Video Article

Application of a C. elegans Dopamine Neuron Degeneration Assay for the Validation of Potential Parkinson's Disease Genes

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Abstract

Improvements to the diagnosis and treatment of Parkinson's disease (PD) are dependent upon knowledge about susceptibility factors that render populations at risk. In the process of attempting to identify novel genetic factors associated with PD, scientists have generated many lists of candidate genes, polymorphisms, and proteins that represent important advances, but these leads remain mechanistically undefined. Our work is aimed toward significantly narrowing such lists by exploiting the advantages of a simple animal model system. While humans have billions of neurons, the microscopic roundworm Caenorhabditis elegans has precisely 302, of which only eight produce dopamine (DA) in hemaphrodites. Expression of a human gene encoding the PD-associated protein, alpha-synuclein, in C. elegans DA neurons results in dosage and age-dependent neurodegeneration.

Worms expressing human alpha-synuclein in DA neurons are isogenic and express both GFP and human alpha-synuclein under the DA transporter promoter (Pdat-1). The presence of GFP serves as a readily visualized marker for following DA neurodegeneration in these animals. We initially demonstrated that alpha-synuclein-induced DA neurodegeneration could be rescued in these animals by torsinA, a protein with molecular chaperone activity [1]. Further, candidate PD-related genes identified in our lab via large-scale RNAi screening efforts using an alpha-synuclein misfolding assay were then over-expressed in C. elegans DA neurons. We determined that five of seven genes tested represented significant candidate modulators of PD as they rescued alpha-synuclein-induced DA neurodegeneration [2]. Additionally, the Lindquist Lab (this issue of JoVE) has performed yeast screens whereby alpha-synuclein-dependent toxicity is used as a readout for genes that can enhance or suppress cytotoxicity. We subsequently examined the yeast candidate genes in our C. elegans alpha-synuclein-induced neurodegeneration assay and successfully validated many of these targets [3, 4].

Our methodology involves generation of a C. elegans DA neuron-specific expression vector using recombinational cloning of candidate gene cDNAs under control of the Pdat-1 promoter. These plasmids are then microinjected in wild-type (N2) worms, along with a selectable marker for successful transformation. Multiple stable transgenic lines producing the candidate protein in DA neurons are obtained and then independently crossed into the alpha-synuclein degenerative strain and assessed for neurodegeneration, at both the animal and individual neuron level, over the course of aging.

Video Link

The video component of this article can be found at http://www.jove.com/details.php?id=835

Protocol

A. Expression Plasmid Construction

Two plasmids are required: one for tissue-specific expression of the gene of interest and a second as selectable transformation marker (though the marker plasmid is usually available from within the research community).

Experimental Plasmid

1. Select a promoter that is expressed in the tissue/cell type of interest; in this case, the DA transporter (Pdat-1) promoter is used. This expression plasmid was created as an Invitrogen Gateway system-compatible destination vector (pDEST-DAT-1) to allow the insertion of any gene of interest downstream of the promoter by recombinational cloning [1].
2. The cDNA of the gene of interest is PCR amplified using primers that contain the Gateway att B recombinational sequence. The amplified cDNA is first recombined into the donor vector pDONR201 to create the entry vector and then transformed into E. coli strain DH5α. Following selection of successful recombinants and mini-prep isolation of DNA, the entry vector is recombined into the pDEST-DAT-1 destination vector to create the expression plasmid. Details on recombinational cloning are available from either the Invitrogen Gateway manual or in Caldwell et al. [5].

Selectable Marker Plasmid

A transgenic marker plasmid consists of a vector with a promoter driving the expression of a fluorescent protein in an obvious tissue. In this particular procedure, the unc-54 promoter drives cherry protein expression in body wall muscle (Punc-54::cherry).

B. Generation of Transgenic C. elegans via Microinjection

See related JoVE article: http://www.jove.com/index/details.stp?ID=833

C. Genetic crosses for DA neurodegeneration Analysis

1. Worms are grown using standard procedures [6].
2. Place 10 Pdat-1::α-syn; Pdat-1::GFP males [1, 2] onto a mating plate with 3-4 L4 stage transgenic hermaphrodites (expressing Pdat-1::gene X and Punc-54::cherry). This should be performed individually for each of the three separate stable lines created. After two days, remove the males.
3. Inspect the F1 generation; if there are several male progeny, the mating was successful.
4. Clone out 5 hermaphrodite L4 animals from each cross that exhibit both the Pdat-1::GFP fluorescent marker (inherited from the male parent) and the Punc-54::cherry fluorescent body wall muscle marker (inherited from the hermaphrodite parent). The cloned animals need to be at the L4 stage to ensure that they have not yet mated with male animals present on the plate. Allow them to self-cross and produce F2 progeny.
5. F2 animals produced in step 3 are cloned out. Specifically, transfer ~5-10 animals that exhibit both fluorescent markers to their own individual plates. Screen the F3 generation for plates where 100% of the animals express GFP in DA neurons and some of the animals are express cherry in body wall muscle cells. These animals will be homozygous for the Pdat-1::α-syn; Pdat-1::GFP gene while stably transmitting the newly created transgene (gene X).

D. Dopaminergic Neuron Analysis

1. Prepare a fresh plate for each of the three lines created above, as well as the Pdat-1::α-syn; Pdat-1::GFP alone strain. Allow them to grow to adulthood.
2. Transfer 30-40 transgenic adults from each line onto a fresh plate. Allow them to lay embryos for 4 hours at 20ºC.
3. Remove the adults. Allow the embryos to develop for 3-4 days at 20ºC until they reach the L4 stage.
4. Pick ~100 transgenic L4 animals to a plate containing 0.04 mg/ml 5-fluoro-2'-deoxyuridine (FUDR). This nucleotide analog blocks development of the next generation via inhibition of DNA synthesis, thus preventing the offspring from overwhelming the experimental animals [7]. Protect the FUDR plates from the light since it is light sensitive.
5. Adult animals will be analyzed at various days following egg laying. The appropriate days of analysis are determined empirically. For example, we have determined that 77%, 87%, and 90% of the Pdat-1::α-syn; Pdat-1::GFP animals exhibit degenerated DA neurons at days 6, 7 and 10 post development, respectively. Therefore, if we predict that the transgene of interest might enhance neurodegeneration, we will analyze at day 6 and/or 7. Likewise, transgenes that might suppress neurodegeneration will be analyzed at days 7 and 10.
6. Make 4 fresh agarose pads (unlike the microinjection agar pads, these are used fresh and are not allowed to dry out). Set out two microscope slides that have a piece of tape across them; the tape serves as spacer for equivalently thick pads. Lay a third slide between them on the bench (they are positioned three-abreast). Place a drop of molten agarose on the center slide and quickly lay another slide on top of the agarose, perpendicular to and across all three slides. Make several additional pads, leaving the two slides together until the pad is ready for use.
7. To analyze worm DA neurons, place a 6 µl drop of 3 mM levamisole (an anesthetic) on a 22 x 30 mm cover glass. Pick 40 adult animals (10 extra beyond the 30 to be scored) into the drop, then invert the cover glass onto the agarose pad. Repeat for each of the other lines.
8. Score 30 animals per line for the presence of each CEP and ADE neuron using a compound microscope with epifluorescence and a filter cube that allows visualization of FITC or GFP. We use an Endow GFP HYQ filter cube (Chroma Technology). Record data on the attached scoring sheet. Wild-type animals will retain complete GFP fluorescence in all 4 CEP and 2 ADE neurons. Individual animals exhibiting loss of DA neurons are scored as non-WT or "degenerating". Likewise, the extent of degeneration can be scored by counting the total neurons lost in each animal as well as the population.
9. Repeat the analysis 2 more times (30 more animals per line per trial). The total number of worms exhibiting wild-type DA neurons from the three rounds of analyses is averaged. Statistical analysis for neuroprotection is then performed using the student's t-test ($p < 0.05$) to compare control worms (Pdat-1::α-syn; Pdat-1::GFP) with strains that over-express candidate genes in DA neurons.

Discussion

The age-dependent loss of dopamine neurons is a clinical hallmark of Parkinson's disease and has been associated with the accumulation or misfolding of a protein called alpha-synuclein. Here we demonstrate how to label, via a fluorescent transgene, the dopaminergic neurons of *C. elegans* and mimic the neurodegeneration seen in Parkinson's disease by coexpressing human alpha-synuclein in these cells.

This video depicts the methodology for growth, genetic crossing, mounting, and scoring of transgenic nematodes to evaluate genetic factors that either enhance neurodegeneration or provide neuroprotection over the course of aging. Care is taken to use markers for transgene maintenance appropriately staged male and hermaphrodites to ensure successful genetic crosses, and consistency in scoring neuron loss or protection. In this manner, *C. elegans* facilitates rapid evaluation of genetic factors that may either contribute to neurodegeneration or represent therapeutic targets for enhancing neuron survival.

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