



Neuron-specific proteasome activation exerts cell non-autonomous protection against amyloid-beta (A β) proteotoxicity in *Caenorhabditis elegans*

Eleni Panagiotidou^{a,b,1}, Anna Gioran^{a,1}, Daniele Bano^c, Niki Chondrogianni^{a,*}

^a Institute of Chemical Biology, National Hellenic Research Foundation, 11635, Athens, Greece

^b Department of Biochemistry and Biotechnology, University of Thessaly, 41334, Larissa, Greece

^c German Center for Neurodegenerative Diseases (DZNE), 53127, Bonn, Germany

ARTICLE INFO

Keywords:

C. elegans
Cell non-autonomous regulation
Proteasome
Proteinopathies
Proteostasis

ABSTRACT

Proteostasis reinforcement is a promising approach in the design of therapeutic interventions against proteinopathies, including Alzheimer's disease. Understanding how and which parts of the proteostasis network should be enhanced is crucial in developing efficient therapeutic strategies. The ability of specific tissues to induce proteostatic responses in distal ones (cell non-autonomous regulation of proteostasis) is attracting interest. Although the proteasome is a major protein degradation node, nothing is known on its cell non-autonomous regulation. We show that proteasome activation in the nervous system can enhance the proteasome activity in the muscle of *Caenorhabditis elegans*. Mechanistically, this communication depends on Small Clear Vesicles, with glutamate as one of the neurotransmitters required for the distal regulation. More importantly, we demonstrate that this cell non-autonomous proteasome activation is translated into efficient prevention of amyloid-beta (A β)-mediated proteotoxic effects in the muscle of *C. elegans* but notably not to resistance against oxidative stress. Our *in vivo* data establish a mechanistic link between neuronal proteasome reinforcement and decreased A β proteotoxicity in the muscle. The identified distal communication may have serious implications in the design of therapeutic strategies based on tissue-specific proteasome manipulation.

1. Introduction

Organisms are exposed to various stresses throughout their whole lifespan. To preserve their protein homeostasis (proteostasis) and therefore ensure the integrity of the proteome, cells have developed a complex proteostasis network regulating protein synthesis, folding and protein degradation through either the ubiquitin-proteasome system (UPS) or the autophagy lysosomal pathway (ALP). There are also surveillance stress response pathways that detect and respond to protein damage; the heat shock response (HSR) and the unfolded protein response in mitochondria (UPR^{mit}) and endoplasmic reticulum (UPR^{ER}). The proteostasis mechanisms have been shown to become deregulated during ageing and age-related diseases, including proteinopathies [1]. Given this failure of proteostasis along with the fact that ageing is the major risk factor for neurodegenerative diseases, it is reasonable to assume that the induction of proteostatic mechanisms and the elucidation

of the involved pathways are central to design preventive/therapeutic strategies against both ageing and neurodegenerative diseases [2].

The proteasome is one of the major molecular regulators of cellular proteostasis and one of the main secondary antioxidant mechanisms subjected to tight redox control [3]. It is responsible for the rapid degradation of misfolded and damaged proteins but also the proteolysis of normal cellular proteins [4]. The 26S/30S proteasome consists of two different sub-complexes, the 20S core and the 19S regulatory particle. The 20S complex can be capped by one (26S) or two (30S) 19S regulatory particle(s). The 20S particle is composed of 28 subunits (14 α -type and 14 β -type) and exerts its proteolytic activities through the β 1, β 2 and β 5 subunits that host the caspase-like, trypsin-like, and chymotrypsin-like activities, respectively [5]. There is increasing interest in the upregulation of proteasome function through various means, including the direct stereochemical alterations of the 20S core that might lead to enhanced activity levels due to the opening of the α -gated channel of the core, the activity-modifying phosphorylation of

* Corresponding author. Institute of Chemical Biology, National Hellenic Research Foundation, 48 Vassileos Constantinou Avenue, Athens, 11635, Greece.

E-mail addresses: epanagiotidou@eie.gr (E. Panagiotidou), agioran@eie.gr (A. Gioran), Daniele.Bano@dzne.de (D. Bano), nikichon@eie.gr (N. Chondrogianni).

¹ These authors contributed equally to this work.

Abbreviations

AD	Alzheimer's disease
ALP	autophagy lysosomal pathway
APP	Amyloid precursor protein
A β	amyloid-beta
A β ₁₋₄₂	amyloid-beta amino acids 1 to 42
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
DCVs	dense core vesicles
dFP	dual fluorescent protein
DMSO	dimethyl sulfoxide
GABA	γ -aminobutyric acid
HSR	heat shock response
mFP	monomeric fluorescent protein
OE	overexpression
RNAi	RNA interference
SCVs	small clear vesicles
UPR ^{ER}	unfolded protein response related to the endoplasmic reticulum
UPR ^{mit}	unfolded protein response related to mitochondria
UPS	ubiquitin-proteasome system
wt	wild type

the proteasome itself or the enhanced expression of proteasome subunits; the translational ramifications for proteinopathies are obvious [6, 7]. We and others have previously shown that overexpression of 20S [β 5 subunit and its orthologs/homologs in the nematode *C. elegans* (*pbs-5*), *Drosophila* (*Pros β 5*) and mice (PSMB5)], or 19S [PSMD11 subunit and its homolog in the nematode (*rpn-6*)] subunits is sufficient to enhance proteasome activity in human cells, *C. elegans*, *Drosophila* and mice and to positively modulate ageing progression and protect against proteotoxicity in A β -, amyloid precursor protein (APP)- or polyglutamine-expressing animal models [8–12].

The cell autonomous regulation of various key proteostasis players is increasingly revealed but there are still many molecular processes that need to be elucidated. Notably, the cell non-autonomous regulation of a few, but importantly not all, key players has also emerged the past few years, further pinpointing the need to understand how tissues can exchange signals, thus affecting phenomenically distal tissues and impacting on organismal physiology. In this regard, *C. elegans* has played a central role in the investigation of the proteostatic cell non-autonomous responses *in vivo*. More specifically, regulation of the cellular heat shock response of somatic cells in *C. elegans* is regulated in a cell non-autonomous manner by thermosensory neurons [13]. Mitochondrial deficiency in neurons activates the UPR^{mit} in the intestine of *C. elegans* [14,15]. Likewise, germline proteostasis affects mitochondrial function and protein aggregation across multiple tissues via the UPR^{mit} and long-range Wnt signaling pathway [16], while binding of an aggregation-prone protein expressed in the neurons of *C. elegans* to the mitochondria elicits a global induction of UPR^{mit}, affecting whole-animal physiology [17]. This inter-tissue communication also extends to the UPR^{ER}; overexpression of a UPR^{ER}-related protein in the nervous system of *C. elegans* leads to the activation of UPR^{ER} in non-neuronal tissues of the animal [18,19]. Neuronal and intestinal overexpression of the autophagy-related gene *atg-18* in *C. elegans* leads to lifespan extension in a cell non-autonomous manner [20], while a microRNA produced and secreted by the intestine can regulate the autophagy levels in the body wall muscle of the animal [21]. Cell non-autonomous proteostasis regulation has been also reported in *Drosophila*. Neuron-specific upregulation of AMPK (AMP-activated protein kinase) can elevate autophagy in the intestine of the flies [22]. Similarly, neuronal overexpression of *Gclc* (glutamate-cysteine ligase catalytic subunit) increases longevity in *Drosophila* and causes

longevity-associated gene transcription changes in the thorax [23]. Despite the highly important role of the proteasome, it is noteworthy that no reports exist so far on its potential cell non-autonomous regulation and the potential downstream effects on animal physiology.

With the knowledge that proteasome activity can be pharmacologically modulated and, therefore, may be exploited for novel therapeutic approaches, we sought to unravel the potential cell-non autonomous regulation of the proteasome and the downstream effects on proteotoxicity in a distal tissue. To this end, we generated transgenic nematodes overexpressing the *pbs-5* proteasome subunit in the nervous system and examined its effects in the muscle tissue of the animal. Our results reveal that *pbs-5* overexpression in the nervous system is sufficient to cell non-autonomously increase the proteasome activity and function in the muscle. We also provide evidence that this interconnection occurs via a specific type of synaptic vesicles, the small clear vesicles (SCVs). Furthermore, we identify glutamate as at least one of the responsible neurotransmitters and we reveal that the dense core vesicles (DCVs), a different type of synaptic vesicles, are also involved in the proteasome-regulating signal transmission. Together, our *in vivo* findings may significantly contribute to shaping our current perception of how the proteasome should be manipulated in therapeutic strategies targeting proteinopathies.

2. Materials and Methods

2.1. *C. elegans* strains and culture

All strains were grown and maintained at 20°C, unless otherwise specified, using standard techniques for maintenance [24] and RNAi experiments [25]. The following *C. elegans* strains were obtained from the Caenorhabditis Genetics Center (CGC): wild type (wt) N2 (Bristol), CL4176 *dvIs27[myo-3p::A β ₁₋₄₂::let-851 3'UTR + rol-6(su1006)]*, DLM9 *uwaEx5[myo-3p::CERULEAN-VENUSlgg-1 + unc-119(+)]*, GMC101 *dvIs100[unc-54p::A β ₁₋₄₂::unc-54 3'UTR + mtl-2p::GFP]*, CB246 *unc-64(e246)*, CB928 *unc-31(e928)*, KR1787 *unc-13(e51)*. The modified ZsProSensor-1 reporter strains YD114 *xzIs2[unc-54p::UIM2ZsProSensor-1]* and YD70 *xzIs1[vha-6p::UIM2::ZsProSensor-1]* were a kind gift from Dr. Carina I. Holmberg (University of Helsinki). The strain expressing A β in glutamatergic neurons UA198 *ban34[eat-4p::sA β 42 + myo-2p::mCherry]; adIs1240[eat-4p::GFP]* [26] was a kind gift from Prof. Guy A. Caldwell (The University of Alabama). The following strains carrying extrachromosomal arrays were also used: Ex[*myo-2p::GFP*], Ex[*let-858p::pbs-5 + myo-2p::GFP*], Ex[*rgef-1p::pbs-5 + myo-2p::GFP*], Ex[*rol-6(su1006)*], Ex[*let-858p::pbs-5 + rol-6(su1006)*] and Ex[*rgef-1p::pbs-5 + rol-6(su1006)*]. A full list of the strains used in this work is documented in Table S1.

2.2. Cloning and *C. elegans* transgenesis

For the construct encoding the *rgef-1p::pbs-5* transgene, the Gateway® Cloning (Invitrogen life technologies, CA, USA) was used. The *rgef-1p* was amplified with a PCR on gDNA of wt nematodes with the attB primers Fw: GGGGACAAGTTTGTACAAAAAAGCAGGCTTactt actgtctatcgtctcgt and Rev: GGGGACAACCTTTGTATACAAAGTTGtc gtttccgatccccctatatca gc and recombined with BP recombinase in pDONR™ 221 P1-P5r. The sequence of *pbs-5* was amplified also from wt gDNA with the attB primers Fw: GGGGACAACCTTTGTATACAAAGTTGTAATG TGGGGCGAGACATTCG and Rev: GGGGACACTTTGTACAAAGCTGGGTTACGT CATCAACACCCAGCC and recombined with BP recombinase in pDONR221™ P5-P2. The two entry clones carrying *rgef-1p* and *pbs-5* were recombined with pGC93 with the LR recombinase to produce an expression clone. This vector was microinjected in the gonads of wt nematodes at 50 ng/ μ L together with either 5 ng/ μ L of the co-injection marker *myo-2p::GFP* or 50 ng/ μ L of the pRF4 plasmid *rol-6(su1006)*. The nematode strain overexpressing *pbs-5* under *let-858p* was generated using a construct made previously [10]

and microinjected at 20 ng/μL. Control strains were microinjected with either co-injection marker only, at the same concentrations. All transgenes were carried in extrachromosomal arrays.

2.3. *C. elegans* treatment with neurotransmitters

All neurotransmitters (acetylcholine; Alfa Aesar, MA, USA, #L02168, GABA; Acros Organics, Belgium, #103280250, glutamate; Alfa Aesar, MA, USA, #A12919, octopamine; Thermo Scientific, MA, USA, #J61281.03, serotonin; Sigma-Aldrich, MO, USA, #H7752, dopamine; Alfa Aesar, MA, USA, #A11136.06, tyramine; Fluorochem, UK, #078887), were dissolved in M9 buffer (42 mM Na₂HPO₄, 22 mM KH₂PO₄, 85 mM NaCl, 1 mM MgSO₄). The stock solutions were diluted with M9 and 200 μL were spread onto plates seeded with UV-inactivated OP50. Nematodes of L3 stage were transferred on these NGM plates containing acetylcholine, GABA, glutamate, octopamine, serotonin, dopamine or tyramine at final concentrations 50 μM - 100 mM, 150 μM - 30 mM, 15 μM - 15 mM, 40 μM - 20 mM, 1.5 μM - 73 μM, 5.2 μM - 158 μM and 14 μM - 143 μM respectively. The nematodes were treated for 20 h and imaged at the stage of young adult for *unc-13(e51)* and of L4 for *unc-64(e246)* mutants.

2.4. *C. elegans* treatment with epoxomicin

Epoxomicin (UBPBio, USA, #F1440-5) was dissolved in DMSO (180 mM stock solution). *C. elegans* embryos were obtained following treatment of gravid adults with a hypochlorite solution and seeding onto NGM plates with UV-killed bacteria. Once the nematodes reached the L3 stage they were transferred into a microcentrifuge tube with 200 μL M9 in which the pellet of 4 mL heat-inactivated OP50 culture had been dissolved, containing 1 mM epoxomicin [27] or DMSO (vehicle). In the case of epoxomicin-treated Muscle ZsProSensor-1 reporter animals, the tubes were stirred for 12 h at 20°C before the animals were imaged. In the case of paralysis experiments with CL4176 animals, the epoxomicin treatment was started at the time of the temperature upshift from 16°C to 25°C and after 12 h the animals were transferred on regular NGM plates seeded with UV-killed OP50. The paralysis assessment was then performed as described below.

2.5. *C. elegans* treatment with chloroquine

Chloroquine (TCI Chemicals, Tokyo Japan, #C2301) was dissolved in M9 buffer (20 mM final working solution). Synchronized day 1 adult nematodes were transferred into a microcentrifuge tube with 200 μL M9 or 200 μL M9+chloroquine. In either case the pellet of 4 mL heat-inactivated OP50 culture had been dissolved in the tube. The tubes were stirred for 16 h at 20°C before the animals were rinsed and collected for protein extraction and western blotting.

2.6. *C. elegans* treatment with oxidative stressors

Paraquat (Sigma-Aldrich, MO, USA, #856177) and NaN₃ (Merck, Darmstadt, Germany, #1.06688) were dissolved in ddH₂O and M9 buffer, respectively. Synchronized L4 nematodes were transferred to plates containing 2 mM paraquat and transferred to freshly prepared plates every other day. Survival was scored until all animals were dead (~8 days after the beginning of the treatment). Synchronized young adult nematodes were transferred to plates supplemented with 180 μM freshly prepared NaN₃ for 24 h and survival was scored after 24 h recovery.

2.7. RNA extraction

~200 young adult nematodes/sample were resuspended in 1 mL Trizol™ (Invitrogen, MA, USA, #15596-026) and underwent 3–4 cycles of freezing (liquid N₂) and thawing (37°C) until intact bodies were no

longer visible. Following addition of 200 μL chloroform (Sigma-Aldrich, MO, USA, #372978) and vigorous mixing, the samples were centrifuged at 13000 rpm for 15 min at 4°C. The upper aqueous phase was pipetted and mixed with 500 μL of chilled isopropanol and centrifuged again at 13000 rpm for 10 min at 4°C. The RNA pellet was washed with 300 μL of 75% ethanol and centrifuged again at 13000 rpm for 10 min at 4°C. Once air dried, the pellet was resuspended in 25 μL RNase-free water and quantified using a spectrophotometer (Quawell, CA, USA, Q5000).

2.8. cDNA synthesis and qRT-PCR

1 μg of RNA was used for reverse transcription using the iScript cDNA synthesis kit (Bio-Rad, CA, USA, #1708891) according to manufacturer instructions. After cDNA synthesis, the reactions were diluted with 80 μL of nuclease-free water and 1 μL of this was used for the qRT-PCR reaction with the iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA, #17251224) on a Bio-Rad CFX Connect Real-Time System (Bio-Rad, CA, USA). Each set of primers was verified through a two-step protocol that included (i) testing of nine combinations of primer concentrations against 10 ng of wt cDNA, choosing the combination which gave the lowest C_T value and only the expected product when the qRT-PCR reaction was run on a 2% agarose gel, and (ii) testing of the linearity of C_T values given by the primer combination identified in step (i) with a serial dilution of cDNA ranging from 50 to 1.56 ng. The following primers were used in this study at 400 nM: *pbs-5*, Fw: GAGATCGTATGGTTGC-CACG, *pbs-5*, Rev: GGAGCTCGTACAAAGTGCAG, *cdc-42*, Fw: CGT TGACGCAGAAAGGACTG, *cdc-42*, Rev: CTTCTTCTCCTGTGTGGTG GGT, *β*, Fw: GCAGAATTCGACATGACTCAGG, *β*, Rev: GCCCAC-CATGAGTCCAATGATT, *dod-24*, Fw: CAAACACTTCACCGAGCCAG, *dod-24*, Rev: AGTCATCGTTCTGGAGCAATTC, *nit-1*, Fw: GCATAGGTGGC CAGATTCAAAA, *nit-1*, Rev: CCAGCTGCTTCTCCACATTT, *skn-1*, Fw: GAGACGAGACGATAA, *skn-1*, Rev: CAGATGAATATGGACGACACTC, *gst-4*, Fw: AAGTTGTTGAACCAGCCC, *gst-4*, Rev: AATCACAATATCA GCCCAAGTC.

2.9. Protein extraction, dot and western blotting

Nematodes were lysed via sonication in RIPA buffer supplemented with proteinase inhibitors. Protein concentration was determined with DC assay (Bio-Rad, CA, USA, #500-0113, -0114, -0115) and 15–20 μg of protein were separated with SDS-PAGE or 5–7 μg of protein were directly loaded on a nitrocellulose membrane mounted on a dot blotter. In the case of western blotting, the separated proteins were transferred onto a nitrocellulose membrane. All membranes were blocked with 5% BSA or 5% non-fat milk for 1 h at RT and incubated overnight with a primary antibody. The secondary antibodies were incubated at RT for 1 h and the membranes were developed using an ECL kit (Bio-Rad, CA, USA, #170-5051) and a Bio-Rad XRS+ Chemidoc (Bio-Rad, CA, USA). Coomassie Brilliant Blue staining was used for equal loading control. The primary antibodies used in this study were: anti-6E10 (BioLegend, CA, USA, #803001), anti-amyloid oligomer (EMD Millipore Corp., MA, USA, #AB9234) and anti-GFP (Cell Signaling, MA, USA, #2956). The secondary antibodies used in this study were: anti-rabbit (Santa Cruz, TX, USA, sc-2357) and anti-mouse (Santa Cruz, sc-516102).

2.10. Proteasome activities

To measure the chymotrypsin-like (CT-L) activity in cell-free assays, 0.5 μg of human purified proteasome (Proteasome Protein Center Kibbutz Ramat-Yohanan, Israel) was mixed with glutamate (Alfa Aesar, MA, USA, #A12919) in concentrations ranging from 15 μM to 15 mM and with Succinyl-Leucine-Leucine-Valine-Tyrosine-7-amino-4-methylcoumarin (Suc-LLVY-AMC, UBPBio, TX, USA, #G1100) at a 100 μM final concentration in a final volume of 100 μL in the wells of a black 96 well plate. 5 technical replicates per sample were used for each assay. SDS at final concentration 0.01% was used as a positive control

and the proteasome inhibitor MG132 (UBPBio, TX, USA, #F1101) at a final concentration 20 μ M was used as a negative control. The fluorescence was measured at 380/460 nm excitation/emission every 5 min for 1 h in a TECAN (Tecan Ltd., Switzerland) plate reader at 37°C.

2.11. Paralysis assays

For transgenic animals crossed with the CL4176 strain: 150–300 animals of L3 stage were shifted from 16°C to 25°C and 16 h later they were scored over the span of 10–14 h for paralysis until the entire population was paralyzed. For transgenic animals crossed with the GMC101 strain: 80–120 animals of L4 stage were transferred on NGM plates with 40 μ M FUDR (Acros Organics, Belgium, #227601000) seeded with UV-inactivated OP50. Once they reached the stage of young adult, the animals were shifted from 20°C to 25°C. Scoring of paralyzed nematodes was initiated 12 h after temperature upshift and lasted until the entire population was paralyzed. For both A β models, the animals were scored as paralyzed if they failed to undergo a full body wave propagation upon prodding and each paralysis assay was repeated at least three times by two blinded experimenters.

2.12. Microscopy and image analysis

The animals were mounted on 2% agarose pads on glass slides and immobilized using 50 mM levamisole (Sigma-Aldrich, MO, USA, #L9756) dissolved in M9 buffer. The imaging was performed with a Leica TCS SPE confocal laser scanning microscope. For the animals expressing ZsProSensor-1, images from the anterior part of the animal were acquired using a 20x/0.70 objective and Z-stacks were obtained with stable laser intensity and gain throughout all conditions of every experiment. Regions of interest were determined on maximum intensity projection images of the Z-stacks and their intensity was determined with ImageJ. For transgenic animals crossed with UA198 animals, a 40x/1.15 oil immersion objective was used and the number of neurons in the tail was determined with Z-stacks and maximum intensity projection images.

2.13. Statistical analysis

All statistical analyses were performed with GraphPad Prism Software (San Diego, USA). Depending on the experiment, a variety of statistical tests was used and each is mentioned in the respective figure legend together with the sample size for each experiment. For analyses comparing the mean of two groups, we used the parametric two-tailed Student's *t*-test, provided that each group comprised of at least 10 values. For fewer than 10 values we used the non-parametric test Mann-Whitney. When fewer than 10 replicates were included in an experimental condition, individual points representing each replicate are shown in the bar charts. For the comparison of more than 2 groups with fewer than 10 values per group, we used a non-parametric ANOVA-single factor with a post-hoc correction for multiple comparisons (Kruskal-Wallis test with Dunn's correction). For the comparison of more than 2 groups with more than 10 values per group, we used a parametric ANOVA-single factor with a post-hoc correction for multiple comparisons (Tukey's multiple comparisons test). For paralysis curves we used the log-rank Mantel-Cox test. All bar charts indicate the mean of the independent experiments \pm SEM.

3. Results

3.1. Neuron-specific proteasome overexpression increases proteasome activity in the muscle

We have previously shown that ubiquitous overexpression of *pbs-5* proteasome subunit is sufficient to increase the overall proteasome activity and to promote enhanced resistance against A β proteotoxicity in

C. elegans [10]. Given that there are no reports on the potential cell non-autonomous regulation of the proteasome and since the nervous system is one of the main tissues communicating with other tissues, we sought to investigate whether neuron-specific *pbs-5* overexpression affects (a) the proteasome activity in distal tissues and, (b) their resistance/response to proteotoxicity in a cell non-autonomous manner. To generate transgenic animals with proteasome overexpression, we took advantage of our previously published *pbs-5* transgene [10]. To drive proteasome overexpression in the nervous system, we introduced in wt N2 nematodes a transgene (as an extrachromosomal array) composed of the promoter of the neuron-specific gene *rgef-1* [28] fused to *pbs-5* (*rgef-1p::pbs-5*) (referred to *pbs-5*-neuron hereafter; for simplicity we use simplified names for the various strains throughout the text, for complete genotypes please refer to the Materials and Methods section as well as to Table S1). We also created transgenic nematodes overexpressing *pbs-5* under the ubiquitous promoter of the gene *let-858* (*let-858p::pbs-5* strain; referred to *pbs-5*-ubiquitous hereafter). The relative control strain was only overexpressing the co-injection marker (*myo-2p::GFP*; referred to control hereafter). In accordance with our previous results [10], we detected increased transcript levels of *pbs-5* subunit in *pbs-5*-ubiquitous animals (Fig. S1). With regard to the *pbs-5*-neuron animals, although we generated two independent lines using different transformation markers (*myo-2p::GFP* and *rol-6(su1006)*), at most we only observed a slight increase in the levels of the *pbs-5* transcript (Fig. S1), probably due to the tissue-specific expression only in the neurons. Given that overexpression of β 5 subunit in all tissues in *C. elegans* (*pbs-5* [10]) and in neurons in *Drosophila* (*Pros β 5* [8,29]) and mouse (PSMB5 [8]) have been shown to enhance the resistance against proteotoxicity and to prevent cognitive deficits, we validated the neuronal proteasome overexpression with a functional assay. To this end, we crossed the *pbs-5*-neuron strain with the UA198 strain that expresses the human A β ₁₋₄₂ peptide in its GFP-tagged glutamatergic neurons, showing progressive, age-dependent neurodegeneration [26,30,31]. We scored significantly more surviving neurons in UA198 *pbs-5*-neuron animals compared to control nematodes on day 6 of adulthood (Fig. 1), thus suggesting a positive effect of neuronal *pbs-5* overexpression. Furthermore, these results also reveal an anti-proteotoxic effect of proteasome overexpression in a cell autonomous manner (modulation in neurons, positive effects in neurons).

Using the *pbs-5*-neuron strain, we initially addressed the question whether neuron-specific *pbs-5* overexpression affects the proteasome activity in a distal tissue. We took advantage of a well-characterized nematode strain expressing a modified ZsProSensor-1 fluorescent reporter under a muscle-specific promoter (referred to Muscle ZsProSensor-1 hereafter). More specifically, the modified ZsProSensor-1 reporter is fused to ubiquitin-interacting motifs derived from the *C. elegans* proteasome subunit RPN-10. Therefore, decreased fluorescence indicates increased proteasome-mediated degradation and vice versa (Fig. 2A) [32]. We crossed our control, *pbs-5*-neuron and *pbs-5*-ubiquitous animals with the Muscle ZsProSensor-1 strain. The fluorescence levels of ZsProSensor-1 reporter were significantly reduced in the muscle of both *pbs-5*-neuron and *pbs-5*-ubiquitous animals (Fig. 2B), thus revealing proteasome activation. To confirm these results and exclude potential effects due to the co-injection marker, we crossed the Muscle ZsProSensor-1 strain with *pbs-5*-neuron and *pbs-5*-ubiquitous animals carrying the *rol-6(su1006)* dominant transformation marker as a co-injection marker. We detected a consistent phenotype with the previous transgenic animals (Fig. 2C). We further sought to investigate whether neuron specific proteasome activation was also affecting other tissues. To this end, we crossed our *pbs-5*-neuron animals with the nematode strain expressing the modified ZsProSensor-1 fluorescent reporter under a gut-specific promoter (referred to Gut ZsProSensor-1); we did not detect enhanced proteasome activity in the gut, thus suggesting a tissue-specific cell non-autonomous effect (Fig. 2D). In total, our results reveal a cell non-autonomous regulation of the proteasome, since *pbs-5* overexpression in the nervous system led to enhanced proteasome activity levels in the muscle.

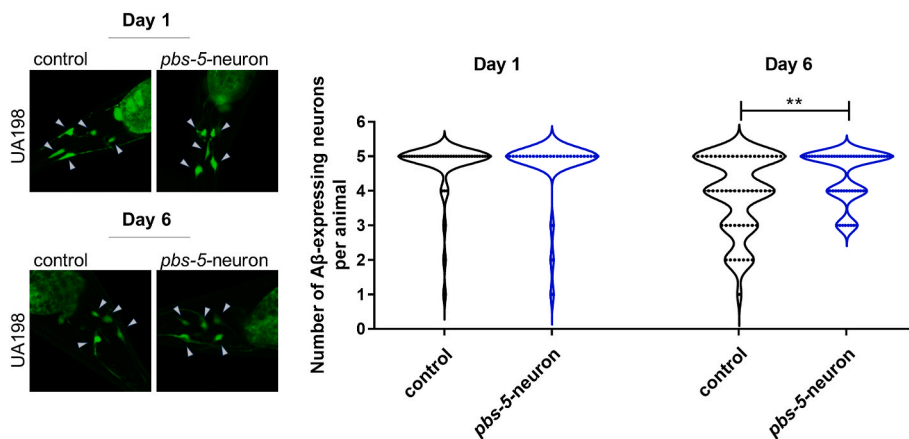


Fig. 1. Delayed A β -mediated glutamatergic neurodegeneration in *pbs-5*-neuron animals. Representative fluorescence micrographs of UA198 control and *pbs-5*-neuron animals expressing the human A β ₁₋₄₂ peptide in their GFP-tagged glutamatergic neurons, on days 1 and 6 of adulthood. The arrows point to intact neurons (left panel). Violin graph showing the number of tail glutamatergic neurons expressing the human A β ₁₋₄₂ peptide in each examined animal from UA198 control and *pbs-5*-neuron populations, on days 1 and 6 of adulthood. Each dot represents an examined animal (right panel). Graph shows the distribution of pooled values from 3 independent experiments, >30 animals/condition, ***p* value < 0.01 (two-tailed Student's *t*-test).

3.2. Neuron-specific proteasome overexpression confers anti-proteotoxic effects in a cell non-autonomous manner

We then sought to investigate whether the cell non-autonomous proteasome activation in the muscle may counteract a proteotoxic threat as it was previously shown for the ubiquitous *pbs-5* overexpression [10]. To this end, we crossed our *pbs-5*-neuron and *pbs-5*-ubiquitous animals with the CL4176 strain, an A β *C. elegans* model. This strain expresses the human A β ₁₋₄₂ peptide under a muscle-specific promoter and upon temperature upshift, they accumulate A β peptide in their body wall muscle cells and become paralyzed in a few hours [33]. Ubiquitous as well as neuron-specific proteasome overexpression significantly delayed the proteotoxic effects of A β peptide leading to delayed paralysis (Fig. 3A, Table S2). To confirm this protective effect, we evaluated the effects of ubiquitous and pan-neuronal proteasome overexpression in an additional A β nematode model, the GMC101 strain. These animals constitutively express the human A β ₁₋₄₂ peptide under a muscle-specific promoter and manifest age-progressive paralysis [34]. Consistent with our previous data, paralysis was also significantly delayed in GMC101 *pbs-5*-neuron and *pbs-5*-ubiquitous animals (Fig. 3B, Table S2). Moreover, ubiquitous and neuron-specific proteasome activation significantly reduced the levels of A β oligomers (Fig. 3C, Fig. S2). A tendency for reduced total A β levels was also recorded. Lack of significant changes in the transcript levels of A β peptide in our transgenic animals verified that the observed differences in the protein levels of A β forms are unlikely to arise from changes in the transcription of the transgene (Fig. 3D). To verify that the activation of the proteasome is responsible for the observed positive outcomes, we have then sought to inhibit this activation. We attempted to pharmacologically alter the proteasome activity by using epoxomicin, a highly specific proteasome inhibitor [35]. Following a series of dose-response experiments, we found that 1 mM epoxomicin (constant treatment for up to 12 h) could decrease proteasome activity in the Muscle ZsProSensor-1 strain (Fig. S3) without compromising survival. We then treated both CL4176 *pbs-5*-neuron and control animals with DMSO (vehicle) and 1 mM epoxomicin and paralysis assays were performed. Despite a small developmental delay exhibited by the animals treated with epoxomicin, we found that epoxomicin not only abrogated the delayed paralysis of *pbs-5*-neuron animals but it accelerated it compared to control animals treated with epoxomicin (Fig. 3E). This strongly suggests that proteasome activation underlies the observed beneficial effects on A β -mediated paralysis.

To further prove the link between proteasome activation and protection against proteotoxicity, we attempted to perform RNAi of *pbs-5* subunit in GMC101 *pbs-5*-neuron animals. Nonetheless, this proved technically impossible as the GMC101 *pbs-5*-neuron animals lost their delayed paralysis rates over their control counterparts, once grown on HT115 bacteria used for RNAi experiments (Fig. S4A). To cross-validate

these results, we evaluated the proteasome activity in Muscle ZsProSensor-1 *pbs-5*-neuron and control animals grown on HT115 bacteria. Indeed, the signal was not reduced anymore (Fig. S4B), as opposed to the observed reduction when the animals are grown on OP50 bacteria (Fig. 2B), thus suggesting that HT115 bacteria interfere with either the function of the proteasome and/or the communication between the neurons and the muscle. Nevertheless, we performed paralysis assays in GMC101 control animals in the presence of *pbs-5* RNAi; the paralysis was significantly accelerated (Fig. S4C), thus verifying the importance of the proteasome function on the paralysis phenotype.

As a proof of concept and to verify the link between proteasome activation in the muscle and the protection against A β proteotoxicity in the muscle, we created transgenic nematodes overexpressing *pbs-5* subunit under the promoter of the muscle-specific gene *myo-3* (*myo-3p::pbs-5* strain; referred to *pbs-5*-muscle hereafter). The relative control strain carried only the co-injection marker (*myo-2p::GFP*; referred to control hereafter). Quantitative PCR analysis confirmed *pbs-5* overexpression (Fig. 4A). To test whether muscle-specific *pbs-5* overexpression results in enhanced proteasome activity in the same tissue (i. e. cell autonomously), we initially crossed the *pbs-5*-muscle strain with the strain expressing the modified ZsProSensor-1 fluorescent reporter in the muscle. As expected, we found reduced levels of fluorescence indicating proteasome activation in Muscle ZsProSensor-1 *pbs-5*-muscle animals (Fig. 4B). We then crossed the *pbs-5*-muscle strain with the GMC101 animals. Paralysis was significantly delayed in GMC101 *pbs-5*-muscle animals, thus verifying that proteasome activation is sufficient for protection against A β proteotoxicity (Fig. 4C). Moreover, this result reveals a cell autonomous anti-proteotoxic effect (modulation in the muscle, positive effects in the muscle).

Mounting evidence suggests that the ALP is implicated in the clearance of misfolded or aggregated A β forms in various models, including *C. elegans* [36,37]. Moreover, the cross-talk between the UPS and ALP is well-accepted although not fully elucidated [38]. We therefore examined the potential involvement of autophagy in our setting. We took advantage of DLM9 strain that expresses under the muscle-specific promoter *myo-3p* a dual fluorescent protein (dFP) fused to LGG-1, a key protein in autophagosome formation. Upon docking and fusion to the lysosome, a monomeric fluorescent protein (mFP) is released, easily distinguishable from dFP via immunoblotting [39]. The ratio mFP/dFP gives an indication of the rate of autophagosome fusion with lysosomes, while a flux assay in the presence of chloroquine that inhibits the acidification of the lysosome thus, blocking the turnover of autolysosomes, reveals if the increased mFP/dFP ratio originates from increased autophagosome formation or block of autolysosome turnover (Fig. 4D). We crossed this strain with *pbs-5*-neuron and control animals. Autophagic flux was found unchanged in the muscle of DLM9 *pbs-5*-neuron animals (Fig. 4E), thus suggesting that the anti-proteotoxic effect of neuron-specific proteasome overexpression was independent of ALP

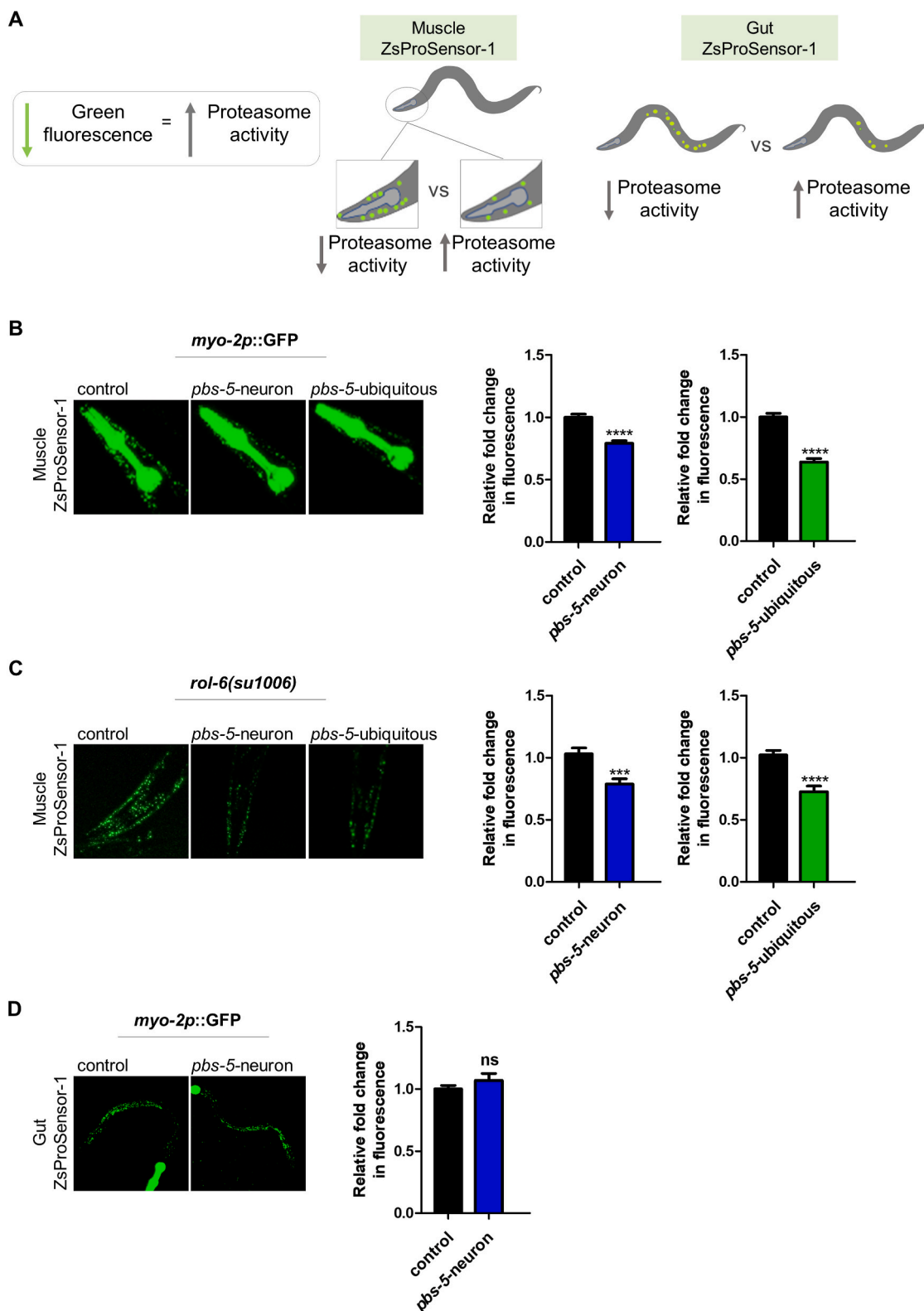
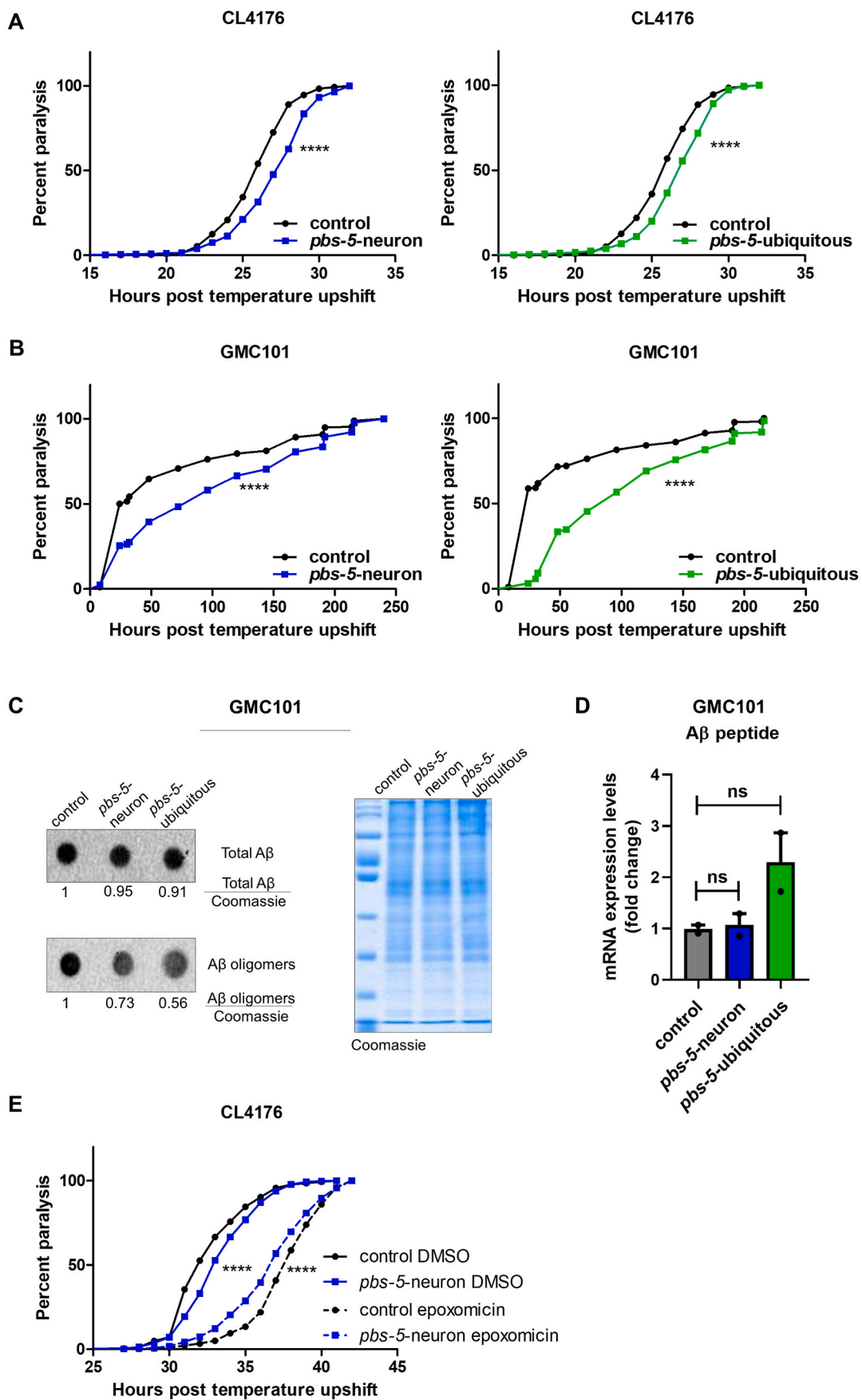


Fig. 2. Enhanced proteasome activity in the muscle of *pbs-5-neuron* animals. (A) Schematic describing the interpretation of the changes in the ZsProSensor-1 signal while monitoring proteasome activity in the muscle (Muscle ZsProSensor-1) or the gut (Gut ZsProSensor-1) of transgenic nematodes. Decreased fluorescence indicates increased proteasome activity and vice versa. (B, C, D) Representative fluorescence micrographs of animals expressing the ZsProSensor-1 reporter (left panel) and quantification of the ZsProSensor-1 signal (right panel) in (B, C) the muscle and (D) the gut, crossed with control, *pbs-5-neuron* or *pbs-5-ubiquitous* animals (only control and *pbs-5-neuron* animals in D) carrying (B, D) the *myo-2p::GFP* co-injection marker and, (C) the *rol-6(su1006)* co-injection marker. The mean value of fluorescence in control animals was set to 1. All values are reported as the mean of at least 3 independent experiments \pm SEM, >20 animals/condition, ****p* value < 0.001, *****p* value < 0.0001, ns, not significant (two-tailed Student's *t*-test).



(caption on next page)

Fig. 3. Neuron-specific proteasome overexpression confers protection against A β proteotoxicity in the muscle of *pbs-5*-neuron animals (cell non-autonomous effect). (A, B) Paralysis curves of control, *pbs-5*-neuron (left panels) and *pbs-5*-ubiquitous (right panels) animals expressing the human A β_{1-42} peptide in their body wall muscle cells, (A) CL4176 strain (temperature-dependent expression of the human A β_{1-42} peptide in the muscle with progressive paralysis within hours), 8 independent experiments with >800 animals, *****p* value < 0.0001 (log-rank Mantel-Cox test) and, (B) GMC101 strain (constitutive expression of the human A β_{1-42} peptide in the muscle with age-progressive paralysis), 3 independent experiments with >140 animals, *****p* value < 0.0001 (log-rank Mantel-Cox test). Curves are the pooled result of the indicated independent experiments. (C) Dot blot analysis (representative blots from 4 independent experiments) of total A β and A β oligomers in GMC101 control, *pbs-5*-neuron and *pbs-5*-ubiquitous animals expressing the human A β_{1-42} peptide in their body wall muscle cells, collected when 50% of the control population was paralyzed. A gel stained with Coomassie brilliant blue was used as a loading control in each experiment. The mean value of signal of the protein of interest to the Coomassie signal in control animals was set to 1. Quantification that appears underneath the blots represents the mean of 4 independent experiments. (D) mRNA expression levels of A β peptide in GMC101 control, *pbs-5*-neuron and *pbs-5*-ubiquitous animals expressing the human A β_{1-42} peptide in their body wall muscle cells on day 1 of adulthood. The mean value of A β peptide mRNA expression in control animals was set to 1. All values are reported as the mean of 2 independent experiments \pm SEM, ns, not significant (Kruskal-Wallis test). (E) Paralysis curves of CL4176 control and *pbs-5*-neuron animals expressing the human A β_{1-42} peptide in their body wall muscle cells treated with DMSO or 1 mM epoxomicin, 4 independent experiments with >450 animals, *****p* value < 0.0001 (log-rank Mantel-Cox test). Curves are the pooled result of 4 independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

activation.

In conclusion, neuron-specific proteasome activation can exert protection against A β proteotoxicity in the muscle in a cell non-autonomous manner.

3.3. The cell non-autonomous protective effects following neuronal proteasome activation are not related to protection against oxidative stress

Given the established link between oxidative stress and AD [40,41] along with the interplay between the proteasome and oxidative stress response [42–44], we sought to investigate whether the observed beneficial effects against A β proteotoxicity were linked to enhanced oxidative stress response. We thus challenged our *pbs-5*-neuron and *pbs-5*-ubiquitous animals with two different oxidative stressors, namely paraquat and NaN₃. Although proteasome activation in all tissues induced protection against oxidative stress (Fig. 5A-B; *pbs-5*-ubiquitous) in accordance with our previous results [10], notably, no resistance against oxidative stress was recorded in the *pbs-5*-neuron animals (Fig. 5A-B; *pbs-5*-neuron). Our results suggest that the neuronal proteasome activation confers cell non-autonomous protective effects in the muscle specifically against proteotoxic stress and not against oxidative stress. Given that the transcription factor SKN-1 is pivotal in oxidative stress resistance while it triggers strong responses to proteasome perturbations [45–47], we also investigated whether SKN-1 activation could account for the protective effects against A β -proteotoxicity. We therefore revealed the mRNA expression levels of *skn-1* as well as of three of its target genes, namely *gst-4*, *dod-24* and *nit-1* [47], in control, *pbs-5*-neuron and *pbs-5*-ubiquitous animals in wt and GMC101 backgrounds. Although in the *pbs-5*-ubiquitous animals, *gst-4* was significantly upregulated (statistically significant in wt background, exhibiting a tendency in GMC101 background without reaching statistical significance), none of the *skn-1* target genes or *skn-1* itself were upregulated in *pbs-5*-neuron animals in either background (Fig. 5C). In total our results suggest that the distal protective effects we score upon neuronal proteasome activation are specific for the A β proteotoxic stress and not for the related oxidative stress.

3.4. The cell non-autonomous activation of the proteasome in the muscle is mediated by SCVs

Our results suggested that one or more neuronal-derived signals prompt proteasome reinforcement in a non-neuronal tissue such as the muscle. The communication between the nervous and the muscle tissues is accomplished via synaptic transmission [48]. In *C. elegans*, neurotransmitters are secreted from neurons in two types of vesicles classified as SCVs and DCVs, based on their size and appearance in electron micrographs [49]; Fig. 6A). We hypothesized that neurons that overexpress the *pbs-5* subunit may affect proteasome activation in the muscle through a neuroendocrine signaling pathway mediated by SCVs and/or DCVs. To address this hypothesis, we crossed our *pbs-5*-neuron animals

with *unc-64* mutants that are deficient in the priming or exocytosis of both DCVs and SCVs [50]. To obtain a fast readout, the derived transgenic animals (*unc-64(e246)*; *pbs-5*-neuron) and their controls were further crossed with the Muscle ZsProSensor-1 strain. We found that the loss-of-function *unc-64(e246)* allele completely abolished the proteasome-activating effect of neuronal *pbs-5* overexpression on muscular proteasome activity (Fig. 6B), indicating that the activating signal is most likely contained in SCVs and/or DCVs.

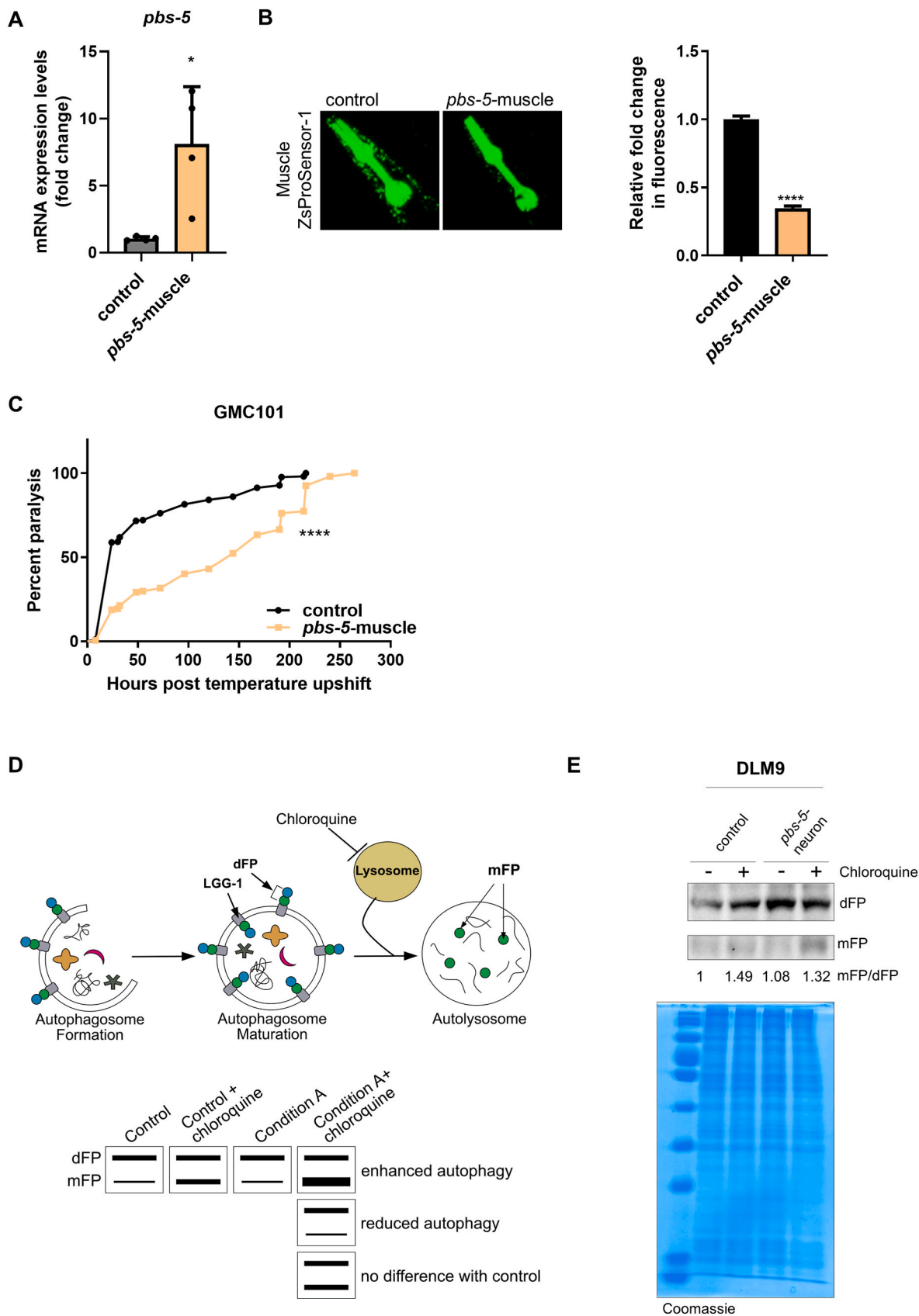
We then sought to tease apart the roles of the two vesicle types and to reveal which one is responsible for the cell non-autonomous communication of neuronal proteasome activation. The *unc-31* gene is known to participate in the exocytosis of DCVs while the *unc-13* in the priming of SCVs [51] (Fig. 6A). Although the *unc-31(e928)* loss-of-function allele did not inhibit the cell non-autonomous effect of proteasome activation in the muscle (Fig. 6C), the *unc-13(e51)* loss-of-function allele completely abolished the proteasome activation occurring in the muscle of ZsProSensor-1 *pbs-5*-neuron animals (Fig. 6D). Our results show that SCVs are responsible for the cell non-autonomous proteasome activation in the muscle following neuronal proteasome overexpression.

Next, we asked whether disruption of the SCVs-mediated communication between neurons and muscle is also translated into loss of protection against A β proteotoxicity. Since *unc-13(e51)* mutants are almost completely immobile, performing paralysis assays in *unc-13(e51)*; *pbs-5*-neuron animals was impossible. Nevertheless, *unc-64(e246)* mutants are not immobile. They have defects in both DCVs and SCVs but our results had already excluded the involvement of DCVs in the cell non-autonomous proteasome activation in the muscle (Fig. 6C). The *unc-64(e246)*; *pbs-5*-neuron animals did not exhibit decreased paralysis rates in neither CL4176 (Fig. 6E) nor GMC101 (Fig. 6F) backgrounds (Table S2). Together, our findings demonstrate that *pbs-5* overexpression in neurons stimulates proteasome activation in the muscle through SCVs, resulting in protection against A β proteotoxicity.

3.5. Glutamate can restore proteasome activity levels in the muscle of *pbs-5*-neuron animals lacking SCVs

We then sought to identify the potential neurotransmitter(s) contained in SCVs that could transmit the signal from the neurons leading to proteasome activation in the muscle. SCVs contain classical neurotransmitters such as glutamate, γ -aminobutyric acid (GABA) and acetylcholine [49]. Since the *unc-13(e51)* mutants are unable to produce SCVs, we anticipated that external supplementation of the responsible molecule(s) would restore the effects of neuronal *pbs-5* overexpression in the muscle of *unc-13(e51)*; *pbs-5*-neuron animals. We therefore tested various concentrations of the three main neurotransmitters, namely glutamate, GABA and acetylcholine in Muscle ZsProSensor-1 *unc-13*; *pbs-5*-neuron animals and their controls (Fig. S5). Proteasome activity was enhanced only upon glutamate supplementation (Fig. 7A, Fig. S5A).

Since we could not perform paralysis assays in *unc-13(e51)* mutants due to their immobile phenotype, we tested whether glutamate



(caption on next page)

Fig. 4. Proteasome activation in the muscle confers protection against A β proteotoxicity in the muscle (cell autonomous effect). (A) mRNA expression levels of *pbs-5* proteasome subunit in control and *pbs-5*-muscle animals. The mean value of *pbs-5* mRNA expression in control animals was set to 1. All values are reported as the mean of 4 independent experiments \pm SEM, **p* value < 0.05 (Mann-Whitney test). (B) Representative fluorescence micrographs of control and *pbs-5*-muscle animals carrying the Muscle ZsProSensor-1 reporter (left panel) and quantification of the Muscle ZsProSensor-1 signal (right panel). The mean value of fluorescence in control animals was set to 1. All values are reported as the mean of at least 3 independent experiments \pm SEM, >25 animals/condition, *****p* value < 0.0001 (two-tailed Student's *t*-test). (C) Paralysis curves of GMC101 control and *pbs-5*-muscle animals expressing the human A β ₁₋₄₂ peptide in their body wall muscle cells, 3 independent experiments with >140 animals, *****p* value < 0.0001 (log-rank Mantel-Cox test). Curves are the pooled result of 3 independent experiments. (D) Schematic describing the potential outcomes and their interpretation when performing a flux assay to a strain expressing a dual fluorescent protein (dFP) fused to LGG-1. LGG-1dFP is localized on the membrane of the autophagosome. During autophagosome maturation some mFP is released but most of the mFP is released by the lysosomal proteases once the autophagosome fuses with the lysosome. However, the mFP released in the autolysosome is rapidly degraded, hence very little is detected in steady-state levels. The steady-state amount of mFP can reflect both the efficiency of the lysosomal proteolysis as well as the rate of autophagosome formation. Chloroquine inhibits the acidification of the lysosome, thus inhibiting lysosomal proteases and blocking lysosomal proteolysis. Hence addition of chloroquine can induce the accumulation of mFP. Depending on the accumulated mFP levels in the sample compared to control, the results from the sample treated with chloroquine indicate the origin of mFP, thus the status of autophagic flux. (E) Immunoblot analysis (representative blots from 5 independent experiments) of dFP and mFP in lysates from control and *pbs-5*-neuron animals expressing LGG-1 tagged with dFP, in the absence (-) or presence (+) of chloroquine for evaluation of the autophagic flux. A gel stained with Coomassie brilliant blue was used as a loading control. The mean value of signal of mFP/dFP in control (-) animals was set to 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

supplementation could restore the proteasome activity in the muscle of *unc-64(e246)* mutants. Surprisingly, we found that external glutamate supplementation did not restore the proteasome activity in the muscle of *pbs-5*-neuron animals in an *unc-64* mutant background (Fig. 7B). Given that the *unc-64* mutants have defects in both SCVs and DCVs, the inability of glutamate alone to restore the proteasome activity in the muscle in these mutants suggests that glutamate can compensate for the lack of SCVs only in the presence of functional DCVs, thus attributing a role to the DCVs and their cargo in this inter-tissue communication. We therefore investigated whether combined supplementation of glutamate (mainly found in SCVs) and other neurotransmitters that are most probably found in DCVs, could restore the proteasome activity in the muscle of *unc-64* mutants and thus the neuron-muscle communication. To this end, we treated Muscle ZsProSensor-1 *unc-64*; *pbs-5*-neuron animals, with glutamate and various concentrations of the 4 biogenic amines that are packaged in DCVs, namely octopamine, serotonin, dopamine and tyramine [49]. We did not detect any restoration of the proteasome activity with the combined supplementation of glutamate and biogenic amines in *unc-64* mutants (Figs. S6A–D). Consequently, the observed results might be due to the action of neuropeptides found in DCVs and therefore the potential cargo of DCVs that plays a role in the neuron-muscle communication needs further investigation. Although GABA and acetylcholine did not restore muscular proteasome activity in *unc-13* mutants (SCVs-defective animals; Figs. S5B–C), *unc-64* mutants have defects in both SCVs and DCVs and therefore, we reasoned that in these animals, neurotransmitters commonly found in SCVs could take over and exhibit additional regulatory roles. We therefore treated Muscle ZsProSensor-1 *unc-64*; *pbs-5*-neuron animals with a combination of glutamate and GABA or acetylcholine. Interestingly, we found that the double supplementation with glutamate and either GABA or acetylcholine results in reduction of proteasome activity in Muscle ZsProSensor-1 *unc-64*; *pbs-5*-neuron animals (Figs. S6E–F). This suggests that GABA and acetylcholine may have proteasome inhibitory roles that are counteracted by DCVs-dependent signals, as we never observed proteasome inhibition by GABA or acetylcholine in animals that are defective for SCVs but have intact DCVs (Figs. S5B–C).

To dissect the mechanism behind glutamate-mediated proteasome activation, we assessed whether glutamate may have direct stereochemical effects on the proteasome complex resulting in its activation. Incubation of purified proteasome with different concentrations of glutamate did not reveal any proteasome activating properties (Fig. 7C), thus suggesting that the glutamate effect on proteasome activity is most likely indirect. We have also checked whether glutamate addition in animals can phenocopy the results of neuronal *pbs-5* overexpression on proteasome activity. Glutamate supplementation in Muscle ZsProSensor-1 control animals did not affect their proteasome activity in the muscle (Figs. 7D, 2 first columns). Likewise, glutamate supplementation of Muscle ZsProSensor-1 *pbs-5*-neuron animals did not further

alter the already enhanced proteasome activity due to the *pbs-5* overexpression (Figs. 7D, 2 last columns). These results suggest that proteasome activation in the muscle of *pbs-5*-neuron animals is not due to a generic or unspecific proteasome-activating effect of glutamate but rather to a specific regulated process.

In conclusion, we show that proteasome activity is subjected to cell non-autonomous regulation since *pbs-5* overexpression in the neurons promotes proteasome activation in the muscle of *C. elegans*. Glutamate contained in SCVs is necessary for this activation but the presence of intact DCVs is also required. This cell non-autonomous proteasome activation is translated into efficient prevention of A β -mediated proteotoxic effects in the muscle of *C. elegans*.

4. Discussion

Proteinopathies including AD have been linked to inefficient protein degradation machineries, such as the UPS or the ALP [52,53]. This inefficiency is further accentuated during ageing which in turn is a top risk factor for aggregation-related diseases [2,54]. Consequently, reinforcement of the proteolytic part of proteostasis during ageing and upon disease progression has been suggested as a pivotal potential preventive and/or therapeutic approach. Here, we report the cell non-autonomous communication of neuronal proteasome activation. We show that overexpression of the *pbs-5* proteasome subunit in the nervous system can promote proteasome activation in the muscle tissue of *C. elegans*. The result is enhanced resistance to A β proteotoxicity in the distal tissue (muscle) but not enhanced resistance to oxidative stress. This communication depends on intact SCVs and glutamate, thus establishing a mechanistic link between neuronal proteasome reinforcement and decreased A β proteotoxicity in the muscle. Furthermore, we show that in the absence of SCVs, glutamate can compensate only in the presence of functional DCVs, thus suggesting a role of the DCVs' cargo in this distal tissue communication as well. Cell autonomous anti-proteotoxic effects are also revealed upon neuron- and muscle-specific proteasome activation.

Our *in vivo* approach conclusively demonstrates the cell non-autonomous control of proteasome function as previously described for other players of proteostasis such as the HSR [13], UPR^{ER} [18,19,55], UPR^{mit} [17] and autophagy [20]. Among the proteolytic pathways, the lysosome-mediated degradation [18] has been already shown to be affected in other tissues once proteostatic mechanisms such as the UPR^{ER} are modulated in neurons [18,55] or in the intestine [18]. Neuron-specific *Prosβ5* overexpression in *Drosophila* has been shown to slow down age-related deficits in learning, memory, and circadian rhythmicity [29]. A follow-up of this study showed that neuronal-specific overexpression of β5 subunit in *Drosophila* (*Prosβ5*) and in mice (PSMB5) was sufficient to ameliorate disease-related phenotypes in the respective AD models [8]. Notably, despite the attempts

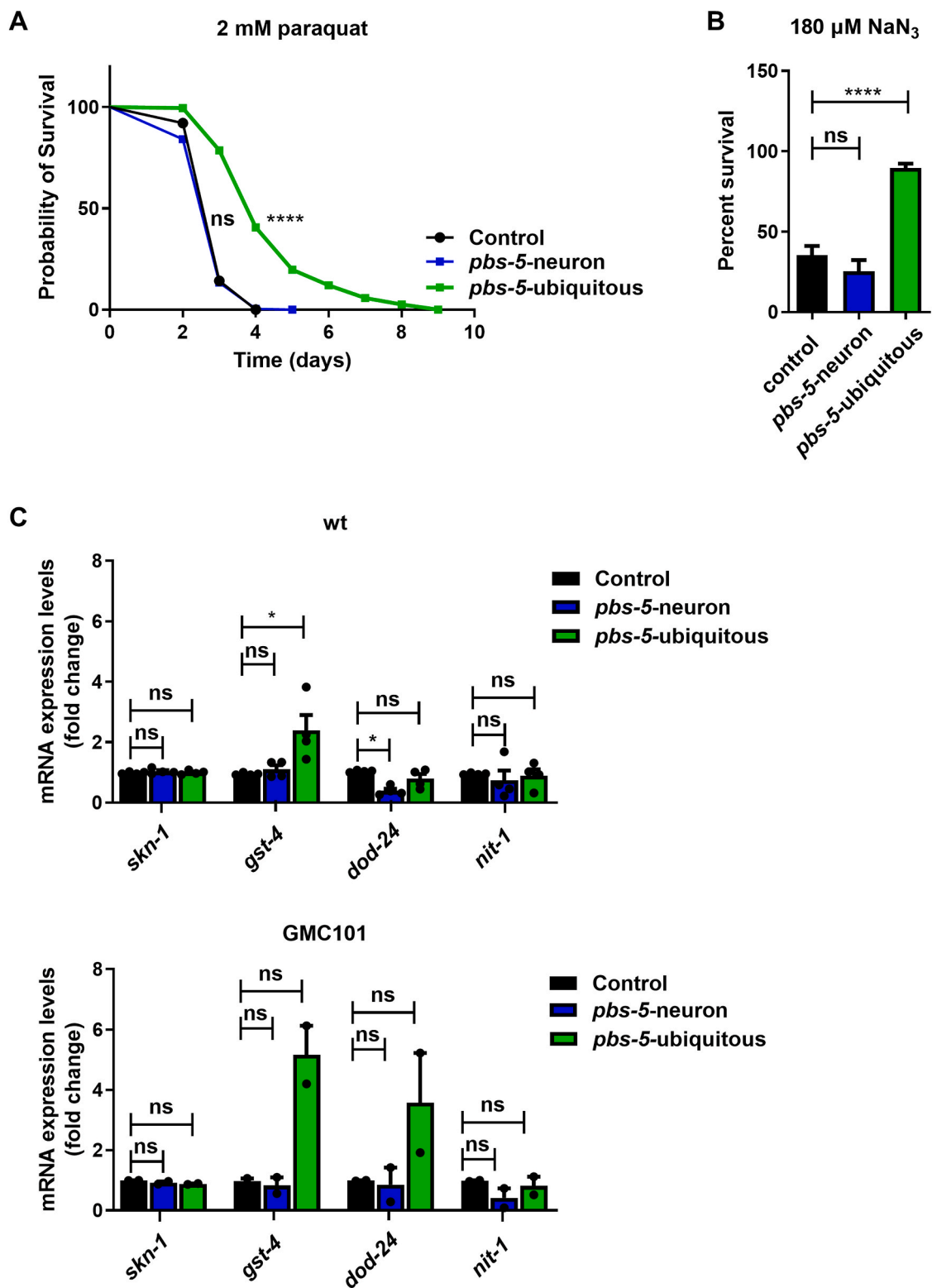
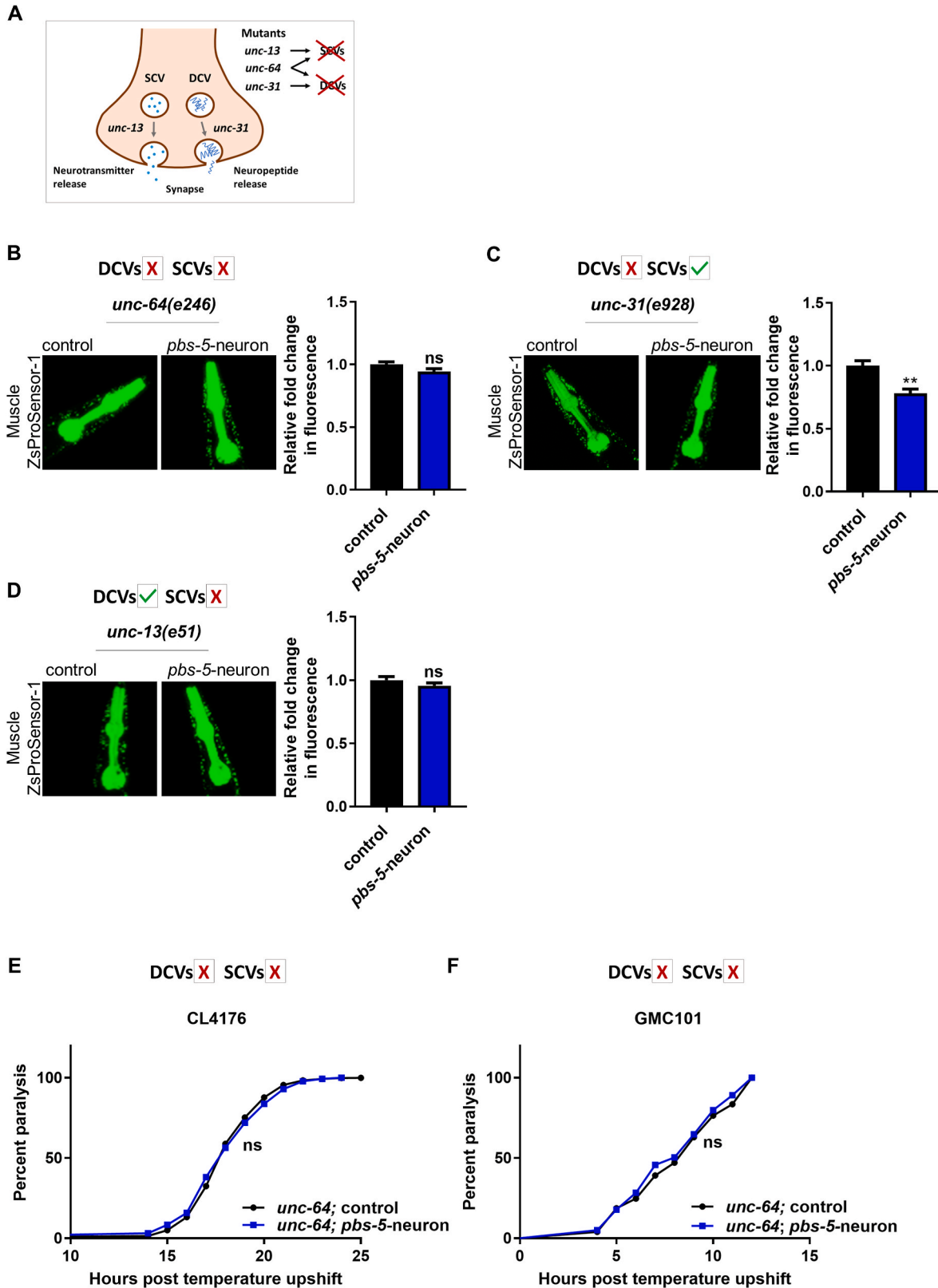


Fig. 5. Neuron-specific proteasome overexpression does not confer protection against oxidative stress as opposed to ubiquitous proteasome overexpression. (A) Lifespan curve of control, *pbs-5-neuron* and *pbs-5-ubiquitous* animals treated with 2 mM paraquat, 3 independent experiments with >190 animals per condition, *****p* value < 0.0001, ns, not significant (log-rank Mantel-Cox test). Curves are the pooled result of 3 independent experiments. (B) Survival percentage of control, *pbs-5-neuron* and *pbs-5-ubiquitous* animals 24 h after exposure to 180 μM NaN_3 , 3 independent experiments with >350 animals per condition, *****p* value < 0.0001, ns, not significant (one-way ANOVA, Tukey's multiple comparisons test). (C) mRNA expression levels of *skn-1* and *skn-1* target genes (*gst-4*, *dod-24*, *nit-1*) in wt (upper panel) and GMC101 (lower panel) control, *pbs-5-neuron* and *pbs-5-ubiquitous* animals in the young adult stage. The mean value of the mRNA expression of each gene in control animals was set to 1. All values are reported as the mean of 4 (for the wt animals) and 2 (for the GMC101 animals) independent experiments \pm SEM, **p* value < 0.05, ns, not significant (Mann-Whitney test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

for tissue-specific proteasome activation, no data exists on its cell non-autonomous regulation and the outcomes in distal tissues.

Tissue-specific proteotoxic stress has been shown to trigger various

proteostatic mechanisms cell non-autonomously in distal tissues as a compensatory response to stress. For example, neuronal overexpression of an aggregation-prone protein (polyQ40) and its binding on neuronal



(caption on next page)

Fig. 6. The cell non-autonomous effects of neuron-specific proteasome overexpression is dependent on SCVs. (A) Schematic describing the involvement of UNC-13 and UNC-31 in the release of neurosecretory vesicles. UNC-13 participates in the priming of SCVs containing small-molecule neurotransmitters. UNC-31 participates in the exocytosis of DCVs primarily containing neuropeptides. (B, C, D) Representative fluorescence micrographs of animals expressing the Muscle ZsProSensor-1 reporter crossed with control or *pbs-5*-neuron animals carrying the *myo-2p::GFP* co-injection marker in (B) *unc-64*, (C) *unc-31* and, (D) *unc-13* mutant backgrounds (left panels) and quantification of the Muscle ZsProSensor-1 signal for each set of experiments (right panels). The mean value of fluorescence in control animals was set to 1. All values are reported as the mean of at least 3 independent experiments \pm SEM, >20 animals/condition, ***p* value < 0.01, ns, not significant (two-tailed Student's *t*-test). (E, F) Paralysis curves of control and *pbs-5*-neuron animals expressing the human A β_{1-42} peptide in their body wall muscle cells in an *unc-64* mutant background, (E) CL4176 strain (temperature-dependent expression of the human A β_{1-42} peptide in the muscle with progressive paralysis within hours), 9 independent experiments with >1000 animals per condition, ns, not significant (log-rank Mantel-Cox test) and, (F) GMC101 strain (constitutive expression of the human A β_{1-42} peptide in the muscle with age-progressive paralysis), 3 independent experiments with >250 animals per condition, ns, not significant (log-rank Mantel-Cox test). Curves are the pooled result of the indicated independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mitochondria has been shown to elicit a global induction of a mitochondrial-specific unfolded protein response (UPR^{mit}), affecting whole-animal physiology [17]. With regard to the proteasome, silencing of various proteasome subunits (i.e. proteasome stress) in the skeletal muscle of *Drosophila* has been shown to mount an adaptive response in the brain that finally hinders retinal and brain ageing [56]. Nevertheless, our study is the first to show that a positive modulation of the proteasome (activation) is communicated to a distal tissue resulting in tissue protection (increased resistance to A β proteotoxicity).

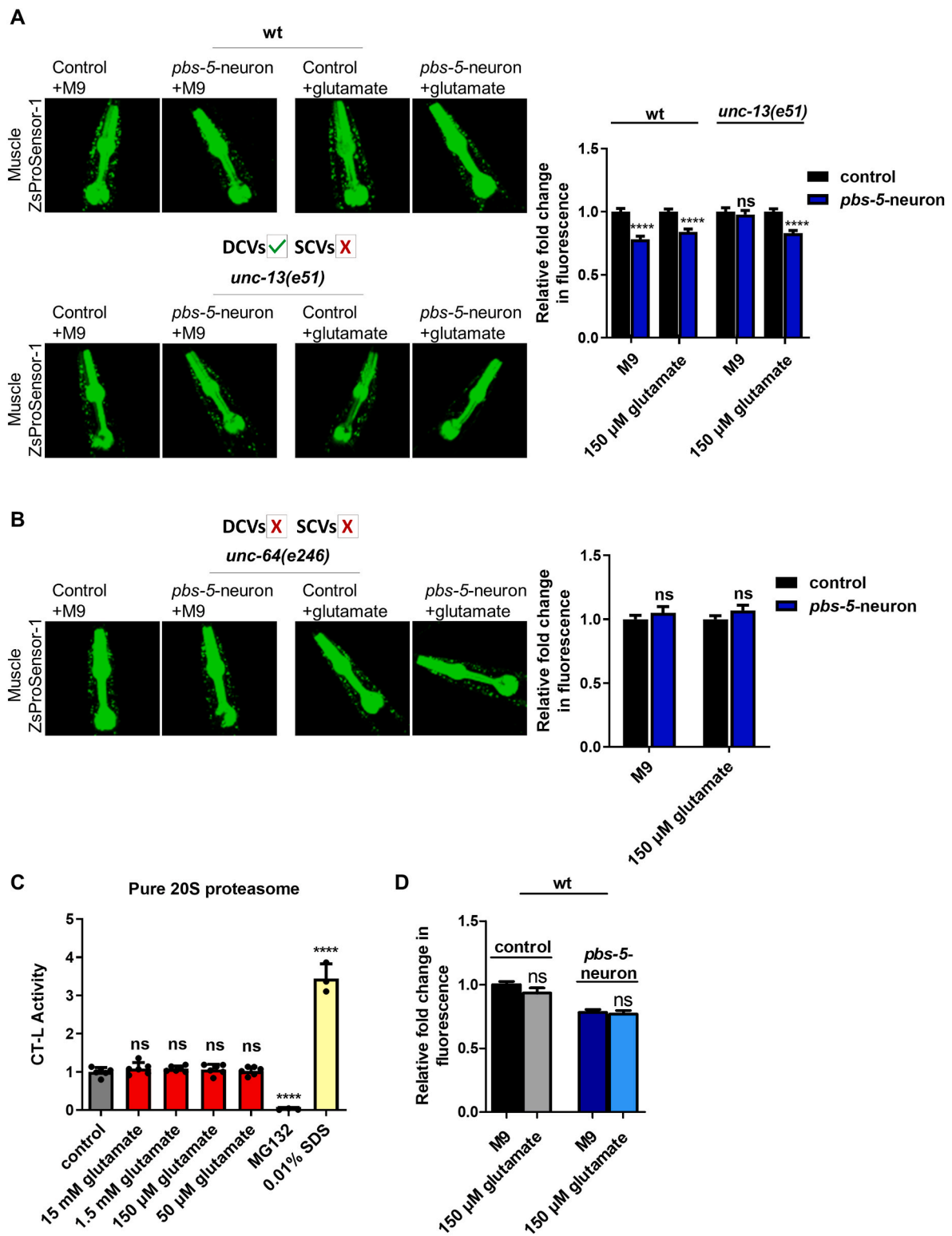
Our data clearly show that neuronal overexpression of the *pbs-5* subunit leads to proteasome activation in a distal tissue such as the muscle and its resistance to A β proteotoxicity. Although we excluded the implication of the intestine since we did not detect enhanced proteasome activity in this tissue upon neuronal proteasome overexpression, we cannot rule out that other tissues like hypodermis or the somatic gonad could be also affected by the proteasome activation in the nervous system. It would be interesting to assess if non-neuronal tissues that are affected by the nervous system also respond to the neuronal proteasome activation and further communicate with other tissues thereby creating an inter-tissue network of higher complexity.

One could ask how proteasome activation confers protection against A β proteotoxicity. Our results revealed significantly lower oligomeric A β levels (and a tendency for reduced total A β levels) in *pbs-5*-neuron animals. This suggests that proteasome activation may promote the degradation of A β species before they become highly aggregated and thus inaccessible and inhibitory to the proteasome [57,58]. In our previous work, proteasome activation through genetic means (systemic *pbs-5* overexpression) [10] or through natural compounds with proteasome-activating properties [59,60], had elicited similar results. Likewise, in a very recent work, genetic and pharmacological proteasome augmentation also resulted in enhanced clearance of APP in flies, cultured cells and mice [8]. Even more importantly, a negative correlation between proteasome activity and protein levels of APP and soluble A β_{42} levels in human hippocampal tissues of AD patients was revealed, thus strengthening the biological relevance of the findings in the organismal models [8]. Finally, combined supplementation of 18 α -glycyrrhetic acid (that activates the proteasome through enhanced transcription of several proteasome subunits [59,61]) and omega-3 fatty acids (that have been shown to directly positively modulate proteasome activity [62]) also resulted in a significant reduced percentage of A β_{42} coverage in the parietal cortex as well as in the hippocampus of 5xFAD (transgenic mice carrying 5 familial AD-associated mutations) mice [62]. It is well established in yeast and mammalian cells that the proteasome crosstalks with autophagy [63] while the same has been demonstrated in *C. elegans* [64,65]. Although we did not detect ALP activation in our setting, thus attributing a major role to the proteasome system for the observed results, one cannot rule out the potential implication of the ALP possibly in a later stage, when heavily aggregated proteins are formed. This was also suggested recently by an elegant study on small molecule VCP/p97 activators where both proteasome and autophagy routes shared a role in the clearance of different forms of misfolded species [66]. In total, our findings suggest that proteasome-related protection against A β

proteotoxicity emerges from enhanced clearance of various A β forms.

In cases where tissue-specific manipulation of a proteostatic mechanism has been achieved, studies mainly focused on the overall organismal fitness and longevity without focusing on the effects on specific distal tissues. Only a few studies have dealt with the effects of proteostasis manipulation in specific tissues and the downstream distal effects in models for protein aggregation [18,67]. Most importantly, none of them has investigated the role of the proteasome. We clearly show the beneficial effects of neuronal proteasome overexpression against A β proteotoxicity in the neuronal tissue but also in the periphery. Mechanistically, the “nervous system-to-periphery” cell non-autonomous communication usually occurs via extracellular signaling molecule release [68,69]. The distal activation of stress responses upon interventions in the nervous system has been shown to be mediated by neurons [13,19,55] and recently by glial cells [67]. Depending on the proteostatic mechanism that is modulated, either SCVs [55,70] or DCVs [17,67] have been shown to be responsible for the distal effects. We clearly show that SCVs are required for the cell non-autonomous communication of the neuronal proteasome activation which was rather surprising. Given that DCVs contain neuropeptides that are potential proteasome substrates and since in our setting the proteasome activity is enhanced, we had speculated that neuropeptides (and thus DCVs) could be the ones to be responsible for the observed effects. Moreover, a 20S proteasome form that is tightly associated with the neuronal plasma membrane and exposed to the extracellular space has been suggested to exist. This proteasome type has been shown to mediate the 20S-dependent degradation of intracellular proteins into bioactive extracellular peptides that modulate neuronal function [71,72]. As our results indicate the implication of DCVs as well, it would be interesting to investigate further their role and to examine whether the observed enhanced proteasome activity in our *pbs-5*-neuron animals affects this particular type of proteasomes and therefore the extracellular peptides produced by those proteasomes.

We also identified glutamate as a central player, without however being able to exclude the involvement of other neurotransmitters. How can glutamate restore this “proteasome activating” signal in *pbs-5*-neuron animals that lack SCVs? A direct or an indirect impingement of glutamate on proteasome activity could be speculated. We ruled out a direct effect on the proteasome complex since glutamate was not able to activate the 20S core proteasome in a cell-free assay. Moreover, direct administration of glutamate in wt background animals was not sufficient to promote proteasome activation whereas glutamate supplementation in animals with neuronal proteasome activation did not further enhance the proteasome activity. It seems that neuron-specific proteasome overexpression should precede before glutamate can function as a messenger provided however that DCVs are also functional. Given the multiple roles of glutamate, primarily as a neurotransmitter ([73] but also as a precursor to the antioxidant glutathione [74] or as a modulator of the tricarboxylic acid cycle once converted into α -ketoglutarate [74], glutamate could contribute to redox or energy status alterations that may significantly impact proteasome activity and function. More extensive studies on the metabolic effects of *pbs-5* neuronal overexpression are needed.



(caption on next page)

Fig. 7. Glutamate supplementation restores the cell non-autonomous activation of the proteasome in *unc-13* mutants. (A, B) Representative fluorescence micrographs of animals expressing the Muscle ZsProSensor-1 reporter crossed with control or *pbs-5*-neuron animals carrying the *myo-2p::GFP* co-injection marker, in (A) wt and *unc-13* mutant backgrounds treated with M9 (vehicle) or 150 μ M glutamate (left panel) and quantification of the Muscle ZsProSensor-1 signal (right panel). The mean value of fluorescence in control animals was set to 1. All values are reported as the mean of 3 independent experiments \pm SEM, >20 animals/condition, *****p* value < 0.0001, ns, not significant (two-tailed Student's *t*-test) and, (B) *unc-64* mutant background treated with M9 (vehicle) or 150 μ M glutamate (left panel) and quantification of the Muscle ZsProSensor-1 signal (right panel). The mean value of fluorescence in control animals was set to 1. All values are reported as the mean of 3 independent experiments \pm SEM, >20 animals/condition, ns, not significant (two-tailed Student's *t*-test). (C) CT-L proteasome activity of pure 20S proteasome in the presence of various concentrations of glutamate in cell-free assays. MG132 (proteasome inhibitor) was used as a negative and SDS (proteasome activator) as a positive control. The mean value of CT-L activity in control sample (no glutamate) was set to 1. All values are reported as the mean of 2 independent experiments \pm SEM, *****p* value < 0.0001, ns, not significant (one-way ANOVA with Dunnett's Multiple Comparison). (D) Quantification of data from (A) comparing the relative fold change in fluorescence between wt control animals carrying the *myo-2p::GFP* co-injection marker (left columns) and *pbs-5*-neuron animals (right columns) supplemented with M9 (vehicle) or 150 μ M glutamate. The mean value of fluorescence in control animals supplemented with M9 was set to 1. All values are reported as the mean of 3 independent experiments \pm SEM, >20 animals/condition, ns, not significant (two-tailed Student's *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

There are limitations in our study mainly linked to constraints imposed by our model. For example, although we partially respond to the question, we were not able to fully elucidate the mechanistic details through which glutamate triggers the increase of the proteasome activity in the muscle. This is mainly due to the fact that in *C. elegans* it is virtually impossible to isolate and analyse the different involved tissues in order to understand tissue-specific dysregulation in an inter-tissue communication context. Finally, we cannot investigate the role of the nervous system in the regulation of the proteasome activity of the muscle under more physiological conditions, i.e. in the absence of *pbs-5* OE. This would suggest the comparison of wt nematodes to mutants for the genes we have identified as responsible for the distal communication, i.e. *unc-64* and *unc-13*. Since these mutants have severe feeding defects (reduced pharyngeal pumping), this comparison is not adequate as these defects may significantly affect the proteasome activity. Nevertheless, our results emphasize on functional insights and are important for potential future applications.

In this study, we show that cell non-autonomous proteasome activation can contribute to anti-proteotoxic effects in nematode models for β toxicity. This could be a common regulatory mechanism in other metazoans, as well as in other protein aggregation-related diseases. This point is of particular significance as it demonstrates how cell non-autonomous proteostasis regulation can have a functional outcome in distal tissues. It is highly important since various neurodegenerative diseases are rarely restrained to a single tissue and are rather accompanied by peripheral maladies exhibited as metabolic alterations and deficiencies in non-neuronal tissues [75,76]. Deciphering this cell non-autonomous communication further in invertebrates along with complementary studies in vertebrates, may have serious implications in the design of tissue-specific proteasome manipulation strategies with therapeutic interest. Our results may contribute significantly to the optimization of proteostatic manipulation to fight proteinopathies through tissue-targeting. It is however also important to reveal all the potential cell non-autonomous affected aspects of proteostatic mechanisms, not only to optimize a tissue-specific manipulation *per se* but also to guarantee that one takes into account all the anticipated side effects, whether positive or negative. Moreover, the identification of the proteasome cell non-autonomous regulation may affect the translation of results of groups working in various fields where the proteasome is affected or manipulated, including the cancer and malaria fields where proteasome inhibition is aimed and tissue-specific manipulation is targeted as anti-cancer and anti-malaria therapeutic avenues, respectively. Finally, our results are also important for physiological processes in which the proteasome and its manipulation are important such as ageing. In total, our study provides the context for elucidating the precise mechanism through which cell non-autonomous proteasome regulation occurs and indicates potential tissue-specific avenues against, but not exclusively, proteinopathies.

5. Conclusions

Our study demonstrates that proteasome activation in the nervous system can enhance the proteasome activity in the muscle of *C. elegans*, thus revealing for the first time a cell non-autonomous regulation of the proteasome function. We show that this distal communication is mediated through SCVs and glutamate. More importantly, we demonstrate that this cell non-autonomous proteasome activation results in effective prevention of the human β -mediated proteotoxicity in the distal tissue (muscle). Notably, this distal effect does not apply to all kinds of stress since neuronal proteasome activation did not confer resistance to oxidative stress. These distal effects should be considered when designing tissue-specific interventions targeting the proteasome as they might dampen the effects that are anticipated through the tissue-specific manipulation.

Author contributions

NC conceived the idea. AG and NC conceptualized and designed the work. AG and DB designed the constructs for *pbs-5* overexpression. EP and AG performed experiments and analyzed the data. EP, AG and NC interpreted the data. AG and NC prepared the first manuscript and all authors reviewed and edited the text. AG and NC supervised the work.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Acknowledgements

Nematode strains used in this study were provided by the Caenorhabditis Genetics Center (CGC) which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). We are grateful to Prof. Guy A. Caldwell for the UA198 strain and to Dr. Carina I. Holmberg for the ZsProSensor-1 strains. EP was supported by the Hellenic Foundation for Research and Innovation (HFRI) under the HFRI PhD Fellowship grant (Fellowship Number: 1540) (salaries only). With the exception of the HFRI PhD Fellowship grant to EP, this research did not receive any other specific grant from funding agencies in the public, commercial, or, not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2023.102817>

References

- [1] W.E. Balch, R.I. Morimoto, A. Dillin, J.W. Kelly, Adapting proteostasis for disease intervention, *Science* (New York, N.Y.) 319 (5865) (2008) 916–919, <https://doi.org/10.1126/science.1141448>.
- [2] R.C. Taylor, A. Dillin, Aging as an event of proteostasis collapse, *Cold Spring Harbor Perspect. Biol.* 3 (5) (2011), a004440, <https://doi.org/10.1101/cshperspect.a004440>.
- [3] M. Lefaki, N. Papaevgeniou, N. Chondrogianni, Redox regulation of proteasome function, *Redox Biol.* 13 (2017) 452–458, <https://doi.org/10.1016/j.redox.2017.07.005>.
- [4] G.A. Collins, A.L. Goldberg, The logic of the 26S proteasome, *Cell* 169 (5) (2017) 792–806, <https://doi.org/10.1016/j.cell.2017.04.023>.
- [5] N. Chondrogianni, I. Petropoulos, S. Grimm, K. Georgila, B. Catalgol, B. Friguet, T. Grune, E.S. Gonos, Protein damage, repair and proteolysis, *Mol. Aspect. Med.* 35 (2014) 1–71, <https://doi.org/10.1016/j.mam.2012.09.001>.
- [6] B. Boland, W.H. Yu, O. Corti, B. Mollereau, A. Henriques, E. Bezard, G.M. Pastores, D.C. Rubinsztein, R.A. Nixon, M.R. DuChen, G.R. Mallucci, G. Kroemer, B. Levine, E.-L. Eskelinen, F. Mochel, M. Spedding, C. Louis, O.R. Martin, M.J. Millan, Promoting the clearance of neurotoxic proteins in neurodegenerative disorders of ageing, *Nat. Rev. Drug Discov.* 17 (9) (2018) 660–688, <https://doi.org/10.1038/nrd.2018.109>.
- [7] E. Njomen, J.J. Tepe, Proteasome activation as a new therapeutic approach to target proteotoxic disorders, *J. Med. Chem.* 62 (14) (2019) 6469–6481, <https://doi.org/10.1021/acs.jmedchem.9b00101>.
- [8] S.E. Chocron, E. Munkácsy, S.K. Harper, P. Karpowicz, N. Jiang, C. Van Skike, N. DeRosa, A.Q. Banh, J.P. Palavicini, P. Wityk, L. Kalinowski, V. Galvan, P. A. Osmulski, E. Jankowska, M. Gaczynska, A.M. Pickering, Genetic and pharmacologic proteasome augmentation ameliorates Alzheimer's-like pathology in mouse and fly APP overexpression models, *Sci. Adv.* 8 (23) (2022) eabk2252, <https://doi.org/10.1126/sciadv.abk2252>.
- [9] N. Chondrogianni, C. Tzavelas, A.J. Pemberton, I.P. Nezis, A.J. Rivett, E.S. Gonos, Overexpression of proteasome beta5 assembled subunit increases the amount of proteasome and confers ameliorated response to oxidative stress and higher survival rates, *J. Biol. Chem.* 280 (12) (2005) 11840–11850, <https://doi.org/10.1074/jbc.M413007200>.
- [10] N. Chondrogianni, K. Georgila, N. Kouritis, N. Tavernarakis, E.S. Gonos, 20S proteasome activation promotes life span extension and resistance to proteotoxicity in *Caenorhabditis elegans*, *FASEB (Fed. Am. Soc. Exp. Biol.) J. : Off. Publ. Fed. Am. Soc. Exp. Biol.* 29 (2) (2015) 611–622, <https://doi.org/10.1096/fj.14-252189>.
- [11] N.N. Nguyen, A. Rana, C. Goldman, R. Moore, J. Tai, Y. Hong, J. Shen, D. W. Walker, J.H. Hur, Proteasome $\beta 5$ subunit overexpression improves proteostasis during aging and extends lifespan in *Drosophila melanogaster*, *Sci. Rep.* (2019), <https://doi.org/10.1038/s41598-019-39508-4>.
- [12] D. Vilchez, I. Morante, Z. Liu, P.M. Douglas, C. Merkwirth, A.P.C. Rodrigues, G. Manning, A. Dillin, RPN-6 determines *C. elegans* longevity under proteotoxic stress conditions, *Nature* 489 (7415) (2012) 263–268, <https://doi.org/10.1038/nature11315>.
- [13] V. Prahlad, T. Cornelius, R.I. Morimoto, Regulation of the cellular heat shock response in *Caenorhabditis elegans* by thermosensory neurons, *Science* (New York, N.Y.) 320 (5877) (2008) 811–814, <https://doi.org/10.1126/science.1156093>.
- [14] J. Durieux, S. Wolff, A. Dillin, The cell-non-autonomous nature of electron transport chain-mediated longevity, *Cell* 144 (1) (2011) 79–91, <https://doi.org/10.1016/j.cell.2010.12.016>.
- [15] Q. Zhang, X. Wu, P. Chen, L. Liu, N. Xin, Y. Tian, A. Dillin, The mitochondrial unfolded protein response is mediated cell-non-autonomously by retromer-dependent Wnt signaling, *Cell* 174 (4) (2018) 870–883.e17, <https://doi.org/10.1016/j.cell.2018.06.029>.
- [16] G. Calcutti, H.J. Lee, K. Shen, U. Pham, M. Herholz, A. Trifunovic, A. Dillin, D. Vilchez, Systemic regulation of mitochondria by germline proteostasis prevents protein aggregation in the soma of *C. elegans*, *Sci. Adv.* 7 (26) (2021), <https://doi.org/10.1126/sciadv.abg3012>.
- [17] K.M. Berendzen, J. Durieux, L.-W. Shao, Y. Tian, H.-E. Kim, S. Wolff, Y. Liu, A. Dillin, Neuroendocrine coordination of mitochondrial stress signaling and proteostasis, *Cell* 166 (6) (2016) 1553–1563.e10, <https://doi.org/10.1016/j.cell.2016.08.042>.
- [18] S. Imanikia, N.P. Özbek, C. Krueger, M.O. Casanueva, R.C. Taylor, Neuronal XBP-1 activates intestinal lysosomes to improve proteostasis in *C. elegans*, *Curr. Biol. : CB* 29 (14) (2019) 2322–2338.e7, <https://doi.org/10.1016/j.cub.2019.06.031>.
- [19] R.C. Taylor, A. Dillin, XBP-1 is a cell-nonautonomous regulator of stress resistance and longevity, *Cell* 153 (7) (2013) 1435–1447, <https://doi.org/10.1016/j.cell.2013.05.042>.
- [20] J. Minnerly, J. Zhang, T. Parker, T. Kaul, K. Jia, The cell non-autonomous function of ATG-18 is essential for neuroendocrine regulation of *Caenorhabditis elegans* lifespan, *PLoS Genet.* 13 (5) (2017), e1006764, <https://doi.org/10.1371/journal.pgen.1006764>.
- [21] Yifei Zhou, X. Wang, M. Song, Z. He, G. Cui, G. Peng, C. Dieterich, A. Antebi, N. Jing, Y. Shen, Y. Shen, A secreted microRNA disrupts autophagy in distinct tissues of *Caenorhabditis elegans* upon ageing, *Nat. Commun.* 10 (1) (2019) 4827, <https://doi.org/10.1038/s41467-019-12821-2>.
- [22] M. Ulgherait, A. Rana, M. Rera, J. Graniel, D.W. Walker, AMPK modulates tissue and organismal aging in a non-cell-autonomous manner, *Cell Rep.* 8 (6) (2014) 1767–1780, <https://doi.org/10.1016/j.celrep.2014.08.006>.
- [23] A. Moskalev, Z. Guvatova, M. Shaposhnikov, E. Lashmanova, E. Proshkina, L. Koval, A. Zhavoronkov, G. Krasnov, A. Kudryavtseva, The neuronal overexpression of Gclc in *Drosophila melanogaster* induces life extension with longevity-associated transcriptomic changes in the thorax, *Front. Genet.* 10 (2019) 149, <https://doi.org/10.3389/fgene.2019.00149>.
- [24] S. Brenner, The genetics of *Caenorhabditis elegans*, *Genetics* 77 (1) (1974) 71–94, <https://pubmed.ncbi.nlm.nih.gov/4366476>.
- [25] R.S. Kamath, M. Martinez-Campos, P. Zipperlin, A.G. Fraser, J. Ahringer, Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*, *Genome Biol.* 2 (1) (2000), <https://doi.org/10.1186/gb-2000-2-1-research0002> research0002.1.
- [26] B.A. Martinez, H. Kim, A. Ray, G.A. Caldwell, K.A. Caldwell, A bacterial metabolite induces glutathione-tractable proteostatic damage, proteasomal disturbances, and PINK1-dependent autophagy in *C. elegans*, *Cell Death Dis.* 6 (10) (2015), e1908, <https://doi.org/10.1038/cddis.2015.270>.
- [27] S. Nayak, M. Fiaschi, D. King, E.R. Tabakin, L. Wood, D.A. Hunt, Development of small molecular proteasome inhibitors using a *Caenorhabditis elegans* screen, *Int. J. Med. Chem.* (2014), 237286, <https://doi.org/10.1155/2014/237286>, 2014.
- [28] Z. Altun-Gultekin, Y. Andachi, E.L. Tsalik, D. Pilgrim, Y. Kohara, O. Hobert, A regulatory cascade of three homeobox genes, *ceh-10*, *ttx-3* and *ceh-23*, controls cell fate specification of a defined interneuron class in *C. elegans*, *Development* 128 (11) (2001) 1951–1969, <https://doi.org/10.1242/dev.128.11.1951>.
- [29] E. Munkácsy, E.S. Chocron, L. Quintanilla, C.M. Gendron, S.D. Pletcher, A. M. Pickering, Neuronal-specific proteasome augmentation via Pro $\beta 5$ overexpression extends lifespan and reduces age-related cognitive decline, *Aging Cell* (2019), <https://doi.org/10.1111/acel.13005>.
- [30] E.F. Griffin, X. Yan, K.A. Caldwell, G.A. Caldwell, Distinct functional roles of Vps41-mediated neuroprotection in Alzheimer's and Parkinson's disease models of neurodegeneration, *Hum. Mol. Genet.* 27 (24) (2018) 4176–4193, <https://doi.org/10.1093/hmg/ddy308>.
- [31] E.F. Griffin, S.E. Scopel, C.A. Stephen, A.C. Holzhauser, M.A. Vaji, R.A. Tuckey, L. A. Berkowitz, K.A. Caldwell, G.A. Caldwell, ApoE-associated modulation of neuroprotection from β -mediated neurodegeneration in transgenic *Caenorhabditis elegans*, *Disease Models & Mechanisms* 12 (2) (2019), <https://doi.org/10.1242/dmm.037218>.
- [32] O. Matilainen, S. Jha, C.I. Holmberg, Fluorescent tools for in vivo studies on the ubiquitin-proteasome system, *Methods Mol. Biol.* 1449 (2016) 215–222, https://doi.org/10.1007/978-1-4939-3756-1_12.
- [33] C.D. Link, A. Taft, V. Kapulkin, K. Duke, S. Kim, Q. Fei, D.E. Wood, B.G. Sahagan, Gene expression analysis in a transgenic *Caenorhabditis elegans* Alzheimer's disease model, *Neurobiol. Aging* 24 (3) (2003) 397–413, [https://doi.org/10.1016/s0197-4580\(02\)00224-5](https://doi.org/10.1016/s0197-4580(02)00224-5).
- [34] G. McColl, B.R. Roberts, T.L. Pukala, V.B. Kenche, C.M. Roberts, C.D. Link, T. M. Ryan, C.L. Masters, K.J. Barnham, A.I. Bush, R.A. Cherny, Utility of an improved model of amyloid-beta (β ₄₂) toxicity in *Caenorhabditis elegans* for drug screening for Alzheimer's disease, *Mol. Neurodegener.* 7 (2012) 57, <https://doi.org/10.1186/1750-1326-7-57>.
- [35] K.B. Kim, J. Myung, N. Sin, C.M. Crews, Proteasome inhibition by the natural products epoxomicin and dihydroeponepycin: insights into specificity and potency, *Bioorg. Med. Chem. Lett.* 9 (23) (1999) 3335–3340, [https://doi.org/10.1016/s0960-894x\(99\)00612-5](https://doi.org/10.1016/s0960-894x(99)00612-5).
- [36] H. Lin, Y. Gao, C. Zhang, B. Ma, M. Wu, X. Cui, H. Wang, Autophagy regulation influences β -amyloid toxicity in transgenic *Caenorhabditis elegans*, *Front. Aging Neurosci.* 14 (2022), 885145, <https://doi.org/10.3389/fnagi.2022.885145>.
- [37] M.A. Rahman, M.D.H. Rahman, A.N.M. Mamun-Or-Rashid, H. Hwang, S. Chung, B. Kim, H. Rhim, Autophagy modulation in aggresome formation: emerging implications and treatments of Alzheimer's disease, *Biomedicines* 10 (5) (2022), <https://doi.org/10.3390/biomedicines10051027>.
- [38] V.I. Korolchuk, F.M. Menzies, D.C. Rubinsztein, Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems, *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 584 (7) (2010) 1393–1398, <https://doi.org/10.1016/j.febslet.2009.12.047>.
- [39] H.C. Chapin, M. Okada, A.J. Merz, D.L. Miller, Tissue-specific autophagy responses to aging and stress in *C. elegans*, *Aging* 7 (6) (2015) 419–434, <https://doi.org/10.18632/aging.100765>.
- [40] D.A. Butterfield, A.M. Swomley, R. Sultana, Amyloid β -peptide (1–42)-induced oxidative stress in Alzheimer disease: importance in disease pathogenesis and progression, *Antioxidants Redox Signal.* 19 (8) (2013) 823–835, <https://doi.org/10.1089/ars.2012.5027>.
- [41] H.J. Forman, H. Zhang, Targeting oxidative stress in disease: promise and limitations of antioxidant therapy, *Nat. Rev. Drug Discov.* 20 (9) (2021) 689–709, <https://doi.org/10.1038/s41573-021-00233-1>.
- [42] I. Korovila, M. Hugo, J.P. Castro, D. Weber, A. Höhn, T. Grune, T. Jung, Proteostasis, oxidative stress and aging, *Redox Biol.* 13 (2017) 550–567, <https://doi.org/10.1016/j.redox.2017.07.008>.
- [43] A.M. Pickering, A.L. Koop, C.Y. Teoh, G. Ermark, T. Grune, K.J.A. Davies, The immunoproteasome, the 20S proteasome and the PA28 $\alpha\beta$ proteasome regulator are oxidative-stress-adaptive proteolytic complexes, *Biochem. J.* 432 (3) (2010) 585–594, <https://doi.org/10.1042/BJ20100878>.
- [44] A.M. Pickering, T.A. Staab, J. Tower, D. Sieburth, K.J.A. Davies, A conserved role for the 20S proteasome and Nrf2 transcription factor in oxidative stress adaptation in mammals, *Caenorhabditis elegans* and *Drosophila melanogaster*, *J. Exp. Biol.* 216 (Pt 4) (2013) 543–553, <https://doi.org/10.1242/jeb.074757>.
- [45] N.W. Kahn, S.L. Rea, S. Moyle, A. Kell, T.E. Johnson, Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in *Caenorhabditis elegans*, *Biochem. J.* 409 (1) (2008) 205–213, <https://doi.org/10.1042/BJ20070521>.

- [46] X. Li, O. Matilainen, C. Jin, K.M. Glover-Cutter, C.I. Holmberg, T.K. Blackwell, Specific SKN-1/Nrf stress responses to perturbations in translation elongation and proteasome activity, *PLoS Genet.* 7 (6) (2011), e1002119, <https://doi.org/10.1371/journal.pgen.1002119>.
- [47] R.P. Oliveira, J. Porter Abate, K. Dilks, J. Landis, J. Ashraf, C.T. Murphy, T. K. Blackwell, Condition-adapted stress and longevity gene regulation by *Caenorhabditis elegans* SKN-1/Nrf, *Aging Cell* 8 (5) (2009) 524–541, <https://doi.org/10.1111/j.1474-9726.2009.00501.x>.
- [48] J. Richmond, Synaptic function, in: E.M. Jorgensen, J.M. Kaplan (Eds.), *WormBook*, The C. elegans Research Community, WormBook, 2007, <https://doi.org/10.1895/wormbook>.
- [49] F. Calahorra, P.G. Izquierdo, The presynaptic machinery at the synapse of *C. elegans*, *Invertebr. Neurosci.* 18 (2) (2018) 4, <https://doi.org/10.1007/s10158-018-0207-5>.
- [50] C.-W. Tien, B. Yu, M. Huang, K.P. Stepien, K. Sugita, X. Xie, L. Han, P.P. Monnier, M. Zhen, J. Rizo, S. Gao, S. Sugita, Open syntaxin overcomes exocytosis defects of diverse mutants in *C. elegans*, *Nat. Commun.* 11 (1) (2020) 5516, <https://doi.org/10.1038/s41467-020-19178-x>.
- [51] S. Speese, M. Petrie, K. Schuske, M. Ailion, K. Ann, K. Iwasaki, E.M. Jorgensen, T.F. J. Martin, UNC-31 (CAPS) is required for dense-core vesicle but not synaptic vesicle exocytosis in *Caenorhabditis elegans*, *J. Neurosci.* 27 (23) (2007) 6150, <https://doi.org/10.1523/JNEUROSCI.1466-07.2007>. LP – 6162.
- [52] L. Hong, H.-C. Huang, Z.-F. Jiang, Relationship between amyloid-beta and the ubiquitin-proteasome system in Alzheimer's disease, *Neurol. Res.* 36 (3) (2014) 276–282, <https://doi.org/10.1179/1743132813Y.0000000288>.
- [53] W. Zhang, C. Xu, J. Sun, H.-M. Shen, J. Wang, C. Yang, Impairment of the autophagy-lysosomal pathway in Alzheimer's diseases: pathogenic mechanisms and therapeutic potential, *Acta Pharm. Sin. B* 12 (3) (2022) 1019–1040, <https://doi.org/10.1016/j.apbs.2022.01.008>.
- [54] N.S. Ilyinsky, S.V. Nesterov, E.I. Shestoperova, A.V. Fonin, V.N. Uversky, V. I. Gordeliy, On the role of normal aging processes in the onset and pathogenesis of diseases associated with the Abnormal accumulation of protein Aggregates, *Biochemistry. Biokhimiia* 86 (3) (2021) 275–289, <https://doi.org/10.1134/S0006297921030056>.
- [55] J.R. Daniele, R. Higuchi-Sanabria, J. Durieux, S. Monshietehadi, V. Ramachandran, S.U. Tronnes, N. Kelet, M. Sanchez, M.G. Metcalf, G. Garcia, P.A. Frankino, C. Benitez, M. Zeng, D.J. Esping, L. Joe, A. Dillin, UPR(ER) promotes lipophagy independent of chaperones to extend life span, *Sci. Adv.* 6 (1) (2020), <https://doi.org/10.1126/sciadv.aaz1441> eaz1441.
- [56] M. Rai, Z. Coleman, M. Curley, A. Nityanandam, A. Platt, M. Robles-Murguía, J. Jiao, D. Finkelstein, Y.-D. Wang, B. Xu, Y. Fan, F. Demontis, Proteasome stress in skeletal muscle mounts a long-range protective response that delays retinal and brain aging, *Cell Metabol.* 33 (6) (2021) 1137–1154.e9, <https://doi.org/10.1016/j.cmet.2021.03.005>.
- [57] N.F. Bence, R.M. Sampat, R.R. Kopito, Impairment of the ubiquitin-proteasome system by protein aggregation, *Science (New York, N.Y.)* 292 (5521) (2001) 1552–1555, <https://doi.org/10.1126/science.292.5521.1552>.
- [58] T.A. Thibaut, R.T. Anderson, D.M. Smith, A common mechanism of proteasome impairment by neurodegenerative disease-associated oligomers, *Nat. Commun.* 9 (1) (2018) 1097, <https://doi.org/10.1038/s41467-018-03509-0>.
- [59] N. Papaevgeniou, M. Sakellari, S. Jha, N. Tavernarakis, C.I. Holmberg, E.S. Gonos, N. Chondrogianni, 18 α -Glycyrrhetic acid proteasome activator decelerates aging and Alzheimer's disease progression in *Caenorhabditis elegans* and neuronal cultures, *Antioxidants Redox Signal.* 25 (16) (2016) 855–869, <https://doi.org/10.1089/ars.2015.6494>.
- [60] M.A. Vasilopoulou, A. Gioran, M. Theodoropoulou, A. Koutsaviti, V. Roussis, E. Ioannou, N. Chondrogianni, Healthspan improvement and anti-aggregation effects induced by a marine-derived structural proteasome activator, *Redox Biol.* 56 (2022), 102462, <https://doi.org/10.1016/j.redox.2022.102462>.
- [61] S. Kapeta, N. Chondrogianni, E.S. Gonos, Nuclear erythroid factor 2-mediated proteasome activation delays senescence in human fibroblasts, *J. Biol. Chem.* 285 (11) (2010) 8171–8184, <https://doi.org/10.1074/jbc.M109.031575>.
- [62] A.N. Mladenovic Djordjevic, M. Kapetanou, N. Loncarevic-Vasiljkovic, S. Todorovic, S. Athanasopoulou, M. Jovic, M. Prvulovic, E. Taoufik, R. Matsas, S. Kanazir, E.S. Gonos, Pharmacological intervention in a transgenic mouse model improves Alzheimer's-associated pathological phenotype: involvement of proteasome activation, *Free Radical Biol. Med.* 162 (2021) 88–103, <https://doi.org/10.1016/j.freeradbiomed.2020.11.038>.
- [63] J.L. Sun-Wang, S. Ivanova, A. Zorzano, The dialogue between the ubiquitin-proteasome system and autophagy: implications in ageing, *Ageing Res. Rev.* 64 (2020), 101203, <https://doi.org/10.1016/j.arr.2020.101203>.
- [64] S. Jha, C.I. Holmberg, Tissue-specific impact of autophagy genes on the ubiquitin-proteasome system in *C. elegans*, *Cells* 9 (8) (2020), <https://doi.org/10.3390/cells9081858>.
- [65] S.A. Keith, S.K. Maddux, Y. Zhong, M.N. Chinchankar, A.A. Ferguson, A. Ghazi, A. L. Fisher, Graded proteasome dysfunction in *Caenorhabditis elegans* activates an adaptive response involving the conserved SKN-1 and ELT-2 transcription factors and the autophagy-lysosome pathway, *PLoS Genet.* 12 (2) (2016), e1005823, <https://doi.org/10.1371/journal.pgen.1005823>.
- [66] L. Wrobel, S.M. Hill, A. Djajadikerta, M. Fernandez-Estevez, C. Karabiyik, A. Ashkenazi, V.J. Barratt, E. Stamatakou, A. Gunnarsson, T. Rasmussen, E. W. Miele, N. Beaton, R. Bruderer, Y. Feng, L. Reiter, M.P. Castaldi, R. Jarvis, K. Tan, R.W. Bürl, D.C. Rubinsztein, Compounds activating VCP D1 ATPase enhance both autophagic and proteasomal neurotoxic protein clearance, *Nat. Commun.* 13 (1) (2022) 4146, <https://doi.org/10.1038/s41467-022-31905-0>.
- [67] A.E. Frakes, M.G. Metcalf, S.U. Tronnes, R. Bar-Ziv, J. Durieux, H.K. Gildea, N. Kandahari, S. Monshietehadi, A. Dillin, Four glial cells regulate ER stress resistance and longevity via neuropeptide signaling in *C. elegans*, *Science (New York, N.Y.)* 367 (6476) (2020) 436–440, <https://doi.org/10.1126/science.aaz6896>.
- [68] N.A. Bishop, L. Guarente, Two neurons mediate diet-restriction-induced longevity in *C. elegans*, *Nature* 447 (7144) (2007) 545–549, <https://doi.org/10.1038/nature05904>.
- [69] C.A. Wolkow, K.D. Kimura, M.S. Lee, G. Ruvkun, Regulation of *C. elegans* life-span by insulinlike signaling in the nervous system, *Science (New York, N.Y.)* 290 (5489) (2000) 147–150, <https://doi.org/10.1126/science.290.5489.147>.
- [70] R. Higuchi-Sanabria, J. Durieux, N. Kelet, S. Homentcovschi, M. de Los Rios Rogers, S. Monshietehadi, G. Garcia, S. Dallarda, J.R. Daniele, V. Ramachandran, A. Sahay, S.U. Tronnes, L. Joe, A. Dillin, Divergent nodes of non-autonomous UPR (ER) signaling through serotonergic and dopaminergic neurons, *Cell Rep.* 33 (10) (2020), 108489, <https://doi.org/10.1016/j.celrep.2020.108489>.
- [71] H.-Y. He, A. Ahsan, R. Bera, N. McLain, R. Faulkner, K.V. Ramachandran, S. S. Margolis, H.T. Cline, Neuronal membrane proteasomes regulate neuronal circuit activity in vivo and are required for learning-induced behavioral plasticity, *Proc. Natl. Acad. Sci. U. S. A.* 120 (3) (2023), e2216537120, <https://doi.org/10.1073/pnas.2216537120>.
- [72] K.V. Ramachandran, S.S. Margolis, A mammalian nervous-system-specific plasma membrane proteasome complex that modulates neuronal function, *Nat. Struct. Mol. Biol.* 24 (4) (2017) 419–430, <https://doi.org/10.1038/nsmb.3389>.
- [73] Y. Zhou, N.C. Danbolt, Glutamate as a neurotransmitter in the healthy brain, *J. Neural. Transm.* 121 (8) (2014) 799–817, <https://doi.org/10.1007/s00702-014-1180-8>. Vienna, Austria : 1996.
- [74] S. Magi, S. Piccirillo, S. Amoroso, V. Lariccia, Excitatory amino acid transporters (EAATs): glutamate transport and beyond, *Int. J. Mol. Sci.* 20 (22) (2019), <https://doi.org/10.3390/ijms20225674>.
- [75] H. Ilieva, M. Polymenidou, D.W. Cleveland, Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond, *J. Cell Biol.* 187 (6) (2009) 761–772, <https://doi.org/10.1083/jcb.200908164>.
- [76] S.A. Liddel, Modern approaches to investigating non-neuronal aspects of Alzheimer's disease, *FASEB (Fed. Am. Soc. Exp. Biol.) J. : Off. Publ. Fed. Am. Soc. Exp. Biol.* 33 (2) (2019) 1528–1535, <https://doi.org/10.1096/fj.201802592>.