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ORIGINAL ARTICLE

Running and swimming prevent the deregulation of the BDNF/TrkB neurotrophic signalling at the neuromuscular junction in mice with amyotrophic lateral sclerosis

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Abstract

Nerve-induced muscle contraction regulates the BDNF/TrkB neurotrophic signalling to retrogradely modulate neurotransmission and protect the neuromuscular junctions and motoneurons. In muscles with amyotrophic lateral sclerosis, this pathway is strongly misbalanced and neuromuscular junctions are destabilized, which may directly cause the motoneuron degeneration and muscular atrophy observed in this disease. Here, we sought to demonstrate (1) that physical exercise, whose recommendation has been controversial in amyotrophic lateral sclerosis, would be a good option for its therapy, because it normalizes and improves the altered neurotrophin pathway and (2) a plausible molecular mechanism underlying its positive effect. SOD1-G93A mice were trained following either running or swimming-based protocols since the beginning of the symptomatic phase (day 70 of age) until day 115. Next, the full BDNF pathway, including receptors, downstream kinases and proteins related with neurotransmission, was characterized and motoneuron survival was analysed. The results establish that amyotrophic lateral sclerosis-induced damaging molecular changes in the BDNF/TrkB pathway are reduced, prevented or even overcompensated by precisely defined exercise protocols that modulate TrkB isoforms and neurotransmission regulatory proteins and reduce motoneuron death. Altogether, the maintenance of the BDNF/TrkB signalling and the downstream pathway, particularly after the swimming protocol, adds new molecular evidence of the benefits of physical exercise to reduce the impact of amyotrophic lateral sclerosis. These results are encouraging since they reveal an improvement even starting the therapy after the onset of the disease.

Keywords ALS · Skeletal muscle · Exercise · NMJ · Neurotransmission · Motoneuron loss

Abbreviations

ALS	Amyotrophic lateral sclerosis
BDNF	Brain-derived neurotrophic factor
NMJ	Neuromuscular junction
NT4	Neurotrophin-4
$p75^{NTR}$	p75 neurotrophin receptor
PDK1	Phosphoinositide-dependent kinase-1
PKA	Protein kinase A
PKC	Protein kinase C
SM	Sec1/Munc18-like
SNAP-25	Synaptosomal-associated protein 25
SNARE	SNAP (Soluble NSF Attachment Protein) receptor
TrkB.T1	Truncated tropomyosin-related kinase B receptor
TrkB.FL	Full-length tropomyosin-related kinase B receptor
WT	Wild type

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Introduction

Amyotrophic lateral sclerosis (ALS) is the most frequent chronic motoneuron disease characterized by progressive motor weakness, atrophy and selective motoneuron loss. In spite of the frequency of the disease, its pathogenesis remains unknown, though neuromuscular junction (NMJ) degeneration and synaptic molecular alterations precede and may be responsible for the motoneuron loss [1–4]. Abnormal muscle cell metabolism and trophism, synaptic molecular changes and axonal transport disruption have been proposed to explain NMJ alteration in ALS [4–9]. These and other evidences [10] indicate that skeletal muscles promote a retrograde signalling pathway necessary to keep the synapses and, consequently, motoneurons healthy.

The brain-derived neurotrophic factor/tropomyosin-related kinase B (BDNF/TrkB) signalling, which is modulated by muscle activity [11–14], is one of the most implicated in the maintenance of neurons and synapses in the brain, but also in the NMJ of the peripheral nervous system [12, 15–18]. There are different isoforms of this receptor which are generated by alternative splicing. It results in full-length receptors (TrkB.FL) with strong survival effects for nervous cells and truncated receptors (TrkB.T1), without intracellular tyrosine kinase domain. At the NMJ, the ratio between TrkB.FL and TrkB.T1 modulates the BDNF triggering of a signalling pathway that influences presynaptic protein kinase C (PKC) activity and, therefore, the acetylcholine release by affecting the phosphorylation state of different proteins directly related with neurotransmission such as Munc18-1 and SNAP-25 [12, 19–21]. Recently, we have demonstrated that this signalling is perturbed in SOD1-G93A mice muscles even before disease onset, especially regarding neurotrophin levels and neurotrophin receptors misbalance [4]. Interestingly, the suppression of the expression of both p75 neurotrophin receptor ($p75^{NTR}$) [22], which is a low affinity transmembrane receptor, and TrkB.T1 [23, 24] BDNF receptors is advantageous in ALS in animal models.

There are many evidences that BDNF is involved in exercise-mediated neuroprotective actions in the brain [25–28]. Therefore, it also may play a role in the benefits derived from moderate exercise in ALS, when it can trigger its signalling properly. However, despite increasing evidences that sustained moderate exercise is neuroprotective in ALS [29–34], its recommendation is still controversial, probably due to differential effects depending on exercise types, protocols and intensities [32, 35–37] that induce a differential activation of motoneuron subpopulations [38, 39], being the small motoneuron innervating slow muscle fibres more resistant to ALS [40–42].

Here, we aimed to investigate the molecular effect of two well-defined training protocols. On the one hand, we use a running-based training which is a low-amplitude and frequency exercise that preferentially triggers slow motor units integrated by small motoneurons, and on the other hand, a swimming-based training which is a high-amplitude and frequency exercise that, in addition, recruits fast motor units integrated by large motoneurons [39, 43]. We hypothesize that accurately defined physical exercise protocols can prevent the profound alterations observed in presymptomatic and symptomatic ALS muscles in the BDNF/TrkB downstream pathway, directly involved in the acetylcholine release modulation to avoid the development of the disease [4].

Results show that many changes from the ALS molecular phenotype are reduced or prevented by the swimming- and running-based training protocols. In addition to unveil the differences among the protocols, this study adds new evidence that exercise is beneficial until the late stages of ALS disease and reveals the molecular changes that are not prevented by training, pointing to a central role of the BDNF/TrkB downstream signalling in the pathogenesis of the disease.

Materials and methods

Animals

Transgenic male B6SJL-Tg(SOD1*G93A)1Gur/J (Stock No. 002726) mice from The Jackson Laboratory (Bar Harbor, ME, USA) were crossed with wild-type (WT) B6/SJL females (Janvier, le Genest, France) and only male descendants were used for comparison (1) to reduce variability in the results because these mice present gender differences in the onset of the disease, life expectancy and response to exercise [37, 44] and (2) because incidence and prevalence of ALS are greater in men than in women [45]. Mice were kept in the animal facility under standard conditions: constant temperature (22 ± 2 °C), relative humidity ($50 \pm 10\%$), a 12-h light/dark schedule and unrestricted access to food and water, 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 experimental procedures which included minimizing the number of animals and their suffering were reviewed and approved by the Animal Research Committee of the Universitat Rovira i Virgili and by the Institutional Animal Care and Use Committee protocols of 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.

ALS onset was defined as the time corresponding to the first observation of myotonia symptoms in the mice hind limb and the disease progression was assessed by a trained observer who evaluated myotonia symptoms and weighing. All the experiments using mice were performed in a blind systematic fashion to minimize bias.

Training protocol

ALS mice were trained from 70 until 115 days of age, 30 min a day, 5 days a week as previously described [43]. Five ALS mice were submitted to a moderate running-based training on a speed-regulated treadmill (max.13 m min^{-1}) (Run ALS) which is a low-amplitude and frequency exercise that preferentially triggers slow motor units integrated by small motoneurons; and five ALS mice were submitted to a high-frequency and amplitude swimming-based training in an adjustable-flow swimming pool (max.5L min^{-1}) (Swim ALS) which is a high-amplitude and frequency exercise that also recruits fast motor units integrated by large motoneurons [43]. These groups were compared to five Untrained ALS mice and five Untrained WT mice that only displayed an exploratory activity during the training time of the first and second groups. Sample size was calculated using previously established criteria [46, 47] to optimize the number of animals used.

Western blotting

The animals were euthanized 4 h after the training was complete (P115). Then, the plantaris muscles were dissected and immediately frozen in liquid nitrogen. The plantaris muscles were used because they are fast-twitching extensors of the ankle that are preferentially affected in ALS due to the vulnerability of the fast IIb fibres in ALS. Therefore, the plantaris muscles are a good model to study skeletal muscle degeneration in this disease [48]. Muscles from both hind limbs of the same animal were processed and analysed together.

Western blotting procedure was performed as previously described [12, 19, 49]. Primary and secondary antibodies conjugated to horseradish peroxidase (HRP) are specified in Table 1. Primary antibodies were omitted from some samples during the procedure as controls, and they did not reveal bands of the appropriate molecular weight. All antibodies specificity had been previously determined [12, 20, 21, 49, 50].

Immunohistochemistry

The spinal cord of the P115 mice was dissected after the animals were anaesthetized by intraperitoneal injection of 3.5% chloral hydrate and perfused transcardially with buffered saline and 4% paraformaldehyde. Then, the spinal cords were post-fixed in 4% PFA and rinsed two times in PBS azide 0.01% buffer. The L1–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

Table 1 List of primary and secondary antibodies used

Target	Source	Reference	Dilution	Target	Source	Reference	Dilution
BDNF	Rb pAb	Sc-20981	1/500	PKA Cβ	Rb pAb	Sc-904	1/1000
NT4	Rb pAb	Sc-545	1/500	PKA RIα	Ms mAb	Sc-136231	1/1000
$p75^{NTR}$	Rb pAb	07-476	1/800	PKA RIβ	Rb pAb	Sc-907	1/1000
TrkB	Ms mAb	Sc-377218	1/1000	PKA RIIα	Rb pAb	Sc-909	1/1000
pTrkB (Tyr816)	Rb pAb	ABN1381	1/1000	PKA RIIβ	Ms mAb	Sc-376778	1/1000
PDK1	Ms mAb	Sc-17765	1/1000	Munc18-1	Rb mAb	13414	1/1000
pDPK1 (Ser241)	Rb pAb	#3061	1/1000	pMunc18-1 (Ser313)	Rb pAb	Ab138687	1/1000
cPKCα	Rb pAb	Sc-208	1/800	SNAP-25	Rb mAb	#5309	1/1000
pcPKCα (Ser657)	Rb pAb	06-822	1/1000	pSNAP-25 (Ser187)	Rb pAb	Ab169871	1/1000
cPKCβI	Rb pAb	Sc-209	1/1000	pSNAP-25 (Thr138)	Rb pAb	Orb163730	1/1000
pcPKCβI (Thr642)	Rb pAb	Ab75657	1/1000	ChAT	Gt pAb	AB144	1/800
nPKCε	Rb pAb	Sc-214	1/1000	HRP-conjugated	Dk a-Rb pAb	711-035-152	1/10.000
pnPKCε (Ser729)	Rb pAb	Sc-12355	1/1000	HRP-conjugated	Rb a-Ms pAb	A9044	1/10.000
PKA Cα	Rb pAb	Sc-903	1/1000	Alexa fluor 568	Dk a-Gt pAb	A-11057	1/500

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) and involved the measurement of at least 100 motoneurons per animal and ≥ 3 animals per group.

Statistical analysis

All values are expressed as mean ± standard deviation (SD) within each group. Statistical significance of the differences between the experimental groups was evaluated under a non-parametric Kruskal–Wallis test followed by Dunn's post hoc test (GraphPad Prism software, San Diego, USA). The criterion for statistical significance was: $*p<0.05$, $**p<0.01$, and $***p<0.001$.

Results

Previous results show that the BDNF-NT4/TrkB signalling at the NMJ is strongly affected (at its three levels of study: diffusible molecules and receptors, targeted kinases and phosphorylated exocytotic proteins) in SOD1-G93A mice plantaris muscles since the presymptomatic stage of the disease [4]. Nerve-induced muscle contraction regulates this neurotrophic signalling to retrogradely modulate neurotransmission [12, 51] and protect NMJ and motoneurons [16]. Therefore, here we aimed to investigate whether running and swimming-based training protocols could prevent the molecular misbalances to provide evidence that physical exercise is effective to slow down ALS progression in SOD1-G93A, in accordance with the improved survival and reduced phenotype severity found by Deforges et al., 2009 [38].

Neurotrophins and TrkB signalling

In concordance with previous results, in Fig. 1a, we show that mBDNF and neurotrophin-4 (NT4) are significantly increased in ALS muscles. The running protocol reduces, while swimming fully prevents, this strong increase of mBDNF. However, NT4 is even more upregulated in trained ALS animals than in Untrained ALS mice. Additionally, proBDNF remains unchanged after both training. Therefore, the mBDNF/proBDNF ratio decreases in Run and is fully normalized in Swim ALS animals, whereas the mBDNF/NT4 ratio is strongly reduced in both training protocols (Fig. 1a).

We next analysed the receptors $p75^{NTR}$ and TrkB (TrkB.T1 and TrkB.FL). Figure 1b shows that both protocols significantly increase $p75^{NTR}$ in relation with Untrained ALS mice to achieve Untrained WT values. Moreover, running fully prevents TrkB.T1 increase and TrkB.FL and pTrkB.Fl decrease. On the other hand, swimming normalizes TrkB.Fl and pTrkB.FL, but does not reduce TrkB.T1 (Fig. 1b). Consequently, running maintains the pTrkB.FL/TrkB.FL ratio and recovers the TrkB.FL/TrkB.T1 ratio, while swimming reduces the pTrkB.FL/TrkB.FL ratio and partially recovers the TrkB.FL/TrkB.T1 ratio, in relation to the Untrained WT values.

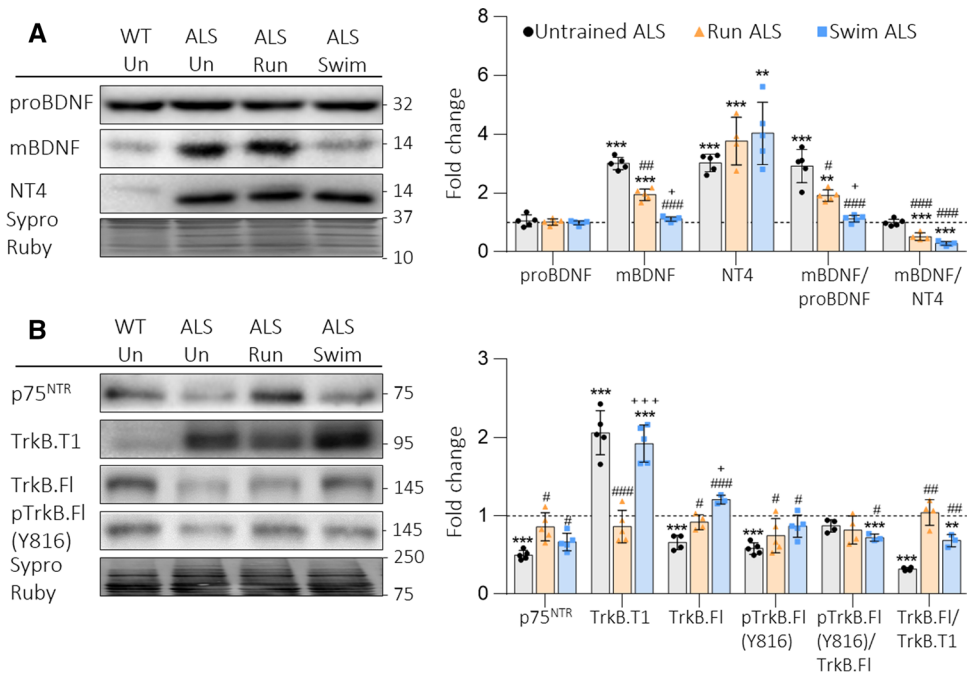

Fig. 1 BDNF, NT4 and receptors in plantaris muscles of trained ALS mice. **a** Run reduces and swim normalizes upregulated mBDNF levels, while any of them normalizes NT4. proBDNF is not modified. **b** Run completely avoids all the alterations present in Untrained ALS animals. Swim avoids protein alterations except TrkB.T1. Data are mean value ± SD, $*p<0.05$, $**p<0.01$, $***p<0.001$. *Versus Untrained WT mice; #versus Untrained ALS; +versus Run ALS mice

Altogether, the first conclusion is that the two training protocols have differential influence over molecular changes of ALS to maintain the protein levels inside the control values to limit disease progression. Interestingly, the alterations that the trainings could not prevent are the ones that were already found at the presymptomatic stage [4].

Serine-threonine kinases

We also investigated how exercise affects the ubiquitous cPKCα, the exclusive presynaptic cPKCβI and nPKCε, the priming kinase phosphoinositide-dependent kinase-1 (PDK1) (Fig. 2) and the different catalytic and regulatory subunits of the protein kinase A (PKA) (Fig. 3). Both protocols increase above the Untrained WT value the total levels of cPKCα, cPKCβI and nPKCε, independently of their affectation in Untrained ALS. Phosphorylated pcPKCα, which is unaffected in Untrained ALS, is decreased after running and so it is the pcPKCα/cPKCα ratio, indicating that cPKCα is downregulated. On the other hand, in Swim ALS, the pcPKCα/cPKCα ratio also decreases because of total protein increase (Fig. 2a). Regarding cPKCβI, swimming reduces pcPKCβI increased levels and normalizes the extremely high pcPKCβI/cPKCβI ratio. In Run ALS it is also fully normalized because of total protein increase (Fig. 2b). Finally, nPKCε and pnPKCε increase proportionally due to training. Because of that, the pnPKCε/nPKCε ratio does not change in relation with Untrained ALS but, as there is more nPKCε available, its activity can increase (Fig. 2c). These changes in the balance of PKC isoforms may be related with the imbalance of the TrkB isoforms directly or through the effect of other presynaptic metabotropic receptors that

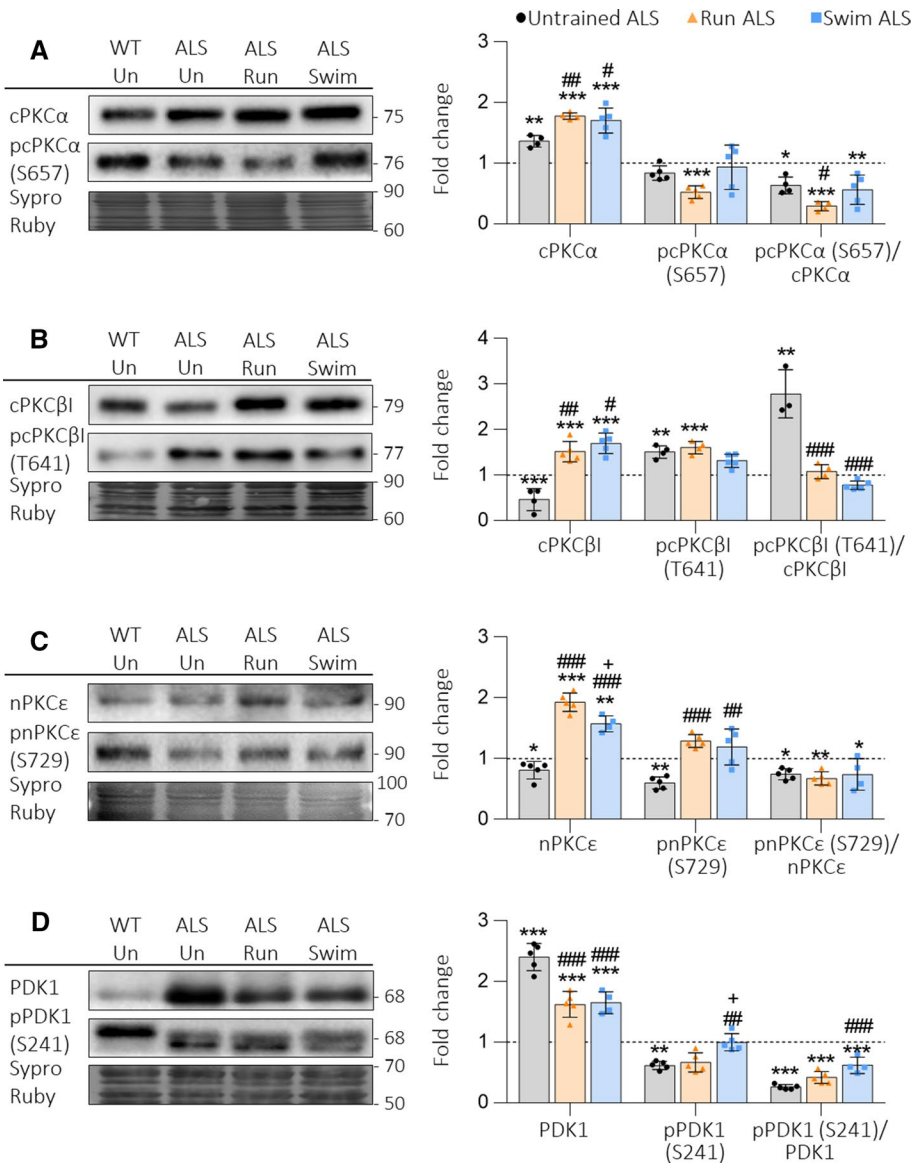

Fig. 2 cPKCα, cPKCβI, nPKCε and PDK1 in plantaris muscles of trained ALS mice. **a–c** Physical activity increases total PKC levels in ALS animals, the values being always above Untrained WT and ALS values. Regarding the phosphorylation ratios, only the pcPKCβI/cPKCβI is normalized. **d** Neither running nor swimming normalizes PDK1 or pPDK1 levels. Consequently, also the ratios are still decreased as in Untrained ALS animals. Data are mean value ± SD, $*p<0.05$, $**p<0.01$, $***p<0.001$. *Versus Untrained WT mice; #versus Untrained ALS; +versus Run ALS mice

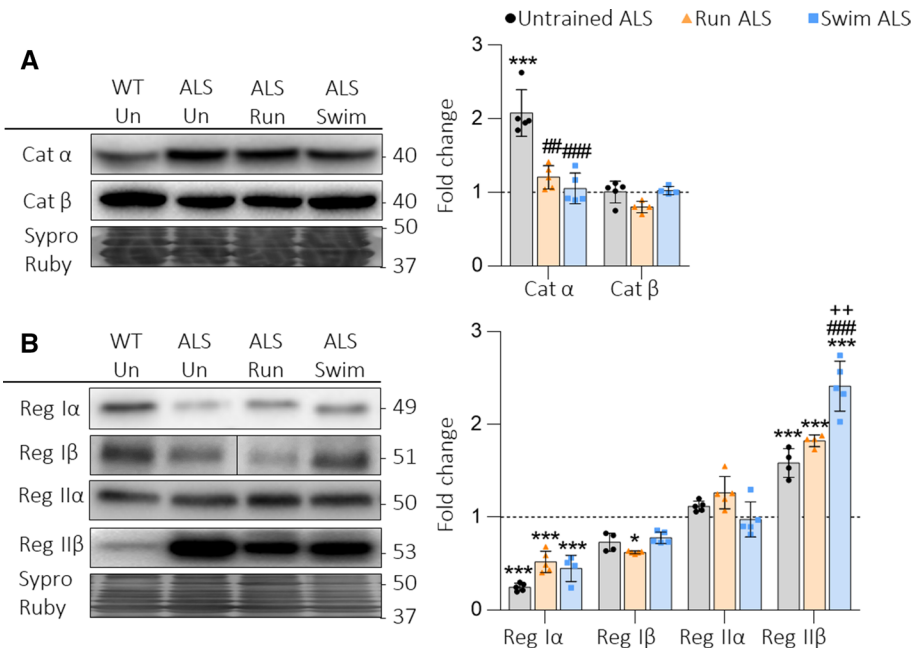

Fig. 3 Catalytic and regulatory PKA subunits in plantaris muscles of trained ALS mice. **a** Catalytic Cα still increases despite physical trainings, while Cβ is not modified. **b** Regulatory RIα decreases and RIIβ increases despite physical training, while RIβ is reduced by run. Data are mean value ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. *Versus Untrained WT mice; #versus Untrained ALS; +versus Run ALS mice. RIβ image is made using images taken from the same gel

modulate TrkB such as muscarinic and purinergic receptors [18, 52–54].

Furthermore, both types of training slightly increase the pPDK1/PDK1 ratio observed in Untrained ALS muscles by reducing total PDK1 and increasing pPDK1 (Fig. 2d), indicating the possibility of being more active, especially after swimming, which is in accordance with the recovery of exclusive presynaptic PKC activity.

We also analysed the two catalytic PKA subunits (Cα, Cβ) and the four regulatory subunits (RIα, RIβ, RIIα and RIIβ). Results show that both protocols normalize the ALS-induced increase of Cα, while Cβ remains unchanged (Fig. 3a). On the other hand, any protocol prevents neither the reduction of RIα nor the increase of RIIβ regulatory subunits caused by ALS. Finally, RIβ, which is unchanged in Untrained ALS, is slightly reduced in Run ALS (Fig. 3b).

Snare/SM proteins

ALS strongly enhances the phosphorylation of two key proteins of the synaptic vesicle exocytotic machinery, Munc18-1 from the regulatory Sec1/Munc18-like (SM) family and the synaptosomal-associated protein 25 (SNAP-25) a component of the soluble NSF attachment protein receptor (Snare) [4]. Figure 4 shows that both Run and Swim protocols fully prevent the increase of pMunc18-1 and, in concordance, normalize the pMunc18-1/Munc18-1 ratio (Fig. 4a). However, the training does not reduce either pSNAP-25 S187 (PKC target) or T138 (PKA target). Therefore, the ratio pSNAP-25/SNAP-25 (for both phosphorylation sites) is as increased (Run ALS) or even significantly higher (Swim ALS) as it is in Untrained ALS (Fig. 4b).

Altogether, it seems that exercise preferentially modulates proteins that regulate exocytosis rather than the SNARE proteins directly implicated on it, probably in relation with the selective modulation of cPKCβI.

Motoneuron survival

Finally, we aimed to investigate whether running- and swimming-based training protocols could prevent motoneuron death in relation with the improvement they provoke over the BDNF/TrkB neurotrophic signalling pathway. Because ALS preferentially affects larger motoneurons innervating faster muscle fibres [4, 38, 41], in Fig. 5 we analysed the changes in the proportion between fast (larger) and slow (smaller) motoneurons in the spinal cord of the symptomatic untrained and trained ALS mice (115 days old, which represents the end stage of the disease in Untrained ALS) compared with WT Untrained animals (Fig. 5).

In Untrained and Run ALS mice, the proportion of smaller (and mainly slow) α motoneurons (300–900 μm^2 of area) becomes significatively higher, while the proportion of bigger (and mainly fast) motoneurons strongly decreases (900–1200 μm^2 of area). These results indicate that big motoneurons are lost in both groups in comparison with the Untrained WT mice. On the other hand, in Swim ALS mice, the proportion of small motoneurons increases like in Untrained and Run ALS groups but less significantly, while the proportion of big motoneurons (900–1200 μm^2 of area) remains equivalent to the control population, indicating that the loss of this population of motoneurons is prevented in these animals. However, the proportion of the biggest motoneurons (> 1200 μm^2

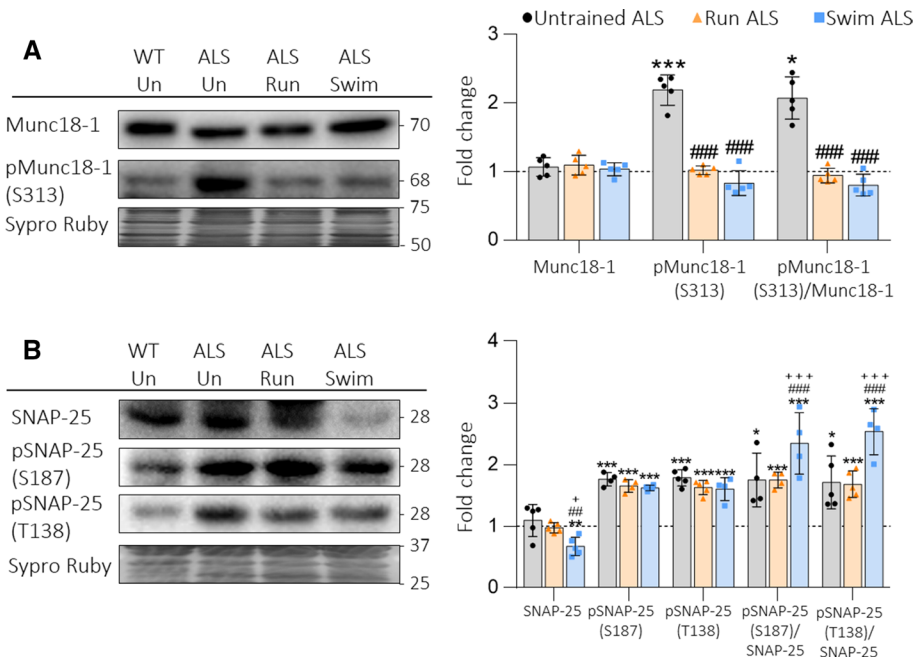

Fig. 4 The SNARE/SM Munc18-1 and SNAP-25 in plantaris muscles of trained ALS mice. **a** Run and swim normalize upregulated pMunc18-1, while Munc18-1 is never modified. The ratio pMunc18-1/Munc18-1 is also normalized after both protocols. **b** SNAP-25 is decreased by swim and pSNAP-25 (S187) and (T138) are significatively increased despite the trainings. Data are mean value ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. *Versus Untrained WT mice; #versus Untrained ALS; +versus Run ALS mice

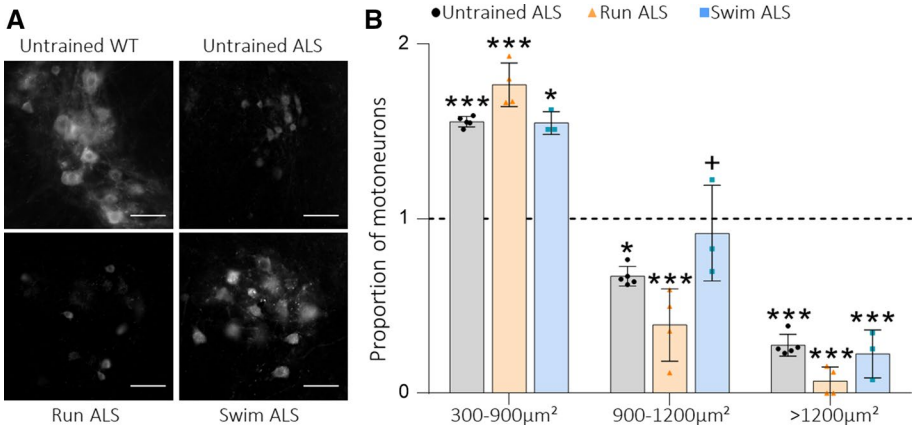

Fig. 5 Motoneuron population proportions change depending on the training protocol. **a** Representative images of motoneurons identified with ChAT immunolabeling in the ventral horn of the lumbar spinal cord (scale bar: 50 µm). **b** Proportion of motoneuron populations in relation to the WT untrained group by soma area at P115. Data are mean ± S.D $***P < 0.001$, $**0.001 < P < 0.01$, $*0.01 < P < 0.05$), *Versus Untrained WT mice; +versus Run ALS mice

of area) is significatively decreased in the three groups (Fig. 5a, b), indicating that they are lost despite the training, while the proportion of sensitive γ motoneurons (< 300 µm^2 of area) increases (data not shown). Altogether, it confirms that ALS specially damages fast motoneurons, corresponding to somas bigger than 900 µm^2, but that a swimming-based protocol started after disease onset can reduce it while running exacerbates the pattern of Untrained ALS.

Discussion

Because the impairment of the NMJ is crucial in ALS onset and progression, strategies that structurally and functionally preserve it would improve the health of the neuromuscular cell partners. Indeed, in SOD1-G93A mice, maintaining neuromuscular activity extends motor units survival along ALS progression in partially denervated

muscles [55] and, in ALS patients, moderate exercise ameliorates the disease symptoms and delays the progression [29, 56–58]. This may be, among others, due to neurotrophic factors expression [59], which prevents motoneuron degeneration, muscle denervation and atrophy [60–62].

The BDNF/TrkB signalling is one of the most implicated pathways in the NMJ stability and it is essential for neurotransmission [12, 15, 16, 63, 64]. Recently, we have demonstrated that this signalling is altered in the muscles of SOD1-G93A mice even before disease onset [4], suggesting that its dysfunctionality contributes to motor impairment in ALS. Now, the present results add new evidence that the two training protocols that have been analysed are capable of inducing activity-dependent adaptations to reduce or fully prevent most of the profound molecular alterations observed in the BDNF/TrkB pathway in Untrained ALS mice.

Neurotrophins and TrkB signalling

Exercise training, through synaptic activity and muscle contraction, increases BDNF in spinal cord and skeletal muscle of rodents and humans [12, 59, 65–67]. This normal situation, which is beneficial not only for the peripheral nervous system [68] but also for the central nervous system [66, 67], is altered in ALS disease, where mBDNF is significantly increased [4, 69] despite the reduction of neuromuscular activity. Contrarily, we show that running and swimming reduce or completely normalize the BDNF accumulation, respectively. These apparent contradictions, together with previous results showing that exogenous BDNF therapy is not beneficial for ALS [70, 71], drives us to the hypothesis that, in ALS, BDNF and other neurotrophins are synthesized by myocytes affected by ALS in an attempt to ameliorate NMJ activity. However, it is not useful because of TrkB.FL downregulation and TrkB.T1 upregulation [4, 72, 73]. Indeed, the adverse effect of TrkB.T1 on ALS mice has been extensively demonstrated [24, 74] and it seems that its overpresence limits the BDNF action because of its negative effect over TrkB.FL that results in pTrkB.FL not transducing the intracellular signalling [74]. In contrast, in trained animals, TrkB.FL is able to trigger the pathway that results in the recovery of the downstream signalling. Indeed, a similar modulation of TrkB receptors has been reported by physical training in WT mice spinal cord [75] and the same effect is found in the WT diaphragm when muscle contraction is increased [12]. Furthermore, in the first stages of ALS, activated microglia synthesize BDNF, which exerts anti-inflammatory and protective effects. However, at later stages, neither toxic microglia nor astrocytes express BDNF [76, 77]. Because this occurs in the central nervous system, it is probably also directly related with the loss of motoneurons in this disease.

In accordance with previous studies [78], we found that $p75^{NTR}$ is reduced in the NMJ of Untrained ALS animal limb muscles and it is reestablished after training. Indeed, it is possible that exercise enhances $p75^{NTR}$ levels to potentiate its myogenic effect as it plays a crucial role in muscle repair [79, 80], it is co-expressed with embryonic myosin heavy chain, indicating muscle fibre regeneration [78] and regulates neuronal survival and differentiation by interacting with Trk receptors [81]. Altogether, it seems that $p75^{NTR}$ could be a marker of improvement in the skeletal muscles.

Interestingly, the only alteration that training could not prevent at all is the great increase of NT4 which is observed yet in the presymptomatic stage [4, 69]. Indeed, it has been reported that NT4 prevented motoneuron death [82] and that avoids the disassembly of postsynaptic acetylcholine receptors (AChR) clusters [83]. Thus, it seems that NT4 could be postsynapticaly synthesized to further increase after exercise and modulate the presynaptic activity in ALS muscles when TrkB receptors are correctly balanced. This would have the final objective to better preserve motoneurons.

Serine-threonine kinases and SNARE/SM targets

We previously demonstrated that induced muscle contraction retrogradely upregulates presynaptic nPKCε and cPKCβI through BDNF/TrkB signalling in WT animals and that they regulate neuromuscular transmission by controlling Munc18-1 and SNAP-25 phosphorylation [12, 19, 20, 49, 84]. Particularly, Munc18-1 phosphorylation is regulated by both cPKCβI and nPKCε at the NMJ [20], while SNAP-25 phosphorylation is exclusively regulated by nPKCε [19]. Here, we prove that in ALS the differential effects of the training over cPKCβI and nPKCε is directly related with the results seen in pMunc18-1 and pSNAP-25 modulation. Thus, the normalization of pMunc18-1/Munc18-1 ratio coincides with the normalization of the activity balance between cPKCβI and nPKCε. On the other hand, SNAP-25 phosphorylation in the residue S187 is still upregulated despite the training because the phosphorylating efficacy of nPKCε is increased enough to guarantee an optimal recruitment of ready-releasable vesicles [85] that may help to maintain neurotransmission. Alternatively, dephosphorylation of pSNAP-25 may be impaired in ALS, as even in untrained muscles pSNAP-25 is elevated despite nPKCε minor activity in Untrained ALS mice.

Regarding PKA, both trainings normalize the ALS-induced increase of Cα subunit, while the complex change in the stoichiometry of the regulatory subunits RIα and RIIβ persists. Adenosine receptor A2 (A2AR) has neuroprotective effects mediated by the PKA signalling [86]. Indeed, PKA activation neuroprotected motoneurons, while its inhibition did not [87]. Altogether, it seems that an increased PKA activity has a neuroprotective effect. Interestingly, this

coincides with the found high levels of pSNAP-25 T138, pointing to the change in regulatory subunits as a major change in ALS that cannot be normalized by training. Therefore, it seems that the potentiation of the PKA pathway could be of great interest in the treatment of ALS patients.

Run and swim have different effects

Since ALS preferentially affects fast-twitching muscles, it is conceivable that the effect of exercise depends on its type, the protocol followed and the intensity [32, 35–38] and that these differences would also be found in the molecular field. Recently, we differentiated the molecular changes in BDNF/TrkB signalling that could be explained or not by the fast-to-slow transition [4] described in ALS plantaris muscles [38, 41]. Deforges et al. [38] shows that, while running exacerbated the fast-to-slow functional transition, swimming preserved the normal phenotype in the plantaris. However, our molecular results reveal that the changes in ALS that presumably reflect the fast-to-slow transition (see Table 2 for protein classification) are always equally modulated by both protocols. On the one hand, some of them are unaffected by the trainings, suggesting that they are unavoidable, perhaps because they are directly related with the pathogenesis of the disease or because they are a strategy to increase motoneuron survival, such as we hypothesized for NT4 or PKA activity. On the other hand, some molecules are normalized by both protocols, which could be related to the coincident benefits of both protocols such as the avoidance of muscular atrophy [38].

Also, there are molecules that do not follow a fast-to-slow transition (Table 2). Among them, the specific effect of swimming over mBDNF and phosphorylated PKCβI and PDK1 coincides with the functional benefits described by Deforges et al. Despite that TrkB.T1 is still upregulated after swimming, the ratio TrkB.FL/TrkB.T1 is strongly increased, while the ratio mBDNF/proBDNF is restored. Therefore, the triggering and the function of the downstream kinases of the signalling are reestablished. As a result, NMJ and the neuromuscular cell partners are better preserved and communicated, which is reflected by the decrease of motoneuron loss, from a 49 and 45% in untrained and running animals, respectively, to only a 28% in swimming animals [38]. As motoneuron death precedes ALS onset [1, 2], their preservation delays it, increases mean survival of the animals and maintains body weight and motor function for longer [33, 36–38, 88]. Indeed, previous studies found that swimming maintains the population of fast myofibres and improves motor performance [38]. Altogether, here we found that swimming softens the drastic loss of big and fast motoneurons by retrogradely modulating presynaptic signalling, which reinforces the idea that neuroprotection is bidirectional. However, swimming exclusively preserves big motoneurons, with a soma area between 900 and 1200 μm^2. This may be due to the higher requirements of the biggest ones ($> 1200\ \mu m^2$), which are more susceptible to oxidative stress and excitotoxicity, which is promoted by physical training [5, 89–91]. Indeed, running is a high-impact exercise which only recruits small motoneurons and generates more oxidative stress than swimming, which is a low-impact exercise that recruits both small and big motoneurons. Therefore, it is

Table 2 Classification of proteins

	Effect			
	No	Equal	Swim	Run
Fast-to-slow transition	NT4	TrkB.FL		
	cPKCα	pTrkB.FL (Tyr816)		
	PKA RIIβ	cPKCβI		
	pSNAP-25 (Ser187)	pnPKCε (Ser729)		
		PKA Cα		
No Fast-to-slow transition	PKA RIα	nPKCε	mBDNF	TrkB.T1
	pSNAP-25 (Thr138)	PDK1	pDPK1 (Ser241)	
		pMunc18-1 (Ser313)	pcPKCβI (Thr642)	
		$p75^{NTR}$		
No change in ALS Untrained	pcPKCα (Ser657)		SNAP-25	PKA RIβ
	PKA Cβ			
	PKA RIIα			
	Munc18-1			

Depending on (1) the changes that follow in ALS mice untrained (rows) and (2) which training protocol has effect on them (columns). The proteins that followed a fast-to-slow transition acquired values similar to the soleus of WT mice and the ones that did not follow a fast-to-slow transition did not. Finally, some proteins do not change in ALS mice untrained

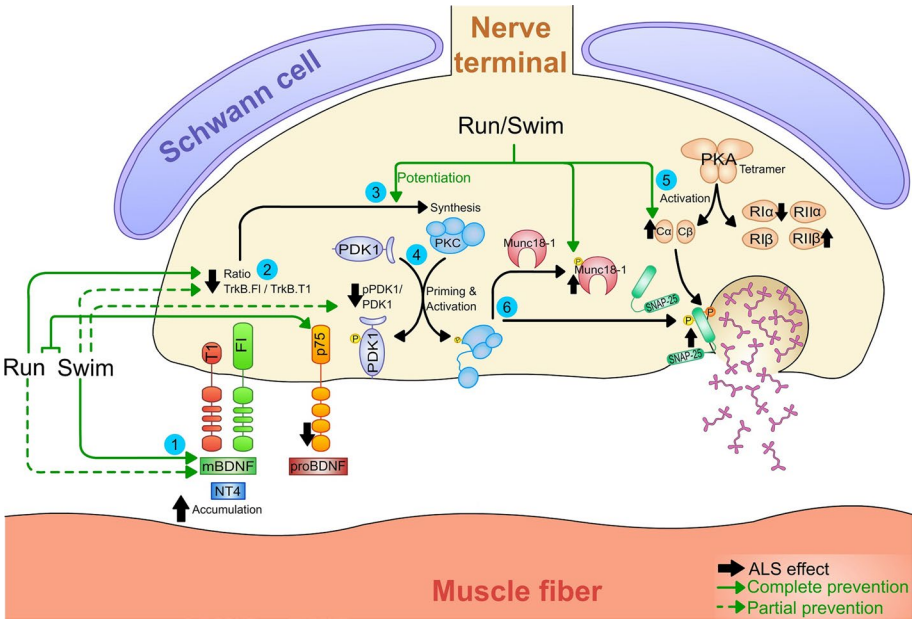

Fig. 6 Graphical representation of the effect of physical training over the BDNF signalling in plantaris muscles with ALS. Despite that any training reduces the accumulation of NT4, both, and especially swimming, reduce mBDNF levels (1). However, the fall of the ratio TrkB.FL/TrkB.T1 is better prevented by running than by swimming (2). The PKC pathway is potentiated by both protocols (3) in contrast to PDK1, whose ratio is only partially normalized by swimming (4). On the other hand, the trainings exclusively modulate catalytic but not regulatory PKA subunits (5). In accordance with global serine/threonine kinase activity affectation, the target related with vesicle exocytosis SNAP-25 is upregulated; while the regulator protein Munc18-1 is normalized after the trainings (6). Altogether, many molecular changes in ALS muscles are prevented or reduced and sometimes overcompensated by the training protocols. Code: green solid arrows indicate a complete prevention of the alteration produced by ALS (represented with strong black arrows next to the proteins) in the studied system, while green dashed arrows indicate a partial prevention. Numbers inside blue circumferences are mean to focus attention into important steps explained in the legend of the figure

comprehensible that, despite both protocols induce molecular changes at the motoneuron terminal, swimming prevents motoneuron loss while running does not. In accordance with the benefits of low-impact exercise, [87, 92] found that voluntary physical activity motivated by the change of the environment induces similar benefits to those of swimming, probably in relation with less oxidative stress production in comparison with forced running protocols. Altogether, this adds new evidence of how susceptible is to determine the effect of physical training and justifies the controversy around it.

The molecules that do not follow a fast-to-slow transition but are modulated equally by both protocols and the ones that do not change could participate in the benefits shared by both running and swimming or may be part of the unavoidable molecular alterations induced by the pathology. In fact, both SNAP-25 phosphorylations (S187 and T138) are unsensitive to any activity training despite that the first one follows the fast-to-slow transition and the second one does not. However, vesicle refilling and ready-releasable vesicle pool size are controlled by them [85, 93], and therefore their upregulation can be a requirement for the maintenance of useful neurotransmission and NMJ structure.

Concluding remarks

In summary, many molecular changes in ALS muscles are prevented or reduced and sometimes overcompensated by the training protocols (for a summary see the Graphical abstract in Fig. 6). They partially prevent the neurotrophic signalling alteration, directly related with neurotransmission and neuromuscular activity protection. Indeed, these molecular changes are contemporary with improvements in animals' condition, especially regarding swimming, where motoneuron loss is reduced [38]. Altogether, despite the controversial opinions on physical exercise as therapy in ALS, it seems that it is always beneficial, but with precise exercise-dependent outcomes. Thus, this study contributes to the understanding of the variable role of exercise in ALS and adds new evidence that both run and swim trainings are beneficial as they diminish the profound molecular alterations observed in the BDNF/TrkB pathway in Untrained ALS mice muscles. Altogether, despite being still far of clinic application, further investigation in this field would be useful as the differences among protocols are essential to target specific molecular changes.

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Compliance with ethical standards

Conflicts of interest The authors declare no competing interests.

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