Excitotoxicity and genetics of amyotrophic lateral sclerosis: effects of intracellular calcium accumulation on proteins encoded by the major genes underlying the disease

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Abstract

The aetiology of Amyotrophic Lateral Sclerosis (ALS), a fatal and incurable disease caused by motor neuron degeneration, is still poorly understood. The discovery of genetic forms of ALS helped to shed light on the mechanisms underlying this pathology, but also showed how complex these mechanisms are. Excitotoxicity is one of the processes strongly suspected to play a role in motor neuron degeneration in ALS. This process consists in neuron damage due to excessive intake of calcium ions (Ca^{2+}) by the cell. This study aims to find a relationship between the proteins coded by the most relevant genes associated with ALS and excitotoxicity. In detail, the profile of eight proteins (TDP-43, C9ORF72, p62/SQSTM1, matrin3, VCP, FUS, SOD1 and profilin-1), was analysed in three different cell types induced to raise their cytoplasmic amount of Ca^{2+}.

Intracellular Ca^{2+} accumulation causes a significant decrease in the levels of TDP-43, C9ORF72, matrin3, VCP, FUS, SOD1 and profilin-1 and an increase in p62/SQSTM1. These events are associated to the proteolytic action of two proteases, calpains and caspases, as well as to the activation of autophagy, a process responsible for the degradation and recycling of cytoplasmic components. Interestingly, Ca^{2+} appears to both favour and hinder autophagy. The discovery of when Ca^{2+} levels become toxic for the cell, as well as understanding why the physiological processes of calpain proteolysis and autophagy become pathological, may elucidate the mechanisms responsible for ALS and help discover new therapeutic targets.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive and fatal disease leading to a rapid degeneration of motor neurons in brain cortex, brain stem and spinal cord, with onset usually in late middle age. Currently, there are neither reliable biomarkers nor effective pharmacological treatments for the disease and its pathogenesis is still poorly understood. The discovery of genetic aetiology in some ALS patients helped to shed light on motor neurons degeneration. However, ALS cases linked to genetic mutations are less than 10–15% and these mutations affect more than 30 genes encoding for proteins that have disparate functions [1, 2]. In fact, some of these proteins act by binding DNA and/or RNA [TAR DNA binding protein-43 (TDP-43), fused in sarcoma/translocated in liposarcoma (FUS), matrin-3 [3–5]]; some retain enzymatic activity [Cu/Zn superoxide dismutase 1 (SOD1), and valosin-containing protein (VCP) [6, 7]]; others are implicated in protein degradation (p62/sequestosome-1) [8], contribute to the formation of cytoskeleton (profilin-1) [9] or regulate intracellular trafficking pathways [chromosome 9 open reading frame 72 (C9ORF72)] [10].

A lot of evidence supports the hypothesis that excitotoxicity is one of the toxic conditions at the heart of motor neuron degeneration in ALS [11, 12]. The excitotoxic process consists in neural cell damage caused by an abnormal intake of calcium ions (Ca^{2+}) due to the hyperactivation of ionotropic glutamate receptors [13]. This Ca^{2+} overload may contribute to necrotic or apoptotic cell death through

mitochondrial dysfunctions, aberrant production of reactive oxygen species and/or endoplasmic reticulum stress [14–16].

The aim of this study is to analyse the effects of excitotoxicity on proteins linked to ALS. In particular, this study assesses the consequences of intracellular Ca^{2+} overload on the proteins encoded by the most relevant genes associated with ALS, in order to find metabolic processes common to all or most of these proteins. For this purpose, the protein profile of TDP-43, FUS, matrin-3, SOD1, VCP, p62/sequestosome, profilin-1 and C9ORF72, the codifying genes accounting for the majority of ALS genetic forms [1, 2], will be evaluated in three different cell types induced to accumulate Ca^{2+} in their cytoplasm. Accumulation will be reached by inducing an excessive ion intake or by altering ion flux between intracellular storage structures and cytoplasm.

Materials And Methods

Reagents

TDP-43 polyclonal antibody (10782-2-AP) was purchased from ProteinTech Group (Chicago, IL, USA). Matrin-3 (A300-591A), FUS (A300-293A) polyclonal antibodies were from Bethyl Laboratories (Montgomery, TX, USA). C9ORF72 (sc-138763), VCP (sc-20799), SOD1 (sc-11407), PARP (sc-7150) polyclonal antibodies and GAPDH (sc-47724), PFN-1 (sc-137235) monoclonal antibodies, as well as ionomycin calcium salt (sc-3592), thapsigargin (sc-24017) and calpeptin (sc-202516) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-mouse IgG (7076P2) and anti-rabbit IgG (7074P2) antibodies conjugated to horseradish peroxidase (HRP) were from Cell Signaling Technology (Leiden, The Netherlands). The active human recombinant caspase set IV (K233-10- 25) was purchased from BioVision (Milpitas, CA, USA). Active calpain-1 (208712) and calpain-2 (208718) were from Calbiochem (La Jolla, CA, USA). MINI-PROTEAN TGX 4–15% precast gel (4561086SP5), TBT RTA Transfer Kit PVDF mini (1704272), Protein molecular markers (SM0671) and the RC DC Protein Assay Kit (500 – 0119) were purchased from Bio-Rad Laboratories (Milan, Italy). The WesternBright Enhanced chemiluminescent substrate (ECL) for HRP (K-12045-D50) was purchased from Advansta (Menlo Park, CA, USA). Nitrocellulose membranes (RPN303D) were purchased from Amersham (Milan, Italy). Lymphoprep® (1114545) was purchased from Axis-Shield (Oslo, Norway). p62 (P0067) and LC3B (L7543) polyclonal antibodies, chloroquine (C6628-25G) as well as high grade versions of all other chemicals and cell media used in this study were purchased from Sigma-Aldrich (Milan, Italy).

Cell culture

Human neuroblastoma SK-N-BE(2) adhesion cell lines were cultured in RPMI 1640 supplemented with 10% foetal bovine serum (FBS), sodium pyruvate (1×) and an antibiotic cocktail (1×) at 37°C and 5% CO_2. Human carcinoma of the uterine cervix (HeLa) cell lines were cultured in High glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and an antibiotic cocktail (1×). Peripheral blood mononuclear cells (PBMC) from a healthy 35-year old donor (who signed a written informed

consent) were collected in EDTA-coated tubes as previously described [17] and then incubated in RPMI 1640 supplemented with 10% FBS and an antibiotic cocktail (1×). These three cell types were incubated in multiwell plates at 37°C and 5% CO_2.

Treatment of SK-N-BE(2) lysate with calpains

SK-N-BE(2) cells ($1x10^6$ cells for each sample) were lysed by repeated passage through a 26-G syringe in a reaction solution composed of 50 mM Tris-HCl pH 7.5, 30 mM NaCl, 5 mM DTT and 1 mM calcium chloride and then separately incubated with 2 U of active human calpain-1 or -2 for 10 min or 180 min at 37°C. Furthermore, two samples were treated for 180 min with both calpain-1 or -2 and 20 mM calpeptin, a calpain inhibitor. The cleavage reaction was stopped by adding a buffer constituted by 50 mM Tris pH 6.8, 5% (w/v) SDS, 8 M deionized urea, and 2% (v/v) 2-mercaptoethanol and 10 mM EDTA. Samples were then frozen at − 70°C.

Treatment of SK-N-BE(2) lysate with caspases

SK-N-BE(2) cells ($1x10^6$ cells for each sample) were lysed by repeated passage through a 26-G syringe in the reaction solution recommended by the manufacturer (50 mM HEPES pH 7.2, 50 mM NaCl, 0.1% CHAPS, 10 mM EDTA, 5% glycerol and 10 mM DTT) and then separately incubated with 2 U of active human caspase-3, -6, -7 or -8 for 180 min at 37°C. The cleavage reaction was terminated by adding a buffer constituted by 50 mM Tris pH 6.8, 5% (w/v) SDS, 8 M deionized urea, and 2% (v/v) 2-mercaptoethanol. Samples were then frozen at − 70°C.

Cell treatments

SK-N-BE(2), HeLa cells and PBMC ($1x10^5$ cells for each sample) were treated with ionomycin (1 μM or 5 μM) or thapsigargin (1 μM or 5 μM) for 24 h. Time-dependent experiments were carried out by incubating SK-N-BE(2) with 1 μM ionomycin or 1 μM thapsigargin for 2 h, 8 h and 24 h. Two-hour treatments of SK-N-BE(2) with 1 μM ionomycin or 1 μM thapsigargin were also performed following 2h preincubation with 100 μM chloroquine.

Western immunoblot analysis

Samples were subjected to SDS-PAGE using 4–15% precast gels as previously described [17]. Resolved proteins were then electro-transferred onto nitrocellulose membrane by using the Trans-Blot Turbo Blotting System (Bio-Rad) with the transfer buffer included in the TBT RTA Transfer Kit nitro mini supplemented with 20% (v/v) ethanol. Membranes were blocked with 2% bovine serum albumin (BSA) in a TBST buffer consisting of 0.02 M Tris–HCl pH 7.6, 0.14 M NaCl, and 0.02% (v/v) Tween 20. Membranes were then exposed to different antibodies in in TBST buffer with 5% BSA. Next, membranes were washed with TBST buffer, incubated with 15 ng/ml of appropriate HRP-conjugated secondary antibodies at 4°C, washed again and then exposed to the enhanced chemiluminescence HRP substrate. The immunostained bands were visualized using a C-DiGit® Blot Scanner gel imaging system and Image Studio™ software ver 5.0 (LI-COR, Bad Homburg, Germany). When longer exposures were required, bands were detected using Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, UK).

Statistical analysis

Statistical analyses were performed using SPSS software ver. 17.0 (IBM, Armonk, NY, USA). Data were analysed using the Student's t test. Significant differences were set at $p < 0.05$.

Results

Protein cleavage by calpains and caspases

Firstly, we investigated whether the proteins linked to ALS considered in this study are substrate of two of the major classes of proteases activated by intracellular Ca^{2+} overload, i.e. calpains and caspases [18, 19]. To this aim, whole lysates of SK-N-BE(2) cells were treated with active recombinant human calpains-1 and − 2 or active recombinant human caspases-3, -6, -7 and − 8.

TDP-43

Western immunoblot analysis showed that TDP-43 is cleaved by calpains-1 and − 2 (Fig. 1, Table 1). In fact, in lysates treated with calpains, a marked decrease in the full-length protein was accompanied by the formation of at least four fragments with a molecular mass of about 36, 32, 25 and 18 kDa. These results are in keeping with previous observations in which TDP-43 calpain products of 36, 32, 25 kDa are referred to correspond to the amino acid sequences 1-324, 1-286, 1-243, respectively [20].

Table 1
Calpains and caspases involved in the proteolysis of proteins codified by genes linked to ALS

	calpain-1*	calpain-2*	caspase-3	caspase-6	caspase-7	caspase-8
TDP-43	++++	+++	+++	++++	++++	+++
C9ORF72	++++	++++	+	++	++	+++
p62	++++	++++	++	++++	++	++++
MATR3	++++	++++	++	++++	++++	++++
VCP	+++	+	–	++	–	++
FUS	++++	++++	++	+++	+++	+++
SOD1	–	–	–	–	–	–
PFN1	–	–	–	–	–	–
* = at 10 minutes of treatment						
– = no decrease in full-length protein (≤ 5%)						
+ = 5%< decrease in full-length protein ≤ 10% or < 5% decrease in full-length protein with visible cleavage products						
++ = 10%< decrease in full-length protein ≤ 50%						
+++ = 50%< decrease in full-length protein ≤ 90%						
++++ = decrease in full-length protein > 90%						

As previously observed in studies performed by us [21] and others [22–26], TDP-43 is a substrate of caspases-3, -6, -7 and − 8 (Fig. 2, Table 1). The most important fragments generated by caspase cleavage have a molecular mass of 35 and 25 kDa. The first one corresponds to the amino acid sequence 90–414 at the C-terminus of the protein; the second one should be the 220–414 proteolytic product described by Zhang et al [26] or the 170–414 fragment reported by us and others [21, 27, 28].

C9ORF72

The C9ORF72 antibody used in our study recognized a band of approximately 55 kDa, consistent with the predicted molecular mass of the protein. After 10 minutes of treatment with calpains-1 or -2, this band completely disappeared and, in parallel, two proteolytic products at ≈ 45 and 25 kDa emerged (Fig. 1, Table 1).

Treatment of the cell lysate with caspases-3, -6 or -7 caused a decrease in the amount of full-length protein of less than 50%. The decrement in the full-length protein after treatment with caspase-8 was of slightly more than 50% (Fig. 2, Table 1).

p62/sequestosome-1

p62 was already completely degraded at 10 minutes of treatment with calpains-1 or -2 (Fig. 1, Table 1). The protein was also a good substrate for caspases-6 and − 8 and, to a lesser extent, for caspases-3 and − 7 (Fig. 2, Table 1).

All these findings are in agreement with a previous study [29].

Matrin-3

Calpains-1 and − 2 had already fully degraded matrin-3 at 10 minutes of treatment (Fig. 1, Table 1). The matrin-3 antibody barely identified some proteolytic fragments, of which the most visible had a molecular mass of ≈ 70, 25 and 15 kDa (Fig. 1).

Matrin-3 was also a substrate for caspases-3, -6, -7 and − 8. In fact, treatment with the three latter caspases determined an almost complete loss of the full-length protein (Fig. 2, Table 1). This loss was associated with the formation of a fragment at about 20 kDa that was detectable also following exposure to caspase-3 (Fig. 2). These results overlap with those reported by Valencia and colleagues [30]. According to their observations, the ≈ 20 kDa fragment observed by us is consistent with the 681–847 C-terminal fragment generated by caspase cleavage at the consensus site $DETD^{680}$.

VCP

VCP was subjected to degradation by both calpains, although cleavage appeared to be slower by calpain-2 than by calpain-1. Interestingly, treatment with calpain-2 at 10 minutes did not determine any appreciable decrease in the full-length protein, but gave rise to an evident proteolytic band at ≈ 50 kDa (Fig. 1, Table 1). It is conceivable that this proteolytic fragment is better recognized than the full-length protein by the antibody used.

Only caspases-6 and − 8 were seen to cleave VCP, as they caused a slight decrease in the full-length protein paralleled by the generation of a proteolytic fragment at about 70 kDa (Fig. 2, Table 1). These findings are in agreement with a previous paper which reported that the cleavage by the two active caspases occurs at the consensus site $VAPD^{179}$ [31].

FUS

Full-length FUS was completely degraded at 10 minutes of incubation with either calpains-1 or -2 (Fig. 1, Table 1). The protein degradation was accompanied by the formation of various proteolytic products with a molecular mass ranging from few kDa less than the full-length protein to ≈ 20 kDa. The most evident of these fragments had a molecular mass of ≈ 40 kDa (Fig. 1).

Exposure to caspases-3, -6, -7 and − 8 was followed by a decrease in the full-length protein (Fig. 2, Table 1). However, identification of the caspase-proteolytic products was difficult with the FUS antibody used (Fig. 2).

SOD1

Neither the calpains nor the caspases here considered induced an appreciable decrease in SOD1 (Figs. 1, 2, Table 1).

Profilin-1

Neither the calpains nor the caspases here considered induced an appreciable decrease in profilin-1 (Figs. 1, 2, Table 1).

Evaluation of protein profiles in cells treated with ionomycin or thapsigargin

The profile of the proteins considered in the study was then evaluated in cells induced to accumulate Ca^{2+} in their cytoplasm. To this aim, three different cell types, SK-N-BE(2), HeLa and PBMC, were treated with ionomycin or thapsigargin. Ionomycin is a Ca^{2+} ionophore that triggers intracellular Ca^{2+} overload through an excessive ion intake from the extracellular environment [32]. Thapsigargin is a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibitor that raises the concentration of cytosolic Ca^{2+} by blocking the ion re-uptake in the intracellular storage structures [33].

TDP-43

Exposure of SK-N-BE(2) cells to ionomycin gave rise to the formation of fragments with a molecular mass of ≈ 36, 32 and 25 kDa (Fig. 3), which are similar to those observed *in vitro* following treatment with calpains (see above). The decrease in the full-length protein amounted to about 35% and 45% for treatment with 1 μM and 5 μM, respectively. Incubation with thapsigargin was followed by the formation of the same 35 and 25 kDa fragments detected *in vitro* after treatment with caspases. Additionally, the 32 kDa band typical of *in vitro* calpain treatment was appreciable, although barely, through 1 μM thapsigargin exposure (Fig. 3). The decrease in the full-length protein was of 30% and 75% after treatment with 1 μM and 5 μM, respectively.

In HeLa cells, incubation with the two ionomycin concentrations mentioned above did not cause an appreciable decrease in the full-length protein, nor did it trigger the formation of the calpain-dependent fragments. Instead, the effects of incubation with thapsigargin at the two previously mentioned concentrations fully overlapped with the ones observed in SK-N-BE(2) cells (Figs. 3, 5).

In PBMC, the effects of the treatment with ionomycin were the same as the ones obtained in SK-N-BE(2) cells. However, the treatment of PBMC with thapsigargin triggered the formation of calpain-dependent fragments, instead of the caspase proteolytic products observed in SK-N-BE(2) cells (Figs. 3, 5). Thus, in PBMC treatments with both ionomycin and thapsigargin induce cleavage of TDP-43 by calpain.

C9ORF72

Incubation of SK-N-BE(2) cells with ionomycin was associated with a marked decrease in full-length C9ORF72 (Fig. 3) of about 67% and 90% for treatment with 1 μM and 5 μM, respectively (Fig. 4). No cleavage products were identified (Fig. 3).

The decrease in C9ORF72 was of approximately 35% and 65% in cells exposed to 1 μM and 5 μM of thapsigargin, respectively. Here too, proteolytic products were hardly appreciable. (Figs. 3, 4).

The C9ORF72 profile found in HeLa cells was very similar to the one obtained in SK-N-BE(2) cells with ionomycin or thapsigargin treatment (Figs. 3, 5).

In untreated PBMC, the intensity of the full-length C9ORF72 band was weaker than that at ≈ 45 kDa, with the latter being compatible with one of the proteolytic products caused by calpain (see above). The full-length C9ORF72 band was no longer appreciable in cells incubated with the highest concentration of ionomycin or thapsigargin here applied. The ≈ 45 kDa proteolytic product was detected in all types of treatment (Fig. 5).

p62/sequestosome-1

Treatment of SK-N-BE(2) cells with 1 μM ionomycin caused an increase in the protein level of about 30% compared to untreated cells. Incubation with 5 μM ionomycin resulted in a ≈ 30% decrease in the protein, although this percentage is not significant. Treatment with 1 μM thapsigargin doubled the level of the protein. In cells exposed to 5 μM thapsigargin, the amount of p62/sequestosome-1 was similar to that observed in untreated cells (Figs. 3, 4).

The p62/sequestosome-1 profile recorded in HeLa cells was very similar to that obtained in SK-N-BE(2) cells with ionomycin or thapsigargin treatment (Figs. 3, 5).

With respect to untreated PBMC, the level of p62/sequestosome-1 remained approximately unaltered in cells exposed to 1 μM ionomycin, whereas it decreased following treatment with 1 μM thapsigargin. The protein was no longer detected in cells incubated with the highest concentration of ionomycin or thapsigargin here applied (Fig. 5).

Matrin-3

Exposure of SK-N-BE(2) cells to ionomycin led to a decrease in matrin-3 of about 50% and 80% for treatment with 1 μM and 5 μM, respectively. In both cases, a ≈ 70 kDa product, compatible with the one observed *in vitro* following treatment with calpains, was detected, although faintly (Figs. 3, 4). Incubation with thapsigargin was followed by a decrement in the protein of 55% and 85% after treatment with 1 μM and 5 μM, respectively (Figs. 3, 4). The ≈ 20 kDa caspase-dependent fragment found *in vitro* was appreciable after incubation with the highest concentration of thapsigargin here used (Fig. 3).

The decrease in matrin-3 in HeLa cells exposed to ionomycin or thapsigargin closely reproduced the one described above for SK-N-BE(2) cells (Fig. 5).

In PBMC, both ionomycin and thapsigargin, at the highest concentrations used, determined a dramatic decrease in matrin-3. The ≈ 70 kDa calpain-dependent product was recorded in cells treated with 1 μM and 5 μM ionomycin, as well as with 5 μM thapsigargin (Fig. 5).

VCP

Exposure of SK-N-BE(2) cells to 1 μM and 5 μM ionomycin induced a decrease in VCP of about 40% and 78%, respectively. A ≈ 50 kDa fragment, consistent with a calpain-dependent cleavage and probably better recognized by the VCP antibody here used than the full-length protein (see above), was clearly detected in cells treated with 5 μM ionomycin (Figs. 3, 4). The decrease in VCP in SK-N-BE(2) cells treated with 1 μM and 5 μM thapsigargin was of about 14% (not significant) and 30%, respectively (Figs. 3, 4). The ≈ 50 kDa calpain-dependent fragment was barely detectable in cells incubated with 1 μM ionomycin (Fig. 3).

In HeLa cells, a marked decrease in VCP was observed with both concentrations of ionomycin. Only the highest concentration of thapsigargin induced an appreciable decrease in the protein level. Proteolytic products of VCP were poorly detected following all treatments (Fig. 5).

In PBMC, all treatments applied caused a slight decrease in VCP. The ≈ 50 kDa calpain-dependent fragment was clearly evident in cells exposed to the highest concentrations of ionomycin or thapsigargin (Fig. 5).

FUS

Treatment of SK-N-BE(2) cells with ionomycin or thapsigargin caused a decrease in the protein level of about 60% at 1 μM and 90% at 5 μM, while thapsigargin induced a decrease of 50% at 1 μM and 75% at 5 μM (Figs. 3, 4). Among the calpain-dependent fragments observed in *in vitro* experiments (see above), only one with a molecular mass of few kDa smaller than the full-length protein was detected. This fragment was visible in cells treated with 1 μM and 5 μM ionomycin as well as with 1 μM thapsigargin (Figs. 3, 4).

HeLa cells, exposed to 1 μM or 5 μM ionomycin as well as to 5 μM thapsigargin, reproduced the decrement in FUS observed in SK-N-BE(2) cells (Figs. 3, 5), while proteolytic products were not detected in treated HeLa cells (Fig. 5).

In PBMC, both ionomycin and thapsigargin elicited an almost complete loss of FUS at the highest concentrations used. Such a loss was paralleled by the formation of a fragment compatible with the calpain-dependent one observed in treated SK-N-BE(2) cells (Figs. 3, 5).

SOD1

Exposure of SK-N-BE(2) cells to ionomycin led to a decrease in SOD1 of about 68% and 97% for treatment with 1 μM and 5 μM, respectively. Incubation with thapsigargin was followed by a decrease in the protein

level of about 54% and 84% after treatments with 1 μM and 5 μM, respectively. No proteolytic products were detected in cells exposed to either of the compounds (Fig. 3).

In HeLa cells as well as in PBMC treated with ionomycin or thapsigargin the SOD1 profile was similar to that obtained in similarly treated SK-N-BE(2) cells (Figs. 3, 5).

Profilin-1

Exposure of SK-N-BE(2) cells to ionomycin at 1 μM and 5 μM led to a decrease in profilin-1 of about 73% and 93% respectively. Incubation with thapsigargin at 1 μM and 5 μM was followed by a decrease in the protein level of about 57% and 85% respectively (Figs. 3, 4). Proteolytic products were not detected in treated cells (Fig. 3).

The profilin-1 profile in HeLa cells as well as in PBMC incubated with ionomycin or thapsigargin was similar to that found in SK-N-BE(2) cells (Figs. 3, 5).

Other proteins

The following proteins are not coded by genes linked to ALS, but an evaluation of their profile could be useful to identify pathways involving proteins directly linked to ALS.

PARP-1

PARP-1 is a marker of apoptosis. In fact, during the apoptotic process, caspases cause PARP-1 cleavage, producing an 89 kDa C-terminal fragment [34]. Treatment of SK-N-BE(2) cells with ionomycin at the two concentrations here applied induced a decrease in the protein level, but no caspase-dependent fragment was detected. On the contrary, such a fragment was observed in the same cell line when exposed to thapsigargin at 1 μM and was even more evident, together with a drastic loss of the full-length protein, when treated with 5 μM thapsigargin (Fig. 6).

LC3

Conversion of LC3 from the non-lipidated (LC3-I) to the lipidated (LC3-II) form, with the latter being detectable at a lower mass than the former, is widely used to monitor autophagy [35]. In untreated SK-N-BE(2) cells, the LC3-I/LC3-II ratio was > 1, whereas it was < 1 when treated with ionomycin or thapsigargin. The higher the concentration of these compounds, the lower the ratio. Furthermore, treatment with ionomycin or thapsigargin was associated with a decrease in the total amount (LC3-I + LC3-II) of LC3 (Fig. 6).

Time-dependent treatments and preincubation with chloroquine

For a better comprehension of the pathways triggered by intracellular Ca^{2+} overload and the consequent effects on the proteins linked to ALS, the profile of some of the latter was analysed in SK-N-BE(2) cells

exposed for 2, 8 and 24 h to ionomycin or thapsigargin at the lowest of the concentrations previously applied. Furthermore, preincubation with chloroquine, an inhibitor of the autophagic process, was performed for 2-h treatments with ionomycin or thapsigargin.

TDP-43

TDP-43 calpain-dependent fragments were detected already at 2 h of ionomycin exposure. Instead, the 35 kDa caspase-dependent fragment was appreciable only after 24 h of thapsigargin treatment (Fig. 7a). Preincubation with chloroquine did not affect the cleavage of the protein by calpains (Fig. 7b).

p62/sequestosome-1

In untreated cells, p62/sequestosome-1 levels decreased during the time interval considered (2–24 h). Treatment with ionomycin caused an even more drastic decrease in the protein level at 2 h compared to untreated cells. However, it raised the overall protein amount in the time interval considered (2–24 h). This profile was closely reproduced by thapsigargin treatment (Fig. 7a).

Pretreatment with chloroquine resulted in an increase in p62/sequestosome-1 levels in both ionomycin- and thapsigargin-treated cells (Fig. 7b).

PARP-1

The decrement of PARP-1 induced by ionomycin was already appreciable after 2 h of treatment. The 89 kDa caspase-dependent proteolytic product was observed after 24 h of thapsigargin treatment (Fig. 7a).

LC3

In untreated cells, the LC3-I/LC3-II ratio was > 1 and did not significantly vary during the time the measurements were taken. Incubation with ionomycin for 2 h was followed by a decrease in the total amount of LC3 as well as by a decrease in the LC3-I/LC3-II ratio compared to the ratio calculated in untreated cells. As reported above, the ratio was < 1 in cells treated with ionomycin for 24 h. The results obtained in cells incubated with thapsigargin overlapped with the ones found in cells incubated with ionomycin (Fig. 7a). Preincubation with chloroquine prevented the decrease in the total amount of LC3 and favoured the formation of the lipidated form of the protein both in ionomycin- and thapsigargin-treated cells (Fig. 7b).

Profilin-1

A drastic decrease in the protein was already clearly evident in cells incubated with ionomycin for 2 h. Treatment with thapsigargin replicated, although to a lesser extent, the decrease in the protein level detected in cells treated with ionomycin (Fig. 7a). Preincubation with chloroquine partially prevented the loss of the protein caused by ionomycin treatment and totally blocked the decrease in the protein amount determined by thapsigargin treatment (the analysis of SOD1 gave a similar result, Fig. 7b).

Discussion

The discovery of genes associated with ALS is in constant progress and undoubtedly important for the comprehension of the causes underlying the disease. However, the numerous functions of the proteins coded by these genes shows how complex the mechanisms involved in the pathology are. This study attempts to find a link between the proteins coded by the most relevant genes associated with ALS and excitotoxicity, one of the processes strongly suspected to play a role in motor neuron degeneration.

TDP-43 is a protein, coded for by the gene *TARDBP* [2], that acts as splicing regulator and transcription factor by binding single-stranded DNA and RNA [36]. The importance of TDP-43 is due to the presence of alterations of this protein in motor neurons of most ALS-affected individuals. Alterations consist in abnormal nuclear/cytoplasmic distribution, aggregation to form inclusions, aberrant phosphorylation and ubiquitinylation as well as proteolytic cleavage [37, 38]. The products of TDP-43 proteolytic degradation are the consequence of the action of caspases [23, 24, 26] as well as of calpains [20]. The results of this study show that an excessive Ca^{2+} influx into the cell triggers TDP-43 cleavage by calpains and determines a decrement in the protein level. Cytoplasmic Ca^{2+} accumulation due to the alteration of intracellular Ca^{2+} storage structures determines a cleavage of TDP-43 by caspases. The more severe the insult, the more relevant is the TDP-43 decrease and its proteolysis by caspases. This condition causes also a weak cleavage by calpains. The role of TDP-43 fragments in the pathogenesis of ALS is still debated. On the one hand, the propensity of the fragments to aggregate and form inclusions may be a determinant for motor neuron toxicity [39, 40]. On the other hand, proteolysis of TDP-43 may be an attempt of the cell to attenuate the damage caused by excessive levels of full-length protein [39, 28].

C9ORF72 is a component of a protein complex that has guanine nucleotide exchange factor (GEF) activity and regulates endosomal trafficking linked to protein degradation [10]. A mutation of *C9ORF72* is the most common genetic cause of ALS [2]. However, this mutation consists in a hexanucleotide repeat within a non-coding region of the gene and thus it is difficult to understand the way in which the genetic alteration affects the protein and, in turn, determines the disease. Our study showed that C9ORF72 is an excellent substrate for calpains and, to a lesser extent, for caspases. An increase in intracellular Ca^{2+} determines a decrement in the protein amount, which is more evident if caused by an excessive ion influx. It has been demonstrated that the repeat expansion in *C9ORF72* is linked to reduced levels of the coded protein in neurons and in other cell types, which has been associated with neurodegeneration [41–43]. This study suggests that the pathological decrease of C9ORF72 caused by the repeat expansion can also be determined by intracellular accumulation of Ca^{2+}.

p62/sequestosome-1 is a cargo protein that binds to proteins targeted for degradation through autophagy and the ubiquitin-proteasome system [44, 8]. This study confirmed that p62/sequestosome-1 is a good substrate for calpains and caspases (in particular caspases-6 and − 8) [29]. However, intracellular Ca^{2+} accumulation, induced either by massive ion intake or by impaired intracellular storage, produces an increase in p62/sequestosome-1 levels. More precisely, high amounts of intracellular Ca^{2+} determine an initial decrease in the protein amount, which is then followed by accumulation. When

autophagy occurs, p62/sequestosome-1 is itself degraded, together with the proteins it carries [8]. Instead, when autophagy is blocked, the levels of p62/sequestosome-1 rise and LC3, another protein linked to autophagy, is converted from non-lipidated to the lipidated form [45, 46]. Therefore, the levels of p62/sequestosome-1 appear to be modulated by Ca^{2+} through autophagy rather than proteolysis by calpains and caspases. Interestingly, motor neuron damage has been associated to either a decrement or an increase of p62/sequestosome-1 [47, 48].

Matrin-3 is a nuclear protein involved in chromatin organization, DNA replication, transcription, repair, and RNA processing and transport [4]. It shows structural and functional similarities with TDP-43 and can aggregate with the latter to form the neuronal inclusions typical of ALS [49]. Herein, matrin-3 has been revealed to be an excellent substrate for calpains and caspases. Additionally, its levels decrease following intracellular Ca^{2+} accumulation. In this respect, neurodegeneration has been associated with both increases and decreases in matrin-3 levels [50].

VCP is an ATPase that plays a role in a wide variety of cellular functions including cell signalling, cell cycling, organelle biogenesis and some aspects of intracellular proteolysis, such as autophagy and the ubiquitin proteasome system [51]. *VCP* mutations may account for ~ 1–2% of familial ALS cases [52, 2]. We found that VCP is a substrate for calpains as well as for caspases-6 and − 8. Furthermore, intracellular Ca^{2+} increase is responsible for a decrement in the protein amount. Since VCP is involved in several cellular processes, it is likely that its decrement determines cell damage by altering different biological pathways. For example, a loss of VCP hampers protein turnover by interfering with the ubiquitin proteasome system and autophagy [53].

Similarly to TDP-43, FUS is a protein involved in transcription regulation, RNA splicing, RNA transport and DNA repair [54]. Mutations of *FUS/TLS* gene account for about 4% of familial ALS cases [2]. This study revealed that FUS is a good substrate for calpains and caspases. In addition, intracellular Ca^{2+} overload is responsible for a decrease in the protein levels. A loss of FUS in motor neurons has been reported to alter RNA metabolism, cellular morphology and axonal function [55].

SOD1 is an enzyme that converts superoxide radicals to molecular oxygen and hydrogen peroxide, thus providing a defence against oxygen toxicity [56]. Among the several genes associated to ALS, *SOD1* was the first identified [57] and is by far the most extensively studied. Our study shows that SOD1 is a substrate for neither calpains nor caspases. However, intracellular Ca^{2+} accumulation leads to a relevant decrement in the protein levels. This decrement is, at least partially, prevented by an autophagic inhibitor. By determining a decrease in the amount of SOD1, it is reasonable to believe that a Ca^{2+} overload may cause oxidative stress, another event associated with motor neuron degeneration in ALS.

Profilin-1 is a protein implicated in cytoskeletal dynamics through the regulation of actin polymerization [58]. Mutations of *PFN1*, the gene coding for profilin-1, account for less than 1% of ALS cases, but their discovery suggested a new cellular mechanism in the pathogenesis of the disease. Similarly to what observed for SOD1, profilin-1 is a substrate for neither calpains nor caspases, despite the relevant

decrease caused by intracellular Ca^{2+} accumulation and partially prevented when autophagy is inhibited. A reduction in the amount of profilin-1 might damage motor neurons by disrupting their cytoskeletal architecture. In this regard, there is increasing evidence that cytoskeletal defects have a major role in motor neuron diseases [59].

The investigations here reported disclose that elevated intracellular Ca^{2+} concentrations result in a decrease in the levels of the proteins examined except for p62/sequestosome-1. Calpain- and caspase-mediated proteolysis as well as autophagy take a part in this decrement (although the involvement of other pathways cannot be ruled out). The predominance of one of the above processes depends on the cell type. In fact, calpain activity was poorly appreciable in a cervical cancer cell line (HeLa), whereas caspase activity was not found in blood mononuclear circulating cells.

Calpains belong to a class of thiol proteases whose catalytic activity is strictly dependent on Ca^{2+} [18]. Here, cytoplasmic Ca^{2+} accumulation caused by a massive ion influx or, to a smaller extent, by internal storage impairments, was seen to activate calpains. Calpain-1 seems to play a role in the early phase and during progression of ALS [60]. In addition, a selective inhibitor of calpains has been demonstrated to be neuroprotective in a mouse model of ALS [61].

Caspases are a class of thiol proteases essential for apoptosis, a form of programmed cell death [62]. Differently from calpains, caspases are not strictly dependent on Ca^{2+} for their activity, but Ca^{2+} is one of the stimuli that trigger the mechanisms that result in the activation of these proteases. A cytoplasmic Ca^{2+} accumulation caused by internal storage alterations activates, later in time with respect to calpains activation mediated by Ca^{2+} influx, the apoptotic caspases-3 and − 7, but not caspase-6 (i.e. lack of caspase-dependent fragments of VCP). However, activation of caspase-6 occurs later than that of caspases-3 and − 7 [21, 63] and therefore the consequences of its activity might become appreciable over a longer period of time. An implication of caspases in the neurodegenerative processes underlying ALS has been documented [64–66], although caspase-6 appears to play a neuroprotective role [67].

Autophagy is a degradation/recycling process that plays a wide variety of roles in the cell, including regulation of protein turnover, elimination of unwanted components, defence towards invading microorganisms, and provision of nutrient elements [68]. The link between Ca^{2+} and autophagy is well documented but controversial. In fact, a rise in intracellular Ca^{2+} levels can activate but also inhibit the autophagic flux [69]. The findings of this work indicate that intracellular Ca^{2+} accumulation initially enhances autophagy, but later blocks the process. Accordingly, the agents that increase cytosolic Ca^{2+} levels block the autophagic flux in its intermediate or even in its latest stages [70, 71]. When autophagy is active, all the proteins linked to ALS here considered are degraded. However, the subsequent block of the process is not associated with a recovery of the degraded proteins, with the notable exception of p62/sequestosome-1. A possible explanation is that, in the persistence of intracellular Ca^{2+} accumulation, the cell attempts to maintain the autophagic activity (even if the process is blocked), thus continuing to synthetize the necessary proteins. At the same time, the synthesis of the proteins degraded

by autophagy is arrested. Autophagy appears to be an important factor in the pathogenesis of ALS, but its role is extremely complex if not contradictory. In fact, both an excessive and an insufficient autophagic flux has been linked to ALS, and autophagy may either exacerbate or alleviate the disease processes at different stages [72, 73].

Calpain-mediated proteolysis, apoptosis and autophagy are tightly connected. In fact, calpains can both regulate the autophagic flux [69, 74] and activate or inactivate caspases [75, 76]. Furthermore, a block of autophagy can trigger apoptosis [77–79]. Moreover, some of the proteins linked to ALS here analysed, such as VCP and C9ORF72 (besides p62/sequestosome-1), play themselves an important role in the control of the processes that determine their degradation [80–82].

Thus, accumulation of Ca^{2+} in the cell, which is likely to be at the core of motor neuron degeneration in ALS, causes the alteration of a complex balance that leads to the activation of proteolytic processes targeting proteins coded by genes linked to the pathology (Fig. 8). A better understanding of when Ca^{2+} levels become toxic for the cell as well as how and why calpain proteolysis and autophagy, which are physiological processes, become pathological may elucidate the mechanisms responsible for ALS and help discover novel biomarkers and therapeutic targets.

Declarations

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Conflict of interests

Giovanni De Marco, Annarosa Lomartire, Umberto Manera, Antonio Canosa, Maurizio Grassano, Federico Casale, Giuseppe Fuda, Paolina Salamone, Maria Teresa Rinaudo, Sebastiano Colombatto, Cristina Moglia: no competing interest.

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Consent to participate and ethics approval

The subject whose PBMC were analysed in this study signed a written informed consent before blood drawn as a part of a study supported by the European Commission's Health Seventh Framework Programme (FP7/2007–2013 under grant agreement 278611) and approved by the Ethics Committee of the Azienda Ospedaliero-Universitaria Città della Salute e della Scienza di Torino in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Consent for publication

All authors have read and approved the submitted version of the manuscript.

Availability of data and material (data transparency)

All data generated or analysed during this study are included in this published article.

Code availability (software application or custom code)

Not applicable.

Author contributions

Giovanni De Marco designed the study, performed the experiments and generated the data along with Annarosa Lomartire. Giovanni De Marco, Annarosa Lomartire and Maria Teresa Rinaudo analysed and interpreted all the results. Sebastiano Colombatto participated in discussion of results and design of some experiments. Giovanni De Marco, Annarosa Lomartire, Maria Teresa Rinaudo and Umberto Manera wrote the manuscript. Umberto Manera, Maurizio Grassano, Federico Casale aided in interpreting the results. Antonio Canosa, Maurizio Grassano, Federico Casale, Giuseppe Fuda, Paolina Salamone and Cristina Moglia worked on the manuscript. Andrea Calvo and Adriano Chiò obtained funding and supervised the study. All coauthors read and approved the manuscript.

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Figures

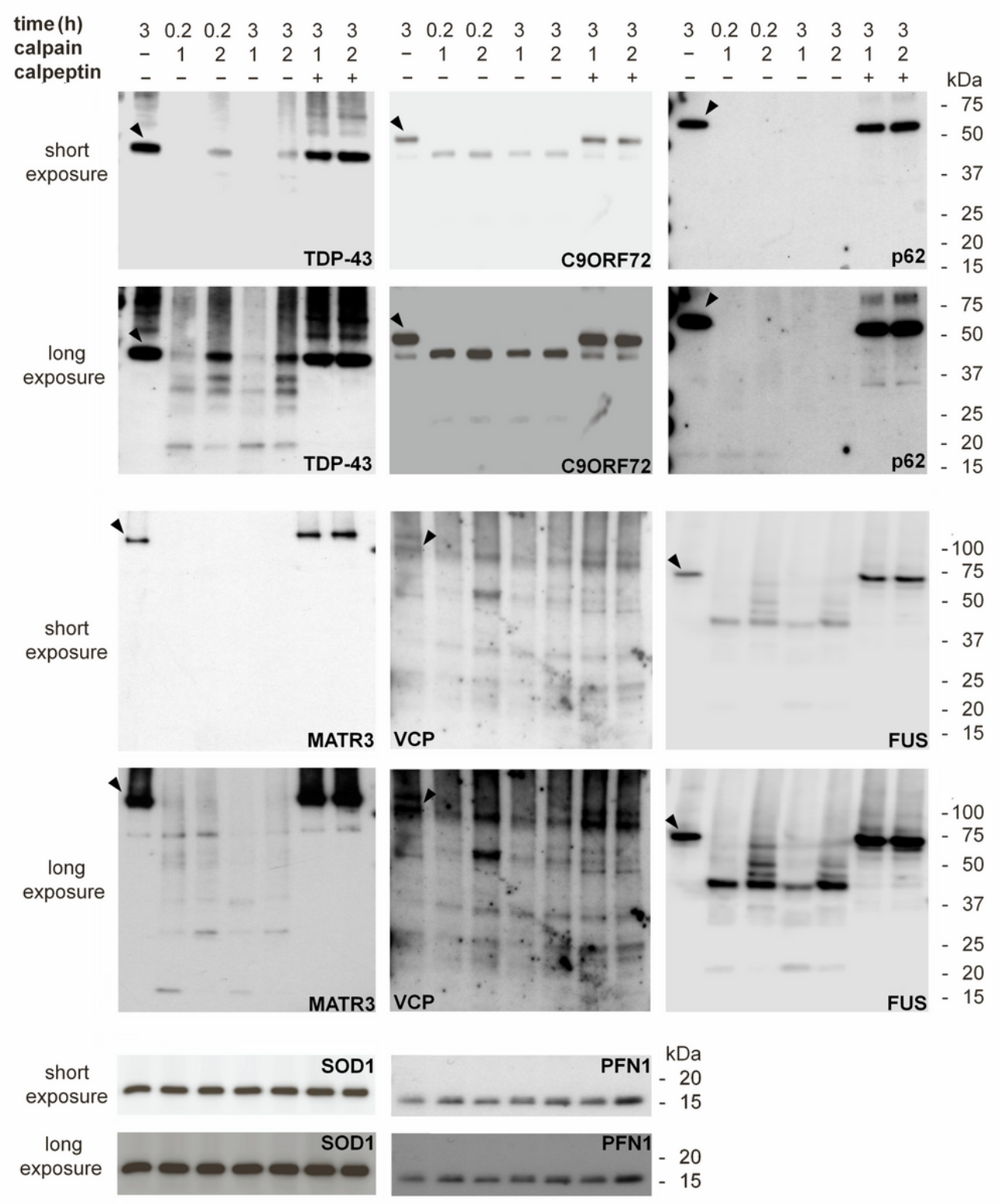

Figure 1

Calpain cleavage of proteins coded by genes linked to ALS. Western immunoblot analysis of the eight proteins here considered in SK-N-BE(2) cell lysate separately incubated with 2 U of recombinant active human calpains-1 or -2, at 37 °C for 10 or 180 minutes. Incubation of the cell lysate in presence of 20 mM calpeptin, a calpain inhibitor, was also performed. Blots are representative of three independent experiments. The arrowhead indicates the immunoreactive band corresponding to the full-length protein

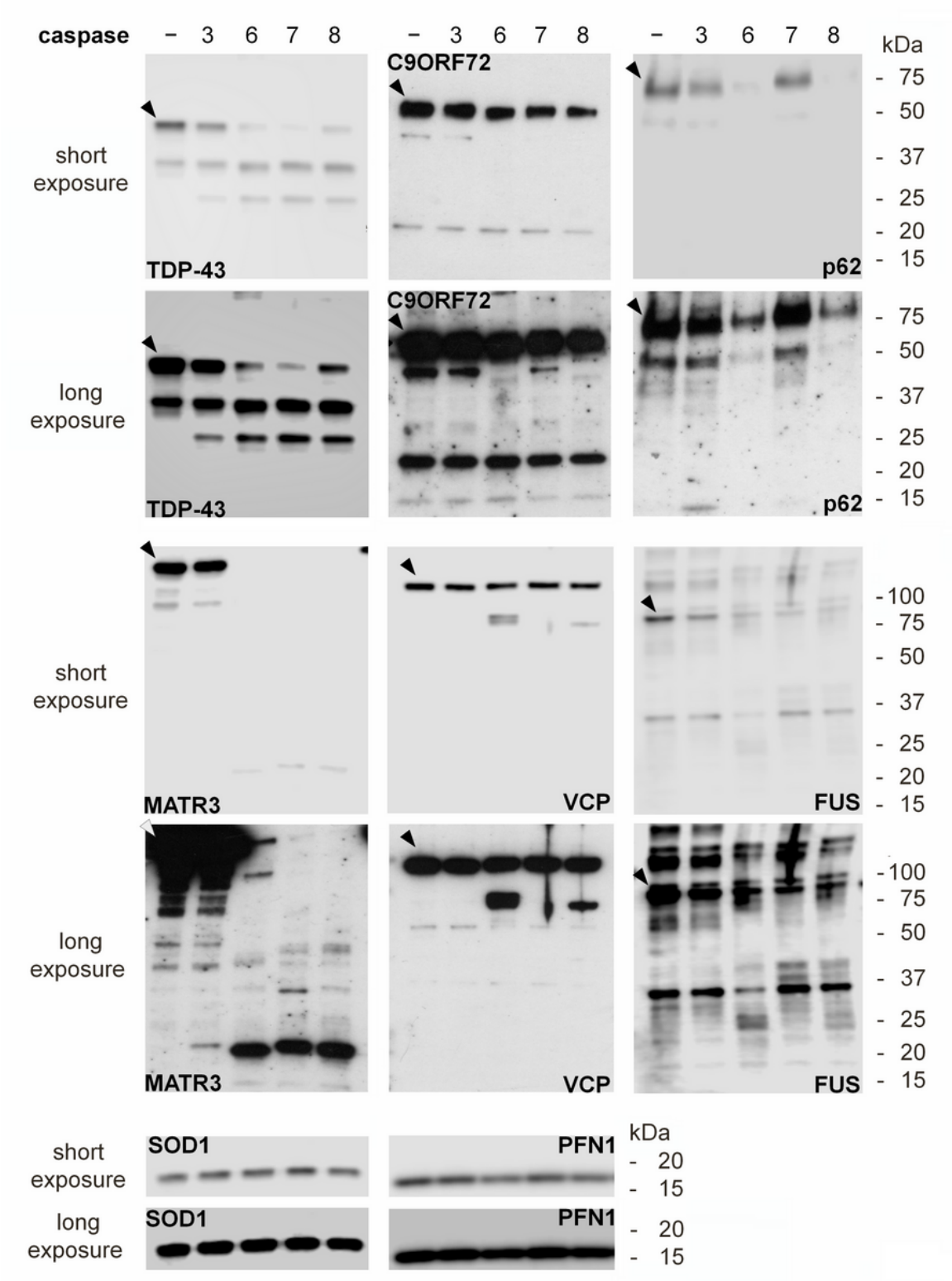

Figure 2

Caspase cleavage of proteins coded by genes linked to ALS. Western immunoblot analysis of the eight proteins here considered in SK-N-BE(2) cell lysate separately incubated with 2 U of recombinant active human caspases-3, 6, 7 or -8, for 3 h at 37 °C. Blots are representative of three independent experiments. The arrowhead indicates the immunoreactive band corresponding to the full-length protein

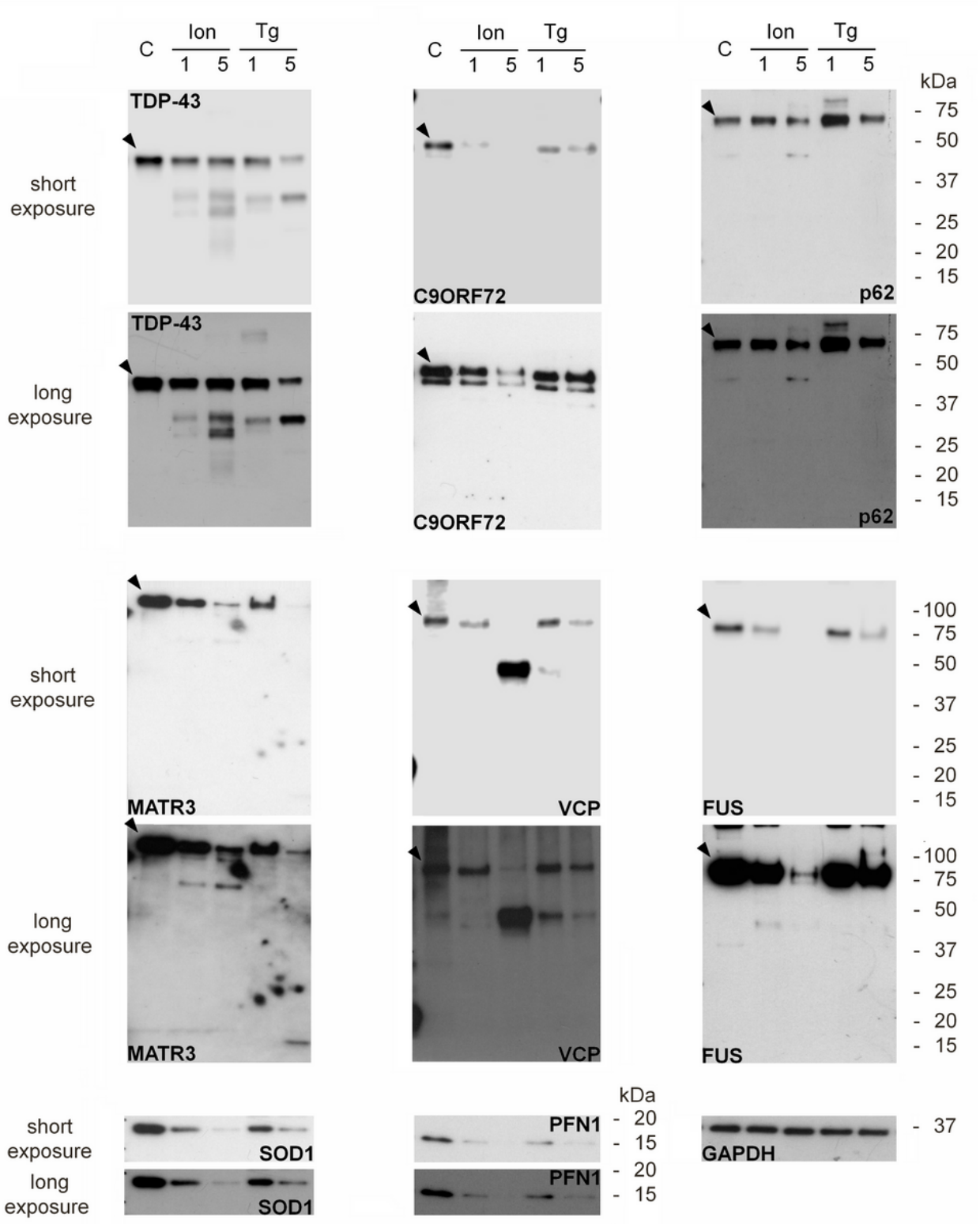

Figure 3

Effects of increased cytoplasmic Ca2+ in SK-N-BE(2) cell line. Western immunoblot analysis of the eight proteins here considered in SK-N-BE(2) cell line treated with ionomycin (Ion) or thapsigargin (Tg) at two different concentrations (1 mM and 5 mM) for 24 h. GAPDH expression is used as a measure of equal protein loading. The arrowhead indicates the immunoreactive band corresponding to the full-length protein. Blots are representative of three independent experiments. C = control, untreated cells

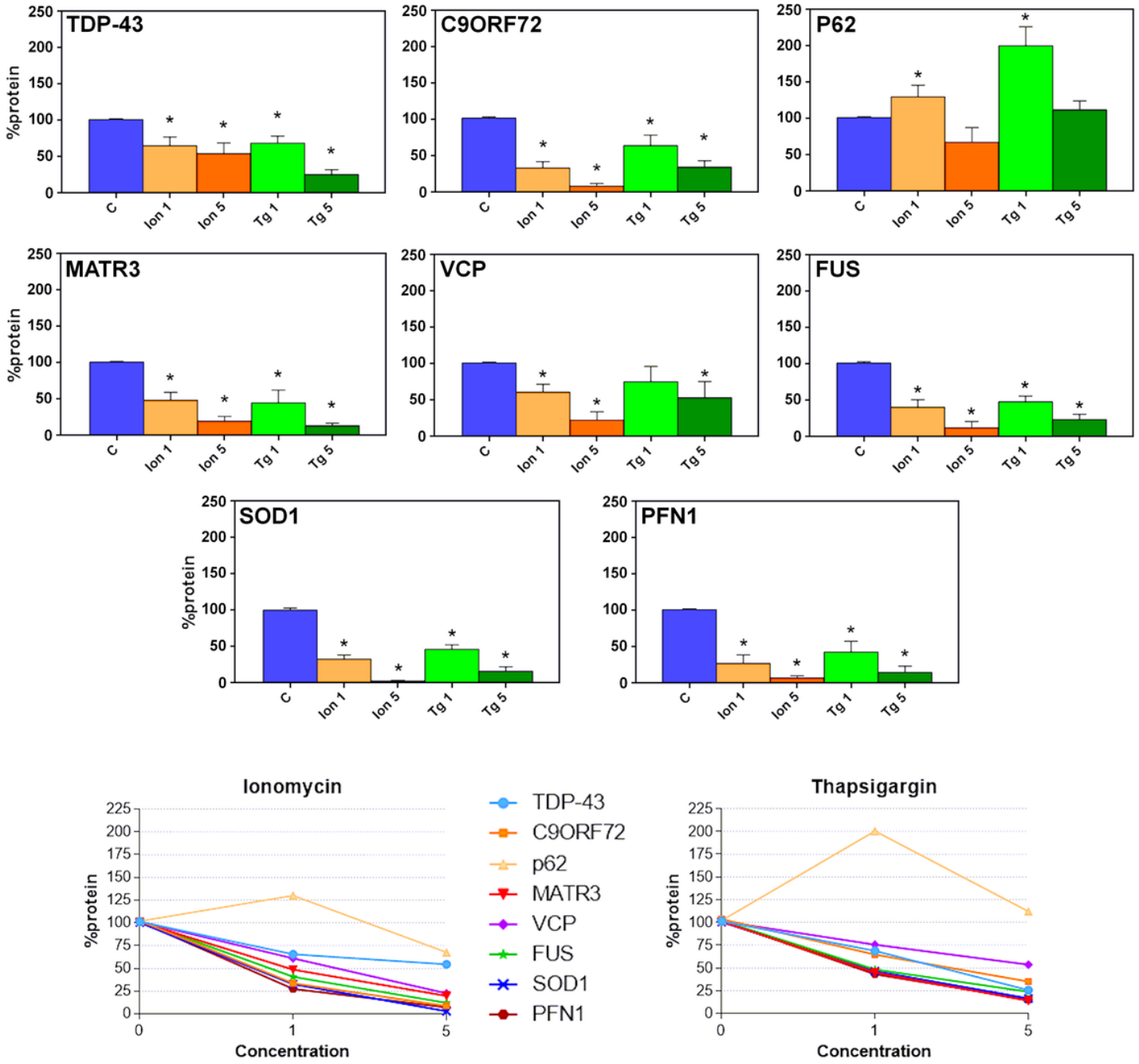

Figure 4

Trend of the eight proteins here analysed in SK-N-BE(2) cells forced to increase their cytoplasmic Ca2+ levels. The bar graphs illustrate the expression of each protein treated with ionomycin (Ion) or thapsigargin (Tg) compared to the expression in untreated cells (C), with the latter being arbitrarily considered as 100%. The line graphs show a comparison among the quantity trends of the proteins here analysed for treatments with ionomycin or thapsigargin. Data are representative of three independent experiments. * $p<0.05$; error bars= standard deviations

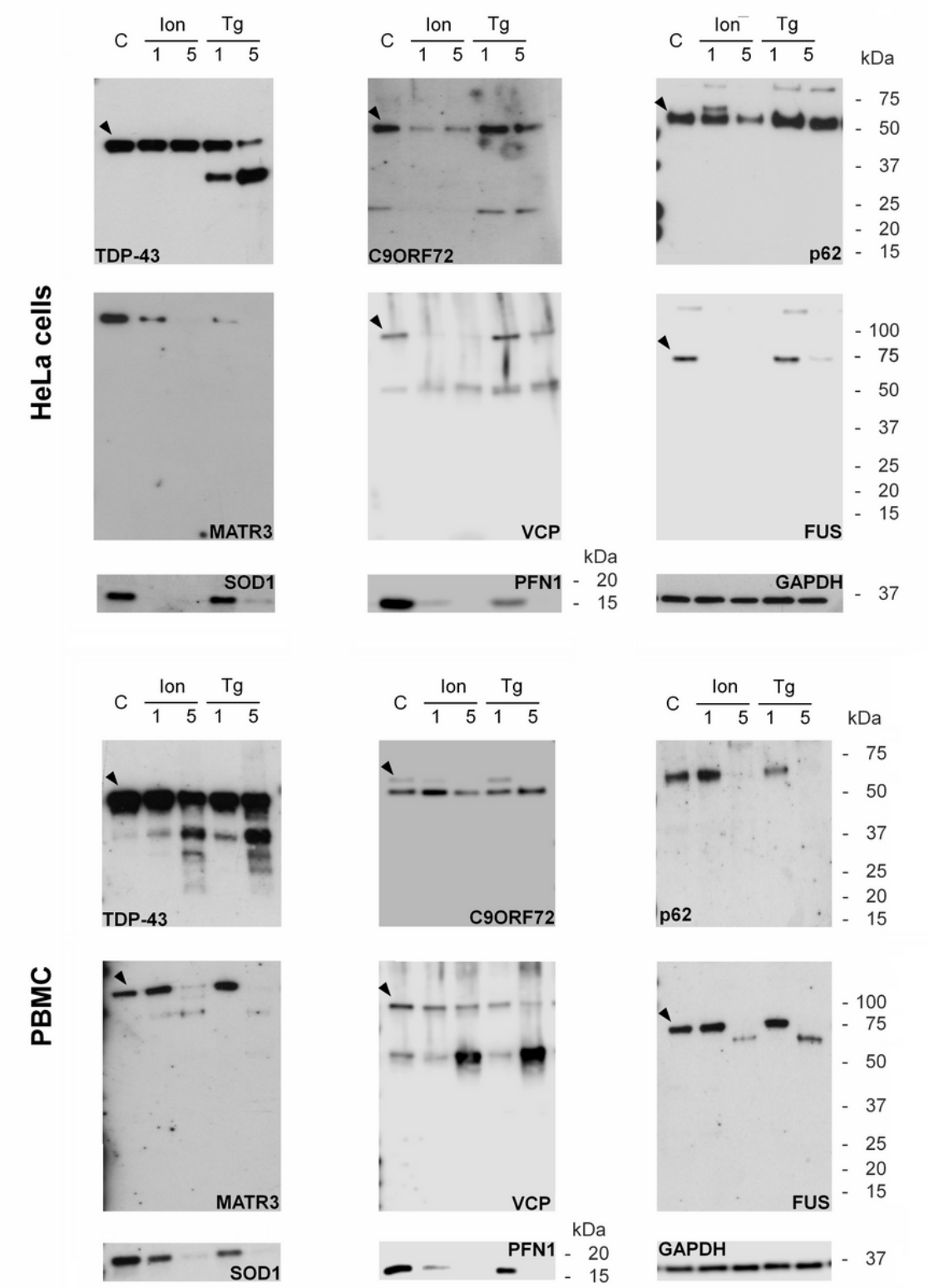

Figure 5

Effects of increased cytoplasmic Ca2+ in HeLa cell line and in peripheral blood mononuclear cells (PBMC). Western immunoblot analysis of the eight proteins here considered in HeLa as well as PBMC treated with ionomycin (Ion) or thapsigargin (Tg) at two different concentrations (1 mM and 5 mM) for 24 h. GAPDH expression is used as a measure of equal protein loading. The arrowhead indicates the

immunoreactive band corresponding to the full-length protein. Blots are representative of three independent experiments. C = control, untreated cells

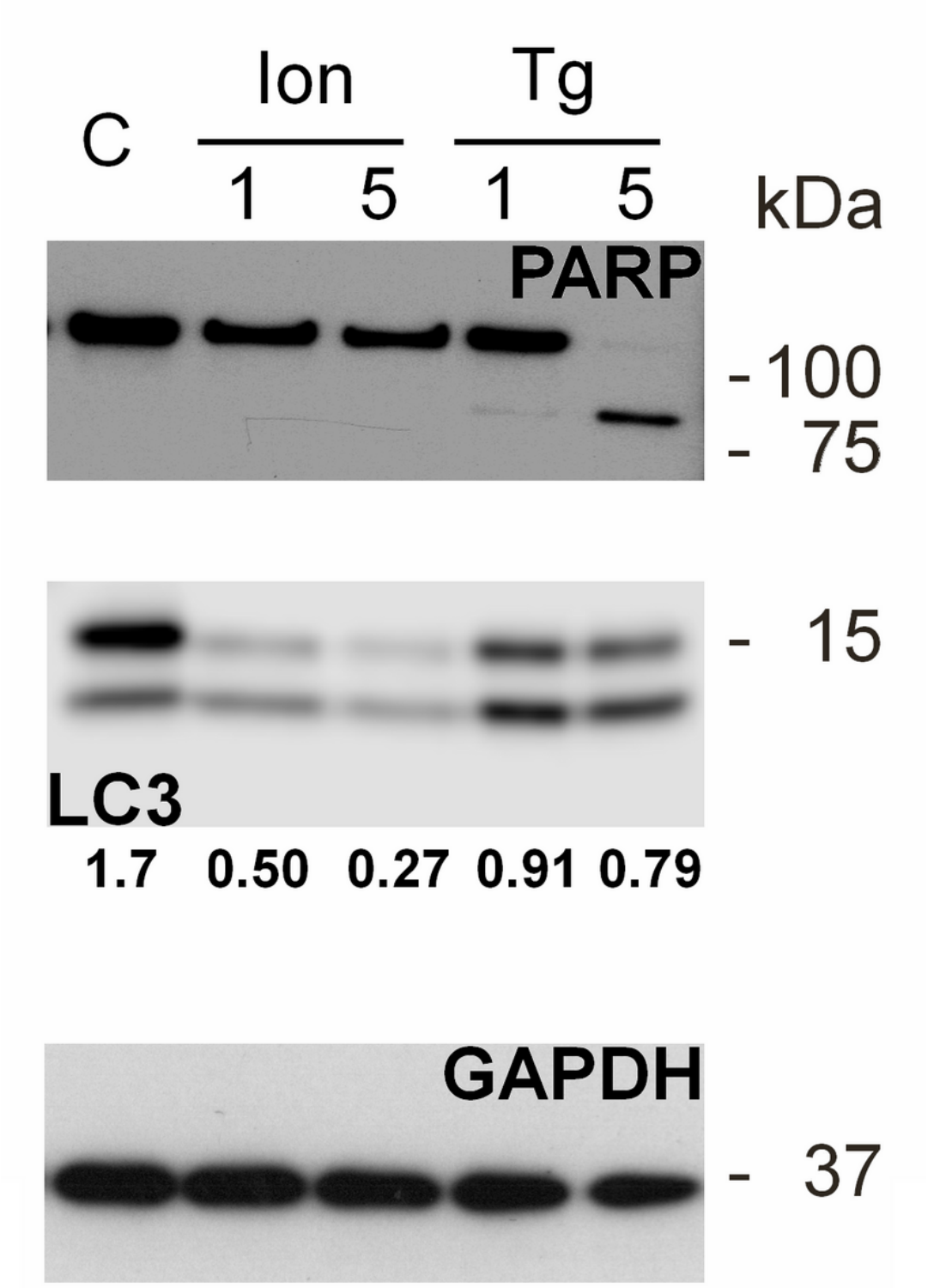

Figure 6

Effects of increased cytoplasmic Ca2+ on proteins not directly linked to ALS. Western immunoblot analysis of a protein linked to apoptosis (PARP) and a protein linked to autophagy (LC3) in SK-N-BE(2) cell line treated with ionomycin (Ion) or thapsigargin (Tg) at two different concentrations (1 mM and 5

mM) at 37 °C for 24 h. The ratio between LC3-I (non-lipidated form) and LC-II (lipidated form) is reported. GAPDH expression is used as a measure of equal protein loading. Blots are representative of three independent experiments

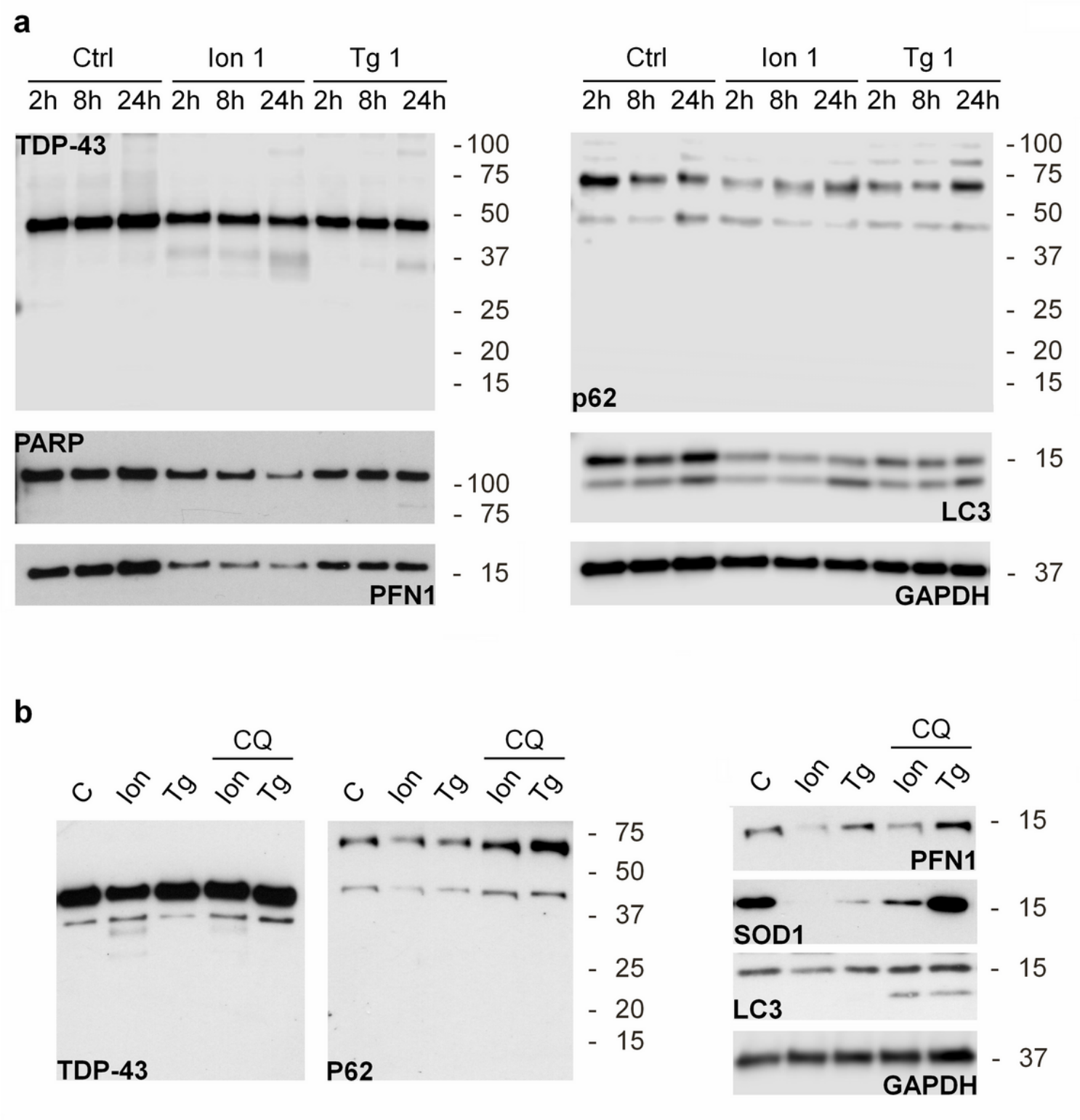

Figure 7

effects of intracellular Ca2+ accumulation over time as well as in presence of chloroquine in SK-N-BE(2) cells. Western immunoblot analysis of TDP-43, p62/SQSTM1, PFN1, PARP and LC3 in SK-N-BE(2) cell line

treated with 1 mM ionomycin (Ion) or thapsigargin (Tg) for 2, 8 and 24 h (a). Western immunoblot analysis of TDP-43, p62/SQSTM1, PFN1, SOD1, PARP and LC3 in SK-N-BE(2) cell line treated with 1 mM ionomycin (Ion) or thapsigargin (Tg) for 2 h with and without preincubation with 100 μM chloroquine (CQ) (b). GAPDH expression is used as a measure of equal protein loading. Blots are representative of three independent experiments

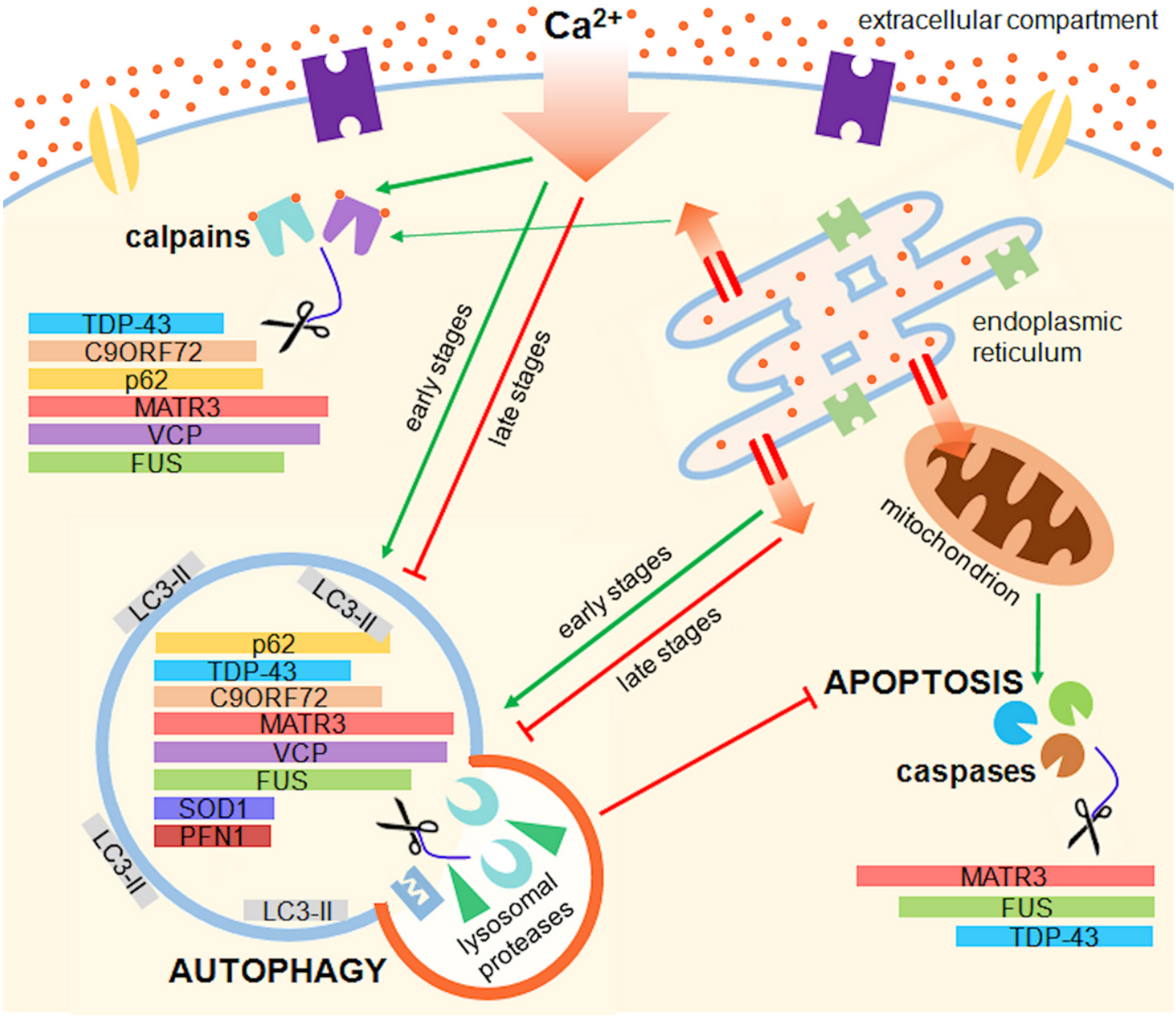

Figure 8

Effects of intracellular Ca2+ accumulations on proteins linked to ALS. Excessive influx as well as abnormal release from intracellular storages (i.e., endoplasmic reticulum) of Ca2+ causes the activation of proteolytic processes including calpain and caspase cleavage as well as autophagy. In the early stages, the raise in intracellular Ca2+ levels triggers the activation of calpains and favours autophagy. Over a longer period, intracellular Ca2+ accumulation leads to a block of the autophagic process (which

is accompanied by an increase in p62/SQSTM1 levels), which, in turn, results in the activation of apoptotic caspases. Apoptosis can also be triggered by Ca2+ accumulation in mitochondria