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Genetic Analysis of KillerRed in *C. elegans* Identifies a Shared Role of Calcium Genes in ROS-mediated Neurodegeneration

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Abstract

In *C. elegans*, neurodegeneration induced by excitotoxicity or aggregation of misfolded proteins is dependent on genes involved in calcium release from the endoplasmic reticulum. Reactive oxygen species (ROS) can also induce neurodegeneration, but the relationship between ROS-mediated neurodegeneration and calcium has not been established. We activated KillerRed in the GABA neurons of *C. elegans* to produce ROS that leads to functional loss and structural degeneration of these neurons and demonstrated that the severity of neurodegeneration was dependent on extent of KillerRed activation. To genetically examine the role of calcium in ROS-mediated neurodegeneration, we measured functional neurodegeneration in *itr-1* (inositol triphosphate receptor), *crt-1* (calreticulin), and *unc-68* (ryanodine receptor) mutants. Similar to other neurotoxic conditions, neurodegeneration triggered by KillerRed was reduced in *itr-1* and *crt-1* mutants. Somewhat unexpectedly, genetic or pharmacological disruption of *unc-68* had a minimal effect on neurodegeneration. Our results indicate ROS-mediated neurodegeneration occurs through a conserved calcium regulated mechanism and suggest that components of the degeneration process have different sensitivities to ROS.

Keywords

C. elegans; neurodegeneration; reactive oxygen species; calcium

Introduction

Degeneration of neurons is a hallmark of neurodegenerative diseases and can be caused by factors such as stroke, physical trauma, or aging. Reactive oxygen species (ROS) and oxidative stress are associated with age and degenerating neurons. Because ROS are normal by-products of metabolism and neurons are highly metabolically active, these cells are particularly susceptible to oxidative damage caused by ROS. Excessive cellular ROS can result in protein oxidation, increased DNA mutations, and lipid peroxidation, all of which have a detrimental effect on neuronal function (Emerit, Edeas, & Bricaire, 2004; Pollari, Goldsteins, Bart, Koistinaho, & Giniatullin, 2014). Although ROS are associated with

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neurodegeneration, the cellular pathways involved in initiation of degeneration due to ROS are not well understood.

C. elegans has been used to identify genes and molecular mechanisms of degeneration triggered by various genetic and environmental insults. Initial studies focused on degeneration resulting from gain-of-function mutations that result in hyperactive ion channels. These channels, such as *mec-4*, *mec-10* and *deg-1*, belong to the DEG/ENaC family of conserved ion channels that cause necrotic-like degeneration of touch receptor neurons when mutated to a constitutive open state (Driscoll & Chalfie, 1991; Syntichaki & Tavernarakis, 2004; Waldmann, Champigny, Voilley, Lauritzen, & Lazdunski, 1996). Similarly, dominant alleles that disrupt desensitization of the nicotinic acetylcholine receptor *deg-3* also cause neurodegeneration (Treinin & Chalfie, 1995). In addition to hyperactive ion channel toxicity, motor neurons of the ventral cord degenerate in response to constitutive activity of a heterotrimeric G protein ($G\alpha_s$), encoded by *gsa-1* (Berger, Hart, & Kaplan, 1998; Korswagen, Park, Ohshima, & Plasterk, 1997). Other experiments have initiated degeneration using pharmacological treatments that model human neurodegenerative diseases or heterologous expression of human disease genes in *C. elegans*. Worms treated with the neurotoxins 6-hydroxydopamine or 1-methyl-4-phenylpyridinium display similar degeneration of dopaminergic neurons as observed in mammalian models of Parkinson's disease (Nass, Hall, Miller, & Blakely, 2002; Pu & Le, 2008). Transactive response DNA-binding protein 43 (TDP-43) has been associated with amyotrophic lateral sclerosis and expression of mutant TDP43^{A315T} in GABA neurons results in functional and structural degeneration of these neurons (Vaccaro et al., 2012). Because of the genetic and molecular tools available in *C. elegans*, the worm is an attractive model organism for genetic dissection of neurodegeneration and identification of cellular mechanisms of degeneration.

Genetic and pharmacological analysis of neurodegeneration in *C. elegans* indicates that calcium (Ca^{2+}) signaling has an important functional role in degeneration triggered by various toxic conditions. Forward genetic screens isolated *crt-1* loss-of-function alleles that suppress neurodegeneration induced by hyperactive degenerin channels or constitutively active $G\alpha_s$ (Barbagallo, Prescott, Boyle, Climer, & Francis, 2010; Xu, Tavernarakis, & Driscoll, 2001). *crt-1* encodes calreticulin, a multi-functional endoplasmic reticulum (ER) resident protein that maintains ER Ca^{2+} levels via a high-capacity Ca^{2+} binding domain (Lee, Singaravelu, Park, & Ahnn, 2007; Nakamura et al., 2001). Suppression of neurodegeneration by disruption of *crt-1* is likely due to altered Ca^{2+} homeostasis in the ER as knock-down of *cnx-1* (calnexin) or pharmacological manipulation of Ca^{2+} can also suppress neurodegeneration (Xu et al., 2001). In addition, disruption of the ryanodine receptor (*unc-68*) or the inositol trisphosphate receptor (*itr-1*) that are involved in Ca^{2+} release from the ER can reduce neurodegeneration (Barbagallo et al., 2010; Xu et al., 2001). Genetic and pharmacological perturbation of Ca^{2+} -regulated calpain and aspartyl proteases also reduce neurodegeneration induced by hyperactive ion channels or protein misfolding (Aggad, Vérièpe, Tauffenberger, & Parker, 2014; Syntichaki, Xu, Driscoll, & Tavernarakis, 2002). Collectively, these results have led to a model in which diverse neurotoxic conditions trigger Ca^{2+} release from the ER that leads to proteolytic cell destruction.

KillerRed is a genetically encoded photosensitizer that produces toxic levels of ROS in response to photoactivation (Bulina et al., 2006). Illumination of transgenic worms expressing KillerRed in specific neurons results in cell-autonomous neuronal cell death (Williams et al., 2013). The neuronal cell death and degeneration due to KillerRed-mediated ROS toxicity resembles other means of inducing neurodegeneration in *C. elegans*. First, the cell bodies of affected neurons exhibit cell swelling and alterations in nuclear structure when visualized by differential interference contrast microscopy. This abnormal neuronal morphology is similar to the effects caused by *mec-4(gf)* and *deg-1(gf)* mutations (Hall et al., 1997). Second, GFP expressing neurons that have been damaged by KillerRed exhibit fragmented neuronal processes and blebbed fluorescent puncta that is also observed in dopaminergic neurons that degenerated in response to manganese treatment (Ijomone, Miah, Peres, Nwoha, & Aschner, 2016). Finally, neurodegeneration triggered by KillerRed and other neurotoxic conditions, such as TDP43^{A315T} or ion channel gain-of-function alleles, is caspase independent and proceeds via a necrosis-like process (Aggad et al., 2014; Barbagallo et al., 2010). Because the degeneration induced by KillerRed is morphologically and genetically similar to other neurotoxic insults, this optogenetic method provides a novel means to investigate the molecular mechanisms and cellular pathways of ROS-mediated neurodegeneration.

The experiments described here utilize worm strains that express soluble KillerRed in the GABA neurons of *C. elegans*. The cell bodies of 19 GABA neurons are located longitudinally within the ventral nerve cord and send circumferential commissures to the dorsal nerve cord. These neurons innervate ventral and dorsal body-wall muscles and are inhibitory motor neurons at the neuromuscular junction (Richmond & Jorgensen, 1999). Worms lacking GABA neuron function cannot relax their body-wall muscles and exhibit a characteristic “shrinker” phenotype when touched on the head (McIntire, Jorgensen, Kaplan, & Horvitz, 1993). Illumination of worms expressing KillerRed specifically in GABA neurons causes neurotoxic production of ROS and the affected animals shrink (Williams et al., 2013; Young & Williams, 2015). Using an optimized method of KillerRed activation and measuring functional degeneration using the shrinker assay, we genetically test the role of genes involved in calcium storage and release from the ER in ROS-mediated neurodegeneration.

Methods

C. elegans strains

Worms were cultured on Nematode Growth Media (NGM) at 20°C using established methods (Brenner, 1974; Stiernagle, 2006). Strains expressing KillerRed and GFP in GABA neurons (*wplIs15[unc-47p::KillerRed]*, *juIs76[unc-25p::GFP]*) have been previously described (Jin, Jorgensen, Hartweg, & Horvitz, 1999; Williams et al., 2013). The alleles *crt-1(bz30)*, *itr-1(sa73)*, and *unc-68(r1161)* were obtained from the *Caenorhabditis elegans* Genome Center. Relevant strains were generated by crossing and verified by direct visualization of KillerRed and GFP, phenotypic characterization of *crt-1* and *itr-1*, or PCR of *unc-68(r1161)* as previously described (Young & Williams, 2015). The *r1161* allele deletes

~7.2kb of the 5' end of *unc-68* and represents the null allele based on allelic interaction analysis (Maryon, Coronado, & Anderson, 1996).

Illumination of KillerRed animals

Ten to fifty L4 to young-adult aged worms were placed in 20 μ l of M9 buffer that was layered on 30 μ l of 1% agarose in the lid of a 200 μ l PCR tube. Animals were exposed to green light using a 10x objective lens of an Olympus BX51 fluorescent microscope via TRITC filters and a 100 W mercury bulb. The power of each illumination was measured using an optical power meter (ThorLabs, Newton, NJ) at 585 nm and was constant for each experiment. After illumination, worms were recovered overnight on a NGM plate with OP50 bacteria before scoring for the shrinker phenotype. For dantrolene experiments, after illumination, animals were recovered on plates containing 50 μ M dantrolene or vehicle (50% DMSO) and scored 24 hours later.

Scoring of shrinker

Recovered worms were analyzed for loss of GABAergic neuron function by gently tapping them on the head and scoring the response. Animals that fail to move backward, but instead exhibit body-wall muscle contraction were scored as shrinker (Jorgensen, 2005). Data are presented as percentage of animals that display the shrinker phenotype $\pm$ 95% confidence intervals and significance was determined using two-tailed Fisher's exact test (GraphPad Quick Calcs). Videos were captured using a SZX10 microscope and DP27 digital camera (Olympus) with cellSens software.

Microscopy and fluorescence intensity measurements

Worms were mounted on 8% agarose pads and immobilized using 0.1 μ m polystyrene beads (Polysciences Inc.). Individual worms expressing GFP in GABA neurons were visualized using an Olympus BX51 microscope with GFP filters. Commissures that were markedly thin or had multiple blebbing or beaded areas were scored as damaged. Images of KillerRed were obtained using TRITC filter set and DCF images were obtained with GFP filters and captured with a Q-Color 3 CCD (Olympus). Mean pixel intensity of individual neuronal cell bodies was measured using ImageJ.

Single worm RT-qPCR

Established methods for single worm RT-qPCR were used (Ly, Reid, & Snell, 2015). In brief, individual worms were lysed in 5 μ l of lysis buffer containing 5 mM Tris - pH 8.0, 0.5% Triton X-100, 0.5% Tween-20, 25 mM EDTA, and 1 mg/ml proteinase-K. Equal volume lysates of individual worms were separated and 10 μ l RT-qPCR reactions were run using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad). Amplification of KillerRed (92bp) used (5' to 3') FW (GATGGAGGACTTATGATGGGAC), REV (TTGTAACGAAATGTGGTCCTGG) and the reference gene *act-1* (111bp) (Ly et al., 2015) used FW (ACGCCAACACTGTTCTTTCC) and REV (CGATGATCTTGATCTTCATGGTTG). Samples were run on the Bio-Rad CFX96 Real-time System and melting curve analysis was performed over a range of 45-95°C to verify amplification of a single product.

Measurement of ROS production

KillerRed expressing animals were treated in the dark with 10 μ M H₂DCFDA (Invitrogen) suspended in M9 buffer for 3 hours. To observe DCF fluorescence during an illumination, worms were mounted on a microscope slide and illuminated and visualized with a 60x objective on an Olympus BX51 microscope. At timepoints within the illumination, KillerRed images (TRITC filter) and DCF fluorescence (GFP filter) were obtained. To compare DCF fluorescence between strains, H₂DCFDA treated animals were illuminated under a 10x objective for 15 minutes and then recovered on an NGM plate before mounting and imaging of KillerRed and DCF fluorescence. Mean pixel intensity of DCF present in cell bodies was determined with ImageJ using regions of interest defined by KillerRed fluorescence.

Results

Optimization of KillerRed induced neurodegeneration for genetic analysis

Illumination of KillerRed results in ROS production and the phototoxic effects of KillerRed are dependent on the duration of illumination (Bulina et al., 2006). We sought to optimize illumination conditions that reduce KillerRed activity to moderate levels and allow identification of changes in ROS-mediated neurodegeneration based on genetic background. We illuminated wild-type worms expressing KillerRed in GABA neurons with green light and scored these animals for loss of GABA neuron function after a 24-hour recovery period. After being gently touched on the head with a thin platinum wire, wild-type animals respond by moving backwards away from the stimulus, while animals that lack GABA neurons contract their body-wall muscles and exhibit the shrinker phenotype (Figure 1 and Supplemental Video 1 & 2). We determined the percentage of shrinker animals from populations that were illuminated at constant power for varying amounts of time (Figure 2A). There was an illumination dependent increase in shrinker frequency. Illumination times of 5 minutes at 0.19 W resulted in ~70% animals having the shrinker phenotype, while increasing illumination times led to saturating effects with almost all of the 20 minute illuminated animals displaying the shrinker phenotype. This effect was dependent on KillerRed because illumination of wild-type animals that lack KillerRed failed to produce shrinker animals (data not shown and Williams et al. 2013). These results indicate the functional effects of KillerRed can be experimentally regulated by altering the levels of illumination.

We assessed the effects of KillerRed on neuron structure by imaging GABA neurons that express GFP. Animals that express KillerRed and GFP in the GABA neurons were illuminated for varying amounts of time and then visualized by fluorescent microscopy 24 hours later (Figure 2B). In the absence of illumination, the neuron cell bodies had a normal oval morphology and the nerve cords and commissures were intact. We observed signs of neuronal damage that increased with longer illumination times. Short illumination times produced GABA neuron commissures that appeared thinner when compared to unilluminated animals. These affected commissures often had large accumulations of fluorescence at various locations along the process. Longer illumination times resulted in more extensive neuronal damage. The commissures of affected neurons were often much

thinner than illuminated controls and were broken at multiple locations with numerous blebs of fluorescence. In addition, neuronal cell bodies of these animals were often rounded relative to unilluminated controls. Severely damaged neurons had commissures that were barely visible and consisted of small fluorescence puncta. We quantified the structural effects of KillerRed, by scoring commissures of worms that were illuminated for varying amounts of time (Figure 2C). Individual commissures that were thin, fragmented, and had multiple blebs were scored as damaged. Illumination conditions that resulted in functional degeneration, as measured by shrinker frequency, caused increased structural damage to the GABA neurons. Because the effects of KillerRed are dependent on extent of illumination, these results indicate neurodegeneration can be modulated to a baseline level that will allow testing the requirement of specific genes for ROS-mediated neurodegeneration.

Disruption of calcium signalling suppresses KillerRed neurodegeneration

We previously demonstrated similar effects of KillerRed in wild-type and *unc-68* mutants, which suggested distinct pathways of neurodegeneration based on cellular injury (Young & Williams, 2015). After establishing an illumination protocol associated with intermediate neuronal damage, we expanded our genetic analysis of ROS-mediated neurodegeneration by testing the requirement of other genes associated with calcium signalling. We generated KillerRed expressing animals in an *itr-1*, *crt-1*, or *unc-68* mutant background and compared the effects of KillerRed to wild-type animals. In the absence of illumination, these genetic backgrounds do not confer a shrinker phenotype (Supplemental Figure 1) and thus, neurodegeneration can be measured using the shrinker assay. Animals were illuminated, then scored for the shrinker phenotype after 24 hours. Relative to wild-type animals, there was a statistically significant reduction in the shrinker frequency of both *itr-1* and *crt-1* mutants after illumination (Figure 3A). Consistent with previous results, we found no significant difference in the shrinker frequency between wild-type and *unc-68* mutant animals (Figure 3A). In addition to genetic disruption, we tested pharmacological perturbation of *unc-68* by treating animals with the ryanodine receptor agonist dantrolene (Fruen, Mickelson, & Louis, 1997; Song, Karl, Ackerman, & Hotchkiss, 1993). Dantrolene treatment failed to have a marked effect on the percentage of illuminated animals that were shrinker (Figure 3B).

itr-1 and *crt-1* mutations do not affect KillerRed activity

One possibility for the reduction of ROS-mediated neurodegeneration seen in *itr-1* and *crt-1* backgrounds is that these genes could influence KillerRed activity. To test this, we first measured KillerRed mRNA and fluorescence in these different genetic backgrounds. We performed single-worm RT-qPCR to assess KillerRed mRNA levels (Figure 4A and Supplemental Figure 2). Using *act-1* as a reference gene (Ly et al., 2015), we found no difference in KillerRed mRNA levels between wild-type and *itr-1* or *crt-1* mutant animals. This result suggests the protein products of *itr-1* and *crt-1* do not have a functional role in transcription or stabilization of KillerRed mRNA. To more directly assess KillerRed expression, we also measured KillerRed fluorescence in living animals (Figure 4B and C). Images of KillerRed expressing animals were collected and fluorescence intensity was determined within individual neurons. Consistent with the RT-qPCR results, we found similar average fluorescence intensity in *itr-1* and *crt-1* animals compared to wild type.

To test whether ROS production by KillerRed is affected by genetic background, we measured fluorescence of a ROS sensing molecular probe. Animals expressing KillerRed in the GABA neurons were treated with 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA), which is a membrane-permeant reporter that is converted to the fluorescent dichlorofluorescein (DCF) when oxidized by ROS (Labuschagne & Brenkman, 2013). Illumination of treated animals resulted in green fluorescence that overlapped with KillerRed fluorescence (Figure 5A). The intensity of fluorescence increased with longer illumination times. Notably, tissues that surround the GABA neurons, including other neuronal cell bodies along the ventral cord, did not have any detectable DCF fluorescence. This demonstrates that ROS production is dependent on both KillerRed and illumination and is consistent with the absence of neurodegeneration in illuminated animals that do not express KillerRed. We measured DCF fluorescence intensity in neuronal cell bodies of wild-type and mutant animals after illumination. The amount of DCF fluorescence was similar in all genetic backgrounds tested (Figure 5 bottom). Collectively, these data demonstrate that *itr-1* or *crt-1* do not affect KillerRed activity and therefore indicate the suppression of neurodegeneration in these genetic backgrounds is not due to reduced ROS production.

Discussion

In this paper, we used the photosensitizer KillerRed to produce ROS in the GABA neurons of *C. elegans*. Illumination of living animals resulted in functional and structural degeneration of these neurons that was dependent on the extent of KillerRed activation. Using illumination times that caused a moderate level of neurodegeneration, we tested the requirement of genes involved in calcium storage and release from the ER in ROS-mediated neurodegeneration. We observed significantly lower levels of functional degeneration, as measured by the shrinker assay, in calreticulin and inositol trisphosphate receptor mutants. Importantly, we demonstrated similar levels of KillerRed expression in these mutant backgrounds. Genetic or pharmacological perturbation of the ryanodine receptor had a negligible effect on levels of neurodegeneration. Collectively, our results add KillerRed to models of toxicity that triggers neurodegeneration in *C. elegans* and suggest that degeneration induced by various cellular injuries occurs through a calcium regulated process.

Calcium and neurodegeneration

Calcium signalling is associated with neurodegeneration that is triggered by various intrinsic and exogenous neurotoxic insults. In mammalian systems, degeneration via glutamate induced excitotoxicity is caused by excessive Ca²⁺ influx through hyperactive ionotropic glutamate receptors (Arundine & Tymianski, 2004; Brassai, Suvanjev, Bán, & Lakatos, 2015). Accumulation of misfolded proteins is associated with many neurodegenerative diseases and can induce the ER unfolded protein response and result in aberrant Ca²⁺ release from the ER (Hetz & Mollereau, 2014; Soto, 2003). Genetic analysis in *C. elegans* has identified Ca²⁺ related genes as being required for neurodegeneration induced by diverse neurotoxic conditions. Initially a genetic selection for mutations that suppress the paralyzed phenotype associated with ectopic expression of *mec-4(d)* ion channels resulted in the isolation of *crt-1* (calreticulin) alleles (Xu et al., 2001). Similarly, deletion of *crt-1* is able to

partially suppress motor neuron loss that is associated with dominant mutations in nicotinic acetylcholine receptors that display increased activation and slow inactivation (Barbagallo et al., 2010). Ectopic expression of TDP-43^{A315T} results in age dependent neurodegeneration of GABA neurons that is delayed in *crt-1* mutants (Aggad et al., 2014; Vaccaro et al., 2012). The suppression of neurodegeneration by *crt-1* is likely due to altered release of calcium from the ER as degeneration induced by mutations in the plasma membrane acetylcholine receptor *deg-3* that leads to increased Ca²⁺ influx is not affected by *crt-1* mutations (Treinin & Chalfie, 1995; Xu et al., 2001). Subsequent studies have demonstrated that neurodegeneration is also reduced by mutations in genes that encode calcium regulated proteases (Aggad et al., 2014; Syntichaki et al., 2002). Collectively, these results have led to a model in which neurotoxic injuries cause inappropriate release of Ca²⁺ from the ER that causes activation of cytoplasmic proteases leading to necrotic cell death.

KillerRed causes ROS-mediated neurodegeneration

We used the ROS producing fluorescent protein KillerRed to cause neurodegeneration of GABA neurons. Illumination of living animals expressing KillerRed results in cell-autonomous ROS production that induces functional and structural degeneration of these neurons. Loss of GABA function is measured by scoring illuminated animals for the shrinker phenotype (Figure 1). Fluorescence microscopy of affected neurons that express GFP reveals abnormal morphology; the nerve cords and commissures become thin fragments and contain numerous fluorescent accumulations. The most damaged neuronal processes are comprised of numerous tiny blebs that are separated by thin wisps of fluorescence. Because the degenerative effects of KillerRed are similar to other neurotoxic conditions, KillerRed expands the panel of insults that trigger neurodegeneration in *C. elegans* and allows for further dissection of the molecular pathways and cellular mechanisms of neurodegeneration that is due to ROS. Furthermore, the effects of KillerRed can be controlled by varying the amount of time animals are illuminated, allowing for identification of genetic conditions that influence degeneration.

Calcium and ROS-mediated neurodegeneration

In this study, we optimized using KillerRed in *C. elegans* to test the role of genes involved in Ca²⁺ homeostasis and release from the ER in ROS-mediated neurodegeneration. We found that mutations in *itr-1* or *crt-1* reduced the toxic effects of KillerRed on GABA neuron function. This reduction in neurodegeneration is not due to altered KillerRed activity as we observed similar levels of KillerRed mRNA and fluorescence in *itr-1* and *crt-1* mutants when compared to wild-type animals. In addition, the amount of ROS produced was not affected in these genetic backgrounds as measured by oxidation of a fluorescent ROS probe. The effects of KillerRed-mediated neurodegeneration by *itr-1* and *crt-1* mutants is similar to suppression of degeneration induced by other neurotoxic conditions, such as *mec-4(d)*, ACR-2(L/S) and TDP-43^{A315T}. Genetic analysis in *C. elegans* leads to a model by which various neurotoxic insults induce Ca²⁺ release from the ER and subsequent activation of Ca²⁺ dependent proteases (Aggad et al., 2014; Syntichaki et al., 2002). Although our results are consistent with this model, future studies examining structural neurodegeneration induced by KillerRed in *crt-1* and *itr-1* mutant backgrounds are necessary to rule out suppression of KillerRed by an alternative mechanism.

The extent of neurodegeneration suppression by *crt-1*, *itr-1*, or *unc-68* mutations is variable with different neurotoxic insults. Nonsense mutations in *crt-1* (*bz29* or *bz30*) almost completely abolish loss of touch receptor neurons observed in *mec-4(u231)* animals while loss of *itr-1* or *unc-68* results in a ~50% reduction in of *mec-4*-induced neurodegeneration (Xu et al., 2001). The *bz30* allele likely represents the null phenotype because missense mutations of *crt-1* show less suppression of *mec-4(u231)* than *bz30* (Xu et al., 2001) and *bz30* suppresses TDP-43^{A315T} degeneration to the same extent as the deletion allele *jh101* (Aggad et al., 2014). By contrast, degeneration of cholinergic motor neurons caused by ACR-2(L/S) channels is partially suppressed by the deletion mutation *crt-1(ok948)* (Barbagallo et al., 2010). Our results with the *crt-1*-dependent suppression of KillerRed-mediated neurodegeneration are similar to the partial suppression of ACR-2(L/S) toxicity (Barbagallo et al., 2010). However, genetic or pharmacological disruption of *unc-68* does not affect KillerRed-mediated neurodegeneration (Figure 3 and Young and Williams, 2015), but does affect ACR-2(L/S) neurodegeneration (Barbagallo et al., 2010). Together, these data could suggest different mechanisms of degeneration based on neuronal type (touch receptor neurons vs. motor neurons), or alternatively distinct degenerative effects based on neurotoxic conditions. We prefer the latter hypothesis because of similarities in the genetic requirements for *mec-4(d)*-induced degeneration of touch receptor neurons (Xu et al., 2001) and TDP-43^{A315T}-induced degeneration of GABAergic neurons (Aggad et al., 2014). Degeneration of both these neuronal types is more sensitive to *crt-1*-dependent suppression than to suppression by *itr-1*. By contrast, we find that GABAergic neurons subject to KillerRed-induced degeneration are equally affected by *crt-1* and *itr-1*.

In contrast to the suppression by *crt-1* and *itr-1* mutations, genetic or pharmacological disruption of the *C. elegans* ryanodine receptor (*unc-68*) had minimal effect on KillerRed-mediated neurodegeneration. Our results suggest that ROS induces Ca²⁺ release from the ER to initiate neurodegeneration via the inositol trisphosphate receptor and that the ryanodine receptor does not have a role in ROS-mediated neurodegeneration. Although Ca²⁺ release from the ER could be a generic response to neurotoxic conditions, such as oxidative stress, the inositol trisphosphate receptor is a potential target of ROS signalling. In vertebrate cells, addition of ROS generating compounds can induce Ca²⁺ release from ER stores in a manner is that dependent on expression of specific inositol trisphosphate isoforms (Bánsághi et al., 2014). Similar levels of KillerRed-mediated neurodegeneration in wild-type and *unc-68* mutants is surprising as disruption of *unc-68* is able to suppress neurodegeneration caused by *mec-4(d)*, ACR-2(L/S), or TDP-43^{A315T} (Aggad et al., 2014; Barbagallo et al., 2010; Xu et al., 2001). Lack of KillerRed suppression by *unc-68* could reflect the cell-autonomous activity of KillerRed and absence of UNC-68 in GABA neurons. Expression of *unc-68* is primarily in body-wall, pharyngeal, and vulval muscles based on antibody staining, however neuronal expression of a *lacZ* reporter is observed with truncated *unc-68* promoter transgenes (Maryon, Saari, & Anderson, 1998; Sakube, Ando, & Kagawa, 1997). By contrast staining with anti-ITR-1 antibodies or visualization of GFP under the *itr-1* promoter revealed broad tissue expression of *itr-1* with signal present in unidentified neurons in the ventral nerve cord, where the GABA cell bodies are located (Baylis, Furuichi, Yoshikawa, Mikoshiba, & Sattelle, 1999). Alternatively, the differential requirement of *unc-68* for neurodegeneration induced by other neurotoxic conditions compared to KillerRed could

reflect the inducible nature of KillerRed-mediated neurodegeneration. Our experiments activated KillerRed in L4 to young adult animal and we scored neurodegeneration 24 hours after induction. By contrast other neurotoxic conditions are due to expression of mutant channels or mis-folded proteins under promoters that are active in embryos and early larval stages (Vaccaro et al., 2012). Notably, ACR-2(L/S)-mediated neurodegeneration is observed in L1 larva soon after hatching (Barbagallo et al., 2010). Future experiments examining neurodegeneration induced by KillerRed activation during embryonic development could test this hypothesis.

Conclusions

KillerRed can be used to induce neurodegeneration within targeted cells and adds ROS to the list of neurotoxic insults available in *C. elegans*. Moreover, KillerRed activity can be experimentally controlled allowing for investigations into genes or drugs that affect neurodegeneration due to excessive ROS. Like other neurotoxic conditions, KillerRed-mediated neurodegeneration is reduced by disruption of *crt-1* or *itr-1*, indicating a shared Ca^{2+} dependent mechanism of neurodegeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Illumination of GABA::KillerRed animals results in shrinkers. Sequential images of control and illuminated GABA::KillerRed worms in response to gentle touch on the head.

Illuminated animals were exposed to green light at 0.16 W for 30 minutes to induce a robust shrinker phenotype. Animals were touched in Frame Number 0 and each frame represents one second after stimulation. Dashed lines indicate position of the animal's head (thick) and tail (thin) in Frame Number 0.

Figure 2.

KillerRed-mediated neurodegeneration varies with illumination time. (A) Functional neurodegeneration. Populations of GABA::KillerRed animals were exposed to green light at 0.19 W for the indicated illumination times and then scored for the shrinker phenotype after a 24-hour recovery period. At least 15 animals were scored for each illumination condition and data represents the percentage of shrinker animals ($\pm$ 95% confidence intervals). $P < 0.0001$ for each illumination condition compared to no illumination control (Fisher's exact test, two-tailed). (B) Structural neurodegeneration. Representative images of GABA::KillerRed worms illuminated at 0.14 W for the indicated times. After a 24-hour recovery period, animals were visualized for GFP fluorescence to assess structural integrity of GABA neuronal processes (arrows). Asterisks indicate neuron cell bodies and arrowheads mark the ventral nerve cords that are out of focus. Scale bar = $5\mu\text{m}$. (C) Quantification of neuronal damage. Worms were illuminated at 0.14 W for the indicated time and after a 24-hour recovery period, neuronal commissures processes were scored for damage. Commissures that were significantly thin, fragmented, or displayed blebbing were scored as damaged. Data represents the total percentage of damaged commissures ($\pm$ 95% confidence intervals) from at least 10 animals for each illumination condition. $P < 0.005$ for each illumination time compared to no illumination control (Fisher's exact test, two-tailed).

Figure 3. Genetic and pharmacological analysis of KillerRed-mediated neurodegeneration. (A) GABA::KillerRed worms of the indicated genotype were illuminated for 5 minutes at 0.17 W and scored for the shrinker phenotype. Data represents the percentage of shrinkers +/- 95% confidence intervals of the indicated number of animals pooled from at least 3 independent illuminations. Significance was determined using Fisher's exact test (two-tailed) for each genotype vs. wild type, ** = $P < 0.01$, * = $P < 0.05$. (B) Shrinker frequency of animals treated with dantrolene or vehicle (DMSO). Data represents the percentage

shrinkers $\pm$ 95% confidence intervals. At least 15 animals were scored for each condition.
 $P < 0.001$ for illuminated vs. dark (Fisher's exact test, two-tailed)

Figure 4. KillerRed expression is normal in *itr-1* and *crt-1* mutants. (A) RT-qPCR analysis of KillerRed mRNA. Average ΔCq values between KillerRed and the reference gene *act-1* were determined from individual worms. Bars represent mean ΔCq values $\pm$ SEM. Circles represent the ΔCq value determined from individual worms. (B) Representative images of KillerRed expressing GABA neuron cell bodies captured with fluorescence microscopy and (C) average neuronal fluorescence intensity. Images were collected from at least 9 worms

and mean pixel density determined from at least 3 neuron cell bodies per image. Data represents mean pixel density from over 50 neurons, $\pm$ SEM. Scale bar = 10 μ m.

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Figure 5.

ROS production. (A) Neurons of a GABA::KillerRed animal treated with 10 μ M H₂DCFDA and illuminated with green light under a 60 $\times$ objective. At the indicated time, images of KillerRed or DCF fluorescence were obtained and merged (bottom row). Arrows indicate GABA neuron cell bodies and arrow head indicates ventral nerve cord. Asterisk in DCF images is non-specific gut autofluorescence. Scale bar = 20 μ m. (B) Quantification of DCF fluorescence in different genetic backgrounds. Populations of worms were treated with H₂DCFDA and then illuminated at 0.13 W for 15 minutes and then recovered and mounted for microscopy. Images were collected from at least 6 worms in each genotype and mean pixel density determined from at least 3 neuron cell bodies per image. Data represents mean pixel density from at least 30 neurons, $\pm$ SEM.