Influence of autophagy genes on ion-channel-dependent neuronal degeneration in *Caenorhabditis elegans*

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Summary

Necrotic cell death is a common feature in numerous human neurodegenerative disorders. In the nematode *Caenorhabditis elegans*, gain-of-function mutations in genes that encode specific ion channel subunits such as the degenerins DEG-1 and MEC-4, and the acetylcholine receptor subunit DEG-3 lead to necrotic-like degeneration of a subset of neurons. Neuronal demise caused by ion channel hyperactivity is accompanied by intense degradation of cytoplasmic contents, dramatic membrane infolding and vacuole formation; however, the cellular pathways underlying such processes remain largely unknown. Here we show that the function of three autophagy genes, whose yeast and mammalian orthologs are implicated in cytoplasmic self-degradation, membrane trafficking and the cellular response to starvation, contributes to ion-channel-dependent neurotoxicity in *C. elegans*. Inactivation of *unc-51*, *bec-1* and *lgg-1*, the worm counterparts of the yeast autophagy genes *Atg1*, *Atg6* and *Atg8* respectively, partially suppresses degeneration of neurons with toxic ion channel variants. We also demonstrate that the TOR-kinase-mediated signaling pathway, a nutrient sensing system that downregulates the autophagy gene cascade, protects neurons from undergoing necrotic cell death, whereas nutrient deprivation promotes necrosis. Our findings reveal a role for autophagy genes in neuronal cell loss in *C. elegans*.

Key words: Neurodegeneration, Ion channel subunits, Autophagy, *C. elegans*, Dopamine neurons, TOR signaling

Introduction

Necrotic death of specific classes of neurons is characteristic for many human neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases (Driscoll and Gerstbrein, 2003; Yuan et al., 2003; Rubinsztein et al., 2005). The majority of these pathologies are induced by dominant mutations that cause late-onset loss of the affected nerve cells. Accumulating data suggest that autophagy – a process in which eukaryotic cells self-digest parts of their contents during development and to survive nutrient deprivation (Klionsky and Emr, 2000; Noda et al., 2002; Cuervo, 2004) – is implicated in various pathological states of the nervous system (Rubinsztein et al., 2005). Autophagy involves the formation of subcellular membrane structures termed autophagic vacuoles to sequester cargo for breakdown by acidic lysosomal hydrolases. In some cases, autophagic degradation of cytoplasmic constituents occurs as part of programmed cell death (Muller et al., 2004).

A strong association between the autophagic process and neuronal function results from studies on Lurcher mice bearing a gain-of-function mutation in the orphan delta 2 glutamate receptor (GluRδ2) (Zuo et al., 1997). This mutation causes neuronal degeneration in vivo. Characterization of a protein complex bound to GluRδ2 identified the autophagy protein Beclin1 (Liang et al., 1999) as a component of the complex (Yue et al., 2002). This finding raises the possibility that autophagy contributes to Lurcher pathology. Indeed, degrading Lurcher purkinje cells display intense autophagic activity (Doughty et al., 2000). Neuronal degeneration following traumatic brain injury in mice has also been shown to induce upregulation of Beclin1 (Erlich et al., 2006). Furthermore, accumulation of vacuole-like structures and lysosomes in neurons were reported in patients with a variety of neurodegenerative pathologies (Rubinsztein et al., 2005; Cataldo et al., 1994; Webb et al., 2003; Qin et al., 2003).

In *Caenorhabditis elegans*, gain-of-function mutant alleles of genes encoding specific ion channel subunits result in necrotic-like swelling and death of certain neurons. For example, gain-of-function mutations in *deg-3*, which encodes the α-subunit of the neuronal nicotinic acetylcholine receptor, trigger degeneration of several sensory neurons and PVC interneurons (Treinin and Chalfie, 1995). Gain-of-function alleles of two degenerin ion channel genes, *deg-1* and *mec-4*, cause the degenerative state of the IL1, ASH and PVC neurons, and six touch receptor neurons, respectively, rendering mutants incapable of mechanosensation (Chalfie and Wolinsky, 1990; Driscoll and Chalfie, 1991). In these genetic models, the identity of the dying neurons (Fig. 1A,B) and the time in development at which these cells die are essentially invariant among individuals (Chalfie and Sulston, 1981).

Degeneration of neurons with hyperactive MEC-4, DEG-1 or DEG-3 is partially suppressed by inactivation of specific aspartyl and calpain proteases (Syntichaki et al., 2002). The function of certain proteases is thus likely to contribute to

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necrotic cell death. The execution of degenerative processes in dying neurons is also influenced by acidic intracellular conditions because the vacuolar H+-ATPase, a membrane pump that acidifies the cell interior, has been found to contribute to toxic ion channel-induced necrosis (Syntichaki et al., 2005).

Ultrastructural analysis of dying neurons in *mec-4(gf)* and *deg-1(gf)* mutants has shown extensive degradation of cytoplasmic contents as well as formation of cytoplasmic vacuoles and membranous whorls during mid to late stages of cell death (Hall et al., 1997). Structural changes involving membrane rearrangements are also characteristic features in a wide range of cells with autophagic activity (Meléndez et al., 2003). Furthermore, lysosome function, which is essential for autophagy, is crucial for neuronal degeneration in *C. elegans* (Artal-Sanz et al., 2006). Here we investigate the role of three *C. elegans* autophagy genes, *bec-1*, *unc-51* and *lgg-1* (Meléndez et al., 2003; Kovács et al., 2004; Takács-Vellai et al., 2005), in necrotic-like cell death induced by mutations affecting ion channel subunits or by the neurotoxin 6-hydroxidopamine (6-OHDA). Our results demonstrate that inactivation of these genes influences neuronal degeneration in this organism.

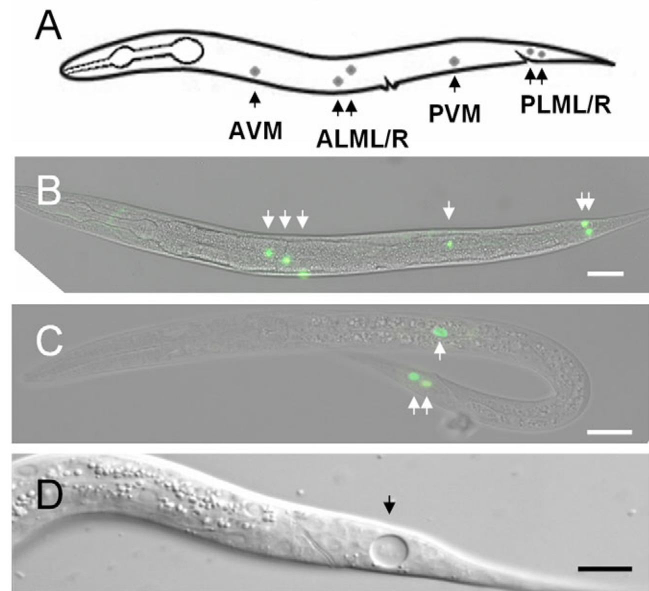

Fig. 1. Gain-of-function mutations in the *mec-4* gene cause a degenerative state of touch receptor neurons in *C. elegans*. (A) Schematic representation of the position of cell bodies of the six touch receptor neurons that express p*mec-4*::GFP. (B) Expression of p*mec-4*::GFP at the L2 larval stage in a wild-type animal. Arrows show the six touch receptor neurons. (C) Expression of p*mec-4*::GFP at the L2 stage in a *mec-4(u231)* mutant. Cell death was inferred when glowing touch receptor neurons were missing from their normal positions. Arrows indicate residual p*mec*-4::GFP expression. (D) Nomarski image shows the morphology of a vacuolated PLM neuron (arrow) undergoing necrosis in a *mec-4(u231)* mutant L1 larva. Bars, 100 μm.

Results

Inactivation of *bec-1/atg6* suppresses toxic ion channel-induced neuronal cell loss

The *C. elegans* genome encodes a defined set of orthologs of the yeast and mammalian autophagy proteins (Meléndez et al., 2003; Kovács et al., 2004). To evaluate whether these components, or at least some of them, are required for structural membrane changes including vacuolization that precede the death of toxic ion-channel-poisoned neurons, we first performed a genetic analysis of degenerin mutants with reduced or no BEC-1 activity. BEC-1 is similar to the yeast and mammalian autophagy proteins Atg6/Vps30 and Beclin1, and essential for development (Meléndez et al., 2003; Takács-Vellai et al., 2005). Two mutations in *bec-1*, *ok691* and *ok700*, result in a highly penetrant (92.7% and 85.1%, respectively) embryonic arrest phenotype (Takács-Vellai et al., 2005). Homozygous *bec-1* mutants that pass embryonic development die at different larval stages or at young adulthood. These animals often fail to emerge from the larval cuticle, are fragile and, if they pass the larval stages, become sterile. We found that in double mutant animals bearing the gain-of-function mutation *u231* in *mec-4*, *bec-1* mutant alleles suppress vacuolarization and swelling of touch receptor neurons (Fig. 2A). *mec-4(u231);bec-1(ok691)* and *mec-4(u231);bec-1(ok700)* double mutant animals had fewer vacuolated neurons than *mec-4(u231)* single mutants. Note that vacuolization is an unambiguous morphological sign of cell death in this neurodegeneration paradigm (Fig. 1D) (Hall et al., 1997; Syntichaki et al., 2002; Syntichaki et al., 2005). We rescued the lethality of *bec-1(–)* mutants by a non-integrated and thus unstable array containing wild-type copies of *bec-1* (this array actually consists of copies of a translational fusion functional BEC-1::GFP reporter: *swEx520*[p*bec-1::BEC-1::GFP + rol-6(su1006)*], see Materials and Methods). According to our data, vacuolization of touch receptor neurons caused by the *mec*-4 alleles *u231* (Fig. 2A) and *e1611* (data not shown) was significantly suppressed in *bec-1(–);Ex*[*bec-1(+)*] genetic background. Together, these data suggest a general requirement for BEC-1 in neuronal degeneration.

We also assayed cell death at the young adult stage in *mec-4(u231)* mutant hermaphrodites deficient for BEC-1 by scoring the number of intact touch receptor neurons that express a p*mec-4*::GFP reporter (Syntichaki et al., 2005). This reporter is expressed consistently in the touch receptor neurons from the late L3 larval stage until the postreproductive period. GFP driven solely by the *mec-4* promoter (p*mec-4*::GFP) was expressed in 5.6 touch receptor neurons on average (n=493) in an otherwise wild-type background (Fig. 1B and Fig. 2B). By contrast, p*mec-4*::GFP accumulated only in 1.92 touch receptor neurons on average (n=635) in *mec-4(u231)* mutants (Fig. 2B). We found an increased number of p*mec-4*::GFP-positive neurons at the young adult stage in homozygous *mec-4(u231); bec-1(–)* mutants, compared with *mec-4(u231)* single mutant animals (Fig. 2B). The expression intensity of both this transcriptional fusion p*mec-4*::GFP reporter and a translational fusion p*mec-4*::MEC-4::GFP reporter in wild-type animals was comparable with that observed in animals with reduced or eliminated BEC-1 activity (Fig. 2C). This implies that suppression of necrotic death by deregulation of *bec-1* was not merely a consequence of a reduction in the quantity of toxic MEC-4. It is also important to note that *mec-4*::GFP expression was not significantly affected in *bec-1(–)* single mutant animals (see the legend of Fig. 2), suggesting that BEC-1 is not required for the development of touch receptor neurons.

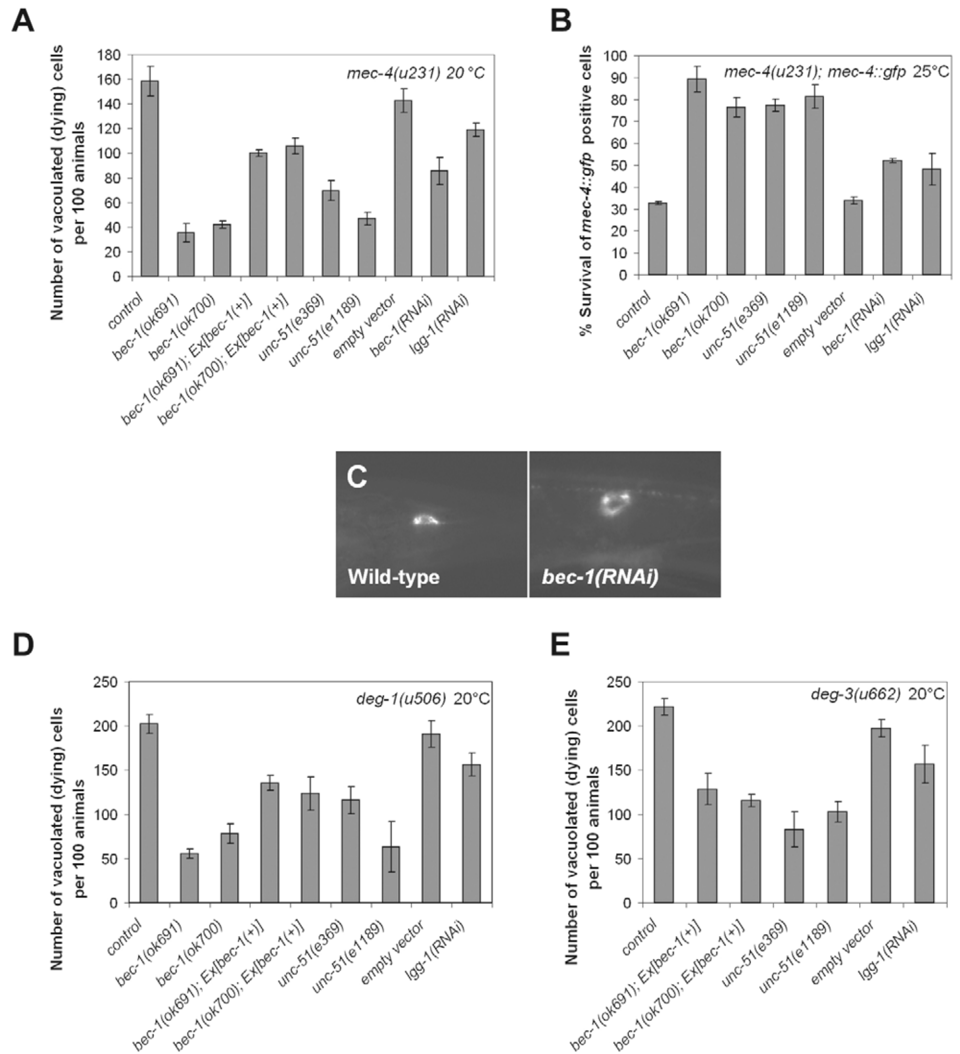

Fig. 2. Inactivation of the autophagy genes *bec-1*, *unc-51* and *lgg-1* suppresses ion-channel-dependent neuronal degeneration in *C. elegans*. (A) Number of vacuolated (dying) touch receptor neurons at the late L1 larval stage per 100 *mec-4(u231)* mutant animals with reduced autophagy gene activity. *bec-1(–)*; *Ex*[*bec-1(+)*]refers to *bec-1* mutant genotype bearing a non-integrated, extrachromosomal array (see Materials and Methods). For homozygous *bec-1* mutants, n=50, P<0.001; for *lgg-1(RNAi)* animals, n=200, P<0.001; for other genotypes, n=200; P<0.0001 (unpaired t-tests). (B) Percentage of p*mec-4*::GFP-positive touch receptor neurons at young adulthood in *mec-4(u231)* mutants with reduced autophagy gene activity. In contrast to panel A, this panel indicates survival rate (%) of cells. For *bec-1* mutants, n=50, P<0.001; for *lgg-1(RNAi)* animals, n=285, P<0.001; for other genotypes, n=200, P<0.0001 (unpaired t-tests). As a control, the expression of *mec-4*::GFP was assayed in *bec-1(ok691)* and *unc-51(e369)* single mutants as well as in *lgg-1(RNAi)* animals. Number of GFP-positive cells: *unc-51(e369)* mutants, 5.38 (n=263); *bec-1(ok691)* mutants, 5.47 (n=37); *lgg-1(RNAi)* animals, 5.67 (n=168); wild-type animals, 5.6 (n=493). These data show that inactivation of *bec-1* and *unc-51* only slightly influenced the development of touch receptor neurons, whereas *lgg-1* RNAi treatment did not affect it at all (it is notable that *lgg-1* RNAi completely abolished the expression of an *lgg-1::gfp* reporter). (C) Expression of a translational fusion p*mec-4*::MEC-4::GFP reporter in a PLM cell in a wild-type (left) and in an affected arrested *bec-1(RNAi)* (right) larva. In the latter, the expression intensity and number of GFP punctae were comparable with the wild type. (D) Number of vacuolated PVC interneurons at the L1 stage per 100 *deg-1(u506)* animals with reduced autophagy gene activity. For homozygous *bec-1* mutants, n=50, P<0.001; for *lgg-1(RNAi)* animals, n=300, P<0.01; for other genotypes, n=300, P<0.0001 (unpaired t-tests). (E) Number of vacuolated IL1 sensory neurons and PVC interneurons at the L1 stage per 100 *deg-3(u662)* mutants with reduced autophagy gene activity. For *lgg-1(RNAi)* animals, n=300, P<0.018; for other genotypes, n=300, P<0.0001; (unpaired t-tests). In RNAi experiments, control animals were fed with *E. coli* HT115 expressing the empty vector under RNAi-inducing conditions. Data are mean ± s.e.m.

We also checked the effects of BEC-1 deficiency on the survival of neurons that express toxic DEG-1 or DEG-3 proteins. By counting the number of vacuolated cells at the L1 larval stage, we found that inactivation and reduced activity of BEC-1 markedly suppress neuronal degeneration in both *deg-1(u506)* and *deg-3(u662)* mutants (Fig. 2D,E). Vacuolization of dying neurons differs from the formation of typical autophagic vacuoles as the latter are only 1 μm in diameter in *C. elegans* (Kovács et al., 2004). Similar but less effective reactions were obtained by RNA-interference-mediated depletion of BEC-1 in mutants with toxic ion channel subunit variants (Fig. 2A, data not shown). In summary, our data demonstrate that downregulation of BEC-1 is protective against necrotic cell death.

Mutational inactivation of *unc-51/atg1* suppresses ion-channel-dependent neuronal degeneration

We next assessed the function of another *C. elegans* autophagy gene, *unc-51*, in neuronal cell loss. *unc-51* encodes a serine/threonine kinase similar to the yeast Atg1 protein (Meléndez et al., 2003; Ogura et al., 1994). Two loss-of-function mutations in *unc-51*, *e369* and *e1189*, result in viable nematodes with uncoordinated movement (Unc). We found that homozygous *mec-4(u231);unc-51(–)* double mutant animals contain fewer vacuolated and more p*mec-4*::GFP-positive cells than *mec-4(u231)* single mutants (Fig. 2A,B). In addition, vacuolated cell corpses were detectable for a slightly longer period in these double mutants than in *mec-4(u231)* single mutant animals [as corpses were eliminated in 6-8 hours

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in *mec-4(u231)* and in 8-10 hours in *mec-4(u231);unc-51(e369)* background]. Mutations in *unc-51* also diminished vacuolization of specific neurons in *deg-1(u506)* and *deg-3(u662)* backgrounds (Fig. 2D,E). Thus, we conclude that UNC-51 contributes to neurotoxicity caused by hyperactive MEC-4, DEG-1 or DEG-3.

LGG-1/Atg8 deficiency influences ion-channel-dependent neurotoxicity

The third autophagy gene we tested in relation to neuronal degeneration was *lgg-1*. LGG-1 is the worm counterpart of the yeast ubiquitin-like protein Atg8, which constitutes a major component of the preautophagosomal complex (Meléndez et al., 2003; Hara et al., 2000). Because LGG-1 has not yet been characterized by mutations, we used RNAi to deplete its function. *lgg-1* RNAi treatment completely abolished the expression of a *lgg-1::gfp* reporter. Our results showed a weaker, but still significant reduction in the number of dying neurons in *mec-4*, *deg-1* and *deg-3* gain-of-function mutant animals treated with *lgg-1* double-stranded RNA, compared with animals fed with bacteria expressing the empty vector (see statistics for *lgg-1* data in the legend of Fig. 2A,B,D,E). This weaker effect of *lgg-1* RNAi on cell survival was probably due to the fact that RNAi is often ineffective for genes expressed in mature neurons. In spite of the weaker reaction, the statistically significant difference (for statistics see the legend of Fig. 2) shows that LGG-1 also influences genetically induced necrotic cell death. In summary, inactivation of *bec-1*, *unc-51* and *lgg-1* suppresses neuronal degeneration in *C. elegans*.

BEC-1 and UNC-51 deficiency restores mechanosensitivity in mutants with toxic ion channel variants

The data showing that BEC-1 contributes to ion channel-dependent neuronal degeneration prompted us to investigate whether inactivation of *bec-1* rescues the mechanosensation-defective phenotype of degenerin mutants. We performed a behavioral assay in which nematodes moving backwards were touched gently with an eyelash at the tail position and scored the percentage of those capable of moving away from the stimulus. While touch sensitivity of randomly chosen *bec-1* mosaic adults was comparable with the wild type, BEC-1 deficiency significantly enhanced the ability of *mec-4(u231)* and *deg-1(u506)* mutants to respond to touch stimulus (Fig. 3). This indicates that the surviving touch receptors may transmit some touch function.

We also tested the touch sensitiveness in *mec-4* and *deg-1* gain-of-function mutant animals bearing a mutation in *unc-51*. Because *unc-51* mutants are strongly restricted in movement (uncoordinated), we monitored whether they are able to shift the anterior or posterior body region upon touching. Both *unc-51* alleles decreased the penetrance of touch insensitivity in *mec-4(u231)* and *deg-1(u506)* mutant nematodes (Fig. 3). Moreover, mutations in *unc-51* suppressed another intriguing aspect of the pleiotropic Deg-1(u506) phenotype. While the cold-sensitive *u506* allele caused developmental arrest at the late embryonic or L1 larval stages at 15°C (0% escapers, *n*=843), a significant portion (6.19%, *n*=407) of *deg-1(u506) unc-51(e1189)* double mutants developed into fertile adults under identical conditions. Similar results were obtained

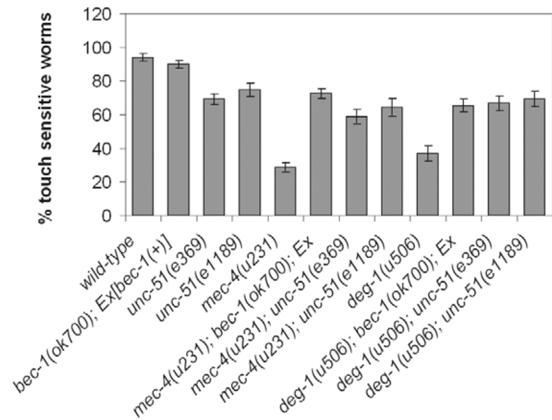

Fig. 3. UNC-51 and BEC-1 deficiency suppresses touch insensitivity in *mec-4(u231)* and *deg-1(u506)* mutants. Ex, *Ex*[p*bec-1*::BEC-1::GFP + *rol-6(su1006)*] genotype. Because *unc-51* mutant animals are paralyzed, we scored their ability to shift the anterior or posterior body region upon touching. For each double mutant genotype *n*=150, $P<0.0001$ (unpaired *t*-test). Data are mean ± s.e.m.

with double mutants bearing the *unc-51* mutation *e369* (4.9%, *n*=870).

Neuronal expression of the *C. elegans* autophagy genes

Because inactivation of *bec-1*, *unc-51* and *lgg-1* protects neurons from ion channel-dependent toxicity, we assumed that these autophagy genes are expressed in the nervous system throughout development. Indeed, UNC-51 was reported to appear in neurons (Ogura et al., 1997). Using a translational fusion GFP reporter driven by *bec-1* or *lgg-1* regulatory sequences, we found that both BEC-1::GFP and LGG-1::GFP are expressed in the cell bodies and processes of neurons, including touch receptor neurons, ventral cord neurons and the nerve ring in the head (Fig. 4A-D). A similar pattern was obtained for the TOR (for target of rapamycin) kinase (Fig. 4E) that is predicted to downregulate the autophagic machinery (Long et al., 2002).

We also tested the intracellular localization of a non-integrated translational fusion GFP::LGG-1 reporter (Meléndez et al., 2003) at the early stages of degenerative processes. GFP::LGG-1 often accumulated in a punctate pattern in neurons showing early signs of vacuolization (Fig. 4F-H). These GFP-positive foci of LGG-1 have been suggested to label preautophagosomal and autophagic structures (Meléndez et al., 2003). Thus, we conclude that autophagy might indeed occur in neurons undergoing necrosis.

Inactivation of *unc-51* or *bec-1* suppresses neurotoxin-induced cell death

Ion channel-induced neurotoxicity depends on the vacuolar H^+-ATPase (Syntichaki et al., 2005). Whether autophagy genes interact with the necrosis process itself or alternatively may affect the activity of ion channels or the vacuolar H^+-ATPase needs clarification. To address this issue we examined the role of UNC-51 and BEC-1 in another necrotic cell death paradigm that is independent of toxic channels. In *C. elegans*, like in mammals, selective degeneration of dopamine neurons can be triggered through exposure to the

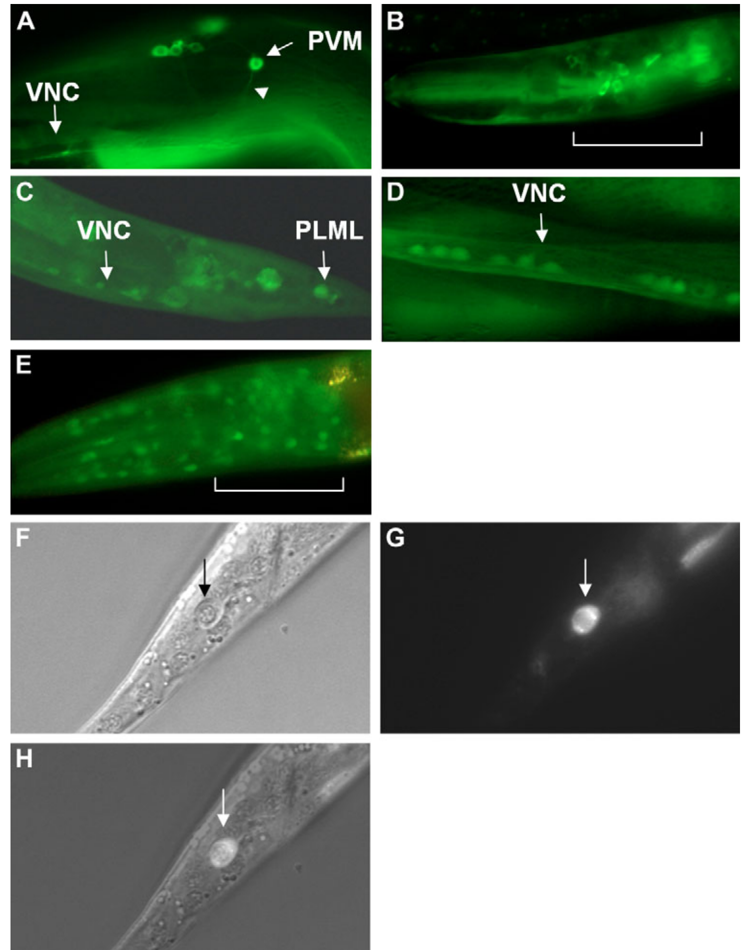

Fig. 4. BEC-1 and LGG-1 are expressed in the nervous system. (A) A non-functional translational fusion BEC-1::GFP reporter accumulates in the PVM of a wild-type animal. Both cell body and processes (arrowhead) of this cell are GFP positive. VNC, ventral nerve cord. (B) BEC-1 expression in the nerve ring (bracket). (C) LGG-1::GFP, which lacks the C terminus of LGG-1 (see Materials and Methods), is expressed in PLML. (D) LGG-1::GFP accumulation in the ventral nerve cord. (E) *CeTor*, which encodes a kinase that downregulates autophagy, is expressed in all neurons. Bracket indicates the nerve ring. (F-H) Nomarski, fluorescence and merged images, respectively, showing the expression of a non-integrated, translational fusion (functional) autophagosome marker GFP::LGG-1 (Meléndez et al., 2003) in a PVC interneuron of a *deg-3(u662)* animal. The GFP-positive punctate areas are inferred to label preautophagosomal and autophagosomal structures (Meléndez et al., 2003). In this specimen, the PVC interneuron is at the early stage of degeneration. Note that GFP becomes gradually diluted as degeneration progresses. The nearly exclusive expression of GFP in PVC is due to a mosaicism of the non-integrated marker.

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neurotoxin 6-hydroxidopamine (Nass et al., 2002). To visualize the three pairs of head dopamine neurons (two pairs of CEP neurons and one pair of ADE neurons) and necrotic processes, we examined hermaphrodites carrying a dopamine transporter (DAT) reporter, p*dat-1*::GFP (Nass et al., 2002). Treatment with 6-OHDA often caused a complete loss of p*dat-1*::GFP expression in the CEP and ADE neurons in the wild-type background (Fig. 5A,B,E). As previously shown by Nass and colleagues (Nass et al., 2002), the loss of GFP signal is unlikely to be a consequence of the transcriptional repression of the p*dat-1*::GFP transgene. Disruption of GFP expression in the head dopamine neurons by 6-OHDA was significantly suppressed in animals defective for UNC-51 or BEC-1 (Fig. 5C-E). Treatment with 6-OHDA caused a similar rate of lethality in wild-type and autophagy mutant genetic backgrounds (data not shown). This suggests that deregulation of *unc-51* and *bec-1* is unlikely to block the uptake of the toxin. Together, our data imply that these autophagy genes contribute to 6-OHDA-induced toxicity of dopamine neurons.

TOR signaling prevents, starvation promotes neuronal cell loss

Autophagy genes are known to be downregulated by a nutrient-sensing system that involves the TOR-kinase-mediated pathway (Klionsky and Emr, 2000). In *C. elegans*, TOR controls life span, reproductive growth and metabolism (Long et al., 2002; Vellai et al., 2003; Jia et al., 2004). We depleted TOR by RNAi and monitored whether this treatment affects neurotoxicity. We found a marked increase in the number of vacuolated (dying) touch receptor neurons in *mec-4(u231)* mutants with reduced CeTOR activity, compared with those treated with the empty vector (Fig. 6). This suggests that TOR signaling affects ion channel-induced neuronal degeneration.

To evaluate whether this effect of TOR on necrotic cell death is realized through a mechanism that involves nutrient signaling, we deprived newly hatched *mec-4(u231)* mutant L1 larvae of food for 24 hours because most cell deaths occur at the L1 stage (Hall et al., 1997). These worms were then transferred into favorable conditions and the survival rate of the six touch receptor neurons at the early adult stage was determined. *mec-4(u231)* mutant animals subjected to starvation contained significantly fewer p*mec-4*::GFP-positive neurons than those grown under well-fed conditions throughout development (Fig. 6). As a control, neither hyperactivation nor downregulation of the RTK-Ras signaling pathway (Fig. 6), which is also implicated in cellular response to nutritional stress, had a significant impact on neuronal loss. Together, environmental insults such as food limitation can make neurons susceptible to fatal necrotic injury. However, it must be noted that TOR has many downstream effectors, thus it cannot be excluded that its effect on neuronal degeneration is independent of autophagy.

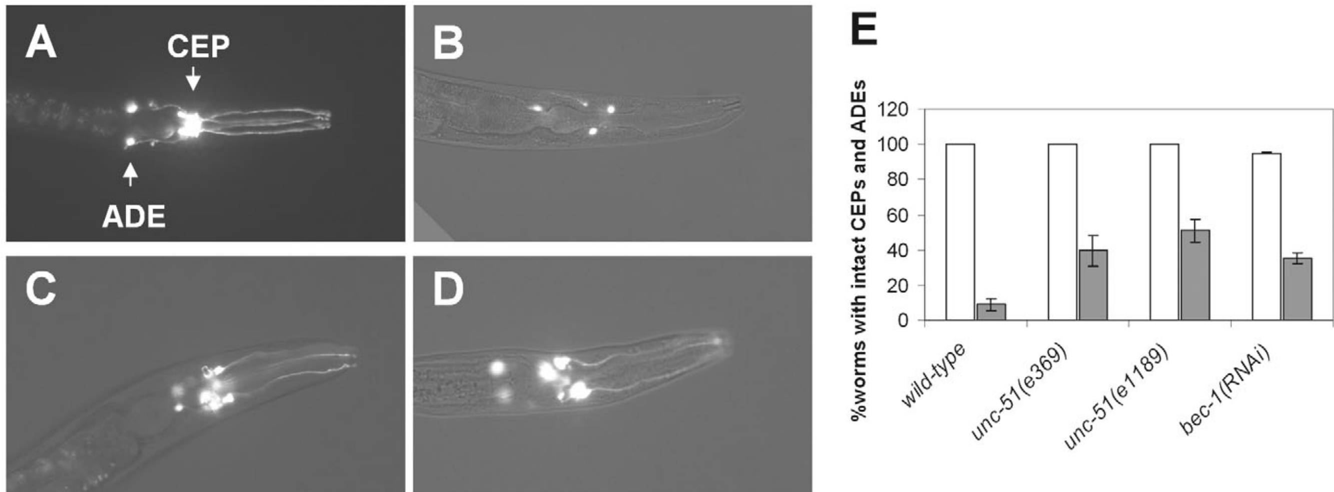

Fig. 5. BEC-1 and UNC-51 interfere with neurotoxin-induced necrotic cell death. (A) Expression of p*dat-1*::GFP in the head dopamine neurons in a wild-type hermaphrodite. (B) p*dat-1::GFP* expression in a wild-type hermaphrodite exposed to the neurotoxin 6-OHDA. 6-OHDA treatment results in severe degeneration of CEP and ADE dopamine neurons, revealed by the loss of processes and/or cell bodies. (C) p*dat-1*::GFP expression in the head dopamine neurons of an *unc-51(e1189)* mutant hermaphrodite. Although in some individuals the running of the processes is slightly affected, GFP expression is similar to that found in the wild type. (D) p*dat-1*::GFP expression in the head dopamine neurons in an *unc-51(e1189)* mutant hermaphrodite exposed to 6-OHDA. (E) UNC-51 and BEC-1 deficiency suppresses 6-OHDA sensitivity of the head dopamine neurons. The percentage of animals with intact CEP and ADE neurons (both cell bodies and processes) was scored. Data are mean ± s.e.m. Empty columns represent control animals, filled columns indicate animals exposed to 6-OHDA. n=100, P<0.01 (unpaired t-test).

Discussion

In this study we demonstrate that three *C. elegans* autophagy genes, *bec-1*, *unc-51* and *lgg-1*, which encode otherwise unrelated proteins, contribute to the degenerative state of dying neurons in a cell death paradigm defined by hyperactive ion channel subunits (Fig. 2). Mutational or RNAi-mediated inactivation of these genes diminishes the genetically introduced or neurotoxin-induced necrotic cell loss. Further studies are needed to determine whether other autophagy genes are also involved in necrotic-like neuronal cell loss. However, at present, only a very limited number of autophagy genes are available as mutant alleles and, as we also show here, their RNAi-mediated silencing is often ineffective or leads to weak reactions as it is generally observed for genes functioning in neurons.

Although autophagy genes seem to be involved in necrotic cell death, it remains an open question whether autophagy itself is also implicated. Various pathological states of the nervous system such as Alzheimer's, Parkinson's and Huntington's diseases have been suggested to involve autophagy (Klionsky and Emr, 2000; Cuervo, 2004). Alzheimer's disease is defined by neuritic plaques consisting of extracellularly aggregated β-amyloid or by intraneuronal fibrillary tangles containing aggregated forms of the microtubule-associated protein tau (Yankner, 1996). In patients with Alzheimer's disease, neurons progressively accumulate lysosomes as they become metabolically compromised. In addition, autophagic vacuoles often contain β-amyloid, suggesting that they may be involved in β-amyloidogenesis. In Parkinson's disease, degeneration of specific dopaminergic neurons results from the accumulation of filamentous cytoplasmic inclusions termed Lewy bodies that consist of mainly α-synuclein. Degradation of normal α-synuclein has been shown to occur via chaperone-mediated autophagy (Cuervo et al., 2004). Furthermore, forced expression of α-synuclein in neuronal cell lines causes a marked induction of autophagy (Stefanis et al., 2001). Huntington's disease is

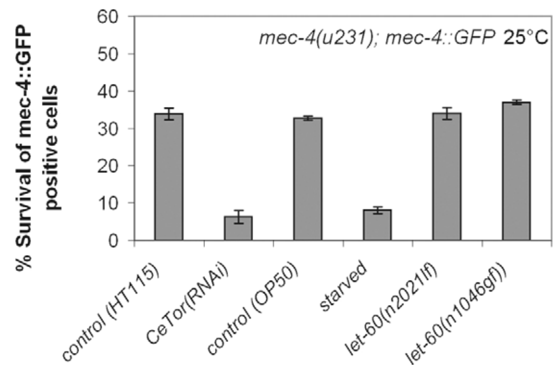

Fig. 6. Influence of nutrient signaling on degenerin-induced neurotoxicity. Survival rate (%) of the six touch receptor neurons at the young adult stage in *mec-4(u231)* mutants with reduced CeTOR activity or exposed to starvation for 1 day at the L1 larval stage. Number of *mec-4*::GFP-positive cells was scored. Note that the percentage of GFP-positive cells was significantly higher in 11-day-old adults well fed during development (20.4%) than in young adults deprived of food for 1 day at the L1 stage (6.2%). Thus the effects of starvation but not starvation regimens extending development by 1 day, resulted in an increased loss of neurons. Control (OP50) animals were fed with *E. coli* OP50. Control RNAi (HT115) animals were fed with *E. coli* HT115 expressing the empty vector under RNAi-inducing conditions. *let-60* encodes a component of the Ras signaling pathway. n=300, P<0.0001 (unpaired t-test). Data are mean ± s.e.m.

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caused by mutations of CAG trinucleotide repeat expansion, resulting in long polyglutamine tracts in the mutant proteins. Inhibition of the autophagic-lysosomal pathway suspends the clearance of mutant huntingtin proteins (Yamada et al., 2002). Moreover, these proteins are often localized into lysosomes. Consistent with these findings, lysosomal function that is required for autophagy has been shown to be crucial for neuronal degeneration in *C. elegans* (Artal-Sanz et al., 2006). Together, these data imply that conditions overactivating the autophagic process contribute to neuronal cell loss. However, it has also been demonstrated that loss of autophagy in the central nervous system causes neurodegeneration in mice (Komatsu et al., 2006; Yokoyama et al., 2006). Therefore, both overactivation and deregulation of autophagy seem to induce neuronal degeneration, suggesting that, depending on the actual cellular environment, autophagy may have a dual role in cell survival, acting both as a contributor and a protector to cell demise (Takács-Vellai et al., 2006). On the one hand, the basal activation of autophagy may protect neurons by providing energy for cell functioning during starvation (self-digestion) and facilitating removal of damaged organelles and misfolded proteins. Autophagy is thus essential for cell survival. On the other hand, prolonged or vigorous activation of autophagy may lead to an effective elimination of the affected cell.

So far autophagic vacuoles have not been demonstrated in the neurodegenerative process in *C. elegans* (Hall et al., 1997). Indeed, a growing number of membrane-mediated functions, such as endocytosis and vesicular trafficking, that are seemingly unrelated to the autophagic process itself appear to involve the product of various autophagy genes (Klionsky and Emr, 2000). It might be possible that autophagy proteins have a role in neuronal degeneration independently of autophagic vacuole formation. The involvement of autophagy in neuronal cell loss and other neuronal functions are important areas for future investigations.

Recently BEC-1 was shown to act both in autophagy and apoptosis (Meléndez et al., 2003; Takács-Vellai et al., 2005). Our study reveals a novel role of autophagy genes in a distinct degenerative process, necrosis. Therefore, the fascinating possibility might emerge that three cell death processes might act in a concerted manner in the cell death machinery that is induced by genetic or environmental factors. Furthermore, identifying autophagy genes as mediators of necrotic-like neuronal cell loss in *C. elegans* will be essential for understanding how genetic lesions affecting ion channel subunits as well as adverse environmental conditions can lead to human neurodegenerative pathologies (Driscoll and Gerstbrein, 2003; Yuan et al., 2003; Rubinsztein et al., 2005).

Materials and Methods

C. elegans strains and genetics

C. elegans strains were maintained as described (Brenner, 1974). Wild-type worms were *C. elegans* var. Bristol (N2). Other strains were: FR758 *swEx520*[p*bec-1*::BEC-1::GFP + *rol-6(su1006)*], FR853 *bec-1(ok691)IV*; *swEx520*[p*bec-1*::BEC-1::GFP + *rol-6(su1006)*], FR854 *bec-1(ok700)IV*; *swEx520*[p*bec-1*::BEC-1::GFP + *rol-6(su1006)*], CB369 *unc-51(e369)V*, CB1189 *unc-51(e1189)V*, [p*mec-4*::MEC-4::GFP + *rol-6(su1006)*], [p*mec-4::gfp* + *rol-6(su1006)*], TU1747 *deg-3(u662)V, mec-4(u231)X*, CB1611 *mec-4(e1611)X*, TU1366 *deg-1(u506)X*, MT4866 *let-60(n2021)IV*, MT2124 *let-60(n1046gf)IV*, BY200 [p*dat-1*::*gfp* + *rol-6(su1006)*], *Ex*[p*lgg-1*::GFP::LGG-1 + *rol-6(su1006)*], BU070 Ex[p*lgg-1*::LGG-1::GFP + *rol-6(su1006)*], FR297 *swEx226*[*CeTor::gfp* + *rol-6(su1006)*].

RNA interference and plasmid construction

Specific cDNA fragments were amplified by RT-PCR, and cloned into the vector pPD129.36 (kindly provided by A. Fire, Stanford University, Stanford, CA). Sequences of the forward and reverse primers used were as follows: *lgg-1*, 5′-CAT GCC ATG GCA TGT GGG CTT ACA AGG AGG AGA AC-3′ and 5′-CAT GCC ATG GCA TGT TCC CTT CTT TTC GAC CTC TCC-3′; *let-363/CeTor*, 5′-CAT GCC ATG GCA TGA ACA ATT GGC AAA TTT CGT G-3′ and 5′-CAT GCC ATG GCA TGT GCA CGT AAC GAT GGA GAA C-3′. RNAi experiments were carried out as described (Kamath et al., 2001). To generate a translational fusion BEC-1::GFP reporter, a 9 kb genomic fragment was amplified with the following forward and reverse primers: 5′-GCT ACTCCT GCA GGC ATA GCG CGT AAT TAC TAT TGC GTT CTC G-3′ and 5′-CGG GAT CCC GAA TAG GCG ATC TGA GAG CAT CG-3′. The PCR product was digested with *Sbf*I and *Bam*HI, and cloned into pPD95.75. This construct contains 5.6 kb upstream sequences of the ATG translation initiation site and the full-length *bec-1* coding region fused with *gfp* reporter gene. Transgenic worms were generated by injecting 50 μg/ml DNA construct with 30 μg/ml co-transformation marker pRF4 [*rol-6(su1006)*] into wild-type hermaphrodites. To examine the expression of *lgg-1*, we also generated a *lgg-1::gfp* reporter. A 3746 bp-long genomic fragment was amplified by the following forward and reverse primers: 5′-AAC TGC AGA ACC AAT GCA TTG GCC GAG GGA AAA GAC GAA GAG-3′ and 5′-TCC CCC GGG GGA CGA CCT CTC CTC CAT ACA CAC-3′. The PCR product was cloned into pPD95.75 (kindly provided by A. Fire) by *Pst*I and *Sma*I. Germ-line transformation was performed according to standard protocols. This strain, BU070 *Ex*[p*lgg-1*::LGG-1::GFP], showed an expression profile consistent with that previously reported (Meléndez et al., 2003), using a functional GFP::LGG-1 reporter.

Cell death assays

Nematodes were treated and assayed as described (Syntichaki et al., 2002). Mutations in *bec-1* arrest development at different developmental stages (Takács-Vellai et al., 2005). Thus, homozygous *mec-4(d);bec-1(–)* mutant animals were selected among the progeny of hermaphrodites with *mec-4(d/d);bec-1(–/+)* genotype. In addition, lethality caused by *bec-1* mutant alleles was rescued by transforming a non-integrated transgene, *swEx520*[p*bec-1*::BEC-1::GFP + *rol-6(su1006)*], that carries the wild-type copy of *bec-1*. The number of vacuolated cells was scored at specific developmental stages in animals of *bec-1(–)*; *Ex*[*bec-1(+)*] mosaic genotype. The percentage of intact (p*mec-4*::GFP-positive) touch receptor neurons at the young adulthood in *mec-4(u231)* mutants was also determined. Starvation conditions were as follows. Newly hatched *mec-4(u231)* mutant L1 larvae were deprived of food for 24 hours, and then transferred onto plates containing bacteria where they developed further. GFP-positive cells were scored at young adulthood. Treatment of nematodes with 6-OHDA was performed as described (Nass et al., 2002). Briefly, nematodes were exposed in liquid suspension to 50 mM 6-OHDA in 10 mM ascorbic acid solution for 1 hour, and then transferred to plates lacking the toxin. p*dat*-1::GFP expression was analyzed the following day at 25°C. The percentage of nematodes with intact head dopamine neurons was determined. Control animals were treated with 10 mM ascorbic acid.

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