Title: Selective toxicity of L-DOPA to dopamine transporter-expressing neurons and locomotor behavior in zebrafish larvae

Running title: Selective L-DOPA toxicity in dopamine neurons of zebrafish larvae

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Abstract:

Dopamine signaling is conserved across all animal species and has been implicated in the disease process of many neurological disorders, including Parkinson's disease (PD). The primary neuropathology in PD involves the death of dopaminergic cells in the substantia nigra (SN), an anatomical region of the brain implicated in dopamine production and voluntary motor control. Increasing evidence suggests that the neurotransmitter dopamine may have a neurotoxic metabolic product (DOPAL) that selectively damages dopaminergic cells. This study was designed to test this theory of oxidative damage in an animal model of Parkinson's disease, using a transgenic strain of zebrafish with fluorescent labeling of cells that express the dopamine transporter. The pretectum and ventral diencephalon exhibited reductions in cell numbers due to L-DOPA treatment while reticulospinal neurons that do not express the DAT were unaffected, and this was partially rescued by monoamine oxidase inhibition. Consistent with the MPTP model of PD in zebrafish larvae, spontaneous locomotor behavior in L-DOPA treated animals was depressed following a 24-hour recovery period, while visually-evoked startle response rates and latencies were unaffected.

Keywords: zebrafish, L-DOPA, neurotoxicity, Parkinson's, monoamine oxidase, dopamine transporter

1. Introduction

Dopamine (DA) signaling is conserved across all animal species and is implicated in the neuropathology of Parkinson's disease (PD). PD is a degenerative disorder characterized by motor deficits caused by the death of DA producing cells in the pars compacta of the substantia nigra (SN), and the prevalence of sporadic PD increases substantially with age (de Lau and

Breteler, 2006). Traditional therapies have focused on augmenting DA levels through L-DOPA administration, or using DA receptor agonists to compensate for the reduced stimulation. Although these treatments effectively ameliorate symptoms, they have failed to demonstrate a disease-modifying effect (Rakshi et al, 2002). Monoamine oxidase inhibitors (MAOIs) are a promising adjunctive or monotherapy in PD and are theorized to reduce PD symptoms by limiting the metabolic degradation of DA by MAO (Riederer and Laux, 2011). DA is degraded into dopamine aldehyde (DOPAL) by MAO, and this may occur intracellularly or extracellularly after DA release. DOPAL is selectively toxic to neurons expressing the dopamine transporter (DAT) (Burke et al., 2004), suggesting that extracellular DOPAL enters neurons through the DAT. Once inside a neuron, DOPAL may cause oxidative damage to mitochondria leading to cell death (Burke et al., 2004; Kristal et al, 2001). This model is supported by the finding that ectopic striatal DAT expression results in vulnerability to L-DOPA toxicity in mice (Chen et al, 2008), while DA neurons in DAT knock-out mice were unaffected (Cyr et al., 2003). DAT expression is greatest in the SN (Fearnley and Lees, 1991) rendering these neurons especially vulnerable to dopaminergic toxicity. L-DOPA's therapeutic effect results from increasing brain DA concentrations, but the potential for L-DOPA to cause longer-term damage to DAT-expressing neurons by augmenting the concentration of DOPAL or other oxidative products has raised concern (Olanow 2015; Lipski et al., 2011)

The current study examined L-DOPA effects on dopaminergic neurons and locomotor behavior in transgenic Tg(*dat:eGFP*) zebrafish larvae. The GFP expression in this zebrafish line allowed individual DAT-expressing neurons within identified nuclei to be tracked before and after L-DOPA exposure. In a subset of larvae, L-DOPA was administered together with the MAO inhibitor selegiline to assess the role of DA metabolism in L-DOPA-mediated toxicity.

Levels of protein oxidation were analyzed in the same treatment groups to determine whether oxidative stress could account for any observed neurotoxicity.

Although zebrafish lack a direct homolog to the mammalian SN, the dopaminergic cells of the ventral diencephalon (vDC) have been proposed to perform a similar role in locomotor behavior (Flinn et al., 2009; Schweitzer, Lohr, Filippi, and Driever, 2012; Tay et al., 2011; Xi et al., 2011). vDC neurons are also vulnerable to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity (Lam, Korzh, and Strahl, 2005; Xi et al., 2011) and knockdown of the PD-associated genes *PINK1* (Anichtchik et al., 2008; Xi et al., 2010) and *parkin* (Kitada et al., 1998; Flinn et al., 2009). We hypothesized that L-DOPA administration would damage DAT-expressing cells, disrupt spontaneous swimming, and increase measures of oxidative damage, and that MAOI treatment would partially mitigate these effects.

2. Methods

Transgenic Tg(*dat:eGFP*) zebrafish embryos were obtained from the Center for Advanced Research in Environmental Genomics at the University of Ottawa, and raised as breeding stock. The housing, breeding, the composition of water for adult fish and of ‘egg water’ for larvae, lighting, and feeding, followed standard protocols described in the Zebrafish Book (Westerfield, 2000). All animal procedures were performed in accordance with the Humboldt State University animal care committee’s regulations. The sex of larvae used in experiments was not determined.

2.1. Drug treatments

Larvae aged 5 days post fertilization (dpf) were exposed to 1 mM L-DOPA ethyl ester (Sigma), dissolved in fish water, alone or in combination with 100 μ M selegiline (R-(-)-

deprenylhydrochloride (Sigma), or to a control solution containing regular fish water, for 24 hours in 60 mm petri dishes in an incubator (40 larvae maximum and about 25 mL of solution per dish; $n = 166$ total). Drug concentrations and exposure protocols were based on previous research using the same reagents in larval zebrafish (Sheng et al, 2010, for L-DOPA; McKinley et al., 2005, for selegiline). After incubation, drug was washed out twice by transferring larvae (using disposable 2mL transfer pipets) into new 60mm dishes containing regular fish water. Larvae were then prepared for neuron imaging and behavioral observation.

2.2. Neuron imaging

Larvae (aged 5-7 dpf) were anesthetized in egg water containing MS-222 (0.01% w/v; Sigma) prior to embedding in low melting temperature agar (1.2% w/v, kept moist with fish water) on a glass cover slip for imaging through the dorsal surface of the head. Imaging was done with an Olympus FV1000 confocal microscope system using a 20x .95NA water immersion lens. Automated 3D image series were acquired encompassing the area in which targeted neurons occurred, either within the pretectum or the ventral diencephalon. Consistent imaging settings (laser strength, confocal aperture, and gain) were used to control for equipment effects on image-based measurements.

Pretectal neurons (Figure 1) were imaged in larvae treated with L-DOPA, L-DOPA + selegiline, or a no-drug control solution ($n = 20$ per group), 24 hours after removal from the drug solutions (7dpf). A 100 μ m height, 100 μ m width, 40 μ m depth volume centered on the pretectum was imaged as a series of 25 images through the depth plane. This volume was sufficient to capture the entire pretectum bilaterally. All GFP-expressing cells within the imaged volume were manually labeled by a rater blind to the treatment condition and counted using the 3D Object

Counter plugin for ImageJ (NIH). Cell counts were compared using one-way ANOVA and a post-hoc Tukey's test in R. Neurons in the vDC were counted and analyzed using similar methods but were counted in each larva twice: before drug treatment and 1 hour after drug washout. A 200 μ m height, 200 μ m width, 40 μ m depth volume centered on the vDC was sufficient to capture all GFP-expressing cells within the structure.

Fig. 1. DAT-expressing cells in a 6 dpf zebrafish larvae. The image is a projection of 25 confocal images spanning 40 μ m. OB: olfactory bulb; TC: telencephalon; MHB: midbrain-hindbrain barrier. Inset: prepectum.

To assess the effects of L-DOPA on non-dopaminergic neurons, descending neurons in the hindbrain and midbrain were labeled (in a subset of 10 larvae in which vDC neurons were

also counted) with Alexa Fluor dextran 568 (molecular weight 10,000; Life Technologies) by backfill injection into the caudal spinal cord using a previously described method (Gahtan, Tangier, and Baier, 2005). Cell bodies of all labeled descending neurons, and of vDC neurons in the same animal, were counted before and after L-DOPA treatment. Drug effects on vDC and descending neurons were evaluated statistically by mixed model repeated measures ANOVA using R 3.1.2.

2.3. Behavior

Following washout from drug solutions a subset of larvae ($n = 58$) were transferred into individual wells of a standard 24 well tissue culture plate (~4 mL of regular fish water per well) for behavioral testing. Spontaneous swimming activity and light dimming-evoked startle responses were recorded 1 hour after drug washout and again 24 hours after drug washout. Spontaneous swimming was recorded during the day in an illuminated enclosure using a digital camera (Pixelink PLB-741) positioned below the recording plate. Images were acquired at 1 Hz for 10 minutes each hour over a 6 hour period and analyzed offline with custom scripts in ImageJ (NIH). Successive image pairs were subtracted to reveal changes in a larva's position from one frame to the next, and results were expressed as percentage of time in motion during the recording period.

Following spontaneous swimming recordings, 5 light dimming trials were run. A light (13 watt CFL bulb positioned 1m above the recording plate projected through a light diffusing plastic sheet) remained on for 60 minutes and was then extinguished (referred to as dimming) for 1 minute. A high speed digital camera (PhotonFocus D1312) positioned below the recording plate captured 5 seconds of video at 60 frames per second beginning 2 seconds before dimming

onset. IR illumination and a visible light blocking filter on the camera allowed behavior to be imaged in the dark after dimming. Startle images were analyzed in ImageJ using background subtraction and thresholding to resolve the larvae as a group of connected pixels (particle) and tracking the XY position of its center. Response rate and latency was measured for each larva on each dimming trial. A startle response was defined as a movement of the particle's center of more than 5 pixels within 2 seconds after dimming, and startle events detected by the software were confirmed by a manual rater blind to group assignment. Latency was measured for response-positive trials as the time between dimming onset and initiation of turning. For all behavioral experiments, stimuli and cameras were controlled by custom computer scripts that allowed precise timing of experimental events. Recorded behavioral data were analyzed by a mixed model repeated measures ANOVA using R 3.1.2.

2.4. Oxidative stress

An OxyBlot™ kit (InterGen) was used as previously described by Kishi et al. (2003) to measure protein carbonylation in whole body protein extracts. Briefly, protein samples were extracted from whole-body homogenates of 6 dpf larvae. Each sample contained protein extract from 3 larvae within the same treatment group (control, L-DOPA or L-DOPA + selegiline). Protein concentrations were normalized for each sample based on nanodrop results of extracted protein and 10 µL of solution (20 µg protein) was loaded into each assay well. All samples were duplicated with a negative control.

3. Results

3.1. L-DOPA effects on DAT-expressing neurons

Figure 2a shows cell counts in control, L-DOPA, and L-DOPA + selegiline groups 24 hours after treatment, and representative pretectal cells are shown in Figure 3. The number of DAT-expressing cells in the pretectum of 7 dpf zebrafish larvae was influenced by drug exposure ($F(2,55) = 4.32, p = .02, \eta^2 = .14$). Untreated 7 dpf larvae ($n = 25$) had an average of 59.4 ± 11.0 GFP-expressing cells in the pretectum. L-DOPA treated larvae ($n = 20$) showed a slight but significant reduction in cell numbers, with a mean of 51.3 ± 7.3 pretectal cells. The MAOI group ($n = 13$) had an average of 54.9 ± 8.1 cells, which was not significantly different from the control or L-DOPA groups as determined by a post hoc Tukey's test. Laterally-projecting neurites of pretectal DAT+ cells appeared to be less visible after L-DOPA treatment (Figure 3), however, since the ability to resolve small processes was variable across larvae no attempt was made to quantify fluorescence intensity from these neurites.

Fig. 2. DAT+ cell counts before and after treatment with L-DOPA and the MAOI, selegiline. A. Number of DAT+ cells in the pretectum of 7 dpf larvae 24 hours after drug exposure. Asterisks indicate groups that differ significantly from controls. Error bars represent s.e.m. B. Number of DAT+ cells in the vDC before and 24 hours following drug

exposure. Asterisks indicate groups that significantly differ from controls. Error bars represent s.e.m.

Fig. 3. Representative confocal images of pretectal DAT+ cells following drug treatment. Each image is a projection of a depth stack (described in the Methods). A. Control group; B. MAOI and L-DOPA; B. L-DOPA alone. D. Western blot showing protein carbonylation levels (a measure of oxidative stress) in whole body homogenate of zebrafish larvae ($N=3$ per group).

Figure 2b shows vDC cell counts across treatment groups and time points (before and 1 hour after drug washout). vDC cells before and after L-DOPA treatment are shown in Figure 4a. There was no main effect of drug treatment group. There was a significant main effect of time ($F(1,23) = 5.23$, $p = .03$, $\eta^2 = .05$), and a group by time interaction ($F(1,23) = 8.80$, $p < .01$, $\eta^2 = .08$). There were no group differences in vDC cell counts prior to treatment (56.6 ± 8.7 and 58.4 ± 8.5 cells for the control and L-DOPA groups, respectively; $F(1,23) = 0.29$, $p = .59$). One hour after drug washout the number of vDC cells fell by 8.4 ± 10.3 in the L-DOPA group, but

increased by 1.1 ± 11.5 in the control group, a significantly different change ($F(1,23) = 4.74$, $p = .04$, $\eta^2 = .21$). (Figure 2b).

There was no change in the number of non-DAT-expressing reticulospinal neurons before or after L-DOPA treatment, $F(1,9) = 0.13$, $p = .73$, $\eta^2 < .01$ (Figure 4b).

Fig. 4. DAT+ vDC neurons (A) and non-DAT-expressing, injection-labeled hindbrain reticulospinal neurons (B) before and after L-DOPA treatment. L-DOPA had significantly greater effect on integrity of DAT+ cells. Each image is a maximum projections of a depth stack (described in the Methods).

3.2. Behavior

Figure 5 illustrates the effects of L-DOPA with and without selegiline on spontaneous swimming activity. When tested 1 hour after drug washout (after 24 hours of drug incubation), spontaneous swimming was significantly different among the treatment groups ($F(2,163) =$

10.33, $p < .01$, $\eta^2 = .11$). Control fish ($n = 59$) were in motion for $30.5 \pm 25.2\%$ of the time recorded while larvae treated with L-DOPA ($n = 62$) were significantly hyperactive, with $41.9 \pm 30.6\%$ of time spent in motion. Fish treated with both L-DOPA + selegeline ($n = 45$) were significantly hypoactive relative to controls, with only $17.8 \pm 24.1\%$ of time in motion. A second spontaneous activity test for a subset of larva done 24 hours after removal from drug solutions revealed a main effect for treatment group ($F(2,45) = 20.93$, $p < .01$, $\eta^2 = .48$) and time post-exposure ($F(1,45) = 40.66$, $p < .01$, $\eta^2 = .48$) and a treatment by time interaction ($F(2,45) = 11.92$, $p < .01$, $\eta^2 = .35$). Simple effects tests showed that L-DOPA treated fish became hypoactive relative to controls 24 hours after drug exposure (8.6 and 21.8% respectively, $F(1,45) = 55.31$, $p < .01$). Swimming activity in the L-DOPA + selegeline group remained significantly depressed and did not recover 24 hours after treatment, $F(1,45) < .01$, $p = .99$.

Fig. 5. Percentage of time spent in motion 0 and 24 hours following drug exposure in all treatment groups. Asterisks indicate groups that significantly differ from controls. Error bars represent s.e.m.

Response rate of visually-evoked startle was $80.9 \pm 37.1\%$ in the L-DOPA group ($n = 21$), which was not significantly different from the control group ($n = 12$; $95.8 \pm 37.0\%$ response rate). The L-DOPA + selegiline treatment group ($n = 15$) had a startle response rate of $33.3 \pm 37.2\%$, which was significantly lower than controls ($F(2,45) = 24.66$, $p < .01$, $\eta^2 = .52$). Drug treatment did not influence the latency of visually-evoked startle responses. Latency increased from 6 to 7 dpf regardless of treatment group, $F(1,18) = 31.73$, $p < .01$, $\eta^2 = .64$, but there was no interaction between treatment group and time on startle latency, $F(1,18) = 0.10$, $p = .75$, $\eta^2 = .01$.

3.4. Oxidative stress

The OxyBlot assay showed higher levels of protein carbonylation in whole body homogenate of L-DOPA and L-DOPA + selegiline groups relative to the control group (Figure 3d). These western blot results could not be analyzed statistically but suggest that L-DOPA increased oxidative stress and that selegiline failed to protect against L-DOPA-induced oxidative stress.

4. Discussion

L-DOPA is effective in relieving motor symptoms of PD patients and has remained the most commonly prescribed PD drug since its approval by the FDA in 1970, despite persistent concerns that its long-term use may be toxic to dopaminergic neurons. L-DOPA toxicity has been clearly demonstrated in cultured dopaminergic neurons but not in humans or whole-animal models (Olanow, 2015). The current experiments exploited technical advantages of zebrafish larvae, particularly the ability to count DAT-expressing dopaminergic neurons in intact, living

animals before and after L-DOPA treatment, to address the question of whether L-DOPA therapy may be toxic.

The present study provides some evidence that L-DOPA is toxic to DAT-expressing neurons in zebrafish larvae through an oxidative stress mechanism. L-DOPA treatment led to an increase in oxidative stress markers and a decrease in the number of DAT-expressing cells. L-DOPA toxicity appeared to require DAT expression because hindbrain reticulospinal neurons that do not express the DAT, which are likely glycinergic or glutaminergic (Kinkhabwala et al., 2011), showed no decrease in fluorescence or cell numbers after L-DOPA treatment. The lack of change in the visually-evoked startle response after 24 hours of L-DOPA treatment also suggests that toxicity is selective as neurons that initiate movements must be spared. These results agree with previous studies in zebrafish showing that dopaminergic cells of the vDC are selectively vulnerable to DAT-specific neurotoxins such as MPTP (Lam, Korzh, and Strahle, 2005; McKinley et al., 2005; Xi et al., 2011). These findings indirectly support a model in which L-DOPA metabolites, particularly DOPAL, enter neurons through the DAT and disrupt vital intracellular processes such as mitochondrial function by oxidizing proteins they come into contact with.

Several findings in the current study are inconsistent with the model described above. Blocking the metabolic pathway that generates DOPAL with the MAOI selegiline did not prevent L-DOPA-induced cell loss or the increase in protein oxidation. The lack of a neuroprotective effect of selegiline cannot be explained by a failure of the drug's administration because locomotor activity was dramatically suppressed in the selegiline-treated group. Zebrafish possess only one isoform of MAO, and as a result MAO-B inhibitors like selegiline have broader effects in zebrafish than in mammals, including increasing serotonin levels

(Anichtchik, Sallinen, Peitsaro and Panula, 2006). Serotonin is known to modulate locomotor activity in zebrafish (Brustein, Chong, Holmqvist, and Drapeau, 2003; Sallinen et al., 2009; Airhart et al., 2007; Yokogawa, Hannan, and Burgess, 2012), and this likely explains the behavioral effects of selegiline in the current study. Our findings of behavioral suppression after selegiline are consistent with previous research in zebrafish larvae that reported no recovery in locomotor activity until 48h after treatment with a different MAO inhibitor, deprenyl (Sallinen et al, 2009). This locomotor suppression by serotonin was independent of the dopaminergic system. Therefore, the observed hypoactivity after selegiline in the current study cannot be attributed to reductions in dopamine cells alone. A selegiline-only control group was not included because our hypothesis concerned selegiline's effect in the presence of excessive dopamine levels but we expect selegiline alone would suppress locomotor activity and not alter protein oxidation levels in the absence of an oxidative stressor.

If DOPAL generation is not necessary for L-DOPA mediated toxicity, then the overproduction of DA itself, which also has oxidative properties, may account for the apparent loss of DAT-expressing cells in the presence of selegiline. Prior research on methamphetamine-induced neurotoxicity suggests that augmented extracellular DA itself is not sufficient to cause lesions (LaVoie and Hastings, 1999), but the fact that amphetamine acts on available DA stores while L-DOPA provides an exogenous source of additional DA may account for this discrepancy. Studies in cell culture have found increased neuronal death due to L-DOPA (Basma et al., 1995; McLaughlin et. al, 2002; Stansley and Yamamoto, 2013; Ziv et al., 1997), however these findings have not been replicated in vivo (Murer et al., 2004; Perry et al., 1984). The contributions of glial cells to the protection of DA neurons may account for these contradictory results, as the addition of glial tissues to cultured neurons reduces neuronal loss (Mena et al,

1997). Similarly, the introduction of glial cells in a mouse model of PD reduced 6-hydroxydopamine neurotoxicity (Akerud et al., 2001), and the number of glial cells expressing the antioxidative enzyme glutathione peroxidase are augmented in the vicinity of pathogenic DA neurons (Damier et al., 1993), further reinforcing the relationship between glial cells and the pathogenic process. A possible explanation of our results is that the larvae have not yet fully developed neuroprotective glial cells that compensate for L-DOPA induced oxidative stress, which would account for the moderate but incomplete neurotoxicity. Glial cells in the larval CNS are still undergoing development at 5 dpf, the age at which drugs were administered in the present study (Brosamle and Halpern, 2002).

The initial behavioral hyperactivity observed after treatment with L-DOPA is consistent with previous reports on behavioral responses to dopaminergic agents in zebrafish larvae and other animals (Irons, MacPhail, Hunter, and Padilla, 2010; O'Neill and Shaw, 1999). The fact that spontaneous locomotor behavior was depressed in the same larvae 24 hours after removal from L-DOPA, when loss of dopaminergic neurons was also observed, is consistent with previous research showing locomotor inhibition after dopaminergic cell loss (Anichtchik et al., 2008; Flinn et al., 2009; Lam et al., 2005; McKinley et al., 2005; Xi et al., 2010; Xi et al., 2011).

Selegiline failed to protect against protein oxidation, which suggests that DOPAL is not the sole mechanism of oxidative stress after L-DOPA administration, or that selegiline did not completely prevent DOPAL generation. DA is also known to form reactive semiquinones that may contribute to neurotoxicity, and this mechanism is independent of MAO activity (Graham, 1978). Direct measurement of DOPAL in subsequent experiments, or administration of DOPAL itself rather than dopamine, could resolve these questions. As L-DOPA had no detectable effects on non-DAT-expressing neurons, the toxicity observed in DAT-expressing neurons likely

required expression of dopamine-related synthetic or metabolic enzymes in those neurons to transform L-DOPA, or dopamine itself, into the cells. DAT blockade is known to prevent MPTP-induced dopaminergic cell death (Lam, Korzh, and Strahl, 2005; McKinley et al., 2005). Augmented DAT levels also increase dopaminergic cell vulnerability (Masoud et al., 2015) and ectopic DAT expression is sufficient to render non-dopaminergic cells vulnerable (Chen et al., 2008). Demonstrating the same protective effect of DAT-blockade in the current zebrafish L-DOPA toxicity model would provide firm evidence that the same cytotoxic mechanism was at work. Ectopic expression of the DAT in the zebrafish brain could also help resolve the cellular mechanism of L-DOPA toxicity in this model. Ectopic DAT expression in mouse striatal neurons makes these neurons especially vulnerable to MPTP toxicity, presumably because they lack regulatory mechanisms (particularly the expression of antioxidant proteins) that protect dopaminergic neurons against oxidative damage (Chen et al., 2008).

The current study is one of several recent attempts to exploit the zebrafish larva as a model system to study cellular mechanisms of PD (Feng et al., 2014; Lam et al., 2005; McKinley et al., 2005; Sheng et al., 2010; Xi et al., 2010). The main advantages of this approach include relative ease of genetic manipulations and drug delivery, the ability to image and manipulate molecularly-defined populations of neurons in living animals, and the potential for high throughput testing in larvae. Monoaminergic systems develop rapidly in zebrafish, and 5 day old larvae possess the same subsets of these neurons as adults (Rink and Guo, 2004). Similarities in the cellular mechanisms and behavioral function of central dopaminergic systems in human and zebrafish support the use of zebrafish as a model for some elements of PD, and the unique advantages of zebrafish may facilitate investigation of genetic influences or screening of potential therapeutic agents. However, multiple factors may limit their utility in translational PD

research, including differences in MAO isoforms, the range of affected behaviors, age-dependent processes (especially when larval zebrafish are used), and pharmacodynamics. For example, in the current study the concentration of L-DOPA given by bath application to larvae (1 mM) was much higher than estimated brain concentrations achieved after therapeutic L-DOPA in PD patients (3-50 μ M, Lipski et al. 2011), and because absorption of L-DOPA through the skin in zebrafish larvae is not well understood it was not possible to accurately estimate concentrations in the brain. The applicability of spontaneous locomotor activity in the zebrafish larvae to the motor impairments in human PD is also unclear, although deficits observed in genetic and pharmacological models are consistent with those found in mammals. Ablation of individual DAT-expressing neurons, in conjunction with behavioral analysis of lesion effects, could complement these studies to gain a better understanding of the consequences of DA cell loss in zebrafish larvae.

Our results indicate that chronic high doses of L-DOPA can be neurotoxic to DAT-expressing cells *in vivo*, but further research is necessary to fully understand the factors underlying the contradictory findings between cell culture and *in vivo* studies. In particular, understanding how glial cell function affects vulnerability to L-DOPA toxicity in the zebrafish is a promising line of future research. We believe the most productive application of zebrafish research into PD mechanisms will involve bridging the gap between pharmacological models of dopamine neurotoxicity and genetic models of PD. The current study takes steps in this direction by addressing the mechanism of oxidative stress in zebrafish dopaminergic neurons.

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