Protective effects of long-term lithium administration in a slowly progressive SMA mouse model

Francesca Biagioni[1,a], Michela Ferrucci[2,a], Larisa Ryskalin[2,a], Federica Fulceri[2], Gloria Lazzeri[2], Maria Teresa Calierno[1], Carla L. Busceti[1], Riccardo Ruffoli[2], Francesco Fornai[1,2,*]

[1]I.R.C.C.S. I.N.M. Neuromed, via Atinense 18, 86077, Pozzilli (IS), Italy

[2]Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, via Roma 55, 56126, Pisa, Italy

[a]These authors equally contributed to the present work.

*** Correspondence:**
Francesco Fornai, MD PhD,

Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Via Roma 55, 56126 Pisa (Italy)

Tel: +390502218611

Fax: +390502218606

Email: francesco.fornai@med.unipi.it

Keywords: spinal muscle atrophy; survival motor neuron protein; spinal cord; motor activity; motor neuron degeneration; motor neuron heterotopy; motor neuron size; radial glia; hindlimb extension reflex; Paw Grip Endurance test.

Running title: Lithium cures SMA 3 mice.

Abstract

In the present study we evaluated the long-term effects of lithium administration to a knock-out double transgenic mouse model ($Smn^{-/-}$; $SMN1A2G^{+/-}$; $SMN2^{+/+}$) of Spinal Muscle Atrophy type III (SMA-III). This model is characterized by very low levels of the survival motor neuron protein, slow disease progression and motor neuron loss, which enables to detect disease-modifying effects at delayed time intervals. Lithium administration attenuates the decrease in motor activity and provides full protection from motor neuron loss occurring in SMA-III mice, throughout the disease course. In addition, lithium prevents motor neuron enlargement and motor neuron heterotopy and suppresses the occurrence of radial-like glial fibrillary acidic protein immunostaining in the ventral white matter of SMA-III mice. In SMA-III mice long-term lithium administration determines a dramatic increase of survival motor neuron protein levels in the spinal cord.

These data demonstrate that long-term lithium administration during a long-lasting motor neuron disorder attenuates behavioural deficit and neuropathology. Since low level of survival motor neuron protein is bound to disease severity in SMA, the robust increase in protein level produced by lithium provides solid evidence which calls for further investigations considering lithium in the long-term treatment of spinal muscle atrophy.

Introduction

Spinal cord pathology and motor impairment at prolonged time intervals characterize a mouse model of spinal muscle atrophy type III (SMA-III) (Fulceri et al., 2012). This model was generated by Monani et al. (2003) who combined a knock-out (KO) for the mouse Smn gene ($Smn^{-/-}$) with the human SMN1 ($SMN1A2G^{+/-}$) and SMN2 ($SMN2^{+/+}$) genes. This KO, double transgenic mouse model ($Smn^{-/-}$; $SMN1A2G^{+/-}$; $SMN2^{+/+}$) possesses low levels of the survival motor neuron (SMN) protein, which is critically related with the preservation of motor neurons in SMA (Burghes and Beattie, 2009). In fact, a decrease in the amount of SMN protein leads to motor neuron loss and worsens disease severity (Lefebvre et al., 1997; Monani et al., 2000; Wirth et al., 2006). In this mouse model, the absence of the mouse SMN ($Smn^{-/-}$) is mitigated by the human protein produced by SMN1 and SMN2 genes (Monani et al., 2003). This may occur either through a full expression of both SMN1 and SMN 2 genes or through partial expression in the heterozygous double transgene which is characterized by the partial expression of the SMN1 gene ($SMN1A2G^{+/-}$). In this specific model, the levels of the SMN protein are barely detectable (Monani et al., 2003). This model is characterized by delayed disease duration with slowly progressive behavioural and morphological alterations reminiscent of SMA-III patients (Fulceri et al., 2012). This renders the model quite different from most experimental models of motor neuron disease characterized by early and massive neuronal loss, which progress rapidly in condensed time intervals. In fact, the short time window (a few weeks) of motor deterioration occurring in most of these models is likely to recruit molecular mechanisms which differ from those occurring during slowly progressive degeneration (lasting more than a year). This also applies to the occurrence of compensatory mechanisms (Monani et al., 2003). Conversely, during short time intervals it is rather difficult to reliably assess the efficacy of neuroprotective treatments. In the short-time, experimental variability (slight dosing variations, intra-group variability, colony-dependent variability etc.) is likely to determine the disease course more than effects produced by short-term drug administration. This concept is well represented by the short-lasting motor neuron disease duration in the G93A transgenic mouse. In this mouse strain, the protective effects of lithium, an autophagy inducer, were demonstrated in several studies (Sarkar et al., 2005; Shin et al., 2007; Feng et al., 2008; Fornai et al., 2008a, 2008b; Calderò et al., 2010; Ferrucci et al., 2010). When experimental conditions did not allow lithium to produce its pharmacological effects, no protective effects were observed (Pizzasegola et al., 2009). In fact, apart from differences in sex and genetic background, the latter study used low serum steady-state lithium levels (0.05–0.07mM) which are unlikely to produce pharmacological effects as pointed out by Chiu et al. (2013). In contrast, in long-lasting models of motor neuron disorders, allowing almost one year of lithium administration the protection against motor neuron loss was consistent (Shimada et al., 2012).

In the present study we administered lithium for more than a year in the SMA-III mouse model to analyze motor activity, neuropathology and SMN protein levels at prolonged time intervals.

Materials and methods

Animals

We used the KO, double transgenic mouse model (N=20) carrying the genotype $Smn^{-/-}$; $SMN1A2G^{+/-}$; $SMN2^{+/+}$ (SMA-III mice) generated by the Jackson Laboratories (Bar Harbor, Maine, USA, Stock No. 5026). The control mouse (N=20) was the FVB/NJ strain (Jackson Laboratory, Stock No. 1800) which corresponds to the Wild type (Wt) for the KO double transgenic $Smn^{-/-}$; $SMN1A2G^{+/-}$; $SMN2^{+/+}$ mouse. This heterozygous mouse for the SMN1A2G gene owns barely detectable SMN protein levels compared with the homozygous (Monani et al., 2003), as confirmed in the present study. All experimental procedures were carried out following the Guidelines of the European Council (86/609/EEC) for the use and care of laboratory animals and they were approved by the Ethical Committee at the University of Pisa.

Animals received food and water *ad libitum* and were housed under controlled conditions: 12 hours light/dark cycle, 21°C room temperature. Lithium administration was kept constant from day 78 until day 535 by adjusting the volume of injection to the mouse weight. In fact body weight was constantly recorded.

Both Wt and KO double transgenic ($Smn^{-/-}$; $SMN1A2G^{+/-}$; $SMN2^{+/+}$) mice were sacrificed at 18 months of age in order: (i) to enhance the onset and detection of motor defects; (ii) to analyze neuropathology at prolonged time intervals; (iii) to evaluate the long-term effects of prolonged lithium administration (exceeding one year) in such a slowly progressive motor neuron disorder. Mice were sacrifice at 18 months of age in order not to risk further the occurrence of accidental deaths (cage deaths), which in longer time may have reduced mice number and/or bias the experimental findings. In fact , these experiments require an average of two years making it difficult to replicate motor test if the mice number is reduced. This is why a high number of mice (N=10 per group) were selected and for each mouse several replica are stored.

Experimental groups and treatment protocol

The SMA-III and Wt mice were divided into 4 experimental groups (N=10 per group): Wt mice receiving saline; Wt mice receiving lithium carbonate (Sigma, St Louis, MO, USA); SMA-III mice receiving saline; SMA-III mice receiving lithium carbonate.

Mice were injected i.p. in the morning every other day between 09:00 h and 12:00 h with either saline solution (0.9% sodium chloride) or lithium carbonate (1 mEq/Kg, dissolved in saline), starting at day 78 (Fulceri et al., 2012).

Motor activity

Behavioral observations were carried out by three blind observers every fifteen days from day 85 until day 535.

Four sets of motor tests were used to document motor impairment as previously described (Fulceri et al., 2012). In particular, starting at 9:00 a.m. each mouse was monitored using the hind limb extension reflex, Paw Grip Endurance (PaGE) test, stride length and rota-rod.

The extension reflex (clasping reflex, abduction reflex) was evaluated holding the mice by the tail. The normal reflex was considered as the abduction and extension of both limbs occurring when the mouse was suspended by the tail. The impairment in the abduction of one or both limbs was considered as a pathological reflex. Values from each group were obtained counting the number of physiological abduction reflexes from each group of mice (N=10), each mouse being tested by three independent observers. Data from each group are expressed as the percentage of normal abduction reflex occurring out of the total (N=30, 10x3) tail suspension trials at each time interval (every 15 days, starting at 85 days).

The PaGE test was carried out according to Weydt et al. (2003) with modifications. In details, mice were placed individually on a meshed wire lid, which was gently shaken for 5 sec to make the mice holding the grid. Then the lid was slowly turned upside-down and the time interval during which the mouse was holding the grid was recorded. The time interval was extended up to 30 minutes in order to detect the slight strength loss which slowly develops in SMA-III mice (Fulceri et al., 2012). Data are expressed as the means of three consecutive trials carried out for each mouse in each group every 15 days. Consecutive trials were carried out independently by three blind observers.

In the stride length test (Fernagut et al., 2002) mice were allowed to move in open field (80x80x30 cm), in which a runway wide (75x5 cm) was arranged to lead into a dark box (20x15x10 cm). The hind paws of the mouse were painted with blue ink and the mouse was allowed to run on a strip of paper down the brightly runway towards the dark box. Stride lengths were measured as the distance between two paw prints. The three longest stride lengths were measured from each run. Data were obtained as the mean from each group where each mouse was measured considering the three longest stride lengths (N=30 corresponding to 10 mice measured 3 times).

The rota-rod test was carried out to measure latency to fall, using a rod rotating at 15 rpm allowing a maximum time interval of 10 min after a brief period of training. Data were expressed as the mean of each group where each mouse was calculated by selecting the best result out of three consecutive trials.

Spinal cord dissection and tissue preparation

Mice were deeply anaesthetized with chloral hydrate and perfused trans-cardially with 50 ml saline solution (0.9% NaCl in PBS) followed by 150 ml fixing solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3). After perfusion, the whole column was dissected and moved overnight within a solution of 4% paraformaldehyde at 4°C. The day after, the spinal cord was carefully dissected from the column and 1 cm long specimen were rinsed in 70% ethylic alcohol overnight at 4°C, they were dehydrated using increasing alcohol solutions, and they were clarified in xylene to be embedded in paraffin.

For each mouse, the various specimen were cut to obtain 6 μm-thick slices in a serial order and mounted on polylysinated slides. These sections were processed for different staining procedures and immunohistochemistry.

Briefly, sections were dried at 37°C, immersed in xylene to remove paraffin, rehydrated by immersion in decreasing ethylic alcohol and stained using hematoxylin (Sigma) followed by eosin (Sigma) solutions. Sections were then dehydrated, rinsed in xylene, cover-slipped with DPX mounting media (Sigma) and finally observed at light microscope Nikon Eclipse 80i (Nikon Corporation, Tokyo, Japan). These hematoxylin & eosin (H&E) stained sections were used to count motor neurons following the stereological procedure reported below.

Morphometry and stereology

Motor neurons throughout the whole lumbar cord (L_1-L_6) were analyzed in 125 sections per mouse to measure number, size and heterotopy in serial, non-consecutive, 6 μm-thick transverse sections, spaced 60 μm and stained with H&E. The count of motor neurons was carried out within lamina IX referring to the atlas (Watson et al., 2009). Motor neurons within lamina IX were considered according with a cut-off criterion (Martin et al., 2007, recently modified by Ferrucci et al., 2010; Fulceri et al., 2012). In fact, the low size-exclusion criteria used here was established based on a series of pilot experiments and depends on strain-specific neuroanatomy of FVB/NJ compared with other mouse strains (Penet et al., 2006). This strain-dependent motor neuron diameter was further validated by the motor neuron specific staining with Smi-32 antibodies.

The cut-off applied here allows to certainly rule out cells other than motor neurons as well as to avoid counting twice the same cell within consecutive sections. However, small motor neurons are ruled out as well. In fact, we included in the motor neuron count only those neurons with a large cell body, with a diameter of at least 22 μm. Motor neurons were further identified based on general motor neuron morphology (multi-polar cell body, with an intense basophilic cytoplasm, well evident nucleolus and non-condensed nucleus) as previously described (Schaffner et al., 1987; Carriedo et al., 1995; Fornai et al., 2008a, 2008b; Ferrucci et al., 2010; Fulceri et al., 2012).

This procedure, despite ruling out small motor neurons (i.e. α-tonic and γ-motor neurons, which could not be included in this analysis), allows us to correctly identify motor neurons within the lamina IX of each section. In particular, we could count motor neurons specifically within medial, intermediate and lateral lamina IX. This allowed us to determine whether motor neuron death occurs differently for motor neurons innervating medial compared with proximal and distal muscles. Therefore, we reported the total motor neuron number and percentage of cell loss in lateral, intermediate, and medial lamina IX in saline-treated compared with lithium-treated SMA mice.

To determine the neuronal diameter, slices were analyzed at 20× magnification with a motorized system (Marzhauser, Molecular Machine & Industries Smart-CutR, Glattbrugg, Switzerland) that allowed to scan the slice throughout the XY plan and to identify laminae IX within the ventral horn. The image analysis software (Molecular Machine & Industries AG, Glattburgh, Switzerland) allowed to draw the motor neuron diameter and read the numerical value automatically. Since the irregular morphology of motor neurons, the final diameter was obtained as the mean value between the maximal and the minimal diameter. Displacement of motor neurons outside the gray matter and within the ventral white matter of the ventral horn, here referred as heterotopic motor neurons, were counted throughout the whole lumbar tract and expressed for each group as the mean percentage value of heterotopic motor neurons out of the total number of motor neurons counted in mice belonging to the same group.

All measures performed in H&E-stained sections were also replicated using Smi-32 immunostained sections, as described in Supplementary data ("Morphometry of Smi-32 immunostained motor neurons") and shown in Supplementary Fig. S1.

Immunohistochemistry

Immunohistochemistry was carried out by using the following primary antibodies: mouse monoclonal anti-Smi-32 antibody (Covance, Emeryville, CA, USA) diluted 1:300, mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (Sigma) diluted 1:400, mouse monoclonal anti-SMN antibody (BD Bioscience, San José, CA), diluted 1:200. Sections for immunohistochemistry were deparaffined by immersion in xylene, re-hydrated by using decreasing alcohol solutions, and permeabilized with Triton X 0.1% in PBS.

Sections to be exposed to peroxidase were pre-incubated with 3% hydrogen peroxide to inhibit the activity of the endogenous peroxidases. Then slices were incubated with a blocking solution (10% normal goat serum in PBS) for 1 hour at room temperature. Primary antibody solutions were prepared in PBS containing 2% normal goat serum and incubated overnight at 4 °C. For peroxidase staining the reaction with primary antibody was revealed by the appropriate anti-mouse biotynilated secondary antibody (Vector Laboratories, Burlingame, CA, USA) used at a dilution of 1:200 for 1 hour at room temperature, followed by incubation with avidin-byotin complex kit 1 hour at room temperature (Vector Laboratories) and the peroxidase substrate diaminobenzidine (Vector Laboratories). Slices were counterstained with hematoxylin, dehydrated in increasing alcohol solutions, immersed in xylene and coverslipped with the mounting agent DPX (Sigma).

Sections to be revealed by fluorescence were exposed to a primary antibody which was further revealed by using fluorescent secondary antibodies Cy3 (red) (Millipore, Temecula, CA) or Cy2 (green) (Millipore) diluted 1:200 in PBS. After washing in PBS, slices were coverslipped with

glycerol. For SMN immunofluorescence DAPI (1:1000) (Sigma) was added to the solution of the secondary antibody in order to visualize cell nuclei. All sections were observed at light microscopy Nikon Eclipse 80i endowed with a fluorescent lamp and provided with digital camera and software for image analysis.

SDS-PAGE immunoblotting

Spinal cord from each experimental group were sonicated in buffer containing 50mM Tris-HCl (pH=7.5), 150mM NaCl, 5mM EDTA, 1% SDS, 0.1% IGEPAL (NP40) and Complete Protease Inhibitor Cocktail Tablet (1 tablet) (Santa Cruz Biotechnology). An aliquot of supernatant was used to determine the protein concentration by a protein assay kit (Sigma). Samples containing 35 μg total protein were diluted in 1.0M Tris (pH=6.8) and separated on 12% sodium dodecyl sulfate-polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose membrane (BIORAD, Milano, Italy).

Membrane was then immersed in blocking solution containing PBS with 0.05% Tween-20 (PBS-T) and 5% not fat dried milk (Sigma) for 30 min at room temperature and then dehydrated. Second day, the membrane was rehydrated and incubated with primary antibody anti-SMN (BD) diluted 1:500 in PBS-T containing 1% not fat dried milk, for 2 hours at room temperature on an orbital shaker. The blots were probed with anti-mouse horseradish peroxidase-labeled secondary antibody (1:1300, KPL, Maryland, USA) diluted in PBS-T containing 1% not fat dried milk and the bands were visualized with Immun-Star HRP Substrate (BIORAD). Membrane was stripped to remove the primary and secondary antibodies and investigate a loading control, beta-actin. Membrane was incubated with stripping solution (NaCl 5M and acetic acid glacial) for 20 min on an orbital shaker, washed with PBS-T, blocked with blocking solution (5% not fat dried milk in PBS-T) for 30 min at room temperature and then dehydrated. Third day, membrane was rehydrated and incubated with primary antibody anti beta-actin (Abcam; Cambridge, UK) diluted 1:2500 in PBS-T containing 1% bovine serum albumin (BSA, Sigma) for 2 hours at room temperature on an orbital shaker and then with anti-rabbit peroxidase coniugate secondary antibody (1:7000, Merck Millipore, Billerica, Massachusetts) containing 1% BSA. The bands were visualized with Immun-Star HRP Substrate (BIORAD).

Films of Western blots (N=3) were scanned and the optical density of the bands was measured by using IMAGEJ.

Statistical analysis

Behavioral data were expressed as the mean±S.E.M. from three independent observations for each mouse (N=30, 10x3), unless data referred to rota-rod which were expressed as the best result of three trials. Comparisons between groups were made by using the Analysis of Variance (ANOVA test). Null hypothesis was rejected for $P<0.05$.

For morphological analysis, measurements were carried out by two different observers, blind to the specific mouse genotype and treatment. For each mouse, data report number, place, size, and occurrence of heterotopic motor neurons. Data obtained from each observer were used to obtain the mean value±S.E.M. from each experimental group (N=10 mice per group). Comparisons between groups were made by using ANOVA with post-hoc Sheffé's test. Null hypothesis was rejected for $P<0.05$. Data from immunoblotting were expressed as the means±S.E.M. (N=3). Comparisons between groups were made by using ANOVA with post-hoc Sheffé's test.

Results

Analysis of motor activity

Motor activity was analyzed using four main test, and the dose of lithium was adjusted to the weight of mice, which increased between day 85 and day 535 (from a mean of 28.5 g up to 35.9 g), while neither disease progression nor lithium treatment altered body weight (Fig. 1A).

Stride length and rota-rod

The length of the stride was maintained in all groups for the whole observation interval (Fig. 1B). Similarly, no difference between groups at rota-rod test was evident. (Fig. 1C).

Hind limb extension reflex (Fig. 2A)

When SMA-III mice were suspended by the tail we observed a loss of the physiological hind limb abduction starting at day 205 ($P<0.0001$) (Fig. 2A). Lithium administration in SMA-III mice significantly protected from the loss of the reflex.

Paw Grip Endurance (PaGE) test (Fig. 2B)

PaGE was the earliest test to be altered in SMA-III mice (Fig. 2B). In fact, already at day 85, this group performed worse than Wt. The loss of grip endurance was significant since day 100 ($P=0.0009$) and progressed along the observation interval. Lithium-induced protection was evident from day 115 ($P=0.01$) and persisted during the whole observation interval.

Lithium prevents motor neuron loss in SMA-III mice (Fig. 3)

In the spinal cord of 18 months old SMA-III mice we found a severe decrease of motor neurons compared with Wt mice (2047.6±77.8 compared with 3668.8±159.0, respectively) (Fig. 3A). Long-term lithium administration prevented neuronal loss without affecting motor neuron number in Wt mice (3312.6±134.1 and 3418.3±137.1,, respectively). At level L_5 (Fig. 3B) representative pictures are consistent with neuronal counts (Figs. 3C-3F).

Somatotopy of the effects of lithium on motor neurons loss (Fig. 4)

The ventral horn contains motor neurons which are arranged as distinct columns according to peripheral innervation. As reported in Fig. 4A, the lateral and intermediate columns contain motor neurons innervating the distal (Pes 9) and proximal (Hm9 and Gl9) limb muscles, respectively; whereas the medial column (ExA9) contains motor neurons for axial muscles. Motor neuron loss in saline-administered SMA-III mice was more severe in the lateral and intermediate compared with medial columns (Figs. 4B-4D).

Within the medial column of saline-treated SMA-III mice we counted 737.4±19.4 motor neurons compared with 1091.2±92.6 motor neurons from saline-treated Wt mice (Fig. 4B). Within intermediate and lateral columns of saline-treated SMA-III mice we counted 693.8±34.2 and 616.4±37.2 motor neurons, compared with 1266.8±58.9 and 1310.8±73.8 motor neurons, respectively, which were counted in Wt mice (Figs. 4C and 4D, respectively).

Chronic lithium administration in SMA-III mice prevented motor neuron loss mostly in the lateral column (Figs. 4B-4D). In fact, in SMA-III mice receiving lithium the amount of medial, intermediate and lateral motor neurons was 961.9±56.1, 1146.8±53.4 and 1203.9±61.7, respectively. Representative pictures (Figs. 4E-4H) are reported from all experimental groups.

Effects of lithium on disease-induced motor neuron alterations (Fig. 5)

Motor neuron diameter was not significantly modified in Wt mice administered lithium (27.53±0.23 μm), but it was significantly increased in SMA-III mice (30.87±0.42 μm) at prolonged disease

315 stages. In these mice, lithium reduced motor neuron diameter (27.57±0.20 μm) back to values
316 measured in Wt mice (27.14±0.27 μm) (Fig. 5). Lithium administration, also increased basophilic
317 structures within motor neurons (Fig. 5) and prevented the increase of the neurofilament protein Smi-
318 32 occurring in SMA-III mice (Figs. 5 and 6).
319
320 **Lithium prevents motor neuron heterotopy in SMA-III mice (Fig. 7)**
321 In SMA patients a few motor neurons move beyond the border of the grey matter mainly towards the
322 ventral nerve roots (Simic, 2008). As reported in the graph of Fig. 7A motor neuron heterotopy was
323 never described in Wt mice either treated with saline or lithium. On the other hand, a few motor
324 neurons were counted following Smi-32 immunostaining in the ventral white matter of saline-
325 administered SMA-III mice. This misplacement was prevented by lithium. Consistently, this is
326 shown in representative pictures Figs. 7B-7E. Occurrence of heterotopy was concomitant with
327 intense radial GFAP immunofluorescence (Fig. 8).
328
329 **Lithium increases SMN expression in the spinal cord of SMA-III mice (Fig. 9).**
330 The SMN protein was barely detectable in gel from saline-administered SMA-III mice, while lithium
331 administration in SMA-III mice rose protein level up to those measured in Wt (Figs. 9A and 9B). In
332 representative pictures (Figs. 9C-9J) SMN protein is visible in DAPI-counterstained neurons at two
333 levels of magnification.
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Discussion

The present study indicates protective effects of lithium in a mouse model of spinal muscle atrophy (SMA). Lithium reduces both motor impairment and motor neuron loss, and it increases the expression of SMN protein. Since the ($Smn^{-/-}$; $SMN1A2G^{+/-}$; $SMN2^{+/+}$) mouse mimics SMA-III disease, the present data shed new light on the potential effects of lithium in this motor neuron disorder. The prolonged time-course of this motor neuron degeneration allowed to detect consistently (for more than a year) the disease-modifying effects of lithium. Remarkably, lithium-produced a dramatic increase in SMN protein, way in excess compared with that previously reported in the spinal cord after valproic acid (Tsai et al., 2008). Lithium increases SMN both in the ventral and dorsal horn, as expected due to the ubiquitous occurrence of the protein (Monani, 2005; Lee et al., 2012). These findings are critical for SMA since the loss of SMN determines disease severity (Munsat and Davies, 1992; Crawford and Pardo, 1996; Campbell et al., 1997; Coovert et al., 1997; Lefebvre et al., 1997; Wirth et al., 2006; Makhortova et al., 2011). In fact, drugs elevating SMN are intensely sought for SMA therapeutics (SI Discussion on the significance of increase in SMN). SMN levels are increased by inhibition of glycogen syntase kinase (GSK)-3beta (Makhortova et al., 2011; Chen et al., 2012a) and lithium is known to possess such an effect (Klein and Melton, 1996; Pasquali et al., 2010). Lithium-induced increase of SMN was concomitant with reduced neurological impairment. In fact, lithium attenuated the loss of the extension reflex and reduced the loss of paw grip endurance. This confirms what recently published about the efficacy neuroprotection of long-term lithium administration (Shimada et al., 2012).

Consistently, in SMA-III mice lithium prevented motor neuron loss, which occurs more severely than motor impairment, thus confirming the study by Simon et al. (2010). These authors demonstrated that a threshold of 40% of motor neuron loss is needed for initiating motor symptoms. Lithium-induced protection against motor neuron loss was more evident compared with attenuation of neurological deficit. In detail, saline-treated SMA-III mice possessed 55.81% of motor neurons compared with Wt mice, while in lithium-administered SMA-III mice motor neurons were 90.29% of Wt mice. Such a full protection did not occur for specific motor deficit. This discrepancy might relate to a difference between the protective effects of lithium in the spinal cord compared with distal motor axons. Further studies are needed to test whether prolonging lithium administration may halt the distal axonopathy in this strain of mice (Cifuentes-Diaz et al., 2002).

We found a specific somatotopy of lithium-induced neuroprotection. In fact, motor neuron loss in SMA-III mice is more evident in the lateral than intermediate and medial column (spared motor neurons: 47.02%, 54.77%, 67.58% of Wt in the lateral, intermediate and medial column, respectively). Consistently, in lithium-treated SMA-III mice percentage of spared motor neurons were 91.84.%, 90.53%, and 88.15%, of Wt in the lateral, intermediate and medial column, respectively. Thus, protective effects of lithium in SMA-III mice were mostly pronounced just in those areas which were mostly affected.

Lithium fully prevented pathological enlargement of spared motor neurons in SMA-III mice and occluded the increase in basophilic structures and neurofilament protein Smi-32 within motor neuron cytosol in SMA-III mice. At present, we do not know why a motor neuron disorder is accompanied by size enlargement. However, it is worth to note that such an effect is disease-independent, since it is described also in G93A transgenic mice (Martin et al., 2007; Fornai et al., 2008a; Ferrucci et al., 2010), dynactin transgenic mice (Laird et al., 2008), chronic glutamate-induced motor neuron loss (Vargová et al., 2001; Fulceri et al., 2011), and axotomy-induced motor neuron damage (Barr and Hamilton, 1948; Hall and Borke, 1988). The occurrence of motor neuron heterotopy in the white matter concomitant with proliferating radial glia represents a typical feature in the spinal cord of SMA patients (Simic et al., 2008) and it was reproduced in the present work. In SMA patients this follows altered synaptic connectivity of motor neurons (Simic et al., 2008). Thus,

suppressive effects of lithium on motor neuron heterotopy may rely either on maintenance of synaptic connectivity or being the consequence of the inhibitory effects of lithium on glial activation in the spinal cord (Chi et al., 2006, 2007; Su et al., 2007; Fornai et al., 2008a, 2008b; Su et al., 2009).

SI Discussion

Motor neuron size variations.

Wild type strain possesses an average of motor neuron size which is lower compared with common strains used to model motor neuron disorders. In fact, when counting the average of motor neurons larger than the size needed to be included in the count (size inclusion ≥22 μm) we found that, in Wt large motor neurons possess a mean diameter of 27.14±0.27 μm, which is way below the size of motor neurons from C57 Black mice and transgenic SOD1 mice, averaging more than 30 μm, as measured in pilot studies not shown here and partly reported in Fornai et al. (2008a) and Ferrucci et al. (2010). In any case, this criterion is even more selective than that one used by Carriedo et al. (1996), who based their identification of motor neurons on Smi-32 cells with a diameter ≥20 μm.

The significance of increase in SMN protein.

All SMA phenotypes are caused by reduced levels of the Survival Motor Neuron (SMN) protein (Lefebvre et al., 1997). A correlation exists between the decrease in SMN protein and SMA severity (Campbell et al., 1997). Such a correlation is so tight that compounds that counteract the decrease in SMN levels also correct the phenotypic changes occurring in SMA (Makhortova et al., 2011). Survival motor neuron protein is a 38 kD protein of 294 amino acids. In humans this protein derives from two highly homologous genes placed on chromosome 5: SMN1 and SMN2 (Lefebvre et al., 1995). Occurrence of SMA is generally due to a loss/mutation of the SMN1 gene. The loss of SMN1 and concomitant variations in the SMN2 gene produce most SMA phenotypes. In detail, the deleterious effects due to a loss of the SMN1 gene may be counteracted by increasing SMN2 copies (Campbell et al., 1997). This is why in the present model the absence of the mouse protein, compensated by the expression of two human genes, leads to a late disease onset and delayed survival. This model was generated by Monani et al. (2003) who set up *smn* KO in which they added two copies of a human gene (SMN1 and SMN2) which leads to a minimal protein production. Of course being protein levels very low, motor activity and motor neurons degenerate throughout the life span mimicking the prolonged disease duration of SMA-III.

The present data apply beyond SMA since SMN protein levels are critical in counteracting motor neuron injuries also in other motor neuron disorders (Veldink et al., 2005). This was recently demonstrated in amyotrophic lateral sclerosis (ALS) where SMN protein interacts with typical ALS proteins such as Fused in Sarcoma (FUS), TAR-DNA binding protein 43 kDa (TDP-43), and Cu-Zn superoxide dismutase type 1 (SOD 1) (Turner et al., 2008; Shan et al., 2010; Kariya et al., 2012; Yamazaki et al., 2012). This calls for ultrastructural studies aimed at co-localizing SMN protein and TDP-43 at the level of gems and Cajal bodies. Recent studies add further potential roles for SMN. For instance, the survival motor neuron protein seems to be key in counteracting stress-induced cell injury (Zou et al., 2011) and it is activated by a variety of stressors including hypoxia (Bebee et al., 2012) and exposure of motor neurons to glutamate (data in progress in this lab).

From SMN expression to other mechanisms for lithium-induced neuroprotection.

Although the molecular mechanisms which regulate the effects of lithium in this $Smn^{-/-}$; $SMN1A2G^{+/-}$; $SMN2^{+/+}$ mouse SMA model remains hypothetical, the occurrence of increased SMN levels is a clear evidence. This is likely to rely on the inhibition of GSK-3 beta produced by lithium since GSK-3 beta inhibition is known to up-regulate the SMN protein (Chen et al., 2012).

Nonetheless, the effects of lithium on phosphatidyl-inositol (PI) turn-over and the activation of the autophagy machinery (Sarkar et al., 2005; Sarkar and Rubinsztein, 2006) are expected to contribute to neuroprotection. In fact, PI turnover is implicated in spinal and bulbar muscle atrophy (Palazzolo et al., 2007, 2009), where the induction of autophagy causes protective effects (Montie et al., 2009; Tanaka et al., 2012). Again, a defect in the autophagy machinery is more and more evident in motor neuron disorders (Fornai et al., 2008a, 2008b; Kim et al., 2008; Laird et al., 2008; Tamai et al., 2008; Hetz et al., 2009; Madeo et al., 2009; Pasquali et al., 2009; Cox et al., 2010; Crippa et al., 2010a, 2010b; Hadano et al., 2010; Ferrucci et al., 2010, 2011; Fornai et al., 2011; Fulceri et al., 2011; Katona et al, 2011; Penas et al., 2011; Tian et al., 2011; Chen et al., 2012b; Otomo et al., 2012; Schaeffer et al., 2012; Song et al., 2012; Wang et al., 2012a, 2012b) up to a point in which recent evidence indicates that autophagy activators are able not merely to alleviate motor neuron disorders but even rescuing motor activity induced by mutation in the key protein TDP-43 (Wang et al., 2012a, 2012b). This renders the induction of autophagy by lithium seminal for motor neuron survival in the long run which becomes well evident at delayed time intervals, as shown by Shimada et al. (2012) in a motor neuron disorder induced by tauopathy. On the other hand, if one administers an autophagy activator such as trehalose which promotes autophagy in the brain but not in the spinal cord, the damage occurs in motor neurons while it is prevented in the brain (Schaeffer et al., 2012). Recently, the recruitment of autophagy in a motor neuron disorder was monitored *in vivo* through real time brain imaging in GFP-LC3/G93ASOD1 double transgenic mice. This allowed to follow the recruitment of the autophagy pathway along the progression of motor neuron disease (Tian et al., 2011).

In conclusion a slow progressive model of motor neuron death offers the unique chance to magnify slight protective effects along prolonged time intervals of drug administration. At the same time investigators may better discern symptomatic versus disease-modifying effects. To our knowledge there are only a few studies in motor neuron diseases and no study at all in a SMA-III model in which drug administration lasted almost 18 months. This unique setting provided multiple evidence for lithium-induced neuroprotection against motor neuron loss.

Indeed, if one goes in depth in the SMA story, nothing really new is under the sun since a Russian-written paper by Il'ina and collegues reported that lithium carbonate exerts positive clinical effects in patients affected by Kugelberg-Welander's spinal amyotrophy (Il'ina et al., 1980).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

ExA9 = external anal sphintere; FUS = Fused in Sarcoma; GFAP = glial fibrillary acidic protein; Gl9 = gluteal muscles; GSK = glycogen synthase kinase; H&E = hematoxylin & eosin; Hm9 = hamstrings; PaGE = Paw Grip Endurance; Pes9 = pes; SMA = spinal muscle atrophy; PI = phosphatidyl-inositol; SMA-III = spinal muscle atrophy type III; SMN = survival motor neuron;

SOD 1 = Cu-Zn superoxide dismutase type 1; TDP-43 = TAR-DNA binding protein 43 kDa; Wt = Wild type.

Acknowledgments

This work was supported by an educational fellowship (to Fe.Fu.) and research grant (to FF) from A.S.A.M.S.I. (Associazione Studio Atrofie Muscolari Spinali Infantili).

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Figure legends

Figure 1. (**A**) **Body weight analysis**. There are no significant variations in body weight between groups as shown in the line-graph. The weight of all mice (N=40) from each group (N=10) homogeneously increased progressively between day 85 and 535 from an average of 28.5 g to 35.9 g. (**B**) **Stride length.** Mice treated with lithium never significantly differ from saline-treated mice in the length of the stride. Between groups there was no consistent significant variation in the length of the stride for the whole observation period. (**C**) **Rota-rod**. No motor impairment was detected in all experimental groups along the entire observation period. All mice (N=40), kept the place on the 15 rotating rpm rod during the 10 min test interval during three consecutive trials.

All values are expressed as the mean±S.E.M. for each group.

Figure 2. Effects of lithium on the extension reflex and PaGE test.
(**A**) In saline-treated SMA-III mice the loss of the reflex appears at day 205 and worsens to reach the complete loss of the reflex. In lithium-treated SMA-III mice the early loss of the reflex was halted. Values from each group are obtained counting the number of normal abduction reflexes. Data are expressed as the percentage of normal abduction reflexes occurring out of the total tail suspension trials. Thus, each number represents the percentage mean ±S.E.M. from each group of mice (N=10), each mouse being tested by three independent observers.
(**B**) The PaGE test detects the earliest motor alterations in SMA-III mice which perform worse than Wt since the beginning of the behavioural observation (day 85). Lithium administration stabilizes the decay in the paw grip endurance for the whole experimental time.

Values are counted as the time interval (in minutes) spent by mice holding the grip before falling and represent the mean ±S.E.M. obtained from each group of mice (N=10).

X axis starts at 85 days of age since this corresponds to time 0 for behavioral evaluation.

*P<0.05 compared with Wt mice.

$^{\#}$P<0.05 compared with SMA-III mice administered saline.

Figure 3. Effects of lithium on motor neuron survival.
(**A**) Graph reports the mean number of motor neurons ±S.E.M. within the ventral horn of the spinal cord in Wt and SMA-III mice chronically treated with saline or lithium (N=10 mice per group). Representative pictures show the lumbar (L_5) spinal cord corresponding to the atlas plate (**B**), in: saline-treated Wt (**C**), SMA-III mice (**D**), lithium-treated Wt (**E**), and lithium-treated SMA-III mice (**F**).

*P<0.05 compared with other groups.

Scale bar =345 μm.

Figure 4. Lithium preserves lamina IX motor neurons from degeneration in SMA-III mice.
(**A**) Atlas plate shows lamina IX motor neurons belonging to the lateral, intermediate and medial grouping within the ventral horn at L_6 level. The counts of the specific lamina IX motor neurons are reported in the graphs (**B**-**D**), showing the number of medial (**B**), intermediate (**C**), and lateral (**D**). Motor neuron loss found in saline-treated SMA-III mice involves all lamina IX motor neurons, but it is more severe for the lateral motor neurons (see **D** compared with **B** and **C**). Values are expressed as the mean±S.E.M. (N=10 mice per group). Representative pictures from: saline-treated Wt (**E**), saline-

treated SMA-III (**F**), lithium-treated Wt (**G**), lithium-treated SMA-III (**H**). Abbreviations: ExA9 = external anal sphintere; Gl9 = gluteal muscles; Hm9 = hamstrings; Pes9 = pes; 7Sp = lamina 7; 8Sp = lamina 8.

*P<0.05 compared with other groups.

Scale bar=133.4 μm.

Figure 5. Lithium preserves motor neuron size in SMA-III mice.
Representative pictures at high magnification of H&E-(**upper line**) and Smi-32-(**lower line**) stained motor neurons from all experimental groups. Motor neurons from SMA-III mice show pathological enlargement prevented by long-term lithium treatment. The graph reports the mean±S.E.M. of motor neurons diameter measured in all experimental groups (N=10 mice per group).

*P<0.05 compared with other groups.

Scale bar=29.67 μm.

Figure 6. Lithium reduces Smi-32 immunofluorescence in SMA-III mice.
Representative pictures of sections from lumbar spinal cord stained using anti-Smi-32 immunofluorescence. Note intense Smi-32 immunofluorescence detected in motor neurons from a saline-treated SMA-III mouse which was reduced by lithium administration back to what is shown for wild types. Incidentally, in keeping with data reported in the main text, even here is evident the suppression of size enlargement induced by lithium in SMA-III mice.

Scale bar=62.84 μm.

Figure 7. Lithium prevents motor neuron heterotopy in SMA-III mice.
The percentage of motor neuron heterotopy is reported in the graph (**A**). Note that heterotopy is absent in Wt mice and it is prevented in SMA-III mice by long-term lithium administration. Values are expressed as the mean±S.E.M. from each group (N=10 mice per group). Representative Smi-32 immunostaining of the ventral horn: saline-treated SMA-III (**B**); saline-treated Wt (**C**); lithium-treated (**D**), and lithium-treated SMA-III (**E**).

*P<0.05 compared with other groups.

Scale bar=78.48 μm.

Figure 8. Lithium reduces GFAP immunostaining in the spinal white matter of SMA-III mice.
Representative pictures at low (upper line, **A**-**D**) and high (lower line, **E**-**H**) magnification of the spinal cord from Wt and SMA-III mice administered either saline or lithium. In saline-treated SMA-III mice marked GFAP immunostaining is evident mostly as radial fluorescent rods in the peripheral spinal cord corresponding to the white matter. Lithium administrations suppresses glial staining which appears similar to Wt.

Scale bars=(**A**-**D**) 380 μm; (**E**-**H**) 53.29 μm.

Figure 9. Lithium increases SMN expression in SMA-III mice.
(**A**) Representative Western blotting of SMN protein which is barely detectable in saline-treated SMA-III mice while it is dramatically increased following long-term lithium administration. Immunoblotting for the housekeeping protein β-actin is also shown. (**B**) Densitometric analysis. The

values represent the mean±S.E.M. of the densitometry ratio SMN/β-actin, expressed in arbitrary units (N=3). (**C**-**J**) Representative immunofluorescence for SMN protein (green) counterstained with the nuclear marker DAPI (blue) from saline-treated Wt (**C**, **G**) and saline-treated SMA-III mice (**D**, **H**), or lithium-treated Wt (**E**, **I**) and lithium-treated SMA-III mice (**F**, **J**) are shown at low magnification in the left panel (**C**-**F**), whereas the details within the squares are reported at high magnification in the right panel (**G**-**J**). Arrows indicate a spot-like SMN immunostaining.

*P<0.0001 compared with other groups

Scale bars=(**C**-**F**) 63.18 μm; (**G**-**J**) 23.66 μm.