Correction

MEDICAL SCIENCES. For the article “Lithium delays progression of amyotrophic lateral sclerosis,” by Francesco Fornai, Patrizia Longone, Luisa Cafaro, Olga Kastsiuchenka, Michela Ferrucci, Maria Laura Manca, Gloria Lazzeri, Alida Spalloni, Natascia Bellio, Paola Lenzi, Nicola Modugno, Gabriele Siciliano, Ciro Isidoro, Luigi Murri, Stefano Ruggieri, and Antonio Paparelli, which appeared in issue 6, February 12, 2008, of Proc Natl Acad Sci USA (105:2052–2057; first published February 4, 2008; 10.1073/pnas.0708022105), the authors note that portions of Fig. 2e and of supporting information (SI) Figs. 11b, 12c, 13c, 17c, 19a and b, 20e, and 23b were assembled from multiple sources or include images moved from their original orientation. The rearrangements were done to provide clarity and consistency within the figures. We regret not explicitly noting this rearrangement in the text or figure legends. We are providing corrected figures, with the rearrangements indicated by thin vertical lines, in accordance with PNAS policies. In addition, SI on the web site has been updated to include the revised figures. The corrected figures and their legends appear below.

Fig. 2. Neuroprotective effects of lithium on medium-size lamina VII neurons. (a) Representative micrographs of those H&E-stained lamina VII neurons that were selected for the count based on size specificity (diameter from 10 to 20 μm). (b) Graph indicates the severe loss of these neurons in G93A mice, which exceeded the loss of MN. Remarkably, the G93A mice treated with lithium showed a much higher number of lamina VII medium-size neurons, even compared with saline-treated WT mice. (c–e) These results were confirmed by gephyrin immunostaining, as shown here, and by all of the staining procedures summarized in SI Fig. 16. Counts represent the mean ± SEM of 62,000 cells per group (3,100 per mouse in groups of 20 mice). Comparison among groups was made by using one-way ANOVA. *, P ≤ 0.05 compared with WT saline-treated group. #, P ≤ 0.001 compared with G93A saline-treated groups. (Scale bars: 17 μm.)
SI Fig. 11. Lithium treatment decreases GFAP immunostaining. (a) Immunofluorescence of cervical and lumbar spinal cords of WT and G93A lithium-treated mice compared with saline-treated mice showing GFAP staining. At the end of the disease, GFAP immunofluorescence appears highly intense in G93A saline-treated mice, whereas lithium treatment decreases GFAP-positive cells in both tracts of the spinal cord compared with saline-treated G93A mice. No GFAP staining was observed in age-matched, saline- or lithium-treated WT mice. (b) Representative immunoblots and relative densitometric analysis of GFAP expression in the spinal cord of saline- and lithium-treated WT and G93A mice. The significance of these findings is discussed in SI Discussion, 2- The Potential Role of Interference with Glial Activation. Values represent the mean ± SEM of five repeated analyses compared by one-way ANOVA. *, P < 0.05 compared with WT saline-treated group; **, P < 0.01 compared with WT saline-treated group; #, P < 0.05 compared with WT and G93A saline-treated groups. (Scale bars: 28 μm.)

SI Fig. 12. Lithium treatment increases the number of NeuN-positive neurons within Lamina VII. (a) Representative images showing NeuN-positive neurons in the Lamina VII of WT and G93A saline- and lithium-treated mice. (b) Histogram shows the number of NeuN-positive neurons within the Lamina VII of the lumbar spinal cord increased in the G93A mice after lithium treatment. Remarkably, this effect not only consisted of protection from the loss that occurred in the G93A saline-treated mice, but extended to a robust increase in neuron number compared with saline-treated controls. The number of NeuN-positive neurons overlaps that reported within Lamina VII after H&E staining, calbindin 28K, and gephyrin immunostaining. The counting criteria were the same under comparable stereological conditions. (c) Representative Western blot and densitometric analyses of NeuN expression in the lumbar spinal cord of WT and G93A mice, which confirms the increase in NeuN staining after lithium treatment. Values represent means ± SEM, analyzed by one-way ANOVA. *, P < 0.05 compared with WT saline-treated group; #, P < 0.05 compared with WT and G93A saline-treated groups. (Scale bars: 13 μm.)
SI Fig. 13. Lithium treatment increases the number of calbindin 28K-positive neurons in Lamina VII. (a) Lumbar spinal cord sections from saline- and lithium-treated WT and G93A mice immunostained for calbindin 28K. (b) Histogram shows the significant loss of Lamina VII calbindin 28K-positive neurons in the G93A mutant mice compared with WT. Even in this case, the increase in the number of neurons in G93A mice after lithium treatment significantly surpasses that counted in controls. As observed after the other staining procedures, lithium induced no effect on neuron number in WT mice. (c) Representative Western blot and densitometric analyses of calbindin 28K expression in lumbar spinal cord in WT and G93A mice confirm the loss of the calbindin 28K-positivity in G93A saline-treated mice and the increase in the calbindin 28K-positivity after lithium treatment. Values represent means ± SEM, analyzed by one-way ANOVA.

SI Fig. 17. Effects of lithium on α-synuclein immunostaining. (a and b) Representative images of Lamina IX (a) and Lamina VII (b) of α-synuclein immunostaining of WT and G93A mice after saline and lithium treatment. Images suggest an increase in α-synuclein staining in G93A mice, which is reverted by lithium administration. (Scale bars: 18 μm.) (c) Representative Western blot and densitometric analyses of α-synuclein expression confirming the lithium-induced occlusion in α-synuclein increased positivity of G93A mice. Values represent means ± SEM, analyzed by one-way ANOVA. *, P < 0.05 compared with WT saline-treated group; #, P < 0.05 compared with WT and G93A saline-treated groups.

SI Fig. 19. Lithium reduces both α-synuclein and ubiquitin in the spinal cord of G93A mice. Representative Western blots and densitometric analysis of α-synuclein (a) and ubiquitin (b) expression in the cervical and lumbar tracts of WT and G93A saline- and lithium-treated mice. Lithium significantly decreased the expression of α-synuclein in the lumbar tract, whereas the expression of ubiquitin decreased in both the cervical and lumbar tracts. Values represent means ± SEM, analyzed by one-way ANOVA. *, P < 0.05 compared with WT saline-treated group; #, P < 0.05 compared with WT saline-treated mice and G93A lithium-treated mice; ##, P < 0.01 compared with WT saline-treated mice and G93A lithium-treated mice.
SI Fig. 20. Effects of lithium on SOD1 immunostaining. (a–d) Immunostaining for SOD1 is lighter in WT (a and b) compared with G93A (c and d) mice. In particular, G93A mice treated with saline show intense and aggregated SOD1 immunostaining (c), which, similarly to ubiquitin, was also found in the axon of motor neurons (arrow). This SOD1 accumulation was markedly cleared after lithium administration (d). (Scale bars: 33 μm.) (e) Representative Western blot and densitometric analyses of a triplicate of SOD1 expression shows an accumulation of the protein in G93A saline-treated mice, whereas this is suppressed after lithium administration. Values represent means ± SEM, analyzed by one-way ANOVA. * P < 0.05 compared with WT saline-treated group; #, P < 0.05 compared with WT and G93A saline-treated groups.

SI Fig. 23. Effects of lithium administration on mitochondria in motor neuron cell cultures and cell lines. (a) SH-SY5Y cells plated on sterile coverslips were either not treated (saline) or were treated for 72 h with 1 mM lithium (changing the medium and readding the substance every 24 h), stained for 60 min with 300 nM MitoTracker Red or MitoTracker Green (Invitrogen), and observed under the confocal fluorescence microscope. Arrows point to cytoplasmic regions of high fluorescence intensity that indicate the accumulation of mitochondria. (Scale bars: 17 μm.) (b) Immunoblotting of cytochrome C (monoclonal antibody; Alexis) in homogenates of SH-SY5Y cells treated for 72 h with 1 mM lithium or not treated (saline). One blot, representative of three, is shown. Using densitometry, the amount of cytochrome C increased in treated vs. nontreated cells by a factor of 2.5. (c) Flow cytometry analysis of control and lithium-treated (1 mM for 72 h) SH-SY5Y cells labeled with rhodamine-123 (100 nM for 20 min), a fluorescent dye that accumulates specifically in healthy mitochondria. Cytofluorimetry profiles show consistent differences between the two populations. In particular, the proportion of cells showing a higher rhodamine labeling (fluorescence > 10^2 arbitrary units; M2) is increased by 3-fold upon treatment with lithium. Data shown are representative of five independent determinations. (d) Neuronal cultures were treated with 0.5 and 1 mM lithium for 18 h (the time interval used in all of the cell culture experiments), then MitoTracker Red was added to the culture for 1 h (lithium and nontreated (NT)). Labeled mitochondria were quantified on the basis of MitoTracker Red fluorescence in treated and NT cultures. (Scale bars: 40 μm.) (e) Data are expressed in arbitrary units as background-subtracted fluorescence intensity, assessed by selecting three small regions without cells on the coverslip. Images were collected by using a confocal laser microscope (CLSM510; Zeiss) and were analyzed by using the ImageJ analysis program. The values represent means ± SEM, analyzed by one-way ANOVA after Newman–Keuls post hoc test. * P < 0.05 compared with WT.

www.pnas.org/cgi/doi/10.1073/pnas.0807693105
Lithium delays progression of amyotrophic lateral sclerosis

Francesco Fornai*†‡, Patrizia Longone§, Luisa Cafaro†, Olga Kastsiuchenka*, Michela Ferrucci†, Maria Laura Manca‡, Gloria Lazzeri*, Alida Spalloni§, Natascia Bellio†, Paola Lenzi‡, Nicola Modugno†, Gabriele Siciliano§, Ciro Isidoro†, Luigi Murri§, Stefano Ruggieri†, and Antonio Papatelli*

*Department of Human Morphology and Applied Biology, and †Department of Neuroscience, Clinical Neurology, University of Pisa 56100 Pisa, Italy; ‡Istituto Neurologico Mediterraneo, Istituto Di Ricoero e Cura a Carattere Scientifico Neuromed, 86077 Pozzilli (IS), Italy; §Molecular Neurobiology Unit, Santa Lucia Foundation, 00179 Rome, Italy; and †Department of Medical Sciences, University of Novara, 28100 Novara, Italy

Edited by Thomas C. Südhof, University of Texas Southwestern Medical Center, Dallas, TX, and approved December 21, 2007 (received for review August 24, 2007)

ALS is a devastating neurodegenerative disorder with no effective treatment. In the present study, we found that daily doses of lithium, leading to plasma levels ranging from 0.4 to 0.8 mEq/liter, delay disease progression in human patients affected by ALS. None of the patients treated with lithium died during the 15 months of the follow-up, and disease progression was markedly attenuated when compared with age-, disease duration-, and sex-matched control patients treated with riluzole for the same amount of time. In a parallel study on a genetic ALS animal model, the G93A mouse, we found a marked neuroprotection by lithium, which delayed disease onset and duration and augmented the life span. These effects were concomitant with activation of autophagy and an increase in the number of the mitochondria in motor neurons and suppressed reactive astrogliosis. Again, lithium reduced the slow necrosis characterized by mitochondrial vacuolization and increased the number of neurons counted in lamina VI that were severely affected in saline-treated G93A mice. After lithium administration in G93A mice, the number of these neurons was higher even when compared with saline-treated WT. All these mechanisms may contribute to the effects of lithium, and these results offer a promising perspective for the treatment of human patients affected by ALS.

**Results**

Effects of Lithium Treatment on the Renshaw-Like Cell Area (Laminae IV–VII). Autoradiographic analysis of the Renshaw-like cell area revealed an increase in the number of GAD67-positive neurons in the lateral and ventral funiculus of lithium-treated G93A mice (Fig. 1A). After lithium treatment was started at the onset of motor symptoms, the increase in the number of these neurons was still comparable (data not shown).

Effects of Lithium Treatment on Motor Neuron Survival (Lamina IX of the Spinal Cord and Brainstem Motor Nuclei). These effects were followed up in the spinal cord, where lithium treatment delayed the onset of paralysis in a dose-dependent manner (Fig. 2). After lithium treatment was started at the onset of motor symptoms, the number of alpha-MN in the spinal cord was significantly reduced compared with the saline-treated group. Even when lithium treatment was started at the onset of motor symptoms, the increase in disease duration was still comparable (data not shown).

Effects of Lithium on Disease Duration and Survival in G93A Mice. G93A male mice were treated daily with lithium carbonate (1 mEq/kg, i.p.), starting at 75 days of age. Lithium treatment prolonged the mean survival time from 110.8 ± 5.0 days (n = 20) to 148 ± 4.3 (n = 20, ∼36% of the life span of these mice; Fig. 1a; P < 0.001) and, most importantly, increased disease duration (from a mean of 9 days to >38 days, >300%; Fig. 1b; P < 0.05) compared with the G93A mice treated with saline. Even when lithium treatment was started at the onset of motor symptoms, the increase in disease duration was still comparable (data not shown).

Effects of Lithium Treatment on Motor Neuron Survival (Lamina IX of the Lumbar and Cervical Spinal Cord and Brainstem Motor Nuclei). These effects were accompanied by a reduced loss of lumbar MN at 90 days of age (SI Fig. 7). However, at the end of disease (which occurred later following lithium), the number of alpha-MN within lumbar lamina IX of the G93A mice treated with lithium was comparable to that found in the saline-treated mice that had died previously (SI Fig. 8). However, even at this stage, we detected a disease modifying effect of lithium. This consisted of (i) preservation of the size of MN (SI Fig. 8 d and e); (ii) preservation of MN number and size in those areas [i.e., cervical spinal cord (SI Fig. 9) or the nucleus ambiguous (SI Fig. 10)], which degenerate later compared with lumbar lamina IX (23, 24); (iii) decreased astrocytosis (SI Fig. 11); and (iv) decreased alpha-synuclein, ubiquitin, and SOD1 aggregation (see SI Fig. 6 and Discussion in SI Text).

Effects of Lithium Treatment on the Renshaw-Like Cell Area (Lamina VII). Lamina VII contains a larger number of interneurons, defined as Renshaw cells, which form a collateral circuit that
incorporation at immunofluorescence (SI Fig. 14) and by using a...b and c). Remarkably, lithium administration led to an increase in the number of these neurons, which became more abundant even when compared with control mice (151% and 100%, respectively). This net increase in neuron number occurred only when lithium was administered to G93A mice; no such effect occurs in WT (Fig. 2b). All of the staining procedures performed to visualize these neurons within lamina VII confirmed these results: H&E (131% of controls), gephyrin (137% of controls; Fig. 2 c and d), NeuN (132% of controls; SI Fig. 12 a and b) and calbindin 28k (126% of controls; SI Fig. 13 a and b). Immunoblotting confirmed all these staining (Fig. 2e and SI Figs. 7c and 13c). Moreover, the procedure of BrdU incorporation at immunofluorescence (SI Fig. 14) and by using ImmunoGold TEM (SI Fig. 15) confirmed the increase in calbindin 28k positive neurons in lamina VII. This is summarized in SI Fig. 16. The increase in neuron number produced by lithium in the spinal cord is disease dependent, because it did not occur in WT. Lithium is known to produce neurogenesis in physiological conditions in the hippocampus (26) but not in the spinal cord. However, in the absence of lithium, occurrence of neurogenesis in the spinal cord of G93A mice is uncertain, and, when occurring (27), it does not produce a net increase in neuron number compared with controls. In the present study, we combined lithium administration with the ongoing disease state. This combination may be necessary to enhance the neurogenetic effects, leading to a robust increase in the number of neurons (see Discussion in SI Text).

Lithium Treatment Rescues Spinal Cord Mitochondria and Facilitates the Clearance of Alpha-Synuclein, Ubiquitin, and SOD1. In ALS, alpha-synuclein and ubiquitin accumulate in affected neurons (9, 28–30). Lithium treatment reduces the accumulation of alpha-synuclein in both MN of lamina IX (SI Fig. 17a) and in neurons of lamina VII (SI Fig. 17b and c). Similarly, lithium reduces ubiquitin (SI Figs. 18 and 19), and SOD1 aggregates in MN (SI Fig. 20).

In G93A mice, MN undergo vacuolization, a process defined as “slow necrosis” (9), in which they appear to be filled with vacuoles (4, 9) due to mitochondrial swelling (Fig. 3 and SI Figs. 8 and 21). Lithium decreases vacuolization (Fig. 3f and h and SI Fig. 21) and

**Fig. 1.** Effects of lithium treatment on the lifespan and neurological symptoms of G93A mice. (a) Survival curve for saline- and lithium-treated G93A mice. Lithium carbonate (1 mEq/kg, daily, i.p.) treatment significantly increased the survival time of G93A mice compared with saline-treated mice. (b) Effects of lithium on specific symptoms, such as hind limb adduction, gait impairment, and the onset of severe paralysis. (c) Symptomatic effects and prolongation of the life span induced by lithium. Values represent the mean ± SEM of 10 mice per group in two different experiments (total N per group = 20). Comparison was made by using ANOVA with Sheffe’s post hoc analysis. *, P ≤ 0.05 compared with G93A mice administered saline. **, P ≤ 0.001 compared with G93A mice administered saline.

**Fig. 2.** Neuroprotective effects of lithium on medium-size lamina VII neurons. (a) Shows representative micrographs of those H&E-stained lamina VII neurons that were selected for the count based on size specificity (diameter ranging from 10 to 20 μm). (b) Graph indicates the severe loss of these neurons in G93A mice, which exceeded the loss of MN. Remarkably, the G93A mice treated with lithium showed a much higher number of lamina VII medium-size neurons even compared with saline-treated WT mice. (c–e) These results were confirmed by gephyrin immunostaining, as shown here and by all of the staining procedures summarized in SI Fig. 16. Counts represent the mean ± SEM of 62,000 cells per group (3,100 per mouse in groups of 20 mice). Comparison among groups was made by using one-way ANOVA. *, P ≤ 0.05 compared with WT saline-treated group. #, P ≤ 0.001 compared with G93A saline-treated groups. (Scale bars, 17 μm.)
normalizes mitochondrial size (Fig. 3 i and j and SI Fig. 21). Moreover, lithium increases the number of normal mitochondria in both WT and G93A mice. This was counted at ultrastructural level (Fig. 3 k and l) and confirmed by semiquantitative RT-PCR (SI Fig. 22) in vivo. We replicated this effect by cytofluorimetric counts of the mitochondria labeled with MitoTracker Red and MitoTracker Green and by Western blot analysis of cytochrome C (SI Fig. 23 a–c) in SH-SY5Y cells and primary spinal cord cultures (SI Fig. 23 d and e). The increase in mitochondria we found in the spinal cord confirms what recently found in endothelial cells (31). This is very encouraging, considering that the loss of mitochondria may be a risk for drugs acting as autophagy enhancers.

**Lithium Increases the Number of Autophagic Vacuoles.** At the ultrastructural level, we found degenerating mitochondria within autophagic vacuoles and whorl-like autophagosomes within G93A MN (Fig. 4). This led us to explore the hypothesis that lithium may improve MN survival by activating autophagy (18). We stained spinal cord samples with monodansylcadaverine (MDC), a fluorescent dye that incorporates selectively into mitochondria both in cervical (i) and lumbar (j) spinal cord. (k and l) Lithium increases the number of mitochondria both in cervical (k) and lumbar (l) MN both in WT and G93A. Values are the mean ± SEM. Comparison between groups was made by using one-way ANOVA. *P* = 0.05 compared with saline-treated mice. **P** = 0.01 compared with saline treated mice. (Scale bars: a, c, e, and g, 1.8 μm; b, d, f, and h, 0.25 μm.)

In the spinal cord from G93A mice treated with saline (arrowheads). (f and h) This vacuolization is consistently absent in mitochondria of G93A mice treated with lithium. (d and h) Lithium decreases the size of mitochondria both in WT and G93A mice (d and h, respectively) both in the cervical (l) and lumbar (l) spinal cord. (k and l) Lithium increases the number of mitochondria both in cervical (k) and lumbar (l) MN both in WT and G93A. Values are the mean ± SEM. Comparison between groups was made by using one-way ANOVA. *P* = 0.05 compared with saline-treated mice. **P** = 0.01 compared with saline treated mice. (Scale bars: a, c, e, and g, 1.8 μm; b, d, f, and h, 0.25 μm.)

**Effects of Lithium on Spinal Cord Cultures from G93A Transgenic Mice.**

If autophagy deficiency causes neurodegeneration in ALS, then its blockade is expected to worsen MN death. To test this hypotheses, we treated spinal cord cultures with the autophagy inhibitor 3-MA (36). MN survival was evaluated by counting

---

**Fig. 3.** Effects of lithium administration on motor neurons mitochondria in vivo. (a–h) Representative pictures of mitochondria (arrows) in MN from the spinal cord of WT mice treated with saline (a and b) or lithium (c and d) and from G93A mice treated with saline (e and g) or lithium (f and h) and from G93A mice treated with saline (e and g) or lithium (f and h). (g) In G93A mice treated with saline, TEM shows mitochondrial vacuolization (arrowheads). (f and h) This vacuolization is consistently absent in mitochondria of G93A mice treated with lithium. (d and h) Lithium decreases the size of mitochondria both in cervical (l) and lumbar (l) spinal cord. (k and l) Lithium increases the number of beclin and LC3 positive vacuoles in SH-SY5Y cells stably transfected with LC3 (33). By counting these markers and the immunostained structures shows a marked effect of lithium in MN both WT and G93A mice. (h) Likewise, LC3 immunopositive vacuoles increase significantly in G93A and WT mice administered lithium. (i–l) Phase-contrast microscopic images of lithium-induced accumulation of vacuoles in SH-SY5Y cells exposed or not for 72 h to 1 mM lithium (j), or lithium plus 50 mM asparagine (Asn) (a slight autophagy blocker acting downstream of lithium) (k), or 400 nM rapamycin (Rap) (l). Arrows point to cytoplasmic vacuoles that accumulate in cells treated with lithium or Rap (a known autophagy inducer). No vacuolization was observed in control (i) or lithium plus Asn-treated cells. (m–p) A parallel experiment was performed with transfected SH-SY5Y cells stably expressing the GFP-LC3 chimeric fluorescent protein. The images clearly show that both lithium (n) and rapamycin (p) change the cytoplasmic diffuse fluorescence pattern of GFP-LC3 to a punctuated pattern indicative of autophagosome formation. Asparagine (o) inhibited the effect of lithium on GFP-LC3 localization. *P* < 0.05 compared with saline. (Scale bars: a–d, 14 μm; e, 0.1 μm; f, 0.08 μm; i–l, 20 μm; m–p, 50 μm.)

**Fig. 4.** Effects of lithium on autophagy in vivo and in vitro. (a–d) MDC-positive small vacuoles in the lumbar spinal cord of WT (a and c, arrows) and G93A mice (b and d, arrows). (e) Representative picture of beclin immunostained vacuoles in the cytoplasm of alpha MN from a G93A lithium-treated mouse. The ImmunoGold particles (20 nm) are localized on both the membrane (that surround the core) and the electrondense core (arrows). (f) LC3 immunostaining is present on a larger membranous structure; the ImmunoGold particles (20 nm) are randomly localized (arrows). (g) The count of beclin immunostained structures shows a marked effect of lithium in MN both WT and G93A mice. (h) Likewise, LC3 immunopositive vacuoles increase significantly in G93A and WT mice administered lithium. (i–l) Phase-contrast microscopic images of lithium-induced accumulation of vacuoles in SH-SY5Y cells exposed or not for 72 h to 1 mM lithium (j), or lithium plus 50 mM asparagine (Asn) (a slight autophagy blocker acting downstream of lithium) (k), or 400 nM rapamycin (Rap) (l). Arrows point to cytoplasmic vacuoles that accumulate in cells treated with lithium or Rap (a known autophagy inducer). No vacuolization was observed in control (i) or lithium plus Asn-treated cells. (m–p) A parallel experiment was performed with transfected SH-SY5Y cells stably expressing the GFP-LC3 chimeric fluorescent protein. The images clearly show that both lithium (n) and rapamycin (p) change the cytoplasmic diffuse fluorescence pattern of GFP-LC3 to a punctuated pattern indicative of autophagosome formation. Asparagine (o) inhibited the effect of lithium on GFP-LC3 localization. *P* < 0.05 compared with saline. (Scale bars: a–d, 14 μm; e, 0.1 μm; f, 0.08 μm; i–l, 20 μm; m–p, 50 μm.)
SMI32-positive neurons after drug treatment compared with NT cultures (SI Fig. 24 a–f). 3-MA alone (5 mM for 2 h) increased G93A MN death by 58% compared with NT G93A MN and by 51% compared with lithium-treated G93A MN (SI Fig. 24g). Moreover, 3-MA treatment significantly increased SMI32-positive MN death in cultures from G93A compared with WT, thus supporting a vulnerability of the autophagy pathway in ALS degeneration (SI Fig. 24g). When 3-MA was given 2 h before lithium treatment (18 h, 1 mM), the latter could no longer counteract the effects of 3-MA, confirming that lithium neuro-protection occurs, at least partially, through an autophagic route (SI Fig. 24g). As found in vivo, lithium administration (18 h) increased gephyrin-positive G93A neurons dose-dependently up to 136% (SI Fig. 24). We further tested the protective effects of lithium against kainate, because G93A MN are highly vulnerable to excitotoxicity (37), which is also a mechanism involved in the neurodegeneration of ALS (1). Lithium (1 mM) given 20 min before kainate (100 μM 15 min) fully protected G93A SMI32-positive neurons (SI Fig. 25 a and b).

Clinical Trial. The previous results led us to translate the use of lithium carbonate to human patients affected by ALS. Recruitment began in October 2005. Typically, compliance was high or moderate for most individuals. There were no significant differences in demographic variables between groups (mean age and standard error in the treated group, 66.9 ± 1.9 vs. 70.3 ± 1.6 years; mean age at the ALS onset, 63.8 ± 1.7 vs. 67.1 ± 1.7). Similar baseline values between groups for ALSFRS-R scale, Norris scale, forced vital capacity (FVC), and Medical Research Council were selected.

When we measured the progression of the disability score in the group treated with lithium, ANOVA test for repeated measures did not show any statistical difference according to the Norris scale (basal value, 79.4 ± 2.8; final value, 71.0 ± 3.9; Fig. 5a) and ALSFRS-R scale (basal value, 39.9 ± 1.2; final value, 34.3 ± 2.3; Fig. 5b). Remarkably, all patients treated with lithium were alive at the end of the study. By contrast, in the group receiving riluzole only, 29% of subjects did not survive (Fig. 5c). This difference was significant at 12 and 15 months.

Assessment of disease progression with objective measurements such as the pulmonary function (FVC) provided similar data. In fact, FVC did not progress significantly (basal value, 89.0 ± 2.6; final value, 74.9 ± 3.6; Fig. 5d). The only significant progression compared with baseline values was observed for MRC but only at the end of the follow up (138.5 ± 1.6; final value, 113.4 ± 4.9; P < 0.05; Fig. 5e). No significant decrement in the Short Form Health Survey-36 (SF-36) scale for quality of life was detected during the 15 months of lithium administration (96.2 ± 2.0 vs. 93.4 ± 2.1).

These data indicate that subjects receiving lithium progressed very slowly in the disease during the 15 months of the follow-up. By contrast, in patients receiving only riluzole, the Norris scale significantly decreased (86.6 ± 2.1 vs. 55.3 ± 3.2, P < 0.01), starting from the 3rd month (P < 0.05) (Fig. 5a). Similar results were found for ALSFRS-R score, which significantly decreased (P < 0.01) (basal value, 40.2 ± 0.8; final value, 24.2 ± 1.8; Fig. 5b); again, this decrease was already significant at the first 3 months scoring interval (P < 0.05). Also FVC significantly decreased from 91.0 ± 1.9 to 58.0 ± 3.0, P < 0.01 (Fig. 5d).

Similar results were obtained for the MRC scale (140.7 ± 1.2 vs. 92.0 ± 5.3, P < 0.01), starting from the 6th month (P < 0.05) (Fig. 5e).

When we compared the group at the end of the follow up, the mean decrement in the normalized Norris score was 46.1% in patients receiving riluzole only and 10.6% in those receiving lithium. Likewise, the decrease was more marked with respect to the baseline in the normalized ALSFRS-R in patients receiving riluzole only compared with lithium (39.8% vs. 14.3%). This was comparable for MRC, which decreased by 34.6% and 18.0%, in riluzole only- and lithium-treated patients, respectively.

In the Fig. 5f the FVC breakdown between lithium- and riluzole-treated patients is shown, and in Fig. 5g this is further detailed for the bulbar form of the disease.

**Fig. 5.** Effects of lithium treatment on disease symptom progression and survival in patients with ALS. (a, b, d, and e) Symptoms progression (evaluated every 3 months) in controls (riluzole-treated) and treated patients (lithium plus riluzole-treated patients) expressed as raw data, using the Norris (a) and ALSFRS-R (b) rating scales and FVC (d) and MRC (e). There was no significant progression in the lithium-treated patients at any time interval apart from the last two evaluations using the MRC scale. In the riluzole-treated patients, symptoms progressed significantly starting at 3 or 6 months (depending on the scale). (c) Survival curve as normalized data shows the percentage of patients surviving over the 15 months of treatment in the riluzole and lithium groups. None of the patients died in the lithium-treated group; however, in the control group, although the patients had comparable disease severity at enrollment, ~30% died. Intra- and intergroup analyses were performed by unpaired t test or ANOVA followed by the Bonferroni post hoc test. (f) Shows the breakdown between treated and control patients for FVC (g) Shows the breakdown between groups for FVC (calculated at 1 month before death) in those patients affected by the bulbar form. *, P < 0.05; **, P < 0.01 compared with baseline value. #, P < 0.05; ##, P < 0.01 compared with control patients.
Discussion
Our study indicates that lithium delays ALS progression in human patients. In fact, all subjects treated with lithium were alive at the end of the follow up (15 months), and their quality of life, as measured by SF-36, was not modified. By contrast, ~30% of the patients receiving riluzole died during the study. The decreases we observed in the ALS-FRS-R and Norris scales were not statistically significant in the group treated with lithium. The delay in disease progression was also assessed more objectively by quantitative measurement of the muscle strength (by the MRC scale) and the preservation of the pulmonary function (by FVC). By contrast, the disease progressed markedly in the control group from the 3rd month of evaluation.

The analysis in the G93A mice showed that lithium delayed cell death within lamina IX and cranial MN while it increased the number of lamina VII Renshaw-like neurons above control values. In addition, lithium decreased reactive gliosis, rescued spinal cord mitochondria, and produced a marked regression of alpha-synuclein, ubiquitin, and SOD1 aggregates. This latter finding suggests that an increased removal of the mutated SOD1 may contribute to the improvement we observed in G93A mice. Thus, lithium affects multiple targets, all of which are likely to contribute to the improvement we observed in G93A mice. Although not yet explored, numerous data associate autophagy with ALS. Apart from the autophagy impairment we found in the SOD1 model, another form of fALS, which is characterized by a mutation of dynein (38), likely depends on autophagy failure. In fact, now we know that dynein is critical for delivering the autophagosomes to lysosome to remove protein aggregates (22). In keeping with this observation, mutations in the dynein gene cause an autophagy impairment and reduce the clearance of aggregates (39). Another point mutation in the gene coding for dynactin, which participates in the dynamic of phagosomes, is responsible of fALS (40). Another form of fALS is caused by a mutation in the ALS 2 gene, i.e., the protein alsin (41), which is implicated in endosome trafficking. Interestingly, SOD1 is degraded by the autophagy pathway (42).

Thus, a convergence of different etiologies to produce an impairment of the endosomal–lysosomal autophagy pathway in producing MN disease is plausible. This pathway is strongly regulated by IP3 levels, which acts as an endogenous autophagy inhibitor, whereas lithium, by blocking IP3 activity, is a strong promoter of autophagy (18, 43), as confirmed in the present study at the level of the MN in vivo. Again, in primary MN cell cultures, we observed that lithium promoted autophagy at doses (1 mM) corresponding to those necessary to inhibit the IP3 turn-over (16, 17), whereas increasing the dose of lithium up to 2–3 mM (thus recruiting the inhibitory activity on GSK3 beta) produced a dramatic increase in cell adhesion. A defective endosomal–lysosomal autophagy pathway (due to either a primary defect in this pathway or dysfunctional mitochondria) could be the common denominator in various forms of ALS. Increasing autophagy to induce neuroprotection was recently fostered by Rubinsztein (44), who discussed the issue of the risk of decreasing mitochondria as a side-effect. In the present study, we demonstrate that lithium, although it increases autophagy, concomitantly produces a marked increase in the number of newly formed undamaged mitochondria. These effects, together with an increase in the number of neurons in lamina VII, may underlie the neuroprotective effects of lithium in the mouse model. A slight protective effect of lithium was recently described by Shin et al. in G93A mice (51). In this article, high doses of the compound were used, which may lead to additional effects (some of which may be detrimental for MN; see above). In fact, in their study, despite a delayed disease onset, the duration of disease was shortened by lithium administration.

At the same time, focusing only on autophagy may be misleading given the multiple targets we found in the present work. For instance, the suppression of glial cells activation we demonstrated in the spinal cord of lithium-treated G93A mice may be critical in view of the recent reports of the detrimental role of glial on MN survival (6–8). This point is addressed further in Discussion in SI Text, which also provides a multifaceted perspective on the potential mechanisms underlying the effects produced by lithium in ALS.

Materials and Methods

Methods. Behavior. Behavioural observations were made by blind observers once a day for all animal groups (n = 20 per group). For details, see Methods in SI Text.

Tissue preparation staining procedures and histological analyses. See Methods in SI Text.

Electron microscopy. Mice (n = 10 from each group, WT, WT plus lithium, G93A, and G93A plus lithium) were perfused and spinal cords were maintained in situ immersed in fixative solution (2% paraformaldehyde/0.1% glutaraldehyde). For details, see Methods in SI Text.

Primary neuronal cultures, immunocytochemistry, cell labeling, and toxicity. Mixed spinal cord cultures were prepared from 13-day-old embryos of a control female mated with a G93A male as described in ref. 37. Three days after plating, AraC (10 μM) was added. For details, see Methods in SI Text.

SH-SY5Y cell lines and treatments. Human neuroblastoma SH-SY5Y cell line was obtained from the American Type Culture Collection (ATCC) and cultured under standard culture conditions. Cells were seeded and cultivated for 24 h before starting the treatments with 1 mM lithium carbonate (Sigma), 50 mM asparagine (Sigma), and 400 nM rapamycin. Treatments lasted 72 h. We confirmed that the medium and medium changes every 24 h. Detailed protocols are described in Methods in SI Text.

Statistical analysis. Data are given as mean ± SEM. Group mean values were compared by ANOVA, followed by post hoc testing.

Clinical Trial. Study design and patients. We conducted a 15-month parallel-group randomized study of adults with ALS, diagnosed according to the El Escorial criteria. We used the El Escorial diagnostic criteria with a follow-up period of 5 years.

The study protocol was approved by the Neuromed IRCCS Ethical Committee, and all subjects provided written informed consent. Initial statistical analysis determined that at least 40 subjects were needed to determine, with a 95% confidence interval, a survival increase ≥6 months.

The present study was performed on 44 patients (20 male and 24 female). No familial case was present. Eleven patients presented bulbar forms of the disease, and the remaining presented the classic onset. Sixteen patients (eight male and eight female, four of whom had the bulb form) were randomly selected to receive riluzole (Rilutek 50 mg, 1 tablet × 2/day) plus lithium (Carbolutin; two daily 150-mg doses of lithium carbonate), and the remaining (12 male and 16 female, 7 of whom had the bulb form) received riluzole only (48). In this way, we carefully matched lithium-treated and control patients for bulb forms and FVC at the time of their inclusion in the study. In particular, the FVC values were 90 ± 10 and 91 ± 10 for lithium-treated and control patients, respectively. Again, the bulb forms were distributed similarly between the groups (4/16 = 25%) in the treated group and (7/12 = 32%) for controls. One physician was not blind to group assignment; however, clinical evaluation, measurement of FVC, and data analysis were conducted by other physicians who were blind to group identities (single-blind study). In this way, the first physician was able to monitor lithium concentration and to adjust the daily dose from 300 mg up to 450 mg daily when lithium plasma levels were <0.4 mEq/l. In fact, the daily dose was selected to reach a plasma range of 0.4–0.8 mEq/l.

Compliance and adverse effects were monitored throughout the study period. Subjects were assessed six times (at baseline and every 3 months for 15 months). The primary endpoint of the present study was the survival rate. The secondary outcomes measured changes in global function, as scored by the ALSFRS-R (49), a widely used and extensively validated functional scale for ALS (normal score, 48), and by the Norris ALS scale. This disability score includes evaluation of the functioning of upper and lower limbs, also taking into account disease progression.
account bulbar function. This score uses 34 items rated with a value from 0 to 3, and the normal score is 100. Quality of life (SF-36) (50) was also evaluated.

In parallel, we assessed the disease progression with more objective measures, such as quantitative segmental muscle strength (by the MRC scale) and the pulmonary function (FVC). The use of these combined approaches is very useful in small clinical trials (see Discussion in SI Text for a comparison of reliability between different scales).

Data analysis. The study analyzed all subjects who entered in the protocol study. Statistical analyses included a descriptive analysis of each group's data. Kolmogorov–Smirnov test of normality was nonsignificant for all variables. Measures were compared every 3 months with respect to the baseline condition, using an ANOVA for repeated measures. Comparisons between the two groups’ mean scores were made by using the unpaired t test and, if necessary, ANOVA, with Bonferroni as post hoc test. Survival analysis was performed by the Kaplan–Meier curve. The null hypothesis was rejected when P ≤ 0.05 for all tests. Statistical analyses were performed by using MedCalc software (version 9.3.6.0). See Methods in SI Text for the detailed methods of the basic study.
G93A+Saline

G93A+Lithium

b Grip test

Stride test

c Rotarod

** *

Stride length (cm)

Age (days)

Time (sec)

0 100 200 300

90 100 110 120 130 140 150

Time (sec)

0 80 60 40 20

90 100 110 120 130 140 150

Time (sec)

0 100 200 300

90 100 110 120 130 140 150
Lumbar spinal cord-Lamina IX – day 90

WT+Saline

WT+Lithium

G93A+Saline

G93A+Lithium

Number of a motor neurons:

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Lithium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>35</td>
</tr>
</tbody>
</table>

* * *

* * *

* * *

* * *

* * *

* * *

* * *

* * *

WT

G93A

a motor neurons/
section
Supp. Fig. 3

**WT+Saline**
- Diameter: 34.970±0.280 μm
- Area: 854.461±17.409 μm²

**WT+Lithium**
- Diameter: 34.800±0.3 μm
- Area: 854.500±16.5 μm²

**G93A+Saline**
- Diameter: 37.474±0.635 μm
- Area: 1093.423±43.024 μm²

**G93A+Lithium**
- Diameter: 34.222±0.303 μm
- Area: 811.577±28.864 μm²

Supp. Fig. 3
Cervical spinal cord - Lamina IX

(a) WT+Saline  WT+Lithium  G93A+Saline  G93A+Lithium

(b)

(c)

WT
G93A

Supp. Fig. 4
G93A+LithiumWT+LithiumWT+Saline G93A+Saline

a) Nucleus Ambiguus

Motor neurons number

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>G93A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Lithium</td>
<td></td>
<td>#</td>
</tr>
</tbody>
</table>

b) Diagram of brain regions

c) WT+Saline, WT+Lithium, G93A+Saline, G93A+Lithium

d) Magnified images of brain sections with arrows indicating differences
a  Cervical spinal cord
  WT+Saline  WT+Lithium
  G93A+Saline  G93A+Lithium

b  Cervical tract  Lumbar tract
  Saline  Lithium  Saline  Lithium

Densitometry of GFAP (% of control)

GT 50KDa

GFAP

Lumbar tract
  Saline  Lithium  Saline  Lithium

WT  G93A  G93A  WT

**

Lumbar tract
  Saline  Lithium  Saline  Lithium

*  *

0  100  200  300  400  500  600

G93A

Cervical tract
  Saline  Lithium  Saline  Lithium

*  *

Lumbar tract
  Saline  Lithium  Saline  Lithium

**  #
Densitometry of NeuN (% of control)

WT   | G93A   | WT   | G93A
---   | ---    | ---  | ---
Saline |      | Lithium |    

Lumbar tract

Neurons number

WT   | G93A
---   | ---
Saline |      | Lithium |    

The images show immunofluorescence of NeuN in the lumbar tract of WT and G93A mice under Saline and Lithium conditions. The graphs illustrate the densitometry of NeuN and the number of neurons, with significant differences indicated by asterisks (*) and hash marks (#).
a. WT+Saline | WT+Lithium | G93A+Saline | G93A+Lithium

b. 

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>G93A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Lithium</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

Number of Calbindin-28K positive neurons

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>G93A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Lithium</td>
<td>60</td>
<td>80</td>
</tr>
</tbody>
</table>

Densitometry of Calbindin-28K (% of control)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>G93A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Lithium</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

Calbindin-28K

Lumbar tract
WT+Saline  G93A+Saline  WT+Lithium  G93A+Lithium

WT  G93A

Number of BrdU positive cells/section

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Lithium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

Number of Calbindin-28K positive neurons/section

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Lithium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

Number of double marked cells (Calbindin-28K+BrdU)/section

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Lithium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

Saline Lithium
WT+Saline (BrdU)  WT+Lithium (BrdU)  G93A (BrdU)

G93A+Lithium (BrdU/Calbindin-28K)

f

Number of BrdU positive cells
Saline  Lithium

Number of Calbindin-28K positive neurons
Saline  Lithium

Number of double marked cells (Calbindin-28K+BrdU)
Saline  Lithium
Number of Calbindin-28K positive neurons within Lamina VII
G93A+Lithium/G93A+Saline increase of: 72%
G93A+Lithium/WT+Saline increase of: 26%

Number of H&E neurons within Lamina VII
G93A+Lithium/G93A+Saline increase of: 67%
G93A+Lithium/WT+Saline increase of: 31%

Number of Gephyrin-positive neurons within Lamina VII
G93A+Lithium/G93A+Saline increase of: 70%
G93A+Lithium/WT+Saline increase of: 37%

Number of NeuN positive neurons within Lamina VII
G93A+Lithium/G93A+Saline increase of: 68%
G93A+Lithium/WT+Saline increase of: 32%

Number of Calbindin-28K positive neurons within Lamina VII
G93A+Lithium/G93A+Saline increase of: 72%
G93A+Lithium/WT+Saline increase of: 26%
Densitometry of α-synuclein (% of control)

WT+Saline  WT+Lithium  G93A+Saline  G93A+Lithium

Saline Lithium Saline Lithium

WT  G93A

20KDa
Supp. Fig. 13
**a**  
**α-synuclein**  
Cervical tract  
20KDa  
WT G93A WT G93A  
Saline Lithium  
Lumbar tract  
20KDa  
WT G93A WT G93A  
Saline Lithium

**b**  
**Ubiquitin**  
Cervical tract  
8KDa  
WT G93A WT G93A  
Saline Lithium  
Lumbar tract  
8KDa  
WT G93A WT G93A  
Saline Lithium

**Graphs:**  
- **α-synuclein (Cervical tract):**  
  - Saline: WT, G93A  
  - Lithium: WT, G93A  
- **α-synuclein (Lumbar tract):**  
  - Saline: WT, G93A  
  - Lithium: WT, G93A

- **Ubiquitin (Cervical tract):**  
  - Saline: WT, G93A  
  - Lithium: WT, G93A

- **Ubiquitin (Lumbar tract):**  
  - Saline: WT, G93A  
  - Lithium: WT, G93A

**Densitometry of α-synuclein (% of control):**  
- **Saline (Cervical):** WT: 100, G93A: 110  
- **Saline (Lumbar):** WT: 100, G93A: 110  
- **Lithium (Cervical):** WT: 110, G93A: 120  
- **Lithium (Lumbar):** WT: 110, G93A: 120

**Densitometry of ubiquitin (% of control):**  
- **Saline (Cervical):** WT: 100, G93A: 110  
- **Lithium (Cervical):** WT: 110, G93A: 120  
- **Saline (Lumbar):** WT: 100, G93A: 110  
- **Lithium (Lumbar):** WT: 110, G93A: 120

**Legend:**  
- *: p < 0.05  
- #: p < 0.01  
- #: #: p < 0.001
SOD1 – Lamina IX

WT+Saline  WT+Lithium  G93A+Saline  G93A+Lithium

Densitometry of mutated SOD1 (% of G93A)

Supp. Fig. 15
Supp. Fig. 16
Fold increase of mRNA levels of mitochondrial genes

G93A+Saline  G93A+Lithium

Relative levels of mitochondrial DNA vs nuclear DNA

- G93A+Saline
- G93A+Lithium

* Significance level

** Graphs showing:
- Fold increase of mRNA levels of cyt-b and ATP synthase-6 genes
- Relative levels of mitochondrial DNA vs nuclear DNA

Legend:
- Red: G93A+Saline
- Black: G93A+Lithium
**Densitometry of Red-MTR (A.U.)**

- **SMI32 MTR**
  - Saline: M1 (58.8%) M2 (4.4%)
  - Lithium: M1 (51.2%) M2 (12.9%)

**Mitotr Green FM**

**Mitotr Red CMXRos**

**Saline Lithium**

**Mitotr Green FM**

**Mitotr Red CMXRos**

**WT G93A**

**Lithium (mM)**

- **NT**
- **0.5**
- **1**

**Densitometry of Red-MTR (A.U.)**

- WT
- G93A

**Saline: CytC**

**Lithium: CytC**

**Fold increase**: 1X 2.5X
**a**

Images showing immunocytochemical staining for SM132 and GFAP under different conditions: NT, Kainate, Kainate+Lithium, and Lithium.

**b**

Bar graph comparing the percentage of SM132 positive cells in WT and G93A models under Kainate+Saline and Kainate+Lithium treatments. The graph shows a significant difference (*) between the groups.
G93A+Saline

G93A+Lithium

b Grip test

90 100 110 120 130 140 150

0 20 40 60 80 100 120

Age (days)

**

Stride test

Stride length (cm)

90 100 110 120 130 140 150

0 2 4 6 8 10 12

Age (days)

c Rotarod

— G93A+Saline
— G93A+Lithium

90 100 110 120 130 140 150

0 100 200 300 400

Age (days)

* * *
Number of Calbindin-28K positive neurons within Lamina VII
G93A+Lithium/G93A+Saline increase of: 72%
G93A+Lithium/WT+Saline increase of: 26%

Number of H&E neurons within Lamina VII
G93A+Lithium/G93A+Saline increase of: 67%
G93A+Lithium/WT+Saline increase of: 31%

Number of NeuN positive neurons within Lamina VII
G93A+Lithium/G93A+Saline increase of: 68%
G93A+Lithium/WT+Saline increase of: 32%

Number of Gephyrin-positive neurons within Lamina VII
G93A+Lithium/G93A+Saline increase of: 70%
G93A+Lithium/WT+Saline increase of: 37%

Number of Calbindin-28K positive neurons within Lamina VII
G93A+Lithium/G93A+Saline increase of: 72%
G93A+Lithium/WT+Saline increase of: 26%
c

WT+Saline  WT+Lithium  G93A+Saline  G93A+Lithium

Densitometry of α-synuclein (% of control)

Saline  Lithium  Saline  Lithium

WT  G93A

20KDa

WT  G93A
Supp. Fig. 13

UBIQUITIN – Lamina IX

WT G93A

Saline

Lithium
Supp. Fig. 15