Rescue of ATXN3 neuronal toxicity in *C. elegans* by chemical modification of ER stress

Yasmin Fardghassemi†, Arnaud Tauffenberger², Sarah Gosselin³, and J. Alex Parker ¹,², ³*

¹ CRCHUM and Department of Biochemistry, University of Montreal, Montreal, Quebec, Canada
² CRCHUM and Department of Pathology and Cell Biology, University of Montreal, Montreal, Quebec, Canada
³ CRCHUM and Department of Neuroscience, University of Montreal, Montreal, Quebec, Canada

*Corresponding author at:
CRCHUM, Tour Viger, 900, rue Saint-Denis, R09.440
Montréal, Québec, Canada, H2X 0A9
Tel.: +1 514-890-8000 ext. 28826. Fax: +1 514 412-7602
E-mail address: ja.parker@umontreal.ca (J.A. Parker);

†The first author is co-first author

Authors e-mail addresses:
Yasmin Fardghassemi: yasminfghassemi@hotmail.com
Arnaud Tauffenberger: arnaud.tauffenberger@gmail.com
Sarah Gosselin: sarah.gosselin.2@umontreal.ca
J Alex Parker: ja.parker@umontreal.ca
Summary statement

We introduce a novel *C. elegans* model for Machado-Joseph disease and its use in preclinical drug discovery.

Abstract

Background: Polyglutamine expansion diseases are a group of hereditary neurodegenerative disorders that develop when a CAG repeat in the causative genes are unstably expanded above a certain threshold. The expansion of trinucleotide CAG repeats cause 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 spinocerebellar ataxia is the type 3 (SCA3) also known as Machado-Joseph disease (MJD), 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.

Results: We generated transgenic *C. elegans* strains expressing human *ATXN3* genes in motor neurons, 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 ER unfolded protein response (UPR**ER**), reduced global ER and oxidative stress, as well as polyglutamine aggregation.

Conclusions: We introduce novel *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**ER** reduced neurodegeneration and warrants investigation in mammalian models of MJD.
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 including 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 (Schols et al., 2004).

MJD is an autosomal dominant progressive neurologic disorder characterized principally by ataxia, spasticity, peripheral neuropathy and ocular movement abnormalities (Franca 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, Teixeira-Castro et al., 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 (Soong et al., 1997, Wullner 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 highly observed among people of Portuguese/Azorean 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 ubiquitin interacting motif (UIM) in the C-terminus tail (Goto et al., 1997, Li et al., 2015).

To aid the study of Machado-Joseph disease 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 due to its comprehensively detailed neuronal lineage and interconnectivity of synapses that resembles aspects of the vertebrate nervous system (Stiernagle, 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 nucleus and cytoplasm, with higher levels observed in the cytoplasm. It has been shown that loss of atx-3 activity results changes in the expression of genes involved in several different pathways: 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 ER stress response and protect against proteotoxicity in simple models of amyotrophic lateral sclerosis (Vaccaro et al., 2013, Vaccaro et al., 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, as well as increased oxidative and ER stress observed in mutant transgenic animals as an early effort for MJD therapy development.
Results

Expression of full-length human \textit{ATXN3} in \textit{C. elegans}

Using 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 \textit{ATXN3} in \textit{C. elegans}. It has been shown previously that expressing ataxin-3 with 89 CAG repeats (CAG89) results in neurodegeneration, as well as protein misfolding phenotypes in cell culture and \textit{Drosophila} models (Stochmanski et al., 2012). Based on our previous neurodegeneration models (ALS models), we expressed human \textit{ATXN3}, either wild-type \textit{ATXN3-CAG10} (Fig. 1A) or mutant \textit{ATXN3-CAG89} (Fig. 1B) in the worm’s 26 GABAergic motor neurons using the promoter for the gene \textit{unc-47}, which encodes a vesicular GABA transporter (Vaccaro et al., 2012a). In our experience, the \textit{unc-47} modeling 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 \textit{ATXN3} in transgenic \textit{C. elegans} strains by western blotting with a human specific anti-\textit{ATXN3} antibody. A band corresponding to wild type \textit{ATXN3-CAG10}, as well as a larger band for the MJD-associated mutant \textit{ATXN3-CAG89} were detected by western blotting of worm protein extracts. However, the anti-\textit{ATXN3} also detected a band in non-transgenic wild type N2 animals (Fig. 1C). \textit{atx-3} encodes a deubiquitylating enzyme that is the highly-conserved \textit{C. elegans} orthologue of human \textit{ATXN3} (Kawaguchi et al., 1994, Echtermeyer et al., 2011), thus can be detected by western blotting with a human specific \textit{ATXN3} antibody. To confirm this, we conducted western blotting experiments using the anti-\textit{ATXN3} antibody and protein extracts from \textit{atx-3} null mutants. \textit{atx-3(tm1689)} is a loss-of-function mutation consisting of a 660 base pair deletion along with a 6 base pair insertion (Consortium, 2012, Rodrigues et al., 2007), and no signal was observed. These data suggest that the anti-\textit{ATXN3} antibody recognizes \textit{C. elegans} \textit{ATX-3} in N2 wild-type worms and partially obscures the signal for \textit{ATXN3-CAG10} transgenics, but a specific, higher molecular weight signal is visible in extracts from \textit{ATXN3-CAG89} animals (Fig. 1C). Finally, we confirmed the specificity of the anti-\textit{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 if the expression of non-native proteins led to decreased health in the transgenic strains. First 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 to non-transgenic wild-type N2 worms (Fig. 2A). These data demonstrate that increased expression of ATXN3 transgenes have negative consequences on lifespan, but the mutant ATXN3-CAG89 transgene had a more severe phenotype compared to ATXN3-CAG10 controls.

Decreased lifespan can indicate poor health of the animals and one sign of aging 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 defects compared to wild type ATXN3-CAG10 transgenics. Starting during adulthood, ATXN3-CAG89 transgenics displayed uncoordinated motility phenotypes progressing to paralysis over a period of 12 days, and occurred at higher rate compared to ATXN3-CAG10 controls (Fig. 2B).

The nematode C. elegans body wall muscle cells receive excitatory (acetylcholine) and inhibitory (GABA) inputs to coordinate muscle contraction/relaxation and 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 known compound aldicarb results in accumulation of acetylcholine at neuromuscular junctions, leading to a number of different phenotypes: hyperactive cholinergic 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 if 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 to ATXN3-CAG10 transgenics, wild type N2 worms, and unc-64(e246) mutants at day one, five and nine 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 motor neurons, 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 movement for a period of ten minutes on agar plates. We observed that ATXN3-CAG89 had impaired motility phenotypes and explored less of their area compared to wild-type N2, and ATXN3-CAG10 worms at days one, five and nine 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 to controls.

**Mutant ATXN3-CAG89 causes progressive motor neuron degeneration**

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

We investigated if 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).

Since *ATXN3* and mCherry are both expressed under the same promoter (*unc-47*), we wondered if transcription factor depletion could contribute to the motility phenotypes observed in
our ATXN3-CAG89 transgenics. To investigate we turned into a worm tracking system (*Wmicrotracker*, Phylum Tech) able to measure both automatically and simultaneously the movement of a population of worms placed in a 96-well-microtiter 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 microtiter 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 uncp::mCherry; ATXN3-CAG89 worms (Fig. S2). These data suggest that the addition of the uncp::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 and were shown to have beneficial effects against mutant TDP-43 neuronal toxicity in models for amyotrophic lateral sclerosis (Vaccaro et al., 2012a, Vaccaro et al., 2013), as well as having protective effects in models for hereditary spastic paraplegia (Julien et al., 2016). Thus we wondered if these compounds had neuroprotective activity against mutant ATXN3-CAG89. We tested these compounds and found that all three molecules suppressed the age-dependent paralysis phenotype caused by mutant ATXN3-CAG89 (Fig. 3A-C), and extended the lifespan of these transgenic worms (Fig. 3D). Additionally, we wondered if these compounds had any effect on the expression of ATXN3-CAG89 transgenes, and perhaps influencing paralysis and lifespan phenotypes. We confirmed that these compounds did not affect the expression of ATXN3-CAG89 transgenes by western blotting with a human specific anti-ATXN3 antibody (Fig. 3E).

To confirm the neuroprotective activity of these molecules with a separate approach we turned to our automated assay measuring the movement of *C. elegans* grown in liquid culture (Veriepe et al., 2015, 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 to 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 if these compounds had protective effects against motor neuron degeneration. Using the unc-47p::mCherry; ATXN3-CAG89 strain, we visualized the GABAergic motor neurons 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 if 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, Vaccaro et al., 2012a). Global oxidative stress can be detected by staining worms with the fluorescent dye 2’,7’-dichlorofluorescein diacetate (DCF-DA) and we observed increased fluorescence in ATXN3-CAG89 transgenics compared to wild-type N2 controls and ATXN3-CAG10 transgenics (Fig. 6A-C).

Our previous work suggests that oxidative stress may 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 may 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 directly test 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 we observed increased fluorescence in ATXN3-CAG89; hsp-4::GFP animals compared to 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 MB and Sal, suggesting that these molecules may act by stimulating an early ER stress response outcome that may 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 centered 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 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 transgenics worms. We observed that ATXN3-CAG89 mutant worms stained with DHF in presence of ero-1(RNAi) showed a significant decrease of fluorescence compared to the empty vector RNAi controls (Fig. 7E). These data suggest that the
oxidative stress observed in our ATXN3-CAG89 transgenic mutant worms may originate in the ER and is dependent on the activity of ero-1.

**Chemical-genetic approach analysis for the UPR\textsuperscript{ER} pathways**

ER stress leads to activation of the of the UPR by three main signaling 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 UPR\textsuperscript{ER}-associated compounds (Fig. 7), we wondered if these compounds required any specific UPR\textsuperscript{ER} 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 UPR\textsuperscript{ER} pathways such as atf-6/ATF6, pek-1/PERK, and RNAi for ire-1/IRE1.

We investigated the contribution of the pek-1/PERK pathway with the deletion mutant pek-1(ok275). We observed that the rescuing activity of guanabenz was completely dependent on pek-1. Methylene blue continued to suppress paralysis in absence of pek-1 suggesting that this compound does not require the pek-1/PERK branch of the UPR\textsuperscript{ER}. Concerning salubrinal, this compound was partially dependent on pek-1 since we observed a reduced ability to suppress paralysis in ATXN3-CAG89; pek-1(ok275) worms (Fig. 8A).

We examined the neuroprotection activity of atf-6 using atf-6(ok551) mutant worms. We observed that atf-6 was not required for the neuroprotective activity of methylene blue and salubrinal since these compounds maintained their ability to suppress paralysis in ATXN3-CAG89; atf-6(ok551) mutants. However, we observed that guanabenz failed to suppress the paralysis in ATXN3-CAG89 mutants when crossed with atf-6 loss of function mutants confirming dependence on the atf-6 branch of UPR\textsuperscript{ER} (Fig. 8B).

To investigate the role of ire-1 in neuroprotection, we used RNAi against ire-1(zc14). We observed that the rescuing activity of guanabenz was completely dependent on ire-1 and, methylene blue showed a partial dependence toward this branch of the UPR\textsuperscript{ER}. However, salubrinal showed no dependence on this pathway as this compound continued to suppress the paralysis in ire-1(RNAi) worms (Fig. 8C). These data suggest that the three chemical compounds employ distinct, or possibly overlapping branches of the UPR\textsuperscript{ER} to achieve neuroprotection against CAG-mediated proteotoxicity.
Small molecule suppression of ATXN3-CAG89 aggregation

Since ATXN3 have been observed to form protein aggregates in several models including *C. elegans*, we wondered if our transgenics displayed similar aggregation phenotypes (Teixeira-Castro et al., 2015, Teixeira-Castro et al., 2011, Koch et al., 2011, Breuer et al., 2010). To determine if ATXN3 proteins could be detected *in vivo*, we fixed whole ATXN3-CAG10 and ATXN3-CAG89 transgenics and stained them with a human ATXN3 antibody. We detected ATXN3 protein in the motor neurons of ATXN3 transgenics (Fig. 9A). Our cursory visual examination suggests a more intense puncta signal in ATXN3-CAG89 transgenics that may reflect increased aggregation potential of ATXN3-CAG89 protein compared to ATXN3-CAG10.

We investigated further using a biochemical assay to detect protein aggregation. Homogenized protein extracts from transgenic worms were separated into supernatant (detergent-soluble) and pellet (detergent-insoluble). Dot blotting the ATXN3-CAG89 transgenic mutants with human ATXN3 antibody showed a high accumulation of the ATXN3-CAG89 in the pelleted, insoluble fraction, when compared to ATXN3-CAG10 and N2 wild-type worms (Fig. 9B). We then investigated if 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). These data suggest neuroprotective roles for methylene blue, salubrinal, and guanabenz in reducing mutant ATXN3 aggregation.
Discussion

We introduce novel *C. elegans* models for investigating mechanisms of motor neuron toxicity caused by a polyglutamine expansion in ATXN3. To model human disease we expressed full-length human *ATXN3* without additional tags, since the inclusions 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 since impaired activity of these motor neurons can lead to easily identifiable motility phenotypes useful for screening purposes (McIntire et al., 1997, McIntire et al., 1993). Additionally, MJD disease is in part associated with dysfunction of motor neurons (Franca 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 motor neurons 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 to wild-type ATXN3-CAG10 transgenics. The fact that we observed a decrease of lifespan resulting from the expression of ATXN3 transgenes in GABAergic motor neurons suggests that there may 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 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 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 to ATXN3-CAG10 controls. These data demonstrate that the ATXN3-CAG89 transgenics produce phenotypes distinct from wild type ATXN3-CAG10 and that these mutant animals may be
suitable for modifier screening. Our western blotting experiments (Fig. 1C) raise the possibility that the expression 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 since the western blotting data shows 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, since we suspect that the anti-ATXN3 antibody detects ATX-3 and ATXN3 it is difficult to definitively conclude that the levels of endogenous ATX-3 is are meaningfully different in the respective strains. Future investigations with more specific antibodies may help 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 motor neurons resulting in strong phenotypes from a small number of cells. We believe this approach will aid drug screening where any particular compound may only need to be active in a small number of neurons to allow for detection of suppression. Furthermore, motor neuron degeneration has been associated with MJD (Franca et al., 2008, McIntire et al., 1993, McIntire et al., 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 if we could suppress mutant ATXN3-CAG89 phenotypes with known neuroprotective compounds. Considering the evidence linking *ATXN3* containing an expanded CAG repeats to ER stress (Rodrigues et al., 2011, Evers et al., 2014, Costa Mdo 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, Vaccaro et al., 2013, Julien et al., 2016), we investigated if 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., 2011, Vaccaro et al., 2013, Vaccaro et al., 2012a). Encouragingly, we observed that the three compounds rescued motility defects, reduced lifespan, neurodegeneration, and aggregation in animals expressing mutant ATXN3-CAG89. Moreover, these three compounds were also able to prevent the oxidative stress and the ER stress response induced in ATXN3-CAG89 transgenics. Of the three compounds tested, we observed that guanabenz was the most effective at suppressing motility defects, neurodegeneration and protein aggregation phenotypes in ATXN3-CAG89 animals. Finally, we identified the branches of the UPRER pathways essential for neuroprotection against mutant ATXN3. It is worth noting that mutations in individual UPRER genes (ire-1, pek-1, or atf-6) did not exacerbate ATXN3-CAG89 toxicity, suggesting there may 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 help to define the genetic requirements for neuroprotection by small molecules. However, we do predict that a triple mutant strain (ire-1, pek-1, and atf-6) would 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, since guanabenz has FDA approval and is being tested in a clinical trial for multiple sclerosis, it could be rapidly translated 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 may 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 *E. coli*. All strains were scored at 20°C. Strains used for this study were: N2, *atx-3(tm1689), unc-47(e307), unc-64(e246), zcls4[hsp-4::GFP], atf-6(ok551) and pek-1(ok275)*, all obtained from the *Caenorhabditis 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. Guy Rouleau (Montreal Neurological Institute and Hospital, McGill University). The cDNAs were amplified by PCR and cloned into the Gateway vector pDONR221 following the manufacturer’s 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, John Hopkins, Addgene plasmid 17253) and the destination vector pCFJ150 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 behavioural, transgene expression levels and molecular profiles were outcrossed and kept for further analysis. The strains used in this study include: XQ350 *unc-119(ed3); ttTi5605mosII; xqIs350(unc-47p::ATXN3-CAG10; unc-119(+)),* and XQ351 *unc-119(ed3); ttTi5605mosII; xqIs351(unc-47p::ATXN3-CAG89; unc-119(+)).

Age-synchronized populations
To obtain age synchronized population of worms, ~8-10 adult hermaphrodites were placed on ten 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 (1M KH2PO4, 1M Na2HPO4, 1M NaCl, 1M MgSO4) and centrifuged at 4000 rpm (A-4-81 Rotor) for 4min at 4°C. After centrifugation, 3 mL
of the supernatant was taken and replaced with 3 mL of a mix solution containing NaOH 5M and bleach (1:2). 10min vortex followed with a high intensity to degrade the worms leaving a pellet containing only eggs. The pellet was washed 4 times with M9 buffer and centrifuged at 4000 rpm (A-4-81 Rotor) for 4 min at 4°C. The pellet was transferred onto NGM plates without bacteria and kept overnight at 20°C. The following day, L1 worms were transferred using M9 buffer onto plates streaked with OP50 *E. coli* and are kept at 20°C.

**Paralysis assays on solid media**
Worms expressing ATXN3-CAG10, ATXN3-CAG89, *unc-47*p::mCherry; ATXN3-CAG10 and *unc-47*p::mCherry; ATXN3-CAG89 were scored for paralysis from adult day one to adult day twelve. Briefly, 30-40 L4 worms (obtained via synchronization) were transferred onto NGM plates and from adult day one to adult day twelve, 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/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 one 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, extruded gonads, or 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/plate.

**Aldicarb tests**
To evaluate synaptic transmission, synchronised worms were grown on NGM plates for adult day one, five and nine. They’ve been after transferred into NGM plates + 1mM aldicarb. Paralysis was scored at each 30min during 2 hours 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/plate in three independent experiences.

**Tracking the movement on solid media**

Synchronized adult day one, five and nine worms were placed on NGM plates for 10 min. Their movement tracking have been 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 1mM isopropyl-β-D-thiogalactopyranoside (IPTG). RNAi-treated ATXN3-CAG89 worms were fed with *E.coli* containing an empty vector (EV) or expressing dsRNA against *ero-1* (Y105E8B.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 two 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 1X (10 mM Na₂HPO₄, 1.8 mM NaH₂PO₄; 140 mM NaCl, adjust 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 one to adult day twelve 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/plate. All experiments were conducted at 20°C.

**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 five for visualization of motor neurons processes in vivo. For visualization of ER stress response, *hsp-4::GFP, hsp-4::GFP, ATXN3-CAG10 and hsp-4::GFP, ATXN3-CAG89* worms were collected at adult day one and, for DCF-DA experiments, wild-type N2 worms and *ATXN3* transgenic lines.
were collected at adult day two. 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 visualised 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 AxioVs40 4.8.2.0. 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 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 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) and Tocris Bioscience (Ellisville, MO). Briefly, 30-40 worms were picked and plated on the corresponding NGM medium (30-40 worms/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 WMicroTracker machine (Phylum Tech) (Simonetta and Golombek, 2007). Briefly, worms were exposed until day five 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 five (obtained via synchronization). Each condition was done in triplicates and the experiments were repeated for at least three times. The nematodes swimming movements were tracked for ten hours.
Reactive oxygen species measurements

The *in vivo* detection of reactive oxygen (ROS) species in *C. elegans* is previously described (Vaccaro et al., 2013, Vaccaro et al., 2012a). Briefly, age-synchronized worms at adult day two 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 1X (10 mM Na₂HPO₄, 1.8 mM NaH₂PO₄; 140 mM NaCl, adjust pH 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 was collected with M9, centrifuged at 4000 rpm (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 1X (10 mM Na₂HPO₄, 1.8 mM NaH₂PO₄; 140 mM NaCl, adjust pH to 7.4; adding ddH₂O to 1000 mL) and centrifuged at 4500 rpm (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 #: D9663-10mL), 0.2% Triton) for 30 min at room temperature. Worms were centrifuged at 4500 rpm (FA-45-30-11 Rotor) for 3 min at 23°C and the supernatants were discarded. 400 µL 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 1X for 5 min and each time centrifuged at 4500 rpm (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 1X was added to the pellets and remained for 30 min at room temperature. Worms were washed with PBS 1X, centrifuged at 4500 rpm (FA-45-30-11 Rotor) for 1 min at room temperature and the supernatants were discarded. Pellets were washed with 400 µL of TOPRO-3 Iodide dye (1:300, Invitrogen- Cat #: T3605) in PBS 1X for 5 min, centrifuged at 4500 rpm (FA-45-30-11 Rotor) for 1 min at 23°C and then, washed twice with PBS-T 1X (add 1mL of Tween to PBS 1X 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 a HCX PL APO CS 40x/1.25 oil objective. Excitation system was performed using a 633 HeNe laser for TOPRO simultaneously with the 488nm line of an Argon laser for eGFP. Scan speed was 400Hz. Detection bandwidth was 643-800nm for TOPRO-3, and 498-551nm 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**

Cells 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 origins. Cells were grown in IMDM (Gibco) supplemented with foetal bovine serum (10%), penicillin & streptomycin (100 units/ml) and L-glutamine (0.292 mg/ml). Collected cells were centrifuged at 1000 rpm (FA-45-24-11 Rotor) for 2 min at 4°C. The supernatants were discarded and the pellets were transferred into new eppendorf tubes with 1mL cold PBS 1X (10 mM Na₂HPO₄, 1.8 mM NaH₂PO₄; 140 mM NaCl, adjust pH to 7.4; adding ddH₂O to 1000 mL) and centrifuged at 1000 rpm for 5min at 4°C. This step was repeated for 3 times. The media 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, 1% sodium deoxycholate) and 0.1% protease inhibitors (10mg/mL leupeptin, 10 mg/mL pepstatin A, 10 mg/mL chymostatin LPC; 1/1000) depending on pellet size. The samples were put on ice for 10min, and then placed at room temperature for 10 min. The samples were then centrifuged at 12000 rpm (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) was collected with M9, centrifuged at 4000 rpm (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, 1% sodium deoxycholate) and 0.1% protease inhibitors (10mg/mL leupeptin, 10 mg/mL pepstatin A, 10 mg/mL chymostatin LPC; 1/1000). Pellets were passed through a 271/2 G syringe 7 times, sonicated for 5 minutes and, centrifuged at 16,000g for 15min at 4°C. Supernatants were collected in 1.5mL tubes. The supernatants were quantified using the BCA Protein Assay Kit (Thermo Scientific) following the manufacturer protocol and instructions.

Thirty-five micrograms per well of protein were loaded in a 10% polyacrylamide gel for 80 min, transferred to nitrocellulose membranes (BioRad) and immunoblotted. Antibodies used were: rabbit anti-ATXN3 (1:500, Proteintech- Cat #: 13505-1) and mouse anti-Actin (1:2500, MP Biomedical- Cat #: 691001). Blots were visualized using peroxidase-conjugated secondary antibodies and ECL western blotting substrate (Thermo Scientific). N2 wild-type worms and atx-3(tm1689) were used as controls. Ladder used: Precision Plus Protein Kaleidoscope (BioRad). Densitometry was performed with Adobe Photoshop.

**Protein solubility**

Fifteen plates of worms for each strain and for each condition (ATXN-CAG89 worms treated with each compound) was collected with M9, centrifuged at 4000 rpm (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 (1M Tris-HCl pH 8, 0.5M EDTA, 1M NaCl, 10% SDS + protease inhibitors (10mg/mL leupeptin, 10 mg/mL pepstatin A, 10 mg/mL chymostatin LPC; 1/1000). Pellets were passed through a 271/2 G syringe 10 times, sonicated for 5 minutes and, centrifuged at 16,000g for 15min at 4°C. The soluble supernatants were collected in 1.5mL tubes and stored at -80°C. The remaining pellets were resuspended in extraction buffer, sonicated and centrifuged at 10,000g for 5min. The supernatants were discarded and the remaining pellets was resuspended into 100 μL of RIPA buffer, sonicated for around 90min 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 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-Immunoassays procedure and followed immunoblotting procedures. 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 with Image Lab software.

**Statistics**

Paralysis and lifespan curves were generated and compared using the Log-rank (Mantel-Cox) test. All experiments were repeated for at least three times. For neurodegeneration, the drug screening and the fluorescence tests (oxidative stress and ER stress response), Student’s t-tests were realized. For the dot blotting quantification, ANOVA tests were used. Prism 6 (GraphPad Software) was used for all statistical analyses.
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Conflict of Interest Statement
Disclosure statement: The authors declare no potential conflicts of interest.

Abbreviations
MJD: Machado-Joseph disease
ALS: Amyotrophic lateral sclerosis
SCA3: Spinocerebellar ataxia Type 3
UPR: Unfolded Protein Response
ERAD: ER-associated degradation
ATXN3: human ataxin-3
atx-3: C.elegans ataxin-3
References


Figure 1. Transgenic *C. elegans* ATXN3 model.

(A) Full length wild-type human ATXN3 containing 10 CAG trinucleotide repeat expansion or (B) full length human ATXN3 with a polyglutamine expansion (CAG89) were expressed under the control of a *unc-47* promoter. UIM (interaction domains with ubiquitin), CAG10/CAG89 (polyglutamine sequences).

(C) Total protein levels for N2, *atx-3(tm1689)* and transgenic worms expressing ATXN3-CAG10 or mutant ATXN3-CAG89. Antibody detection revealed signals for transgenic and N2 wild-type worms but no corresponding signal was detected in *atx-3(tm1689)* samples. Bands observed at ~42 kDa in size corresponding to full-length ATXN3 were observed in extracts from transgenic ATXN3-CAG10 worms, and a slightly larger band at ~50 kDa in size was observed for ATXN3-CAG89 samples. A band was observed in non-transgenic N2 worms likely representing the
endogenous ATX-3 protein. No band was observed in the atx-3(tm1689) deletion mutant. The atx-3(tm1689) western blots were done independently from the transgenic ATXN3 experiments. For the ATXN3-CAG89 lane, the ratio of ATXN3 to actin was made using the top band. **(D)** Total protein levels from cells derived from healthy (control; CTL) and MJD (SCA3) patients. Staining showed signals for both healthy and MJD patients, having an estimated molecular weight of 42 kDa and 60 kDa respectively. For all experiments, actin staining was used as a loading control and expression ratios ± s.e.m. of ATXN3 to actin was determined from 3 independent experiments. Representative western blots are shown.
Figure 2. Lifespan and neuronal phenotypes in ATXN3 transgenics.
(A) ATXN3-CAG89 worms had reduced lifespan compared to wild type ATXN3-CAG10 or N2 worms (*P < 0.05 and ****P < 0.0001 respectively, by log-rank (Mantel-Cox) test) (N=300-360). ATXN3-CAG10 animals showed reduced lifespan compared to N2 wild-type worms (*P < 0.05, by log-rank (Mantel-Cox) test) (N=300-360). The experiment was repeated 3 times.
(B) CAG89 transgenics had a significantly higher paralysis phenotype compared to wild-type ATXN3-CAG10 transgenics (****P < 0.0001, by log-rank (Mantel-Cox) test) (N=270-300). The experiment was repeated 3 times.
(C) Cholinergic neuronal transmission was measured by determining the onset of paralysis induced by the cholinesterase inhibitor aldicarb. Adult day one unc-47(e307) and ATXN3-CAG89 mutant strains showed a higher hypersensitive phenotype in presence of the aldicarb-induced paralysis comparatively to wild-type N2, ATXN3-CAG10 and unc-64(e246) worms (****P < 0.0001 for unc-47(e307) mutant worms when compared to the controls, and, ****P < 0.0001, *P < 0.05, and ****P < 0.0001 for ATXN3-CAG89 mutants respectively to the controls, by log-rank (Mantel-Cox) test) (N=270-300). Adult day five unc-47(e307) and ATXN3-CAG89 worms also showed a higher hypersensitive phenotype in presence of the aldicarb-induced paralysis comparatively to wild-type N2, ATXN3-CAG10 and unc-64(e246) worms (****P < 0.0001, **P < 0.01, ****P < 0.0001 for unc-47(e307) mutants respectively compared to the controls, and, ****P < 0.0001 for ATXN3-CAG89 mutants when compared to wild-type N2 and unc-64(e246) worms, by log-rank (Mantel-Cox) test) (N=270-300). A hypersensitive phenotype was observed at adult day nine unc-47(e307) and ATXN3-CAG89 mutant strains when compared to wild-type N2, ATXN3-CAG10 and unc-64(e246) worms (****P < 0.0001, **P < 0.01, ****P < 0.0001 for unc-47(e307) mutants when compared to the controls respectively, and, **P < 0.01 and ****P < 0.0001 for ATXN3-CAG89 mutants when compared to the wild-type N2 and unc-64(e246) worms, by log-rank (Mantel-Cox) test) (N=270-300). The experiment was repeated 3 times.
(D) Synchronised adult day one, five and nine worms placed on NGM plates and photographed after 10min of free movement. ATXN3-CAG89 mutant worms showed defects in motility when compared to wild type N2 and ATXN3-CAG10 transgenic worms.
(E-F) show representative photos of living, adult expressing wild-type unc-47p::mCherry, unc-47p::mCherry;ATXN3-CAG10 and unc-47p::mCherry; ATXN3-CAG89 transgenic worms at
day five and day nine of adulthood. Image of the GABAergic motor neurons from an entire *unc-47p::mCherry* and *unc-47p::mCherry; ATXN3-CAG10* worms were taken. Image of an entire *unc-47p::mCherry; ATXN3-CAG89* transgenic worm was taken and then zoomed on the panel on the right representing a magnification of the area indicated. Increased incidences of gaps or breakages were observed in mutant *unc-47p::mCherry; ATXN3-CAG89* transgenics compared to wild-type *unc-47p::mCherry and unc-47p::mCherry; ATXN3-CAG10* controls.

Quantification of neurodegeneration in transgenic worms at day five and day nine of adulthood. 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). These experiments were replicated for 3 times.
Figure 3. Methylene blue, salubrinal or guanabenz suppress paralysis during aging and, extend lifespan in ATXN3-CAG89 worms without affecting the expression level of this transgene.

(A-C) The motor defect phenotype observed in ATXN3-CAG89 worms was significantly decreased in presence of 60 \( \mu \)M methylene blue (MB) (****\( P < 0.0001 \)), 50 \( \mu \)M salubrinal (Sal) (****\( P < 0.0001 \)) or 50 \( \mu \)M guanabenz (Gua) (****\( P < 0.0001 \)) (by log-rank (Mantel-Cox) test, N=270-300). This experiment was replicated for 3 times.

(D) ATXN3-CAG89 worms showed an increase lifespan in presence of 60 \( \mu \)M methylene blue (MB) (****\( P < 0.0001 \)), 50 \( \mu \)M salubrinal (Sal) (*\( P < 0.05 \)) or 50 \( \mu \)M guanabenz (Gua) (****\( P < 0.0001 \)) (by log-rank (Mantel-Cox) test, N=300-360). This experiment was replicated for 3 times.

(E) Total protein levels for transgenic worms expressing mutant ATXN3-CAG89 with and without exposure to salubrinal, guanabenz or methylene blue. Antibody detection revealed signals for all four conditions. No differences were observed between the untreated and treated transgenic worms expression. Actin staining was used as a loading control and expression ratios ± s.e.m. of ATXN3 to actin was determined from 3 independent experiments. Representative western blots are shown.
Figure 4. Methylene blue, salubrinal or guanabenz suppress acute paralysis in ATXN3-CAG89 transgenics.

The swimming activity of ATXN3-CAG89 worms was scored for a period of ten hours and treatment with (A) 60 μM methylene blue (MB) (**) $P < 0.01$), (B) 50 μM salubrinal (Sal) (***) $P < 0.001$) or (C) 50 μM guanabenz (Gua) (****$ P < 0.0001$) (by Student’s $t$-test) significantly rescued the impaired movement phenotype of ATXN3-CAG89 worms. This experiment was done 3 times.
Figure 5. Rescue of motor neuron degeneration by small molecules in ATXN3-CAG89 transgenics.

Shown are representative photos of living, adult unc-47p::mCherry; ATXN3-CAG89 transgenics at day five of adulthood with or without compounds. Images are black and white, photo-reversed.
to aid visualization of neurons.

(A) Image of degenerating GABAergic motor neurons from an entire ATXN3-CAG89 transgenic. The panel on the right is a magnification of the area indicated by the rectangle. Arrows indicate gaps or breaks along neuronal processes. Rescue of neurodegeneration was observed in ATXN3-CAG89 worms in the presence of (B) 60 μM methylene blue (MB), (C) 50 μM salubrinal (Sal) or (D) 50 μM guanabenz (Gua).

(E) Quantification of neurodegeneration in transgenic ATXN3-CAG89 worms at day five of adulthood. Significant rescue of the neurodegeneration morphology was observed in ATXN3-CAG89 transgenics when treated with 60 μM methylene blue (*P < 0.05), 50 μM salubrinal (**P < 0.001) or 50μM guanabenz (****P < 0.0001) (by Student’s t-test, N=100 for each condition). These experiments were repeated 3 times.
Figure 6. Reduction of oxidative stress in ATXN3-CAG89 transgenics by methylene blue, guanabenz or salubrinal.

Shown are representative photos of living, adult (A) wild-type N2 worms, as well as (B) wild-type ATXN3-CAG10 and (C) mutant ATXN3-CAG89 transgenics at day two of adulthood in the...
presence of the oxidative stress marker, 2′,7′-dichlorofluorescein diacetate (DCF-DA). Increased fluorescence is observed in the (C) ATXN3-CAG89 transgenic worms. (D-F) ATXN3-CAG89 transgenics stained with DCF-DA showed decreased fluorescence after treatment with (D) 60 µM methylene blue (MB), (E) 50 µM salubrinal (Sal) or (F) 50 µM guanabenz (Gua).

(G) Quantification of fluorescence in N2 controls, ATXN3-CAG10, or ATXN3-CAG89 transgenics stained with DCF-DA. ATXN3-CAG89 transgenics showed increased fluorescence compared to N2 or ATXN3-CAG10 worms (####P < 0.0001). ATXN3-CAG89 transgenics had less fluorescence when treated with 60 µM methylene blue, 50 µM salubrinal or 50 µM guanabenz in presence of DCF-DA compared to untreated CAG89 controls (****P < 0.0001) (Student’s t-tests, N=17-25 for each condition). This experiment was repeated 3 times.
Figure 7. Methylene blue, guanabenz and salubrinal rescue the ER stress response in ATXN3-CAG89 transgenic worms.

(A) Shown are representative photos of living, adult hsp-4::GFP, hsp-4::GFP; ATXN3-CAG10 and hsp-4::GFP; ATXN3-CAG89 transgenics at day one of adulthood. hsp-4::GFP; ATXN3-CAG89 transgenics show increased GFP expression compared to hsp-4::GFP, or hsp-4::GFP; ATXN3-CAG10 controls (top panels). Treatment with 60 µM methylene blue (MB), 50 µM salubrinal (Sal) or 50 µM guanabenz (Gua) reduces the fluorescence of hsp-4::GFP; ATXN3-CAG89 to control levels. The hsp-4::GFP reporter shows increased fluorescence in the intestine (I) and spermatecha (S) of adult animals.

(B-D) Quantification of fluorescence of transgenics with or without treatment with compounds. An increased fluorescent signal was observed in hsp-4::GFP; ATXN3-CAG89 worms compared to hsp-4::GFP transgenic controls (##P < 0.01). Fluorescence was decreased in hsp-4::GFP; ATXN3-CAG10 transgenic worms when treated with 60 µM methylene blue (*P < 0.05), 50 µM salubrinal (****P < 0.0001) or 50 µM guanabenz (**P < 0.01) (Student’s t-tests, N=17-25 for each condition). However, the decreased fluorescence observed is even more significant in hsp-4::GFP; ATXN3-CAG89 mutants when treated with 60 µM methylene blue, 50 µM salubrinal or 50 µM guanabenz (****P < 0.0001) (Student’s t-tests, N=17-25 for each condition). These experiments were replicated for 3 times.

(E) Shown are representative photos of living, adult day two ATXN3-CAG89 transgenics fed with E. coli containing an empty vector (EV) or expressing dsRNA against ero-1. ATXN3-CAG89 mutant worms showed a high level of fluorescence when stained with 2′,7′-dichlorofluorescein diacetate (DCF-DA) and a significant decrease of this fluorescence when grown in the presence of ero-1 RNAi. Quantification of fluorescence of ATXN3-CAG89 mutant worms on EV or ero-1 RNAi. A significant decrease of fluorescence of ATXN3-CAG89 mutants was observed in the presence of ero-1 RNAi when compared to EV (****P < 0.0001) (Student’s t-tests, N=17-25 for each condition). This experiment was repeated three times.
Figure 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 partially 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 3 times.

(B) ATXN3-CAG89 and ATXN3-CAG89; *atf-6*(ok551) mutant worms 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 who is dependent to the *atf-6* branch of UPRER 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 3 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 partially (**P < 0.001) failed to suppress the paralysis in *ire-1* RNAi worms in opposition to Sal who suppressed paralysis in *ire-1* RNAi worms (**P < 0.01 (by log-rank (Mantel-Cox) test, N=270-300). This experiment was repeated 3 times.
Figure 9. Small molecule suppression of ATXN3-CAG89 aggregation

(A) Fixed whole ATXN3-CAG10 and ATXN3-CAG89 transgenic worms stained with a human ATXN3 antibody (blue) and TOPRO dye (yellow) for the nucleic acids. Arrows indicate ATXN3 protein in the motor neurons of ATXN3 transgenics. Second panel is a color-inverted image to aid visualization. The insets are magnifications of the aggregates indicated in the transgenic worms with a dashed line box.

(B) Total protein levels for N2 and transgenic worms expressing ATXN3-CAG10 or mutant ATXN3-CAG89. Antibody detection revealed high accumulation of the ATXN3-CAG89 in the pelleted, insoluble fraction, when compared to ATXN3-CAG10 and N2 wild-type worms. Decreased of aggregation in ATXN3-CAG89 worms when treated with 60 μM MB, 50 μM Sal and 50 μM Gua. Gua was especially potent in reducing protein aggregation.

(C) Quantification of the intensity of the aggregation in transgenic ATXN3-CAG89 mutants and wild-type N2 and ATXN3-CAG10 worms. Significant increase of aggregation in ATXN3-CAG89 when compared to the wild-type N2 and ATXN3-CAG10 worms. Once treated with 60 μM methylene blue (*P < 0.05), 50 μM salubrinal (*P < 0.05) or 50μM guanabenz (***P < 0.001 (by ANOVA tests)) ATXN3-CAG89 showed a significant decrease aggregation levels.
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 three 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.