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Protein clearing pathways in ALS

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ABSTRACT

In the present review a large amount of experimental and clinical studies on ALS are discussed in an effort to dissect common pathogenic mechanisms which may provide novel information and potential therapeutic strategies for motor neuron degeneration.
Protein clearing systems play a critical role in motor neuron survival during excitotoxic stress, aging and neurodegenerative disorders. Among various mechanisms which clear proteins from the cell recent studies indicate autophagy as the most prominent pathway to promote survival of motor neurons.
Autophagy regulates the clearance of damaged mitochondria, endoplasmic reticulum and misfolded proteins in eukaryotic cells. Upon recruitment of the autophagy pathway, an autophagosome is produced and directed towards lysosomal degradation.
Here we provide evidence that in both genetic and sporadic amyotrophic lateral sclerosis (ALS, the most common motor neuron disorder) a defect in the autophagy machinery is common. In fact, swollen, disrupted mitochondria and intracellular protein aggregates accumulate within affected motor neurons. These structures localize within double membrane vacuoles, autophagosomes, which typically cluster in perinuclear position. In keeping with this, when using autophagy inhibitors or suppressing autophagy promoting genes, motor symptoms and motor neuron death are accelerated. Conversely stimulation of autophagy alleviates motor neuron degeneration.
Therefore, autophagy represents an important target when developing novel treatments in ALS.

Key words
Autophagy • Autophagoproteasome • Mitochondria • Lithium • G93A transgenic mice

Introductory statement

Several mechanisms are involved in motor neuron survival, which at a first glance, provide a scattered scenario where different features do not appear to integrate into a single mechanistic perspective. Therefore, it is important to dissect the weighted influence for each of these mechanisms on motor neuron survival in order to establish what is a robust versus a slight effect. It is expected that when a mechanism exerting a robust influence on motor neuron survival is impaired this leads eventually to serious neurotoxic effects. On the other hand, one would expect that by stimulating the very same mechanism motor neuron survival can be promoted. Strong evidence indicates that protein clearing systems play such a robust effect in promoting motor neurons survival. In fact, the impairment of specific protein clearing systems leads to motor neuron degeneration, while enhancement of cell clearance confers neuroprotection. Again, the fine ultrastructural pathology of degenerating motor neurons in both genetic and sporadic amyotrophic lateral sclerosis (ALS) is characterized by intraneuronal protein

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aggregates and mitochondria. This witnessess for the failure of proteins/organelles clearance in ALS motor neurons. A recent fact is the accumulation of the TAR DNA-binding protein of 43 kDa (TDP-43) which is now widely recognized as a hallmark of ALS since it is mislocalized and accumulated in cytoplasmic aggregates both in sporadic and genetic ALS (Neumann et al., 2006; Yokoseki et al., 2008). This happens also in the absence of TDP-43 mutations. Protein clearing systems are responsible for the clearance of TDP-43 (Wang et al., 2010), thus witnessing again for a protein clearing defect in ALS motor neurons. This is replicated when looking at the autophagy-dependent clearance of other proteins which are clustered in cytoplasmic inclusions into ALS motor neurons (i.e. Cu-Zn superoxide dismutase type 1, SOD1, alpha-synuclein).

This evidence indicates a defect in the autophagy machinery as a common determinant in ALS. In fact, autophagy inhibitors worsen the viability of motor neurons in a variety of ALS models while the stimulation of autophagy alleviates motor neuron degeneration (Pasquali et al., 2009a; Madeo et al., 2009). Moreover, when specific autophagy components are impaired by gene manipulation autophagy failure leads eventually to motor neuron loss (Ferguson et al., 2009).

On the other hand, one might argue that autophagy represents a mere epiphenomenal event within dying motor neurons, which lose their ability to metabolize proteins just as a secondary phenomenon due to other primary mechanisms generating a bulk of degraded cell components. However, the primary loss of protein clearance *per se* leads to motor neuron death while suppressing protein clearance accelerates motor neuron death in genetic ALS. Thus, as it will be emphasized in this review, autophagy failure needs to play a causative role, which cannot be mismatched with an accompanying phenomenon (Pasquali et al., 2009a).

Therefore, it is reasonable to expect that, the tuning of autophagy, upon appropriate pharmacological activation, should protect motor neurons.

This review extends the significance of autophagy beyond ALS to encompass different scenarios characterized by the death of motor neurons.

The autophagy pathway

In every eukaryotic cell, there are two main systems for degradation of cytoplasmic proteins: the ubiquitin-proteasome (UP) system (Ciechanover, 2006) and autophagy (Yorimitsu and Klionsky, 2005). While the UP system mainly degrades short-lived nuclear and cytosolic misfolded proteins (Ciechanover, 2006), autophagy is involved in the clearance of long-lived protein and organelles (mitochondria and endoplasmic reticulum). A certain amount of baseline autophagy activity is required in order to mantain organelles and proteins at turnover, and this slow rate can quickly change upon appropriate requests.

For instance, autophagy is quickly upregulated when cells need to generate intracellular nutrients and energy during starvation or growth factor withdrawal. Thus, nutritional status, as well as hormonal factors, temperature, oxygen concentrations, and cell density, are important autophagy modulators (Klionsky, 2007; Maiuri et al., 2007; Mizushima and Klionsky, 2007; Rubinsztein et al., 2007).

Autophagy is also upregulated during development, to perform structural remodelling, and to remove damaged cytoplasmic components, that are produced in different stressful conditions, such as during oxidative stress, infections, and protein accumulation (Mudher et al., 2004; Rubinsztein, 2006; Yang et al., 2006; Forman et al., 2007). In particular, autophagy is very important for degradation of misfolded proteins and damaged mitochondria (Klionsky and Emr, 2000; Ravikumar et al., 2004; Castino et al., 2005). The pivotal role of autophagy in axonal elongation is demonstrated following axonal injury and results from a massive occurrence of autophagy components within axons compared with cell body and dendrites (Yue, 2007). This explains the important role of autophagy in synaptogenesis (Shen and Ganetzky, 2010).

The first autophagy step is membrane nucleation which leads to the formation of a dynamic structure, deriving from the endoplasmic reticulum (ER) and/or the trans Golgi network. This initial structure evolves into a semi-circled single membrane, the “phagophore”, and then into a double-layered vesicle, named “autophagosome”. Formation of the autophagosome requires the incorporation of phosphatidyletanolamine (PE)-light chain (LC3) I

and the small GTPase rab24 (Fengsrud et al., 1995; Oosthuyse et al., 2001). LC3 is a microtubule-associated protein, indicated as MAP-LC3, which normally participates in the dynamics of the cytoskeleton. This isoform corresponds to the 18 kDa LC3 I. When autophagosome formation begins, the 18 kDa LC3 I dissociates from the microtubules and is lipidated to16 kDa LC3 II isoform, bound to PE (lipidated LC3). This process is carried out by a concerted action of the serine protease Atg4, which removes a small peptide at the C-terminal of the LC3 I, and an ubiquitin-like conjugating enzyme that catalyzes the covalent binding of PE to LC3 II (Cai et al., 2008; Otomo et al., 2008). In line with the association of LC3 I to microtubules, this phase of autophagosome formation normally occurs close to the microtubule organizing centre. Subsequently, the autophagosome converts into the autophagolysosome. This might occur in two way: i) the autophagosome first fuses with early and late endosomes, forming the so-called amphisome to merge with lysosome; ii) the autophagosome fuses directly with lysosome. This latter process, which by-passes the endosomal compartment, leads to a less effective autophagy (lacking the endosomal enzymes). Fusion of autophagosomes with lysosomes requires the lysosomal integral membrane glycoprotein LAMP2 (Petiot et al., 2000). LC3 II can be rescued and recycled back before the autophagosome fuses with acidic organelles or may be degraded by lysosomal hydrolases after autophagolysosome formation.

Both endosomes and lysosomes represent degrading vacuoles which possess a great amount of acidic hydrolases allowing the digestion of a wide range of biomolecules, such as protein macro-aggregates, organelles, membranes and cytoplasmic components. In particular, endosomes require the activity of the endosomal sorting complex required for transport (ESCRT), which plays a critical role in the trafficking of ubiquitinated substrates from the endocytotic vesicles to multivesicular bodies (MVB; Babst et al., 2002).

The ESCRT system, first identified in yeast Vps class E mutants, consists of four major classes of molecules, which act consecutively: the Vps27p/Hrs complex, which first recognizes the ubiquitinated cargo, the ESCRT-I complex, belonging to the endosomal membrane, which delivers the cargo to the ESCRT-II complex and, finally, the ESCRT-III complex, which internalizes the cargo within the MVB. Then, ubiquitin is removed proteolytically and ESCRT machinery is dissociated from the endosomal membrane by the activity of ATPase Vps4p/SKD1 (for a review see Hurley and Emr, 2006; Slagsvold et al., 2006; Williams and Urbe, 2007). MVB merges with the autophagosome to produce an amphisome. Within amphisome the enzymatic apparatus of endosomes is further implemented by melting with the lysosomes which leads to formation of autophagolysosomes allowing to produce a more effective autophagy, where macroaggregates are completely hydrolysed, due to synergistic activities of endosomal and lysosomal enzymes (i.e. the lysosomal cysteine proteases, cathepsins B, D, and L).

A defect in the ESCRT system produces defective endosomes that lead to aberrant MVB, which in turn, do not allow the formation of the amphisome. This was demonstrated in yeast, where alterations of the vacuolar protein sorting 2 (VPS2), a component of the ESCRTIII complex, result in the formation of dysmorphic hybrid vacuole-endosome structures; additionally, disruption of other ESCRTIII components abolishes the ability of MVB to internalize membrane-bound cargoes (Babst et al., 1998; Katzmann et al., 2001; Babst et al., 2002).

Mutations in the ESCRT-III subunit CHMP2B, the mammalian orthologue of Vps2, were recently associated with frontotemporal dementia and ALS (Cox et al., 2010). Similarly, a mutation of alsin, which normally moves the endosomes, produces familial ALS (see below).

Moreover, in mammals the lysosomal transmembrane proteins, LAMP-2 and CLN3, are required for melting lysosomes with autophagosomes (Levine and Kroemer, 2008).

The autophagy pathway is controlled by several signalling molecules, that act at different steps throughout the enzymatic cascade (Castino et al., 2005). The first step of the autophagy pathway is controlled by a hetero-oligomeric complex consisting of the phosphoinositide 3-kinase (PI3k) class III, the mammalian counterpart of yeast Vps34, beclin 1, the mammalian counterpart of yeast Vps30/Atg6, and the protein p150, the mammalian counterpart of yeast Vps15, and Atg14 (Olzmann et al., 2008). These molecules form the so-called autophagy interactome, which is responsible for the induction of the phagophore and formation of a functional

autophagosome (Olzmann et al., 2008). Thus, induction of autophagy passes via beclin 1, PI3k class III and p150 myristylated kinase (Pattingre et al., 2005). The subcellular localization of beclin 1 is controversial. Levine and coworkers found beclin 1 mainly in the ER and mitochondria (Pattingre et al., 2005). However, this placement may be not the primary site but the migration of beclin 1 towards altered organelles targeted for degradation. Analysis of endogenous beclin 1 in other cell types suggests that it resides in the trans-Golgi network or in as yet uncharacterized cytosolic structures (Kihara et al., 2001; Høyer-Hansen et al., 2007).

A key regulator of autophagy is the mammalian Target Of Rapamycin, mTOR. This possesses a kinase activity and regulates a number of cell functions, like protein translation, ribosome biogenesis, localization of transcription factors. mTOR is a regulator of cell growth and differentiation, integrating signalling from both growth factors and nutrients. mTOR also acts as the major inhibitory signal for the autophagy pathway and shuts off autophagy in the presence of growth factors and abundant nutrients. The PI3k class I/Akt signaling molecules link receptor tyrosine kinases to mTOR activation and thereby repress autophagy in response to insulin-like and other growth factor signals (Lum et al., 2005). Other regulatory molecules that control autophagy include 5'-AMP-activated protein kinase (AMPK), which responds to low energy; the eukaryotic initiation factor 2α (eIF2α), which responds to nutrient starvation, double-stranded RNA, and ER stress; BH3-only proteins that contain a Bcl-2 homology-3 (BH3) domain and disrupt Bcl 2/Bcl-XL inhibition of the Beclin 1/PI3k class III complex; the tumor suppressor protein, p53; death-associated protein kinases (DAPk); the ER-membrane-associated protein, IRE1; the stress-activated kinase, c-Jun-N-terminal kinase; the inositol trisphosphate (IP3) receptor (IP3R); GTPases; Erk1/2; ceramide; and calcium (Meijer and Codogno, 2006; Criollo et al., 2007; Maiuri et al., 2007; Rubinsztein et al., 2007).

Autophagy can be pharmacologically induced by inhibiting negative regulators such as mTOR with rapamycin (Rubinsztein et al., 2007), or lithium, a molecule that lowers IP3 levels (Sarkar et al., 2005; Criollo et al., 2007). Pharmacological inhibition of autophagy can be performed by targeting the PI3k class III involved in autophagosome formation with 3-methyladenine or by targeting the fusion of autophagosomes with lysosomes, using the aminoacid asparagine or inhibitors of the lysosomal proton pump such as bafilomycin A1; genetic inhibition of autophagy can be achieved by using viral vector containing the null Vps34 gene, that corresponds to the mammalian PI3k class III (Petiot et al., 2000; Kihara et al., 2001). p62 protein is a selective autophagy substrate, also named sequestosome 1 (Talbot and Ansorge, 2006), and represents an important molecule in both autophagy and ubiquitin-proteasome system. In fact, p62 is a shuttle either to the proteasome (Seibenhener et al., 2004; Babu et al., 2005), or to autophagy (Pankiv et al., 2007).

The merging of protein clearing systems: the autophagoproteasome

Recent evidence provided by our research team (Fornai et al., 2004; Castino et al., 2008; Pasquali et al., 2009a,b), demonstrates that autophagy and proteasome components can melt into a single double membrane limited organelle previous to lysosomal fusion thus making different protein clearing systems not as alternative pathways in the cell but as convergent mechanisms, which eventually, merge into the same organelle. In fact, proteasome components were described within neuronal bodies reminiscent of autophagosomes (Fornai et al., 2004) which we further characterized by using autophagy markers (Castino et al., 2008), thus suggesting the merging of clearing systems into a novel organelle we termed autophagoproteasome (Pasquali et al., 2009a,b). These findings add to the amphisome (the convergence between autophagosome and endosome) the proteasome compartment leading to a poly-catalytic structure, the so-called autophagoproteasome which features misfolded proteins, altered mitochondria within multi-membrane-limited structures, and contains Proteasome Activator 700, the proteasome 20 S subunit (UP components) clustered with LC3 II, beclin and Rab 24 (autophagy components).

The interplay between ER stress and autophagy

Increasing evidence indicates that altered cellular organelles may regulate autophagy, featuring as a key event in neurodegeneration. This includes

damaged mitochondria and the unfolded protein response (UPR), which originates from the ER, a specialized subcellular compartment essential for the secretory pathway.

Mitochondria become targets for autophagy degradation after nutrient deprivation, a process known as mitophagy (Lemasters, 2005), which regulates the physiological turn over of mitochondria (Menzies and Gold, 1971; Pfeifer, 1978).

Although mechanisms targeting mitochondria for mitophagy remain poorly understood, it is likely that this involves the membrane pore transition (MPT) and signaling through PI3k and possibly c-Jun amino-terminal kinases (Rodriguez-Enriquez et al., 2006).

The UPR signalling system is triggered when misfolded proteins accumulate within the ER (Kaufman, 2002). The UPR is an adaptive reaction that aims to restore the ER capacity to produce properly folded proteins (for a review, see Matus et al., 2008). In addition to the UPR, ER-stress leads to a release of Ca from the ER and an increase in cytosolic free Ca (Berridge, 2002). When prolonged, UPR triggers apoptosis by two ER stress-specific cell death signals (Nakagawa et al., 2000; Urano et al., 2000; Hitomi et al., 2004).

Occurrence of chronic ER stress and induction of UPR were described in a number of experimental models of neurodegeneration, linked to protein misfolding and aggregation (the so-called proteinopathies), such as Parkinson's disease, Alzheimer's disease, Creutzfeldt-Jacob disease, and ALS. In these models there is a tight correlation between disease progression, protein accumulation and UPR induction (Lindholm et al., 2006). More interestingly, upregulation of ER stress markers was observed in post-mortem brain tissues from human patients (Lindholm et al., 2006). ER stress is a potent trigger of autophagy (Bernales et al., 2006; Kamimoto et al., 2006; Ogata et al., 2006; Yorimitsu et al., 2006; Criollo et al., 2007; Ding et al., 2007; Fujita et al., 2007; Høyer-Hansen et al., 2007; Kouroku et al., 2007). While the induction of autophagy by ER stress is conserved from yeast to mammals, the signaling pathways responsible for autophagy induction and its cellular consequences appear to vary according to cell type and kind of stimulus.

The direct link between ER stress and autophagy was established in yeast *Saccharomyces cerevisiae* treated with distinct ER stressors. The data show that ER itself is the major autophagosomal substrate during ER stress and suggest that induction of autophagy represents a pro-survival response due to augmented removal of unfolded proteins (Bernales et al., 2006). Additionally, it was proposed that autophagy response provides a negative feedback during the recovery period by removing expanded ER.

Protein clearing pathways are key in regulating motor neuron survival

There is evidence that motor neurons are defective in sustaining a bulk protein misfolding (i.e. during a potent oxidative insult or a genetic defect). In line with this, stressful conditions which are bare for other cells cause the death of motor neurons. This might depend on a high threshold for autophagy induction within motor neurons (Manzerra and Brown, 1992; Brown and Rush, 1999; Mautes and Noble, 2000; Batulan et al., 2003). Since stress response is a major autophagy inducer, these findings suggest that motor neurons may be constitutively deficient to activate autophagy after common injuries.

Confirming the vulnerability to protein misfolding, motor neurons are more vulnerable than non motor neurons to the proteasome inhibitor lactacystin (Urushitani et al., 2002).

Among protein clearing pathways, the UP system in motor neuron seems to have a role in the regulation of axonal survival and degeneration (MacInnis and Campenot, 2005).

Mutant SOD1 also undergoes degradation by proteasomes (Hoffman et al., 1996; Johnston et al., 2000), and recent studies demonstrated decreased proteasome activity in cell lines expressing mutant SOD1 (Hyun et al., 2002; Urushitani et al., 2002) and in lumbar spinal cord from G93A transgenic mice (Kabashi et al., 2004; Cheroni et al., 2005). In G93A transgenic mice the loss of proteasomal function was found to correlate with disease progression (Kabashi et al., 2004) and the accumulation of mutant SOD1 (Cheroni et al., 2005).

On the other hand, as discussed in depth in this article, the most recent studies indicate that autophagy plays the major protective role for spinal motor neurons against neurodegeneration.

Apart from ALS, autophagy is critical for motor neuron survival also in other neurodegenerative dis-

orders such as spinal and bulbar muscular atrophy (SBMA, Kennedy's disease), an X-linked motor neuron disease resulting from the expansion of a polyQ-encoding CAG tract in the 5' end of the androgen receptor (AR) gene (La Spada et al., 1991). Histopathology of SBMA presents many similarities with ALS. In fact, in patients with SBMA, lower motor neurons are markedly depleted through all spinal segments and in brainstem motor nuclei except for the third, fourth and sixth cranial nerves (Sobue et al., 1981, 1989). Nuclear inclusions containing mutant AR and several UP components are found within the surviving motor neurons (Li et al., 1998; Merry et al., 1998; Stenoien et al., 1999; Diamond et al., 2000; Adachi et al., 2005). A very recent study demonstrates that the autophagy pathway is able to degrade cytoplasmically retained mutant AR proteins. In this disease, autophagy inhibition worsen, while autophagy activation alleviates motor neuron death (Montie et al., 2009).

Taken together these findings provide a strong evidence that autophagy plays a central role in motor neuron survival. Since the primary defect is different in different motor neuron diseases, it is intriguing to disclose the biological link bridging different pathological conditions into a common defect of the final pathway. In this view, autophagy does represent the more general protective response suitable for the motor neuron, that independently from the primary cause of degeneration, is recruited due to its efficacy in preserve cell viability.

Protein misfolding in ALS

Protein aggregates in ALS

Motor neuron degeneration in humans ALS is characterized by size variations of the cytoplasm and nucleus, mitochondrial damage (Martin, 2007), and neuronal inclusions which show different morphological features, appearing as basophilic inclusions, skein-like inclusions, Lewy-body-like inclusions, and Bunina bodies. Among them, Bunina bodies are small eosinophilic intraneuronal inclusions which are generally considered to be a specific hallmark of ALS. Bunina bodies were first described in 1962 from Bunina within motor neurons of spinal cord and brainstem in two familial ALS cases and were considered as a neurotrophic virus. One year before publication by Bunina, van Reeth et al. described similar intracellular inclusions in the anterior horn cells of the spinal cord from a patient with Pick's dementia with atypical ALS (van Reeth et al., 1961). Bunina bodies possess pale central areas with a tendency to form chain-like clusters. Despite a variety of immunohistochemical studies, only two proteins are recognized within Bunina bodies: cystatin C12 and transferrin (Mizuno et al., 2006a). Other inclusions such as skein-like inclusions or Lewy body-like/round inclusions, are ubiquitin-positive. Bunina bodies may be negative (Leigh et al., 1991) or positive (Lowe et al., 1988; Murayama et al., 1990) for ubiquitin and for the ubiquitin-related protein p62 (Mizuno et al., 2006b).

Recently, TDP-43, a nuclear protein that is involved in transcriptional repression and alternative splicing, was identified as a major component of neuronal intracytoplasmic inclusions in motor neurons in ALS, as well as in frontotemporal degeneration with ubiquitinated inclusions (Arai et al., 2006). Skein-like and Lewy body-like/round inclusions in ALS are positive for TDP-43, while Bunina bodies are negative (Tan et al., 2007).

SOD1 protein, which is responsible for about 20% of the familial cases of ALS (Rosen et al., 1993), is also described as a constant component of all ALS-associated inclusions in both familial and sporadic ALS (Urushitani et al., 2004; Matsumoto et al., 2005; Tan et al., 2007; Tan et al., 2008).

Transgenic mice, overexpressing a mutant form of the SOD1 protein, linked to familial ALS, have been widely used as animal model of ALS. In particular, SOD1 mutant mice that contain the G93A substitution were created in 1994 from Gurney et al. (1994) and provided milestone information about ALS pathology (Martin and Liu, 2004). The G93A transgenic mice develop a severe motor impairment, beginning at hindlimb level and progressively extending to trunk and forelimb, until complete paralysis and death (Martin and Liu, 2004; Martin et al., 2007; Feng et al., 2008; Fornai et al., 2008a). Recently, these mice were extensively analyzed at level of the motor nuclei of cranial nerves (Ferrucci et al., 2010), which also degenerate in the human ALS (Kusaka et al., 1988; Hartmann et al., 1989), extending the reliability of this animal model to the brainstem pathology.

The degeneration seen in these mice resembles a prolonged necrotic-like process with cellular swell-

ing (Martin et al., 2007; Fornai et al., 2008a,b). Surviving motor neurons from G93A mice analysed at the end of the disease show several morphological alterations, which consist of swelling of cell body and proximal axons, and accumulation of large monodansylcadaverine-positive (autophagy) vacuoles within the cytosol (Fornai et al., 2008a). Immunohistochemical analysis of such cytoplasmic vacuoles reveals alpha-synuclein, ubiquitin and SOD1 (Martin et al., 2007, Fornai et al., 2008a). Similarly to human pathology, electron microscopy shows swelled and vacuoled mitochondria within cytosolic autophagosomes (Fornai et al., 2008a).

Although a defective UP system was supposed to produce ALS-associated protein aggregates (Keller et al., 2000; Kabashi et al., 2004; Koyama et al., 2006; Cheroni et al., 2009), recent knowledge revealed the prominent role of autophagy (Fornai et al., 2008a,b, Laird et al., 2008; Hetz et al., 2009; Madeo et al., 2009; Crippa et al., 2010).

Autophagy plays the major protective role for spinal motor neurons against neurodegeneration. In primary motor neuron cultures obtained from G93A mice, induction of the autophagy pathway (using lithium or rapamycin) clears several autophagy substrates such as SOD1, ubiquitin, and alpha-synuclein, which accumulate in both familial ALS and sporadic ALS. Autophagy inducers such lithium clear these proteins and rescue motor neuron from excitotoxicity both in normal and ALS mice (Fornai et al., 2008a,b; Calderò et al., 2010).

Kabuta and colleagues reported that autophagy reduced mutant SOD1-mediated toxicity and that induction of autophagy decreased mutant SOD1 protein levels (Kabuta et al., 2006).

Moreover, abundant autophagy vacuoles and an increase in autophagy proteins due to a defect in autophagy progression were found in different experimental models of motor neuron death (Kinch et al., 2003; Matyja et al., 2005; Tarabal et al., 2005; Calderò et al., 2007; Morimoto et al., 2007; Fornai et al., 2008a,b; Laird et al., 2008; Li et al., 2008).

The increase in autophagy proteins is due to a lack of autophagy progression as a sort of non effective compensatory mechanism. When autophagy is not impaired to progress, accumulation of autophagosomes does not occur and increased levels of activated autophagy proteins like LC3 II are not accompanied by persistent increase in autophagic vacuoles. In contrast, in the presence of a defective autophagy progression LC3 II levels increase and an excess of misfolded proteins and/or altered organelles is observed.

This is supported by a recent study, which demonstrates that defective autophagy in diseased motor neurons is accompanied by accumulation of large vesicles identified as autophagosomes (Venkatachalam et al., 2008, Laird et al., 2008). In this study the presence of an autophagy defect was related with the onset of motor neuron disease and clinical motor symptoms (Venkatachalam et al., 2008), addinging strong evidence of the pro-survival effect of ongoing autophagy for motor neuron.

These studies are in line with data provided by Klionsky (2007), who reported that defective autophagy can lead to a paradoxical accumulation of autophagosomes filled with autophagy substrates.

Genetic mutations in ALS and autophagy

More insight about the pathogenesis of ALS comes from familial forms of ALS caused by specific defects in *Als* genes.

Familial ALS are approximately 10% of ALS cases (Strong et al., 1991; Majoor-Krakauer et al., 2003). Analysis of Als genes shows that most mutations causing different familial ALS produce distinct molecular defects but all of them provoke impairment of autophagy.

Als1

ALS1 is an adult-onset, fatal, autosomal dominant disorder associated with mutations in the SOD1 gene on chromosome 21q21 (Rosen et al., 1993).

Clinically indistinguishable from sporadic ALS, mutations in SOD1 gene account for about 20% of familial cases of ALS. From the first discover, in 1993 (Deng et al., 1993; Rosen et al., 1993), over 100 missense point mutations have been identified throughout the gene (Bendotti and Card, 2004; Turner and Talbot, 2008), as well as deletions and insertions leading to C-terminal truncated forms (Watanabe et al., 2005). SOD1 is a 153 amino acid cytosolic enzyme, ubiquitously expressed in most aerobic organisms (Rakhit et al., 2004). The biological function of SOD1 consists of catalyzing the dysmutation of superoxide anion to molecular oxygen and hydrogen peroxide (Liochev and Fridovich, 2003). Most SOD1 mutations lead to autosomal

dominant familial ALS which only in a few cases are associated with reduced enzymatic activity, many mutants retaining high levels of dysmutase activity (Deng et al., 1993; Vim et al., 1996). Therefore, it is assumed that mutated SOD1 becomes toxic due to a gain of function that confers to the enzyme an abnormal activity (Vim et al., 1997; reviewed in Xu Zuoshang, 2000; Cleveland and Rothstein, 2001). Supporting this view, null SOD1 mice do not develop motor neuron disease (Reaume et al., 1996) and mice with mutant SOD1 do not have any effects by modulating (increase or decrease) the expression of wild type SOD1 (Bruijn et al., 1998).

Mice expressing various ALS-related mutants SOD1 recapitulate the quick paralysis seen in familial ALS SOD1 human patients, providing an important contribution to define ALS disease mechanisms.

Despite the exact mechanism by which mutated SOD1 cause ALS is not known, it is intriguing to note that many pathological features observed in familial cases of ALS due to SOD1 mutations are also present in sporadic cases of ALS, thus making transgenic SOD1 mice of great interest. Neuropathology includes astrogliosis, axonal disorganization, mitochondrial pathology, fragmentation of Golgi apparatus, as well as precipitation of both mutated and wild type SOD1 into cytosolic aggregates and vacuolar degeneration. All these represent hallmarks of both sporadic and familial ALS (Dal Canto and Gourney, 1995; Wong et al., 1995; Mourelatos et al., 1996; Bruijn et al., 1998; Stieber et al., 2000). In addition, administration of antibodies directed against SOD1 were recently suggested to protect against disease progression (Urushitani et al., 2007).

It is established that mutant SOD1 fails to fold properly, thus implicating accumulation of misfolded SOD1 as a potential toxin in ALS. The misfolded SOD1 forms ubiquitinated cytoplasmic inclusions early in the disease process which increase as disease progresses (Bruijn et al., 1997).

Recently, mutant SOD1 aggregates were found to accumulate in fractions that are enriched in ER membranes and intensify as disease progresses. The ER-associated SOD1 aggregates bind to the ER-luminal polypeptide chain binding protein (BiP) (Kikuchi et al., 2006). This inhibits ER-associated degradation (ERAD). Multiple ALS-associated mutants of SOD1 – including the G93A – but not the wildtype SOD1, interact with Derlin-1, a transmembrane ER protein that is involved in dislocation of misfolded proteins from the ER to the cytosol. Binding of mutant SOD1 to Derlin-1 inhibits ERAD and thereby generates ER stress (Nishitoh et al., 2008).

Once in the cytosol, mutant SOD1 was reported to co-localize with p62 in motor neurons (Gal et al., 2007). This would be a crucial step to deliver mutant SOD1 proteins to the autophagy machinery for degradation.

Interestingly, Saxena et al. (2009) have recently published a method to carry out a longitudinal analysis of three transgenic SOD1 mice, aimed to identify motor neurons that are selectively vulnerable or resistant to motor neuron disease. They found that vulnerable motor neurons are selectively prone to ER stress and show upregulated ER stress markers from birth. This suggests a role for the ER stress in motor neuron degeneration and introduce the novel concept of different sensitivity of different subtypes of mutant SOD1 motor neurons to ER stress (Saxena et al., 2009).

This is likely to explain why different motor neurons possess a variable susceptibility to ALS, being the large phasic motor neurons proner to cell death compared with the small tonic motor neurons (Hegedus et al., 2007). Occurrence of selective changes in ALS motor neurons leading to cell death emerge from the study of Kikuchi et al. (2006). These Authors found that transgenic mice expressing mutant SOD1 exhibit age- and region-specific molecular alterations indicative of a broad recruitment of ER signaling pathways, including caspase 12, a prototypical ER cell death effector (Nakagawa et al., 2000). They also showed that mutant SOD1, and to a lesser extent wild-type SOD1, accumulates in the ER as high molecular weight aggregates which interact with the ER chaperone immunoglobulin-binding protein BiP, a key component of the ER misfolded protein recognition machinery (Schroder and Kaufman, 2005). The binding of mutant SOD1 to BiP (Kikuchi et al., 2006) could mediate SOD1 import into the ER. Another mechanism proposed for mutant SOD1 toxicity is the inhibition of the proteasome activity. This effect of mutant SOD1 is still discussed, since intracellular accumulations in familial or sporadic ALS patients are not immunoreactive for proteasome components (Ii et al., 1997; Watanabe et al., 2001) but have been reported to contain Dorfin, an E3 ubiquitin ligase, which physi-

cally binds and ubiquitinates various SOD1 mutants, thereby enhancing their degradation, without affecting the stability of wild-type SOD1 (Niwa et al., 2002). In agreement with this, biochemical evidence from spinal cords of SOD1 mice has reported decreased activities of the proteasome in lumbar spinal cords of SOD1 mutant mice (Kabashi et al., 2004; Cheroni et al., 2009).

Remarkably, misfolded mutant SOD1 damages mitochondria by its deposition on the cytoplasmic face of the outer membrane of spinal cord mitochondria (Liu et al., 2004; Vande Velde et al., 2008). The damaged mitochondria occur within motor neurons of humans and mice (Martin et al., 2007), and vacuoles containinig mitochondria are present at various stage of degeneration in these mice (Wong et al., 1995). This supports the hypothesis that mitochondrial damage is key in disease initiation (Dal Canto and Gurney, 1994; Kong and Xu, 1998).

Swelled mitochondria are accompanied by elevation of mitochondrial calcium levels and oxidative stress, which appear at presymptomatic stage (Brustovetsky et al., 2003), whereas at the end of the disease decreased activity in the respiratory chain has been found (Mattiazzi et al., 2002). The mitochondrial pathology is more severe at the end of disease, when large and vacuoled mitochondria were found in the cytosol of motor neurons (Fornai et al., 2008a,b).

Other mechanisms of mutant SOD1-induced toxicity consist of: i) extracellular secretion of mutant SOD1 by interaction with chromogranines (Urushitani et al., 2006), which in turn is promoted by proteasomal inhibition, and subsequent microglial activation (Zhao et al., 2009); ii) activation of the multiprotein membrane-associated NADPH oxidase through selective interaction with the catalytic subunit Nox2, thus promoting extracellular formation of superoxide anion (Harraz et al., 2008); iii) interference with axonal cytoskeletal organization and/or inhibition of both anterograde (Williamson and Cleveland, 1999) and retrograde (Murakami et al., 2001; Perlson et al., 2009) axonal transport, probably by altering dynein functions (Kieran et al., 2005; Ligon et al., 2005).

Als2

ALS2 is a juvenile-onset, slowly progressive, autosomal recessive disorder in humans (Yang et al., 2001; Eymard-Pierre et al., 2002) and a mild phenotype in mice (Otomo et al., 2008), that maps to chromosome 2q33 and is associated with mutations in the alsin gene, a putative GTPase regulator (Hadano et al., 2001; Yang et al., 2001). Alsin interacts with Rab5 that is important in many stages of endocytosis and early trafficking of signalling molecules (Gorvel et al., 1991; McLauchlan et al., 1998; Nielsen et al., 1999; Sato et al., 2005). In neurons Rab5 is required for the formation of endocytic pits for internalization of different receptors (Brown et al., 2005). All described mutations in the *Als2* gene are expected to cause premature protein termination and loss of function of the alsin protein (Devon et al., 2003). *Als2*-deficient mice have subtle motor neuron pathology and motor behavior abnormalities and neurons from these mice show marked defects in specific endosomal trafficking pathways with a severe deficit in early endosome fusion stimulating activity *in vitro* (Devon et al., 2006).

Mutation in the alsin gene is concomitant with an increase in autophagy markers due to such a defect in autophagy progression.

Dynein/dynactin mutations

Mutations in the dynactin gene (Munch et al., 2004) leading to a loss of function cause a familial ALS which markedly differs from the SOD1 mutation. However, both ALS-associated mutations lead to defective autophagy.

Transgenic mice that express the human gene encoding for the mutated variant of the dynactin protein associated with familial ALS in humans (Puls et al., 2003; Munch et al., 2004) show an increase in the ratio of LC3 II/LC3 I within motor neurons, which is accompanied by defective autophagy (Laird et al., 2008).

In fact, the mutant form of dynactin produces a deficiency in the autophagy machinery at the level of fusion of the autophagosome/amphisome with lysosome, and increased amounts of autophagy vacuoles, including non cleared autophagosomes and endosomes as well as LC3 II.

Thus, dynactin mutation disrupts the transport of the phagosome, which remains clustered in perinuclear regions. Such an alteration is accompanied by an increase of ubiquitin immunoreactivity.

The dynactin protein is a component of a complex which is involved in vesicle trafficking beyond the autophagosome. In particular, dynactin interacts

with dynein and provides a powerful engine to move the vesicles along the perykarion and axon (Waterman-Storer et al., 1997).

Interestingly, motor neuron loss observed in humans or mice carrying the dynactin mutation can be reproduced by impairing alternatevely this vesicle-moving apparatus.

For instance, dynein which was not yet bound to any familial ALS is part of this vesicle-moving apparatus since it promotes the movement of the autophagosome to the lysosome (Ravikumar et al., 2005; Rubinsztein, 2006; Jahreiss et al., 2008; Kimura et al., 2008; Olzmann and Chin 2008; Olzmann et al., 2008). If a mutant form of dynein is expressed in experimental models, such a mutation produces motor neuron loss (Hafezparast et al., 2003; Teuling et al., 2008).

Again, overexpressing dynamitin, which disassembles dynactin (LaMonte et al., 2002) impair the physiological activity of the dynactin/dynein complex leading to motor neuron loss.

The impairment of all these steps in the autophagy pathway often involves the movements of the autophagosome/amphisome (such as dynactin or alsin), and this is consistent also with alterations in axonal transport, which relies on similar mechanisms. For the same reason, a derangement of axonal transport associates with impaired autophagy. Komatsu et al. (2007) recently showed that deletion of Atg7, an autophagy gene that is essential for the biogenesis of autophagosomes, leads to abnormal swelling and dystrophy of cell axon terminals. Neuronal autophagy is essential for axonal homeostasis, local membrane trafficking, and turnover (Komatsu et al., 2007). Lending substance to this interconnection, it is worth noting that mutant SOD1 alters the transport of membrane-bound organelles (vesicles transported along the axons) and mitochondria, leading to accumulation in perinuclear position (De Vos et al., 2007). These observations confirm the convergence between autophagy, axonal transport, the cellular traffiking of altered organelles and the site of accumulation of misfolded toxic proteins (Tooze and Schiavo, 2008).

Als4

ALS4 (also known as "distal hereditary motor neuronopathy" with pyramidal features, or dHMN) is a rare, childhood- or adolescent-onset, autosomal dominant form of ALS that is characterized by slow disease progression, limb weakness, severe muscle wasting, and pyramidal signs associated with degeneration of motor neurons in the brain and spinal cord. The phenotype of ALS4 includes a long disease duration, absence of overt sensory abnormalities, and sparing of bulbar and respiratory muscles (Chance et al., 1998; Rabin et al., 1999; De Jonghe et al., 2002).

It localizes on chromosome 9q34 (Chance et al., 1998; Rabin et al., 1999; Blair et al., 2000; De Jonghe et al., 2002) and consists of missense mutations in the *Senataxin* gene (*Setx*), that encodes a DNA/RNA helicase (Chen et al., 2004). DNA/RNA helicases are known to be involved in DNA repair, replication, recombination and transcription, RNA processing, transcript stability, and translation initiation (Tanner and Linder 2001). Recent studies showed that several human diseases, including spinal muscular atrophy (SMA), are associated with defects in proteins or protein complexes that possess helicase activity (Campbell et al., 2000; Meister et al., 2000; Grohmann et al., 2001). Pellizzoni et al. (2001) identified RNA helicase A as a novel SMN-interacting protein and showed that this interaction is defective in some SMA mutants, suggesting a critical role for the SMN complex in several aspects of mRNA biogenesis.

Abnormal SETX protein in ALS4 might impair the capacity of neurons to produce error-free mature mRNA, thus leading to ER stress and motor neuron loss.

Tdp43 mutations

TDP-43 is 414 amino acids long, encoded by six exons, widely expressed and predominantly nuclear. This protein contains two RNA-recognition motifs (RRM1 and RRM2) and a C-terminal glycine-rich region that may mediate interactions with other proteins. Patients with TDP-43 mutations develop typical ALS with some variability within families in the site and age of onset (Yokoseki et al., 2008). Although about a half of all ALS patients develop cognitive impairment of varying severity, only one patient carrying a TDP-43 mutation has been reported to develop cognitive deficits (Corrado et al., 2009). Neuropathology of TDP43 mutations consists of inclusions in neurons and glial cells within the spinal cords and throughout the brains

(e.g., Neumann et al., 2006; Van Deerlin et al., 2008). Diffuse granular cytoplasmic staining of TDP-43 (which may represent an earlier stage of inclusion development) and nuclear clearing of TDP-43 have also been described in the spinal cords and brains of ALS patients carrying TDP-43 mutations. It is unclear whether TDP-43 mutations lead to motor neuron loss through a gain of one or more toxic properties or a loss of normal function due to sequestration of the protein in nuclear or cytoplasmic inclusions and the corresponding disruption of its interactions with protein or RNA targets.

On the other hand, TDP-43 is a major component of ubiquitinated protein aggregates found in many patients with sporadic ALS or the most common form of frontotemporal dementia, called FTLD-U (frontotemporal lobar degeneration with ubiquitinated inclusions). In ALS and FTLD-U patients, TDP-43 immunoreactive inclusions are observed in the cytoplasm and nucleus of both neurons and glial cells. The brains and spinal cords of patients with TDP-43 proteinopathy present a biochemical signature that is characterized by abnormal hyperphosphorylation and ubiquitination of TDP-43 and the production of ~25 kDa C-terminal fragments that are missing their nuclear targeting domains (Arai et al., 2006; Neumann et al., 2006). TDP-43 inclusions are now recognized as a common feature of most ALS patients, with the striking exception of patients with familial ALS caused by SOD1 mutations. These findings suggest that TDP-43 shares pathological features with other neurodegenerative disease-associated proteins like huntingtin, SOD1 and alpha-synuclein.

The autophagy-dependent clearance does represent a common feature although it is still unclear whether aggregation of TDP-43 is a primary event in ALS pathogenesis or whether it is a by-product of the disease process.

Knockdown of Tsg101 or Vps24, the component of ESCRT complex, prevents the nuclear transport of wild type TDP-43, resulting in cytoplasmic accumulation of ubiquitinated TDP-43 aggregates (Filimonenko et al., 2007).

Moreover, in diseased brains, TDP-43 forms pathogenic C-terminal fragments, with lower molecular weight species about 25 and 35 kDa (TDP-25 and TDP-35, respectively), which are prone to aggregation (Neumann et al., 2006; Igaz et al., 2008). Overexpression of the truncated fragment TDP-25 forms ubiquitinated and phosphrylated cytoplasmic aggregates (Igaz et al., 2009; Zhang et al., 2009) that are similar to those found in FTLD-U and ALS brains. In a very recent study carried out by Wang and coll. (2010), it was demonstrated that UP system and autophagy degrade both TDP-43 and its pathological fragment TDP-25. In particular, it was found that TDP-25, more than TDP-43, is greatly increased in cells treated with autophagy inhibitors, and it decreases in cells treated with an autophagy enhancer (Wang et al., 2010), suggesting that autophagy is primarily involved in clearing the pathological forms of TDP-43. In agreement with this, TDP-25 was found to colocalize with punctuated LC3 in transfected cells (Wang et al., 2010), thus suggesting that TDP-25 is more specifically regulated by autophagy.

Recent report found that mutant TDP-43, when overexpressed in mice, causes neurodegeneration in the absence of cytosolic aggregation (Wegorzewska et al., 2009). Analogously, overexpression of wild-type TDP-43 in *Drosophila* motor neurons is sufficient to produce cell-autonomous toxicity in the absence of ALS-associated mutations (Hanson et al., 2010). These findings support the hypothesis of the intrinsic toxicity of TDP-43. In particular, given that the majority of TDP-43 is found in the nucleus, where the protein plays its physiological role, the Authors attribute the intrinsic toxicity of wild type TDP-43 to its capacity to interfere with gene expression and/or mRNA splicing within the cell nucleus (Hanson et al., 2010). A recent finding (Cushman et al., 2010) demonstrate that TDP-43 as well as FUS, possesses a protein domain with the structure of a prion protein (see Fornai et al 2011, chapter 15, this issue). Interestingly such a domain is characterized by the repeats of asparagines which is a powerful autophagy inhibitor.

ESCRT mutations

Recently, the ESCRT-III subunit CHMP2B (charged multivesicular body protein 2B)/Vps2B was found to be mutated in a large Danish pedigree with frontotemporal dementia (FTD) (Skibinski et al., 2005), and in patients with ALS (Parkinson et al., 2006). Cell pathology demonstrates accumulation of ubiquitin-positive protein deposits that are also positive for p62/Sequestosome-1, a common com-

ponent of protein inclusions associated with neurodegenerative disease (Talbot and Ansorge, 2006). Filimonenko and coll. (2007) have demonstrated that autophagic degradation is inhibited in cells depleted of ESCRT subunits as well as in cells overexpressing CHMP2B mutants. In these cells they studied the dynamics of the intracellular vacuoles by following the autophagy-related protein LC3 II stained with the green fluorescent protein (GFP). As usual, they found that autophagy impairment was concomitant with an increase in LC3 II-GFP. Moreover, they demonstrated that once the LC3 II-positive autophagosome is formed, the subsequent fusion of the endosome/MVB to produce an amphisome does not occur and production of autophagolysosome is impeded. Inhibition of autophagy by impairment of the ESCRT pathway leads to accumulation of protein aggregates containing ubiquitinated proteins and p62, which is involved in the recruitment of mono-ubiquitinated substrates for autophagy degradation (Filimonenko et al., 2007). These protein aggregates in ESCRT-depleted cells also contain TDP-43, recently identified as the major ubiquitinated protein in FTLD-U and ALS.

PI(3,5)P2 signalling mutations

In humans mutations in the phospfoinisitol-3,5biphosphate (PI-3,5P_2) regulatory complex are associated with ALS (Chow et al., 2009). This mutation affects the conversion of the phosphatidylinositide PI-3P into the signalling lipid PI-3,5P_2 and leads also to Charcot-Marie-Tooth Type 4J disease (Chow et al., 2007). In mouse models, this mutation causes motor neuron loss that begins in fetal life and leads to death in 6 weeks after birth (Zhang et al., 2007; Jin et al., 2008; Chow et al., 2009). This is characterized by accumulation of swollen intracellular vacuoles (Chow et al., 2007; Jin et al., 2008).

The predominant effect of deficient PI-3,5P_2 is to block the progression of autophagy. This view is supported by the recent observation that inhibition of the PI-3P kinase FAB1 (also known as PI-P5K3) in cultured cells results in accumulation of LC3 II (de Lartigue et al., 2009).

Definitive understanding of the role of PI-3,5P_2 in the autophagy pathway is not yet accomplished. Current models suggest that PI-3,5P_2 is synthesized within endosomes and lysosomes in response to specific cargoes (Dove et al., 2009). Membrane-bound PI-3,5P_2 is thought to interact with membrane proteins, and to recruit cytosolic proteins required for trafficking. Through these interactions, synthesis of PI-3,5P_2 directs trafficking of vesicles along the endosome-lysosome axis and promotes recycling of membranes to the trans-Golgi network (Rutherford et al., 2006).

In particular, two models for the specific role of PI-3,5P_2 in autophagy have been proposed (Feguson et al., 2009). In the first model, PI-3,5P_2 belongs to the membranes of autophagy vacuoles, such as autophagosomes, late endosomes and/or lysosomes, where it serves as a molecular signal for vesicle fusion, leading to mature autophagolysosomes. Deficiency of PI-3,5P_2 on these membranes could slow or prevent vesicle fusion events leading to accumulation of vesicles containing p62. Alternatively, PI-3,5P_2 serves for recycling the autolysosome membranes after fusion of the amphisome with the lysosome. In this model, autophagy intermediates could accumulate due to the deficiency of membrane components required for continued generation and maturation of autophagosomes. The second model is supported by the co-localization of p62, a marker of the autophagosme, with LAMP-2, which specifically labels the lysosome, which implies that vesicle fusion has occurred.

Ferguson and coll. (2009) found in two independent mouse mutants, deficient in PI-3,5P_2, elevated brain levels of the autophagy markers LC3-II and p62, which localize within neurons and astrocytes, associated with extended neurodegeneration. In particular, in these mutant brains p62, which typically accumulates in neurodegenerative disease including ALS (Ross and Poirier, 2004), is located within insoluble inclusion bodies that also contain ubiquitinated proteins.

UPR and sporadic ALS

What is emerging is that ER stress and induction of the UPR occur in sporadic ALS, thus placing ER stress centrally in the pathogenesis of a wide spectrum of motor neuron diseases. The UPR is induced at symptom onset and disease end stage in rodents models of familial ALS expressing mutated SOD1 (Kikuchi et al., 2006). Up-regulation of UPR prior to the onset of symptoms in mutant SOD1 rodents implies an active role in the disease. Atkin and coll. (2008) showed that full UPR, including

induction of stress sensor kinases, chaperones and apoptotic mediators, is also present in the spinal cords of patients with sporadic ALS. Furthermore, the UPR chaperone protein disulphide isomerase (PDI) was present in the cerebrospinal fluid and was aggregated and widely distributed throughout motor neurons of these patients (Atkin et al., 2008).

Ilieva et al. (2007) showed both increased phosphorylation of eIF2alpha and increased expression of ER chaperones (PDI and KDEL-containing proteins) in the spinal cord of patients with sporadic ALS. This strongly supports the participation of ER stress in ALS pathogenesis. Among the potential causes of such ER stress the Authors proposed intense oxidative stress, responsible for protein oxidative damage, ALS-specific changes in fatty acid concentrations, and mitochondrial dysfunction leading to increased free radical production (Ilieva et al., 2007).

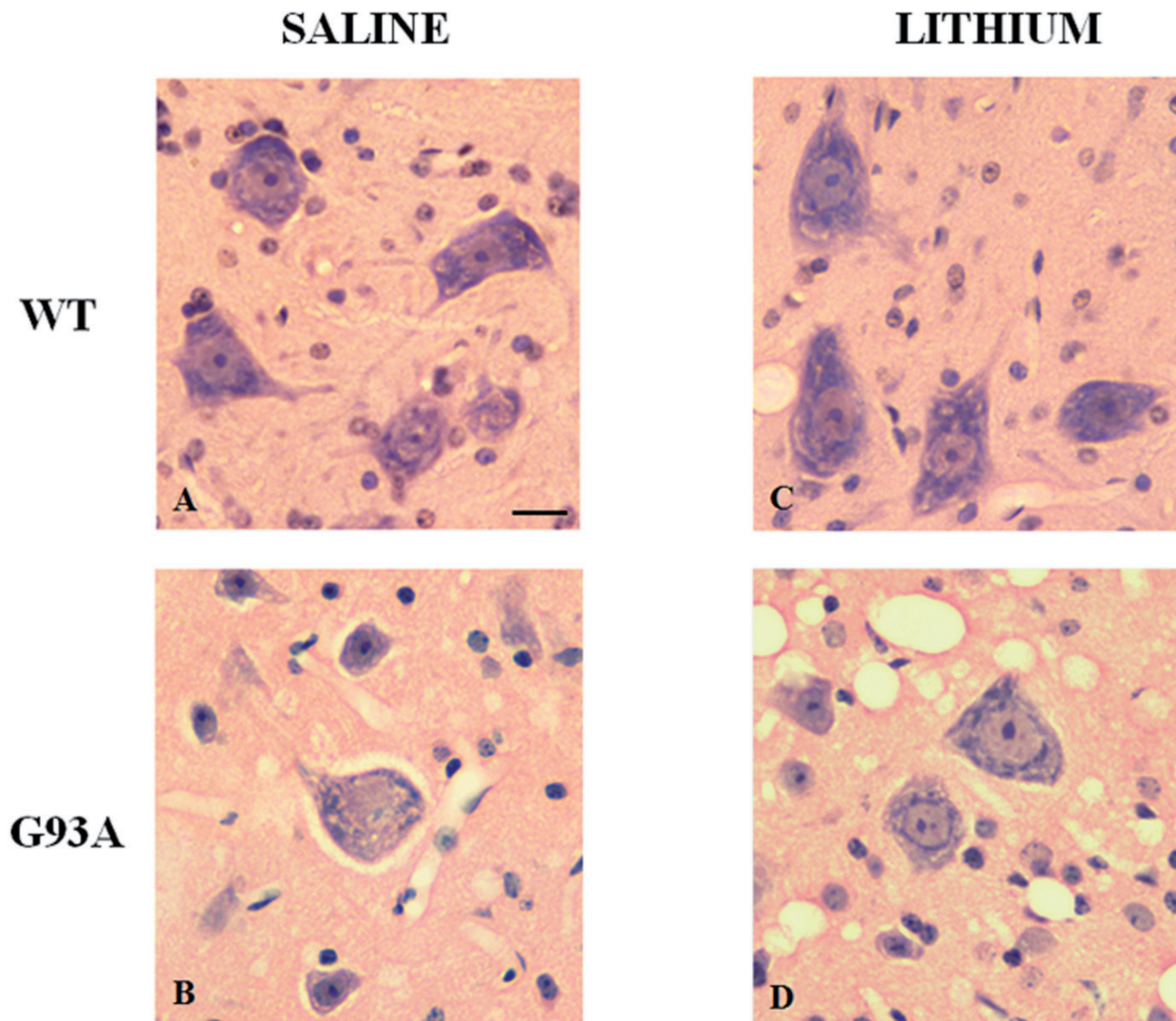

Fig. 1. - Effect of autophagy induction on morphological features of motor neurons from G93A mice. a) Representative image of spinal motor neurons from a saline-treated wt mouse stained with H&E shows euchromatic nucleus, evident nucleolus, and intensely basophilic cytoplasm, due to the abundance of ribosomes bound to endoplasmic reticulum. b) In saline-treated G93A mice, motor neurons exhibit severe morphological alterations, consisting of increased size, loss of basophilic reaction in the cytosol, intracellular vacuolization and nuclear alterations. d) Autophagy induction preserves the morphology of survival spinal motor neurons in G93A transgenic mice at the end of the disease. This effect was obtained by daily administration of lithium carbonate, 1 mEq/kg i.p., starting at 75 days of age (for details, see Fornai et al., 2008). c) No effects of lithium treatment were evident in wt mice. Scale bar = 22 μm.

Autophagy induction and ALS

In recent studies Fornai et al. (2008a,b) enhanced the autophagy pathway by administering either lithium or rapamycin (for the pharmacology of these effects see Pasquali et al., 2009a). Following treatment of motor neuron primary cultures with these drugs, all the autophagy markers appeared upregulated and a recovery in the cell pathology was observed: large autophagosomes occurring in ALS motor neurons were replaced by newly formed, small MVB stained with LC3 II.

In vivo treatment led to the same results: damaged mitochondria in motor neurons of G93A mice given saline were cleared in the motor neurons of ALS mice treated with lithium. In particular, motor neurons that appear severely damaged in the saline-treated G93A mice featuring increased size and packed chromatin in the nucleus, show normal size and normal ultrastructure in the ALS mouse treated with an autophagy enhancer (Fig. 1).

Primary cultures of motor neurons from G93A and wild type mice did not show differences in spontaneous cell death in baseline conditions. However, inhibition of autophagy by 3-methyladenine (3-MA) precipitated cell death only in primary motor neurons from G93A mice. This effect was reverted by the autophagy inductor rapamycin (Fornai et

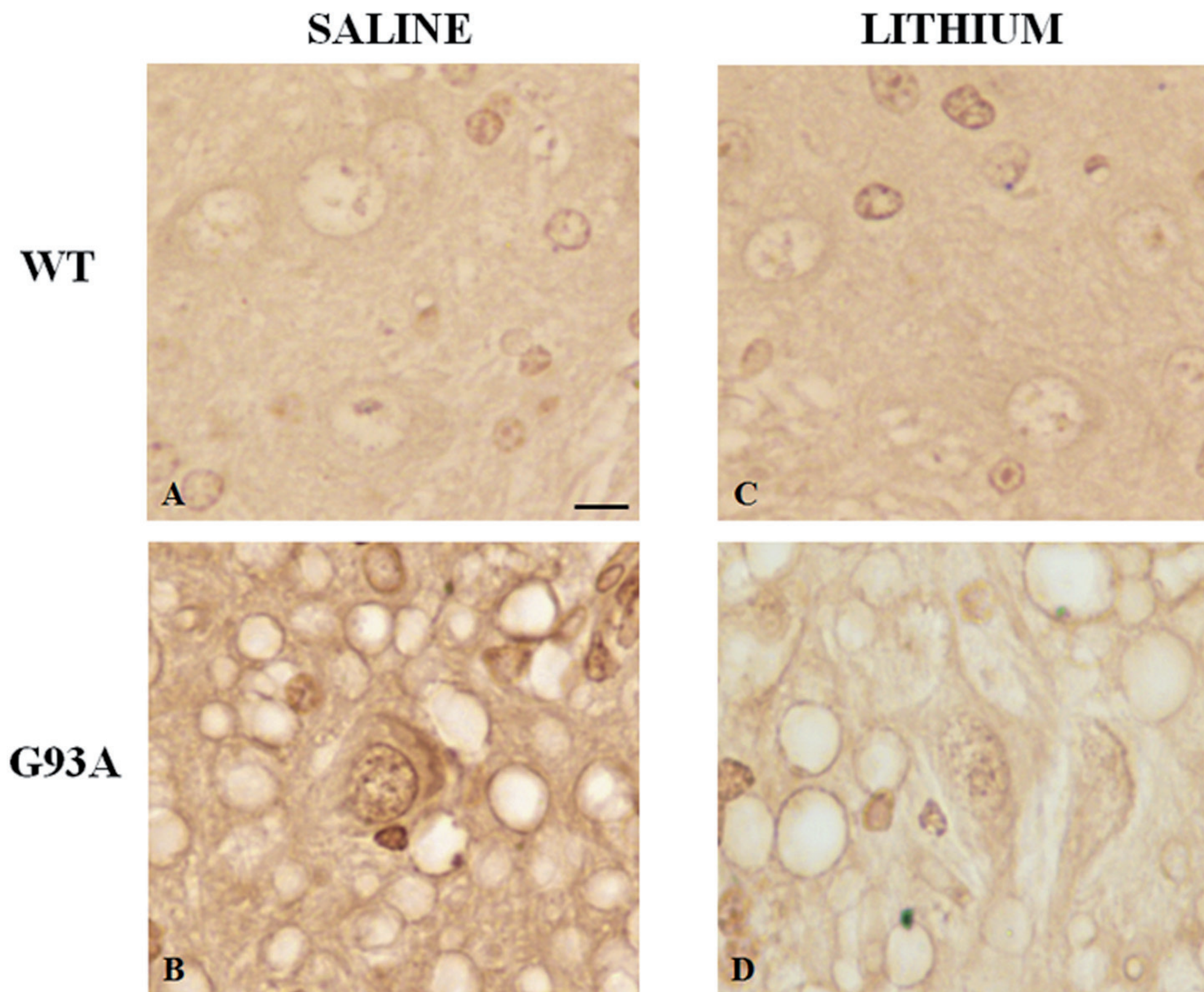

Fig. 2. - Effect of autophagy induction on SOD1 immunopositivity in the ipoglossal nucleus of G93A mice. Deposits of SOD1 immunopositivity within the cytosol of brainstem motor neurons belonging to the ipoglossal nucleus (12 N) of a G93A mice (b), and their reduction in a G93A mouse which received chronic lithium (1 mEq/kg/day i.p., d; Fornai et al., 2008). SOD1 immunostaining in saline- (a) and lithium- (c) treated mice is also shown. Scale bar = 12 μm.

al., 2008a,b). Similarly to what observed in other neurodegenerative disease, these *in vivo* and *in vitro* data demonstrate that autophagy is beneficial role for G93A motor neurons. In line with this, we recently found that following administration of the autophagy blocker asparagine to G93A mice ALS symptoms are precipitated and occur much earlier than in the ALS G93A mutation (unpublished data). The beneficial role of autophagy in the ALS G93A mutation is further confirmed by the data of Kabuta et al. (2006). These Authors found that autophagy is neuroprotective against excitotoxicity and promotes clearance of mutant SOD1, as confirmed also by a very recent study (Crippa et al., 2010). Induction of autophagy in G93A mice provokes a significant clearance of SOD1 aggregates from motor neurons in the spinal cord (Fornai et al., 2008a) and brainstem motor nuclei (Ferrucci et al., 2010; Fig. 2). Moreover, activation of autophagy promotes the clearance of protein aggregates classically described within diseased motor neurons in most forms of familial and sporadic ALS (Fig. 3). In particular, promoting autophagy enhanced the clearance of alpha-synuclein (Fornai et al., 2008a; Fig. 4) which accumulates in the spinal cord of sporadic ALS and produces motor neuron death when it is mutated (Martin et al., 2006).

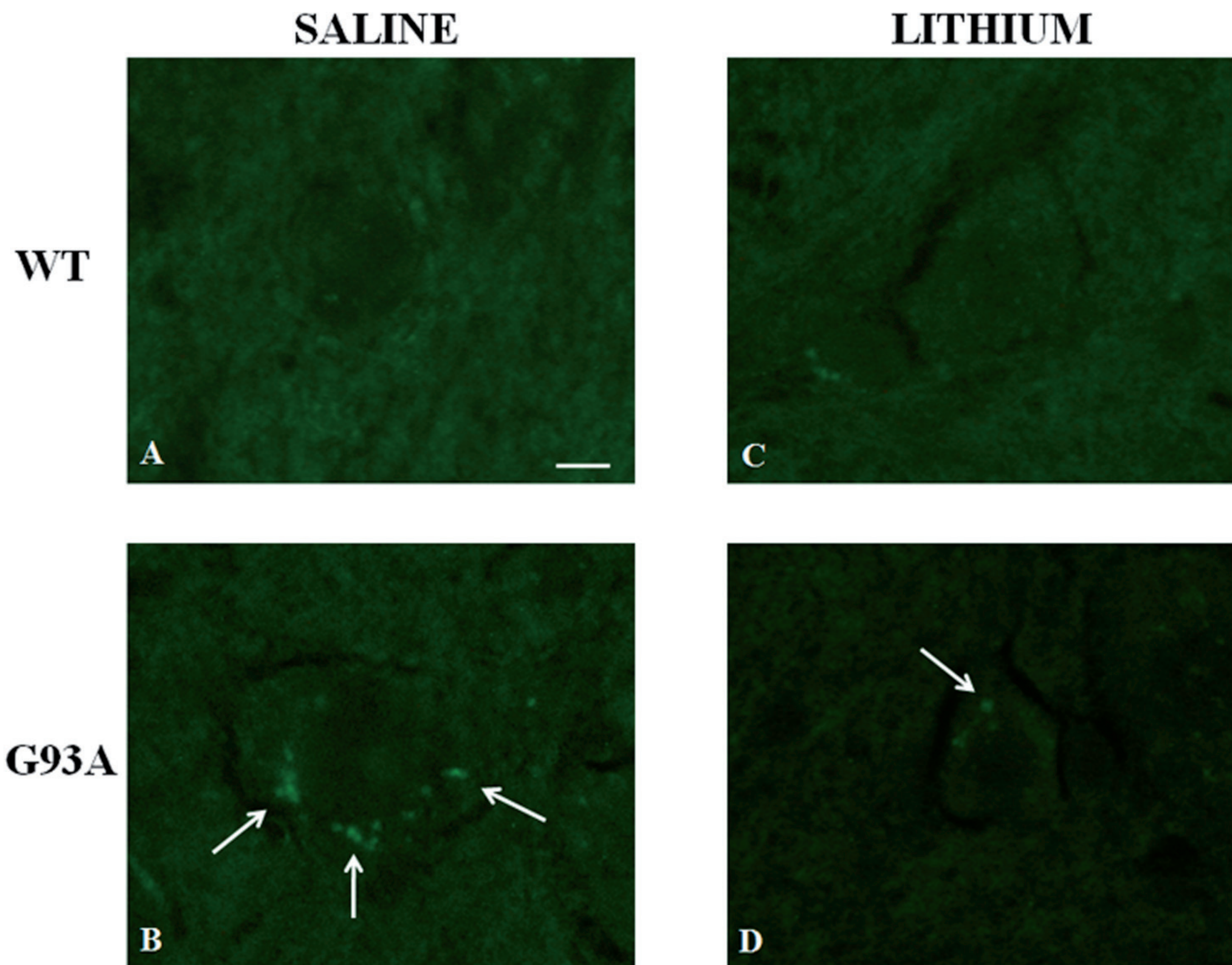

Fig. 3. - Effect of autophagy induction on cytosolic vacuoles in G93A motor neurons. Monodansylcadaverine-positive vesicles featuring large vacuolar structures are evident in G93A motor neurons (b, arrows). Induction of autophagy by chronic lithium (1 mEq/kg/day i.p., Fornai et al., 2008) promotes the clearance of these structures, as attested by reduced amount of vacuoles (arrow) occurring in motor neurons of lithium-treated G93A mice (d). Motor neurons of saline- (a) and lithium-(c) treated wt mice are devoid of these cytosolic vacuoles. Scale bar = 12 μm.

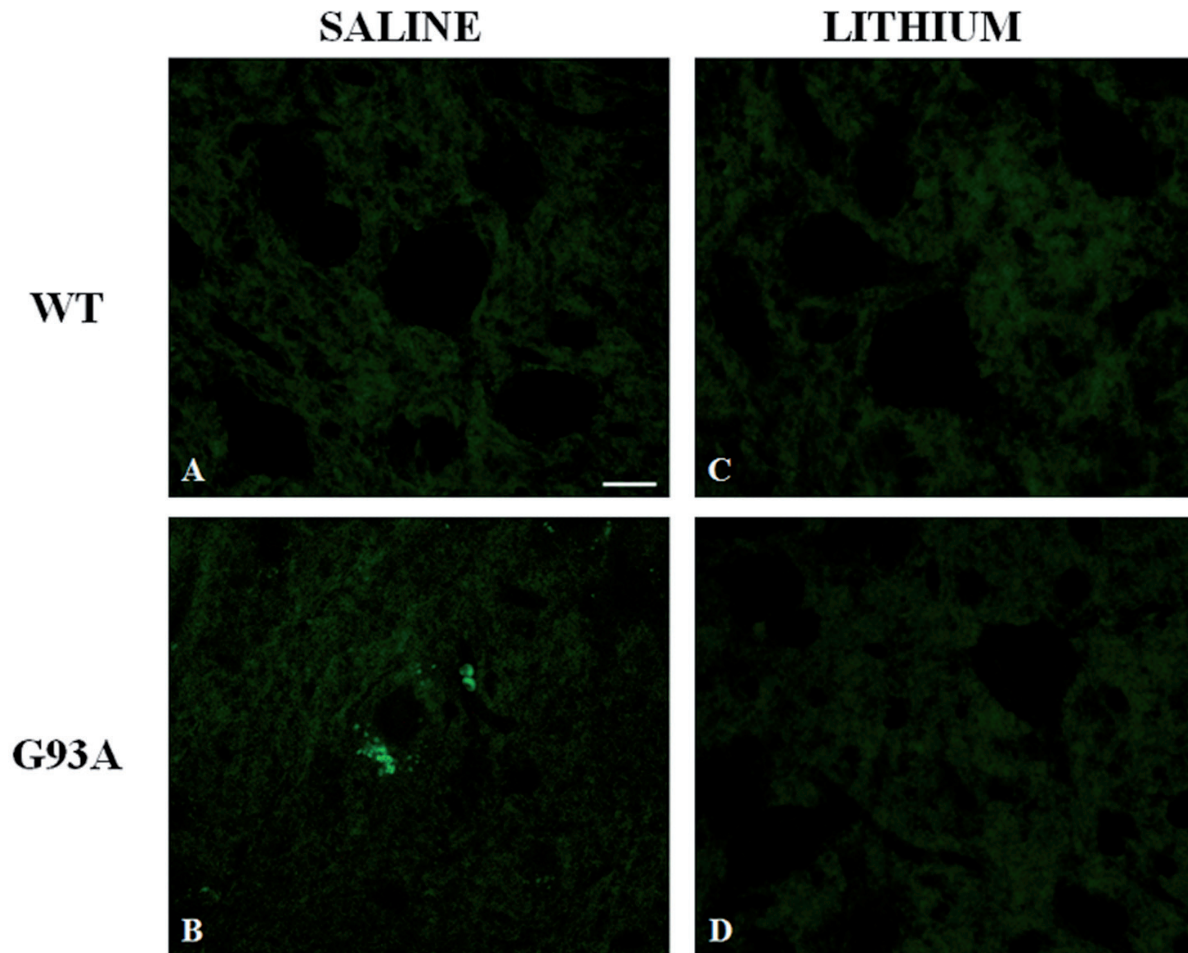

Fig. 4. - Effect of autophagy induction on alpha-synuclein immunofluorescence in G93A motor neurons. Faintly diffused apha-synuclein immunofluorescence in motor neurons from a saline-treated wt mouse (a), features as intensely positive "puncta" in G93A mice administered with saline (b). (d) Induction of autophagy with lithium salts (1 mEq/kg/day i.p., Fornai et al., 2008) eliminates the immunofluorescent aggregates of alpha-synuclein, probably by enhancing the clearance of alpha-synuclein cytosolic deposits. Effect of lithium on alpha-synuclein immunopositivity in wt motor neuron is also shown (c). Scale bar = 16.7 μm.

Autophagy also counteracts ubiquitin aggregates in the SOD1 mouse model and in other forms of ALS (see later Filimonenko et al., 2007). All these proteins, i.e. SOD1 (Rosen et al., 1993; Shibata et al., 1994), alpha-synuclein (Doherty et al., 2004), and ubiquitin (Leigh et al., 1991) represent substrates for the autophagy pathway (Fengsrud et al., 1995; Ferrucci et al., 2008; Vogiatzi et al., 2008 for SOD1, ubiquitin, and alpha-synuclein, respectively) and they accumulate in both familial ALS and sporadic ALS. Such findings confirm the presence of a defective autophagy pathway. Moreover, mitochondrial damage, which represents the primary defect in these G93A mice, appears to be completely reverted by autophagy activation. In fact, autophagy promotes sequestration of mitochondria (mitophagy). Thus, large and vacuolated mitochondria visible in motor neurons of G93A mice were rescued by autophagy activation, which generates high numbers of small and well-structured newly formed mitochondria.

Concluding remarks

Increasing evidence shows that enhanced autophagy counteracts the pathophysiology of ALS. At this regard, ubiquitin-positive aggregates and mutant SOD1 detergent insolubility were significantly

decreased in the mouse motor neuron-like NSC-34 cell line transiently transfected with the human SOD1G93A, in the presence of millimolar concentrations of trehalose, possibly due to its capacity to induce autophagy or to its properties as chemical chaperone (Gomes et al., 2010).

On the other hand, since the multiple biological effects of lithium, neuroprotection observed after lithium treatment in animal models of ALS by several Authors was considered to be a consequence of different lithium-mediated effects, other than induction of autophagy (Shin et al., 2007; Feng et al., 2008). This is the case, for example, of the dramatic effect produced by lithium treatment on mitochondria of G93A mice. In this mouse model big and damaged mitochondria in non-treated ALS motor neurons appeared to be substituted with smaller and well-structured mitochondria in lithium-treated ALS motor neurons (Fornai et al., 2008a,b). This phenomenon, which cannot be explained by mere fission, calls for the occurrence of mitochondriogenesis, which is produced by lithium also in endothelial cell cultures (Struewing et al., 2007). Cytofluorimetry and reverse-transcriptase polymerase chain reaction (RT-PCR) for mitochondrial DNA and RNA confirmed that lithium is able to produce the biogenesis of mitochondria within motor neurons (Fornai et al., 2008a,b).

Summary

The protein clearing systems play a key role in neurodegeneration. In particular, autophagy represents the main pathway which through the clearance of the altered cell components removes the potentially toxic material thus counteracting cell death. There is now a solid evidence that links directly a defective autophagy to ALS. This was firstly demonstrated by Fornai et al (2008a,b) and firmly confirmed by other subsequent papers (Laird et al., 2008; Hetz et al., 2009). Motor neuron degeneration in familial forms of ALS is triggered by the impairment of specific proteins belonging to protein clearing systems, while promoting autophagy improves motor neuron loss (Fornai et al., 2008a,b; Hetz et al., 2009). Despite further studies are needed to clear the actual role of autophagy in motor neuron degeneration, taken together, the reported data indicate a neuroprotective role of autophagy in ALS and help to explain a variety of features involved in motor neuron degeneration.

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