RESEARCH ARTICLE

Rescue of ATXN3 neuronal toxicity in *Caenorhabditis elegans* by chemical modification of endoplasmic reticulum stress

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ABSTRACT

Polyglutamine expansion diseases are a group of hereditary neurodegenerative disorders that develop when a CAG repeat in the causative genes is unstably expanded above a certain threshold. The expansion of trinucleotide CAG repeats causes hereditary adult-onset neurodegenerative disorders, such as Huntington’s disease, dentatorubral–pallidoluysian atrophy, spinobulbar muscular atrophy and multiple forms of spinocerebellar ataxia (SCA). The most common dominantly inherited SCA is the type 3 (SCA3), also known as Machado–Joseph disease (MJD), which is an autosomal dominant, progressive neurological disorder. The gene causatively associated with MJD is *ATXN3*. Recent studies have shown that this gene modulates endoplasmic reticulum (ER) stress. We generated transgenic *Caenorhabditis elegans* strains expressing human ATXN3 genes in motoneurons, and animals expressing mutant *ATXN3-CAG89* alleles showed decreased lifespan, impaired movement, and rates of neurodegeneration greater than wild-type *ATXN3-CAG10* controls. We tested three neuroprotective compounds (Methylene Blue, guanabenz and salubrinal) believed to modulate ER stress and observed that these molecules rescued *ATXN3-CAG89* phenotypes. Furthermore, these compounds required specific branches of the ER unfolded protein response (UPR<sub>ER</sub>), reduced global ER and oxidative stress, and polyglutamine aggregation. We introduce new *C. elegans* models for MJD based on the expression of full-length ATXN3 in a limited number of neurons. Using these models, we discovered that chemical modulation of the UPR<sub>ER</sub> reduced neurodegeneration and warrants investigation in mammalian models of MJD.

KEY WORDS: Ataxin 3, *Caenorhabditis elegans*, Endoplasmic reticulum stress, Guanabenz, Polyglutamine

INTRODUCTION

Polyglutamine (poly-Q) expansion diseases are a class of dominantly inherited neurodegenerative disorders that develop when there is an abnormal expansion, and subsequent translation, of trinucleotide CAG repeats (Gatchel and Zoghbi, 2005; Matos et al., 2011; Shao and Diamond, 2007; Xu et al., 2015). These diseases are characterized by a selective loss of neurons along with physical and psychological complications (Matos et al., 2011). Indeed, the abnormal expansion of polyglutamine induces numerous pathological changes in patients, including modifications of the proteome leading to functional alterations, generation of toxic poly-Q protein species, protein aggregation, transcriptional dysregulation, proteotoxic stress and mitochondrial dysfunction (Shao and Diamond, 2007). However, the exact mechanism of disease pathogenesis is still not well understood. The poly-Q expansion diseases include several neurodegenerative disorders, such as Huntington’s disease, dentatorubral–pallidoluysian atrophy, spinobulbar muscular atrophy and six forms of spinocerebellar ataxia (SCA) (Matos et al., 2011; Teixeira-Castro et al., 2015). SCAs are considered to be rare disorders, with a prevalence ranging from 0.3 to 2.0 per 100,000 (van de Warrenburg et al., 2002). SCA3 (spinocerebellar ataxia type 3), also known as Machado–Joseph disease (MJD), is considered to be the most common form of SCA worldwide (Schöls et al., 2004).

MJD is an autosomal dominant progressive neurological disorder characterized principally by ataxia, spasticity, peripheral neuropathy and ocular movement abnormalities (França et al., 2008). This disease is accompanied by neurodegeneration in selective regions, mainly in the cerebellum, basal ganglia, brainstem and spinal cord (Teixeira-Castro et al., 2011, 2015; Matos et al., 2011). Regarding brain function, it has been shown that metabolism is decreased in several regions of the nervous system, such as the cerebellum, brainstem and cerebral cortex, along with negative perturbations in both dopaminergic and cholinergic neurotransmission (Soon et al., 1997; Wüllner et al., 2005; Yen et al., 2000; Riess et al., 2008; Rub et al., 2008). MJD, constituting the most prevalent subtype of SCA, is more frequently observed among people of Portuguese/Azorean ancestry, with the highest prevalence in the Azorean island of Flores (1/239; Bettencourt et al., 2008). The gene causatively associated with MJD is *ATXN3* (ataxin 3) and is located on chromosome 14 (14q24.3-14q32.45; Kawaguchi et al., 1994; Takiyama et al., 1994). This gene encodes a poly-Q-containing protein named ataxin 3 (Kawaguchi et al., 1994).

Ataxin 3 has 339 amino acid residues, with an estimated molecular weight of 42 kDa for normal individuals (Kawaguchi et al., 1994). Healthy individuals have between 10-51 CAG repeats, which is expanded to 55-87 repeats in the disease state (Maciel et al., 2001; Cummings and Zoghbi, 2000). Ataxin 3 has several functional domains, including the N-terminus of the catalytic Josephin domain that presents a globular and a very conserved structure, followed by two ubiquitin interacting motifs (UIM) that are also considered as conserved regions, the poly-Q domain and finally, depending on the protein isoform, a third atypical UIM in the C-terminus tail (Goto et al., 1997; Li et al., 2015).

To aid the study of MJD, we turned to the model organism *Caenorhabditis elegans*. This nematode is 1 mm long, easy to maintain in laboratory settings, highly amenable to genetic manipulation, and is especially well suited for neuroscience
research because of its comprehensively detailed neuronal lineage and interconnectivity of synapses that resembles aspects of the vertebrate nervous system (Stienagle, 2006). Additionally, the *C. elegans* genome contains an orthologue of ATXN3, named *atx-3*. The *C. elegans* orthologue of human ataxin 3 is localized in both the nucleus and the cytoplasm, with higher levels observed in the cytoplasm. It has been shown that loss of *atx-3* activity results in changes in the expression of genes involved in several different pathways: the ubiquitin–proteasome pathway, signal transduction and cell structure and motility (Rodrigues et al., 2007; Kawaguchi et al., 1994). Ataxin 3 has been identified as participating in the endoplasmic reticulum network (Matos et al., 2011; Echtermeyer et al., 2011; Reina et al., 2010). We previously showed that several compounds, including Methylene Blue, salubrinal and guanabenz, target the endoplasmic reticulum (ER) stress response and protect against proteotoxicity in simple models of amyotrophic lateral sclerosis (ALS; Vaccaro et al., 2013, 2012a) and have beneficial effects in models of the neurological disorder hereditary spastic paraplegia (Julien et al., 2016). In this study, using our transgenic *C. elegans* ATXN3 models, we explored whether small molecules that regulate ER stress response activity were able to rescue locomotor phenotypes, neuronal loss and the increased oxidative and ER stress observed in mutant transgenic animals as an early effort for MJD therapy development.

**RESULTS**

**Expression of full-length human ATXN3 in *C. elegans***

Using Mos1-mediated single copy insertion (MoSCI) transposon-mediated single copy insertion into the genome (Frokjaer-Jensen et al., 2014), we created strains to model MJD by expressing full-length, human ATXN3 in *C. elegans*. It has been shown previously that expressing ataxin 3 with 89 CAG repeats (CAG89) results in neurodegeneration, and in protein misfolding phenotypes in cell culture and *Drosophila* models (Stochmanski et al., 2012). Based on our previous neurodegeneration models (ALS models), we expressed human ATXN3, either wild-type ATXN3-CAG10 (Fig. 1A) or mutant ATXN3-CAG89 (Fig. 1B), in the worm’s 26 GABAergic motoneurons using the promoter for the gene *unc-47*, which encodes a vesicular GABA transporter (Vaccaro et al., 2012a). In our experience, the *unc-47* modelling approach produces animals with strong phenotypes resulting from the expression of disease-associated proteins in a small number of neurons. Thus, in the context of chemical suppressor screens, relatively few neurons need to be exposed to small molecules to detect reversion of phenotypes.

We confirmed the expression of full-length human ATXN3 in transgenic *C. elegans* strains by western blotting with a human specific anti-ATXN3 antibody. A band corresponding to wild-type ATXN3-CAG10 and a larger band for the MJD-associated mutant ATXN3-CAG89 were detected by western blotting of worm protein extracts. However, the anti-ATXN3 also detected a band in non-transgenic wild-type N2 animals (Fig. 1C). *atx-3* encodes a deubiquitylating enzyme that is the highly conserved *C. elegans* orthologue of human ATXN3 (Kawaguchi et al., 1994; Echtermeyer et al., 2011), thus it can be detected by western blotting with a human specific ATXN3 antibody. To confirm this, we conducted western blotting experiments using the anti-ATXN3 antibody and protein extracts from *atx-3* null mutants. *atx-3(tm1689)* is a loss-of-function mutation consisting of a 660 bp deletion along with a 6 bp insertion (C. elegans Deletion Mutant Consortium, 2012; Rodrigues et al., 2007), and no signal was observed. These data suggest that the anti-ATXN3 antibody recognizes *C. elegans* ATX-3 in N2 wild-type worms and partly obscures the signal for ATXN3-CAG10 transgensics, but a specific, higher molecular weight signal is visible in extracts from ATXN3-CAG89 animals (Fig. 1C). Finally, we confirmed the specificity of the anti-ATXN3 antibody by western blotting using protein extracts from cells originating from healthy controls or MJD patients (Fig. 1D).

**Decreased lifespan and impaired neuronal phenotypes in mutant ATXN3 transgenics**

We wondered whether the expression of non-native proteins led to decreased health in the transgenic strains. Initially, we investigated whether lifespan was altered in our ATXN3 transgenics. Using age-synchronized animals we observed that both ATXN3-CAG10 and ATXN3-CAG89 transgenics showed significantly decreased lifespan compared with non-transgenic wild-type N2 worms (Fig. 2A). These data demonstrate that increased expression of ATXN3 transgenics has negative consequences on lifespan, but the mutant ATXN3-CAG89 transgene had a more severe phenotype compared with ATXN3-CAG10 controls.

Decreased lifespan can indicate poor health of the animals, and one sign of ageing in *C. elegans* is decreased motility that can be quantified using assays for progressive, age-dependent paralysis (Collins et al., 2008; Herndon et al., 2002). We observed that the age-synchronized ATXN3-CAG89 transgenics displayed progressive motor deficits compared with wild-type ATXN3-CAG10 transgenics. Starting during adulthood, ATXN3-CAG89 transgenics displayed uncoordinated motility phenotypes, progressing to paralysation over a period of 12 days, and occurred at a higher rate compared with ATXN3-CAG10 controls (Fig. 2B).

The nematode *C. elegans* body wall muscle cells receive excitatory (acetylcholine) and inhibitory (GABA) inputs to coordinate muscle contraction and relaxation and to facilitate movement (McIntire et al., 1993). Activity of the neuromuscular junction can be measured indirectly with the acetylcholinesterase inhibitor aldicarb (Mahoney et al., 2006). It has been shown that treating worms with the known compound, aldicarb, results in accumulation of acetylcholine at neuromuscular junctions, leading to a number of different phenotypes: hyperactive cholineric synapses, muscle hypercontraction and acute paralysis (Mahoney et al., 2006). Mutant strains having a defect in synaptic vesicle release demonstrate resistance for aldicarb. Resistance or hypersensitivity to aldicarb-induced paralysis has been used to identify genes that regulate acetylcholine secretion or inhibitory GABA signalling in different studies, including ours for ALS models (Loria et al., 2004; Vaccaro et al., 2012b). To investigate whether our transgenic ATXN3 strains had abnormal activity at the neuromuscular junction, we exposed them and two control strains, unc-64(e246) (resistant to aldicarb, encodes syntaxin) and unc-47(e307) (hypersensitive to aldicarb), to aldicarb. Transgenic ATXN3-CAG89 strains and unc-47(e307) mutants were hypersensitive to aldicarb-induced paralysis compared with ATXN3-CAG10 transgenics, wild-type N2 worms, and unc-64(e246) mutants at days 1, 5 and 9 of adulthood (Fig. 2C). ATXN3-CAG10 transgenics did show a hypersensitivity to aldicarb-induced paralysis, but this was less severe than what was observed for ATXN3-CAG89 animals. These data suggest that the function of the GABAergic motoneurons, and perhaps their inhibitory signalling function, is impaired in mutant ATXN3-CAG89 transgenics.

Additionally, we tracked the movement of wild-type N2 worms and both ATXN3 transgenics for a period of 10 min on agar plates. We observed that ATXN3-CAG89 worms had impaired motility phenotypes and explored less of their area compared with wild-type N2, and ATXN3-CAG10 worms at days 1, 5 and 9 of adulthood (Fig. 2D). Overall, these data demonstrate that mutant
**ATXN3-CAG89** transgenic worms have increased neuromuscular dysfunction that advances in a progressive manner, leading to increased rates of paralysis compared with controls.

**Mutant ATXN3-CAG89 causes progressive motoneuron degeneration**

Many neurodegenerative diseases are characterized by neuronal dysfunction before degeneration (Saxena and Caroni, 2011). To determine whether the progressive paralysis phenotype observed in **ATXN3-CAG89** worms was accompanied by neurodegeneration, we crossed the transgenic lines with an integrated reporter, **unc-47p::mCherry**, expressing the red fluorescent protein mCherry in GABAergic motoneurons (Petrash et al., 2013). We observed a significant increase of gaps and/or breaks in motoneurons of **ATXN3-CAG89** worms when compared with the wild-type **unc-47p::mCherry** and **ATXN3-CAG10** transgenics at days 5 and 9 of adulthood (Fig. 2E,F). We did not observe neurodegeneration in young adult day 1 mutant **ATXN3-CAG89** transgenic worms (Fig. S1B). These observations suggest a gradual decline of neuronal function that is correlated with age-dependent neurodegeneration as observed in diseases such as MJD.

We investigated whether endogenous **atx-3** contributed to motility and neurodegeneration phenotypes in our **ATXN3-CAG89** transgenics. We observed that the rates of paralysis and neurodegeneration of **atx-3(tm1689)**, **ATXN3-CAG89** animals was indistinguishable from **ATXN3-CAG89** controls, suggesting that **atx-3** does not contribute to **ATXN3-CAG89** phenotypes (Fig. S1).

As **ATXN3** and mCherry are both expressed under the same promoter (**unc-47**), we wondered whether transcription factor depletion could contribute to the motility phenotypes observed in our **ATXN3-CAG89** transgenics. To investigate, we turned to a worm tracking system (**Wmicrotracker**; Phylum Tech) able to measure both automatically and simultaneously the movement of a population of worms placed in 96-well microtitre plates over several hours (Veriepe et al., 2015; Vaccaro et al., 2012a; Therrien and Parker, 2014; Schmeisser et al., 2017). The apparatus makes use of two infrared light beams crossing each microtitre well from top to bottom, and a detector determines how often the light rays are interrupted by worms moving in the well. Each interruption counts as a movement registered by the machine (Schmeisser et al., 2017). We observed no difference in overall movement between **ATXN3-CAG89** and **unc-47p::mCherry**; **ATXN3-CAG89** worms (Fig. S2). These data suggest that the addition of the **unc-47p::mCherry** transgene to **ATXN3-CAG89** transgenics does not influence motility phenotypes.

**Methylene Blue, salubrinal and guanabenz suppress paralysis and extend lifespan in ATXN3-CAG89 transgenics without affecting the expression of this transgene**

Our group previously identified several small molecules, including Methylene Blue, salubrinal and guanabenz, that target the ER stress response. These molecules suppress paralysis and extend lifespan in **ATXN3-CAG89** transgenics (Petrash et al., 2013). To determine whether these molecules also affect the expression of the **ATXN3-CAG89** transgene, we performed western blot analysis in animals treated with Methylene Blue, salubrinal and guanabenz. We observed no difference in the expression of the **ATXN3-CAG89** transgene in animals treated with these molecules compared with untreated animals (Fig. S1). These data suggest that the suppression of paralysis and extension of lifespan by Methylene Blue, salubrinal and guanabenz is not due to changes in the expression of the **ATXN3-CAG89** transgene.
Fig. 2. See next page for legend.
response and were shown to have beneficial effects against mutant TDP-43 neuronal toxicity in models for ALS (Vaccaro et al., 2012a; Therrien and Parker, 2014). We observed that ATXN3-CAG89 animals treated with Methylene Blue, salubrinal or guanabenz had increased motility compared with untreated controls (Fig. 4). These data suggest that molecules regulating the ER stress response can attenuate neuronal dysfunction caused by mutant ATXN3-CAG89.

### Small molecules rescue neurodegeneration in ATXN3-CAG89 transgenics

After observing that Methylene Blue, salubrinal or guanabenz suppressed mutant ATXN3-CAG89-induced paralysis, we examined whether these compounds had protective effects against motoneuron degeneration. Using the unc-47p::mCherry, ATXN3-CAG89 strain, we visualized the GABAergic motoneurons in vivo in day 5 adults and observed a significant decrease of neurodegeneration for worms when treated with any of the three compounds (Fig. 5). These data demonstrate that chemical manipulation of ER stress mechanisms protects neurons against mutant ATXN3 toxicity.

### Methylene Blue, salubrinal and guanabenz prevent the oxidative stress induced by ATXN3-CAG89 transgenics

We wondered whether one mechanism associated with mutant ATXN3-CAG89 toxicity involved elevated levels of oxidative stress, as we have previously observed in other models of neuronal proteotoxicity (Vaccaro et al., 2013, 2012a). Global oxidative stress can be detected by staining worms with the fluorescent dye 2′,7′-dichlorofluorescin diacetate (DCF-DA), and we observed increased fluorescence in ATXN3-CAG89 transgenics compared with wild-type N2 controls and ATXN3-CAG10 transgenics (Fig. 6A-C).

Our previous work suggests that oxidative stress might be linked with activation of the unfolded protein response in the ER (Julien et al., 2016; Vaccaro et al., 2013). Furthermore, small molecule-mediated reduction of the ER stress response was likewise correlated with decreased oxidative stress levels in C. elegans proteotoxicity models. We observed that treatment of ATXN3-CAG89 transgenics with Methylene Blue, guanabenz or salubrinal reduced the fluorescence from DCF-DA staining (Fig. 6D-G). These data suggest that the ER stress response might be involved in neuronal toxicity caused by mutant ATXN3-CAG89.

### Rescue of ER stress response by Methylene Blue, salubrinal and guanabenz in ATXN3-CAG89 transgenic worms

After observing high levels of oxidative stress in ATXN3-CAG89 transgenics, we wanted to test directly for the involvement of the ER stress response. hsp-4 encodes a widely expressed, protective Hsp70/BiP protein induced by ER stress that can be monitored with a transgenic, transcriptional hsp-4::GFP reporter that shows strong fluorescence in the intestine and spermatheca (Urano et al., 2002). We crossed the ATXN3 transgenics with the hsp-4::GFP reporter and observed increased fluorescence in ATXN3-CAG89; hsp-4::GFP animals compared with ATXN3-CAG10; hsp-4::GFP or hsp-4::GFP controls (Fig. 7A, top panels). We recently showed that Methylene Blue, guanabenz and salubrinal rescue the ER stress response in multiple models for another neurological disorder, hereditary spastic paraplegias (Julien et al., 2016). Based on this finding, we examined whether any of these compounds were able to prevent the ER stress response caused by ATXN3-CAG89 transgenics. We found that all three compounds reduced fluorescence of the ER stress hsp-4::GFP reporter transgene in ATXN3-CAG89 transgenics (Fig. 7A-D). A significant reduction of the ER stress was also observed in the ATXN3-CAG10 transgenic worms when treated with...
the compounds. This is consistent with the fact that *ATXN3-CAG10* transgenics have motility and lifespan phenotypes intermediate to N2 worms and *ATXN3-CAG89* transgenics. We observed a significant induction of the GFP signal in *hsp-4*::GFP worms when treated with Methylene Blue and salubrinal, suggesting that these molecules might act by stimulating an early ER stress response outcome that might be neuroprotective in the context of *ATXN3* transgenics, ultimately resulting in lower proteotoxicity followed by decreased ER stress overall. These data suggest that small molecule interventions centred on the ER stress response protect against mutant *ATXN3* toxicity.

If stress within the ER persists, and it is not resolved by the early protective ER unfolded protein response (UPRER) pathways, this can stimulate the clearance of misfolded proteins from the ER through ER-associated degradation (ERAD). The processing of misfolded proteins by ERAD is a redox-intensive process that requires the ER oxidoreductase *ero-1*/*ERO1*, the activity of which can result in the production of peroxides and, consequently, increased reactive oxygen species (Harding et al., 2003). Thus, impairing *ero-1*/*ERO1* activity disrupts the processing of proteins, along with the generation of peroxides and associated oxidative stress (Harding et al., 2003).

Therefore, we predicted that knockdown of *ero-1* by RNAi could reduce the amount of oxidative stress observed in our *ATXN3-CAG89* transgenic worms. We observed that *ATXN3-CAG89* mutant worms stained with DCF-DA in the presence of *ero-1* (RNAi) showed a significant decrease of fluorescence compared with the empty vector RNAi controls (Fig. 7E). These data suggest that the oxidative stress observed in our *ATXN3-CAG89* transgenic mutant worms might originate in the ER and is dependent on the activity of *ero-1*.

**Chemical-genetic approach analysis for the UPRER pathways**

ER stress leads to activation of the UPR by three main signalling branches, resulting in an upregulation of chaperone proteins and a general arrest of protein translation (Walker and Atkin, 2011). Based on our data demonstrating an activation of ER stress in *ATXN3-CAG89* mutants, along with a reduction of this stress by UPRER-associated compounds (Fig. 7), we wondered whether these compounds required any specific UPRER branches for their neuroprotective activities. To identify the mechanisms related to each compound, we opted for a genetic approach using mutants against key components of two branches of the UPRER pathways, such as *atf-6*/*ATF6*, *pek-1*/*PERK*, and RNAi for *ire-1*/*IRE1*. 
these compounds maintained their ability to suppress paralysis in neuroprotective activity of Methylene Blue and salubrinal, because guanabenz failed to suppress the paralysis in mutant worms. We observed that worms (Fig. 8A). ability to suppress paralysis in ATXN3-CAG89 was partly dependent on pek-1 (McIntire et al., 1997, 1993). Additionally, MJD disease is in part associated with dysfunction of motoneurons (França et al., 2008; Wang et al., 2009). We engineered strains expressing ATXN3 in the animal’s 26 GABAergic neurons, because impaired activity of these motoneurons can lead to easily identifiable motility phenotypes useful for screening purposes (McIntire et al., 1997, 1993). Moreover, ATXN3-CAG89 transgenic mutants with human ATXN3 antibody showed a high accumulation of the ATXN3-CAG89 in the pelleted, insoluble fraction, when compared with ATXN3-CAG10 and N2 wild-type worms (Fig. 9B). We then investigated whether Methylene Blue, salubrinal and guanabenz were able to reduce the aggregation observed in our ATXN3-CAG89 transgenics. We observed a significant decrease of aggregation in ATXN3-CAG89 worms when treated with these three compounds, and noted that guanabenz was especially potent in reducing protein aggregation (Fig. 9B,C). These data suggest neuroprotective roles for Methylene Blue, salubrinal and guanabenz in reducing mutant ATXN3 aggregation.

**Small molecule suppression of ATXN3-CAG89 aggregation**

As ATXN3 has been observed to form protein aggregates in several models, including *C. elegans*, we wondered whether our transgenics displayed similar aggregation phenotypes (Teixeira-Castro et al., 2015, 2011; Koch et al., 2011; Breuer et al., 2010). To determine whether ATXN3 proteins could be detected in vivo, we fixed whole C. elegans models for investigating mechanisms of motoneuron toxicity caused by a polyglutamine expansion in ATXN3. To model human disease, we expressed full-length human ATXN3 without additional tags, because the inclusion of tags can mask or enhance the phenotypes of wild-type and mutant proteins (Catoire et al., 2008; Wang et al., 2009). We engineered strains expressing ATXN3 in the animal’s 26 GABAergic neurons, because impaired activity of these motoneurons can lead to easily identifiable motility phenotypes useful for screening purposes (McIntire et al., 1997, 1993). Moreover, MJD disease is in part associated with dysfunction of motoneurons (França et al., 2008; Kanai and Kuwabara, 2009; Pinto and De Carvalho, 2008; Rub et al., 2002). Furthermore, we have previously constructed neurodegeneration models based on the expression of human disease proteins in these same GABAergic motoneurons and found that the transgenic models provide robust phenotypes for experimentation, as we have previously demonstrated in our ALS models (Vaccaro et al., 2012a). Lastly, *C. elegans* has an ATXN3 orthologue, ATX-3, that shares many cellular functions (Kawaguchi
et al., 1994; Rodrigues et al., 2007; Piano et al., 2002), which perhaps extend to the nervous system, making the worm an appropriate model for studying conserved pathophysiological mechanisms of MJD.

*ATXN3*-CAG89 transgenic worms showed a progressive age-dependent paralysis, and reduced lifespan phenotypes compared with wild-type *ATXN3*-CAG10 transgensics. The fact that we observed a decrease of lifespan resulting from the expression of *ATXN3* transgenes in GABAergic motoneurons suggests that there might be some sort of communication from the nervous system to the rest of the organism that negatively modifies health. We and others have observed that cellular stress originating in specific neuronal populations can affect organism-wide stress mechanisms that in turn can affect lifespan (Veriepe et al., 2015; Vaccaro et al., 2012c; Taylor and Dillin, 2013; van Oosten-Hawle et al., 2013). Future studies will help to elucidate how the neuronal expression of *ATXN3* regulates lifespan.

Consistent with the reduced motility observed in *ATXN3*-CAG89 transgenics, we also observed significant neurodegeneration in *ATXN3*-CAG89 transgenics compared with *ATXN3*-CAG10 controls. These data demonstrate that the *ATXN3*-CAG89 transgenics produce phenotypes distinct from wild-type *ATXN3*-CAG10 and that these mutant animals might be suitable for modifier screening. Our western blotting experiments (Fig. 1C) raise the possibility that the expression of *ATXN3*-CAG89 could decrease the expression of the endogenous *C. elegans* atx-3 gene, perhaps accounting for some of the negative phenotypes observed in *ATXN3*-CAG89 transgenics. We think this is unlikely because the western blotting data show that the level of endogenous ATX-3 in non-transgenic N2 worms (ratio 1.43) is lower than that of *ATXN3*-CAG10 controls (ratio 1.84), yet N2 worms do not have motility defects or paralysis phenotypes at a rate higher than the *ATXN3*-CAG10 transgenics. Furthermore, as we suspect that the anti-ATXN3 antibody detects ATX-3 and ATXN3, it is difficult to conclude
definitively that the levels of endogenous ATX-3 are meaningfully different in the respective strains. Future investigations with more specific antibodies might help to resolve this issue.

Several transgenic C. elegans ATXN3 models have been reported, consisting of full-length or fragments of ATXN3, a variety of CAG repeat lengths, with the protein often fused to a fluorescent marker, and high copy expression in body wall muscle cells or the entire nervous system (Khan et al., 2006; Bonanomi et al., 2014; Teixeira-Castro et al., 2011; Christie et al., 2014). The novelty of our approach is that we have expressed full-length, untagged human ATXN3 at low levels based on MoSCI transposon-mediated single copy insertion where transgenics express only two copies of the ATXN3 transgene. Furthermore, these transgenics have been engineered to express wild-type or mutant ATXN3 in a subset of the worm’s motoneurons, resulting in strong phenotypes from a small number of cells. We believe this approach will aid drug screening, where any particular compound might need to be active in only a small number of neurons to allow for detection of suppression. Furthermore, motoneuron degeneration has been associated with MJD (França et al., 2008; McIntire et al., 1993, 1997; Pinto and De Carvalho, 2008; Kanai and Kuwabara, 2009; Rub et al., 2002).

To explore the potential of our ATXN3 transgenics in chemical modifier screens, we tested whether we could suppress mutant ATXN3-CAG89 phenotypes with known neuroprotective compounds. Considering the evidence linking ATXN3 containing expanded CAG repeats to ER stress (Rodrigues et al., 2011; Evers et al., 2014; Costa and Paulson, 2012; Matos et al., 2011; Reina et al., 2010), and our previous findings connecting ER stress to other neurodegenerative diseases (Vaccaro et al., 2012a, 2013; Julien et al., 2016), we investigated whether pharmacological interventions centred on ER stress could protect against mutant ATXN3-CAG89 toxicity. We focused on three compounds, guanabenz, salubrinal and Methylene Blue, for their known neuroprotective activity and their roles as ER stress modulators (Matos et al., 2011; Rodrigues et al., 2016), we investigated whether pharmacological interventions tested activate the UPRER pathway to reduce toxicity, suggesting that there might be redundancy between the pathways. This is consistent with the notion that the compounds tested activate the UPRER pathway to reduce ATXN3-CAG89 toxicity. Thus, disabling individual genes blocks the activity of some molecules and not others, and helps to define the genetic requirements for neuroprotection by small molecules. However, we do predict that a triple mutant strain (ire-1, pep-1 and atf-6) would
Fig. 7. See next page for legend.
enhance ATXN3-CAG89 toxicity and block the rescuing activity of all molecules tested here.

Additional studies are required to extend these findings to mammalian models of MJD. However, from a practical perspective, given that guanabenz has US Food and Drug Administration approval and is being tested in a clinical trial for multiple sclerosis, it could be translated rapidly into clinical settings for MJD.

Future studies will make use of unbiased drug screen approaches to identify additional neuroprotective molecules. The novelty of this approach relies on rapidly identifying molecules that restore movement in ATXN3-CAG89 transgenics, followed by systematic characterization of lifespan, neurodegeneration and aggregation phenotypes. Our C. elegans ATXN3 strains might serve as the initial step of an in vivo drug discovery and development pipeline for MJD and other polyglutamine diseases.

MATERIALS AND METHODS

Nematode strains

Standard methods of culturing and handling worms were used (Stiernagle, 2006). Worms were maintained on standard nematode growth media (NGM) plates streaked with OP50 Escherichia coli. All strains were scored at 20°C. Strains used for this study were as follows: N2, atx-3(m1689), unc-47(e307), unc-64(e246), zcb1[hs4-GFP], af-6(ok551) and pek-1(ok275); all obtained from the C. elegans Genetics Center (University of Minnesota, Minneapolis).

Transgenic ATXN3 worms and plasmid constructs

Human cDNAs for wild-type and mutant ATXN3 were obtained from Dr Gyu Rouleau (Montreal Neurological Institute and Hospital, McGill University). The cDNAs were amplified by PCR and cloned into the Gateway vector pDONR221 following the manufacturer protocol (Invitrogen). Multisite Gateway recombination was performed with the pDONR ATXN3 clones along with clones containing the unc-47 promoter (obtained from Dr Erik Jorgensen, University of Utah), the unc-54 3’UTR plasmid pCM5.37 (obtained from Dr Geraldine Seydoux, Johns Hopkins; Addgene plasmid 17253) and the destination vector pCF3150 to create unc-47p:mCherry, ATXN3-CAG10 and unc-47p:mCherry; ATXN3-CAG89 expression vectors.

Transgenic lines were created by microinjection of unc-119(ed3) worms and screened for MoSCi transposon-mediated single copy insertion of the desired transgene. Several lines were isolated for each transgene of interest, and those showing similar behaviour, transgene expression levels and molecular profiles were outcrossed and kept for further analysis. The strains used in this study include the following: QX350 unc-119(ed3); tiT5605mosi; qxIs350(unc-47p::ATXN3-CAG10; unc-119(+)), and QX351 unc-119(ed3); tiT5605mosi; qxIs331(unc-47p::ATXN3-CAG89; unc-119(+)).

Age-synchronized populations

To obtain an age-synchronized population of worms, ~8-10 adult hermaphrodites were placed on 10 NGM plates for 3-4 days and kept at 20°C. Then, once the plates contained a large number of adult worms, they were collected with M9 buffer containing 1 mM NaCl, 1 mM MgSO4 and centrifuged at 3,250 × g (A-4-81 Rotor) for 4 min at 4°C. After centrifugation, 3 ml of the supernatant was taken and replaced with fresh plates per plate. The pellet was washed four times with M9 buffer for 10 min with a high intensity to degrade the worms, leaving a pellet containing only eggs. The pellet was transferred onto NGM plates with bacteria and kept overnight at 20°C. The next day, L1 worms were transferred using M9 buffer onto plates streaked with OP50 E. coli and kept at 20°C.

Paralysis assays on solid media

Worms expressing ATXN3-CAG10, ATXN3-CAG89, unc-47p:mCherry; ATXN3-CAG10 and unc-47p:mCherry; ATXN3-CAG89 were scored for paralysis from adult day 1 to adult day 12. Briefly, 30-40 L4 worms (obtained via synchronization) were transferred onto NGM plates and from adult day 1 to adult day 12, were scored as paralysed if they failed to move after being prodded with a worm pick. Worms were scored as dead if they were unable to respond to tactile head stimulus. They were transferred to fresh plates every 2 days until the cessation of progeny production. All experiments were conducted at 20°C, and each condition was done in triplicates with 30-40 worms per plate.

Lifespan assays

Approximately 40 L4 worms (obtained via synchronization) were transferred using M9 buffer on new NGM plates and tested daily from adult day 1 until death. Worms were transferred to fresh plates every 2 days until the cessation of progeny production. Worms were scored as dead if they failed to respond to tactile stimulus and showed no spontaneous movement or response when prodded. Dead worms displaying internally hatched progeny or extruded gonads or worms that crawled off the plate were excluded. The transgenic ATXN3 strains were compared with wild-type worms (N2). All experiments were conducted at 20°C, and each condition was done in triplicates with 40 worms per plate.

Aldicarb tests

To evaluate synaptic transmission, synchronized worms were grown on NGM plates until adult day 1, 5 and 9. They were then transferred onto NGM plates plus 1 mM aldicarb. Paralysis was scored every 30 min for 2 h on aldicarb plates. Worms were counted as paralysed if they failed to move upon prodding with a worm pick. All experiments were conducted at 20°C. For each strain, the test was done in triplicates with 30 worms per plate in three independent replicates.

Tracking the movement on solid media

Synchronized adult day 1, 5 and 9 worms were placed on NGM plates for 10 min. Their movement tracking was filmed and photographed using a 12MP Camera installed on a standard laboratory microscope.

RNAi experiments

Synchronized L1 worms were transferred onto NGM plates enriched with 1 mM isoprpyl-b-D-thiogalactopyranoside (IPTG). RNAi-treated ATXN3-CAG89 worms were fed with E.coli containing an empty vector (EV) or expressing dsRNA against ero-1 (Y10588B.8) or ire-1 (C41C4.4).
The RNAi clone was obtained from the ORFeome RNAi library (Open Biosystems) and sequence verified. For ero-1 experiments, age-synchronized worms at adult day 2 were incubated on a slide with 5 µM 2′,7′-dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich) for 30 min at room temperature and washed three times for 5-10 min with PBS 1× (10 mM Na₂HPO₄, 1.8 mM NaH₂PO₄ and 140 mM NaCl, adjusted to pH to 7.4; adding ddH₂O to 1000 ml). Worms were visualized by fluorescence microscopy under 488 nm wavelength excitation.

For ire-1 experiments, worms were scored from adult day 1 to adult day 12 for paralysis assays. They were transferred to fresh plates every 2 days until the cessation of progeny production. Each condition was done in triplicates with 30-40 worms per plate. All experiments were conducted at 20°C.

Fig. 8. See next page for legend.
Fig. 8. Chemical-genetic approach analysis of UPRER pathways in ATXN3-CAG89 mutants. (A) Paralysis assays for ATXN3-CAG89 and ATXN3-CAG89; pek-1(ok275) transgenic mutant worms in presence of 60 μM MB, 50 μM Sal and 50 μM Gua. We observed that Gua failed to suppress the paralysis in ATXN3-CAG89; pek-1(ok275) worms, Sal partly suppressed paralysis (**P<0.001) and MB suppressed paralysis in ATXN3-CAG89; pek-1(ok275) mutant worms (**P<0.0001 by log-rank (Mantel–Cox) test; n=270-300). This experiment was repeated three times. (B) ATXN3-CAG89 and ATXN3-CAG89; atf-6(ok551) mutant worms were treated with 60 μM MB, 50 μM Sal and 50 μM Gua. We observed that Sal and MB suppressed paralysis in ATXN3-CAG89; atf-6(ok551) mutant worms (**P<0.0001 for both compounds), contrary to Gua, which is dependent on the atf-6 branch of the UPR pathway, showing an incapacity to suppress the paralysis in the transgenic mutant worms [by log-rank (Mantel–Cox) test; n=270-300]. This experiment was repeated three times. (C) Paralysis assays for ire-1 RNAi ATXN3-CAG89 worms treated with 60 μM MB, 50 μM Sal and 50 μM Gua. We observed that Gua totally and MB partly (***P<0.001) failed to suppress the paralysis in ire-1 RNAi worms, contrary to Sal, which suppressed paralysis in ire-1 RNAi worms (**P<0.01) by log-rank (Mantel–Cox) test; n=270-300. This experiment was repeated three times. Gua, guanabenz; MB, Methylene Blue; Sal, salubrinal; UPRER, ER unfolded protein response.

Fluorescence microscopy (neurodegeneration, ER stress response and oxidative stress assays)

For scoring of neuronal processes for gaps or breakages, unc-47:mCherry; ATXN3-CAG89, unc-47:mCherry; ATXN3-CAG10 and unc-47:mCherry were collected at adult day 5 for visualization of motoneuron processes in vivo. The expression of the ER stress response, hsp-4::GFP, hsp-4::GFP; ATXN3-CAG10 and hsp-4::GFP; ATXN3-CAG89 worms were collected at adult day 1, and for DCF-DA experiments, wild-type N2 worms and ATXN3 transgenic lines were collected at adult day 2. The nematodes were immobilized using M9 buffer with 60% glycerol and mounted on slides with 2% agarose pads. mCherry was visualized at 595 nm, and GFP was visualized at 488 nm using a Zeiss Axio Imager M2 microscope. Fluorescent expression was visualized with a DIC microscope Zeiss AxioObserver A1. The software used was Axiosvision 4.8.2.0. One hundred worms were scored per condition for the neurodegeneration assays. Approximately 25 worms were visualized per condition for the ER stress response and the oxidative stress experiments. Image processing and quantification were done with Adobe Photoshop. To compare fluorescence in ER stress response and oxidative stress assays, we calculated the changes in the ratio (size/intensity of fluorescence). Student’s unpaired t-test was used for statistical analysis.

Compound testing on solid media

Worms were exposed from hatching (by synchronization) to 60 μM Methylene Blue, 50 μM salubrinal or 50 μM guanabenz incorporated into NGM solid medium, or to NGM solid medium only as a control. All the plates were streaked with OP50 E. coli. Compounds were purchased from Sigma-Aldrich (St Louis, MO, USA) and Tohris Bioscience (Ellissville, MO, USA). Briefly, 30-40 worms were picked and plated on the corresponding NGM medium (30-40 worms per plate for each condition, and each condition was done in triplicates) in order to complete the paralysis and lifespan assays, the neurodegeneration observations (fluorescence microscopy) and the drug screens.

Compound testing in liquid culture

The swimming activity of the nematodes was measured by a WMicroTracker machine (Phylum Tech) (Simonetta and Golombek, 2007). Briefly, worms were exposed until day 5 of adulthood on the corresponding NGM medium plates (drug exposure on solid media) and then were transferred into a 96-well plate. Each well contained a final volume of 100 μl of the drug with the appropriate concentration or M9 buffer used as control, and ~30 worms adult day 5 (obtained via synchronization). Each condition was done in triplicates, and the experiments were repeated at least three times. The swimming movements of the nematodes were tracked for 10 h.

Reactive oxygen species measurements

The in vivo detection of reactive oxygen species in C. elegans has been described previously (Vaccaro et al., 2013, 2012a). Briefly, age-synchronized worms at adult day 2 were incubated on a slide with 5 μM DCF-DA (Sigma-Aldrich) for 30 min at room temperature and washed three times for 5-10 min with PBS 1× (10 mM Na2HPO4, 1.8 mM NaH2PO4 and 140 mM NaCl, with pH adjusted to 7.4; adding ddH2O to 1000 ml). Worms were visualized by fluorescence microscopy under a 488 nm wavelength excitation.

Immunofluorescence (antibody staining for transgenic worms)

Five plates of worms for each strain were collected with M9, centrifuged at 3,250 g (A-4-81 Rotor) for 4 min at 4°C and washed twice with M9 buffer with the same centrifugation conditions. Worm pellets were placed at −80°C overnight. The supernatants were discarded, and 500 μl of cold methanol (stored at −20°C for 5 min) was added to the pellets and remained for 5 min at room temperature (fixation step). The supernatants were discarded, and the pellets were washed twice with PBS 1× (10 mM Na2HPO4, 1.8 mM NaH2PO4 and 140 mM NaCl, with pH adjusted to 7.4; adding ddH2O to 1000 ml) and centrifuged at 2,151 g (FA-45-30-11 Rotor) for 3 min at 23°C. The pellets were then blocked with 300 μl donkey serum solution [0.05 mg/l BSA, 5% donkey serum (Sigma-Aldrich; cat #: D9663; 10 ml), 0.2% Triton] for 30 min at room temperature. Worms were centrifuged at 2,151 g (FA-45-30-11 Rotor) for 3 min at 23°C and the supernatants discarded. Four hundred microliters of the primary antibody rabbit anti-ATXN3 (1:200; Proteintech; cat #: 13505-1) in the donkey serum solution was added to the pellets and stored at 4°C overnight. Worms were washed twice with PBS 1× for 5 min and each time centrifuged at 2,151 g (FA-45-30-11; Rotor) for 1 min at 23°C to discard the supernatants. The supernatants were discarded, and 400 μl of the secondary antibody donkey anti-rabbit IgG (H+L) Alexa Fluor 488 (1:250; Invitrogen; cat #: A-21206) in PBS 1× was added to the pellets and remained for 30 min at room temperature. Worms were washed with PBS 1×, centrifuged at 2,151 g (FA-45-30-11 Rotor) for 1 min at room temperature and the supernatants discarded. Pellets were washed with 400 μl of TOPRO-3 iodide dye (1:300; Invitrogen; cat #: T3605) in PBS 1× for 5 min, centrifuged at 2,151 g (FA-45-30-11 Rotor) for 1 min at 23°C, then washed twice with PBS-T 1× (add 1 ml of Tween to PBS 1× stock) for 5 min at room temperature. Worms were mounted on slides with 20 μl mounting solution (Invitrogen ProLong Antifade Kit; cat #: P7481) and stored at 4°C overnight. Confocal images were acquired on a Leica TCS-SP5 inverted confocal microscope using an HCX PL APO CS 40×/1.25 oil objective. Excitation system was performed using a 633 HeNe laser for TOPRO simultaneously with the 488 nm line of an argon laser for eGFP. Scan speed was 400 Hz. Detection bandwidth was 643-800 nm for eGFP. The software used was LAS Image Analysis. Twenty-five to thirty worms were visualized per condition for the aggregation phenotype. Image processing was done with LAS Image Analysis and Adobe Photoshop.

Cell lysis

Cell lines derived from healthy and MJD patients were obtained from Dr Guy Rouleau (Montreal Neurological Institute and Hospital, McGill University). MJD lymphoblastoid cell lines (LCL) were established from peripheral blood samples of MJD patients of European origin. Cells were grown in IMDM (Gibco) supplemented with fetal bovine serum (10%), penicillin and streptomycin (100 units/ml) and L-glutamine (0.292 mg/ml). Collected cells were centrifuged at 98 g (FA-45-24-11 Rotor) for 2 min at 4°C. The supernatants were discarded and the pellets transferred into new Eppendorf tubes with 1 ml cold PBS 1× (10 mM Na2HPO4, 1.8 mM NaH2PO4 and 140 mM NaCl, with pH adjusted to 7.4; adding ddH2O to 1000 ml) and centrifuged at 98 g for 5 min at 4°C. This step was repeated three times. The medium was aspirated and the pellets were resuspended in an appropriate volume of RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS and 1% sodium deoxycholate) and 0.1% protease inhibitors (10 mg/ml leupeptin, 10 mg/ml pepstatin A and 10 mg/ml chymostatin LPC; 1/1000) depending on pellet size. The samples were put on ice for 10 min, then placed at room temperature for 10 min. The samples were then centrifuged at 142 g (FA-45-24-11 Rotor) for 15 min at 4°C. The pellets were discarded, and the supernatants were collected in 1.5 ml tubes and stored at −80°C.
Western blotting

Fifteen plates of worms for each strain and for each condition (with or without compounds) were collected with M9, centrifuged at 3,250 g (A-4-81 Rotor) for 4 min at 4°C and washed twice with M9 buffer with the same centrifugation conditions. Worm pellets were placed at −80°C overnight. Pellets were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS and 0.1% sodium deoxycholate) and 0.1% protease inhibitors (10 mg/ml leupeptin, 10 mg/ml pepstatin A and 10 mg/ml chymostatin LPC; 1/1000). Pellets were passed through a 27.5-gauge syringe seven times, sonicated for 5 min, and centrifuged at 16,000 g for 15 min at 4°C. Supernatants were collected in 1.5 ml tubes. The supernatants were quantified using the BCA protein assay kit (Thermo Scientific) following the manufacturer’s protocol and instructions.

Protein solubility

Fifteen plates of worms for each strain and for each condition (ATXN-CAG89 worms treated with each compound) were collected with M9, centrifuged at 3,250 g (A-4-81 Rotor) for 4 min at 4°C and washed twice...
with M9 buffer with the same centrifugation conditions. Worm pellets were placed at −80°C overnight. To obtain soluble and insoluble fractions for our transgenics, worms were lysed in extraction buffer (1 M Tris-HCl pH 8, 0.5 M EDTA, 1 M NaCl and 10% SDS) plus protease inhibitors (10 mg/ml leupeptin, 10 mg/ml pepstatin A and 10 mg/ml chymostatin LPC; 1/1000). Pellets were passed through a 27.5-gauge syringe 10 times, sonicated for 5 min, and centrifuged at 16,000 g for 15 min at 4°C. The soluble supernatants were collected in 1.5 ml tubes and stored at −80°C. The remaining pellets were resuspended in extraction buffer, sonicated and centrifuged at 10,000 g for 5 min. The supernatants were discarded and the remaining pellets resuspended in 100 μl of RIPA buffer and sonicated for ~90 min until the pellets were resuspended in solution. The pellets were collected and stored in 1.5 ml tubes at −80°C. The supernatants and pellets were quantified using the BCA protein assay kit (Thermo Scientific) following the manufacturer’s protocol and instructions.

**Dot blotting**

Dot blotting was done using the Bio-Dot SF microfiltration apparatus (cat. #: 170-6542). The assays were done using the manufacturer’s protocol and instructions following the section Protein Slot Blotting-Immunoblotting procedure and followed immunoblotting procedures. The antibody used was rabbit anti-ATXN3 (1:500; Proteintech; cat. #: 13505-1). Blots were visualized using peroxidase-conjugated secondary antibodies and ECL western blotting substrate (Thermo Scientific) via the Bio Rad ChemiDoc MP Imaging System (model #: Universal HOOD III). N2 wild-type worms and ATXN3-CAG10 were used as controls. Quantification was performed using Image Lab software.

**Statistics**

Analysis of synaptic transmission was performed using the Bio-Rad GeneFxDoc MP Imaging System (model #: Universal HOOD III). N2 wild-type worms and ATXN3-CAG10 were used as controls. Quantification was performed using Image Lab software.

**Concluding remarks**

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**Supplementary information**

Supplementary information available online at http://dmm.biologists.orglookup/doc/10.1242/dmm.029736.supplemental

**References**


Supplementary Figure 1. atx-3(tm1689) do not affect the paralysis and neurodegeneration phenotypes observed in ATXN3-CAG89 transgenic worms

(A) No difference between unc-47p::mCherry; CAG89 and (unc-47p::mCherry; CAG89); atx-3(tm1689) transgenic worms were identified for the paralysis phenotype. This experiment was replicated for 3 times.

(B) Quantification of neurodegeneration in transgenic worms at days one, five and nine of adulthood. At adult day one worms, there was none significant neurodegeneration phenotype for the transgenic worms when compared to unc-47p::mCherry. ATXN3-CAG89 transgenics showed a significant increase of neurodegeneration compared to unc-47p::mCherry and ATXN3-CAG10 controls (****P < 0.0001 for day five of adulthood and ***P < 0.001 for adult day nine worms, by Student’s t-test) (N=100 for each condition). No difference between unc-47p::mCherry; CAG89 and (unc-47p::mCherry; CAG89); atx-3(tm1689) transgenic worms were identified for the neurodegeneration phenotype for none of the tree days of the study (days one, five and nine of adulthood). These experiments were replicated for 3 times.
Supplementary Figure 2. unc-47p::mCherry do not affect the swimming activity of ATXN3-CAG89 mutant worms

The swimming activity of ATXN3-CAG89 mutants was compared to unc-47p::mCherry; CAG89 worms for a period of ten hours at (A) day one of adulthood and, (B) day five of adulthood. No differences for the motility was observed between ATXN3-CAG89 and unc-47p::mCherry; CAG89 worms.