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Strengths and limitations of morphological and behavioral analyses in detecting dopaminergic deficiency in *Caenorhabditis elegans*

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ABSTRACT

In order to develop a better understanding of the role environmental toxicants may play in the onset and progression of neurodegenerative diseases, it has become increasingly important to optimize sensitive methods for quickly screening toxicants to determine their ability to disrupt neuronal function. The nematode *Caenorhabditis elegans* can help with this effort. This species has an integrated nervous system producing behavioral function, provides easy access for molecular studies, has a rapid lifespan, and is an inexpensive model. This study focuses on methods of measuring neurodegeneration involving the dopaminergic system and the identification of compounds with actions that disrupt dopamine function in the model organism *C. elegans*. Several dopamine-mediated locomotory behaviors, Area Exploration, Body Bends, and Reversals, as well as Swimming-Induced Paralysis and Learned 2-Nonanone Avoidance, were compared to determine the best behavioral method for screening purposes. These behavioral endpoints were also compared to morphological scoring of neurodegeneration in the dopamine neurons. We found that in adult worms, Area Exploration is more advantageous than the other behavioral methods for identifying DA-deficient locomotion and is comparable to neuromorphological scoring outputs. For larval stage worms, locomotion was an unreliable endpoint, and neuronal scoring appeared to be the best method. We compared the wild-type N2 strain to the commonly used *dat-1*p::GFP reporter strains BY200 and BZ555, and we further characterized the dopamine-deficient strains, *cat-2* e1112 and *cat-2* n4547. In contrast to published results, we found that the *cat-2* strains slowed on food almost as much as N2s. Both showed decreased levels of *cat-2* mRNA and DA content, rather than none, with *cat-2* e1112 having the greatest reduction in DA content in comparison to N2. Finally, we compared and contrasted strengths, limitations, cost, and equipment needs for all primary methods for analysis of the dopamine system in *C. elegans*.

1. Introduction

As the population ages, a global increase in the occurrence of neurodegenerative and neurological diseases and disorders is expected over the next few decades. Neurodegenerative diseases are predicted to become the second leading cause of death by the year 2040 (WHO, 2004). As the etiology of these diseases is increasingly investigated, a common hypothesis that environmental toxicant exposure plays a role in onset and progression has emerged (Cannon and Greenamyre, 2011; Landrigan et al., 2005). One of the major neurodegenerative diseases thought to have a cause rooted in environmental factors is Parkinson's disease (PD). Marked by dysfunction of the dopaminergic system in particular, PD results in the loss of dopamine (DA) neurons in the substantia nigra pars compacta and decreased control of motor function (Shulman et al., 2011). The development of PD is linked to familial factors at only ≈10% (Lesage and Brice, 2009), meaning the cause of ≈90% of cases cannot be explained. Another DA-related disease with possible links to environmental factors is Attention Deficit Hyperactivity Disorder (ADHD) (Banerjee et al., 2007; Swanson et al., 2000). ADHD manifests as hyperactivity and disruption of cognitive disorders, such as memory and inhibiting behaviors (Barkley, 1997). While ADHD is strongly linked to genetics at ≈80%, there is still a ≈20% occurrence from factors that remain largely unknown (Faraone et al., 2005).

The theory that environmental toxicant exposure is a risk factor for

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neurodegenerative disease is gaining empirical support (Chin-Chan et al., 2015; Cicchetti et al., 2009; Coppedè et al., 2006; Modgil et al., 2014; Monte, 2003). Several epidemiological studies have shown correlations between environmental toxicant exposure and dopaminergic neuronal disorders. Elevated serum levels of β-hexachlorocyclohexane (β-HCH) have been found in PD patients, high occupational exposure to pesticides and metals have been linked to an earlier age of PD onset, and organophosphates have been correlated to attention and ADHD in children (Marks et al., 2010; Ratner et al., 2014; Richardson et al., 2009). Environmental release of the pesticide glyphosate has also been correlated to the prevalence of both PD and ADHD (Samsel and Seneff, 2015; Seneff et al., 2015). In a case-control study completed by Tanner et al. (2011), a correlation was discovered between PD and usage of the pesticides rotenone and paraquat. In addition, laboratory research in model systems has confirmed that environmentally relevant toxicants, such as some pesticides, have the ability to disrupt the dopaminergic system (Betarbet et al., 2000; Caito et al., 2013; Jones and Miller, 2008; McCormack et al., 2002). The Betarbet et al. (2000) rat study found that exposure to the pesticide rotenone can induce dopaminergic neurodegeneration and lead to the development of multiple symptoms characteristic of PD. In another study, exposure to the pesticide paraquat was also able to induce dopaminergic neurodegeneration in mice (McCormack et al., 2002). Some of these same toxicants, as well as others, were found to induce morphologically detectable dopaminergic neurodegeneration in the nematode *Caenorhabditis elegans* (Caito et al., 2013; González-Hunt et al., 2014; Hartman et al., 2019; Negga et al., 2012; Ray et al., 2014), and *C. elegans* is increasingly used in toxicological and neurotoxicological studies (Avila et al., 2012; Hunt, 2017; Leung et al., 2008). It may also be possible to investigate whether or not putative neurodegenerative compounds can elicit quantifiable behavioral defects in the worm. Behavioral studies in the *C. elegans* model have been well documented (Hart, 2006); many of the behaviors have been extensively characterized and classified by their mediating neurotransmitter (Chase and Koelle, 2007). This study focuses solely on a subset of the behaviors mediated by DA, which includes basal slowing rate, egg-laying, swimming-induced paralysis, and odor avoidance (Hardaway et al., 2012; Kimura et al., 2010; McDonald et al., 2007; Sawin et al., 2000; Vidal-Gadea et al., 2012). Basal slowing rate (BSR), the dopamine-mediated decrease in locomotion that results from mechanosensory perception of a bacterial food source (Sawin et al., 2000), has generally been considered the most promising method for analyzing behavioral changes after toxicant exposure. The Sawin et al. (2000) study utilized the dopamine-deficient *C. elegans* mutant strain *cat-2*, which carries a mutation in the tyrosine hydroxylase necessary for DA synthesis (Lints and Emmons, 1999), to analyze behaviors characteristic of depleted DA levels. The authors found that the *cat-2* mutant failed to slow forward motion on food. As the decrease in locomotion on food is a characteristic phenotype of wild-type N2 worms and a healthy dopaminergic system in *C. elegans*, our current study utilizes disruptions of this behavior as a proxy for DA dysfunction.

In addition to observation of changes to dopamine-mediated behaviors, a standard method for assessing dopaminergic alterations in *C. elegans* is visualization of morphological changes to DA neurons (Nass et al., 2002). While morphological and behavioral methods work relatively well, there are some caveats to both. To increase the utility of this model organism in neurotoxicological studies, we wanted to compare and contrast these approaches. Quantifying changes to neuronal morphology requires the use of specific transgenic worm strains expressing a fluorescent protein such as GFP in the DA neurons. With this morphological method, in order to test effects of toxicants in conjunction with genetic differences, one must either employ RNAi in the transgenic reporter strain, which can be complicated by the relative recalcitrance of neurons to RNAi knockdown in *C. elegans*, or use strains containing both the fluorescent transgene and the desired mutations, which would need to be created by crossing the transgenic strain with the mutant strain. In addition, there is some concern over whether the visually altered neuronal structure or loss of GFP in the neurons truly corresponds to neuronal degeneration as opposed, for example, to GFP degradation or aggregation. While the first paper to report the use of a transgenic strain to visualize neurodegeneration (Nass et al., 2002) did demonstrate that degeneration identified by fluorescence microscopy was comparable to that observed via transmission electron microscopy, it is not clear that this would be true for all environmental exposures and genetic manipulations. In addition, it is always possible that the transgenic changes to the dopaminergic cells could perturb the normal function of those neurons. Finally and perhaps most importantly, mechanisms of dopaminergic dysfunction that do not involve cellular degeneration would presumably not be detected by this method. With the behavioral method of DA system analysis, possible limitations include the necessity of reaching a threshold of degeneration before physical effects manifest (it is not clear whether behavior or morphological analysis would be more sensitive), uncertain specificity of the assays (i.e., are they controlled only by dopamine), and potential variability in the behaviors due to lab-to-lab variance of environmental factors. Conditions in the environment need to be carefully controlled for when completing behavioral analyses, as worms alter behaviors in response to laboratory conditions, such as light, temperature, and vibrations (Hart, 2006).

In this study, we compare the use of morphological changes to the DA neurons and DA-mediated locomotory behaviors as tools for measuring DA system dysfunction in *C. elegans*, including consideration of sensitivity, specificity, efficiency, and cost-effectiveness. Our initial hypothesis was that behavior would be the most sensitive method for identifying DA dysfunction, because we suspected dopaminergic neurons most likely function at an inadequate level preceding outright morphologically detectable degeneration. We hoped to use the findings in this study to develop a screening tool for assessing the effects of environmental contaminants on the dopaminergic system that can be utilized as a first step prior to mammalian studies, which tend to be costly and time consuming. There is also potential for the assays in this study to be used for screening protective and therapeutic treatments of dopaminergic neurodegeneration. With neurodegenerative diseases increasing in prevalence, uncovering and regulating causative factors has become imperative. We hope that information in this study will inform efforts to discover environmental contributors to dopaminergic neurodegeneration.

2. Materials & methods

2.1. Strains

Strains Bristol N2(WT), *cat-2*(e1112), *cat-2*(n4547), BZ555[egIs1(*dat-1p*::GFP)], *tbh-1*(n3247), *tdc-1*(n3420), *cha-1*(p503), *tph-1*(n4622), and UA57[baIs4(*dat-1*p::GFP + *dat-1*p::CAT-2)] were obtained from the *Caenorhabditis* Genetics Center (CGC). Strain BY200[vtIs1(*dat-1*p::GFP; *rol-6*)] was obtained from the Aschner Lab; studies used the strain in its original form [outcrossed four times: Nass et al. (2002)] or our lab's 8X outcrossed population. For our locomotion studies, it is important to note that the *rol-6* phenotype in BY200 is rarely present. We typically only see this rolling phenotype in severely stressed populations. Under normal conditions, BY200 worms move in a similar fashion to N2. Each experiment began with a synchronized population of L1 worms, obtained by sodium hydroxide/bleach lysis to harvest eggs and overnight liquid hatch in complete K-medium (Boyd et al., 2009). Worms were grown on K-agar OP50, bacterial food source, plates (Williams and Dusenbery, 1988) until the age specified in each experiment. Strains were maintained in 20 °C incubators at all times, unless being used in experimental procedures or transferred at room temperature.

2.2. Video acquisition

Locomotion videos, 10 s each, were recorded using NIS-Elements BR software and a Nikon SMZ1500 stereomicroscope. Before video capture, each agar plate had the lid removed and was allowed to sit on the microscope stage for ≈30 s to permit worm acclimation to placement. Lids were removed to avoid visual interference of internal lid condensation. Worms were allowed to acclimate to avoid video capture of tap-response or lid removal induced locomotion.

2.3. Behavioral analyses

For this study, we developed a low-cost assay we term Area Exploration (AE). This assay functions to calculate the total area covered and measures each newly explored area only once; any areas explored more than once, mostly due to backward and forward motion in the same spot, are not counted as additionally explored areas. The rationale for measuring total AE as opposed to total movement is that worms with normal dopamine function make a large number of small movements within a given patch of food, but rarely leave the area with food until the food is depleted. Thus, if we count this small back-and-forth (in the same area) movement, we would reduce our ability to quantify the inappropriate, away-from-food movement mediated by dopamine deficiency. Therefore, instead, we measure total area explored, which increases as dopamine deficiency reduces the worms' ability to sense food. AE was assessed using a macro (Fig. S1A) created in Fiji (Schindelin et al., 2012) to construct pathways of worms throughout prerecorded 10 s locomotion videos (Fig. 1). The macro functions by first creating a pathway for each worm using the "Z-project…" function. Once pathways were completed, the "Analyze Particles…" function was used to quantify the area of each pathway. The individual sizes of the worms were also quantified using the same prerecorded videos and a similar macro (Fig. S1B). The resulting size data was used for AE normalization purposes; we divided the area values from the AE macro by the correlating worm size values from the Size macro. Normalization to size was used to control for size differences due to treatment (since a growth-delayed/small worm must move more to cover the same area as a large worm), age, light source, and light bending due to the food status/thickness of agar plates.

For adult strain comparison locomotion studies, age-synchronized populations of worms were grown until reaching the gravid adult stage and then recorded. To avoid any interference of transfer-induced stress, videos of these populations were recorded directly on the growth plates. Sample populations consisted of three biological replicates, defined as experiments performed on different days using worms obtained from different bouts of synchronization. For comparison of locomotion with and without a food source, synchronized gravid adult worms were washed from growth plates, gravity settled, and rinsed (supernatant was removed and replaced with fresh K-medium 2X) to remove any transferred bacteria. Sample populations were then divided between two new agar plates, one with OP50 and one without any food source. Transferred worms were allowed to dry and acclimate to new plates for 30–60 m at room temperature before video acquisition. Acclimation during this step is necessary to avoid video capture of increased movement observed after transfer and to ensure agar plates are dry; this increased movement disappears shortly after the liquid transferred along with the worms evaporates or is absorbed. Agar plates containing excessive surface moisture result in thrashing movements (swimming) and not body bends. Sample populations consisted of five biological replicates.

Body bends (BBs) and Reversals were manually quantified from identical worms using the same 10 s locomotion videos as the AE assay. If movement resulted in forward motion and propagated a wave along the body of the worm, it was counted as one body bend. The initiation of backward motion from forward movement or a stopped position was counted as one reversal.

For the DA-mediated swimming induced paralysis (SWIP) (McDonald et al., 2007), ≈10 day 1 adult worms were picked into 300 µl molecular grade water in 48 well plates with a platinum wire pick. All worms in a specific well were transferred all together and the start time on a stopwatch was recorded when worms were placed in the liquid. After a 30 min incubation at room temperature, individual wells were observed under a stereomicroscope, and the number of worms paralyzed and the number of worms in total were recorded. Paralysis was determined when there was a complete absence of movement in both the body and the head; it is typical for the body to paralyze first without paralysis of the head. The percentage of worms paralyzed was calculated per well. This experiment consisted of three biological replicates with three technical replicates each.

As another measure of dopaminergic function, the enhancement of odor avoidance to 2-nonanone by preconditioning was used as described (Kimura et al., 2010), with minor adaptations. ≈600-1000 day 1 adult worms were washed off OP50 plates with K-medium into 15 ml conical tubes, allowed to gravity settle, and the supernatant was removed and replaced with fresh K-medium. This process was repeated three times to remove most bacteria from the worms. The worms were then divided among three 60 mm "pre-exposure" agar plates by transferring 10 µL of pelleted worms to each plate. Each of these "pre-exposure" plates had six agar plugs on the lid (at the five points of a star with one plug in the center; Fig. S2A). Once the liquid transferred with the worms had completely evaporated from the plates, we added 0.1 µL 10% 2-nonanone in ethanol to each plug on one plate lid ("pre-exposed" group), 0.1 µL ethanol to each plug on another plate lid ("mock" group), and nothing on each plug of the final plate lid ("naïve" group). The worms were left on these plates for 60 min. Following this 60 min incubation, they were washed off the plates and immediately plated on avoidance assay plates, 60 mm agar plates divided vertically into six equal sections by lines drawn on the bottom using a 3D-printed stencil. The stencil included two dots, for odorant placement, spaced equidistant from the center in the second section and a dot, for worm placement, in the center of the plate (Fig. S2B-C). The worms were transferred in a drop of liquid (5 µL) to the center and allowed to dry (50–150 total worms per plate). When the worms began to crawl away from the center, 0.3 µL 10% 2-nonanone in ethanol was added to each odorant dot and the plate was closed and inverted. After a 12 min incubation, the plate was transferred to the -20 °C freezer to prevent

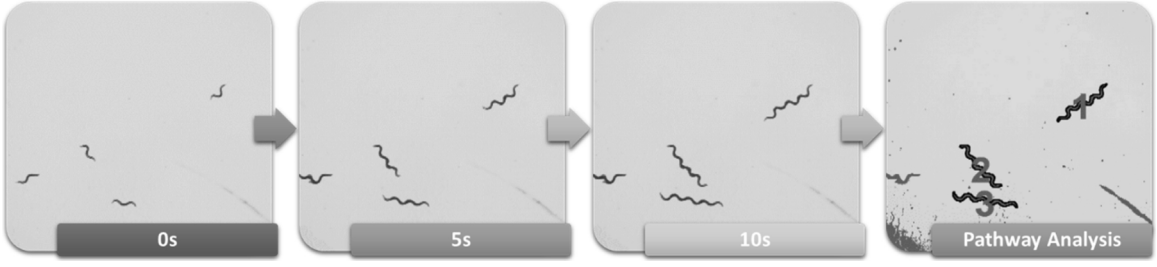


Fig. 1. Depiction of Area Exploration Analysis Process. - Pathways are constructed from 10 s locomotion videos and analyzed for total area coverage.

Table 1
Neuron Scoring Categories – Explanation of each scoring category used for assessing neurodegeneration through morphological alterations in the transgenic BY200 strain.

Neuron Scoring Categories
0 |Normal
1 |Slight Structural Changes (Thin/Crooked/Branched)
2 |Blebs
3 |Breaks
4 |Only CEP Cell Bodies Present
5 |Complete Absence of CEP Neuron

further worm movement. (Note: Incubation in the freezer was brief, and plates were not allowed to freeze completely.) After worms slowed further movement, plates were removed from the freezer, and the number of worms in each section was counted and used to calculate the avoidance index. [The avoidance index is a weighted index, which represents the number of worms in each section multiplied by a weight and then summed; the weighted sum is then divided by the total number of worms on the plate. The weights were −2.5 for Section 1, −1.5 for Section 2 (containing the spots of odorant), −0.5 for Section 3, 0.5 for Section 4, 1.5 for Section 5, and 2.5 for Section 6.] The experiments were done with three technical replicates (plates) per group and per pre-exposure condition for each of three biological replicates. For all replicates, the experimenter was blind to strain or treatment.

2.4. Neuronal scoring

After video acquisition, ≈10 BY200 worms from each treatment group were picked with a platinum wire into one well, per group, of a 96-well plate containing 100 µl K-medium and 10 µl of a paralytic agent, either 300 mM tetramisole hydrochloride (Sigma) or 10 µl 200 mM sodium azide (Sigma). Once paralysis was complete, fluorescence images of the DA neurons in the head region were captured on a Keyence BZ-X All-in-One Fluorescence Microscope. Using the captured images, the four cephalic (CEP) neurons of paralyzed worms were manually scored (Table 1) based on an adapted version of a previously described scoring system used in our laboratory (González-Hunt et al., 2014). Representative images illustrating morphological alterations used for scoring can be found in Supplemental Fig. 3.

2.5. cat-2 mRNA analysis by real-time reverse-transcription PCR

RNA was isolated from frozen samples of 1000–5000 L4 worms using the Qiagen RNeasy Mini Kit with on-column DNase digestion using the Qiagen RNase-Free DNase Set. RNA concentration was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Next, RNA was converted to cDNA with the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. The resulting cDNA samples were diluted to 2 ng/µl in Sigma water and expression levels were quantified using Real-Time PCR with the 7300 Real-time PCR System (Applied Biosystems), PowerSYBR Green PCR Master Mix (Applied Biosystems), and forward/reverse primers (Integrated DNA Technologies) for the *cat-2* gene (Fig. 2B). Primers were positioned downstream of the e1112 point mutation locus (Fig. 2A); this location was chosen to amplify transcripts from all three curated isoforms of *cat-2* in *C. elegans*. Gene expression fold change was calculated by normalizing the cycle threshold (Ct) values for *cat-2* to the combined average expression of housekeeping genes *cdc-42* and *tba-1* (Fig. 2B). Samples from five biological replicates were run in triplicate (n = 5, each sample assayed in technical triplicate).

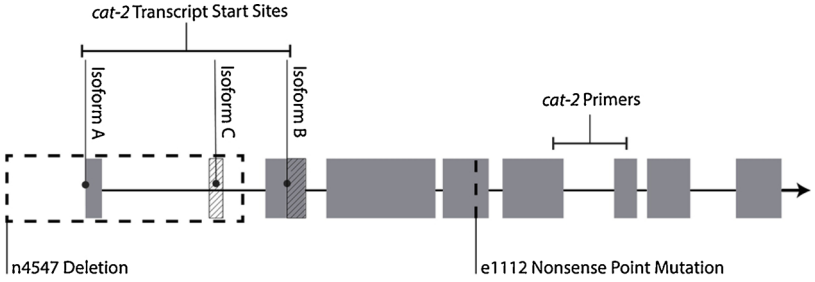
2.6. ELISA analysis of dopamine levels

Frozen samples of ≈2500-11000 L4 worms were thawed and sonicated in TE buffer using a Model 3000 Ultrasonic Homogenizer (Biologics, Inc.). Samples were sonicated at 10% power for 10 s three times, and kept on ice between sonication periods. After sonication, 10 µl aliquots of each samples were frozen for future protein quantification with Pierce BCA Protein Assay Kit (Thermo Scientific). The remaining sample volume was centrifuged, and the supernatant was used for DA quantification with the ABNOVA ultra-sensitive Dopamine ELISA Kit (KA3838). ELISA results were normalized to protein concentration levels. Samples from seven biological replicates were run in duplicate or triplicate.

2.7. 6-OHDA dosing

For larval studies, 1000 synchronized L1 worms were incubated at 20 °C for 1 h in 0, 10, or 15 mM 6-hydroxydopamine hydrochloride (6-OHDA). For adult studies, 300 synchronized L4 worms were incubated at 20 °C for 1 h in 0, 25, 50, or 100 mM 6-OHDA. 6-OHDA (Sigma) doses were from a 100 mM (or 200 mM for high dosing experiment) stock

A



B

Gene	Amplicon	Primer Sequence
cat-2	228bp	F: CCTGGCCTCACTTGCTTTTC
		R: TCCACAATGAACCAATAAACCGT
cdc-42	110bp	F: GAGAAAAATGGGTGCCTGAA
		R: CTCGAGCATTCCTGGATCAT
tba-1	238bp	F: TGATCTCTGCTGACAAGGCTT
		R: CCGACCTTGAATCCAGTTGG

Fig. 2. Information on *cat-2* mRNA quantification. - (A) Schematic of *cat-2* isoforms, locations of *cat-2* strains' mutations, and location of *cat-2* primer set. (B) Size and sequence information for primers sets used in mRNA assay. All primers had an annealing temperature of 60 °C.

solution dissolved in 20 mM (or 40 mM for high dosing experiment) ascorbic acid to prevent oxidation; all samples within individual experimental replicates were dosed in equivalent concentrations of ascorbic acid. Due to the rapid oxidation of 6-OHDA, worms were dosed in closed 1.7 u L microcentrifuge tubes in the absence of bacteria. After 1 h incubation, worms were gently centrifuged [2200 rcf (L1) or 1000 rcf (L4), 2 m (L1) or 30 s (L4)] and rinsed (aspirated to pellet, re-suspended in ≈1 mL K medium and re-centrifuged) six times to ensure removal of 6-OHDA. Worms were then plated and allowed to grow on agar plates. 1, 2, or 3d post-exposure, video of worm locomotion on the growth plates was captured. Immediately following video acquisition, on each day, worms were picked for neuron scoring. Larval studies consisted of sample populations from two biological replicates and adult studies consisted of three to nine biological replicates.

2.8. Statistical analysis

All experiments were analyzed using JMP Pro 13 or 14 for Windows (SAS Institute Inc., Cary, NC). Each statistical analysis began with a 1, 2, or 3-way ANOVA, except for Neuronal Scoring, which began with an Ordinal Logistics Chi Squared Whole Model Test. Statistical significance was set at a p-value less than 0.05. Post-hoc tests include Dunnett, Student's *t*-test, and Tukey HSD. All bar graphs include error bars constructed using standard error of the mean.

3. Results

3.1. Time course of behavioral changes and neurodegeneration after 6-OHDA larval dosing

We created an analysis of "area explored," in which we expected that the area covered by a worm on food would increase as dopamine deficiency reduces the worms' ability to sense the food via mechanosensation. In order to compare sensitivity of AE and neuronal scoring, N2 and BY200 worms were treated with 6−OHDA to induce dopaminergic neurodegeneration. 6-OHDA was our toxicant of choice for inducing neurodegeneration, because it has been shown to selectively target the dopaminergic system (Nass et al., 2002). Worms were dosed at their first larval stage immediately after hatching and monitored over the course of 3 days for changes to locomotion on food and changes to neuron morphology. There were no detectable 6-OHDA-induced effects on locomotion on days 1 and 2 (Fig. 3A). On day 3, a significant increase in AE can be seen at 10 mM 6-OHDA in strain BY200. As expected based on our previous work (González-Hunt et al., 2014), however, 10 and 15 mM 6-OHDA induced significant dose-related changes to neuron morphology at all timepoints (Fig. 3B). Locomotion changes in N2 after 6-OHDA were analyzed simultaneously with BY200. There were no detectable effects of 6-OHDA in N2s (Fig. 3C). Plotting the same data in Fig. 3C as absolute values rather than percent control (Fig. 3D) highlights the differences in locomotion between the two strains without regard to specific 6-OHDA effects. Strain differences

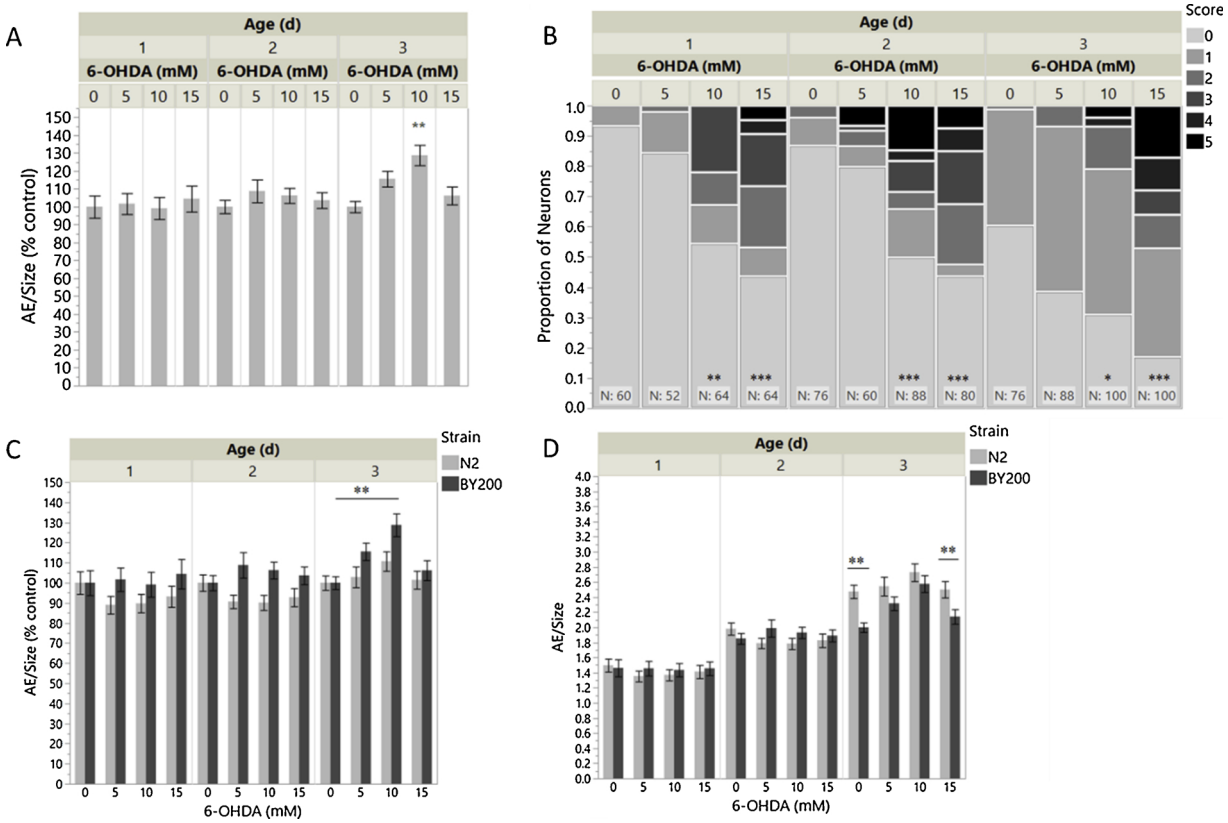


Fig. 3. Time course of behavioral changes and neurodegeneration after 6-OHDA larval dosing. – (A) Area exploration normalized to size and (B) neurodegeneration scores over the course of 3 days following 1 h L1 exposure to 6-OHDA in BY200. (C; D) Area exploration normalized to size in N2 and BY200 over the course of 3 days following 1 h L1 exposure to 6-OHDA. "AE/Size (% control)" shows significance in comparison to strain-matched 0 mM 6-OHDA control. "AE/Size" shows significance in comparison to dose-matched N2 control. All experiments were completed with two biological replicates. (A) n = 777; 2-way ANOVA 6-OHDA*Age p = 0.0006; Tukey HSD [10 mM,3d vs. 0 mM,3d p = 0.0015]. (B) n = 908 neurons/227 worms; 2-way ANOVA 6-OHDA*Age p < 0.0001; Tukey HSD [15 mM,3d vs. 0 mM,3d p < 0.0001; 15 mM,2d vs. 0 mM,2d p < 0.0001; 10 mM,2d vs. 0 mM,2d p < 0.0001; 15 mM,1d vs. 0 mM,1d p < 0.0001; 10 mM,1d vs. 0 mM,1d p = 0.0021; 10 mM,3d vs. 0 mM,3d p = 0.0222]. (C) n = 1489 3-way ANOVA 6-OHDA*Age*Strain p < 0.0001; Tukey HSD [BY200,10 mM,3d vs. BY200,0 mM,3d p = 0.0011]. (D) n = 1489 3-way ANOVA 6-OHDA*Age*Strain p < 0.0001; Student *t*-test [N2,0 mM,3d vs. BY200,0 mM,3d p = 0.0003; N2,15 mM,3d vs. BY200,15 mM,3d p = 0.0043].

in AE were observed at 0 and 15 mM 6-OHDA on Day 3, with N2 moving significantly more than BY200.

3.2. Characterization of locomotion in adult worms

We observed no 6-OHDA-induced behavioral changes in early larval stages, even when neurodegeneration was detectable. It may be that movement in larval stages is disrupted or too variable, possibly due to molting lethargus (Cassada and Russell, 1975) or strain-specific developmental rates, for reliable locomotion analysis. However, because comparable locomotion and neurodegeneration effects were seen in the BY200 strain in adults, we next analyzed AE in adult populations in more depth. To ensure we were capturing effects of DA deficiency, two DA deficient mutants were used as positive controls alongside N2 and BY200. The DA-deficient strains carry mutations in *cat-2*, the tyrosine hydroxylase involved in the production of DA. As locomotion is a DA-dependent behavior, these strains should exhibit altered movement in comparison to WT: specifically, they should exhibit a higher rate of movement in the presence of a food source. The nonsense point mutant *cat-2* e1112 ("cat-2_point") contains a stop codon in the middle of the gene (Fig. 2A), has been the more widely used strain in previous locomotion studies, and is well characterized (Lints and Emmons, 1999; Sawin et al., 2000; Yao et al., 2010); the deletion mutant *cat-2* n4547 ("cat-2_deletion") contains a deletion eliminating two of the three transcriptional start sites (Fig. 2A), and is a less well-studied strain (Omura et al., 2012). Observation of worms on growth plates revealed increased AE in all strains compared to WT, with the *cat-2* point mutation mutant showing the greatest and most significant difference (Fig. 4). To further characterize locomotion among adult worms and to compare our lab's method of locomotion analysis to other established methods, we split the worms from the growth plates into two populations. One-half of the worms were transferred to plates without a food source and the other half were transferred to plates containing a lawn of OP50. All endpoints (AE, BBs, and reversals) were measured using the same video recordings of worm locomotion; BBs and Reversals use the exact same individual worms. In conditions of no food, the *cat-2* point mutation strain exhibited a significantly higher rate of AE than WT (Fig. 5A). In conditions with food, BY200 and both DA-deficient strains exhibit significantly higher rates of locomotion than WT. BSR [(On-Off)/Off], calculated using AE data on/off food, is significantly lower in BY200 and the *cat-2* deletion strain compared to WT, but surprisingly not significantly different in the *cat-2* point mutation strain (Fig. 5B). In conditions of no food, BY200 exhibits fewer body bends in comparison to WT (Fig. 5C). In on-food conditions, BY200 and the DA-deficient strains exhibit a greater number of body bends than WT. The BSR, calculated from BBs on/off food, is significantly less in BY200 and both DA-deficient strains (Fig. 5D). In conditions of no food, BY200 exhibits a greater number of reversals than WT (Fig. 5E). On food, BY200 and the DA-deficient strains exhibit a smaller number of reversals in comparison to WT, with the most significance seen in the DA-deficient strains.

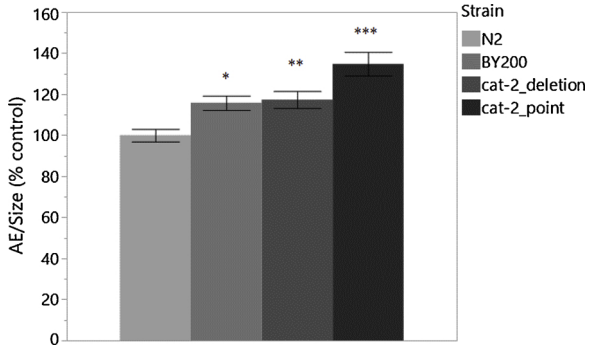


Fig. 4. Area exploration in adult worms. – Comparison of AE in WT, BY200, and DA deficient strains after reaching adulthood normalized to N2 control. Experiment was completed with 3 biological replicates. n = 406 1-way ANOVA Strain p = < 0.0001; Dunnett [BY200 vs. N2 p = 0.0280; cat-2_deletion vs. N2 p = 0.0080; cat-2_point vs. N2 p < 0.0001].

In order test the specificity of the AE assay to DA deficiency, we tested other neuronal mutants using this endpoint. We compared basal locomotion in the presence of food between several strains containing mutations in the cholinergic (*cha-1*), serotonergic (*tph-1*), tyraminergic (*tdc-1*), octopaminergic (*tdc-1* and *tbh-1*), and dopaminergic (*cat-2*_point) neurons. We also included WT and strain UA57, which overexpresses *cat-2,* to this study. The results of this study show that the AE assay has the ability to detect deficiencies in DA and serotonin production pathways (Fig. 6).

In the search for a more DA specific assay, we conducted supplementary experiments analyzing other assays that have been described as dopaminergic-dependent: SWIP (McDonald et al., 2007) and learned 2-nonanone avoidance (Kimura et al., 2010). For SWIP analysis, we compared WT, *cat-2*_point, UA57, *tbh-1*, and *tph-1*. We chose to include UA57 and *tbh-1* in this assay, because both mutants showed increased SWIP in our preliminary assays, and previous studies have shown SWIP is regulated by increased DA function (Hardaway et al., 2012); *tph-1* was chosen due to the results of the neuronal mutation strain comparison AE assay. In the manner we conducted this assay, we were able to identify a deficiency in the octopamine synthesis pathway and an upregulation in the DA synthesis pathway, but were not able to identify a difference between *cat-2*_point and *tph-1* (Fig. S4). (Note: Additional refinement of the SWIP assay parameters, such as age of worms and timepoint of analysis, could potentially lead to circumstances that create a higher baseline level of paralysis in WT and allow for detection of reduced SWIP). For 2-nonanone avoidance strain comparisons, we chose to compare WT, *cat-2*_point, and *tph-1*. The results indicate an enhanced avoidance index after pre-exposure in the WT strain, but neither mutant strain altered their avoidance after pre-exposure to 2-nonanone (Fig. S5B). Effects to WT avoidance were also not seen after 50 mM 6-OHDA exposure (Fig. S5A). It is important to note, several variable methods of analyzing nonanone or nonanol exist (Bargmann et al., 1993; Kaur et al., 2012; Salim et al., 2018; Sammi et al., 2018), such as immediate odor avoidance. However, we decided to use the Kimura et al. (2010) method for assessing DA-mediated learning, because the dopaminergic neurons (which are not chemosensory) play only a supplementary role in immediate avoidance behavior, precluding specificity to dopamine function. Nonetheless, there is potential that immediate nonanone avoidance might function as a nonspecific but sensitive method for detection dopaminergic dysfunction.

3.3. Molecular characterization of BY200 and DA-deficient strains

Previous experiments in this study identified a difference in locomotion between the DA-deficient strains. One explanation for this is a difference in DA production between the strains. As previously mentioned, one strain carries a nonsense point mutation presumably resulting in nonsense-mediated decay of any mRNA produced, and one carries a deletion mutation. While the point mutation (e1112) affects all three isoforms of the *cat-2* gene, the deletion mutant (n4547) only interferes with two of the curated isoforms. Based on the nature of these mutations and the locomotion observations, in which locomotion was generally more altered in the point mutant, we hypothesized the *cat-2* point mutant would have less CAT-2 protein production than the *cat-2* deletion mutant and as a result less DA production, making the *cat-2* point mutant the optimal strain for representation of a true DA deficiency. In order to identify the strain with the greatest DA deficiency, we first tested potential transcript-level changes by quantifying *cat-2*

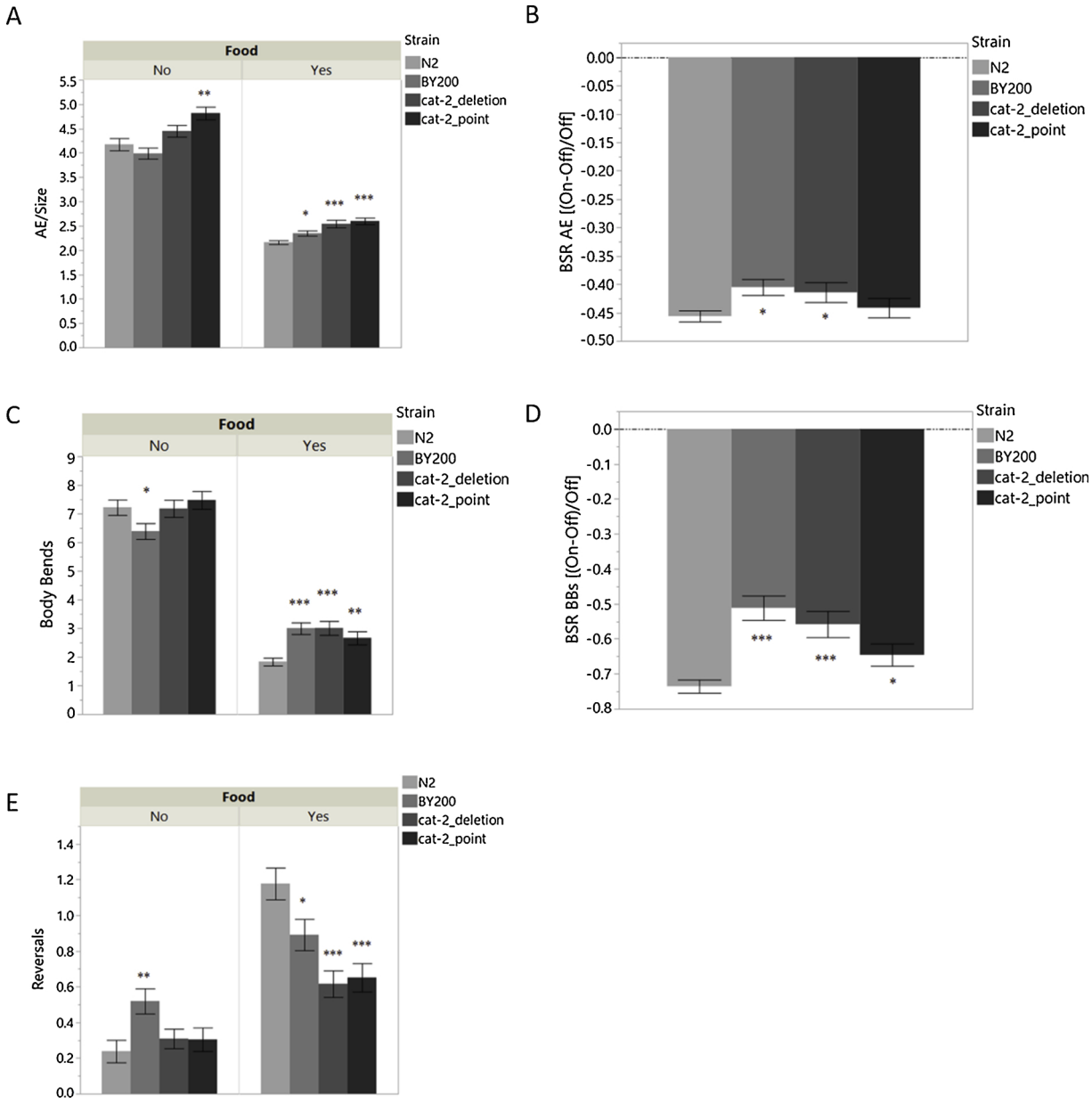


Fig. 5. Characterization of locomotion in adult worms. – (A) AE of adult strains On/Off food source. (B) BSR based on AE values. (C) BBs of adult strains On/Off food source. (D) BSR calculated using BBs values. (E) Reversals in adult strains On/Off food source. All analyses methods utilized the same sets of videos. BBs and Reversals utilized the same exact worms. All experiments were completed with 5 biological replicates. (A) n = 862 1-way ANOVA Strain [Food = No $p < 0.0001$; Food = Yes $p < 0.0001$]; Student *t*-test [(Food = No: cat-2_point vs. N2 $p = 0.0003$) (Food = Yes: cat-2_point vs. N2 $p < 0.0001$; cat-2_deletion vs. N2 $p < 0.0001$; BY200 vs. N2 $p = 0.0257$)]. (B) n = 862 1-way ANOVA Strain $p = 0.0447$; Student *t*-test [BY200 vs. N2 $p = 0.0123$; cat-2_deletion vs. N2 $p = 0.0380$]. (C) n = 651 1-way ANOVA Strain [Food = No $p = .0469$; Food = Yes $p = < 0.0001$]; Student *t*-test [(Food = No: N2 vs. BY200 $p = 0.0492$) (Food = Yes: cat-2_deletion vs. N2 $p < 0.0001$; BY200 vs. N2 $p = 0.0001$; cat-2_point vs. N2 $p = 0.0056$)]. (D) n = 651 1-way ANOVA Strain $p = < 0.0001$; Student *t*-test [BY200 vs. N2 $p < 0.0001$; cat-2_deletion vs. N2 $p < 0.0001$; cat-2_point vs. N2 $p = 0.0473$]. (E) n = 651 1-way ANOVA Strain [Food = No $p = 0.0122$; Food = Yes $p < 0.0001$]; Student *t*-test [(Food = No: BY200 vs. N2 $p = 0.0025$) (Food = Yes: N2 vs. cat-2_deletion $p < 0.0001$; N2 vs. cat-2_point $p < 0.0001$; N2 vs. BY200 $p = 0.0168$)].

mRNA. This experiment shows expression of *cat-2* mRNA is significantly lower in both *cat-2* mutant strains, but contrary to our expectations, the decrease in expression was more pronounced in the deletion mutant (Fig. 7A). This result suggests that the *cat-2* deletion mutant should have lower DA production than the point mutation. To try to understand why our locomotion observations were not as expected, based on *cat-2* mRNA content, we directly measured the DA content of each strain. As expected, DA content was decreased in the DA deficient strains (Fig. 7B). Contrary to *cat-2* expression patterns but consistent with behavioral results, the *cat-2* point mutant has somewhat less DA than the deletion mutant.

The apparent trends towards higher *cat-2* mRNA and DA content in the BY200 were not statistically significant; at least in the case of the mRNA, this may have been in part because expression was highly variable among biological replicates.

3.4. AE and neurodegeneration in adult worms after 6−OHDA L4 dosing

Our results so far indicate that AE seems to be the most sensitive method of locomotion analysis in adult worms; AE results also align better with expected results among the DA-deficient strains, based on molecular observations and mutant characteristics. To further test this

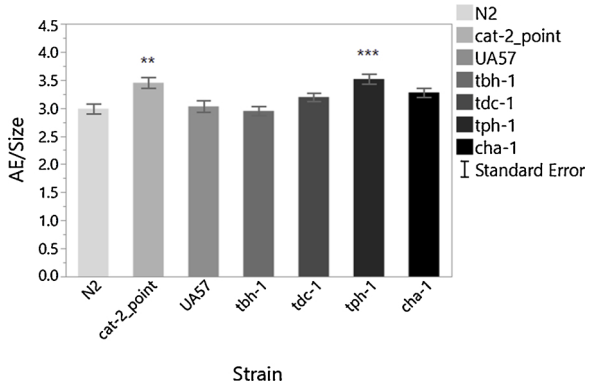


Fig. 6. Area Exploration in mutant strains deficient in neurotransmitter production. – The Area Exploration assay is sensitive to decreases in dopamine (*cat-2*) and serotonin (*tph-1*). n = 778 worms 1-way ANOVA Strain $p < 0.0001$; Dunnett [N2 vs. cat-2_point $p = 0.0006$; N2 vs. tph-1 $p < 0.0001$].

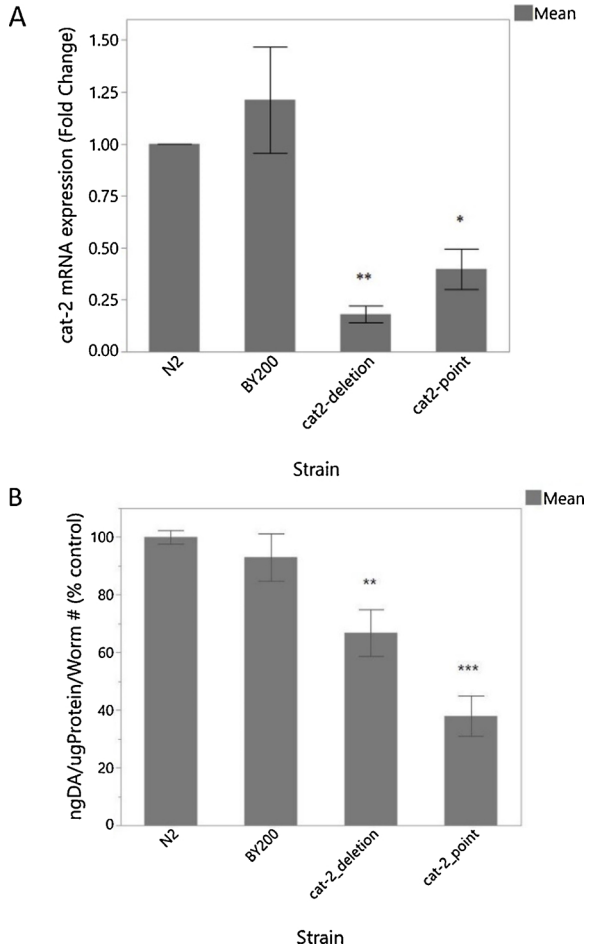


Fig. 7. Molecular characterization of BY200 and DA-deficient strains. – (A) RT-PCR mRNA quantification of *cat-2* gene expression. (B) ELISA quantification of DA content. RT-PCR was completed with 5 biological replicates and ELISA was completed with 4 biological replicates. (A) n = 20 1-way ANOVA Strain $p = 0.0002$; Student *t*-test [N2 vs.cat-2_deletion $p = 0.0007$; N2 vs. cat-2_point $p = 0.0073$]. (B) n = 59 1-way ANOVA Strain $p < 0.0001$; Student *t*-test [N2 vs. cat-2_point $p < 0.0001$; N2 vs. cat-2_deletion $p = 0.0043$].

assay's sensitivity, we compared AE to neurodegeneration in adult worms after toxicant exposure. For this purpose, we dosed N2, BY200, BZ555, and the *cat-2* point mutant with 25 and 50 mM 6−OHDA. We did not use the deletion mutant since it showed weaker phenotypes than did the point mutant. We exposed L4s instead of L1s, because development of DA neurons is not complete until the L2 stage when the two posterior deirid neurons, PDE, are formed (Altun et al., 2002-2018Altun ., - et al., 2018Altun et al., 2002-2018), and we wished to ensure exposure of all dopaminergic neurons. However, we found that these neurons do not seem to be affected by 6-OHDA at these concentrations (data not shown); therefore, scoring reflects CEP neurons only, as they are the primary targets of 6-OHDA exposure. AE and neuronal scoring assays were both able to identify significant differences after 6-OHDA treatment in the BY200 strain (Fig. 8A–B). BZ555 showed a significant response to 6-OHDA treatment in the neuronal scoring assay (Fig. 8B) but did not exhibit any behavioral changes (Fig. 8A). There were no significant dose dependent changes to N2 or *cat-2* e1112 behavior (Fig. 8A).

4. Discussion

4.1. Area exploration vs. neurodegeneration

Significant neurodegeneration was seen during larval stages, but effects of 6-OHDA on locomotion were not easily detected until worms reached adulthood. While the cause of this discrepancy is not currently known, we believe there could be several factors involved. One possibility is that the larvae are not moving much due to molting stage lethargus around the timepoints chosen in this experiment; although, we cannot exclude other possibilities, such as less innate DA signaling mediated behavior at this life stage. Because strains or treatment could result in differential developmental rates and lethargus at different times, AE analysis in larvae could lead to possible false positive or negative results.

In BY200 adults, the effects of 6-OHDA were detectable using either AE or neuronal scoring. There was a similar dose response using both endpoints. This result, along with the basal AE observed in *cat-2* mutants, even off food (although the mechanistic basis for this is unclear), makes a strong case for AE being a dopamine-mediated behavior. In comparison to BY200, neurodegeneration in BZ555 seemed to respond in a similar, although statistically different, dose dependent manner to 6-OHDA treatment but had no detectable behavioral response. The difference in sensitivity, detected by neuronal scoring, between BY200 and BZ555 could be attributed to their genetic difference, as BZ555 does not carry the *rol-6* gene. The lack of behavioral response in AE by BZ555 is not as easily explained, as there was detectable neurodegeneration at levels shown to coincide with increased locomotion in BY200. Interestingly, while *cat-2* mutants move significantly more than WT under control conditions (as expected), increasing 6-OHDA treatment had no further effect on movement, suggesting that despite the residual DA we detected by ELISA, DA deficiency-mediated increased AE may already be maximal in the *cat-2* point mutant.

4.2. Possible non-monotonic dose-response in movement

In larvae, we observed an effect of 6-OHDA at 10 mM but not 15 mM 6-OHDA. This seemingly counterintuitive finding may be explained by processes that have opposite effects on our outcome: decreasing DA function leads to increasing AE, but increasing general toxicity may eventually overcome this increased movement. We were unable to provide concrete proof that this effect is occurring. We attempted several high dose experiments with L4 dosing and reached a 6-OHDA solubility limit before being able to detect whole organism effects of 6-OHDA. While we do not have proof that this is the case in our experiments, we highlight this possibility to alert other researchers about using exposure levels that are too high, particularly with chemicals with greater acute organismal toxicity than 6-OHDA. Bypassing lower doses that could elicit increased locomotion, could lead to toxicants not being identified by chemical screens. It is thus important to use a range of doses and identify doses that are optimal in terms of detecting an effect.

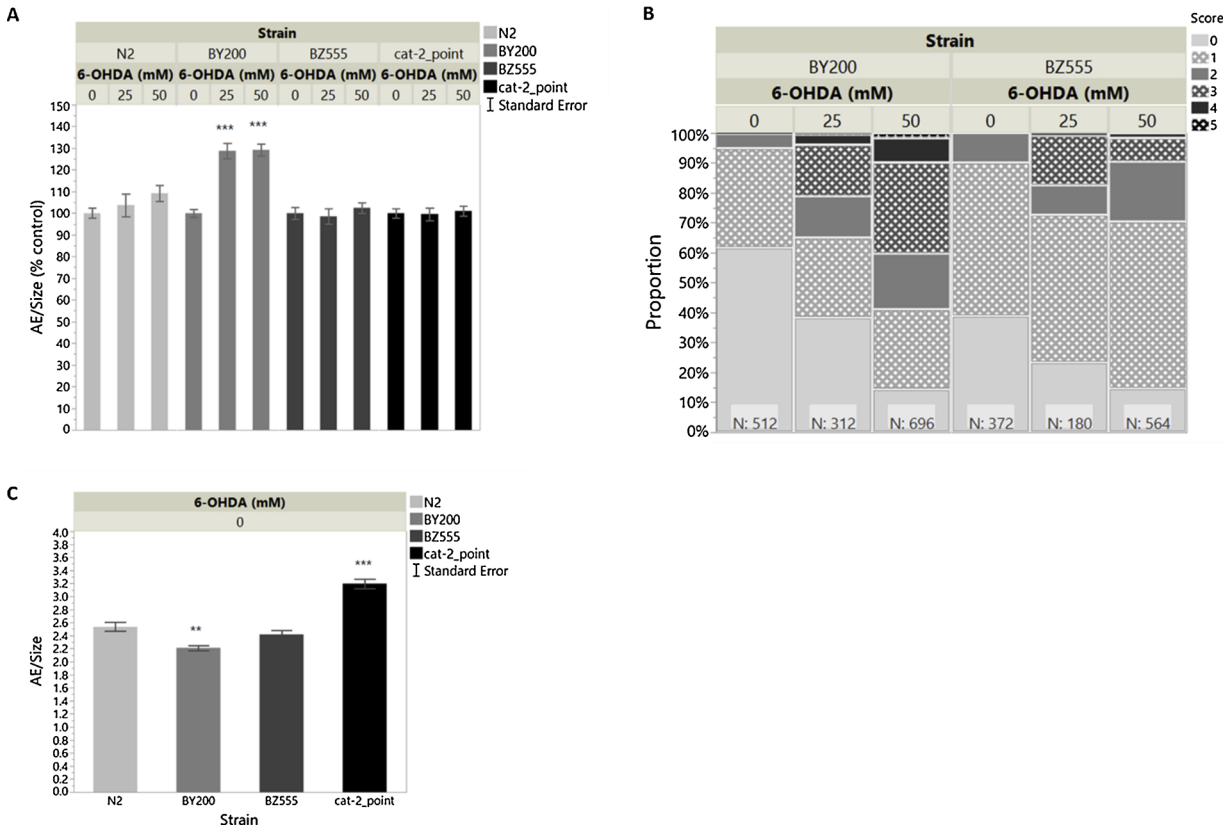


Fig. 8. AE and neurodegeneration in adult worms after 1 h L4 exposure to 6-OHDA. - (A) Area Exploration/Size normalized to strain matched 0 mM 6-OHDA control. "AE/Size (% control)" shows significance in comparison to strain-matched 0 mM 6-OHDA control. n = 1970 worms 2-way ANOVA Strain*6-OHDA $p < 0.0001$; Tukey HSD [BY200,25 mM vs. BY200,0 mM $p < 0.0001$; BY200,50 mM vs. BY200,0 mM $p < 0.0001$]. (B) Neurodegeneration scores of BY200 and BZ555. n = 2636 neurons/659 worms Ordinal Logistics Chi Squared Whole Model Test $p < 0.0001$; Effect Likelihood Ratio Chi Squared Tests [Strain $p = 0.0234$; 6-OHDA $p < 0.0001$; Strain*6-OHDA $p < 0.0001$]. (C) Basal Area Exploration/Size in N2, BY200, BZ555 and the *cat-2* point mutant following 1 h L4 exposure to 0 mM 6-OHDA; these strain underwent the 6-OHDA protocol and were exposure to ascorbic acid only. "AE/Size" shows significance in comparison to dose-matched N2 control. n = 759 worms 1-way ANOVA Strain $p < 0.0001$; Dunnett [N2 vs. BY200 $p = 0.0041$; N2 vs. cat-2_point $p < 0.0001$]. Note: All graphs in this figure are a combination of 3 separate experiments, each completed with 3 biological replicates.

4.3. AE vs. BBs vs. Reversals

All of the tested methods of analyzing basal locomotion on agar plates were adequate for identifying DA deficiency in on-food conditions. However, AE was the sole method, in this study, for identifying DA deficiency off food. While looking at on-food and off-food conditions individually, AE trends aligned more with expected results, based on measured strain DA content. When comparing BSR, which takes into consideration locomotion in both food conditions, the BSR calculated using BBs seems to be a better identifier of DA deficiency. However, neither method of calculating BSR correlated to DA content. It is important to note that these endpoints were obtained using the same videos/population of worms. Whether the rate of movement of the *cat-2* point mutant is higher or lower in comparison to the other strains differs depending on analysis method; the *cat-2* point mutant has a significantly higher AE rate than BY200 and the *cat-2* deletion mutant on food, but fewer BBs in the same conditions. Understanding the basis of the differences between AE and BBs would require further analyses of subtle non-speed related behavioral changes, such as bending angle, bend amplitude, and wavelength, may need to be completed to get a clearer idea of the locomotion differences among strains (Angstman et al., 2016).

4.4. Molecular differences in the DA system among strains

Published results indicate that *cat-2* mutants should not have a difference in locomotion when on food compared to when off food (Sawin et al., 2000). This was not what we observed, perhaps due to differences between lab environments, transfer methods, or the type of bacteria used as a food source. Whatever the reason, we found that the *cat-2* mutants slowed when on food almost as much as did N2 worms. However, despite this rather mild phenotype, the two mutant strains were statistically dissimilar to each other as well. This finding led us to inquire about the differences in *cat-2* expression among the strains, in order to identify the strain with the greater DA deficiency to use as a positive control for future studies.

In contrast to some previous descriptions (Lints and Emmons, 1999; Omura et al., 2012), neither *cat-2* mutant is a null mutant. Of the two mutants, the point mutant shows a stronger phenotype. This is as expected, given that the deletion mutation leaves one of the three isoforms of the *cat-2* gene intact. While we chose ELISA determination of DA levels for this study, previous HPLC analysis has also shown that the *cat-2* point mutant retains about 40% DA levels relative to WT (Sanyal et al., 2004). We hypothesize that the residual DA production could be explained by the presence of tyrosinases, which can bypass the need for *cat-2* in the DA production pathway (Rios et al., 1999). In addition to the unexpected DA quantification results, the relatively high level of *cat-2* mRNA in the point mutant is also surprising, since this nonsense mutation would be expected to cause nonsense-mediated decay of the transcript. We speculate that the detected mRNA may reflect non-functional transcripts that escape nonsense-mediated decay. Laser ablation or other methods to selectively destroy dopamine-producing cells might permit further determination of the behavioral consequences of complete loss of dopamine function.

Table 2
Methods of analyzing dopaminergic system function. – This table lists the endpoints used in this study to examine different aspects of the DA system, from a molecular level to behavioral phenotypes. This purpose of this table is to provide insight into the options available and their advantages and disadvantages when assessing the state of the dopaminergic system. Price range depends on availability of assay specific equipment.

Method	Positives	Negatives	Cost	Assay Specific Reagents	Assay Specific Equipment
Neuron Scoring	• Direct visualization of neurons	• Must use strain with transgene insert *dat-1p*::GFP • Only identifies morphologically-based dopaminergic dysfunction	Low-Very High	• Paralytic agent ($)	• Fluorescence Scope ($$$$)
Area Exploration	• Less prone to user error	• Slightly time-consuming • Difficult to use for multi-strain comparisons at larval stages	Low - High	• None	• Stereomicroscope with Camera and Video Acquisition Software ($$$)
Body Bends	• Published standard method	• Very time-consuming • Identification of BBs can be difficult • Difficult to use for multi-strain comparisons at larval stages	Low - High	• None	• Stereomicroscope with Camera and Video Acquisition Software ($$$)
Reversals	• Easy to quantify	• Low output values • Difficult to use for multi-strain comparisons at larval stages	Low- High	• None	• Stereomicroscope with Camera and Video Acquisition Software ($$$)
cat-2 mRNA Quantification	• Detection of changes at the molecular level	• Requires high number of worms	Moderate - High	• RNeasy Kit with DNase Digestion ($) • cat-2 primers ($) • Power SYBR Green MM ($$)	• Real-time PCR System ($$$) • Thermocycler ($$)
DA ELISA	• Directly quantifies neurotransmitter changes	• Very time-consuming • Requires high number of worms	Moderate - High	• ABNOVA DA ELISA Kit ($$) • BCA Protein Assay Kit ($)	• Microplate Reader ($$$)

4.5. Differences between WT, BY200, and BZ555

A concerning finding in this study was that strain BY200 seems to be differentially affected by 6-OHDA treatment in comparison to the WT strain. In fact, BY200 was the only strain that showed a behavioral change after 6-OHDA treatment at our standard doses [at a higher concentration, 100 mM 6-OHDA, we were able to elicit a response from WT (Fig. S6)]. It is unclear if BY200 is more or less sensitive than WT, as we could have missed the optimal effect dose for behavioral changes in the other strains, but this differential response needs to be taken into consideration when identifying compounds as neurodegenerative. In addition to this difference in treatment response, all three methods of locomotion analysis identified a difference in locomotory behavior between N2 and BY200, which may mean BY200 should not be regarded as the "same as WT" when using it for neuronal scoring experiments. The explanation for these differences is unclear, as BY200 was constructed from the N2 strain (Nass et al., 2002). Potential explanations include, but are not limited to, general interference due to the overexpression of the transgene (or its components, *pdat-1*:GFP and *rol-6*), such as disruption by the transgene of expression of other RNAs, or an interference of cellular function by copious non-native expression of genetic product.

In an attempt to identify a DA neuron reporter strain more similar to WT, we also tested the BZ555 strain. BZ555 also contains a *dat-1*p::GFP transgene but does not have the *rol-6* marker and is listed on the CGC website (https://cgc.umn.edu/strain/BZ555) as less sensitive to 6-OHDA than BY200. We tested this strain in our 6-OHDA AE studies in conjunction with N2, BY200, and *cat-2*_point. We found BZ555 does not have a behavioral response to 6-OHDA treatment. This suggests the *rol-6* gene mutation, only present in BY200, may be linked to the differential behavioral response. The *rol-6* gene mutation leads to a cuticle collagen defect (Kramer et al., 1990), and we speculate it could possibly increase cuticle permeability to toxicant exposure. As previously stated, the reasoning behind the differential behavioral response between BY200 and BZ555 is potentially a little more complex than that, because BZ555 was shown to respond to treatment through neuronal scoring at similar levels of neurodegeneration that induced behavioral changes in BY200. In addition, we observed that BY200 displayed brighter GFP fluorescence than BZ555, and BZ555 neurons tend to appear much thinner in comparison, which is why our scoring system, developed using the bright BY200 strain, detected more minor damage in the control BZ555 groups than BY200.

4.6. Dopamine- and serotonin-mediated behavior

In this study, we used several commonly employed DA-mediated behaviors to attempt to identify one that was truly specific to DA deficiency. AE has been our main behavioral endpoint and readily identified the DA-deficiency in the *cat-2* mutant strains through the decrease in basal slowing in the presence of a food source. To test whether this phenotype was specific to DA deficiency, we compared the locomotion of several neurotransmitter deficient strains using the AE assay and found that deficiencies in serotonin were also detectable. This was surprising because the Sawin et al. (2000) study found that the serotonin-mediated form of BSR is the enhanced slowing rate and is only detectable after the worms have undergone conditions of starvation. Trying to tease apart these two neurotransmitter deficiencies has proven to be difficult. Neither the SWIP assay nor the 2-nonanone avoidance assay were able to identify differential phenotypes between *cat-2*_point and *tph-1*. Thus, the behavioral assays currently available could serve as a first-tier test of potential dopaminergic dysfunction, but additional research would be required to confirm dopaminergic involvement. The similarity in behavior between *cat-2* and *tph-1* mutants could not be explained by a similar decrease in dopamine; in fact, *tph-1* had approximately 30% higher dopamine content than N2 (as determined by DA ELISA in n = 3 experiments; data not shown). The

increased dopamine observed in *tph-1* suggests there is a possibility that mutant strains undergo compensatory processes that lead to altered levels of other neurotransmitters. It is important to note this potential caveat to the use of mutants in interrogating which neurotransmitters mediate which specific behaviors.

4.7. Comparisons among DA system function analyses methods used in this study

Table 2 offers comparisons of the primary methods addressed in this study. It highlights significant points to consider when choosing a method of analyzing the dopaminergic system in *C. elegans* after toxicant exposure. Neuron scoring is generally a low cost, straightforward method of assessing morphological changes to the DA system. In cases where a lab may not have easy access to a fluorescence microscope, the cost would increase significantly. One downside to this assay is the necessity of using strains with transgenic reporter constructs such as the commonly used p*dat-1*::GFP, which could possibly affect the sensitivity or resistance of the strains to toxicant exposure. Area Exploration, Body Bends, and Reversals all analyze locomotion in a similar manner with slightly different effort requirements. The biggest advantages of one of these locomotion analyses over the others is ease of use and increased automation. AE relies on computer-generated data, thereby relatively minimizing user error and increasing throughput, which is ideal for a screening tool. BBs requires manual counting of user defined bends, which can take a considerable amount of time and vary greatly from researcher to researcher based on their definition of what constitutes a body bend. Reversals are not as automated as AE, but identification and manual counting is simple and straightforward and therefore less subject to user differences. Unfortunately, reversals are not as numerous as forward bends per individual worm, resulting in lower output values and a potentially smaller dynamic range. The last two methods described in Table 2, *cat-2* mRNA quantification using RT-PCR and DA content analysis using ELISA, may be less useful as screening tools, considering they both require somewhat costly reagent kits, a high "n," and a considerable amount of time to complete. However, as these two methods analyze molecular function of the DA system, they can be quite useful for more thorough mechanistic studies of the effects that toxicants, already identified through a screening process, may have on the overall dopaminergic system in *C. elegans*.

5. Conclusion

While locomotion analysis is a promising tool, it is quite variable and currently not diagnostic for definitive proof of dopaminergic dysfunction. Analyzing behaviors is a quick and easy screening method, but further analyses would be needed to definitively say which specific neuronal system is disrupted. Morphological neurodegeneration appears to be a less variable and a more reliable method, but requires the use of strains containing a specific transgene for visualization. There are many strengths and limitations to consider when choosing between the two methods; locomotion analysis is effective for screening for behavioral changes, but is probably best used either before or in conjunction with neuronal scoring.

In the process of comparing locomotion and neurodegeneration, we found that quantification of *cat-2* mRNA content by RT-PCR and quantification of DA content by ELISA also show promise as methods of analyzing DA system function; it remains to be tested whether they will be responsive to chemical challenge. The choice of tool may depend on other aspects of experimental design and logistical constraints (Table 2), and for more in-depth mechanistic analysis, all of the methods taken together may tell a more informative story about what is happening to the DA system after toxicant exposure.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2019.07.002.

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