HHS Public Access

Author manuscript

J Vis Exp. Author manuscript; available in PMC 2021 July 08.

Measuring RAN peptide toxicity in *C. elegans*

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Abstract

C. elegans is commonly used to model age-related neurodegenerative diseases caused by repeat expansion mutations, such as Amyotrophic Lateral Sclerosis (ALS) and Huntington's disease. Recently, repeat expansion-containing RNA was shown to be the substrate for a novel type of protein translation called repeat-associated non-AUG-dependent (RAN) translation. Unlike canonical translation, RAN translation does not require a start codon and only occurs when repeats exceed a threshold length. Because there is no start codon to determine the reading frame, RAN translation occurs in all reading frames from both sense and antisense RNA templates that contain a repeat expansion sequence. Therefore, RAN translation expands the number of possible disease-associated toxic peptides from one to six. Thus far, RAN translation has been documented in eight different repeat expansion-based neurodegenerative and neuromuscular diseases. In each case, deciphering which RAN products are toxic, as well as their mechanisms of toxicity, is a critical step towards understanding how these peptides contribute to disease pathophysiology. In this paper, we present strategies to measure the toxicity of RAN peptides in the model system *C. elegans*. First, we describe procedures for measuring RAN peptide toxicity on the growth and motility of developing *C. elegans*. Second, we detail an assay for measuring postdevelopmental, age-dependent effects of RAN peptides on motility. Finally, we describe a neurotoxicity assay for evaluating the effects of RAN peptides on neuron morphology. These assays provide a broad assessment of RAN peptide toxicity and may be useful for performing large-scale genetic or small molecule screens to identify disease mechanisms or therapies.

SUMMARY:

Repeat-associated non-ATG-dependent translational products are emerging pathogenic features of several repeat expansion-based diseases. The goal of the protocol described is to evaluate toxicity caused by these peptides using behavioral and cellular assays in the model system *C. elegans*.

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DISCLOSURES:
The authors have nothing to disclose.

Keywords

neurodegeneration; ALS; Huntington's disease; C9orf72; proteotoxicity; repeat expansion disorders

INTRODUCTION:

The inappropriate expansion of DNA repeat sequences is the genetic basis for several neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and Huntington's disease (HD)[1]. While there are established cellular and animal models for these diseases, mechanisms underlying these conditions are not well defined. For example, HD is caused by the expansions of a CAG repeat sequence in the coding sequence for the Huntingtin protein Htt[2]. Because CAG encodes the amino acid glutamine, the CAG repeat expansion results in the insertion of a polyGlutamine, or polyQ, sequence within Htt. Expanded polyQ proteins form length- and age-dependent protein aggregates that are associated with toxicity[3,4]. Surprisingly, two recent studies suggest that the length of the polyQ sequence is not the main driver of HD disease onset, suggesting that polyQ-independent factors may also contribute to the disease[5,6].

One possible polyQ-independent mechanism involves a newly discovered type of protein translation termed Repeat Associated Non-AUG-dependent (RAN) translation[7]. As its name implies, RAN translation only occurs when an expanded repeat sequence is present and does not require a canonical start codon. Therefore, RAN translation occurs in all three reading frames of the repeat to produce three distinct polypeptides. In addition, because many genes also produce an antisense transcript that contains the reverse complement of the expanded repeat sequence, RAN translation also occurs in all three reading frames of the antisense transcript. Together, RAN translation expands the number of proteins produced from an expanded repeat-containing DNA sequence from one peptide to six peptides. To date, RAN translation has been observed in at least eight different repeat expansion disorders[8]. RAN peptides are observed in postmortem patient samples and only in cases where the patient carries an expanded repeat[9,10]. While these peptides are clearly present in patient cells, their contribution to disease pathophysiology is unclear.

To better define the potential toxicity associated with RAN peptides, several groups have expressed each peptide in various model systems, such as yeast, flies, mice, and tissue culture cells[11–16]. Rather than utilizing the repeat sequence for expression, these models employ a codon-variation approach in which the repeat sequence is eliminated but the amino acid sequence is preserved. Translation initiation occurs through a canonical ATG and the peptide is typically fused to a fluorescent protein at either the N- or C-terminus, neither of which appears to interfere with RAN peptide toxicity. Therefore, each construct overexpresses a single RAN peptide. Modeling the different RAN products in a multicellular organism with simple assays to measure RAN peptide toxicity is vitally important to understand how the different RAN products from each disease-causing repeat expansion contribute to cellular dysfunction and neurodegeneration.

Like other model systems, *C. elegans* provides a flexible and efficient experimental platform that enables studies of new disease mechanisms, such as RAN peptide toxicity. Worms offer several unique experimental attributes that are not currently available in other models of RAN peptide toxicity. First, *C. elegans* are optically transparent from birth until death. This allows for simple visualization of RAN peptide expression and localization, as well as in vivo analysis of neurodegeneration in live animals. Second, transgenic methods for generating RAN peptide expression models are inexpensive and fast. Given the short three-day life cycle of *C. elegans*, stable transgenic lines expressing any given RAN peptide in a cell-type specific manner can be produced in under a week. Third, simple phenotypic outputs can be combined with genetic screening methods, such as chemical mutagenesis or RNAi screening, to rapidly identify genes essential for RAN peptide toxicity. Finally, the short lifespan of *C. elegans* (~20 days) allows investigators to determine how aging, which is the greatest risk factor for most repeat expansion diseases, influences RAN peptide toxicity. Together, this combination of experimental attributes is unmatched in any other model system and offers a powerful platform for the study of RAN peptide toxicity.

Here we describe several assays that leverage the experimental advantages of *C. elegans* to measure the toxicity of RAN peptides and to identify genetic modifiers of this toxicity. The codon-varied ATG-initiated RAN peptides are tagged with GFP and expressed individually in either muscle cells under the *myo-3* promoter or in GABAergic motor neurons under the *unc-47* promoter. For expression in muscle cells, it is important that toxic RAN peptides are tagged with green fluorescent protein (GFP), or other fluorescent protein (FP) tag that can be targeted with an RNAi feeding vector. This is because toxic RAN peptide expression usually blocks growth, rendering such strains nonviable. The use of *gfp(RNAi)* conditionally inactivates RAN peptide expression and allows strain maintenance, genetic crosses, etc. For assays, these animals are removed from *gfp(RNAi)*, which allows expression of the RAN peptide and the resulting phenotypes. In addition to the molecular strategy for designing codon-varied RAN peptide expression constructs, we describe assays for measuring developmental toxicity (larval motility and growth assay), post-developmental age-associated toxicity (paralysis assay), and neuron morphological defects (commissure assay).

PROTOCOL:

1. Generating codon-varied RAN peptide expression constructs

1.1. Design the individual RAN peptide coding sequence utilizing synonymous codons to eliminate the fundamental repetitive DNA/RNA structure but preserve the overlying amino acid sequence.

1.2. Order the custom codon sequences commercially at the repeat lengths needed for the studies (typically 5–100 repeats). Include a HindIII restriction site at the 5’ end and a BamHI restriction site at the 3’ end to facilitate cloning into *C. elegans* expression vectors, such as pPD95.79.

NOTE: If synthesis of larger constructs proves difficult, smaller building block sequences can be synthesized and then built into larger repeats using a directional ligation strategy[17].

1.3. Subclone the peptide sequence into a *C. elegans* expression vector using standard T4 ligation methods.

1.4. Subclone a cell type-specific promoter sequence generated by PCR in front of the RAN peptide sequence to drive tissue-specific RAN peptide-GFP expression.

1.5. Microinject the RAN peptide construct into the gonad of wild type *C. elegans* to generate transgenic strains containing extrachromosomal arrays using standard methods[18]. For a co-injection marker, the muscle-specific RFP reporter (*myo-3p::RFP* (*pCFJ104*)) was utilized, although other markers can be utilized as well.

1.6. Integrate a stable transgene into the genome using standard *C. elegans* integration screening methods[19].

1.7. If the RAN peptide is toxic and transgenic animals do not survive, feed animals *gfp(RNAi)* expressing bacteria pre- and post-injection to silence the expression of the toxic protein.

NOTE: Removal of animals from *gfp(RNAi)* enables RAN peptide expression for phenotypic analyses using the assays described below.

2. Measuring the developmental toxicity of RAN peptides following RNAi-based gene knockdown: Video speed analysis protocol

2.1. Pour standard nematode growth medium (NGM) RNAi 24 well plates with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 25 μg/mL carbenicillin.

2.2. Streak out RNAi-feeding clones in HT115 bacteria to be tested onto Luria broth (LB) and carbenicillin (25 μg/mL) agar plates and grow bacteria at 37 °C for 24 h. Include *empty vector(RNAi)* as the positive control for toxicity and *gfp(RNAi)* as the negative control.

2.3. For each clone, pick a single colony into 1 mL of LB + carbenicillin liquid media and grow bacteria overnight for 18 h at 37 °C while shaking at 250 rotations per minute.

2.4. Spot four individual wells of the NGM RNAI 24 wells with 20 μL of the following overnight bacterial cultures: column 1 wells = *gfp(RNAi)*; column 2 wells = *(EV)RNAi*; columns 3–6 wells = RNAi against genes to be tested. Allow RNAi bacteria to induce dsRNA production overnight at room temperature (RT).

2.5. Isolate eggs from day one adult *C. elegans* expressing the integrated RAN peptide transgene using the standard hypochlorite method and seed ~30 *C. elegans* eggs into each well of the NGM RNAi 24 well plate.

2.6. Incubate the 24 wells at 20 °C for 7 days.

NOTE: This incubation time is for strains expressing toxic C9orf72 RAN dipeptides, such as PR50 or GR50. Other strains may require shorter incubation times to prevent exhaustion of the bacterial food source and subsequent starvation.

2.7. Acquire transmitted light videos using a stereo dissecting microscope fitted with a monochrome camera connected to a computer running video acquisition software.

2.7.1. Acquire two videos from two separate wells for each RNAi condition using consistent video acquisition settings between wells. For each video, acquire 10 s at 800 × 600 pixel resolution and 13.16 frames per second (time voxel dimension = 0.076 s) using 18.6x magnification (1.49 zoom setting in AF software). Set the image exposure time to 4 ms. Label each video immediately with the strain, RNAi condition, and well number.

2.8. Blind the experimenter to the genotype associated with each video file by randomizing the order of the videos and changing the name of the videos to numbers. Save this group of videos as a new file. Unblind the experimenter after the analysis is complete.

2.9. Measure the distance traveled and the length of each animal for 20 different animals from each video, examining a total of ~40 worms for each RNAi condition.

2.9.1. In the software, select the annotation tools button, then the draw text tool. Click on a point in the center of the worm being measured in the first frame of the video and mark it as a number. Advance the video to the end while visually tracking the movement path of the worm. In last frame, click on a point in the center of the worm being measured and mark the next corresponding number. Then, using the draw scalebar tool, draw a line from the first number to the second number to record the “distance traveled.”

2.9.2. “Distance Traveled” is the distance between the two centroid points. This distance is shown adjacent to the measurement line. Record this measurement in a spreadsheet. Repeat this measurement for 19 other worms in the video while preserving each individual measurement in the analysis window.

2.10. Make measurements from animals exhibiting the most robust movement to avoid the selection of animals exhibiting no movement and biasing the data towards paralysis. Once the measurements are complete, take a snapshot of the final video frame illustrating all of the measurement lines so that the data can be traced back to the animal from which they originated.

2.11. Analyze the data using a two column spreadsheet. Column one is the “Worm Identifier” (name or number), column two is the “Speed” (distance traveled/time).

2.11.1 The time of each video can be found by right clicking on the video under the experiment and going to the properties of the video.

2.12. Normalize the speed measurement by finding the length of each animal by selecting Quantify, then Statistics on the software. Clicking on the annotation tool icon on the video display should allow for the use of the “Draw Polyline” tool. This tool can then be used to freely trace the *C. elegans* from the video from the tip of the head to the tip of the tail. The length of the line will be recorded under statistics.

2.12.1 Take a snapshot of the final video frame illustrating all of the polyline's used for sizing the *C. elegans* so that the data can be traced back to the animal from which they originated.

2.13 Analyze the data through adding two additional columns to the spreadsheet. Column three is the "Animal Length", and column four is the "Normalized Speed" (speed/animal length). Analyze the normalized speed data using a One-way ANOVA with post-hoc testing versus *empty vector(RNAi).*

3. Measuring developmental toxicity of RAN peptide: Growth assay

3.1. Pick ~30 gravid animals into a 50 μL spot of hypochlorite solution (10 mL of bleach, 2.5 mL of 10 N NaOH, 37.5 mL of dH_2O) on either a *gfp(RNAi), (EV)RNAi,* or *(gene-specific)* 6 cm *RNAi* plate.

3.2. After 24 h, move six transgenic progeny that have crawled out of the hypochlorite solution spot to a new 6 cm *RNAi* plate identical to the RNAi conditions on the plate on which they were subjected to the hypochlorite solution treatment. Put these progeny (F1) at 20 °C to grow and reproduce.

3.3. After 24–48 h, pick 10 transgenic L4 animals onto 6 cm RNAi matching the RNAi conditions that the animals have been growing on. Allow the animals to pulse lay eggs for 24 h in a 20 °C incubator.

3.4. After 24 h, remove the adult animals and discard them. Count the number of eggs and L1 larvae laid during the 24 h period using a mechanical handheld counter. This is the total brood size.

3.5. Over the next 72 h, count the number of animals that reach the L4 or older stage. As each animal is counted, remove it from the plate.

3.6. Quantify the "Percent Growth" as the percentage of animals that reach the L4 or older stage out of the total brood size.

3.7. Perform a 2 × 2 Fisher's exact test versus *empty vector(RNAi)* to determine if the percent growth is statistically significant. The categories for comparison are "Growth" (# of animals that reached the L4 or older stage in 72 h) and "No Growth" (total brood size minus the number of animals reaching L4 or older stage in 72 h).

4. Post-developmental RAN peptide paralysis assay

4.1. Maintain integrated *C. elegans* transgenic strains expressing RAN peptide-GFP in the muscle on *gfp*(RNAi) by placing 4–6 transgenic L4's on a 6 cm *gfp*(RNAi) plate and grow the worms at 20 °C.

4.2. If the experiment is utilizing RNAi to test for genetic effects on RAN peptide toxicity, move 10 transgenic gravid adults from an unstarved plate to each of two 6 cm *empty vector*(RNAi) (positive control for paralysis, negative control for genetic effects), *gfp(RNAi)* (negative control for paralysis, positive control for genetic effects), *gene-specific*(RNAi) (i.e., 10 gravid adults per 6 cm plate).

4.3. If mutants are being used to analyze genetic effects on RAN peptide toxicity, place 10 gravid WT or mutant animals expressing the same RAN transgene on each of two 6 cm *empty vector*(RNAi) or *gfp(RNAi)* plates. Grow the worms at 20 °C for 48 h.

4.4. Pick 10 sets of 10 transgenic L4's from the 6 cm RNAi plates and place each set on a 3 cm RNAi plate (i.e., n = 100 animals for each genotype). Ensure that the L4's selected for the assay all have superficially normal motility. Place the plates within a zipper storage bag to retain moisture.

4.5. Place the bagged plates with L4's in the 25 °C incubator.

4.6. Remove the animals 24 h later from the 25 °C incubator one strain at a time to minimize the amount of time they are out at RT.

4.7. Tap the plates on the dissecting microscope to check for movement. If the animals move more than a body length, count the animal as mobile and transfer it to a new 3 cm RNAi plate. Use a platinum pick to tap the remaining worms on the head or tail. If the animal moves more than a body length, count the animal as mobile and transfer it to a new 3 cm RNAi plate.

NOTE: Do not exceed 10 worms per plate when transferring. Be careful to avoid moving progeny to the new RNAi plate because the assay depends on following aged animals only.

4.8. Group all of the nonmoving worms together so that movement of more than a body length can easily be detected. Give the animal at least one minute to move more than one body length. If it still does not move, it is paralyzed, bagged (i.e., larvae hatched inside mother), or dead.

4.9. Censor animals that exhibit bagging, desiccate on the sides of the plate, exhibit extruded intestines, burrow, become lost, or die from the assay at the time of detection. Paralyzed worms are counted, left on the old RNAi plate, and discarded.

4.10. Score the animals for paralysis every day for 5–7 days as described in steps 4.6–4.9.

4.11. Analyze paralysis data with a log-rank test identical to the one used to analyze lifespan. In this statistical analysis, moving worms are scored as "Alive", paralyzed worms are scored as "Dead", and dead, bagged, gut extruded, desiccated, burrowed, or otherwise lost worms are scored as "Censored".

NOTE: In this analysis, the "Percent Alive" number indicates the "Percent Moving" animals. The inverse represents the "Percent Paralyzed". Use the online analysis tool OASIS (https://sbi.postech.ac.kr/oasis2/) to perform the log-rank statistical analyses.

5. Measuring neuron pathology: Commissure assay

5.1. Generate transgenic *C. elegans* expressing the RAN peptide of interest in the GABAergic neurons using the *unc-47* promoter. Also express *unc-47p::GFP* or *unc-47p::RFP* to reveal the cellular morphology of the GABA neurons.

5.2. Pick 50 transgenic L4 animals and place them on a 6 cm OP50 plate at 25 °C for 24 h.

5.3. Transfer the 50 transgenic animals onto a new 6 cm OP50 plate at 25 °C for 24 h.

5.4. Make agarose pads for imaging the animals under widefield microscopy.

5.4.1. Place two pieces of tape on top of two microscope slides. Place a cleaned slide without tape in the middle of the two taped slides.

5.4.2. Dispense ~100 μL (1 drop from a disposable plastic Pasteur pipette) of 3% molten agarose onto the middle slide with a disposable sterile transfer pipette.

5.4.3. Immediately place a second clean slide across the drop of molten agarose so that it rests on the taped slides and creates a thin and even layer of agarose between the slides.

5.4.4. After the agarose has cooled and solidified, carefully remove the two slides with the layer of agarose between them, without separating them, and place them in a plastic bag with a damp piece of paper under them. Make one slide per 10 worms to be examined along with three extra slides.

5.5. Remove the transgenic *C. elegans* from the 25 °C incubator and pick 10 transgenic *C. elegans* into a 100 μL drop of 10 mM levamisole in a glass depression slide. Incubate for 10 min or until animals are paralyzed.

5.6. Remove a slide pair from the plastic bag and carefully separate them. Label the slide with the agarose with the genotype and add 2 μL of 10 mM levamisole to the middle of the agarose. Move the 10 animals into the levamisole on the agarose pad. Cover the animals with a #1 thickness coverslip.

5.7. Image animals in which the vulva is oriented on the right side of the animal. In this orientation, the commissures of the motor neurons are nearest to the coverslip and are clearly visible on an inverted widefield fluorescence microscope. If the vulva is on the left side of the head, the commissures of the motor neurons will be underneath the animals and not as clearly visible, making accurate quantification difficult. Omit quantification of these animals.

5.7.1. Visualize the neuron commissures expressing GFP with an inverted widefield fluorescence microscope with a 63× 1.4x NA oil immersion lens and a GFP filter set (Ex480/40 nm; Em527/30 nm). If the neuron commissures express RFP instead of GFP, utilize a red fluorescent protein (RFP) filter set (Ex560/40 nm; Em 645/75 nm).

5.8. Image the worms within 45 min of placing the coverslip on the worms to minimize toxicity from immobilization.

5.9. For each worm, count the number of visible commissures. There are 16 visible commissures in wild type animals. Also count the number of commissures that have large beads (i.e., blebs) in the commissures as well as the number of commissures that are broken. To confirm the commissure is broken, follow the commissure from the dorsal cord to the ventral cord by adjusting the focal plane.

NOTE: Commissures in which the *unc-47p::GFP* fluorescence exhibits a gap are considered broken. A broken commissure typically has a bleb on either side of the break, helping show that the commissures is broken. Count the amount of blebbing and breaks for 20 worms.

5.10. Calculate the fraction of commissures with blebs or breaks over the total number of observed commissures for each animal. The absolute number of commissures counted per animal (including wild type, blebbed, and broken events) can also be measured.

5.11. For each category, calculate the mean ± SD and statistically analyze with either a Student's t-test for comparison between two populations, or a one-way ANOVA for comparisons between 3 or more populations.

REPRESENTATIVE RESULTS:

We used the assays described here to evaluate the effect of different gene inhibitions on the toxicity of RAN dipeptides that are found in ALS patients with a G_4C_2 repeat expansion. Using the growth assay to measure developmental toxicity, we analyzed the effects of several genetic knockout mutants identified in a genome-wide RNAi screen suppressors of muscle-expressed PR50-GFP toxicity. While expression of PR50-GFP alone resulted in a completely penetrant growth arrest, loss of function mutations in several genes suppressed PR developmental toxicity from 12–94% (Figure 1A).

We also measured the effect of specific gene knockdowns on the developmental motility of PR50-expressing animals using the video speed analysis method. As expected, *gfp(RNAi)* resulted in a large increase in motility compared to *empty vector(RNAi)* due to inhibition of PR50-GFP expression. We also discovered that RNAi against the proteasome subunit *rpn-7* resulted in a significant increase in PR50 motility (Figure 1B).

ALS, like many neurodegenerative diseases, occurs in adults. Therefore, we analyzed adult phenotypes using the age-dependent paralysis assay. PR50-GFP exhibited up to 80% paralysis by 5 days of age. However, RNAi directed at the gene *cul-6* significantly delayed paralysis, suggesting that *cul-6* is required for PR50-GFP toxicity (Figure 1C). This effect was specific to *cul-6(RNAi)* because *cul-1(RNAi)* did not alter PR50-GFP toxicity.

Neurodegenerative proteins, such as toxic RAN peptides, are commonly modeled in the body wall of *C. elegans*[4,20–22]. However, it is also important to determine if RAN proteins cause neuropathology when expressed in *C. elegans* neurons, because neuron-specific toxicity is a common feature of many neurodegenerative diseases. We examined neuron-specific toxicity using the commissure assay. In day 2 adults, PR50-GFP expression in the motor neurons led to a significant increase in motor neuron blebbing. This neuropathology was significantly suppressed by a mutation in the insulin/IGF receptor gene homolog *daf-2* that delays the toxic properties of several neurodegenerative proteins[23] (Figure 2B).

DISCUSSION:

Here we report methods that can be used to assay RAN peptide toxicity modeled in the muscle or in the neurons of *C. elegans*. While neurodegenerative proteins have an age onset phenotype in human patients, they can also exhibit developmentally toxicity when overexpressed in model systems. Overexpression has significant interpretive limitations, but it also provides a powerful starting point for genetic or pharmacological screens aimed at identifying genes or drugs that can reverse toxic phenotypes. This is especially important given that most precise animal models of disease have either no phenotype or weak phenotypes not suitable for unbiased screening approaches[24–26]. Our *C. elegans* RAN peptide models and assays represent a powerful, complementary approach to other RAN peptide model systems, such as yeast and *Drosophila*, for understanding the cellular pathways important for the toxicity of these newly described proteins.

Conditional toxicity of muscle-expressed RAN peptides

Measuring RAN peptide toxicity requires a chase period for elimination of the effects of *gfp(RNAi)*. However, the time between removal from *gfp(RNAi)* and the emergence of phenotypes can be inconsistent if care is not taken to precisely time the initiation of experiments, selection of staged animals, temperature shifts, etc. As we have become more experienced with these assays, the timing and penetrance of RAN peptide phenotypes has become relatively consistent. One of the most critical steps in these assays is the proper shift of animals to 25 °C. Without this shift, RAN peptides exhibit significantly weaker toxicity. However, animals cannot be continually grown at 25 °C because they do not grow and reproduce, presumably due to higher baseline expression of the RAN peptide. This is not unlike the situation in yeast or *Drosophila* where strains expressing toxic RAN peptides are kept under permissive conditions (i.e., low temperature) but then acutely shifted to restrictive conditions (i.e., higher temperature) prior to the assay[13,15] to enhance peptide expression. In such conditional expression systems, genetic conditions that enhance or suppress toxicity could result from changes in peptide expression levels. To determine if genetic modifiers of RAN peptide toxicity act via alterations in transgene expression levels, we take two approaches. First, many of our transgenic strains express a second RFP reporter under the control of the same promoter used to drive expression of the RAN peptide. Changes in promoter activity that cause reduced or increased RAN peptide expression cause similar reductions or increases in RFP levels. We consider mutants or RNAi conditions that significantly alter RFP levels to act nonspecifically. Second, we perform quantitative PCR and Western blotting to determine if the levels of RAN peptide mRNA or protein are altered between conditions. However, Western-based detection of RAN peptides can sometimes prove difficult or impossible, as we and others have observed with the C9orf72-derived RAN peptide PR50-GFP. In such cases, we quantify in vivo GFP levels to determine if protein expression is altered between the conditions being compared.

Developmental toxicity: Advantages and limitations

Forced overexpression of toxic RAN peptides in muscle often leads to developmental arrest in *C. elegans*. While this clearly does not mimic human disease pathology, it provides a powerful starting point for genetic suppressor screens, because RAN toxicity suppressors are

easily identified as animals that grow and reproduce in the absence of *gfp(RNAi)*. Some suppressors will facilitate growth due to reduction of transgene expression. To differentiate between these possibilities, we generally include a second RFP reporter driven by the same promoter in our RAN peptide transgenic strain. Suppressors that reduce or silence transgene expression will also exhibit reduced RFP expression levels. On the other hand, suppressors that exhibit normal or elevated RFP levels are unlikely to function via reduction of transgene expression. Much like the use of yeast growth or *Drosophila* eye morphology[13,15,27], these approaches, while not precisely modeling aspects of the disease in humans, provide a powerful screening tool for the initial identification of RAN peptide modifiers.

Post-developmental toxicity (paralysis assay): Advantages and limitations

The advantage of the paralysis assay is that a relative large number of animals (50–100) can be measured with tools present in any worm lab. Paralysis is also a severe phenotype that is easily detected. The main limitation of this assay is that is insensitive to movement defects that do not cause complete paralysis. Such defects may require more sensitive and quantitative approaches to measure, such as the thrashing assays or kinematic movement assays[28]. Paralysis assays should be performed blinded to genotype if possible and care should be taken to ensure that worms are not undergoing any other type of stress (e.g., contamination, starvation), that could significantly impact the assay results. *C. elegans* expressing toxic RAN peptides sometimes fail to exhibit a robust paralysis phenotype. This is likely due to persistent effects of *gfp(RNAi)* continuing to suppress RAN peptide expression. In these cases, if we fail to observe significant paralysis (>10%) in the *empty vector(RNAi)* control animals after day 2, we typically terminate the assay and initiate another replicate. One modification of this assay could involve the use of alternative inducible RAN peptide expression systems. The only other commonly used inducible expression system for *C. elegans* is the heat shock promoter. However, the required heat shock can cause stress responses that might affect the toxicity of the proteins. The future application of other conditional expression systems, such as the auxin-inducible degron (AID) system[29], could significantly enhance our ability to study RAN peptide toxicity.

Neurodegeneration/commissure assay: Advantages and limitations

Motor neuron commissures in *C. elegans* represent the axon of one motor neuron passing between the ventral and dorsal nervous cords. Blebbing and breakage can be easily detected in the isolated commissures, allowing neurodegeneration to be quickly quantified in live animals (Figure 2). While examining the animals, it is important they do not incubate in levamisole for an extended period of time as this can cause premature animal death, which leads to neuronal blebbing and breakage in the absence of any toxic RAN peptide. In some cases, commissures are completely degenerated and no longer visible. Therefore, they cannot be scored for neuropathological features because our assay measures the percentages of commissures broken or blebbing out of the total number of observed commissures. In these cases, the commissure assay might underestimate the effect of the RAN peptide.

In conclusion, the assays described in this paper are useful for measuring toxicity caused by RAN peptides in *C. elegans*. Using *gfp(RNAi)* to regulate expression of the proteins allows for post-developmental phenotypes to be observed. Our approaches can be easily adapted to

perform large-scale genetic screens for suppressors or enhancers of toxicity. Secondary assays, such as the paralysis assay and commissure assay can confirm that toxicity is suppressed post-developmentally and test if the mechanism of suppression is conserved in neurons.

ACKNOWLEDGMENTS:

NIH R21NS107797

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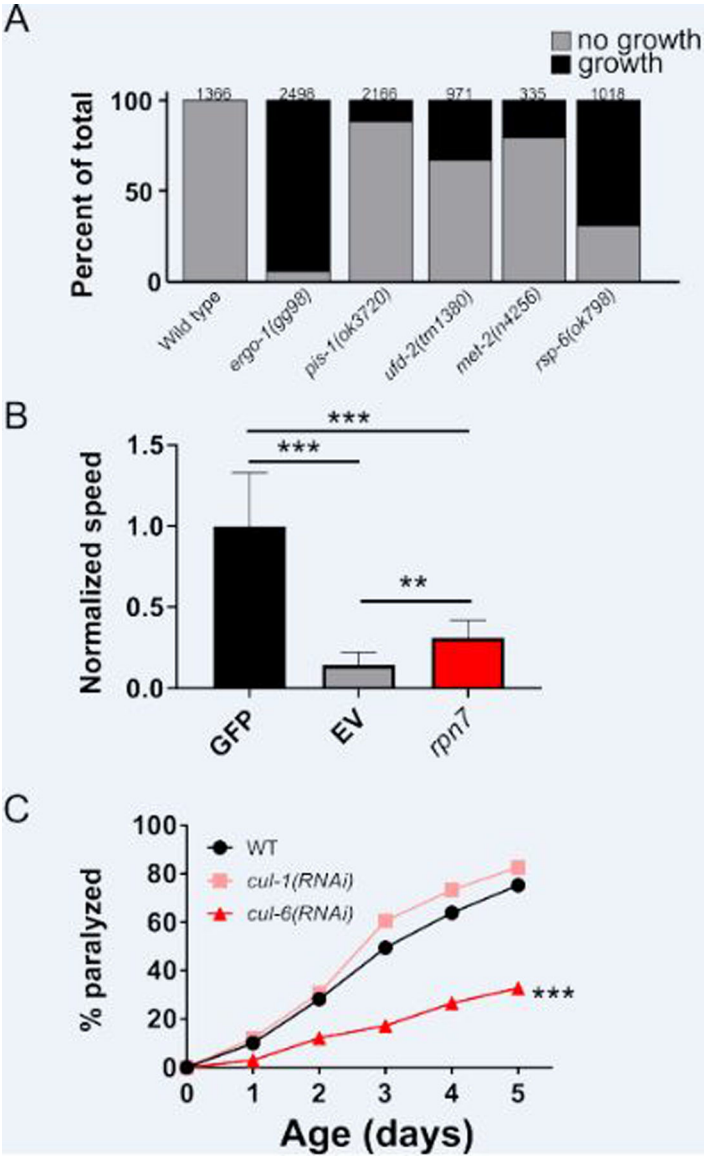

Figure 1: Assays to evaluate the toxicity of muscle-expressed RAN peptides.

(**A**) RAN peptide growth assay. The indicated mutants were crossed into the *drIs34* (*myo-3p::PR50-GFP*) genetic background under *gfp(RNAi)* conditions. Numbers above each genotype indicate the number of progeny scored for growth. All genotypes have $p < 0.05$ using the Fisher's exact test as compared to wild type. (**B**) Video speed analysis to measure RAN peptide toxicity during development. *drIs34 (myo-3p::PR50-GFP)* animals were grown under *gfp(RNAi)*, *empty vector(RNAi)*, or *rpn-7(RNAi)* conditions and then scored for motility as described. Speed was normalized to *gfp(RNAi)* treated animals. Data shown are mean ± SD, n = 40 for each genotype. **-$p < 0.01$, ***- $p < 0.001$, one-way ANOVA with Tukey post-test. (**C**) Paralysis assay to measure age onset toxicity. *drIs34 (myo-3p::PR50-GFP)* animals were grown on *empty vector(RNAi)* ('WT'), *cul-1(RNAi)*, or *cul-6(RNAi)* and paralysis was scored as described every 24 h. ***-$p < 0.001$, log-rank test with Bonferroni post-test correction vs. WT. n = 100 animals per genotype.

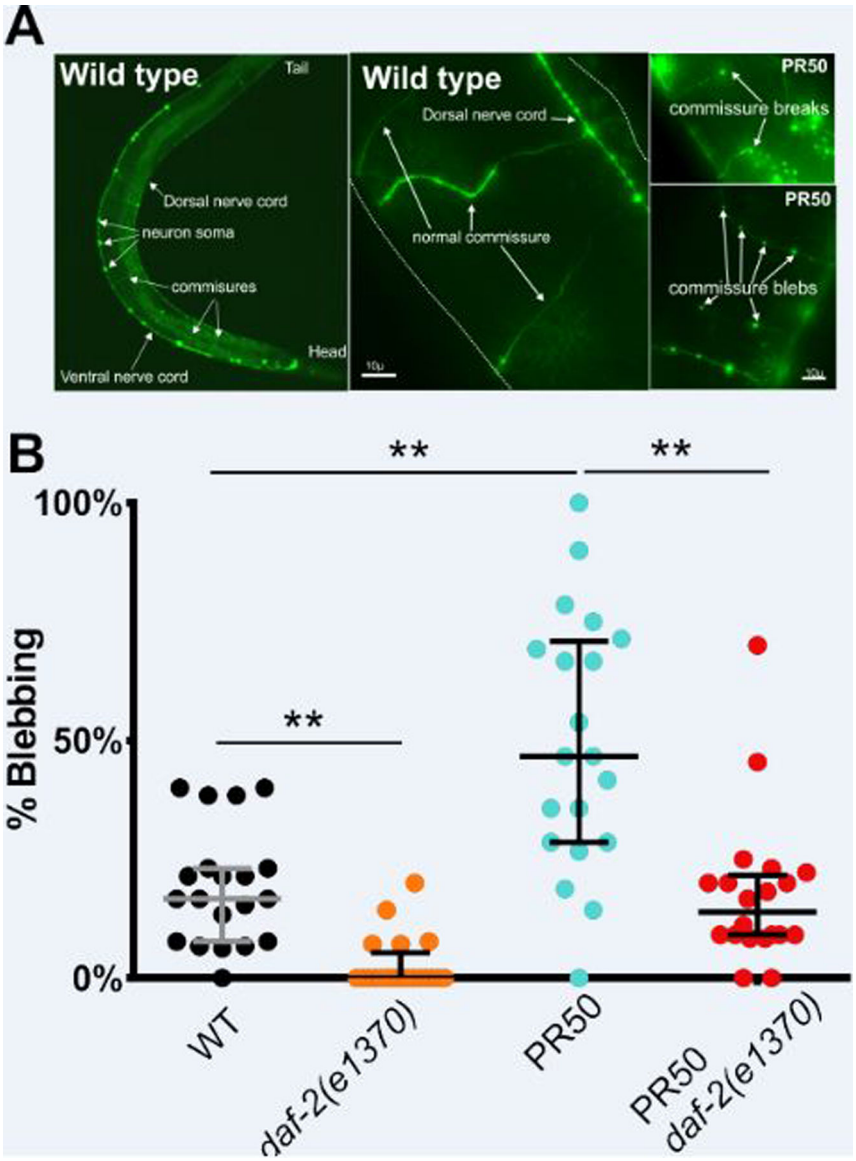

Figure 2: Assay for measuring neuropathology of motor neuron expressed RAN peptides.

(**A**) Images of adult *C. elegans* expressing the *unc-47p::GFP* motor neuron reporter in wild type or *unc-47p::PR50-GFP* expressing animals. The images for wild type illustrate normal commissure morphology. Images represent flattened Z-series stacks obtained by widefield fluorescence microscopy. PR50 animals demonstrate examples of commissure breakage and blebbing. (**B**) Effect of *daf-2(e1370)* on PR50 commissure blebbing. Points represent the percent blebbing commissures from a single animal, with the mean and SD shown. n = 20 animals per genotype **-$p < 0.01$, one-way ANOVA with Dunn's post-hoc test.

Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
PETRI DISH,60X15MM,500/CS	CORNING LIFE SCIENCES PLASTIC	FB0875713A	Nematode growth plates and RNAi
35mm x 10mm Petri Dish, Sterile	CELLTREAT Scientific Products	50–202-036	Nematode growth plates and RNAi
AGAR GRANULATED 2KILOGRAM	BD DIAGNOSTIC SYSTEMS	DF0145070	Nematode growth plates and RNAi
IPTG BIOTECH 10G	THERMO SCI FAIRLAWN CHEMICALS	BP162010	Nematode growth plates and RNAi
CARBENICILLIN 5G	THERMO SCI FAIRLAWN CHEMICALS	BP26485	Nematode growth plates and RNAi
TISSUE CULT PLATE 24WEL 50/CS	CORNING LIFE SCIENCES DL	87721	Nematode growth plates and RNAi
Gibco™ Bacto™ Peptone	Gibco™	DF0118–17-0	Nematode growth plates and RNAi
COVER GLASSES NO 1 22MM 1OZ/PK	THERMO SCI ERIE	12542B	Imaging for commissure assay
Fisherbrand™ Superfrost™ Plus Microscope Slides	THERMO SCI ERIE	12–550-15	Imaging for commissure assay
LEVAMISOLE HYDROCHLORIDE 10GR	THERMO SCI ACROS ORGANICS	AC187870100	Imaging for commissure assay
Leica Immersion type N (Oil)	W NUHSBAUM INC	NC9547002	Imaging for commissure assay
FEMOTIPS DISPSBL MICROINJ 20CS	EPPENDORF NORTH AMERICA BIOTOOLS	E5242952008	Microinjection to generate RAN peptide transgenic strains
MICROLOADER TIPS 2 X 96 PCS	EPPENDORF NORTH AMERICA BIOTOOLS	E5242956003	Microinjection to generate RAN peptide transgenic strains
FF COV GLASS NO1 40X22MM 1OZPK	THERMO SCI ERIE	125485C	Microinjection to generate RAN peptide transgenic strains
AGAROSE ULTRAPURE	LIFE TECHNOLOGIES	16500500	Microinjection to generate RAN peptide transgenic strains
HALOCARBON OIL 700	SIGMA-ALDRICH INC	H8898–50ML	Microinjection to generate RAN peptide transgenic strains
Leica Advanced Fluorescence imaging software	Leica Microsystems	LAS-AF	Image acquisition software for video speed analysis and commissure assay