Chapter 9

Modeling Dopamine Neuron Degeneration in *Caenorhabditis elegans*

Michelle L. Tucci, Adam J. Harrington, Guy A. Caldwell, and Kim A. Caldwell

Abstract

Ongoing investigations into causes and cures for human movement disorders are important toward the elucidation of diseases, such as Parkinson's disease (PD). The use of animal model systems can provide links to susceptibility factors as well as therapeutic interventions. In this regard, the nematode roundworm, *Caenorhabditis elegans*, is ideal for age-dependent neurodegenerative disease studies. It is genetically tractable, has a short life span, and a well-defined nervous system. Fluorescent markers, like GFP, are readily visualized in *C. elegans* as it is a transparent organism; thus the nervous system, and factors that alter the viability of neurons, can be directly examined in vivo. Through expression of the human disease protein, alpha-synuclein, in the worm dopamine neurons, neurodegeneration is observed in an age-dependent manner. Furthermore, application of a dopamine neurotoxin, 6-hydroxy-dopamine, provides another independent model of PD. Described herein are techniques for *C. elegans* transformation to evaluate candidate neuroprotective gene targets, integration of the extrachromosomal arrays, genetic crosses, and methods for dopamine neuron analysis that are applicable to both types of neurotoxicity. These techniques can be exploited to assess both chemical and genetic modifiers of toxicity, providing additional avenues to advance PD-related discoveries.

Key words: *Caenorhabditis elegans*, Parkinson's disease, Alpha-synuclein, Dopamine, Neurodegeneration, Neurotoxicity

1. Introduction

The sense of urgency to define causes and cures for neurodegenerative diseases cannot be overstated in terms of the devastation these disorders have on individuals, families, and our society. Among the most devastating diseases that result from degeneration and loss of neurons within the brain include both Alzheimer's disease and Parkinson's disease (PD). While extensive studies on the genetics and cellular pathology involved with these diseases

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have been conducted, effective therapies to prevent associated progressive neurodegeneration remain elusive (1, 2). This highlights the need to investigate means to more rapidly accelerate the discovery process. In this context, establishment of methods to evaluate factors impacting neurodegeneration in an experimentally reliable and inexpensive model organism that can be easily genetically altered to identify potential therapeutic targets for treatment of these diseases represents an attractive strategy.

The use of the nematode *Caenorhabditis elegans* as a model system has allowed the study of various pathologies associated with diseases in a simple, multicellular organism (3). Notably, the transparent anatomy of this animal allows for dynamic visualization of cells, including neurons, through expression of fluorescent proteins (i.e., GFP) in living animals. Here, we describe two methods used to study a key pathological hallmark of PD, dopaminergic (DA) neuron degeneration, in *C. elegans*. DA neurodegeneration can be achieved by genetic overexpression of the PD-associated gene, alpha-synuclein (α-syn), or treatment of worms with the DA neuron-specific neurotoxin, 6-hydroxy-dopamine (6-OHDA) (4, 5). Additional methods described here include microinjection of candidate gene targets, integration of extrachromosomal transgenic arrays, and genetic crosses to evaluate potential genetic modifiers of neurotoxicity. Strategies designed to optimize effective scoring of DA neuron survival in the nematode model are also discussed. These assays can be used to identify both genetic and chemical modifiers of DA neurotoxicity in vivo (6).

2. Materials

Materials below are separated based on the sequential steps of the methods described.

Common materials used throughout experimentation

35-, 60-, and 100-mm nonvented Petri dishes.

2.1. Microinjection

1. Calibrated pipettes with aspirator (needle loader).
2. Borosilicate glass capillaries (injection needles).
3. Standard mouth pipetter tubing (included with borosilicate glass capillaries).
4. Young adult/gravid worms N2 (wild type (WT)) worms.
5. Series 700 Halocarbon oil.
6. Narishige PP-830 needle puller.
7. Microinjection system with microscope manipulator, DIC, or Hoffman modulation contrast optics is required.
8. Compressed nitrogen gas tank.

9. 2% agarose injection pads (agarose and 22 × 50-mm coverslips) (see Note 1).
   (a) Prepare a 2.0% agarose solution with water and microwave until molten.
   (b) Using a Pasteur pipette, place a small drop of agarose on a 22 × 30-mm coverslip.
   (c) Immediately place a second coverslip 90° to the first coverslip and gently press down.
   (d) Wait ~30 s for the agarose to solidify and then remove the second coverslip.
   (e) Repeat this process to make multiple injection pads.
   (f) Allow the coverslips to dry overnight on the benchtop and then store in the original coverslip box.

2.2. Integration

UV cross-linker.

2.3. Genetic Crosses

1. *C. elegans* N2 males.
2. Transgenic hermaphrodites containing genes of interest.

2.4. Neurodegeneration Analysis

1. Worm strains used in neurodegeneration experiments (see Note 2):
   BY200 *vtIs1*($P_{dat\text{-}1}$::GFP, pRF4(*rol-6*(*su1006*)))
   UA44 *baIn11*($P_{dat\text{-}1}$::α-synuclein, $P_{dat\text{-}1}$::GFP)
2. *Escherichia coli* OP50 strain (*Caenorhabditis* Genetics Center).
3. Nematode growth medium (NGM) for plates.

975 mL	ddH_2O
2.5 g	Peptone
3.0 g	NaCl
17.0 g	Bacto agar

Autoclave media to sterilize and allow to cool, and then add the following sterile solutions aseptically:

1.0 mL	Cholesterol (dissolve 0.125 g in 25 mL 100% ethanol)
1.0 mL	1 M $CaCl_2$
1.0 mL	1 M $MgSO_4$
25.0 mL	1 M KPO_4, pH 6.0
0.567 mL	Streptomycin sulfate (0.36 g/mL)
1.0 mL	Nystatin (11.2 mg dissolved in 1.0 mL 100% ethanol; prepare this immediately before adding to media)

After NGM plates have been poured and solidified, *E. coli* OP50 bacteria are seeded onto the plates. OP50 bacteria

Table 1
NGM plate volumes

Plate size	35 mm	60 mm	100 mm
NGM media volume	4 mL	10 mL	25 mL
Amount of OP50 bacteria seeded onto each plate	50 µL	100 µL	750 µL

should be inoculated into LB liquid media the evening before they are needed. See Table 1 for appropriate media volumes and amount of LB broth + OP50 bacteria per NGM plate used for seeding. Once seeded with LB broth + OP50 bacteria, incubate overnight at 37°C (12–18 h). Store inverted at 4°C until use (see Note 3).

4. 5-fluoro-2′-deoxyuridine (FUDR) worm plates:
   Prepare NGM media as above. After autoclaving, however, do not add streptomycin sulfate. Add 0.04 mg/mL FUDR directly to the media. FUDR is light sensitive. Concentrated OP50 (see below) is used to seed FUDR plates.
5. LB broth:

10.0 g	Tryptone
5.0 g	Yeast extract
10.0 g	NaCl

   Add up to 1,000 mL with ddH_2O and adjust pH to 7.0. Aliquot into 75-mL working volumes and autoclave to sterilize; store at room temperature.
6. Production of concentrated OP50 using 2XTY

16.0 g	Tryptone
10.0 g	Yeast extract
5.0 g	NaCl

   Add up to 1,000 mL with ddH_2O and adjust pH to 7.0. Autoclave to sterilize and allow the solution to cool to room temperature. Upon reaching room temperature, add the following to the 2XTY medium:

1.0 mL	Cultured LB + OP50 bacteria
3.0 mL	1 M $MgSO_4$ (sterile)
0.567 mL	Streptomycin sulfate (0.36 g/mL)

Incubate at 37°C with shaking for 12–18 h before harvesting the cells. Aseptically aliquot the cultures into four, sterile, 250-mL centrifuge tubes. Centrifuge at 3,800 × *g* for 10 min. Pour off supernatant and add 25.0 mL M9 buffer (see item 5 in Subheading 2.5). Resuspend pellet by gentle vortex and store at 4°C until time of use.

7. Levamisole.
8. Glass microscope slides.
9. Glass coverslips (22 × 30 mm).
10. 2% agarose.

2.5. Chemical Manipulation

1. Sterilized 10.0-mL glass conical tubes.
2. Sterilized glass Pasteur pipettes.
3. 6-OHDA.
4. Ascorbic acid.
5. M9 buffer:

22 mM	KH_2PO_4
90 mM	Na_2HPO_4
85.6 mM	NaCl

Add 3.0 g KH_2PO_4, 12.8 g Na_2HPO_4, and 5.0 g NaCl to 900 mL of ddH_2O, bring volume to 1 L, and stir for 1 h at room temperature. Aliquot into 75-mL working volumes and autoclave to sterilize. Cool to room temperature and add 75 µL of separately autoclaved 1 M $MgSO_4$. Store at room temperature.

6. 20% bleach/NaOH solution: Add 1.0 mL 5 N NaOH and 2.0 mL bleach (sodium hypochlorite; preferably from a fresh bottle) to 7 mL of ddH_2O. This solution is light sensitive. Prepare fresh before each use.

3. Methods

3.1. PD-Related Genes (Genetic Manipulation)

Several genetic factors have been linked to PD (7). Most notably, α-syn was identified to have a detrimental effect on PD patients through either multiplication of the α-syn gene locus or through genetic mutations (8, 9). Although *C. elegans* lacks conservation of this gene, multiple worm PD models have been established through overexpression of the human α-syn gene in worm DA neurons or expressed pan-neuronally (10–12). Furthermore, these models have been used in large-scale genomic screens to identify genetic factors that attenuate α-syn-induced toxicity. These genetic factors have worm orthologs and can be further studied to identify

mechanisms underlying neuroprotection (6, 13, 14). The methods below describe the experimental process of injecting *C. elegans* with the gene of interest, integration of extrachromosomal DNA, genetic crossing into a GFP-illuminated DA neuron strain expressing human α-syn ($P_{dat\text{-}1}$::α-syn + $P_{dat\text{-}1}$::GFP), and analysis of the DA neurons. *C. elegans* transformation can also be performed by ballistic methods (15).

The methodology for cloning the gene of interest (either worm or human cDNA) into the appropriate expression vector is assumed in this protocol. Although traditional cloning is an acceptable approach, recombinational cloning using Gateway technology is available for a variety of vectors that enable neuronal subtype-specific expression in the worm community. The *C. elegans* ORFome developed by Marc Vidal has facilitated the cloning of approximately 19,000 *C. elegans* ORFs into Gateway vectors (ORFeome v3.1) (16). Gateway technology uses the lambda recombination system to move the gene of interest from one vector to the next. Fragments that contain recombination sites use recombinase machinery to make the cloning process simple (17). In this study, the expression vector utilized consists of the dopamine transporter (*dat-1*) promoter driving expression in the eight worm DA neurons.

3.1.1. Microinjection

The procedures described in this section can be viewed in video format from the *Journal of Visualized Experiments* (JoVE) (18).

1. To generate transgenic animals, two plasmids are injected simultaneously. One is an expression vector that contains the gene of interest and the second serves as an injection marker plasmid (see Note 4).
2. Prior to microinjection, it is advisable to purify the DNA. In this regard, DNA isolated from the Qiagen mini-prep DNA isolation kit can be used (see Note 5).
3. Quantitate both plasmids and mix together in a 1:1 ratio of 50 ng/μl each for a final concentration of 100 ng/μl (see Note 6).
4. To prepare needle loaders, take the calibrated pipettes and heat the middle over an open flame.
5. Rapidly pull the ends apart to approximately twice the total length, removing from the flame as you pull them apart.
6. Snap the two halves apart once the capillary tube cools.
7. Store upright and out of the way to prevent personal harm and/or damage to capillary tubes.
8. To prepare injection needles, place the borosilicate glass capillaries into a needle puller. For the Narishige PP-830 needle puller, a heat setting of 24.8 is recommended (see Note 7).
9. Prior to loading DNA into loading needles, centrifuge injection DNA at 13,100 × *g* 10–30 min to pellet any particulate matter that may clog the injection needle.

10. Using a standard mouth pipetter tubing, insert the needle loader and draw up approximately 1–2 μl of injection mixture.
11. The needle loader is placed at the back end of the injection needle and inserted all the way until the tip comes in contact with the internal needle tip.
12. Gently blow with the mouth pipetter to release the DNA solution into the tip of the injection needle tip. Remove the loader carefully (see Note 8).
13. To prepare equipment, open the main valve of the compressed nitrogen tank that is connected to the microinjection arm and holder. The regulator valve must be closed to allow the gas to enter the line. The pressure should be about 35 psi. The foot pedal is used to release the pressure each time it is stepped on.
14. Place the injection needle in the microinjection arm. Position the needle in the center of the viewing field at 4× under bright-field illumination.
15. Place an injection pad on the stage and focus the edge of the pad to the center of the viewing field.
16. Increase the magnification to 40×, keeping both the needle and edge of agarose injection pad in the center and in focus.
17. Slowly lower the injection needle until it is in view and gently move the needle down to touch the agarose injection pad.
18. To break the needle, gently press the foot pedal down (this causes a slight movement in the needle) and slowly apply the needle to the edge of the agarose injection pad. Repeat this until a small amount of injection mix visibly expels. For a proper break, a small, but visible, amount of injection mix expels each time the pedal is pressed (see Note 9).
19. Do not change the X or Y positions on the microinjection arm of the scope, but raise the needle arm up (Z position) to provide room to remove the agarose injection pad. Slide the stage out from underneath the needle.
20. Prepare the agarose injection pad by placing a small amount of halocarbon oil on the surface (see Note 10).
21. With a small amount of halocarbon oil on the worm pick (acts as glue), transfer a worm onto the agarose injection pad and gently stroke it to adhere the worm to the pad. The worm should not move.
22. Quickly place the agarose injection pad back onto the stage and center the worm in the field of view, with the gonad distal tips toward you.
23. Lower the needle into the same plane of focus as the worm. Use the fine focus to position the injection needle to the same plane as the gonad.

24. If using the microinjection microscope system, switch the scope filters to Hoffman Illumination (high contrast). As an alternative, DIC may be used to increase contrast.
25. Using the 40× objective, focus on the syncytial center of the gonad (it should appear "grainy"). This is below the "honeycomb" pattern of the germ nuclei.
26. Use the fine focus to position the tip of the injection needle to the same plane as the gonad.
27. Insert the needle slowly and apply a short pulse of gas pressure. A proper injection looks like a small wave of liquid spreading across the distal gonad (see Note 11).
28. If the other gonad arm is readily accessible to the needle and less than 30–45 s have passed, repeat steps 27 and 28. Injection of the worm (one or both gonads) should not take longer than 1 min (see Note 12).
29. After one or both gonad arms are injected, quickly remove the worm by placing a small drop of M9 buffer on top of the worm using a mouth pipetter. The liquid "drops" underneath the worm and release the worm from the injection pad.
30. Using the mouth pipetter, slowly draw up the worm into the loading needle and dispense onto a seeded agar plate.
31. Place the worm near the lawn of food to allow them to recover.
32. Repeat this injection process until you have injected roughly 30–50 worms successfully, with each injected worm segregated out onto individual 35-mm plates.
33. Allow the P0 to reproduce for 2–3 days and then search for F1 progeny expressing the injection marker.
34. After 2–3 days, segregate out single F1 worms expressing the injection marker to individual small plates and wait for an additional 3–4 days to allow for the F2 generation to grow (see Note 13).
35. Filter through the plates and keep the ones that have a high penetrance of the transgenic marker. Worms expressing the transgenic array are considered a "stable" line (see Note 14).
36. Obtain at least three stable lines to control for the variability in the gene copy number among transgenic animals.
37. Maintain each stable line separately and analyze the lines for the phenotype of interest (see Subheading 3.1.4). In this example, the phenotype is neurodegeneration (see Note 15).

3.1.2. Integration

Due to the fact that stable transgenic worm lines consist of extrachromosomal arrays, random segregation of the transgenic DNA occurs during cell division processes, resulting in variable copy number within the progeny. Integration of the transgenic constructs

within the chromosomes avoids this issue and enables establishment of isogenic populations for analysis. Various methods of DNA integration have been used, such as gamma and UV irradiation and integration by injection (19). Irradiation causes chromosomal breaks, whereby the injected DNA is ligated during repair. Mutations can arise with exposure to irradiation, thus outcrossing to wild-type animals is necessary (see Subheading 3.1.3). The procedure below describes UV irradiation. It is advisable to analyze the three stable lines prior to integration to obtain preliminary phenotypes of interest (in this example, enhanced neurodegeneration or neuroprotection) (see Subheading 3.1.4). Once this is completed, select the line that is most representative of all the stable lines and begin integration.

1. Grow and select for approximately 200 L4-stage worms (see Note 16). These worms should all express the genetic marker that indicates expression of the injected constructs (such as fluorescence).
2. Wash the worms with M9 buffer. Using a sterilized glass Pasteur pipette, rinse the worms off the NGM plate with ~1.0 mL M9 buffer into a sterile 10.0-mL glass conical tube (see Note 17).
3. Centrifuge in a clinical centrifuge at $145 \times g$ for 1 min.
4. Remove supernatant and suspend worm pellet in 1.0 mL of M9 buffer. Repeat centrifugation and wash (steps 2–4) until supernatant is clear of bacteria (approximately three times).
5. Pipette the worms onto unseeded NGM plates and allow M9 buffer to absorb into the plate (see Note 18).
6. Place the plate inside the cross-linker with the Petri dish lid off (see Note 19).
7. Close the cross-linker door, press the energy button, and input energy level to 400–450 J/m^2 (see Note 20).
8. Press start and when the irradiation is finished, remove the Petri plate from cross-linker. Add 100 μL of concentrated OP50, cover with lid, and allow to incubate at 20°C for at least 4 h, but do not exceed 12 h (see Notes 21 and 22).
9. Transfer two healthy irradiated worms to 60-mm plates. The dead worms are visible on the plate (see Note 23).
10. Repeat step 7 until 50 plates (100 worms) are transferred. Label the plates 1–50.
11. Grow until starved +2 days to allow dauer formation. At 20°C, this takes approximately 10 days.
12. If a fluorescent marker was used, check under a fluorescent stereomicroscope and take a small chunk from each plate that contains a high concentration of worms carrying the transgenic

marker. This should be performed using a sterilized inoculating loop. Transfer the chunk to a 60-mm-sized plate (repeat for the remaining 49 plates). Approximately 100–500 transgenic larvae should be transferred onto each plate. Label the plates 1–50 to correspond to the plates of origin.

13. At room temperature, grow until almost starved (2–4 days).
14. From each plate, pick eight young larvae transgenic animals, one worm per 35-mm plate. Keep track of which worms came from the original 50 plates (label individual plates A–H, along with the original plate number). This allows one to determine independent lines (see Notes 24 and 25).
15. After progeny have developed (~4 days), check for 100% transmission of the transgenic marker.
16. If successful, clone three animals to single plates and confirm successful integration by examining the next generation for 100% transmission of the transgenic marker.
17. Once an integrated line is obtained, outcrossing must be performed to remove any extraneous mutations due to the irradiation (see Note 26).

3.1.3. Genetic Crosses

The procedures described in this section can be viewed in video format from JoVE (20).

Males arise in a WT population at a very low frequency via chromosomal nondisjunction (~0.2%). To generate *C. elegans* males, L4 hermaphrodites can be placed for an extended "heat shock" in 34°C incubator for 2–4 h, and then back to room temperature to allow for propagation. Once males are obtained, a simple genetic cross should be set up to maintain a stock, whereby each plate contains a single hermaphrodite with three to five males. Following a successful cross, ~50% of the F1 population is male.

The process given below describes the cross of the transgenic lines (stable or integrated) that express the gene of interest for neuronal analyses and the transgenic line expressing α-syn in the DA neurons.

1. Place three to four L4-staged transgenic (integrated or stable) hermaphrodites expressing your gene of interest on a 35-mm Petri dish with a small bacterial lawn (see Note 27). Set up individual plates for each independent line expressing the gene of interest.
2. Place 10–15 males expressing $P_{dat\text{-}1}$::GFP + $P_{dat\text{-}1}$::α-syn on the mating plates.
3. Propagate worms for 2 days and then remove the males to prevent the P0 males from interacting with the F1 generation.
4. Monitor the F1 generation and look for male progeny. If males are present, the cross was successful.

5. On five 35-mm Petri dishes, clone out individual L4 hermaphrodites from each cross that expressed both the $P_{dat\text{-}1}$::GFP marker and the injection marker used with the gene of interest (see Note 28).
6. Allow to self-fertilize and produce F2 progeny. This takes 2–3 days.
7. Similar to step 5, clone out the F2 generation (~5–10 animals) to their own individual plates.
8. Monitor the F3 generation to search for plates with 100% worms that express both GFP and the transgenic marker for the gene of interest.

3.1.4. Dopamine Neurodegeneration Analysis

The procedures described in this section can be viewed in video format from JoVE (20).

1. Synchronize worms by transferring ~50 gravid adult hermaphrodites to 100-mm seeded NGM plates, and place worms at 20°C for 5 h for egg laying (see Note 29).
2. Remove gravid adult worms from the plates, leaving only the eggs. Grow worms at 20°C for 4 days.
3. Four days posthatching, transfer ~100 transgenic young adult worms to 60-mm seeded FUDR plates (see Note 30).
4. On days of DA neuronal analysis, prepare molten 2.0% agarose solution (see Note 31).
5. Prepare agarose pads for analysis by placing a piece of tape on two microscope slides to act as a spacer. A drop of molten agarose is deposited on a third microscope slide that is placed between the two "spacer" slides. A fourth microscope slide is placed on to the top of and perpendicular to the agarose slide, pressing down slightly, to create a pad. See Fig. 1 (see Note 32). When the agarose has solidified (for a few seconds), the perpendicular slide should be carefully removed so that the exposed agarose pad can be used for mounting worms.
6. Transfer 35–40 worms to an 8.0 μL drop of 3 mM levamisole (an anesthetic) on a 22 × 30-mm cover glass. Invert cover glass and place it onto the 2% agarose pad prepared in step 5. Repeat this process for each worm strain that is analyzed.
7. Score the six anterior dopaminergic neurons (4 CEP and 2 ADE) as normal (i.e., wild-type) or degenerative for 30 worms/strain (Fig. 2). Degenerating neurons may exhibit several morphological defects, including neurite retraction and complete cell loss (see Fig. 3a–c) (see Notes 33 and 34).
8. Repeat each experiment in triplicate (30 worms/screen × 3 screens = 90 total worms analyzed/strain).

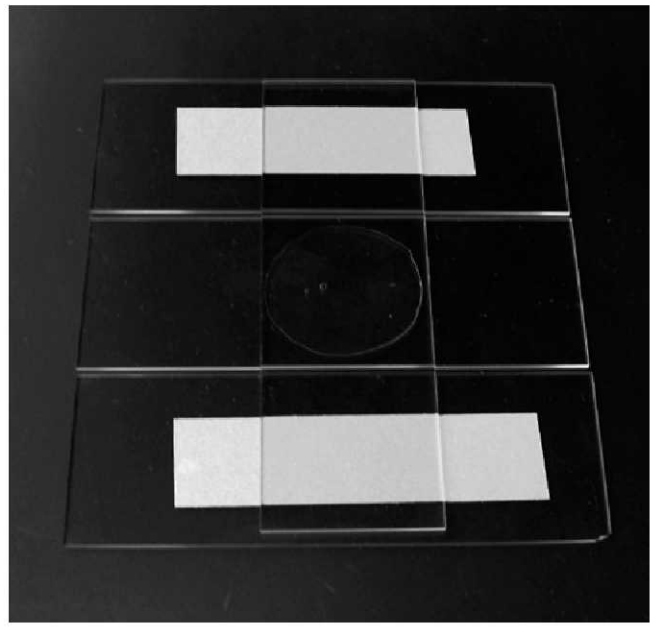

Fig. 1. Image of the microscope slide arrangement used when creating agarose pads. Removal of the top slide yields a pad that worms can be mounted on for microscopy analysis.

Screen: **Date:**

Transgenic line:

worm no.	WT neurons	Degenerating neurons		
		ADE	CEP	
			ventral	dorsal
1	○	○○	○○	○○
2	○	○○	○○	○○
3	○	○○	○○	○○
4	○	○○	○○	○○
5	○	○○	○○	○○

Fig. 2. Example of a scoring sheet that can be used in the analysis of the anterior DA neurons in *C. elegans*. Each neuron within an animal exhibiting degenerative changes can be individually noted. This allows for quantitative data on both the total number of neurons degenerating in a population (each worm has six anterior DA neurons × number of worms analyzed), as well as the number of worms within a population that exhibit any degenerating neurons.

3.2. 6-OHDA-Induced Toxicity (Chemical Manipulation)

Alternative methods to induce DA neurotoxicity may also be used to observe neuronal cell death. The DA analogue, 6-OHDA, is readily taken up into dopaminergic neurons through the dopamine transporter, DAT-1, where it forms free radicals and causes oxidative stress, resulting in cell death (4, 5). For this degeneration assay,

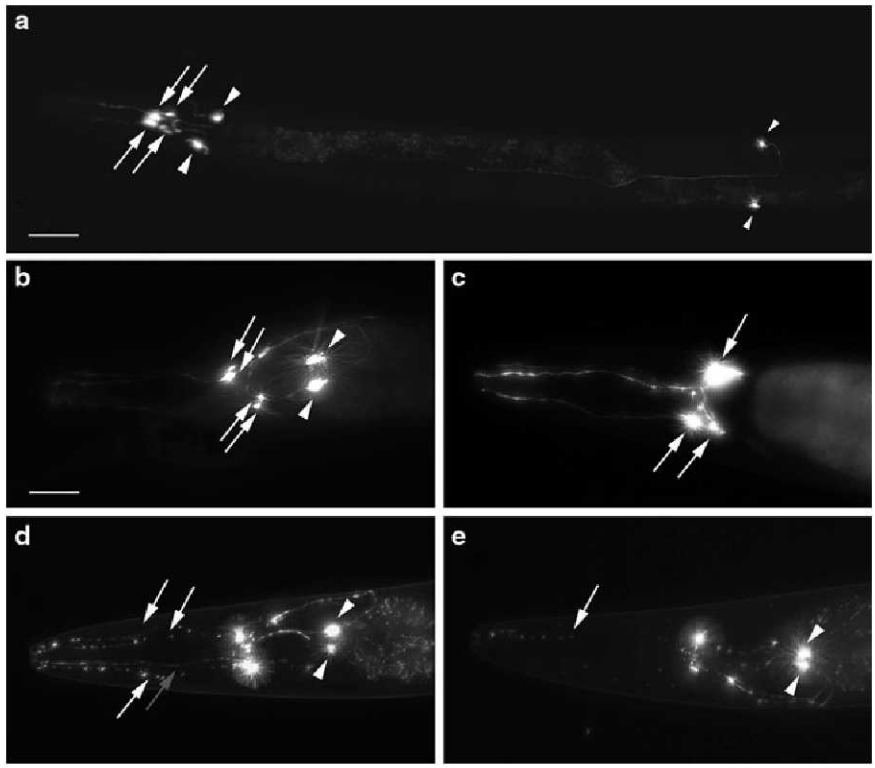

Fig. 3. *C. elegans* DA neurons are shown using GFP driven from the DA transporter promoter ($P_{dat\text{-}1}$::GFP). (**a**) The 6 anterior DA neurons include two pairs of cephalic (CEP) neurons (*arrows*) and one pair of anterior deirid neurons (ADEs; *large arrowheads*); the cell bodies and processes are highlighted. Two posterior deirid neurons are also present in each hermaphrodite (PDEs; *small arrowheads*). (**b**). The anterior region of *C. elegans* is magnified, displaying the six anterior-most DA neurons. The cell bodies of the four CEP neurons and the two ADE neurons are labeled with *arrows* and *arrowheads*, respectively. (**c**) A 7-day-old worm coexpressing GFP and α-syn in DA neurons; most worms within this population are missing anterior DA neurons when they are adults. In this example, three of the four CEP neurons (*arrows*) remain while there are no ADE neurons. (**d, e**) Following exposure to 6-OHDA, CEP and ADE neurons progressively degenerate as shown in these examples. (**d**) In this example, three of four CEP cell processes are present, but degenerating (*white arrows*). The fourth CEP neuronal process is not yet degenerating. The two ADE neurons in this animal are still intact (*arrowheads*). (**e**) This worm exhibits further degeneration, whereby only one CEP cell process remains; the two ADE neurons in this animal are still intact (*arrowheads*). Scale bar in (**a**) = 100 μM. Scale bar for (**b**–**e**) = 50 μM.

late L3–L4 worms expressing GFP under the *dat-1* promoter [BY200, *vtIs1*($P_{dat\text{-}1}$::GFP), pRF4(*rol-6*(*su1006*))] are treated with various concentrations of 6-OHDA. Younger worms should not be treated due to increased lethality while older worms have slightly higher resistance to the toxin than L4-stage worms.

1. Worms should by synchronized either through bleaching or egg laying (see Note 35).
2. To bleach, perform the following steps:
   (a) Wash gravid adult hermaphrodites off a 60-mm NGM plate with 1.0 mL ddH_2O and transfer them into a 10.0-mL glass conical tube.

(b) Centrifuge tube at 145 ×*g* for 1 min and decant supernatant, leaving worm pellet at bottom.

(c) Add an additional 1.0 mL ddH_2O to the worm pellet, centrifuge, and decant supernatant. Repeat twice.

(d) Add 1.0 mL 20% bleach solution to each tube of worms and incubate at room temperature for 5–7 min with gentle agitation.

(e) After bleaching, centrifuge tube and decant supernatant.

(f) Wash worms with 1.0 mL M9 buffer, centrifuge, and decant supernatant. Repeat wash for three additional times.

(g) After last centrifugation, leave ~100 μL M9 buffer in each tube, resuspend pellet (worm eggs), and transfer eggs to 100-mm seeded NGM plate using a Pasteur pipette. Store at 20°C.

3. Allow worms to grow at 20°C for 65–70 h, until they reach the late L3–L4 larval stage.
4. Wash worms off the 100-mm plates with 3.0 mL ddH_2O into 10.0-mL glass conical tubes, centrifuge, and decant supernatant.
5. Wash worms with 1.0 mL ddH_2O, centrifuge, and decant supernatant. Repeat wash for three additional times (see Notes 36 and 37).
6. Decant ddH_2O, add 1.0 mL 6-OHDA solution (see Table 2) to each tube, and incubate for 1 h at 20°C with gentle agitation every 10 min (see Note 38).
7. After the 1-h incubation, centrifuge worms and decant supernatant.
8. Wash worms with 1.0 mL M9, centrifuge, and decant supernatant. Repeat wash for three additional times.
9. After the last centrifugation, leave ~100 μL M9 buffer in each tube, resuspend worm pellet, and transfer worms to 60-mm seeded NGM plates. Place the worm plates at 20°C until time of analysis (see Table 2).

Table 2
6-OHDA concentrations and times of analysis

6-OHDA concentration	10 mM 6-OHDA, 2 mM ascorbic acid	30 mM 6-OHDA, 6 mM ascorbic acid	50 mM 6-OHDA, 10 mM ascorbic acid
Analysis times	2, 6, 24, 48, and 72 h	2, 6, 12, 24, and 48 h	1, 2, 3, and 6 h

3.2.1. Dopamine Neurodegeneration Analysis

Scoring of the anterior dopaminergic neurons is conducted using the same procedure as mentioned above. Degenerating neurons may exhibit several morphological defects, including neurite blebbing, cell body rounding, and cell loss/death (see Fig. 3a, b, d, e). Degeneration from 6-OHDA is most often seen in the CEP neurons while the ADE neurons are less sensitive to this type of toxicity (see Note 39).

4. Notes

1. The injection pads have a long shelf life once made. However, if too dry, worms will desiccate.
2. For general worm maintenance, see ref. 21.
3. To grow LB + OP50 for seeding NGM plates, inoculate 75 mL of LB broth with *E. coli* OP50 and 50 μL of streptomycin sulfate (0.36 g/mL). Incubate in 37°C incubator for 12–18 h. Store at 4°C.
4. There are a variety of injection marker plasmids that provide a readily visible phenotype for successful transgenic animal production. Examples include fluorescent tissue markers, such as pharyngeal muscle ($P_{myo\text{-}2}$::mCherry) and body wall muscle ($P_{unc\text{-}54}$::GFP). Other phenotypic markers include the plasmid pRF4 which encodes a dominant collagen mutation (*rol-6*(*su1006*)) that elicits a roller phenotype. Care should be taken, however, to avoid choice of a marker that may obfuscate scoring of dopaminergic neurons.
5. A good DNA prep should have a 260/280 absorbance ratio range between 1.7 and 2.0.
6. Injection marker plasmids that express RFP (i.e., mCherry, dsRed) may need to be injected at a lower injection due to lethality with high expression. The injection plasmid $P_{myo\text{-}2}$::mCherry is one example, where the injection concentration should be 1–5 ng/μl.
7. Multiple injection needles can be made and stored in a small box. These needles are very fragile and are easily broken if not stored properly; one method is to lay them on top of molding clay.
8. Several injection needles should be loaded per transgenic construct to save time. After several worm injections, the needle can break or become clogged, thus a new needle is required. Since bubbles can prevent proper plasmid flow, allow time for any bubbles to float out of the needle tip. This is best done when the needle is held with the tip facing down.
9. If the needle opening is too large, this may cause damage to the worm. Conversely, too small of a break (and opening) prevents proper flow of DNA.

10. Injection pads that are too dry can desiccate the worm quickly. To prevent this, breathe on the pad one time. If the pad is too moist (immediate use after making the injection pads), the worms will have difficulty adhering.
11. Injecting too much can cause the liquid to flow into the proximal bend of the gonad arm and shut down oocyte production.
12. If the worm is on the injection pad too long, the worm will desiccate and die.
13. The DNA is not integrated in the chromosome of the worm and it exists as an extrachromosomal array, which segregates randomly; this can be inherited (also randomly).
14. Usually, 10% of the F1 progeny yield a stable transgenic line.
15. Since gene copy can be variable across transgenic lines, when selecting a line for chromosomal integration (see Subheading 3.1.2), it is important to choose a transgenic worm line that is representative of the other (≥2) stable lines and has >50% transmission frequency.
16. Upon UV irradiation, half of the L4 population may die.
17. *C. elegans* have a propensity for sticking to plastic tubes, thus glass tubes are recommended.
18. Bacteria can act as a shield against the UV irradiation.
19. The plastic of the Petri dish can act as a shield against the UV irradiation.
20. Repetitive use of the UV bulbs causes the intensity to decrease over time, and therefore this energy level may need to be incrementally increased by ~50–100 J/m^2, if chromosome integration is no longer observed (and bulbs should be replaced accordingly).
21. The machine self-monitors until the dose is complete; this takes approximately 20 s. However, as described in Note 20, the time to completion is dependent on bulb usage.
22. After irradiation, sufficient time should be allowed for worms to recover because transferring worms too early (before ~4–6 h) causes extra stress on the animals and results in lethality.
23. Healthy worms can be identified by the worms moving in a wild-type manner. Other worms are sluggish and typically die over time.
24. At least three independent lines are necessary to account for variation in gene copy number.
25. It is critical at this stage that only a single worm is transferred/plate and that there are no small larvae or eggs attached to the single worm.
26. By crossing with an N2 WT animal, recombination through the genetic cross helps reduce the number of these mutations. For genetic cross procedures, (see Subheading 3.1.3).

27. A small bacterial lawn forces closer contact between the hermaphrodite and males.
28. The cloned hermaphrodite animals need to be at the L4 stage to ensure that they have not mated with the P0 males.
29. If stable transgenic lines are used in crosses instead of chromosomally integrated lines, the transmittance rate of the transgene within these stable lines will dictate how many adults are needed. For example, low transmittance lines may require more adults to lay eggs in order to obtain ~100 worms for analysis.
30. FUDR blocks DNA synthesis and does not allow the eggs that have been laid to develop, thus eliminating the need to transfer animals throughout their life span prior to analysis.
31. The day you choose to analyze is dependent on your hypothesis for the overexpressed gene. For example, if the coexpressed gene might enhance neurodegeneration, then analysis should begin on day 6 of development. However, if the overexpressed gene might be neuroprotective, then analysis should be performed on days 7 and 10 (see Fig. 4).

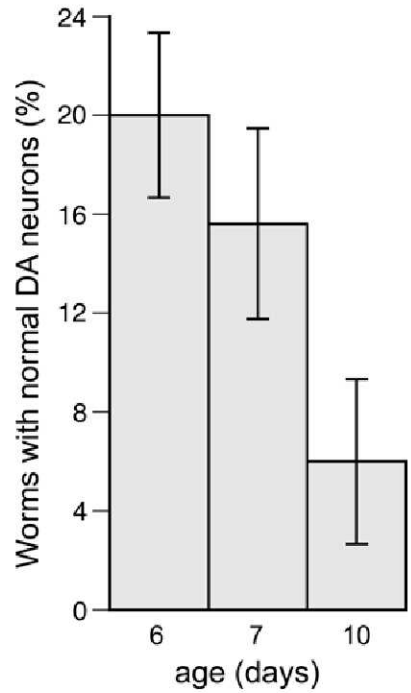

Fig. 4. DA neurons within *C. elegans* expressing both $P_{dat\text{-}1}$::GFP + $P_{dat\text{-}1}$::α-syn exhibit age-dependent degeneration. When these worms are 6 days old, approximately 20% of the population displays a full complement of normal DA neurons while only 5% of the population has normal DA neurons in 10-day-old animals. This information can be used to examine the consequences of expressing potential neurodegenerative or neuroprotective proteins (in 6 and 10-day-old animals, respectively).

32. Do not make the pad too thick as this could cause a microscope objective to place too much pressure on the pad during analysis, causing the worms to burst and die.
33. Do not score dead worms or those that have exploded, as the neurons degenerate quickly and skew the results.
34. It might be useful to score ventral and dorsal CEPs separately because the dorsal CEP neurons synapse onto the ADE DA neurons while the ventral CEP neurons do not. In P*dat-1*::α-syn worms, dorsal CEPs degenerate significantly more often than ventral CEPs. ADEs degenerate significantly more than either dorsal or ventral CEP neurons (Hamamichi and Caldwell, personal communication) (see Fig. 5).
35. Synchronizing by egg laying increases the percentage of transgenic animals; this is important when working with stable (and not chromosomally integrated lines) because bleaching a total population of transgenic and nontransgenic animals results in nontransgenic offspring that unnecessarily overcrowd the plate.
36. Make sure to wash off most of the bacteria from the worms. The presence of bacteria increases the oxidation of 6-OHDA and decreases the potency of the chemical.
37. Do not use M9 buffer to wash bacteria off; it oxidizes 6-OHDA quickly.

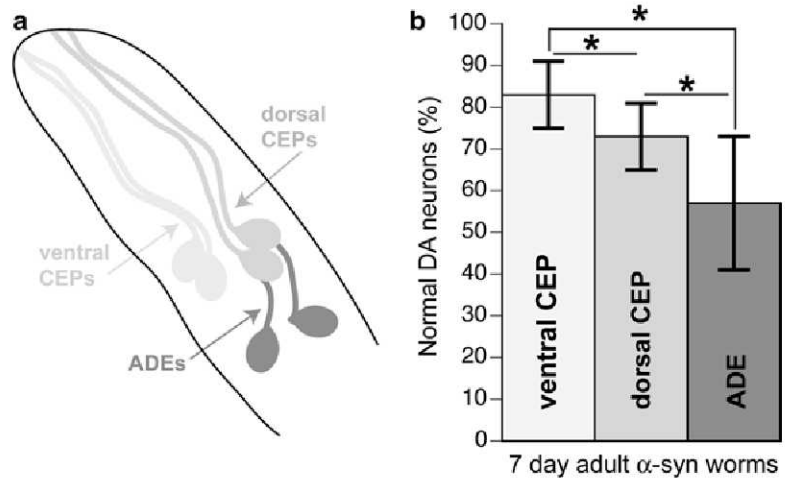

Fig. 5. Anterior DA neurons exhibit differential sensitivity to α-syn-induced degeneration. (**a**) There are six DA neurons in the anterior region of a *C. elegans* hermaphrodite; these neurons can be subclassified in pairs as two dorsal CEP neurons, two ventral CEPs, and two ADE neurons. The dorsal CEPs are postsynaptic to the ADE neurons while the ventral CEPs are not postsynaptic to the ADEs. (**b**) In worms expressing both $P_{dat\text{-}1}$::GFP + $P_{dat\text{-}1}$::α-syn, dorsal CEPs degenerate significantly more often than ventral CEPs. ADEs degenerate significantly more than either dorsal or ventral CEP neurons in this strain ($P < 0.05$; Fisher Exact Test).

38. Fresh 6-OHDA solution should be made immediately prior to adding the solution to the worms. If the solution turns pink immediately, the chemical will not cause DA neurodegeneration, as it is already oxidized. In general, this usually occurs after the hour incubation. When making the solution, mix water and ascorbic acid before adding the 6-OHDA because the ascorbic acid helps stabilize 6-OHDA in solution.
39. It is important to use the same 6-OHDA chemical supplier and lot number, as changes to either of these factors may elicit differences in the amount of degeneration observed. Each batch or lot number of 6-OHDA should be tested for efficiency to induce neurotoxic effects before a large-scale experiment is performed.

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