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Autophagy activation in glutamate-induced motor neuron loss

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ABSTRACT

Recent literature demonstrated that exposure to excitatory amino acid in specific experimental conditions might produce a defect in the autophagy pathway. Such an effect was observed in motor neurons exposed chronically to glutamate agonists. On the other hand, it is well known that glutamate induces motor neuron death and this is supposed to play a key role in the physiopathology of motor neuron loss in amyotrophic lateral sclerosis (ALS). Similarly, a defective recruitment of autophagy was recently documented in ALS. In the present study we found that exposure of motor neurons to kainic acid produces intracellular changes associated with defective autophagy. In this experimental conditions, pharmacological activation of autophagy rescues the loss of motor neurons.

Key words

Kainic acid • Excitotoxicity • Lithium • Motor neuron • Amyotrophic lateral sclerosis

Introduction

The term excitotoxicity was introduced to describe the neurotoxic effect of glutamate and its derivatives in 1957, when it was found that high doses of glutamate, given to mice systemically, induced neuronal degeneration (Lucas and Newhouse, 1957).

Glutamate receptor-mediated excitotoxicity is involved in the pathogenesis of many neurologic disorders such as stroke, ischemia and slowly progressing neurodegenerative diseases (reviewed in Dingledine et al., 1990; Meldrum and Garthwaite, 1990; Beal, 1992; Choi, 1992). This was further extended to motor neuron degeneration in amyotrophic lateral sclerosis (ALS) (Rothstein et al., 1992; 1995; Bruijn et al., 2004). High levels of glutamate have been detected in the cerebrospinal fluid of ALS patients (Rothstein et al., 1992; 1995), suggesting an abnormal intracellular transport of glutamate. This might depend on a deficient glutamate clearance since the spinal cord is deficient of astroglial glutamate transporter in ALS transgenic models (Howland et al., 2002) and in sporadic ALS patients (Rothstein et al., 1995). Motor neurons are vulnerable to excitotoxicity mostly mediated by AMPA receptors (Hugon et al., 1989; Ikonomidou et al., 1996). Intrathecal or intraspinal administration of AMPA receptor agonists induces motor neuron degeneration (Ikonomidou et al., 1996; Kruman et al., 1999). Direct application of glutamate agonists to organotypic spinal cord cultures as well as motor neuron cultures induces motor neuron loss, which is prevented by glutamate antagonists (Rothstein et al., 1993; Estevez et al., 1995; Carriedo et al., 1996; Vandenberghe et al., 1998; Urushitani et al., 1998; Fryer et al., 1999; Saroff et al., 2000; Van Den Bosch and Robberecht, 2000; Urushitani et al., 2001). High sensitivity of motor neurons to excitotoxicity is associated with abundance of ionotropic glutamate receptors allowing the entry of high Ca^{2+} levels in the absence of Ca^{2+} buffering proteins

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(Van Den Bosch et al., 2000; Van Den Bosch et al., 2002). Therefore, glutamate causes a high Ca^{2+} influx, which is in excess and permeate mitochondria (Van den Bosch et al., 2006). In this way, a number of altered mitochondria need to be cleared to keep the cell working properly. The clearance of mitochondria is regulated by the autophagy pathway, which in turn is involved in excitotoxicity (Borsello et al., 2003; Matyja et al., 2005; Shacka et al., 2007; Wang et al., 2008) as well as in ALS (Fornai et al., 2008b; Madeo et al., 2009; Pasquali et al., 2010).

The point which remains unclear is whether autophagy counteracts or mediate excitotoxicity. In fact, during excitotoxicity degenerating motor neurons exhibit activation of autophagy with protein aggregation within the endoplasmic reticulum (ER) (Tarabal et al., 2005; Calderò et al., 2007). In detail, electron microscopy of degenerating motor neurons evidences sub-cellular alterations reminiscent of deficient recruitment of the autophagy machinery such as swollen mitochondria, alterations of Golgi apparatus and ER, and abundant large cytoplasmic vacuoles containing accumulated proteins and mitochondria (Calderò et al., 2007). Indeed, these morphological findings suggest that it is rather a defective autophagy which characterizes the death of motor neurons (Madeo et al., 2009; Pasquali er al., 2009). In fact autophagy markers tend to accumulate during an autophagy failure as occurs during excitotoxicity, while the same markers are expected to be cleared by an overactive autophagy. In this way there is a clear overlap between glutamate-induced and ALS-related motor neuron loss (Fornai et al., 2008a,b; Madeo et al., 2009; Pasquali et al., 2009).

In the present study we analyzed whether AMPA receptor stimulation via kainic acid (KA) produces activation of autophagy and whether this is defective compared with what it is required to rescue motor neurons. Once we confirmed that excitotoxicity recruits autophagy, which remains defective to clear altered proteins and organelles, we provided a pharmacological stimulation of such a pathway in order to ameliorate motor neuron survival.

Methods

Primary motor neuron cultures

Mixed spinal cord were obtained from 14-days-old mice embryos as previous described (Carriedo et al., 1996). Pregnant (14 days gestation) Swiss Webster female Albino mice were purchased from Charles River Laboratories (Calco, Lecco, Italy). The spinal cords were dissected and both meninges and dorsal root ganglia were removed. The cords were then incubated for 10 min in 0.025% trypsin and then dissociated. The cell cultures were plated at a density of 3 "spinal cords" per well plate (35 mm dishes) on poly-D-lysine-coated glass coverslips and maintained in D-MEM supplemented with 5% FBS and 5% HS. Twenty four hours after plating the medium was replaced with Neurobasal supplemented with B-27 and 0.5 mM glutamine. Three days after plating cytosine arabinoside (10 μM) was added and the medium was replaced with Neurobasal supplemented with B-27 and changed every 3 days.

For analysis at light microscopy, motor neurons grown on glass coverslips, inside 35 mm diameter dishes, were transferred into cell culture plates 24 well, to perform immunohistochemistry.

Motor neuron assigned to electron microscopy were grown in a cell culture plates 6 well where at the end of the experiment they were fixed and then scraped off to perform ultrastructural analysis.

Pharmacological treatments

Spinal cord cultures at 8-9 days in vitro (DIV) were used for toxicity experiments. The cells were treated with 1 mM lithium carbonate 30 min before the exposure to kainic acid (50 μM for 15 min). The *N*-methyl-D-aspartic acid (NMDA) receptor antagonist MK-801 (10 μM) was added during all KA exposure. After this time, the cultures were rapidly washed and maintained in the free medium. In other experiments, 1 mM lithium carbonate was administered 30 min before KA (50 μM for 15 min), the cell were washed and lithium was added again for 24 h in order to persist for a longer time in the culture previously exposed to KA. At the end of the treatment, the cultures were processed for light and electron microscopy.

Western blot analysis

Motor neurons from each experimental group were lysed in TEN buffer (50 mM Tris, 2 mM EDTA, 150 mM NaCl, 1% NP-40 at pH 7.6, containing 0.1% of phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml of protease inhibitors) and centrifuged at 15,000 rpm for 20 min at 4°C. An aliquot of supernatant

was used to determine protein concentration by a protein assay kit (Sigma). Samples containing 40 μg total protein were separated by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel. The proteins were transferred to PVDF membrane (Millipore, Bedford, MA, USA). The membrane was immersed in blocking solution (5% not fat dried milk in 20 mM Tris, 137 mM NaCl at pH 7.6 containing 0.05% Tween-20) for 3 h at 4°C. Subsequently, the membrane was incubated with primary antibody anti-beclin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA USA) at 4°C overnight. The blot was probed with horseradish peroxidase-labelled secondary antibody (1:2000; Amersham Pharmacia Biotech, Buckinghamshire, UK,) and the bands were visualized with enhanced chemiluminescence reagents (Amersham).

Densitometric analysis

Densitometry was used to measure immunoreactive bands. Western blots were scanned by using Adobe PHOTOSHOP CS2 version 9.0 (Adobe Systems, Mountain View, CA), and the optical density (OD) of the bands was measured by using NIH IMAGE 1.61. The relative values of each immunoreactive band were calculated by subtracting the background OD value from the measured OD of the bands. Groups used for statistical analyses were always determined within the same Western blot. The results were confirmed by triplicate.

Immunohistochemistry on primary cultures of motor neurons

To assess the effects of treatments on motor neuron survival, primary cultures were stained with the motor neuron antibody SMI32. Immunostaining was extended to Beclin-1 as an autophagy protein.

At first, cells were fixed in a solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3 (10 min); after washing in PB cell cultures were incubated with Triton-X 0.1% in PB (15 min), followed by 3% hydrogen peroxide (10 min).

Before incubation with primary antibodies, a blocking solution (10% normal goat serum in PBS) was added for 1 hour at room temperature.

All solutions containing the primary antibodies were prepared in PB containing 2% normal goat serum and incubated overnight at 4°C; we used primary antibodies against beclin-1 (mouse, 1:50; Santa Cruz) and SMI-32 (mouse, 1:1000; Covance, Emeryville, CA, USA).

For the immunoperoxidase procedure, the secondary biotinylated antibody (anti-mouse IgG; Vector Laboratories, Burlingame, CA USA) was used at a dilution of 1:200 for 1 hour at room temperature, followed by incubation with ABC kit (1 hour at room temperature, Vector Laboratories) and diaminobenzidine (Vector Laboratories).

For the immunofluoresce staining, we used anti-mouse IgG conjugated to Cy3 (red, 570 nm; Chemicon, Billerica, MA USA) diluted 1:400 and the anti-mouse IgG conjugated to fluorescein (green, 510-520 nm; Vector Laboratories) at a dilution of 1:100 (1 and a half hour, at room temperature).

Finally, glass coverslips containing cells were mounted on polylysinated slides by using glycerol (fluorescence stained motor neurons) or DPX plastic mounting media (Sigma Aldrich, St. Louis, MO USA) after dehydration in alcohol (peroxidase stained motor neurons).

Immunohistochemical reactions were analysed by using a light microscope (Nikon Eclipse 80i, Japan) equipped with a fluorescent lamp.

Densitometry of beclin1-immunofluorescent motor neurons was carried out by drawing the cell body profile using an image analysis software (Molecular Machine & Industries AG, Glattburgh, Switzerland) and measuring the optical density of the selected area as described. In particular, for each treatment, we drew the profile of 50 motor neurons and we measured signal intensity by using NIH IMAGE 1.61 to evaluate beclin-1 immunostaining. This analysis was carried out at 20x magnification by two independent observers, blind to treatments. The values given for optical density represent the mean ± SEM obtained by three independent experiments and was expressed as arbitrary units.

Cell count and morphometric analysis

The effects of each treatment on motor neuron survival were measured by counting the number of SMI-32-positive motor neurons in a total of 3 slides per group. Measurement of motor neuron diameter (n = 500 each experiment) was performed by an image analysis software (Molecular Machine & Industries AG, Glattburgh, Switzerland) which allowed to draw the diameter of the cell under observation and read the value.

In particular, SMI-32 immunoperoxidase was used to count motor neuron number, whereas SMI-32 immunofluorescence was used to measure motor neuron diameter.
All measurements were carried out at 20x magnification by two different observers (M.F, A.B), blind to treatments.

Electron microscopy

Fixation of primary motor neurons was carried out directly into the culture dishes rinsing the cells into the fixing solution (0.1% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS, pH 7.4) for 2 h. The fixed cells were scraped from the culture dishes and centrifuged at 15,000 g for 20 min. Pellets were post-fixed in 1% OsO_4 buffered solution, dehydrated in ethilic alcohol and embedded in Epon-araldite. Ultrathin sections of culture cells were stained with uranyl acetate and lead citrate, and finally examined at Jeol Jem 100SX transmission electron microscope (Jeol, Tokyo, Japan).

Statistical analysis

Cell counts, morphometric and densitometric data are expressed as mean ± SEM obtained by three independent experiments. Cell count is expressed as the percentage of SMI-32 positive motor neurons compared with control. Statistical analysis was carried out by using analysis of variance (ANOVA) followed by post-hoc tests (Fisher or Sheffè). The differences among experimental groups were considered statistically significant for $P < 0.05$.

Results

Lithium protects against kainic acid-induced motor neuron loss
Kainic acid (50 μM) induced a dramatic reduction in the percentage of SMI-32-positive motor neurons (13.74% ± 3.62) compared with controls (100% ± 10.07) (Figs. 1D, 1G and 1A, 1G respectively).
Lithium (1 mM) given 30 min before KA and left in the medium for further 24 hours protected the motor neurons from death as revealed by counting SMI32-positive motor neurons (57.46% ± 2.31) (Figs. 1F, 1G). In contrast, pre-treatment with lithium lasting only 30 min before KA administration was unable to protect motor neurons from KA-induced cell death (20.92% ± 1.87) (Figs. 1E, 1G).

The effects of 24 hours lithium exposure on KA also produce the preservation of the motor neurons size. In fact after KA exposure motor neurons increase their diameter (29 ± 0.35 μm) (Figs. 2D, 2G) compared with control (21.23 ± 0.29 μm) (Figs. 2A, 2G). A similar increase in motor neuron size was observed when lithium exposure was limited for 30 minutes before KA (27.59 ± 0.91 μm) (Figs. 2E, 2G). In contrast, this was absent when lithium was left in the cultures 24 hours after KA washed out (20.86 ± 1.08 μm) (Figs. 2F, 2G).

Kainic acid-induced motor neuron loss is caused by a defective autophagy

To asses the involvement of autophagy in the excitotoxic effect of KA we evaluated the expression of beclin 1, which is a marker of the autophagy pathway, by immunoblotting and immunofluroscence. As shown in the gel of Fig. 3H, KA produced the highest expression of beclin-1. Similarly, immunohistochemistry showed that in KA-treated motor neurons (Figs. 3D, 3G) immunofluorescence for beclin 1 was more intense than in control cultures (Figs. 3A, 3G).
The ultrastructural analysis of motor neurons treated with KA revealed damaged swollen mitochondria with matrix diluted and broken and disorganized cristae. In addition in the cytoplasm of these motor neurons close to altered mitochondria it is possible to observe autophagy-related structures (Fig. 4C).

Autophagy activation is protective against KA-induced neurotoxicity

Immunoblotting for the autophagy protein beclin-1 showed that lithium exposure for 24 h after KA decreased the expression of beclin 1 compared with KA (Fig. 3H). In contrast, when lithium was given only for 30 min before KA expression of beclin-1 was unchanged compared with KA alone (Fig. 3H). The same data were observed at immunofluorescence (Figs. 3E, 3G) which confirmed that 24 h of lithium exposure after KA were needed to decrease beclin 1 expression (Figs. 3F, 3G). Note that lithium given alone does not alter the levels of beclin 1 (Figs. 3B, 3C, 3G).
Again, ultrastructural alterations induced by KA were prevented only by prolonged lithium administration (24 h), which led to a decrease of damaged mitochondria (Fig. 4D). Motor neurons treated with

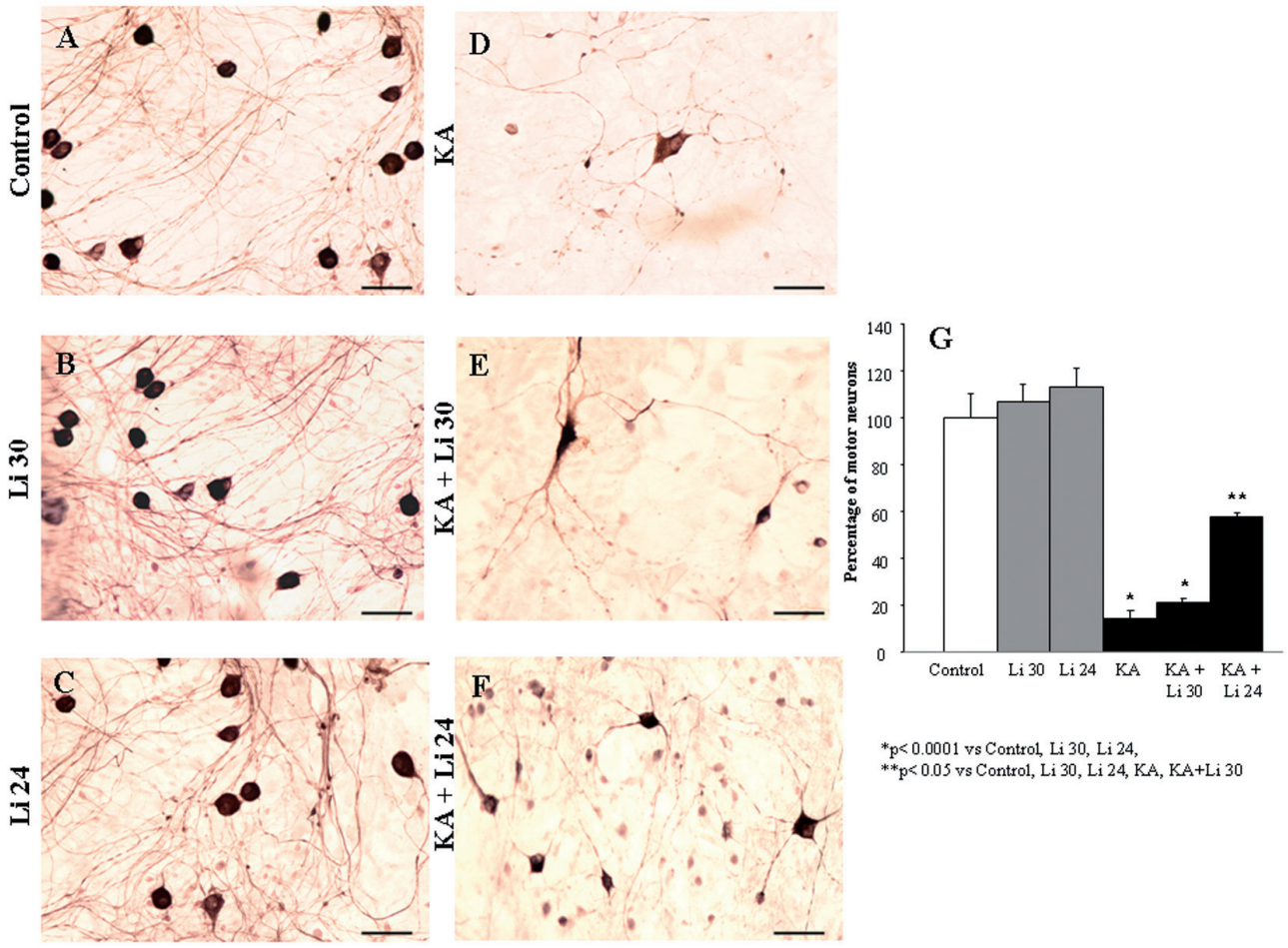

Fig. 1. - Lithium protects motor neurons from KA-induced excitotoxicity. Immunoperoxidase with the anti-SMI 32 antibody selective for motor neurons either in the absence (A-C) or in the presence (D-F) of KA. KA destroys motor neurons (D, G), that is attenuated by lithium administration lasting 24 h after KA (F, G). Lithium administered only for 30 min before KA is not effective (E, G). (A) Control; (B, C) motor neurons culture treated with lithium for 30 min and for 24 h, respectively. (G) Number of motor neurons in the culture after each treatment; (data are expressed as the percentage of motor neurons number respect to the control). $^{*}P < 0.0001$ compared with control, Li 30, Li 24; $^{**}P < 0.05$ compared with control, Li 30, Li 24, KA, KA + Li 30. Scale bar: 83 μm.

lithium for prolonged time (24 h) revealed cytoplasmatic authophagosomes surrounded by well preserved mitochondria (Fig. 4D). In contrast, when lithium was given only 30 min before KA mitochondria remained aberrant (data not shown).

Discussion

In the present work we confirm that exposure of motor neurons to the glutamate agonist KA produces a massive cell death. This effect is associated with severe alterations persisting im spared motor neurons. For instance, a significant increase in cell size measured as motor neuron diameter was constantly observed after a brief exposure to KA. This effect when analyzed in depth using electron microscopy consisted of enlargement of ER and Golgi apparatus with formation of aberrant large autophagy vacuoles often containing altered mitochondria. In these motor neurons the mitochondria appear as giant organelles with severe alteration in their inner trim. This ultrastructural features are reminiscent of a non effective recruitment of the autophagy pathway. In fact, when the expression of the autophagy protein beclin-1 was analyzed a huge increase was observed in the absence of autophagy progression. Since beclin-1 is part of the autophagy machinery and get

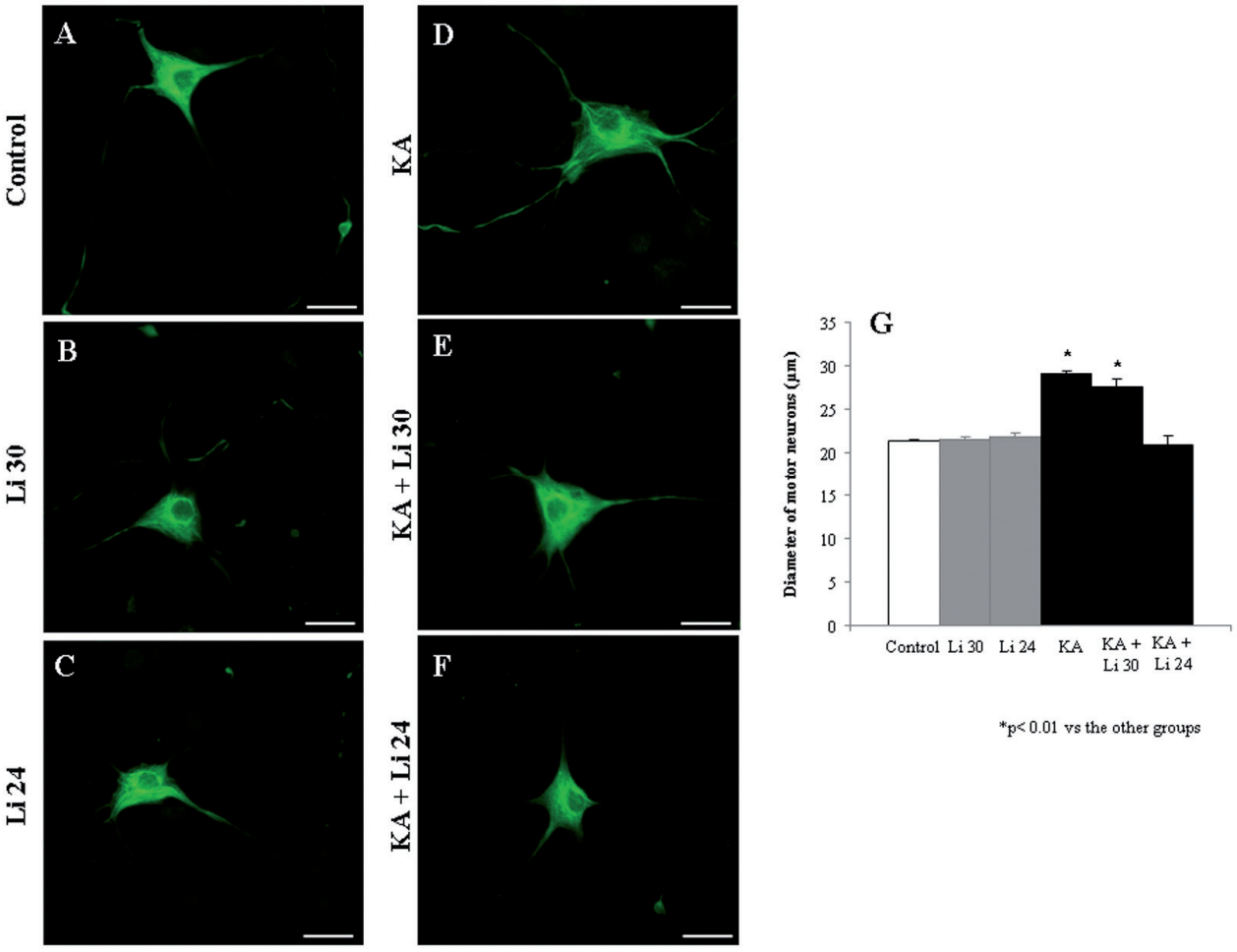

Fig. 2. - Lithium prevents increase of motor neuron size induced by KA. Immunofluorescence with the anti-SMI 32 antibody of motor neurons culture. Note that KA increases motor neurons diameter (D, E, G) as compared with either control (A, G), or with lithium 30 min (B, G) and lithium 24 h (C, G). Lithium administered for 24 h prevents this effect (F, G). Data represent the mean ± SEM of three independent experiments. $^{*}P < 0.01$ compared with the other groups. Scale bars: 28 μm.

metabolized upon its recruitment in the autophagy process, such a beclin-1 increase was very likely to be the consequence of an autophagy failure. In fact, upon autophagy stimulation we could no longer observe the persistence of high amount of beclin-1 which was associated with the rescue of autophagy progression at ultrastructural level. Such a stimulation of autophagy occurs by administration for prolonged time (24 h) of lithium to motor neurons. In this experimental conditions before observing spared neurons at ultrastructural level we could detect the protection of KA-induced motor neuron loss. This protective effect of lithium was obtained by administering low doses of lithium starting 30 min before KA and lasting 24 h after KA. In these experimental conditions protection against KA-induced motor neuron death was impressive (almost a half). As mentioned above this was accompanied by preservation of motor neuron morphology. In, fact, lithium-treated motor neurons exhibited a normal size and a high amount of neuronal processes. It is known that lithium enhances axonal regeneration after spinal cord injury (Huang et al., 2003; Yick et al., 2004; Wada et al., 2005) and increases collateral axon branching and giant growth cones in different neuronal types (Lucas et al., 1998; Williams et al., 2002). This effect of lithium on preserving neuronal morphology was also described in G93A transgenic mice both in the spinal cord and brain stem (Fornai et al., 2008a; Ferrucci et al., 2010). Since lithium

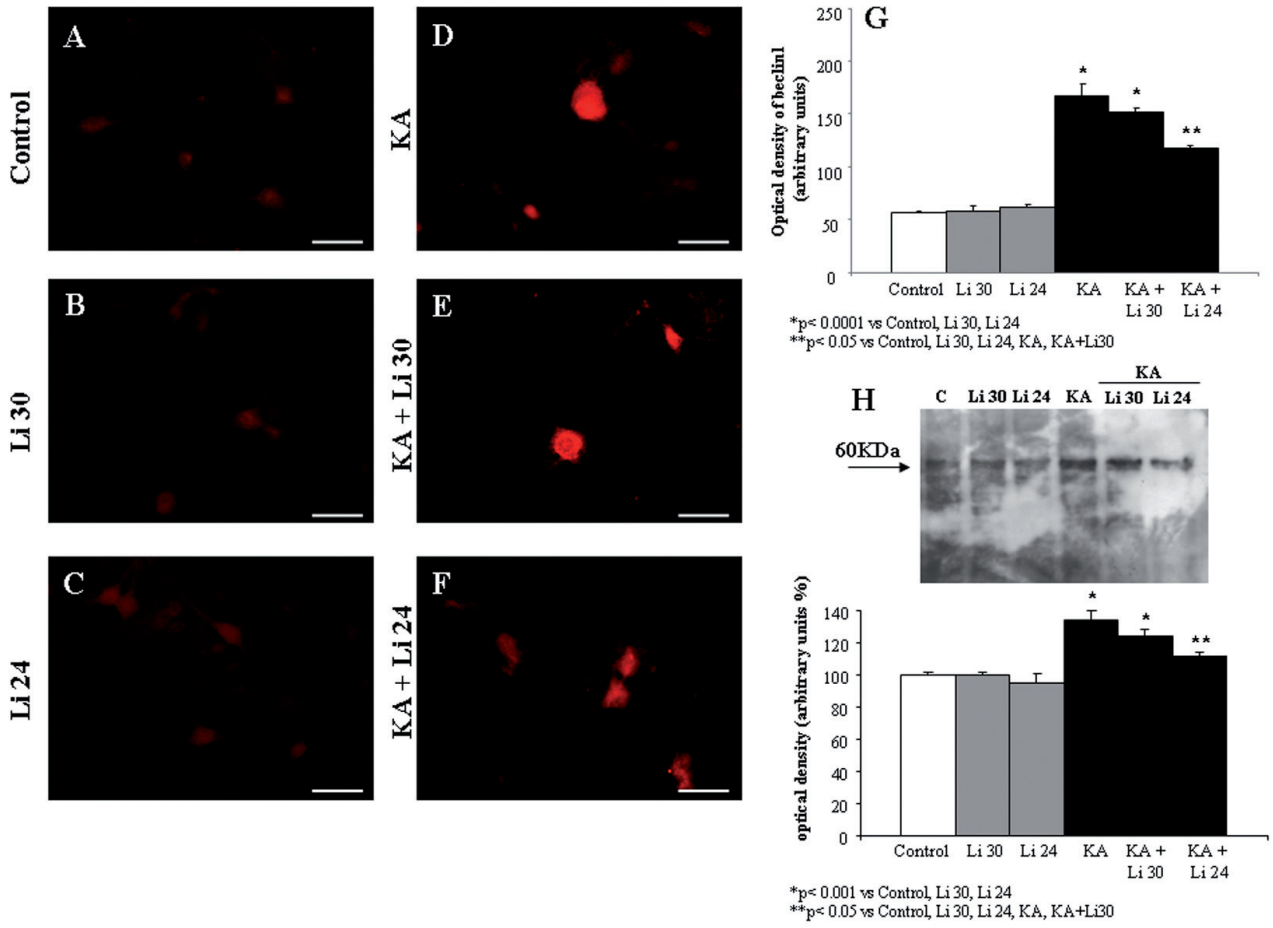

Fig. 3. - Beclin-1 in motor neurons. After KA beclin-1 increases and this effect is occluded when lithium is administered for 24 h after KA but not when lithium persists only 30 min in the cell culture. Immunofluorescence images shows beclin-1 in control (A), lithium 30 (B) lithium 24 h (C) after KA (D) or following combined administration Lithium + KA with Lithium persisting for 30 min (E) or 24 h (F). The graph in G shows the densitometric analysis of beclin-1 immunofluorescence. Data represent the mean ± SEM of three independent experiments. $*P < 0.0001$ compared with control, Li 30, Li 24; $**P < 0.05$ compared with control, Li 30, Li 24, KA, KA + Li30. In (H) immunoblotting. Data represent the mean ± SEM of three independent experiments. $*P < 0.001$ compared with control, Li 30, Li 24; $**P < 0.05$ compared with control, Li 30, Li 24, KA, KA + Li30. Scale bar: 62 μm.

is known to promote the differentiation of newly formed neural cells toward neuronal phenotype in the hippocampus (Kim et al., 2004; Wexler et al., 2008) and spinal cord (Juan et al., 2007; Fornai et al., 2008a), it remains to be established whether occurrence of a higher motor neuron number is produced by preventing cell death and/or increasing cell proliferation and differentiation. In our experimental conditions, we did not investigate the occurrence of neuronogenesis after lithium exposure, The beneficial effects of lithium on motor neurons survival are attributed to its antioxidant and antiapoptotic properties (Shin et al., 2007), the inhibition of the glycogen synthase kinase-3 (GSK-3; Feng et al., 2008), or the activation of autophagy (Fornai et al., 2008a). Typical autophagic vacuoles, containing altered mitochondria and other cytoplasmic material, were found within remaining motor neurons of G93A mice at the end of disease (Fornai et al., 2008a,b) and defective autophagy is now widely recognized as a common phenomenon in ALS. In particular, even when an increase in both autophagy-related formations and autophagy proteins are found in motor neurons (Kinch et al., 2003; Morimoto et al., 2007; Li et al., 2008), this represents the consequence of defective autophagy progression. In fact, as demonstrated by

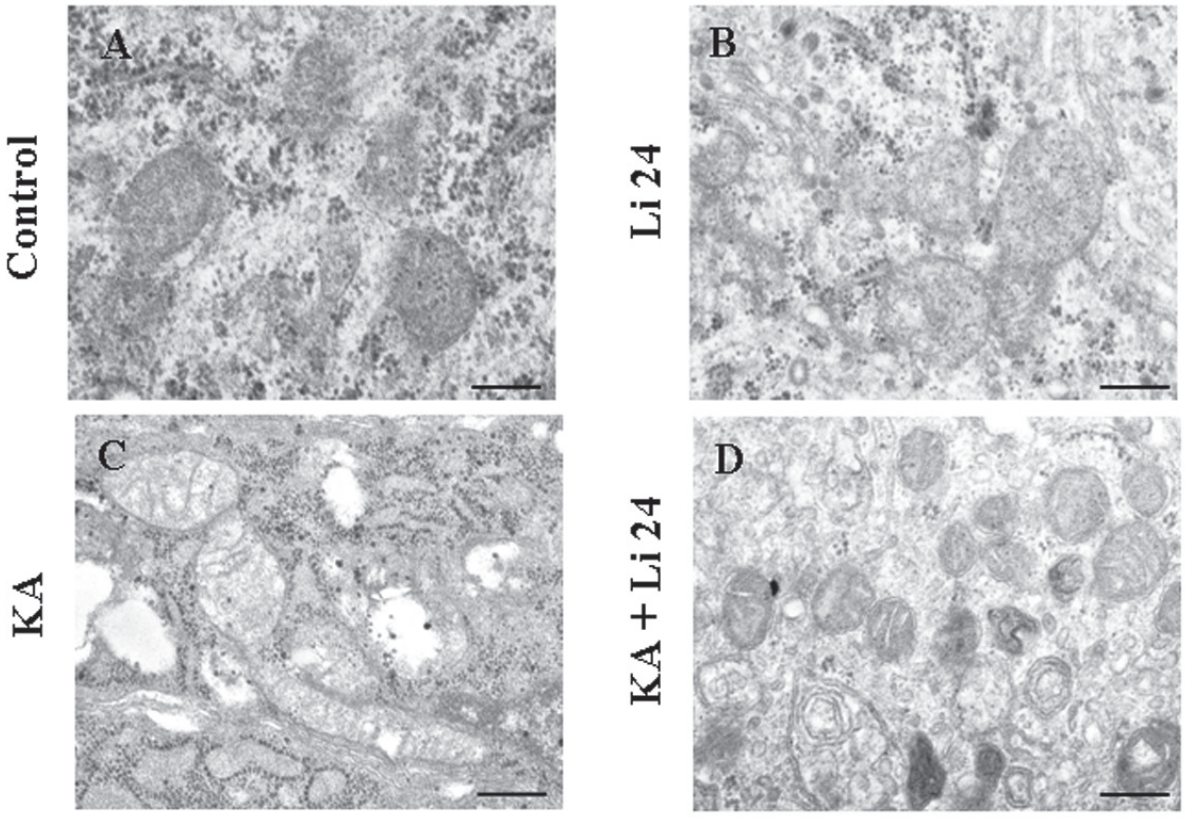

Fig. 4. - Ultrastructural alterations induced by KA are prevented by lithium. Representative pictures of motor neurons ultrastructure from control culture (A), from culture treated with lithium for 24 h (B), from culture treated with KA (C) and from culture treated with Lithium + KA with Lithium persisting 24 h (D). In culture treated with KA TEM micrograph shows mitochondrial vacuolation (C), prevented by treatment with Lithium persisting 24 h (D). Normal mitochondria morphology in control culture (A) and after Lithium for 24 h alone (B). Scale bar: A, B, D = 0.3 μm, C = 0.4 μm.

Venkatachalam et al. (2008) defective autophagy in diseased motor neurons is accompanied by accumulation of large vesicles identified as autophagosomes (Venkatachalam et al., 2008).

Neuropathological features of ALS both sporadic and familial are now recognized as a consequence of defective autophagy pathway.

Ultrastructural analysis provided also in the present study demonstrates that activation of autophagy produces the clearance of large stagnant autophagy vacuoles, and damaged mitochondria accumulating in diseased motor neurons.

In the last years increased evidence indicates that lithium protects neuronal cells against different toxic insults, such as ischemia and excitotoxicity. In particular, lithium prevents ischemic-induced brain injury (Nonaka and Chuang, 1998; Xu et al., 2003; Cappuccio et al., 2005), and it was found to prevent KA-induced excitotoxic cytopathological changes and motor neuron death in chicken embryo (Calderò et al., 2010). The mechanisms underlying such a neuroprotection are under investigation, and it is likely that multiple pathway are involved. In fact, lithium is known to target different molecules, thus affecting multiple (and often opposite) pathways, and the final effect critically depends on the dose (for a dedicated review see Pasquali et al., 2010).

In particular, lithium at low doses inhibits inositol monophosphatase (Ki = 0.8 mM) thus causing a reduction of inositol 1,3,5-triphosphate IP_3 (Sarkar et al., 2005; Sarkar and Rubinsztei, 2006) and activation of autophagy (Criollo et al., 2007).

Interestingly, activation of glutamate receptors has been reported to increase the levels of IP_3, by hydrolyzing PIP2 (Ruiz et al., 2009) suggesting that glutamate produces an autophagy deficiency that might be crucial for motor neuron survival. Thus, lithium through inhibiton of IMPase is able to directly coun-

teract this effect, allowing the autophagy pathway to normally proceed within the motor neurons.

This data suggest the occurrence of a defective autophagy in motor neurons after KA exposure that produces motor neurons death and utrastructural alterations. These consist of increased motor neurons size, damaged mitochondria, protein accumulation, large cytoplasmic vacuoles placed in perinuclear positions. Intrìguingly these cellular pathology is reminiscent of ALS, which in turn is characterized by a defective autophagy. We report that pharmacological activation of the autophagy machinery is able to counteract KA-mediated motor neuron damage.

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