Research

Lysosomal enzyme cathepsin D protects against alpha-synuclein aggregation and toxicity

Liyan Qiao1, Shusei Hamamichi2, Kim A Caldwell2,3,4,5, Guy A Caldwell2,3,4,5, Talene A Yacoubian3,4,5, Scott Wilson3, Zuo-Lei Xie1, Lisa D Speake1, Rachael Parks1, Donna Crabtree1, Qiuli Liang1, Stephen Crimmins1, Lonnie Schneider1, Yasuo Uchiyama6, Takeshi Iwatsubo7, Yi Zhou3, Lisheng Peng8, YouMing Lu8, David G Standaert3,4, Ken C Walls1, John J Shacka1,5,9, Kevin A Roth1,3,5 and Jianhua Zhang*1,3,5

Address: 1Department of Pathology, University of Alabama at Birmingham, Birmingham, USA, 2Department of Biological Sciences, The University of Alabama, Tuscaloosa, USA, 3Department of Neurobiology, University of Alabama at Birmingham, Birmingham, USA, 4Department of Neurology, University of Alabama at Birmingham, Birmingham, USA, 5Center for Neurodegeneration and Experimental Therapeutics, University of Alabama at Birmingham, Birmingham, USA, 6Department of Cell Biology and Neurosciences, Osaka University, Osaka, Japan, 7Department of Neuropathology, Graduate School of Medicine, Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan, 8Biomolecular Science Center, Burnett College of Biomedical Sciences, Orlando, USA and 9Department of Veterans Affairs, Birmingham VA Medical Center, Birmingham, AL35294, USA

Email: Liyan Qiao - lqiao@uab.edu; Shusei Hamamichi - hamam@bama.ua.edu; Kim A Caldwell - kncaldwell@ua.edu; Scott Wilson - wilson@nrc.uab.edu; Zuo-Lei Xie - xiezl1969@yahoo.com.cn; Lisa D Speake - ldspake@bsu.edu; Rachael Parks - rparks1@mvnu.edu; Donna Crabtree - donna.crabtree@gmail.com; Qiuli Liang - liangqili02@gmail.com; Stephen Crimmins - scrimmins@nrc.uab.edu; Lonnie Schneider - lonnie11@uab.edu; Yasuo Uchiyama - y-uchi@anat1.med.osaka-u.ac.jp; Takeshi Iwatsubo - iwatsubo@mol.f.u-tokyo.ac.jp; Yi Zhou - yzhou@nrc.uab.edu; Lisheng Peng - youming@mail.ucf.edu; YouMing Lu - youming@mail.ucf.edu; David G Standaert - dstanden@uab.edu; Ken C Walls - kcwalls@uab.edu; John J Shacka - shacka@uab.edu; Kevin A Roth - karoth@uab.edu; Jianhua Zhang* - zhanja@uab.edu

* Corresponding author

Abstract

α-synuclein (α-syn) is a main component of Lewy bodies (LB) that occur in many neurodegenerative diseases, including Parkinson’s disease (PD), dementia with LB (DLB) and multi-system atrophy. α-syn mutations or amplifications are responsible for a subset of autosomal dominant familial PD cases, and overexpression causes neurodegeneration and motor disturbances in animals. To investigate mechanisms for α-syn accumulation and toxicity, we studied a mouse model of lysosomal enzyme cathepsin D (CD) deficiency, and found extensive accumulation of endogenous α-syn in neurons without overabundance of α-syn mRNA. In addition to impaired macroautophagy, CD deficiency reduced proteasome activity, suggesting an essential role for lysosomal CD function in regulating multiple proteolytic pathways that are important for α-syn metabolism. Conversely, CD overexpression reduces α-syn aggregation and is neuroprotective against α-syn overexpression-induced cell death in vitro. In a C. elegans model, CD deficiency exacerbates α-syn accumulation while its overexpression is protective against α-syn-induced dopaminergic neurodegeneration. Mutated CD with diminished enzymatic activity or overexpression of cathepsins B (CB) or L (CL) is not protective in the worm model, indicating a unique requirement for enzymatically active CD. Our data identify a conserved CD function in α-syn degradation and identify CD as a novel target for LB disease therapeutics.
Introduction

Patients with α-synuclein (α-syn) A53T, A30P, E46K mutations or gene amplification develop typical Parkinson’s disease (PD) and often an associated dementia[1]. However, in > 90% of PD cases, and almost all dementia with Lewy body (DLB) and Lewy body variant of Alzheimer’s disease (LBVAD) cases, α-syn aggregation occurs yet without a clear evidence of mutation or up-regulation of α-syn mRNA transcription. Therefore, impaired α-syn clearance may play a more important role than α-syn overexpression in neuronal α-syn accumulation and neurodegenerative disease pathogenesis.

Experiments in vitro have shown that α-syn can be cleared by the cytosolic ubiquitin-proteasome system (UPS), and/or lysosome-mediated autophagic pathways[2]. The UPS degrades short-lived, misfolded and/or damaged proteins via an ubiquitin-dependent signaling pathway. Macrouautophagy is initiated by de novo synthesis of double membrane vesicles in the cytoplasm. These vesicles encircle long-lived or damaged proteins or organelles by an unknown signaling mechanism and deliver these cargos to lysosomes for degradation. Chaperone-mediated autophagy (CMA) is initiated by chaperones binding to KFERQ-consensus sequence-containing cytosolic proteins followed by delivery to the lysosomes via lysosomal membrane LAMP-2a receptors. Wildtype α-syn has a pentapeptide sequence that can serve as a CMA recognition motif and can be translocated to the lysosome, while dopamine modified or pathogenic A53T and A30P mutant α-syn block CMA [3,4].

Lysosomal function declines with age in the human brain[5]. Accumulation of autophagic vacuoles (AVs) has been reproducibly observed in postmortem AD and PD patient brains compared to normal controls, consistent with either an overproduction of AVs or a deficit in autophagolysosomal clearance[6]. Enhancing macroautophagy by either mTOR-dependent or independent mechanisms can help clear aggregation-prone proteins, including huntingtin, A53T and A30P mutant α-syn [7,8]. However, because both macroautophagy and CMA are dependent on intact lysosomes, enhancing macroautophagy may not be effective in clearing potentially neurotoxic proteins if lysosomal function is impaired. Understanding the role of lysosomal enzymes in α-syn clearance may provide new strategies for LBVAD, DLB, and PD therapy.

Cathepsin D (CD) is the principal lysosomal aspartate protease and a main endopeptidase responsible for the degradation of long-lived proteins [9,10], including α-syn[11]. CD is expressed widely in the brain, including the cortex, hippocampus, striatum, and dopaminergic (DA) neurons of the substantia nigra (SNr)[12]. CD is synthesized as a precursor with a signal peptide cleaved upon its insertion into endoplasmic reticulum[13]. The CD zymogen is activated in an acidic environment by cleavage of the pro-peptide. CD is also up-regulated in AD brains as an early event, but whether CD up-regulation is coincidental, disease promoting, or compensatory is unclear[14].

CD gene (Ctsd) homozygous inactivation was reported to cause human congenital neuronal ceroid lipofuscinosis (NCL) with postnatal respiratory insufficiency, status epilepticus, and death within hours to weeks after birth[15]. These patients had severe neurological defects at birth and neuronal α-syn accumulation was not examined. Another patient with significant loss of CD enzymatic function (7.7% Vmax from patient fibroblast lysates compared to controls) due to compound heterozygous missense mutations developed childhood motor and visual disturbances, cerebral and cerebellar atrophy, and progressive psychomotor disability[16]. Conceivably, milder forms of CD deficiency could predispose to late onset neurodegenerative disorders, including AD and PD. Parkinsonism has been noted in lysosomal tripeptidyl peptidase I deficient patients[17], adult forms of NCL patients[18], and Gaucher disease patients[19]. α-syn aggregation has been reported in both neurons and glia in several lysosomal disorders, such as Gaucher disease[20], Niemann-Pick disease[21], GM2 gangliosidosis, Tay-Sachs, Sandhoff disease, metachromatic leukodystrophy, and beta-galactosidosis[22].

Significant α-syn accumulation has not been previously reported in mouse models of proteolytic disorders involving proteasomes, autophagy or other lysosomal proteases [23-26]. Here we report a robust α-synucleinopathy in CD deficient mice, despite the compensatory up-regulation of other lysosomal proteases, and the absence of an increase of α-syn mRNA expression. We found that proteasome activities are significantly reduced in the CD-deficient brain, whereas several key UPS factors are either normal or up-regulated, indicating cross-talk between lysosomal and proteasomal activities at the levels of signaling rather than a reduction of protein levels. Finally, we demonstrate that CD, but not Cathepsin B (CB) or Cathepsin L (CL), overexpression reduces α-syn aggregation and provides potent neuroprotection from α-syn-induced neuron death in vitro and in vivo.

Results

CD deficient mice exhibit extensive accumulation of α-syn in neurons

To investigate the involvement of lysosomal functions in α-syn clearance, we analyzed mice deficient in CD, previously generated by a targeted insertion of the neo marker in exon 4[27]. CD-deficient (Ctsd-/-) mice die at approxi-
mately postnatal day 26 (p26) due to a combination of nervous system and systemic abnormalities. Extensive neuron death resulting from activation of both apoptotic and non-apoptotic pathways has been observed in these mice [27-31]. We examined brains from p21 and p25 Ctsd/- mice and found significant α-syn accumulation in neuronal cell bodies in p25 Ctsd/- but not wildtype cortex (Fig. 1a). In contrast to the brains of human lipidoses patients[32] where α-syn aggregates are found in both neurons and glia and co-localize with lipids, in Ctsd/- brains α-syn accumulations do not co-localize with autofluorescent lipofuscin (data not shown).

Furthermore, α-syn accumulations in Ctsd/- brains were present in cells co-expressing the neuron marker, neuronal nuclear antigen (NeuN), but not in glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes (Fig. 1b–c). Accumulation of ubiquitinated proteins also occurs in Ctsd/- cortex (Fig. 1a). A relatively small fraction of neurons (~5%) with intense accumulated α-syn immunostaining also exhibit co-localization with intense accumulated ubiquitin staining, consistent with the observation that a small fraction of α-syn in LB is ubiquitinated [33-36]. Correspondingly, we found elevated levels of high molecular weight but not monomeric α-syn, and high molecular weight ubiquitinated proteins in both TritonX-100 soluble and insoluble extracts from the cortex of Ctsd/- mice by western blot analyses compared to Ctsd+/+ mice, similar to what occurs in LB diseases (Fig. 1d–e). Truncated 12 kDa and 10 kDa α-syn fragments are reduced in Ctsd/- extracts (Fig. 1d–e).

Antibody pulldown experiments indicate that a small fraction of accumulated α-syn in Ctsd/- brains is mono-ubiquitinated (Fig. 1f). These results are consistent with a small fraction of accumulated α-syn in Ctsd/- brains being mono-ubiquitinated [33-36]. Ubiquitin immunostaining is present in nearly all neurons with variable intensity. 5% of those neurons with intense accumulated ubiquitin staining also exhibit intense accumulated α-syn immunostaining in Ctsd/- brain sections. Conversely, 5% of neurons with intense accumulated α-syn immunostaining also exhibit intense accumulated ubiquitin staining. p21 Ctsd+/+ and Ctsd/- brains do not exhibit considerable difference in α-syn immunostaining intensity or protein level by western blot analyses. However, ubiquitin accumulation occurs as early as p21 in Ctsd/- cortical extracts compared to wildtype cortical extracts (Fig. 1g).

Immunostaining with synphilin antibodies have not detected "aggregation-like" structures in the cell body where α-syn is. Nor did we observe increased synphilin in western blot analyses. Thus the α-syn accumulation is unlikely to be induced by synphilin accumulation in Ctsd/- brains. The cytoplasmic microtubule-associated protein, tau, did not accumulate in Ctsd/- cortex at p25 compared to wildtype cortex at p25 (data not shown), suggesting that CD deficiency does not have a general effect on the accumulation of all cytoplasmic proteins.

In human LB, 95% of the insoluble α-syn is phosphorylated [33-35,37,38]. We found that anti-phospho-α-syn antibody (from Dr. Iwatsubo [33,36,38]) did not recognize the intensely immunoreactive accumulated α-syn in Ctsd/- brains, although numerous neurons in both wildtype and Ctsd/- brains exhibited labeling with this antibody. Whether this antibody lacks specificity when used in mouse brain sections or the accumulated α-syn in Ctsd/- brain is simply not phosphorylated cannot be determined from this immunohistochemical study. Western blot analyses indicate that a few immunoreactive bands are increased in Ctsd/- extracts compared to wildtype extracts.

### Intense α-syn immunoreactive accumulations are outside of autophagosomes and lysosomes in affected neurons

Prior studies found autophagosomes start to accumulate in Ctsd/- brains as early as p1, compared to Ctsd+/+ age-matched controls [28,30]. Furthermore, CB immunostaining as well as enzymatic activities are increased as early as p21 in Ctsd/- brains [28,30]. Electron microscopy studies demonstrated that CB was associated with irregularly shaped and membrane-bound structures containing electron-dense materials, characterizing them as lysosomes in Ctsd/- brains [28,30]. We found that α-syn accumulation does not become prominent until near p25 in Ctsd/- brains. α-syn accumulation occurs in only a subpopulation of neurons that exhibit enhanced Atg8 (AuTophaGy8)/LC3 (light chain 3) staining, indicating that AV accumulation precedes α-syn accumulation (Fig. 2a). We also found that α-syn accumulations are adjacent to, but do not overlap with, immunoreactivity for the AV-associated protein Atg8/LC3 or the lysosomal associated protein CB, suggesting that α-syn accumulation formed outside of autophagosomes and lysosomes (Fig. 2b). Neuronal populations immunoreactive for the apoptotic marker, cleaved caspase-3, are distinct from those with intense α-syn accumulation (Fig. 2c).

α-syn accumulation is not due to up-regulation of its mRNA, and appears despite compensatory up-regulation of other proteases

While bulk protein degradation appears to be normal in Ctsd/- mice [27], we found that α-syn mRNA is down-regulated in Ctsd/- brains at p25 when α-syn accumulation occurs (Fig. 3a). This is consistent with the finding that α-syn mRNA is either unchanged or down-regulated in the majority of sporadic PD cases [39], indicating that α-syn accumulation is unlikely to be the consequence of ele-
Figure 1 (see legend on next page)
vated gene expression. Prior studies reported that CB but not CL protein is up-regulated in Ctsd-/- brains at p23[28]. To better understand the role of CD in selective protein degradation and PD, we analyzed the expression of genes encoding other brain-enriched lysosomal proteases, autophagy-associated factors, proteasome subunits, and genes linked to familial PD. Interestingly, genes encoding other lysosomal cathepsins B, L, F and H (Ctsb, Ctsl, Ctsf, and Ctsh) mRNAs are all up-regulated at p25 (Fig. 3a). This result may suggest a common transcription regulatory mechanism for these cathepsins in response to CD deficiency. Alternatively, the influx of macrophages or microglia into the Ctsd-/- brain at this age may lead to an increase in cathepsin mRNA expression[40]. We also determined that accumulation of autophagosomes in Ctsd-/- neurons is accompanied with transcriptional up-regulation of Atg7 but not Atg12 (Fig. 3a). Both Atg7 and Atg12 are involved in an ubiquitin-like activity important for autophagosome expansion. Up-regulation of Atg7 may indicate an increase of autophagosome production in addition to a blockade of autophagy completion [28,38,40]. mRNA of Park2 which encodes Parkin, mutation of which has been found in a subset of autosomal recessive PD;UCHL1, mutation of which has been found in a subset of autosomal dominant PD; and Psmb7 which encodes proteasome 20S core β2 subunit are also modestly up-regulated in response to CD deficiency, indicating a compensatory response to CD deficiency at the level of gene transcription (Fig. 3a).

**CD deficiency reduces proteasome activities**

In addition to deficient macroautophagy, we found an accumulation of GAPDH, a substrate of CMA (Fig. 3b). Interestingly, we also found reduced proteasome activity in Ctsd-/- brain extracts (Fig. 3c), suggesting a functional interaction between the two major α-syn clearance machineries, lysosomes and proteasomes. Accumulation of ubiquitinated proteins in Ctsd-/- brain appears at p21 compared to that in wildtype brains (Fig. 1g, 3e), when proteasome activities are unaltered as determined by the proteasome activity assay (data not shown). So far, none of the proteasome-related proteins we examined, including UCHL1, a gene mutated in familial PD and a ubiquitin hydrolase and E3 ligase; Usp14, a key deubiquitination enzyme; Rpt3, an ATPase regulatory subunit, a4 subunit that is important for the gating into the 20S core particle, and b1 subunit that is part of the proteasome core, were significantly changed as determined by western blot analyses in Ctsd-/- brains (Fig. 3d).

**Overexpression of CD reduces α-syn aggregation in mammalian cells**

To further understand how CD activity influences α-syn homeostasis, we examined whether enhancing CD expression can reduce α-syn aggregation. We adopted the simple culture system developed by McLean and colleagues in which an α-syn-green fluorescent protein (GFP) fusion protein (α-syn-GFP) forms visible aggregates in cells when co-expressed with synphilin[41]. We transfected H4 neuroglioma cells with α-syn-GFP, synphilin, and CD. As a control, cells were transfected with α-syn-GFP, synphilin and empty vector pcDNA3.1. Approximately 50% of control transfected cells exhibited α-syn aggregates, consistent with published findings[41]. Remarkably, transfection of CD together with α-syn-GFP and synphilin led to less than 20% of transfected cells exhibiting α-syn aggregates (Fig. 4a). To address the issue of whether CD could affect synphilin levels, we performed immunocytochemistry
α-syn accumulation does not co-localize with LC3 or CB immunoreactivity and is independent of active caspase-3 immunoreactivity.

a. Immunostaining with Atg8/LC3 and α-syn antibodies shows that LC3 staining is increased in Ctsd−/− mice compared to Ctsd+/+ mice, and intense α-syn immunoreactivity does not colocalize with LC3 immunoreactivity (arrows, p25). LC3 = FITC (green). α-syn = Cy3 (red). Arrowheads point to cells with high LC3 immunoreactivity but without intense α-syn immunostaining.

b. α-syn accumulation does not overlap with CB immunoreactivity. CB = FITC (green). α-syn = Cy3 (red). Arrow points to α-syn intense immunoreactive staining adjacent to CB staining.

c. Neurons with active caspase-3 immunoreactivity do not exhibit intense α-syn immunoreactivity in Ctsd−/− brains. α-syn = FITC (green). Active caspase-3 = Cy3 (red). Arrows point to active caspase-3 immunoreactivity. Scale bar = 10 micron. n = 3 mice each genotype.
Deficits of proteasomes in CD deficient mice. a. α-syn (Snca) mRNA is down-regulated in CD deficient brains compared to wildtype control brains. Ctsb, Ctsl, Ctsf, Cth, Atg7, UCHL1, Park2, and Psmb7 mRNA levels are up-regulated. Atg12 and Psmb6 mRNA levels appear to be normal. b. Western blot analyses show an increase of steady state GAPDH, a CMA substrate. n = 3 p25 brain. *p < 0.05 by Student t-test, compared between Ctsd+/+ and Ctsd−/− brains. c. Extracts from Ctsd−/− cortex exhibit reduced proteasome activities compared to Ctsd+/+ as indicated by assays with trypsin-like fluorogenic substrate (VGR-AMC, reaching maximum at 60 min), chymotrypsin-like fluorogenic substrate (Z-GGL-AMC, reaching maximum at 120 min), and peptidylglutamyl peptide-like fluorogenic substrate (Suc-LLVY-AMC, reaching maximum at 120 min). The activities that are inhibitable by the proteasome inhibitor lactacystin were quantified. n = 3 mice each genotype. *p < 0.05 by Student t-test. d. Normal expression of proteins involved in UPS. Western blot analyses of UCHL1, Usp14, Rpt3, α4 and β1 indicate that these UPS factors are expressed normally in Ctsd+/+ and Ctsd−/− cortical extracts. Actin immunoblotting was used as a loading control. n = 3 mice each genotype. e. A diagram regarding onset of relative pathologies.
Figure 4 (see legend on next page)
CD reduces α-syn aggregation. a. CD reduces α-syn aggregation in an aggregation assay. 50% of the cells transfected with α-syn-GFP, synphilin and empty vector exhibited visible α-syn aggregates. Co-transfection of CD together with α-syn-GFP and synphilin reduced the number of cells with visible α-syn aggregates to 20%. n = 3 independent transfection, each in quadruplicate. p < 0.05 compared to absence of exogenous CD by Student t-test. b. Double immunostaining for CD and α-syn in the aggregation assay after transfection of H4 cells with α-syn+synphilin+vector and α-syn+synphilin+CD. Many more cells transfected with synphilin+α-syn+vector have α-syn aggregates than cells transfected with synphilin+α-syn+CD. α-syn = red; CD = green. Higher magnification images of the boxed areas are shown at the left-most panel. α-syn aggregates tended to be found in cellular areas with low levels of CD staining, in H4 cells with either α-syn+synphilin+vector or α-syn+synphilin+CD. Scale bar = 25 micron. Arrows point to α-syn aggregates. c. Double immunostaining for CD and the V5 tag of synphilin in the aggregation assay after transfection of H4 cells with α-syn+synphilin+vector and α-syn+synphilin+CD. Many more cells transfected with synphilin+α-syn+vector have α-syn aggregates than cells transfected with synphilin+α-syn+CD. Synphilin = red; CD = green. The levels of synphilin immunostaining were indistinguishable in cells transfected with synphilin+α-syn+vector versus cells transfected with synphilin+α-syn+CD. This is true in cells with or without aggregates. Scale bar = 25 micron. Arrows point to α-syn aggregates.

Overexpression of CD is neuroprotective in C. elegans
To further investigate the role of CD activity in α-syn clearance in vivo, we generated transgenic C. elegans expressing a human α-syn and GFP fusion protein in body wall muscle cells. In these worms, human α-syn::GFP forms aggregates as worms develop and age (Fig. 6a). As in mammalian cells[46], co-expression of the worm TOR-2 protein chaperone ameliorated the formation of α-syn::GFP aggregates (Fig. 6b). Importantly, this established a genetic background within which enhancement of α-syn aggregation could be more readily visualized by RNA interference (RNAi). Using bacterial RNAi feeding to specifically target the C. elegans ortholog of Ctsd, we knocked down Ctsd in α-syn::GFP + TOR-2 transgenic worms. RNAi targeting of Ctsd led to a return of fluorescent aggregates over time (Fig. 6c), and RNAi of Ctsd did not affect tor-2 mRNA levels as examined by real-time RT-PCR analyses.
Figure 5 (see legend on next page)
mature product are shown. Actin immunoblot was used as a loading control.

A53T and A30P may be via alteration of their intracellular targeting rather than direct cleavage. CD 53 kDa and 47 kDa precur-
sors and the 32 kDa mature product are shown. Actin immunoblot was used as a loading control.

mutated "syn monomers. CD is synthesized as a prepropeptide (53 kDa); the signal peptide is cleaved upon CD
insertion into the endoplasmic reticulum (47 kDa). The CD zymogen is then activated in the acidic lysosomal environment to
produce the 32 and 14 kDa products [13,47]. d. Enhanced CD expression reduces A53T and A30P mutant "syn-induced cell
death, but does not reduce Y125A mutant "syn-, 10 μM chloroquine-, or 2 μM staurosporine-induced cell death. For a-d, *p
< 0.05 compared to control (CTL); † p < 0.05 compared to otherwise identical transfection except without CD. n = 3 transfec-
tion for each experimental conditions. Student t-test was used. e. Increased protein levels correlate with transfection of
respective cDNAs. SHSY5Y cells were transfected with control vector, or respective cDNA in each lane. The respective anti-
bodies used for the immunoblots are at the left side of the gel images. Mutated "syn-GFP were produced. CD transfection did
not produce significant truncation intermediate products on the mutated "syn-GFP, suggesting that the protection against
A53T and A30P may be via alteration of their intracellular targeting rather than direct cleavage. CD 53 kDa and 47 kDa precur-
sors and the 32 kDa mature product are shown. Actin immunoblot was used as a loading control.

PCR (Fig. 6f). Thus, Ctsd deficiency led to "syn aggrega-
tion in both mice and worms.

We further investigated whether human CD attenuates the
loss of dopaminergic neurons in a C. elegans model of "syn-induced neurodegeneration[44]. Overexpression of
"syn led to DA neuron death, as evidenced by the finding that only 16% of 7 d old adult "syn-expressing worms (n
= 270 worms analyzed) displayed normal numbers of DA
neurons (Fig. 6d, g). In contrast, co-overexpression of human CD significantly protected against DA neurode-
generation, since 30% of same-staged animals (n = 270)
exhibited wildtype DA neurons (Fig. 6e, g; p < 0.001,
Fisher Exact Test). Overexpression of enzymatic mutants
of CD (D295R and F229I) [16,47], or related human
cathepsin gene products, CB or CL, did not attenuate DA
neuron death in this in vivo assay (Fig. 6g), thereby sug-
gest a specific role of CD in neuroprotection against "syn-induced DA cell death, as well as an essential role of
CD enzymatic activity in this neuroprotection.

Discussion
Our results demonstrate that CD deficiency leads to sig-
nificant accumulation of "syn that is concentrated in
neuronal cell bodies, despite the up-regulation of multi-
ple lysosomal cathepsin genes. "syn-synucleinopathy arises
from CD dysfunction, either directly due to deficient pro-
teolytic cleavage, and/or indirectly as a consequence of
compromised proteasome activity. Most significantly, we
further demonstrated that CD overexpression protects
neurons from "syn aggregation and "syn-induced death
both in mammalian cells and in C. elegans. These findings
suggest that targeted CD therapy may inhibit or reverse LB
disease-associated neuropathology.

The "syn accumulations in Ctsd-/- brains are neuronal
and perinuclear. Although Ctsd-/- brain extracts exhibited
increased immunoreactive protein species recognized by
the anti-phospho-"syn antibody that specifically recog-
nizes human LB[36], "syn accumulations in Ctsd-/-
brains could not be detected by this antibody that specific-
ically recognizes human LB despite that they were success-
fully used in our hands to demonstrate LB-specific
staining of human PD specimen (data not shown). Only
5% of neurons exhibited intense immunoreactivity for
both "syn and ubiquitin suggesting their accumulation,
an observation in accordance with that only a small frac-
tion of "syn is ubiquitinated in human LB [33-36]. "syn accumulations are adjacent to but not overlapping with
autophagosomal or lysosomal markers, suggesting an
attenuation of these complexes and organelles in encir-
cling accumulated "syn in the Ctsd-/- brains. Where "syn
unfolds and forms oligomers, and whether CD reduces a specific sub-population of "syn protofibrils or
other small assemblies of "syn require further investiga-
tion.

α-syn gene triplication is causative in a subset of PD, which
suggests that increasing α-syn gene dosage pro-
motes DA neuron death in these patients[1]. Overproduc-
tion of α-syn via AAV delivery induces DA neuron death
in rodents[45]. CD deficiency induced by aging, genetic
polyorphism, or environmental toxins may predispose
animals to an earlier onset of spontaneous neuron death,
or exacerbate neurotoxin or α-syn overproduction-
induced DA neuron loss, in comparison to wildtype con-
trol mice. Furthermore, although Ctsd-/- mice die approx-
imately p26 without apparent tau or beta amyloid
accumulation in the brain (data not shown), we cannot
Figure 6 (see legend on next page)
exclude the possibility that partial loss of CD may also affect tau or beta amyloid clearance.

We found significant down-regulation of proteasome activities in Ctsd-/- brains. The reduction of proteasome activity may be due to reduced expression of UPS regulators and proteasome subunits, inactivating post-translational modification of these proteins, or specific inhibitory signaling by the accumulated ubiquitinated proteins. Crosstalk between protein degradation pathways is potentially important in neurodegenerative disease pathogenesis since inhibition of proteasomes up-regulates lysosomal enzymes[48], and enhancing proteasome function alleviates autophagy defects[49]. Furthermore, α-syn overexpression in mammalian cells affects proteasome activity, macroautophagy as well as lysosomal functions[50]. Lysosomal storage disorders and lysosomal cysteine protease inhibitor E64 treatment were previously shown to down-regulate UCHL1[51], however, such is not the case in Ctsd-/- brains in our study, indicating a distinct mechanism of CD deficiency-induced proteasome dysfunction. GAPDH accumulation may be a consequence of impaired CMA[3], because Ctsd-/- cells are compromised in lysosomal function[3]. Alternatively, GAPDH elevation may represent an ultimately futile compensatory signal that elevates macroautophagy initiation as previously suggested in HeLa cells[52]. Additionally, GAPDH elevation may promote the formation of protein inclusions [53,54].

The overproduction of α-syn has been shown to cause cell death in many studies through a variety of mechanisms, including a compromise in synaptic vesicle function, the induction of ER stress, Golgi fragmentation, mitochondrial dysfunctions and proteasome dysfunction [44,55-58]. However, cell death in these studies required massive overproduction of α-syn. Even in these conditions, the challenge has been to determine whether the intermediate species of α-syn are the most toxic to neurons. It remains possible that the α-syn aggregates in sporadic PD are not causative to DA neuron death, but rather a compensatory activity to attenuate DA neuron death, or a mere bystander effect in DA neuron death. The comparison of different mutant forms of α-syn in their propensity in forming fibrils and their toxicity was carried out in yeast and the rate of fibrillization did not positively correlate with toxicity[59]. α-syn can interact with many other proteins[60], but whether these interactions elicit cytoprotective versus death-inducing effects remains to be elucidated. Other observations suggest that LB formation may be a mechanism to sequester toxic α-syn species and thus exert a protective function [61-63]. Our observation that α-syn accumulates in Ctsd-/- brains does not distinguish its role in promoting or attenuating death in Ctsd-/- brains in vivo. We did not observe neurons with concurrent activation of caspase-3 and α-syn accumulation (Fig. 2). This may indicate that α-syn accumulation and caspase-3 activation are with different time course. Alternatively, our observation may suggest that certain forms of α-syn accumulation induce non-apoptotic cell death, or even compensatory neuroprotection.

α-syn knockout mice are resistant to MPTP-induced DA neuron death [64-66]. Reduction of α-syn is neuroprotective in multiple animal models [67,68]. Regardless of the precise mechanism for CD deficiency induced α-synucleinopathy, our finding that enhanced expression of CD is neuroprotective against α-syn aggregation and toxicity, both in worms and in mammals, provides a basis for future investigation of whether delivery of CD to the central nervous system in animal models of LB diseases can help alleviate protein aggregation and toxicity, and thereby serve as a previously unexplored target for disease therapy.
**Methods**

**Mice**

We genotyped littermates from $\text{Ctsd}^{+/+}$ breeding. We used $\text{Ctsd}^{+/+}$, $\text{Ctsd}^{+/n}$ and $\text{Ctsd}^{-/-}$ littermates on C57BL6 background for all experiments.

**Immunohistochemistry**

Brains were placed in Bouin’s fixative overnight at 4°C followed by paraffin embedding. 5 μm thick sections were used for immunohistochemical studies. The following antibodies were used: mouse anti-NeuN (Chemicon), mouse anti-α-syn (BD Transduction Lab), sheep anti-α-syn (Chemicon), goat anti-CD (Santa Cruz), mouse anti-GFAP (Chemicon), rabbit anti-Ub (Dako), mouse anti-Ub (FK2, Biomol), mouse anti-Ub (Chemicon, clone Ub1), mouse anti-Ub (Zymed), mouse anti-synaptophysin (Chemicon), goat anti-cathepsin B (Santa Cruz), rabbit anti- active caspase 3 (Chemicon), and rabbit-anti-GAPDH (Cell Signaling). Horseradish peroxidase conjugated donkey derived secondary antibodies were used at 1:2000 (Jackson ImmunoResearch). The sections were then incubated with tyramide signal amplification (TSA) plus detection solution with Cy3 or fluorescein tyramide according to the manufacturer’s instruction (PerkinElmer Life Sciences), followed by bisbenzimide staining of DNA. For chromogenic immunohistochemistry, we incubated the sections with a biotinylated secondary antibody (Vector Laboratories) followed by ABC reagent (ABC kit, Vector Laboratories). We visualized the immunoreaction by treating the sections in 0.05% diaminobenzidine (DAB) according to the manufacturer’s instruction (PerkinElmer Life Sciences), followed by bisbenzimide staining of DNA. We then centrifuged homogenates at 15,000 g for 15 min at 4°C to separate supernatants (fractions soluble in 1% TritonX-100) and pellets (TritonX-100-insoluble fractions)[43]. Pellets were resuspended in lysis buffer containing 2% SDS. Western blotting for each sample was done at least twice. The antibodies used were described in Immunohistochemistry.

**Quantitative PCR**

Total brain RNA was isolated from p25 mice using RNA-STAT60 (Tel-Test, Friendswood, TX). Total RNA (2 μg) was then reverse transcribed using Applied Biosystems GeneAmp Gold RNA PCR Reagent Kit (Foster City, CA). Real-time PCR reactions were setup in duplicate using TaqMan gene assays and amplified in an Applied Biosystems Step-One instrument. ΔΔCT curves were generated using 18S TaqMan gene assays as internal standards. Quantitative PCR results are shown as standard deviation of from 3 different amplifications from RNA reverse transcribed from 3 different animals. Individual gene assay kits were purchased from Applied Biosystems for each of the RNAs analyzed. Paired t-tests were conducted on RQ (relative quantity) values for each group to determine significance.

**Proteasome activity assays**

We analyzed the proteasome activities using the TritonX-100-soluble fractions. The assay buffer consists of 50 mM Tris (pH7.5), 2.5 mM EGTA, 20% glycerol, 1 mM DTT, 0.05% NP-40, 50 μM substrate. Lactacystin was used at a final concentration of 10 μM to block proteasome activities as negative controls. Fluorescence was read at 5 min intervals for 2 h, at an excitation wavelength of 380 nm
and an emission wavelength of 460 nM. Assays were done in triplicate, each using \( n \geq 3 \) mice per genotype.

**C. elegans experiments**  
Nematodes were maintained following standard procedures[69]. Worms expressing \( \alpha \)-syn alone UA49 [\( baIn2; P_{unc-54}:\alpha\text{-syn}\text{-gfp}, rol-6 \text{(su1006)} \)] or with \( tor-2 \) [UA50; \( baIn3; P_{unc-54}:\alpha\text{-syn\text{-gfp}}, P_{unc-54}:tor-2, rol-6 \text{(su1006)} \)] were created, integrated into the genome to generate an isogenic line, and out-crossed four times. We used the worm line that overexpresses \( TOR-2 \) protein (a worm homolog of human torsinA) and \( \alpha \)-syn fused to GFP in the body wall muscle cells because these cells are much larger than neurons for detecting \( \alpha \)-syn aggregation. Moreover, \( C. \text{ elegans} \) dopaminergic neurons have been shown to be refractory to RNAi. Using this isogenic line, we knocked down the worm Ctsd ortholog by RNAi, and scored for the return of \( \alpha \)-syn aggregates over the course of development and aging.

RNAi was performed by bacterial feeding as described[70] with the following modification. A Ctsd-specific RNAi feeding clone targeting a distinct portion of the *C. elegans* open reading frame [R12H7.2 (asp-4)]; e-value = 1.8e-108] with highest homology to human *Ctsd* (Geneservice) was grown for 14 h in LB broth with 100 \( \mu \)g/ml ampicillin and seeded onto NGM agar plates containing 1 mM isopropyl \( \beta \)-D-thiogalactoside. After 4 h incubation at 25°C to dry the plates, five gravid adults were then placed onto the corresponding RNAi plates and allowed to lay eggs for 9 h; the resulting age-synchronized worms were analyzed at the indicated stage. RNAi knockdown was performed in duplicate sets of animals and enhancement \( \alpha \)-syn misfolding was scored as positive if at least 80% of worms displayed an increased quantity and size of \( \alpha \)-syn::GFP aggregates. For each trial, 20 worms were transferred onto a 2% agarose pad, immobilized with 2 mM levamisole, and analyzed using Nikon Eclipse E800 epifluorescence microscope equipped with Endow GFP HYQ filter cube (Chroma Technology). Images were captured with a Cool Snap CCD camera (Photometrics) driven by MetaMorph software (Universal Imaging).

**Quantitative PCR from *C. elegans***  
The procedure for total RNA isolation, cDNA preparation, and semi-quantitative RT-PCR was described previously[71]. The following primers were used for the PCR:  
*cdk-5* Primer 1: 5' ggg-gat-gag-ggt-gtt-cca-agc 3'; Primer 2: 5' ggc-gac-cgg-cat-ttg-aga-tct-c 3'; *tor-2* Primer 1: 5' caa-tta-tca-tgc-gtt-ata-caa-ag 3'; Primer 2: 5' ctc-act-tcg-ata-agt-att-g 3'.

For the DA neurodegeneration analysis, strain UA54 [\( baEx45; P_{dat-1}:\alpha\text{-syn}, P_{dat-1}:\text{gfp}, P_{dat-1}:CD, rol-6 \text{(su1006)} \)], UA90 [\( baEx69; P_{dat-1}:\alpha\text{-syn}, P_{dat-1}:\text{gfp}, P_{dat-1}:CD \text{D295R, rol-6 (su1006)} \)], UA91 [\( baEx70; P_{dat-1}:\alpha\text{-syn}, P_{dat-1}:\text{gfp}, P_{dat-1}:CD \text{F229I, rol-6 (su1006)} \)], UA53 [\( baEx44; P_{dat-1}:\alpha\text{-syn}, P_{dat-1}:\text{gfp}, P_{dat-1}:\text{CB, rol-6 (su1006)} \)], and UA55 [\( baEx46; P_{dat-1}:\alpha\text{-syn}, P_{dat-1}:\text{gfp}, P_{dat-1}:\text{CL, rol-6 (su1006)} \)] were generated by injecting 50 \( \mu \)g/ml of expression plasmid containing the human cathepsin cDNA and 50 \( \mu \)g/ml of rol-6 into an integrated line of UA44 [\( baIn1; P_{dat-1}:\alpha\text{-syn}, P_{dat-1}:\text{gfp} \)] [72]. Three stable lines were randomly selected for neurodegeneration analysis. The 6 anterior DA neurons (4 CEP and 2 ADE neurons) of 30 animals/trial were examined for neurodegeneration when the animals were 7 days old. 90 animals from each of three CD (or CD D295R, CD F229I, CB, and CL) transgenic lines were analyzed (3 lines \( \times \) 3 trials of 30 animals/trial = 270 total animals scored). Worms displaying at least one degenerative change (dendrite, axon, or cell body loss) were scored as exhibiting degenerating neurons as previously reported [44,72].

**Abbreviations**  
\( \alpha \)-syn: alpha-synuclein; PD: Parkinson’s disease; CD: Cathepsin D; GFP: green fluorescent protein; RNAi: RNA interference; UPS: ubiquitin-proteasome system.

**Competing interests**  
KAC and GAC are scientific advisors to QRxPharma, Ltd. from whom they receive monetary compensation and a sponsored research agreement. The other authors have declared that no conflict of interest exists.

**Authors’ contributions**  
LQ performed the majority of the experiments of immunohistochemistry, western blot analyses, proteasome activity assays, and cell culture studies. SH, KAC and GAC contributed to all the findings in the worm. TAY contributed the \( \alpha \)-syn aggregation assay in H4 cells. SW contributed the real-time PCR. ZLX, RP, LDS, DC, QL, SC and LS assisted with various experiments. YU provided the LC3 antibody. TI provided the anti-phospho-\( \alpha \)-syn antibody. YZ constructed mammalian expression plasmids for GFP-\( \alpha \)-syn. LP and YML constructed the mammalian expression plasmid for CD. KCW, JJS and KAR contributed the original CD mutant mice, brain sections and extracts for our initial findings, helped with setting up western and immunohistochemistry techniques, and provided discussions on autophagy and CD. KAR was essential for identification of the \( \alpha \)-syn aggregates after immunostaining and provided neuropathology expertise, discussions, and assistance in writing the manuscript. DGS provided discussions and expertise in PD, \( \alpha \)-syn metabolism, and confocal microscopy. JZ directed the project and wrote the manuscript.
Acknowledgements

We thank Songsong Cao for generating the α-syn::GFP transgenic worms, Dr. Pam McLean for plasmids used for α-syn aggregation assay in H4 cells, Drs. M Brenner and Y-F Chen for critically reading the manuscript, Drs. J Wu and J Tucholski, and members of our laboratories for technical assistance and discussions. Csd mutant mice were generously provided by Dr. P Safi (University of Kiel). We thank Genta Ito for handling the shipping of the phospho-α-syn antibody. This work is funded by the American Parkinson’s Disease Association and Michael J. Fox Foundation (GAC, KAC, DGS), Batten Disease Support and Research Association, VA career development award, and UAB Alzheimer Disease Research Center (JJS). NIH grants NS535107 and NS41962 (KAR), a UAB faculty development and startup fund, UAB Alzheimer Disease Research Center, the American Parkinson’s Disease Association, and Michael J. Fox Foundation (ZJ). This work is also supported by UAB Neuroscience Core Facilities (NS47466 and NS57098). This work was also supported by the American Heart Association (0553413, YL), NIH/NINDS (R01NS501383, YL) and NIH/NIH (R01AG033282, YL).

References

1. Morris HR: Genetics of Parkinson’s disease. Ann Neurol 2005, 57:85-96.
2. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC: Alpha-Synuclein is degraded by both autophagy and the proteasome. J Biol Chem 2003, 278:25009-25013.
3. Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Rubinsztein DC: Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science 2004, 305:1292-1295.
4. Martinez-Vicente M, Talloccy Z, Kaischik S, Massey AC, Mazzilli J, Mosharor EV, Hodara R, Frendburg R, Wu DC, Follenzi A, et al.: Diamine-modified alpha-synuclein blocks chaperone-mediated autophagy. J Clin Invest 2008.
5. Cuervo AM, Dice JF: When lysosomes get old. Exp Gerontol 2000, 35:119-131.
6. Marino G, Lopez-Otin C: Autophagy: molecular mechanisms, physiological functions and relevance in human pathology. Cell Mol Life Sci 2004, 61:1439-1454.
7. Yamamoto A, Cremona ML, Rothman JE: Autophagy-mediated clearance of huntingtin aggregates triggered by the insulin-signaling pathway. J Cell Biol 2006, 172:719-731.
8. Berger Z, Ravikumar B, Menzies FM, Oroz LG, Underwood BR, Pansegrau W, Nukina N, Nukina Y, Rubinsztein DC: Cathepsin D deficiency induces lysosomal storage with ceroid lipofuscinosis. J Biol Chem 2003, 278:13086-13096.
9. Schapira AH, Blau N, Towers CH, Rubinsztein DC: Impaired degradation of mutant α-synuclein in Huntington’s disease. J Neurochem 2002, 81:960-971.
10. Nijssen PC, Brusse E, Leyten AC, Martin JJ, Teepen JU, Roos RA: Autosomal dominant adult neuronal ceroid lipofuscinosis: parkinsonism due to both striatal and nigral dysfunction. Mov Disord 2002, 17:482-487.
11. Zimmaro A, Neudorfer O, Elstein D: The glucocerebrosidase gene and Parkinson’s disease in Ashkenazi Jews. N Engl J Med 2005, 352:728-731.
12. Wong K, Sidransky E, Verma A, Moxon T, Sandberg GD, Wakefield LJ, Morrison A, Lwin A, Colegail C, Allman JH, et al.: Neuropathology provides clues to the pathophysiology of Gaucher disease. Mol Genet Metab 2004, 82:192-207.
13. Nakamura K, Ito M, Nakahara Y, Suzuki-Migita R, Yokoyama M, Mishima K, Saito T, Osumi M, et al.: Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 2006, 441:885-889.
14. Hara T, Nakamura K, Mutai T, Nakamoto A, Nakahara Y, Suzuki-Migita R, Yokoyama M, Mishima K, Saito T, Osumi M, et al.: Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 2006, 441:880-884.
15. Eskelinen EL, Illert AL, Tanaka Y, Schwarzmann G, Blanck J, von FK, Safatig P: Role of LAMP-2 in lysosome biogenesis and autophagy. Mol Biol Cell 2002, 13:3353-3368.
16. Covey AM, Dice JF: When lysosomes get old. Exp Gerontol 2000, 35:119-131.
17. Wassmann K, Schapira AH, Rubinsztein DC: Cathepsin D deficiency is associated with a human neurodegenerative disorder. Ann Neurol 2003, 53:988-998.
36. Fujiwara H, Hasegawa M, Doehmae N, Kawashima A, Masliah E, Gold- 
berg MS, Shen J, Tsukuo T, Iwatsubo T: Alpha-Synuclein is phospho-
rylated in synucleinopathy lesions. Nat Cell Biol 2002, 4:160-168.
37. Neumann M, Kuijle PJ, Giasson BI, Ozmen L, Borroni E, Spooren W, 
Muller V, Odys S, Fujiwara H, Hasegawa M, et al.: Misfolded protei-
nease K-resistant hyperphosphorylated alpha-synuclein in 
aged transgenic mice with locomotor deterioration and in human 
alpha-synucleinopathies. J Clin Invest 2002, 110:1429-1437.
38. Hirai Y, Fujita SC, Iwatsubo T, Hasegawa M: Phosphorylated 
alpha-synuclein in normal mouse brain. FEBS Lett 2004, 572:227-232.
39. Cantuti-Castelvetri I, Klucken J, Ingelsson M, Ramasamy K, McLean PJ, 
Frosch MP, Hyman BT, Standaert DG: Alpha-synuclein and chap-
erones in dementia with Lewy bodies. J Neuropathol Exp Neu-
rology 2005, 64:1058-1066.
40. Nakashii H, Zhang J, Koike M, Nishioku T, Okamoto Y, Kominami E, 
Nakanishi H, Zhang J, Koike M, Nishioku T, Okamoto Y, Kominami E, 
Hashimoto M, Takeda A, Sagara Y, Sisk A, Mucke L: Dopaminergic 
and autophagy preserve survival after apoptotic cytochrome 
c release in the absence of caspase activation. Cell 2007, 129:983-997.
41. McLean PJ, Kawamata H, Hyman BT: Alpha-synuclein-enhanced 
green fluorescent protein transgenes form protein- 
sensitive inclusions in primary neurons. Neuroscience 2001, 
104:901-912.
42. Masliah E, Rockenstein E, Weinberg M, Hashimoto M, Takeda A, Sagar a Y, Sisk A, Mucke L: Dopaminergic loss and 
motor changes of cathepsin D-deficient mice. J Neurosci 2001, 
21:7526-7533.
43. Giasson BI, Duda JE, Quinn SM, Zhang B, Trojanowski JQ, Lee VM: 
Neuronal alpha-synucleinopathy with severe movement dis-
order in mice expressing A53T human alpha-synuclein. Ne-
uron 2002, 34:521-533.
44. Cooper AA, Gitter AD, Cashikar A, Haynes CM, Hill JK, Bhullar B, Liu 
X, Ku, Strathearn KE, Liu F, et al.: Alpha-synuclein blocks ER-
Golgi traffic and Rabi rescue s neuron loss in Parkinson's 
mice. PLoS Biology 2006, 4:3234-3248.
45. St. Martin JL, Klucken J, Outeiro TF, Nguyen P, Keller-McGandy C, 
Cantuti-Castelvetri I, Grammatopoulos TN, Standaert DG, Hyman BT, 
McLean PJ: Dopaminergic neuron loss and up-regulation of 
cell cycle proteins by Tm RNA induced by targeted over-expression 
of alpha-synuclein in mouse substantia nigra. J Neurochem 2007, 
100:1449-1447.
46. McLean PJ, Kawamata H, Shariﬀ S, Hewett J, Sharma N, Ueda K, 
Breakefield XO, Hyman BT: TorsinA and heat shock proteins 
are involved in the expression of A53T human alpha-synuclein 
aggregation. J Neurochem 2002, 83:846-854.
47. Tyynela J, Sohar I, Sleet DE, Gin RM, Donnelly RJ, Baumann M, Halta 
M, Lobel P: A mutation in the ovine cathepsin D gene causes a 
genetic lysosomal storage disease with profound neuro-
developmental defects. J Biol Chem 2004, 279:2761-2769.
48. Rideout HJ, Lang-Rollin I, Stefanis L: Involvement of macroau-
tophagy in the dissolution of neuronal inclusions. Int J Biochem 
Cell Biol 2004, 36:2531-2562.
49. Pandey UB, Nie Z, Batley Y, McCray BA, Risotto GP, Nedelsky NB, 
Schwartz SL, DiProspero NA, Knight MA, Schuldiner O, et al.: 
HDAC6 rescues neurodegeneration and provides an essen-
tial link between autophagy and the UPS. Nature 2007, 
447:859-863.
50. Stefanis L, Larsen KE, Rideout HJ, Sulzer D, Greene LA: Expression 
of A53T mutant but not wild type alpha-synuclein in PC12 
cells induces alterations of the ubiquitin-dependent degrada-
tion system, loss of dopamine release, autophagic cell death. J 
Neurosci 2001, 21:9549-9560.
51. Bifsha P, Lamas K, Ashmarina L, Durand S, Sevyantepe V, Trudel S, 
Quinnou C, Chembotob S, Xu Y, Gravel RA, et al.: Altered gene 
expression in cells from patients with lysosomal storage dis-
orders suggests impairment of the ubiquitin pathway. Cell 
Death Differ 2007, 14:511-523.
52. Coeell A, Ricci JE, Taia S, Milastra S, Mauher U, Boucher-Hayes L, Fitz-
gerald P, Guio-Carrion A, Waterhouse NJ, Li CW, et al.: GAPDH 
and autophagy preserve survival after apoptotic cytochrome 
c release in the absence of caspase activation. Cell 2007, 
129:983-997.
genes in a Parkinson's disease model. Proc Natl Acad Sci USA 2008, 105:728-733.
72. Cao S, Gelwix CC, Caldwell KA, Caldwell GA: Torsin-mediated protection from cellular stress in the dopaminergic neurons of Caenorhabditis elegans. J Neurosci 2005, 25:3801-3812.