motoneuron; NMJ, neuromuscular junction; NT4, neurotrophin-4; $p75^{NTR}$, p75 neurotrophin receptor; PDK1, phosphoinositide-dependent kinase-1; PKA, protein kinase A; PKC, protein kinase C; PLCγ, gamma phospholipase C; SM, Sec1/Munc18-like; SNAP-25, Synaptosomal-associated protein 25; SNARE, SNAP (Soluble NSF Attachment Protein) receptor; TrkB, tropomyosin-related kinase B receptor; WT, Wild Type.

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LEGENDS TO THE FIGURES

Figure 1. BDNF, NT4 and receptors in plantaris muscles of ALS mice at P50 and P115. Panels **(A-B)** show Western blot bands and quantification. **(A)** ALS disease increases NT4 yet at P50, and mBDNF at P115 but never affects proBDNF. **(B)** ALS disease increases TrkB.T1 yet at P50 and decreases $p75^{NTR}$, TrkB.Fl and pTrkB.Fl at P115. Statistical significance was evaluated under a non-parametric Kruskal-Wallis test followed by Dunn's post hoc test. Data are mean percentage ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (n=5; 3 repeats).

Figure 2. cPKCα, cPKCβI, nPKCε and PDK1 in plantaris muscles of ALS mice at P50 and P115. Panels **(A-D)** show Western blot bands and quantification. **(A)** cPKCα increases at P115. **(B)** cPKCβI decreases at P115 while pcPKCβI decreases at P50 and increases at P115. (**C**) nPKCε and nPKCε decrease at P50 and P115. (**D**) PDK1 increases yet at P50 but pPDK1 decreases at P115. Statistical significance was evaluated under a non-parametric Kruskal-Wallis test followed by Dunn's post hoc test. Data are mean percentage ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (n=5; 3 repeats).

Figure 3. Catalytic and regulatory PKA subunits in plantaris muscles of ALS mice at P50 and P115. Panels **(A-B)** show Western blot bands and quantification. **(A)** Catalytic Cα increases yet at P50 and Cβ is increased only at P50. **(B)** Regulatory RIα decreases and RIIβ increases at P115 without any change in the other subunits. Statistical significance was evaluated under a non-parametric Kruskal-Wallis test followed by Dunn's post hoc test. Data are mean percentage ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (n=5; 3 repeats).

Figure 4. The SNARE/SM Munc18-1 and SNAP-25 in plantaris muscles of ALS mice at P50 and P115. Panels **(A-B)** show Western blot bands and quantification. **(A)** pMunc18-1 increases at P115. **(B)** SNAP-25 is decreased only at P50; pSNAP-25 (S187) and (T138) increase at P115. Statistical significance was evaluated under a non-parametric Kruskal-Wallis test followed by Dunn's post hoc test. Data are mean percentage ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (n=5; 3 repeats).

Figure 5. Motoneurons in the spinal cord of WT and ALS mice at P115. Panel **(A)** shows MN stained with ChAT in a WT mice (right) and in a SOD1-G93A mice (left). The bar indicates 50 μm. Panel **(B)** shows the proportion of large (fast) and small (slow) MN in the spinal cord of the symptomatic (115 days-old) SOD1-G93A mice compared with the WT ones at the same days old. The percentage of 300-900 μm^2 MN (slow) increase in the ALS spinal cord whereas the percentage of >900 μm^2 MN (mainly fast) diminishes. Statistical significance was evaluated under a non-parametric U Mann-Whitney test followed by Holm-Sidak post hoc test. Data are mean percentages ± SD, *** $p < 0.001$. (n=5; 6 sections per n)

Figure 6. Overview of the molecular changes in the ALS muscles at P50 and P115. (A and B.1) In red, the molecules that are decreased; in green, the ones that are increased and in white the ones that are not changed. In **(A)** the molecular changes at P50 are represented. Most molecules are unaffected despite some changes begin to appear. In **(B.1)** the molecular changes at P115 are represented. There are profound alterations in the BDNF/TrkB receptor complex, in their coupled serine-threonine kinases and in the main related synaptic vesicle fusion protein targets. **(B.2)** In blue, the molecules that would have followed a fast-to-slow transition (and, therefore, resemble WT soleus) and in yellow the ones that have not (different to WT soleus), at P115. This dissimilarity may indicate an adaptation of the motor units beyond the fast-to-slow transition in the ALS context. Alternatively, they may represent a molecular alteration related to the primary cause of the disease. The molecules that do not change are indicated in white. In **(A, B.1 and B.2)** the black arrow indicates the normal downstream signalling, from the receptors to the exocytotic machinery going through the different serine-threonine kinases.