Video Article

A *Caenorhabditis elegans* Model System for Amylopathy Study

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URL: http://www.jove.com/video/50435
DOI: doi:10.3791/50435

Keywords: Neuroscience, Issue 75, Medicine, Neurobiology, Cellular Biology, Molecular Biology, Genetics, Developmental Biology, Cell Physiological Phenomena, neurosciences, beta-amyloid, *C. elegans*, *Caenorhabditis elegans*, apoptosis, amylopathy, amyloid beta, Alzheimer's disease, AD, neurons, cell culture, transgenic worm, animal model

Date Published: 5/17/2013

Citation: Duan, Z., Sesti, F. A *Caenorhabditis elegans* Model System for Amylopathy Study. *J. Vis. Exp.* (75), e50435, doi:10.3791/50435 (2013).

Abstract

Amylopathy is a term that describes abnormal synthesis and accumulation of amyloid beta (Aβ) in tissues with time. Aβ is a hallmark of Alzheimer's disease (AD) and is found in Lewy body dementia, inclusion body myositis and cerebral amyloid angiopathy [1-4]. Amylopathies progressively develop with time. For this reason simple organisms with short lifespans may help to elucidate molecular aspects of these conditions. Here, we describe experimental protocols to study Aβ-mediated neurodegeneration using the worm *Caenorhabditis elegans*. Thus, we construct transgenic worms by injecting DNA encoding human $A\beta_{42}$ into the syncytial gonads of adult hermaphrodites. Transformant lines are stabilized by a mutagenesis-induced integration. Nematodes are age synchronized by collecting and seeding their eggs. The function of neurons expressing $A\beta_{42}$ is tested in opportune behavioral assays (chemotaxis assays). Primary neuronal cultures obtained from embryos are used to complement behavioral data and to test the neuroprotective effects of anti-apoptotic compounds.

Video Link

The video component of this article can be found at http://www.jove.com/video/50435/

Introduction

Amyloid beta (Aβ) is a peptide of 36-43 amino acids that is formed after sequential cleavage of the amyloid precursor protein (APP) by β and γ secretases [1]. The γ secretase processes the C-terminal end of the Aβ peptide and is responsible for its variable lengths [5]. The most common forms of Aβ are $A\beta_{40}$ and $A\beta_{42}$, the latter being more commonly associated to pathologic conditions such as AD [5]. At high concentrations Aβ form β-sheets that aggregate to form amyloid fibrils [6]. Fibrils deposits are the main component of *senile plaques* surrounding neurons. Both plaques and diffusible, non-plaque Aβ oligomers, are thought to constitute the underlying pathogenic forms of Aβ.

Laboratory study of neuronal amylopathies is complicated by the fact that these conditions progress with time. Therefore, it is important to develop genetically tractable animal models-complementary to mice-with short life span. These models can be used to elucidate specific aspects of amylopathies-typically cellular and molecular-and by virtue of their simplicity, help to capture the essence of the problem. The worm *Caenorhabditis elegans* falls is this category. It has a short life span, ~20 days and in addition basic cellular processes including regulation of gene expression, protein trafficking, neuronal connectivity, synaptogenesis, cell signaling, and death are similar to mammalian [7]. Unique features of the worm include powerful genetics and lack of a vessel system, which enables to study neuronal damage independently of vascular damage. On the other hand, the lack of a brain limits the use of *C. elegans* to studying many aspects of neurodegeneration. In addition, the reproduction and identification of anatomical distributions of lesions cannot be performed in this organism. Other limitations include the difficulty to assess both differences in gene expression profiles and impairment of complex behavior and memory function. Here we describe methods to generate *C. elegans* models of amylopathies.

Protocol

1. Construction of Transgenic Worms

1. Transformation.
    1. Prepare injection pads. Place a drop of hot, 2% agarose dissolved in water, onto a glass coverslip. Quickly place a second coverslip on the drop and lightly tap it. After the agarose is solidified, slide coverslips apart, and bake the coverslip-pad in a vacuum oven at 80 °C O/N.
    2. Pull pipettes. We use a Sutter P-97 puller to pull 1/0.5 mm O.D./I.D. borosilicate capillaries with filament. Pipettes are forged with closed tip which is broken open at a later stage.
    3. Prepare injection mix. Make the injection mixture containing the DNA of interest (20 ng/µl per construct) plus empty plasmid DNA to a final concentration of 150 ng/µl. Centrifuge the injection mixture to remove any contaminant. This step is crucial because contaminants

reduce transformation efficiency. Transfer the top 5 µl (debris and other contaminants collect in the bottom) to a fresh tube to be used in injection.
4. Load injection mix by capillarity. The bottom of the pipette is immersed in the injection mix. Typically 0.5 µl are sufficient to inject 100 worms.
5. Load injection pipette. Insert the pipette onto the holder of the micromanipulator and break it open by rubbing its tip against the edge of a cover slip mounted on a glass slide or alternatively against debris on the agarose pad. This is a crucial step as too large tips damage the worm and too small tips are easily clogged. The quality of the broken tip can be judged by the shape and most importantly by the flow rate. The flow rate can be assessed by the size of the bubbles flowing from the open tip immersed in a drop of halocarbon 700 oil in response to an injection pressure.
6. Transfer worms to injection pad. Place a drop of 700 halocarbon oil on an injection pad and transfer several (1 or 2 for beginners) worms to the oil. Use a worm pick to push the worms down onto the pad until they adhere to the agarose. Worms should be oriented in rows with ventral sides facing the same direction. Avoid touching worm's head. If after several attempts the worms fail to adhere to the pad, replace with a fresh pad or increase agarose thickness and/or concentration.
7. Insert the pipette into the worm. By moving the stage, position the worm under the pipette. Position the pipette in the central core of the gonad because its cytoplasm is shared by many germ cell nuclei. This increases the likelihood to deliver injected DNA to many progeny. The pipette should lie almost parallel to the worm (~15°-25° angle). Push vertically the tip of the pipette down the worm's body until the skin is depressed. Then gently tap on the manipulator to induce tip's insertion. For best results inject both gonads.
8. Inject the DNA solution. Apply pressure to the pipette until the gonad swells. Stop the flow and pull the worm off the pipette. Test the needle for flow and then move to the next worm and repeat injection steps.
9. Recover the worms: Add a drop (~10 µl) of recovery buffer on the worms and incubate until the worms begin swimming. Add an equal volume of M9, wait until worms resume swimming and repeat several times until the solution is mostly M9. Individually transfer worms to seeded plates.

2. Integration.
1. Place 40 healthy, well-fed, L4 worms into a fresh plate
2. Irradiate with γ-ray with 4,000 rads for 40 min. Transfer irradiated worms (P0) in fresh, OP50 seeded plates (4 worms/plate). Worms can alternatively be irradiated with a dose of 300 J/m^2 UVs.
3. Transfer 10-20 F1 transformants from each plate to individual plates, label the plates with the corresponding P0 origin.
4. Single out 2-4 F2 transformants for each F1 to separate plates. Check the F3 progeny for 100% transmission of the transformation marker. Typically, 1-3% of progeny from an irradiated worm will have an integration event.
5. Make stocks of three or more independent transformant lines.

2. Behavioral Assays

1. Age-synchronization.
1. Grow worms in standard 10 cm NGM plates + OP50 *E. coli* until a large population of gravid adults is reached (3-5 days).
2. Collect the worms in 50 ml Falcon tubes by suspending them in 1 ml M9 buffer.
3. Add 5 ml M9 buffer and centrifuge at 450 x g for 3 min. Discard supernatant. Repeat 3-4x.
4. At the end of the last centrifugation, remove the supernatant and add 10 volumes of basic hypochlorite solution (0.5 M NaOH, 1% hypochlorite freshly mixed) to the pelleted worms. Incubate at RT for ~10 min. The lysis reaction can be monitored by placing a drop of the lysis reaction on a coverslip and examining the worms under a microscope. When roughly 80% of worms are broken the reaction should be stopped.
5. Stop the lysis reaction by adding the same volume of sterile egg buffer.
6. Collect the eggs (and carcasses) by centrifugation at 450 x g for 5 min.
7. Wash the eggs with sterile egg buffer 2-3x.
8. Incubate the eggs O/N in M9 buffer and seed them on standard NGM plates.

2. Chemotaxis assay.
1. Prepare several pieces of agar roughly 0.5x0.5x0.5 cm. We deposit the agar in a 10-cm plate and we cut the agar chunks from there.
2. Soak the agar chunk in a solution containing the desired attractant (at saturating, near-saturating concentrations) for 2 hr. Typical attractants are lysine (0.5 M), biotin (0.2 M).
3. Deposit an agar chunk in a 10 cm test plate in which the location of a test spot and a control spot have been marked (**Figure 1A**). Allow equilibration and formation of a gradient O/N (**Figure 1B**). Prepare 5 plates for a single experiment.
4. Prior the experiment add 10 µl of 20 mM NaN_3 (anesthetic) to each spot.
5. Place 20 age-synchronized worms in the center of the plate. Place the plate in the incubator at 20 °C.
6. After 1 hr, count the animals on the test/control spots and calculate the chemotaxis index, (C.I.) as follow: $C.I. = \frac{N_{Test} - N_{Cnt.}}{N}$ where N, N_{test} and $N_{Cnt.}$, indicate the total number of animals, the number of animals in the test spot and the number of animals in the control spot.

3. Primary Embryonic Cell Culture

1. Lyse worms as described in age-synchronization.
2. Stop the lysis reaction by adding the same volume of sterile egg buffer and centrifuge at 450 x g for 5 min. Gently discard supernatant being careful to not lose pelleted eggs. Repeat 2-3x or until supernatant is clear.
3. Resuspend pelleted eggs (and carcasses) in 2 ml sterile egg buffer and add 2 ml of sterile 60% sucrose in egg buffer. Mix this solution until eggs are completely resuspended (as they tend to form clumps under centrifugation) by hand or by vortexing.

4. Centrifuge at 450 x g for 15 min.
5. Carefully transfer supernatant (containing the eggs) to a sterile tube. Discard pellet which contains carcasses and other by-products of lysis.
6. Remove residual sucrose by resuspending the eggs in egg buffer and centrifuging at 450 x g for 5 min. Gently collect and discard the supernatant. Repeat 3x.
7. Under a laminar hood resuspend pelleted eggs in sterile egg buffer containing 1 U/ml chitinase at RT to digest the eggshells. After 30 min start to monitor the reaction (under an inverted cell culture microscope). Each batch of chitinase has a slightly different activity. Typically digestion is completed in 1 hr.
8. When roughly 70-80% eggshells are digested by chitinase add CM-15 (L-15 cell culture medium containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin). Dissociate cells using a syringe with a 27 gauge. Filter the cell suspension with a 5.0 µm filter to remove intact embryos, clumps of cells and larvae.
9. Pellet the dissociated cell suspension by centrifugation at 450 x g for 15 min. Remove the supernatant and resuspend the pellet in CM-15 cell culture medium.
10. Plate dissociated cells on glass cover slips previously coated with peanut lectin (0.1 mg/ml) dissolved in water. **Note:** cells must adhere to the substrate in order to differentiate.
11. Cells can be maintained at RT (16-20 °C) in air for more than 2 weeks.

Representative Results

With our protocols we study the effects of human $A\beta_{42}$ oligomer on neuronal function [8]. A fragment encoding human $A\beta_{42}$ and the artificial signal peptide coding sequence of Fire vector pPD50.52 was amplified from construct PCL12 [9] using primers that introduced a Sma 1 restriction endonuclease site at the ends. The fragment was then inserted into a construct containing a 2,481-bp *flp-6* promoter sequence in the pPD95.75 Fire vector between the unique Sma 1 site [10]. Using the transformation techniques described in protocol 1 we constructed a transgenic worm expressing $A\beta_{42}$ in the ASE neurons (FDX(ses25) strain) [8]. To mark positive transformants we used the $P_{gcy\text{-}5}$::GFP reporter which specifically drives GFP expression in the ASE right (ASER) neuron [11]. Worms stick to the pad because the dry agarose absorbs their water. Therefore it is crucial that the animals are placed onto the injection pad and injected relatively quickly, because otherwise they will desiccate and die. The percentage of F1 progeny that carries a transmissible extrachromosomal array may vary. Typical values are in the 3-7% range. It is important that the injection mix (1.1.3) contains non-encoding DNA sharing sequence homology with the transgenic DNA (usually empty vector) because the DNAs undergo homologous recombination with each other. However, if overexpression of the transgene is a problem the injection mix should be supplemented with 50-100 ng/µl of genomic DNA digested with Sca 1. ASE neurons detect water soluble attractants such as biotin and therefore their function can be assessed in behavioral assays (chemotaxis assay, protocol 2 and **Figures 1A and 1B**) [12]. In a typical experiment, we tested seven day old worms expressing $P_{gcy\text{-}5}$::GFP alone (DA1262 strain) or with $A\beta_{42}$ (FDX(ses25)) for chemotaxis to biotin. In young worms (3-4 day old) the effects of $A\beta_{42}$ expression are modest but already detectable (~ 10% decrease in chemotaxis index, see ref. [8]). Representative results of this experiment are shown in **Figures 1C and 1D**. Most DA1262 worms were found in, or nearby, the attractant spot (**Figure 1C**). By contrast only a few worms expressing $A\beta_{42}$ could find the attractant spot (**Figure 1D**). We tested 100 animals/genotype distributed in 5 test plates/genotype obtaining a chemotaxis index for biotin 0.68±0.09 and 0.12±0.04 for DA1262 and FDX(ses25), respectively. Active worms were individually picked and transferred to the center of the test plate. It is important to not only quickly, but also gently transfer the worms because otherwise they may remain inactive for several minutes and fail to track the attractant. At the end of the experiment we suggest monitor worms activity by looking at their paths. If only a few tracks are visible we usually discard the plate.

GFP fluorescence in the ASER neurons of FDX(ses25) worms disappears within the first eleven days of life (not shown). This suggests that these cells undergo apoptosis due to the presence of $A\beta_{42}$. Therefore we determined whether a broad-spectrum inhibitor of apoptosis such as caspase inhibitor N-(2-Quinolyl)valyl-aspartyl-(2,6-difluorophenoxy)methyl Ketone (Q-VD-OPh) [13] could stop the loss of ASER cells. To this end we employed cultured primary ASER neurons from embryos [14] , which were prepared as described in protocol 3. Representative images of an ASER neuron in a young (4 day old) FDX(ses25) worm along with images of neurons in culture are shown in **Figures 2A-C**. Cultured ASER cells were short-lived (**Figure 2D**). As expected incubation with 0.3 µg/ml Q-VD-OPh freshly supplemented daily, completely stopped the loss of fluorescent ASER neurons. When working with primary neurons in culture it is important to maintain an opportune cell density. This parameter mainly depends on the number of worms used to extract the eggs. We measure cell density with a standard hemocytometer and take particular care to maintain cell density constant from cell culture to cell culture. For pharmacological experiments such as those described here we worked with ~200,000 cells/cm^2 which was obtained by harvesting four confluent 10 cm plates. Cells were plated in 12 wells of a 24-well plate. For optimal results it is also important to dissociate the embryonic cells before seeding as they tend to form clumps. We use a syringe with a 27 gauge needle and gentry aspirate the suspension back and forth a couple of times. This is generally sufficient to dissociate most of the cells (especially in the beginning we suggest to check the suspension under a microscope).

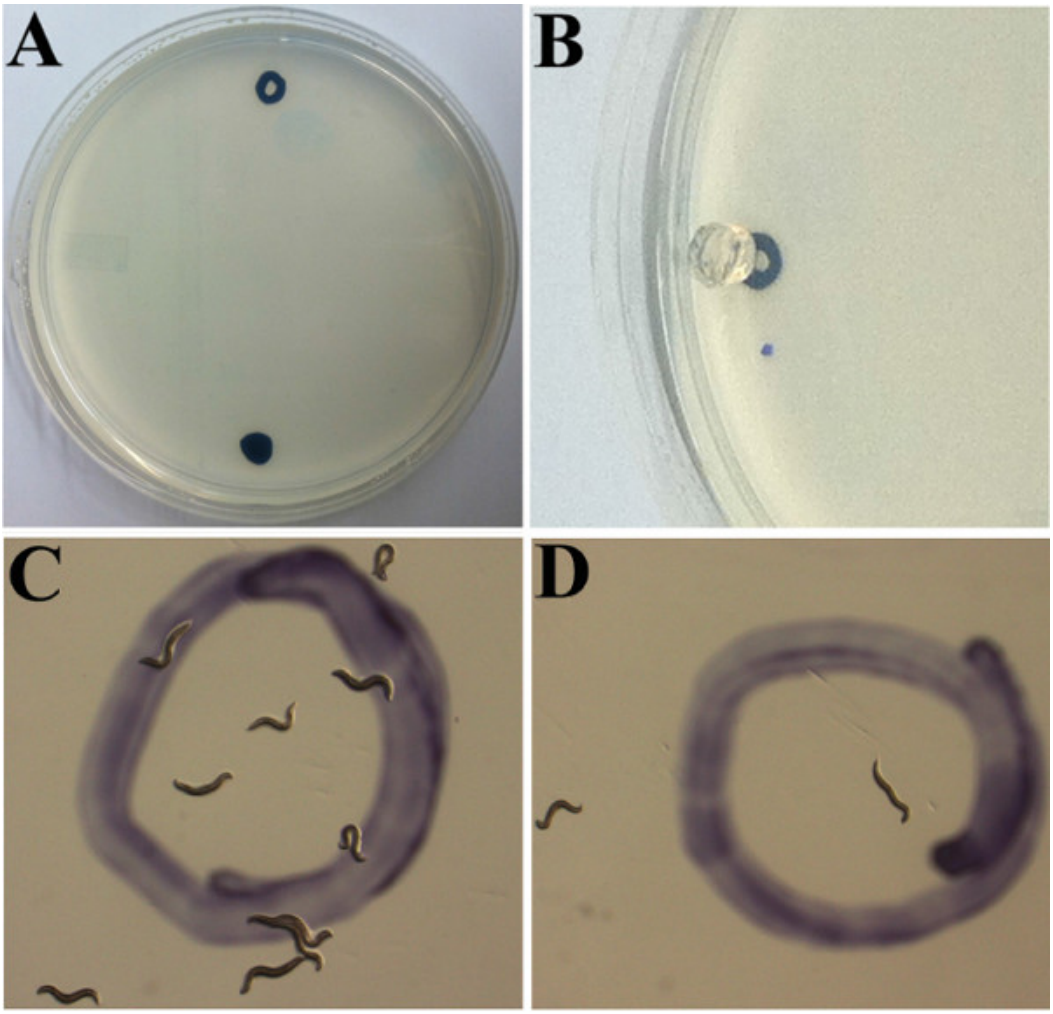

Figure 1. Chemotaxis assay. A. Representative chemotaxis plate. Attractant (biotin) and control spots are marked with an hollow and a filled circle. **B.** A chemotaxis plate loaded with a 0.5-cm diameter piece of agar used to establish a gradient of biotin. The piece of agar was cut from a 10-cm plate using the top side of a glass Pasteur pipette. **C.** Representative distribution of worms around the attractant spot. In this example the majority of DA1262 worms were able to localize the source of attractant (biotin). **D.** As in **C.** for FDX(ses25) worms. These biotin-insensitive worms exhibited a scattered distribution around the plate and only few were found near the attractant spot.

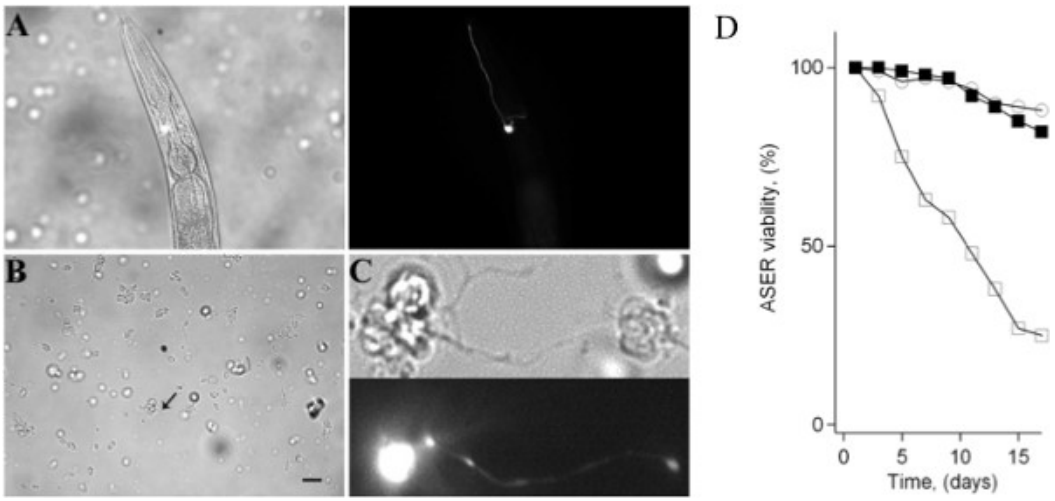

Figure 2. Culture of *C. elegans* embryonic cells. A. Fluorescence microscopy image (left picture) and bright light (right picture) taken from a FDX(ses25) transgenic worm head. This worm expresses GFP in the ASER neuron driven by the *gcy-5* promoter. **B.** Bright light image of a culture of FDX(ses25) embryonic cells. Scale bar is 5 µm. **C.** Fluorescence microscopy image of a cultured FDX(ses25) ASER neuron. Images were taken with an Olympus BX61 microscope equipped with a digital camera. **D.** Representative experiment testing the viability of cultured, age-synchronized, ASER neurons. Cells were obtained from DA1262 embryos (hollow circles) or FDX(ses25) embryos maintained in the absence/presence of 300 ng/ml Q-VD-Oph (hollow and filled squares, respectively). The disappearance of GFP fluorescence was used as a measure of a neuron's viability. The experiment started with ~300 fluorescent ASER neurons. Viability was calculated as 100*(number of fluorescent cells at day X divided by number of fluorescent cells at day 1). Click here to view larger figure.

Discussion

Here we describe a combined approach, to study cellular and molecular aspects of amylopathies using *C. elegans*. The advantages of this approach include: 1) low cost. *C.elegans* is maintained in normal Petri dish seeded with bacteria, at room temperature. 2) Powerful genetics. Transgenic animals can be obtained in few months and a wide array of promoter sequences is available to drive expression of the desired gene in specific neurons. 3) Simple, well-characterized, nervous system. *C. elegans* possesses a remarkably simple nervous system (302 neurons). This simplicity has afforded extensive characterization of the worm's nervous system including cell lineage, specific function/role of each neuron and its synaptic connections. The limitations of *C. elegans* include the small size of the cells, which hinders the application of standard biochemical techniques such as immunohistochemistry and a thick skin (cuticle) which insulates neurons form the external environment. Therefore, pharmacological approaches may be of limited efficacy in *C. elegans.* Cultures of primary cells represent a valid strategy to partially ameliorate this problem.

The critical steps in these experimental protocols include starting with large quantities of worms and monitoring the preparations in order to not lose eggs, embryos *etc*. It is also crucial to learn how to handle the animals during injection. Keeping worms in the agarose pad too long, punching them with a large pipette or injecting too much DNA mix can irreversibly damage them. Transformation efficiency, reproducibility and transgene expression may vary. Efficiency is related to the purity of the injection mix and its composition (presence of non-coding DNA). Extrachromosomal arrays vary from animal to animal therefore it is critical to establish at least 2-3, independent lines per transgene. On the other hand, the addition of digested genomic DNA to the mix represents a valid strategy to reduce transgenic over-expression. Chemotaxis assays are relatively trouble-free. However it is crucial to maintain consistency in the concentration gradient in order to avoid false results. Use pieces of agar of the same size-for example cut them with a Pasteur pipette-and maintain the equilibration time constant. If any, remove excess solution with a cotton swab.

In conclusion, here we provide an example of how a simple, genetically tractable organism can be exploited to investigate molecular aspects of amylopathies. The same experimental techniques could be applied to the study of other neuronal proteins and to the generation of new animal models of disease.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

We thank Dr. Shuang Liu for critical reading of the manuscript. The PCL12 construct was a gift form Dr. Christopher D. Link. This work was supported by two National Science Foundation grants (0842708 and 1026958) and an AHA grant (09GRNT2250529) to FS.

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