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Motoneuron survival is promoted by specific exercise in a mouse model of amyotrophic lateral sclerosis

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Several studies using transgenic mouse models of familial amyotrophic lateral sclerosis (ALS) have reported a life span increase in exercised animals, as long as animals are submitted to a moderate-intensity training protocol. However, the neuroprotective potential of exercise is still questionable. To gain further insight into the cellular basis of the exercise-induced effects in neuroprotection, we compared the efficiency of a swimming-based training, a high-frequency and -amplitude exercise that preferentially recruits the fast motor units, and of a moderate running-based training, that preferentially triggers the slow motor units, in an ALS mouse model. Surprisingly, we found that the swimming-induced benefits sustained the motor function and increased the ALS mouse life span by about 25 days. The magnitude of this beneficial effect is one of the highest among those induced by any therapeutic strategy in this disease. We have shown that, unlike running, swimming significantly delays spinal motoneuron death and, more specifically, the motoneurons of large soma area. Analysis of the muscular phenotype revealed a swimming-induced relative maintenance of the fast phenotype in fast-twitch muscles. Furthermore, the swimming programme preserved astrocyte and oligodendrocyte populations in ALS spinal cord. As a whole, these data are highly suggestive of a causal relationship not only linking motoneuron activation and protection, but also motoneuron protection and the maintenance of the motoneuron surrounding environment. Basically, exercise-induced neuroprotective mechanisms provide an example of the molecular adaptation of activated motoneurons.

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Abbreviations ALS, amyotrophic lateral sclerosis; CA II, carbonic anhydrase II; ChAT, choline acetyl-transferase; GFAP, glial fibrillary acidic protein; IGF-1, insulin-like growth factor 1; MyHC, myosin heavy chain; NPCs, neural progenitor cells; ROS, reactive oxygen species; SOD1, superoxide dismutase 1.

Amyotrophic lateral sclerosis is a chronic neurodegenerative disease characterised by a progressive motor weakness originating from selective motoneuron cell death. On average, mortality occurs within the 4 years following the occurrence of the first clinical symptoms. The currently available therapy extends survival in humans by approximately 3 months. Thus, developing new therapeutic strategies for ALS is of paramount importance. Mutations in superoxide dismutase 1 (SOD1) have been observed in about 20% of familial ALS patients (Rosen, 1993). SOD1 normally converts superoxide ion, a by-product of mitochondrial metabolism, to water and hydrogen peroxide. Despite the fact that SOD1 activity impairment has been ruled out as the causal event of the disease (Shefner *et al.* 1999), there is some evidence for a gain in toxic function with the mutant form of SOD1 (Boillée *et al.* 2006). The clinical and morphological abnormalities are common to familial and other forms of ALS, suggesting a common degeneration mechanism. Yet, in spite of the wide variety of possible causes for ALS, including environmental agents, oxidative stress, disturbance of the glutamatergic neurotransmission, a large amount of literature data correlates neuronal cell death to glutamatergic excitotoxicity (Heath & Shaw, 2002). Interestingly, the deleterious effects of glutamatergic excitotoxicity might be decreased by submitting mice to physical exercise training (Carro *et al.* 2000, 2001). These beneficial effects have been linked to

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an exercise-induced increase in circulating IGF-1 uptake by neurons (Carro *et al.* 2001). Furthermore, several groups have reported beneficial effects of a moderate running-based training in ALS mouse models including a 10- to 24-day increase in the life span of mutant mice submitted to training in comparison to their sedentary counterparts (Kirkinezos *et al.* 2003; Veldink *et al.* 2003; Liebetanz *et al.* 2004; Kaspar *et al.* 2005). It should be noted, however, that one study reported deleterious effects of high-intensity exercise in ALS mice (Mahoney *et al.* 2004).

Whether there is an exercise-induced neuroprotection is still a matter of debate. According to Veldink *et al.* (2003), the analysis of the spinal cord anatomy of trained *vs* untrained mice revealed no difference in neuron distribution and survival. In contrast, Kaspar *et al.* (2005) reported that physical exercise significantly protected motoneurons from death. These contradictory data concerning the effects of exercise in neuroprotection outline the specific effect exerted by any given exercise protocol i.e. treadmill running for Veldink *et al.* (2003) and wheel running for Kaspar *et al.* (2005). Furthermore, although the molecular mechanism(s) underlying the exercise-induced effects is still unknown, the latter results do show that the effect of IGF-1 delivery and exercise are mediated through different molecular mechanisms, which in combination result in synergistic survival (Kaspar *et al.* 2005). If the exercise-induced neuroprotection is not reflecting the action of diffusible factors, such as IGF-1, then, which mechanism(s) could account for both the generally increased resistance of exercised motoneurons to cell death and the specific effect of a given exercise protocol? One cue for resolving this controversy may be to consider a causal link between the motoneuron activation, the adaptation of neuron intrinsic properties and neuroprotection. Indeed, the activation level of selected motoneuron sub-populations is dependent on the exercise type as shown by Grondard *et al.* (2008). Briefly, a swimming-based program was associated with high hindlimb movement amplitude and frequency exercise (4.86 ± 0.40 cm and 373.9 ± 47.6 cycles min^{-1}) preferentially activating a sub-population of large motoneurons (with soma area greater than 700 μm^2) and a running-based training, described as a low hindlimb movement amplitude and frequency exercise (3.79 ± 0.23 cm and 234.2 ± 24.5 cycles min^{-1}), preferentially activating a sub-population of small motoneurons (with soma area less than 300 μm^2). Furthermore, only specific sub-populations of small lumbar motoneurons (<300 μm^2) adapted their electrophysiological properties in running rats (Beaumont & Gardiner, 2002). Thus, an essential requirement would be to test whether motoneuron activation leads to their specific resistance to ALS-induced cell death, in contrast to resting neurons.

To directly address the importance of exercise intensity regulation and the existence of an exercise-induced neuroprotection in ALS mice, we have compared the efficiency of either a daily swimming- or running-based training, on the basis of our previous experiments on control mice (Grondard *et al.* 2008). We provide experimental evidence suggesting a relationship between exercise-induced motoneuron activation and their subsequent protection. Furthermore, our data revealed an unexpected link between exercise-induced neuroprotection and the maintenance of the motoneuron cell environment, in particular, the astrocytes that have been recently pointed out as regulators of ALS progression (Yamanaka *et al.* 2008).

Methods

Mice

The care and treatment of animals followed the national authority (Ministere de la Recherche et de la Technologie, France) guidelines for the detention, use and the ethical treatment of laboratory animals. All experimental procedures which include minimizing the number of animals used and their suffering were approved by the policies of the French Agriculture and Forestry Ministry.

Transgenic male mice with the G93A human SOD1 mutation B6SJL-Tg (SOD1-G93A) 1Gur/J (ALS mice) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Male mice B6SJL (Janvier, le Genest, France) served as control for this mutant strain.

Disease onset was defined as the time corresponding to the first observation of myotony symptoms in the mice hind limb. Death was scored when mice were unable to stand on their feet 30 s after having been placed on their side. Then, the moribund mice were killed with CO_2.

All the experiments using mice were performed in a blind systematic fashion so as to minimize bias.

Training protocol

At 70 days of age, colour randomly assigned transgenic male were trained 30 min a day, 5 days a week as performed in previous studies using ALS mice (Kirkinezos *et al.* 2003; Veldink *et al.* 2003). Training was performed until 115 days of age or until death. Twenty ALS mice (running ALS) were submitted to a running-based training on a speed-regulated treadmill (max. 13 m min^{-1}) and 25 ALS mice (swimming ALS) were submitted to a swimming-based training in an adjustable-flow swimming pool (max. 5 l min^{-1}), as previously described (Grondard *et al.* 2008). A third group of 50 ALS mice (sedentary ALS) and 10 control mice were placed in the pool without flow (25 ALS

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mice and 5 controls) and floated at the water surface accordingly exhibiting a poor activity or on the treadmill without speed (25 ALS mice and 5 controls). Likewise, these untrained animals which were studied in parallel only displayed an exploratory activity for the whole duration of training of the previously described animal groups.

Core body temperature was determined using a calibrated digital thermometer (Fischer Scientific Company) equipped with a rectal probe designed for rodents.

Behavioural study

General characteristics of mice were given using weight, forelimb grip strength and spontaneous activity ($n = 8$ in each group). Sedentary and trained mice between 70 and 126 days old were tested once a week and all of the tests were performed blindly (the group assignment being unknown to the observers). The time during which the animals were able to sustain their weight holding onto a metal rail suspended in midair was recorded with the maximum time being 180 s. Each mouse was subjected to three trials with at least a 10 min resting period between tests. The ambulatory behaviour was assessed in an open-field test. The apparatus consisted of a wooden box measuring 50 cm × 50 cm × 20 cm. The floor of the arena was divided into 25 equal squares of 10 cm. Squares adjacent to walls were referred to as periphery and the nine remaining squares were referred to as centre. The mice were tested individually and the open field was washed after each session. Each mouse was placed in a central square on the open field and was allowed to move freely for 5 min. The number of peripheral and central square crossings was scored manually by the experimenter.

Histological evaluation

Mice at 115 days old ($n = 8$ in each group) were anaesthetized by intraperitoneal injection of 3.5% chloral hydrate and perfused transcardially with buffered saline and 4% paraformaldehyde. The L1 to L5 lumbar region of the spinal cord was processed for vibratom sectioning at 40 μm thickness and one out of every six sections was subsequently processed for immunostaining on free-floating sections. The immunohistochemical analysis was based on detection of choline acetyl-transferase (goat anti-ChAT, 1/800, Chemicon International, Temecula, CA, USA) to stain motoneurons, glial fibrillary acidic protein (rabbit anti-GFAP, 1/100, Dako, Glostrup, Germany) to stain astrocytes, carbonic anhydrase II (rabbit anti-CA II, 1/100; Ghandour *et al.* 2000) to stain oligodendrocytes and nestin (mouse anti-nestin, 1/300, Chemicon International) to stain neural progenitor cells (NPCs). Secondary antibodies used were donkey anti-species-specific antibodies associated with the fluorophores Alexa Fluor 568 anti-goat IgG (1/500, Molecular Probes), Alexa Fluor 488 anti-rabbit (1/500, Jackson-Immunoresearch laboratories, West Grove, PA, USA) and Cy2 anti-mouse (1/500, Jackson-Immunoresearch laboratories). Sections were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and visualized using a BX51 Olympus microscope and Q Imaging Fast 1394 Retiga 2000 R camera.

For each mouse, 10 sections of lumbar spinal cord were studied. All cells were counted from within the ventral horn below a lateral line across the spinal cord and from the basis of the central canal. Only neuronal cells with the presence of a nucleolus located within the nucleus were counted. Motoneuron soma areas were evaluated using Image J v1.33 software (National Institutes of Health, Bethesda, MD, USA).

Muscle typology immunofluorescence

At 115 days old ($n = 5$), soleus, plantaris and tibialis muscles were frozen in isopentane cooled in liquid nitrogen and transverse 10 μm cryostat sections were used for muscle typology. Fresh-frozen sections of each muscle were fixed with acetone and incubated with mouse antibodies raised against myosin heavy chains (MyHC): NCL-MyHCs (anti-MyHC I, slow), NCL-MyHCf (anti-MyHC II, fast) (Novocastra, Newcastle, UK), A4.74 (anti-MyHC IIa) and BF.F3 (anti-MyHC IIb) (DSHB, IA, USA) at a dilution of 1 : 20. Secondary antibodies used were rabbit anti-species-specific antibodies conjugated with Cy2 or Cy3 (1 : 20, Dako). Sections were visualized as described above. For each mouse, three sections of each muscle were studied. Each MyHC count and soma areas were evaluated using Image J v1.33 software. The percentage of fibre IIx was determined as the difference between the total number of type II fibres and the sum of type IIa and IIb fibres [IIx = II − (IIb + IIa)].

Apoptosis evaluation

For immunohistochemical analysis, the activated caspase-3 was detected by a rabbit anti-activated caspase-3 antibody (1 : 20, Chemicon International) and motoneurons were detected as described above. Donkey anti-species-secondary antibodies were associated with the fluorophores Alexa Fluor 568 anti-goat IgG (1 : 500, Molecular Probes) and Alexa Fluor 488 anti-rabbit (1 : 500, Jackson-Immunoresearch).

For Western blot analysis, the ventral horn part of the lumbar spinal cord of 115-day-old mice ($n = 3$ in each group) was frozen in isopentane and lysed

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with RIPA buffer supplemented with protease inhibitors (20 mM orthovanadate, 25 mM pyrophosphate; Sigma, Saint Quentin, Fallavier, France). Protein concentration of the clarified homogenates (4°C, 15 min, 12 000g) was determined using the Bradford protein microassay procedure (Biorad Laboratories, Hercules, CA, USA). After addition of a loading buffer, protein samples were electrophoresed on a 10% SDS–polyacrylamide gel and transferred on to PVDF membranes (Biorad Laboratories). The activated caspase-3 was detected with an antibody specific for cleaved forms of caspase-3 (1 : 1000, rabbit anti-activated caspase-3, Promega, Madison, WI, USA) and revealed with a goat peroxidase-conjugated secondary antibody directed against rabbit immunoglobulins (1 : 7500, Jackson Immunoresearch). Bound antibody complexes were developed using the ECL plus kit and exposed to hyper-film ECL-plus X ray film (Amersham Biotech, Les Ulis, France). After stripping, membranes were incubated with a mouse anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (1 : 5000, Chemicon International) and revealed with a goat peroxidase-conjugated secondary antibody directed against mouse immunoglobulins (1 : 5000, Jackson Immunoresearch).

Statistical analysis

For the behavioural study, data are presented as mean and standard error of mean ($n = 8$). A one-way ANOVA for repeated measures with Tukey's *post hoc* analysis was performed on the data. For the other studies, all values are displayed as means and standard deviation within each group. A Kolmogorov–Smirnov normal distribution analysis was performed on all data followed by either a Student's t test for normally distributed data or a non-parametric Kruskal–Wallis test, to verify significant differences between groups (Systat v 8.0, SPSS Inc., Chicago, IL, USA). All the analyses have been statistically powered to ensure significant scientific statements. All the data presented in this study have a statistical power of 90% (AnaStats.fr, France).

Results

Life span extension and behavioural improvement depend on the exercise type in ALS mice

We submitted two populations of ALS mice to a daily swimming- or running-based training, on the basis of our previous experiments on control mice (Grondard *et al.* 2008). These protocols started in the asymptomatic phase of the disease (70 days). The swimming programme resulted in the significant delay of 16 days ($P < 0.001$) for the appearance of symptoms, from 93 ± 8 days in sedentary ALS mice to 109 ± 15 days in swimming ALS mice (Fig. 1*A*). Furthermore, the mean survival increased from 127 ± 9 days in sedentary ALS mice to 153 ± 24 days in swimming ALS mice, corresponding to an improvement of 25 days ($P < 0.01$) in the survival of ALS mice (Fig. 1*B*). In contrast, no effect on the appearance of symptoms or in survival could be recorded with the running-based training.

In order to evaluate the motor function benefits induced by each exercise programme, we subjected the mice of each group to a grip test and to an open field test. All ALS mice presented a reduced body weight compared to controls (Fig. 1*C*). The weight of sedentary and running ALS mice decreased from 112 days until death. In contrast, the weight of the swimming ALS mice remained constant until 120 days ($P < 0.05$). In the grip test, the average time sedentary and running ALS mice could sustain their weight using their forelimb muscles decreased from 105 days and these mice were unable to grip after 119 days (Fig. 1*D*). In contrast, the swimming ALS mice had enough strength to grip for a time comparable to controls, up to 119 days ($P < 0.05$). Near death, the swimming ALS mice showed difficulties to sustain the grip.

We then compared the spontaneous activity of the mice in an open field test (Fig. 1*E*). A significant decrease in locomotion activity was found in running ALS from 91 days of age and in sedentary ALS mice from 105 days of age when compared to controls ($P < 0.05$). In contrast, the swimming ALS mice maintained a near to normal locomotion activity until 112 days of age.

Motoneuron protection depends on the exercise type

The comparison of the lumbar motoneuron populations in the trained and sedentary ALS mice at 115 days of age showed remarkable differences in the neuroprotective potentials of each exercise (Fig. 2). A dramatic motoneuron loss was recorded in the ventral horns of the sedentary and the running ALS mice with a 49% and 45% reduction, respectively (Fig. 2*B*). Likewise, the motoneuron distribution in sedentary and the running ALS mice exhibited a decrease in the proportion of motoneurons exhibiting medium (between 300 and 700 μm^2) and large (greater than 700 μm^2) soma in comparison to control mice, with a slight reduction in the absolute number of motoneurons exhibiting soma larger than 500 μm^2 (Fig. 2*C*). As a consequence, an increased proportion of motoneurons exhibiting small soma area (less than 300 μm^2) was observed. In contrast, the swimming-based training in ALS mice exhibited a neuroprotective potential with only a 28% reduction of motoneurons in comparison to controls. Interestingly, the swimming-induced protection from neuronal loss was readily apparent for motoneurons exhibiting medium size soma (between 300 and 700 μm^2).

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The relative maintenance of the muscle phenotype is also dependent on the exercise type

Since muscle weakness is a major cause of disability in ALS, we investigated whether the running- and the swimming-induced protection of motoneurons led to the maintenance of the muscle phenotype. Our count of neurons was performed in the lumbar spinal cord from which the sciatic nerve originates, then we analysed the phenotype of three muscles of the calf innervated by the sciatic nerve, namely two extensor muscles of the ankle, the slow-twitch soleus and the fast-twitch plantaris, and a flexor muscle of the ankle, the fast-twitch tibialis. According to all the phenotypic criteria we used (i.e. the number of myofibres, their cross-section area and the muscle typology), only the swimming-based training efficiently preserved the ALS muscle phenotype close to the corresponding control muscles (Fig. 3). The ALS muscles displayed a significant hypoplasia that appeared particularly severe for the slow-twitch soleus, suggesting that the level of muscular impairment was related, at least in part, to the muscle type (Fig. 3*A*). Unlike running, swimming efficiently counteracted the hypoplasia in the soleus. Furthermore, the ALS soleus muscle displayed a dramatic atrophy that was rescued by both training programmes (Fig. 3*D*). In the fast-twitch muscles, only the fast IIb fibres were atrophied in ALS mice. Both exercises limited this atrophy. In sedentary ALS fast-twitch muscles, we found a significant fast-to-slow transition from fast-twitch type II fibres to slow-twitch type I fibres and, within the type II fibre population, from type IIb/IIx to IIa fibres, concurring with the findings of Hegedus *et al.* (2008) (Fig. 3*C*). In contrast, the typology of the slow-twitch soleus indicated a slow-to-fast transition. The running programme induced opposite effects on the three muscle typology, counteracting the fast-to-slow transition in the tibialis, but worsening those of the

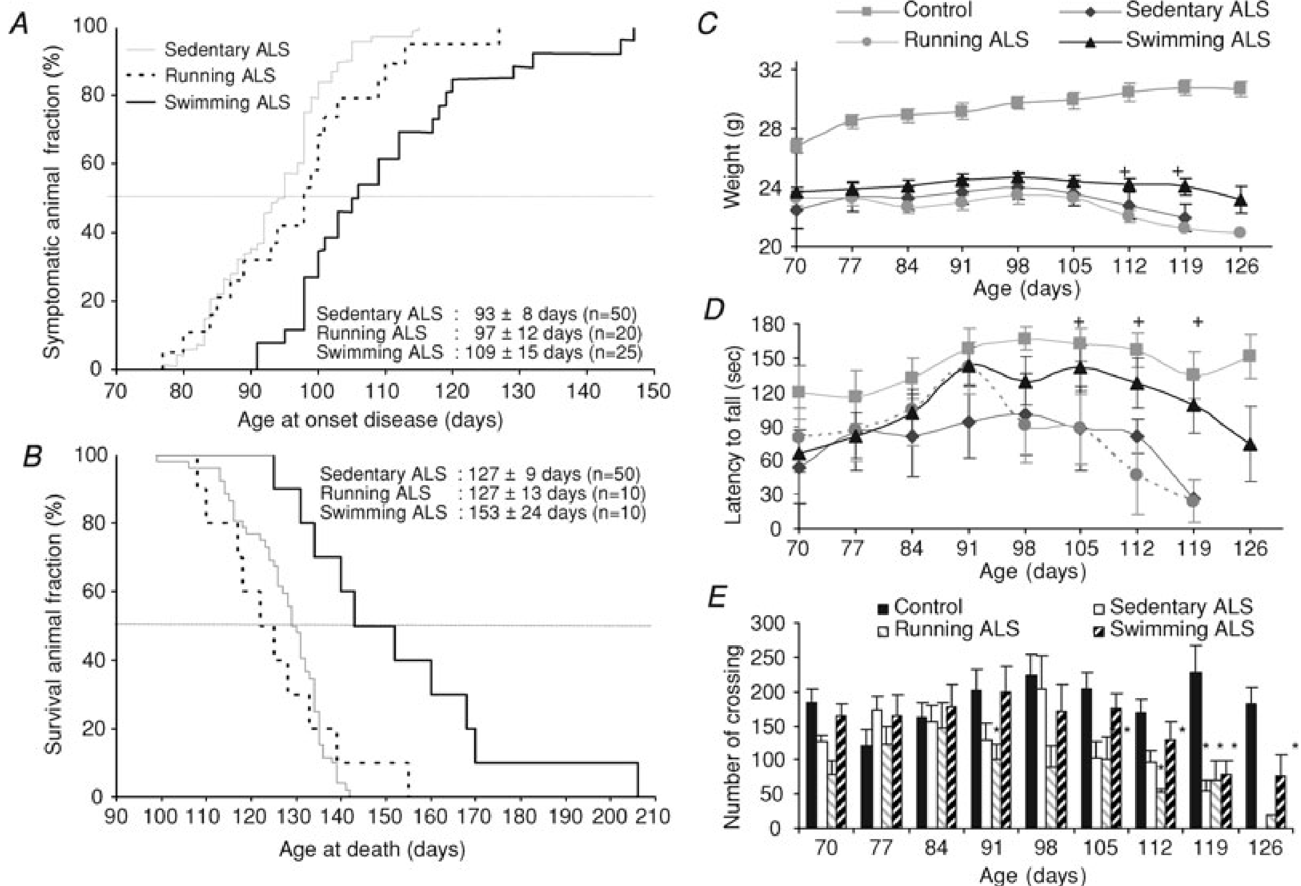

Figure 1. Differential effects of either a swimming- or a running-based training in ALS mice
Only the swimming programme delays disease onset (*A*) and extends survival (*B*) in ALS mice as shown by Kaplan–Meier curves. The mean time to disease onset was delayed by 2 weeks ($P < 0.001$) and the mean time to death was delayed by 3 weeks ($P < 0.01$) for the swimming ALS mice compared to the sedentary and the running ALS mice. Swimming delays the onset of disease symptoms, including a stabilization of the body weight (*C*), a delay in disease-related weakness as shown by the performance assessment of forelimb strength (grip test) (*D*) and the level of spontaneous exploratory activity (open field) (*E*). The behavioural data are shown as mean ± S.E.M. ($n = 8$; *$0.01 < P < 0.05$), *control *versus* ALS mice or $^+$swimming ALS *versus* other ALS groups).

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plantaris, leading to a decrease of type IIb fibres and being unable to correct the slow-to-fast transition in the soleus. However, the swimming-based training led to the maintenance of muscle typology in all three muscles.

Exercise-induced control of non-neuronal spinal cell populations in ALS mice

Several studies support the hypothesis that multiple cell types drive the motor degeneration in ALS mice, in association with a direct damage of motoneurons (Hall *et al.* 1998; Yamanaka *et al.* 2008). We therefore analysed the influence of each exercise mode on the cell types that lay beyond the motoneurons in ALS mice at 115 days of age. We first analysed the astrocyte population in the spinal cord of control mice and sedentary, running and swimming ALS mice (Fig. 4*A*). Astrogliosis and astrocytic activation as assessed by using GFAP immunostaining were significantly increased in sedentary and running ALS mice. A significant increase in astrocyte density was noted throughout the lumbar spinal cord (about 40% in sedentary and running ALS mice and only 3% in swimming ALS mice), with a high concentration in the ventral horn (Fig. 4*B*). Furthermore, compared with the long, thin, filament-like GFAP staining observed in controls, GFAP staining in ALS astrocytes was more condensed and brightly fluorescent, although the filaments often appeared shorter in length. In contrast, no significant astrogliosis was detected in the swimming ALS mice. We secondly evaluated the oligodendrocyte population, on a type II carbonic anhydrase immunostaining basis (Fig. 4*C*). We found a 30% decrease in oligodendrocytes in the ventral spinal cord of sedentary and running ALS mice compared with controls (Fig. 4*D*). Interestingly, the swimming-based training led to the maintenance of the oligodendrocyte population in the ALS spinal cord. Finally, we evaluated the neural progenitor cells (NPC) population through the count of nestin-positive cells (Fig. 4*E*). We observed a significant increase in NPCs throughout the spinal cord of sedentary ALS mice compared to a very discrete presence of NPCs in controls, in agreement with previous studies (Chi *et al.*

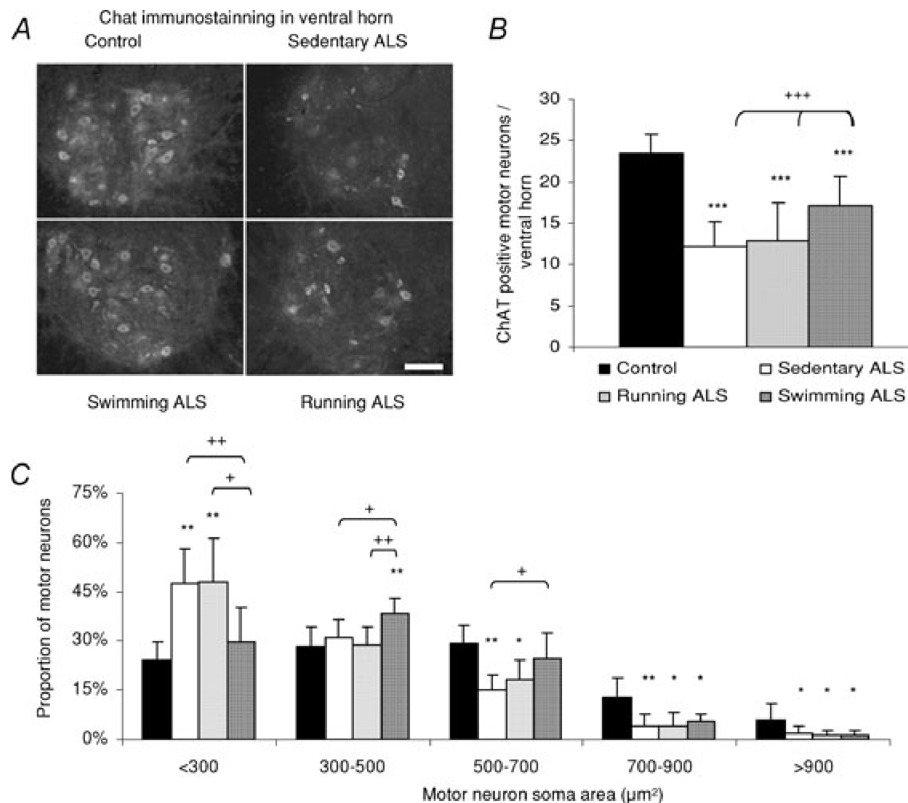

Figure 2. Only the swimming-based training significantly protects lumbar motoneurons in ALS mice
A, representative images showing motoneuron populations identified using ChAT immunostaining in the ventral horn of the lumbar spinal cord from control mice and sedentary, running and swimming ALS mice (scale bar: 50 μm). *B*, quantification of surviving motoneurons in control mice and sedentary, running and swimming ALS mice at 115 days of age. Data are shown as mean ± S.D. *C*, morphometric evaluation of surviving motoneurons in control mice and sedentary, running and swimming ALS mice at 115 days of age. Percentage of motoneurons by soma area (distribution: 200 μm^2) are shown as mean ± S.D. ($n = 8$; $^{***}P < 0.001$, $^{**}0.001 < P < 0.01$, $^{*}0.01 < P < 0.05$), *control *versus* ALS mice or $^{+}$swimming ALS *versus* other ALS groups.

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2006). The running- and the swimming-based training induced a significant decrease in the number of NPCs, with, respectively, a 39% and a 58% reduction compared to sedentary ALS mice.

The swimming programme limits apoptosis in the lumbar spinal cord of ALS mice

To verify whether the swimming-based training limits the apoptosis process in the spinal cord of ALS mice, we evaluated the activation of the caspase pathway at 115 days of age. The quantification of the active form of caspase-3 in the ventral spinal cord of sedentary ALS mice showed that the apoptosis process was limited in swimming-trained compared to sedentary and running-trained ALS spinal cords (Fig. 5).

Exercise protocols do not modify body core temperature

A difference between the two exercise conditions could be a change in core temperature during exercise and this could influence neuroprotective mechanisms (Dietrich *et al.* 2009). The core body temperature was determined in 12 trained control animals. No significant difference in body temperature of the trained control mice was found before (T0) and after training (T30) (Fig. 6).

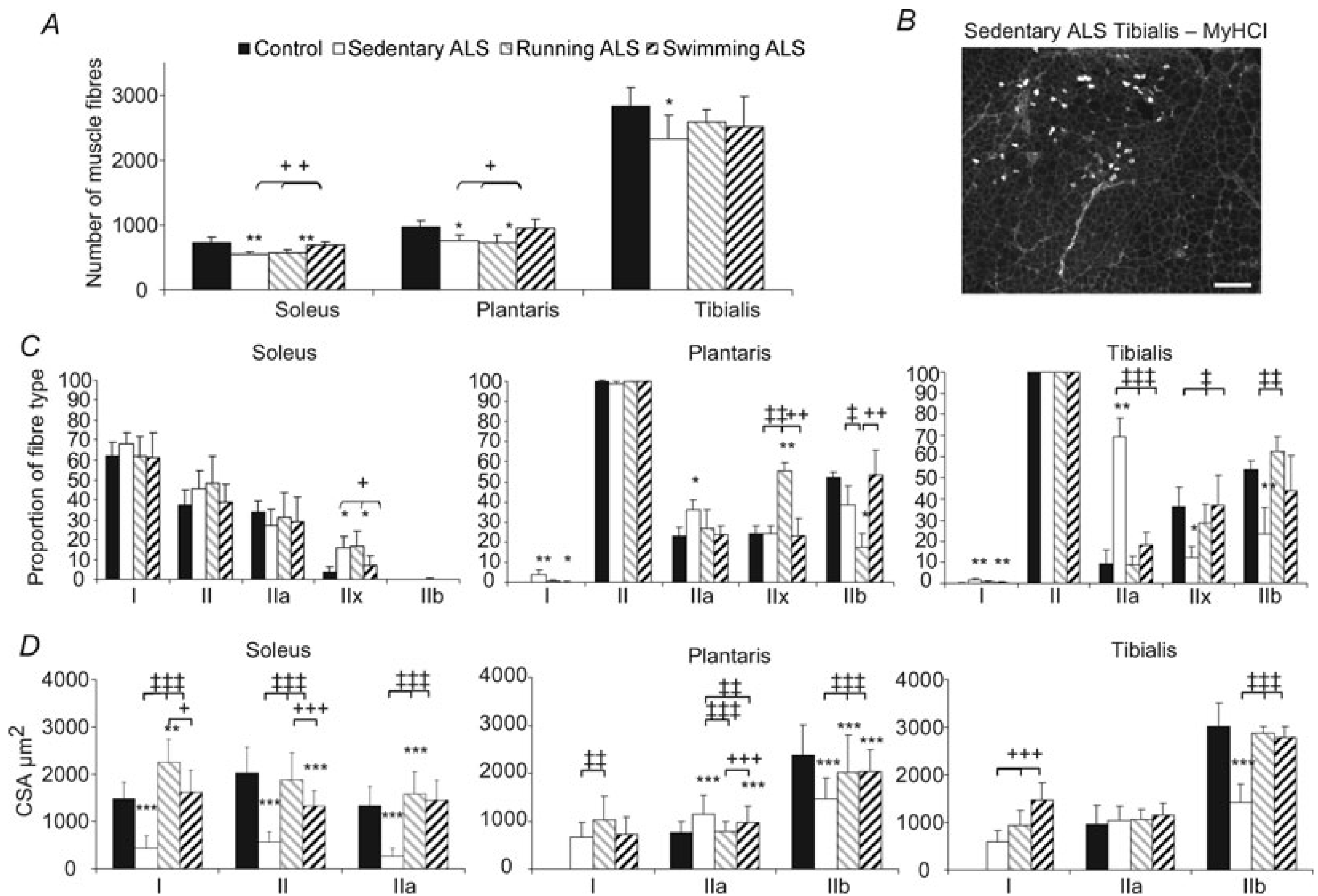

Figure 3. Differential effects of swimming- and running-based training programmes in sustaining the skeletal muscle phenotype in ALS mice

Only the swimming programme limits the hypoplasia affecting the two extensor muscles, namely the slow-twitch soleus and the fast-twitch plantaris from ALS mice at 115 days of age (*A*). Both exercises limit the hypoplasia detected in the fast-twitch flexor tibialis. *B*, representative image showing a type I fibre appearance in a fast-twitch tibialisin sedentary ALS mice. Scale bar: 200 μm. Analysis of the muscle typology in ALS muscles compared to controls (*C*) reveals a slow-to-fast myofibre transition in the slow-twitch soleus, and a fast-to-slow myofibre transition in the fast-twitch plantaris and tibialis that are significantly limited by the swimming programme. The running- and the swimming-based training programmes significantly decrease the severe muscular atrophy detected in all myofibre types in soleus, and in IIb myofibre in plantaris and tibialis ALS muscles as shown by the evolution of the cross-section area (CSA) of each fibre type (*D*). Data are shown as mean ± S.D. ($n = 5$; an average of 3 sections of each muscle per animal at 115 days old was used; *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$P < 0.001$, *control *versus* ALS groups, ‡sedentary ALS *versus* training ALS groups, +swimming ALS *versus* other ALS groups).

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Discussion

This study provides evidence for significant beneficial effects of forced swimming on the life span of ALS mice as well as on the associated clinical symptoms. Survival was extended by about 20% of life span, which can be considered as one of the best treatments tested on ALS animals when starting at the onset of symptoms (Carri *et al.* 2006).

In our exercise conditions, swimming can be described as a high movement amplitude and frequency exercise, and running as a low movement amplitude and frequency exercise (Grondard *et al.* 2008). Accordingly, swimming preferentially activates large motoneurons, belonging to fast motor units (i.e. a medium- and large-size soma motoneuron and a great number of large diameter muscle fibres, of the faster type II) and, in contrast, running preferentially activates small motoneurons belonging to slow motor units (i.e. a motoneurons of small soma area and a low number of type I muscle fibres). Here, we show that swimming, in striking contrast to running, preferentially protects medium-size motoneurons (between 300 and 700 μm^2) in ALS spinal cord but not the smaller ones (less than 300 μm^2). Taken together, these data strongly suggest that the exercise-activated neurons are selectively protected against cell death, in contrast to resting neurons. Accordingly, the running programme led to a limited neuroprotection that is mainly observed for the motoneurons of small soma area (less than 300 μm^2). These data account for the evolution of the muscular phenotype in three skeletal muscles differing in their nature and function. Only the swimming-based training was able to completely counteract the hypoplasia detected in all muscles and notably in the soleus that unexpectedly appeared affected by a severe lost of muscle fibres. Furthermore, only the swimming-based training led to the maintenance of the faster myofibre population to a level comparable to

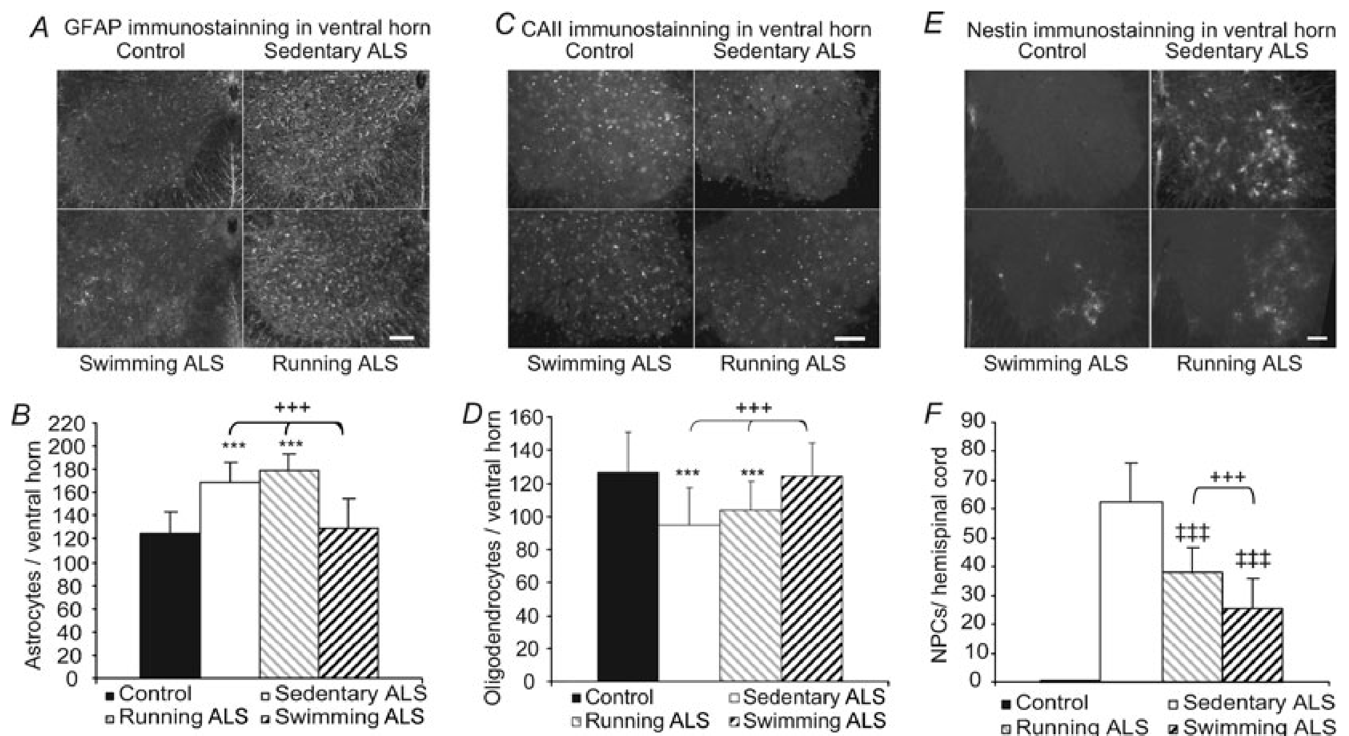

Figure 4. Differential effects of swimming- and running-based training programmes in maintaining the non-neuronal cell populations in the lumbar spinal cord of ALS mice

A, representative images showing a population of astrocytes in control mice and in sedentary, running and swimming ALS mice using glial fibrillary acidic protein (GFAP) immunostaining.The quantification of astrocytes in the ventral horn of the lumbar spinal cord reveals a severe astrogliosis in sedentary and running ALS mice that is limited by swimming exercise (*B*). *C*, representative images showing a population of oligodendrocytes in control, sedentary, running and swimming ALS mice using CAII immunostaining. The unexpected ALS-induced decrease of oligodendrocyte population is limited by swimming exercise (*D*). *E*, representative images showing a population of neural progenitor cells (NPC) in control mice and sedentary, running and swimming ALS mice using nestin immunostaining. Cell quantification in hemi-spinal cord reveals a limited NPC proliferation in swimming-exercised mice (*E*). All scale bars: 100 μm. Number of cells are shown as mean ± S.D.; ($n = 5$; 10 sections of spinal cord per animal. $^{***}P < 0.001$, *control *versus* ALS mice or +swimming ALS *versus* other ALS groups or ‡sedentary ALS *versus* training ALS groups).

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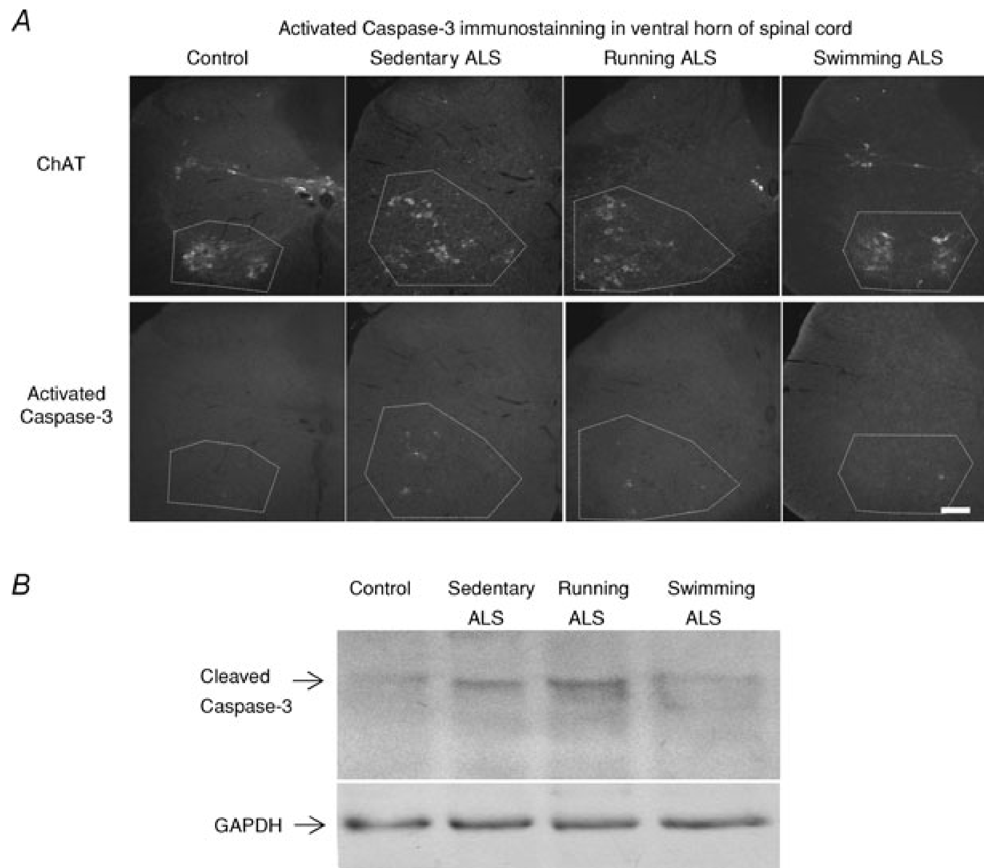

Figure 5. The swimming-based training limits cell death
The swimming programme induces a reduction of apoptotic cells as shown by immunodetection of active caspase-3 in the ventral horn of the lumbar spinal cord from control mice and sedentary, running and swimming ALS mice at 115 days of age (*A*) and by Western blot (*B*) ($n = 3$ in each group). Scale bar: 100 μm.

controls in the three tested muscles. Taken together, these results account for the swimming-induced enhancement of the fast motor-unit activity, probably leading to their maintenance and finally to their protection. Interestingly, both exercise programmes displayed comparable potential to counteract the muscular atrophy favouring the hypothesis of an exercise-induced production of a muscle trophic diffusible factor.

Consequently, the molecular mechanism(s) underlying swimming-induced neuroprotection reflects not so much the modulated expression of a diffusible factor, such as IGF-1, which would have indiscriminately protected the whole neuron population by an endocrine or paracrine way, but an intrinsic modification of activated neurons. This concurs with the result of Kaspar *et al.* (2005) showing distinct molecular mechanisms of action between the IGF-1 delivery and exercise, which, in combination, results in synergistic survival. In addition, the differential exercise effects found in the present study are unlikely to come from a difference in exercise intensity. Indeed, our swimming protocol, a high movement frequency and amplitude exercise when compared to our running protocol (Grondard *et al.* 2008), should be detrimental according to Mahoney *et al.* (2004). In striking contrast with this assumption, swimming induced beneficial effects whereas running did not. Repeated electrical activation of the same neuronal network might induce an intrinsic

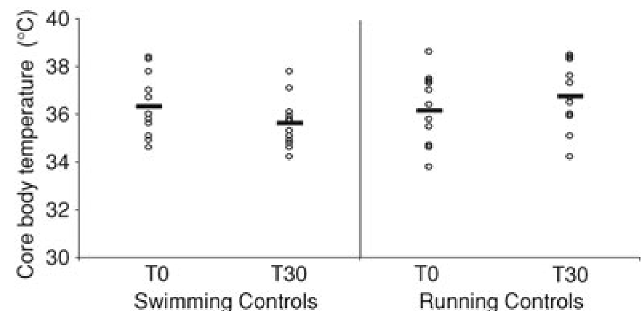

Figure 6. Effects of swimming and running-based training programmes on the core body temperature of control mice
Monitoring of core body temperature before (T0) and after training (T30). Chart representation of core body temperature for each animal ($n = 12$, ○); means are indicated by a bar. No significant difference could be observed before and after swimming- ($P = 0.190$) or running-based ($P = 0.264$) training.

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modification of the transcriptome of activated cells, as previously reported in a study of running-induced adaptations in rats showing that only the subpopulation of motoneurons of small soma area displayed an adaptation of their electrophysiological properties (Beaumont & Gardiner, 2002). According to our present results, it can be assumed that this neuronal subpopulation probably corresponds to the motoneurons specifically activated by the running exercise. In the specific case of ALS, the transcriptome modification of the selectively activated motoneuron may lead to an efficient protection against degeneration.

An alternative and non-exclusive mechanism that could be suggested to explain the specific neuroprotection induced by the swimming-based exercise implies an intrinsic modification of the energy metabolism in recruited muscles. On the one hand, the energy metabolism was shown to be highly dysfunctional in the whole organism of ALS mice (Dupuis *et al.* 2004; Fergani *et al.* 2007). On the other hand, the muscle might be a primary target site for ALS (Dobrowolny *et al.* 2008). In addition, the neuromuscular disruption, especially the post-synaptic cleft, appeared as the most prominent event that induces motoneuron death (Frey *et al.* 2000; Fischer *et al.* 2004). Thus, a strength training programme would modify the metabolism of the whole organism, more particularly in the muscle, where the swimming-induced preferential utilization of the glycolytic metabolism would entail a decrease in the reactive oxygen species (ROS) production that could be highly relevant in the ALS context of SOD1 mutation. Accordingly, a running-based training might preferentially use the aerobic metabolism that could worsen the ALS phenotype in particular conditions, such as those used by Mahoney *et al.* (2004). This hypothesis is substantiated by recent evidence suggesting beneficial effects of ROS reduction for skeletal muscles specifically expressing the mutated SOD1(G93A) protein (Dobrowolny *et al.* 2008).

Furthermore, we reported here, for the first time, that exercise impacts on all neural cell distribution in ALS spinal cord with an efficiency dependent on the nature of the exercise. Only the swimming-based training was able to limit the ALS-induced astrogliosis, reducing both proliferation and hypertrophic processes. Importantly, mutant SOD1 damage in astrocytes was recently shown to accelerate ALS disease progression (Yamanaka *et al.* 2008). Our data are indicative of a strong relationship between neuroprotection rate and astrocyte preservation, suggesting that the swimming-based training promotes cell survival not only on the motoneuron, but also on its surrounding cellular environment resulting in the maintained integrity of functional neuronal circuits. We also observed that the swimming-based training specifically promoted a significant maintenance of the oligodendrocyte population, which displayed a 30% loss in sedentary and running ALS mice. While the role of oligodendrocytes in motoneuron and axonal degeneration in ALS remains elusive, a significant loss of large myelinated fibres in the corticospinal tract and ventral roots was observed in human ALS (Delisle & Carpenter, 1984). Furthermore, myelin degradation in endstage mice has furthermore been observed in the SOD1(G93A) model (Neusch *et al.* 2007). It is thus tempting to speculate that the corticospinal fibres involved in the neuronal network specifically activated by the swimming-based training are also protected. In contrast to the differentiated neural cells, neurons and glia, the neural progenitor cell (NPCs) proliferation, which was enhanced in ALS spinal cord, as previously described (Chi *et al.* 2006; Juan *et al.* 2007), was strongly inhibited by both running and swimming programmes. The fact that both exercises have the same impact on the exercise-induced loss of NPCs suggested a common mechanism, probably associated with the emission of a diffusible factor.

The importance of physical exercise in maintaining nervous system health and function has consistently emerged as a key factor of plasticity and cell survival but the mechanisms underlying the exercise-induced benefits still remain unclear. The present data strongly suggest that some of the exercise effects are specifically and directly exerted on activated motoneurons which probably adapt their proteome, becoming thus selectively more resistant to apoptosis. This concept has been recently substantiated in a spinal-muscular-atrophy model mouse in our laboratory (Biondi *et al.* 2008). Thus, analysing the molecular response of activated motoneurons to exercise and, more precisely, the modulation and activation of the cell-surface receptors is most likely to constitute a milestone in discovering relevant means of preventing motoneuron death in ALS.

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Author contributions

S.D. conducted, designed and analysed the majority of experiments. J.B. and O.B. conducted the Western blot experiments. C.G. designed, and conducted partly, the training programme and the behavioural analyses. S.L. and C.P. assisted in the majority of experiments and C.P. helped in writing the manuscript. P.L. and P.P.V. helped in the analysis of behaviour data and P.L. helped in writing the manuscript. C.C. helped in the analysis of muscle data. F.C. supervised the project and wrote the manuscript.

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