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Selective toxicity of L-DOPA to dopamine transporter-expressing neurons and locomotor behavior in 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-h recovery period, while visually-evoked startle response rates and latencies were unaffected.

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 et al., 2012; Tay et al., 2011; Xi et al., 2011). vDC neurons are also vulnerable to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

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(MPTP) neurotoxicity (Lam et al., 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 μM selegiline (R-(−)-deprenyl hydrochloride (Sigma), or to a control solution containing regular fish water, for 24 h 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 2 mL transfer pipets) into new 60 mm 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 20× .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 (Fig. 1) were imaged in larvae treated with L-DOPA, L-DOPA + selegiline, or a no-drug control solution ($n = 20$ per group), 24 h after removal from the drug solutions (7 dpf). A 100 μm height, 100 μm width, 40 μm 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 h after drug washout. A 200 μm height, 200 μm width, 40 μm depth volume centered on the vDC was sufficient to capture all GFP-expressing cells within the structure.

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 et al., 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.

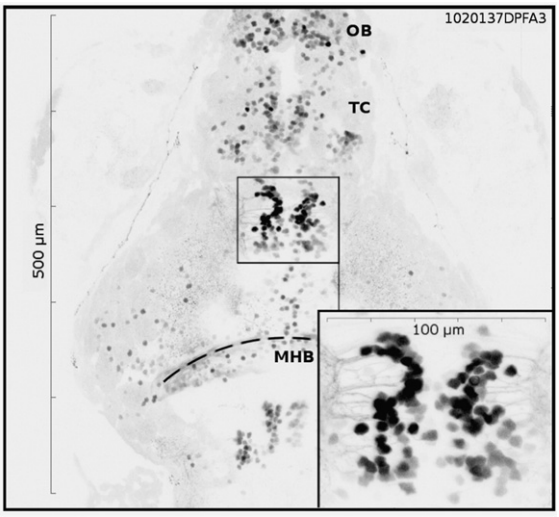

Fig. 1. DAT-expressing cells in a 6 dpf zebrafish larvae. The image is a projection of 25 confocal images spanning 40 μm. OB: olfactory bulb; TC: telencephalon; MHB: midbrain-hindbrain barrier. Inset: pretectum.

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 h after drug washout and again 24 h 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 min each hour over a 6 h 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 1 m above the recording plate projected through a light diffusing plastic sheet) remained on for 60 min and was then extinguished (referred to as dimming) for 1 min. A high speed digital camera (PhotonFocus D1312) positioned below the recording plate captured 5 s of video at 60 frames per second beginning 2 s 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 were 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 s 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.