

# Reactive Oxygen Species-Mediated Neurodegeneration is Independent of the Ryanodine Receptor in *Caenorhabditis elegans*

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Despite the significant impacts on human health caused by neurodegeneration, our understanding of the degeneration process is incomplete. The nematode *Caenorhabditis elegans* is emerging as a genetic model organism well suited for identification of conserved cellular mechanisms and molecular pathways of neurodegeneration. Studies in the worm have identified factors that contribute to neurodegeneration, including excitotoxicity and stress due to reactive oxygen species (ROS). Disruption of the gene *unc-68*, which encodes the ryanodine receptor, abolishes excitotoxic cell death, indicating a role for calcium ( $\text{Ca}^{2+}$ ) signaling in neurodegeneration. We tested the requirement for *unc-68* in ROS-mediated neurodegeneration using the genetically encoded photosensitizer KillerRed. Upon illumination of KillerRed expressing animals to produce ROS, we observed similar levels of degeneration in wild-type and *unc-68* mutant strains. Our results indicate that ROS-mediated cell death is independent of *unc-68* and suggest multiple molecular pathways of neurodegeneration.

## Introduction

Neurons are the primary functional cell of the nervous system. The human brain contains ~86 billion neurons (Azevedo et al., 2009) that form countless synapses and mediate complex processes such as physiological regulation, behavior, and consciousness. Neurons of the central nervous system are subject to neurodegeneration, which results in the progressive loss of neuronal function and ultimately neuronal death. Many neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, are characterized by neurodegeneration of selective brain regions and are becoming increasingly common. Although genes and environmental factors that contribute to neurodegeneration have been identified (Xie et al., 2014), the cellular pathways and molecular mechanisms that underlie the degenerative process are poorly understood.

Reactive oxygen species (ROS) are implicated in many different neurodegenerative diseases, but their contribution to the degenerative process is not well established (Labunsky and Gladyshev, 2013). ROS are a diverse family of oxygen molecules with unpaired electrons produced as natural byproducts of mitochondrial electron transport. Because of the unpaired electron, ROS are highly reactive and oxidize macromolecules such as proteins, nucleic acids, and lipids resulting in disruption of cellular function. Cells possess multiple lines of defense against ROS, such as detoxifying enzymes and small molecule antioxidants (Chang et al., 2008). ROS-mediated neurodegeneration results from oxidative stress due to increased levels of ROS or decreased ability to respond to increased ROS production (Chakraborty et al., 2013). Although ROS can be damaging to cells and are associated with degeneration, signaling pathways involved in ROS-mediated neurodegeneration have not been identified.

*Caenorhabditis elegans* is an established model organism that is well-suited to identify and characterize molecular mechanisms of neuronal function and dysfunction (Li and Le, 2013). This nematode has a short life cycle of three days and can be easily propagated in the laboratory. In addition, worms have a simple, yet well-defined nervous system of 302 neurons that mediate readily observable behaviors (Hart, 2006). The worm is transparent and

transgenes that drive tissue-specific fluorescent reporters can be introduced and used to visualize neuronal morphology in living animals. There are a number of strains that have mutations in specific genes, allowing researchers to use a genetic approach to infer the function of specific genes by examining the phenotypic consequences of gene mutation. Finally, many genes are conserved between worms and humans. Because of these features, the worm has been used to model different aspects of human neurodegenerative diseases (Dimitriadis and Hart, 2010).

Using worms as a model of neurodegeneration, multiple labs have demonstrated that calcium ( $\text{Ca}^{2+}$ ) has an important functional role in the degenerative processes. The constitutively open ion channel *mec-4(d)* causes degeneration in a manner that resembles excitotoxic neurodegeneration (Driscoll and Chalfie, 1991). Mutations that affect calcium release from ER stores or pharmacological perturbation of cytoplasmic  $\text{Ca}^{2+}$  levels prevent *med-4(d)* degeneration (Xu et al., 2001). Genetic ablation of coenzyme Q biosynthesis causes degeneration of a specific class of motor neuron in a  $\text{Ca}^{2+}$  dependent manner (Earls et al., 2010). In addition, pharmacological manipulation of  $\text{Ca}^{2+}$  release from the ER delays neurodegeneration in a worm model of amyotrophic lateral sclerosis (Aggad et al., 2014). Collectively, these experiments suggest that  $\text{Ca}^{2+}$  signaling is part of the degenerative process, but the role of  $\text{Ca}^{2+}$  signaling has not been established for ROS-mediated neurodegeneration.

We examined the requirement of  $\text{Ca}^{2+}$  in ROS-mediated neurodegeneration using optogenetic production of ROS in specific neurons of *C. elegans*. Specifically, we expressed the genetically encoded photosensitizer KillerRed in the GABA nervous system of worms. Killer Red is a modified fluorescent protein that produces large amounts of ROS when illuminated (Bulina et al., 2006). Illumination of worms that express KillerRed in neurons results in cell autonomous neurodegeneration and cell death (Williams et al., 2013). We used KillerRed to trigger ROS-mediated neurodegeneration of neurons that use the neurotransmitter  $\gamma$ -aminobutyric acid (GABA). These motor neurons form inhibitory neuromuscular junctions with body-wall muscles and mediate muscle relaxation during locomotion

(McIntire et al., 1993; Schuske et al., 2004). When wild-type worms are lightly tapped on the head, they respond by moving backwards. By contrast, worms that are defective in GABAergic neurotransmission lack the ability to relax muscles. This results in a distinctive shrinker phenotype when lightly tapped on the head, due to longitudinal muscle contraction. Because of this distinct phenotype due to GABA neuron loss, we used the shrinker phenotype to assay ROS-mediated neurodegeneration that resulted from KillerRed activation.

A genetic approach was used to determine the role of  $\text{Ca}^{2+}$  in neurodegeneration by assaying neurodegeneration in animals that have mutations in a gene necessary for  $\text{Ca}^{2+}$  homeostasis. The *unc-68* gene encodes for the ryanodine receptor, which is critically involved in  $\text{Ca}^{2+}$  release from the ER (Maryon et al., 1996). Mutations in *unc-68* alter  $\text{Ca}^{2+}$  release and are able to suppress excitotoxic neurodegeneration caused by the open ion channel *mec-4(d)* (Xu et al., 2001). We employed a similar genetic approach to test the involvement of  $\text{Ca}^{2+}$  in ROS-mediated degeneration by comparing KillerRed-mediated neurodegeneration in wild-type and *unc-68* mutant animals.

## Methods

### *C. elegans* maintenance and strains

Worms were cultured at 20°C and maintained on NGM using standard techniques (Stiernagle, 2006). Strains expressing KillerRed and GFP in GABA neurons have been described previously (McIntire et al., 1997; Williams et al., 2013) and were obtained from Marc Hammarlund, Yale University. The alleles of *unc-68* (*r1161* and *e540*), as well as the *unc-47(e307)*, which encodes the vesicular GABA transporter (McIntire et al., 1997), were obtained from the *Caenorhabditis* Genome Center, University of Minnesota. Relevant strains were generated by crossing, verifying genotype by direct visualization of fluorescence (GFP and KillerRed) and by observation of uncoordinated phenotype (*unc-68*). The *unc-68(r1161)* allele (Maryon et al., 1996) was verified by polymerase chain reaction using the primers *unc-68* FW (5' AAATTGCAGCCCGAAAGACG 3') and *unc-68* REV (5' CAACCAATCGCCAATCCTGC 3').

### Worm illumination to activate KillerRed

Worms were illuminated using an Olympus BX51 microscope equipped with an arc-lamp and TRITC filters. L4 worms were placed in the lid of an inverted PCR tube containing 29µl of 8% agarose and 21µl of 1X M9. Samples were placed on a microscope stage and focused using white light. Using the TRITC filter, worms were illuminated with green light for 10 minutes. After illumination, worms were recovered on new NGM plates and analyzed for neurodegeneration 24 hours after illumination.

### Scoring by GABA phenotype

The shrinker phenotype was used to assay GABA neuron function and quantify neurodegeneration. Worms on NGM plates were lightly tapped on the head and scored for response. Normal

backward motion was scored as a wild-type response, while a contraction of the body in response to stimuli was scored as a shrinker response. Data presented is the percentage of animals displaying the shrinker response  $\pm$  95% confidence intervals.

## Results

### Activation of KillerRed causes neurodegeneration

To establish that activation of KillerRed leads to ROS-mediated neurodegeneration, we measured degeneration of KillerRed expressing worms upon illumination. Worms that express KillerRed in the GABA neurons were subject to illumination, or kept in the dark. One day after treatment, animals were analyzed for loss of GABA neuron function by analyzing the shrinker phenotype. Animals were scored by determining the percentage that responded with normal backwards movement (nonshrinker) or displayed longitudinal body muscle contractions (shrinker) when lightly tapped on the head. We observed a higher percentage of animals that displayed the shrinker phenotype after illumination compared to unilluminated controls (Figure 1A). Upon illumination, 60% of the worms displayed the shrinker phenotype, compared to 25% of worms kept in the dark. This result indicates that KillerRed activation causes a reduction in neuronal function that is likely due to neurodegeneration. Although KillerRed animals have a slight shrinker phenotype in the absence of illumination (compare Figure 1 to Figure 2), this is likely a consequence of the KillerRed transgene (see discussion).

To verify that KillerRed elicits neurodegeneration, we examined neuronal structure by fluorescence microscopy. Animals that express both KillerRed and GFP in the GABA neurons were illuminated or kept in the dark, and then the structural integrity of the nervous system was analyzed by visualization of GFP. The neurodegenerative effects of KillerRed activation were seen in illuminated animals (Figure 1B). The nerve cords of these animals were disrupted and fragmented, while the cell bodies had a rounded morphology that is characteristic of neurodegeneration (Hall et al., 1997). In addition, the commissures of these animals were very thin and often had breaks in them (data not shown). By contrast, control animals kept in the dark had intact nerve cords and cell bodies with normal oval-shaped morphology. Thus, activation of KillerRed induces functional and structural neurodegeneration and provides a tool to genetically dissect ROS-mediated neurodegeneration by comparing the effects of KillerRed in different genetic backgrounds.

### *unc-68* mutants are not GABA defective

In order to test whether *unc-68* mutants have defective neurodegeneration we first had to establish that *unc-68* mutants have normal GABA function in the absence of KillerRed. As we measure neurodegeneration by scoring ablation of GABA neurons via the shrinker phenotype, we tested whether *unc-68* mutants are shrinker (Figure 2). Wild-type, *unc-68(r1161)* and GABA- (*unc-47(e307)*) animals were scored by determining the percentage of age-matched animals that were shrinker. 6% of wild-type worms were shrinker, while GABA- worms displayed a 79% shrinker frequency. Consistent with normal GABA function, *unc-68* mutant

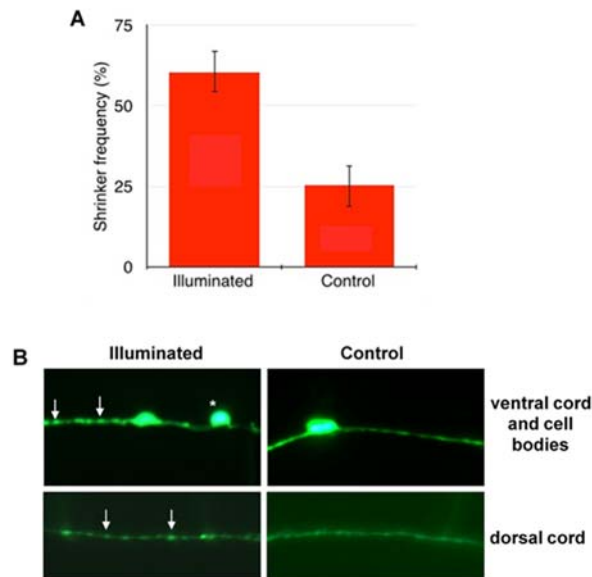


Figure 1. KillerRed activation causes neurodegeneration. (A) KillerRed expressing animals were illuminated or kept in the dark (Control), then scored for GABA neuron function by determining the percentage of animals that displayed the shrinker phenotype. Data presented is the percentage of shrinkers  $\pm$  95% confidence intervals. Illumination resulted in a significant increase in the shrinker frequency. (B) Representative images of the nervous system from illuminated and control animals obtained imaged by fluorescent microscopy. Illumination resulted in beading of ventral and dorsal cords (arrows) as well as rounding of cell bodies (asterisk) that is characteristic of neurodegeneration.

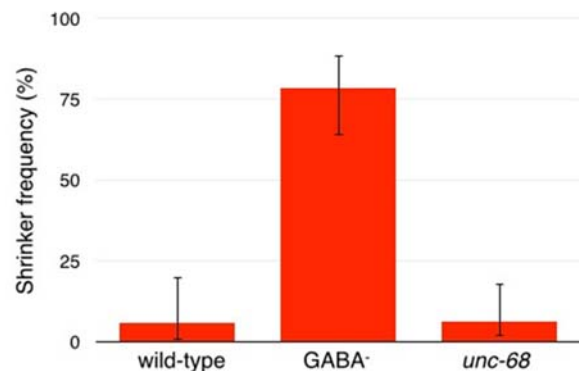


Figure 2. *unc-68* mutants are not GABA defective. The indicated types of worms were scored for the shrinker phenotype. Data presented is the frequency of shrinkers  $\pm$  95% confidence intervals. At least 30 worms were scored for each experiment. Wild-type and *unc-68* mutant worms do not display the shrinker phenotype. By contrast GABA- worms, *unc-47(e307)* mutants, have a robust highly penetrant shrinker phenotype.

animals showed a 5% shrinker rate. Because *unc-68* mutant animals do not have a GABA defect, we can determine whether ROS-mediated neurodegeneration requires *unc-68* function using KillerRed expressed in GABA neurons.

### Neurodegeneration is not affected by mutations in *unc-68*

To assess the role of *unc-68* in ROS-mediated neurodegeneration, we generated *unc-68* mutants that express KillerRed in the GABA neurons. Genotypic verification was done by single worm PCR reactions to amplify the *unc-68* gene in both wild-type and *unc-68(r1161)* animals. The expected 8172 base pair product was seen upon amplification of wild-type animals. A much smaller product of  $\sim$ 970 base pairs was obtained when *unc-68(r1161)* animals were amplified (Figure 3), consistent with the  $\sim$ 7.2 kilobase pair deletion of the *r1161* allele (Maryon et al., 1996). Because the molecular lesion of *unc-68(e540)* has not been determined, strains of this genotype were verified by visual observation of the uncoordinated phenotype.

After verifying the genotype of generated strains, we assayed KillerRed-mediated neurodegeneration of wild-type worms and *unc-68* mutants (Figure 4). Illumination resulted in a similar percentage of shrinker animals in wild-type (134/222 = 60%) and *unc-68* animals (116/221 = 52%). Both alleles of *unc-68* that were tested (*r1161* and *e540*) had similar levels of neurodegeneration. Because there is a similarity of the shrinker frequencies between illuminated animals of wild-type and *unc-68* mutants, these results indicate there are similar levels of neurodegeneration in both strains and that ROS-mediated neurodegeneration is independent of *unc-68*.

### Discussion

*C. elegans* is an ideal system to study ROS-mediated neurodegeneration due to ease of propagation, well-characterized nervous system with specific behavioral outcomes, and ability to express heterologous transgenes in a tissue specific manner. Worms are small, easy to culture in a laboratory setting, and large numbers of genetically identical animals can be readily obtained. The nervous system of *C. elegans* is simple compared to mammalian nervous systems and specific behaviors can be attributed to specific neuron types, allowing for direct study of neuronal function (Hart, 2006). Many genes and molecular mechanisms are conserved between worms and mammals. In addition, the ability to express heterologous transgenes allows targeting of neurodegenerative insults, such as ROS, in a tissue-specific manner. Because of this, *C. elegans* provides a simple manipulation platform to investigate neurodegeneration in a system with similar neuron structure and complex pathways with humans (Alexander et al., 2014).

To test whether  $\text{Ca}^{2+}$  signaling is involved in ROS-mediated neurodegeneration, we used KillerRed expressed in GABA neurons to specifically produce ROS in these cells and measured degeneration in wild-type and *unc-68* mutants. Neurodegeneration was assayed by the shrinker phenotype that is caused by loss of GABA neurons. We can use this assay because *unc-68* mutants do not have the shrinker phenotype in the absence of KillerRed. Activation of KillerRed results in a robust shrinker phenotype in

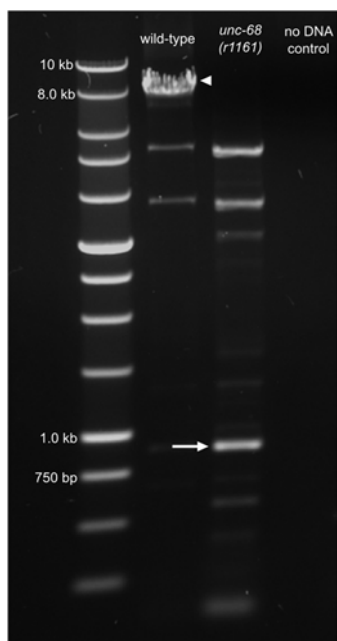


Figure 3. Molecular verification of *unc-68(r1161)* allele. Parallel single worm PCR reactions were performed to amplify the *unc-68* gene using wild-type and *unc-68(r1161)* animals as template. The expected 8172 bp product was obtained from wild-type (arrowhead) animals. The *r1161* allele is an ~7.2 kb deletion of *unc-68* and the expected ~950 bp product was obtained upon amplification of *unc-68(r1161)* animals (arrow). Other bands are likely non-specific amplification products as no-template controls were empty.

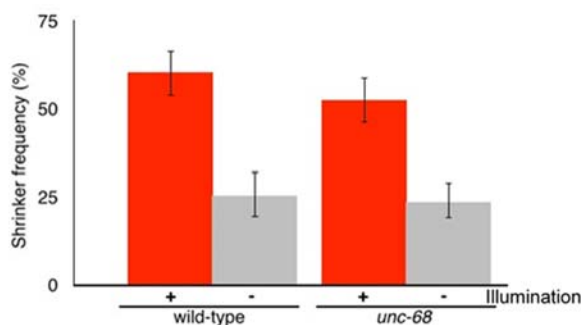


Figure 4. *unc-68* mutants are not defective in KillerRed-mediated neurodegeneration. Wild-type and *unc-68* mutant animals were illuminated (+) or kept in the dark (-), then scored for the shrinker phenotype. Data presented in the frequency of shrinker animals  $\pm$  95% confidence intervals. At least 100 animals were scored for each condition and *unc-68* mutants represent a pool of *e540* and *r1161* alleles, both of which had similar levels of neurodegeneration.

both wild-type and *unc-68* mutant backgrounds. There is a slight shrinker phenotype to KillerRed worms that are kept in the dark (~25%), compared to worms that do not express KillerRed (~6%). This is likely due to the presence of the KillerRed transgenic construct that is made up of multiple copies of the GABA specific

promoter driving KillerRed. Other transgenes that have the same promoter fused to other neutral reporter genes, such as GFP, also have a slight shrinker phenotype (D. Williams, personal observations).

When we compared wild-type to *unc-68* mutants, similar levels of neurodegeneration were seen in both genetic backgrounds, indicating that UNC-68 is not required for ROS-mediated neurodegeneration. Our results are in contrast to previous studies that indicated a role for  $\text{Ca}^{2+}$  signaling as part of a neurodegenerative pathway by demonstrating *unc-68* mutations suppress neurodegeneration (Earls et al., 2010; Xu et al., 2001). This suggests there are multiple pathways of neurodegeneration that differ in their  $\text{Ca}^{2+}$  dependence (Figure 5). The targets of  $\text{Ca}^{2+}$  during excitotoxic degeneration are  $\text{Ca}^{2+}$  dependent calpain proteases that mediate autophagy like processes as the neuron degenerates (Syntichaki et al., 2002). As the cellular pathology of ROS mediated neurodegeneration and excitotoxic degeneration are similar (Hall et al., 1997; Williams et al., 2013), our results suggest that ROS could activate calcium independent proteases. Alternatively, there could be other means of  $\text{Ca}^{2+}$  release from the ER in the absence of UNC-68. Further genetic analysis with mutations in other genes that affect  $\text{Ca}^{2+}$  or pharmacological manipulation of  $\text{Ca}^{2+}$  levels could be used to distinguish between these possibilities.

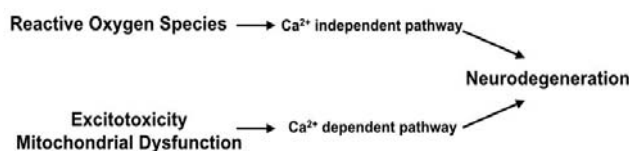


Figure 5. Multiple pathways of neurodegeneration in *C. elegans*. ROS, excitotoxicity, and mitochondrial dysfunction can lead to neurodegeneration through molecular pathways that differ in their dependence on  $\text{Ca}^{2+}$ .

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## Notes and references

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