

Transgenic Zebrafish Expressing mCherry in the Mitochondria of Dopaminergic Neurons

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Abstract

Genetic mutations and environmental toxins are known to affect mitochondrial health and have been implicated in the progressive degeneration of dopaminergic neurons in Parkinson's disease. To visualize mitochondria in dopaminergic neurons of live zebrafish, we used the regulatory elements of the *dopamine transporter (dat)* gene to target a reporter, mCherry, after fusion with the mitochondrial localizing signal (MLS) of Tom20. Immunoblot analysis of mitochondrial and cytosolic fractions from Tg(*dat:tom20 MLS-mCherry*) larvae shows that mCherry is efficiently targeted to the mitochondria. Confocal imaging of live fish was carried out from 1 day postfertilization (dpf) to 9 dpf. We also colocalized *dat* mRNA expression with the mCherry protein in the olfactory bulb (OB), subpallium (SP), pretectum (Pr), diencephalic clusters 2 and 3 (DC2/3), caudal hypothalamus (Hc), locus coeruleus (LC), anterior preoptic area (POa), retinal amacrine cells (RAC), caudal hypothalamus (Hc), and preoptic area (PO). Treating Tg(*dat:tom20 MLS-mCherry*) larvae with the dopaminergic neurotoxin MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine) at 2 or 3 dpf resulted in a decrease in mCherry fluorescence in the pretectum, olfactory bulb, subpallium, diencephalic clusters 2 and 3, and the caudal hypothalamus. Labeling of mitochondria in nigrostriatal dopaminergic neurons of zebrafish could allow their visualization *in vivo* following genetic or pharmacological manipulations.

Introduction

MITOCHONDRIA ARE RESPONSIBLE for energy production, apoptosis, calcium storage, signaling events, and metabolism. Dysfunctional mitochondria and oxidative metabolism are two theories that may explain nigrostriatal dopaminergic (DA) neurodegeneration in Parkinson's disease (PD). Mitochondria may contribute to DA neuron cell death in PD due to changes in their membrane potential or in their structure and dynamics.^{1,2} A defect in cellular energy production could predispose the DA neurons to death following exposure to other toxin or genetic defects, all together increasing the neuron's susceptibility to apoptosis. In a healthy neuron, the production and clearance of harmful oxidants, which are produced from cellular metabolism, are tightly regulated.³ It has been suggested that in PD patients there is an abundance of reactive oxygen species and increased oxidative stress.

Methods of visualizing mitochondria in living animal models, especially in cells relevant to PD, are crucial to the development of an *in vivo* model, which will allow us to test paradigms of DA neuron death or neuroprotection. The tar-

geting of fluorescent markers to the mitochondria of transgenic zebrafish has facilitated the study of mitochondrial function under specific physiological and/or genetic conditions.⁴⁻⁶ However, these transgenic models labeled the whole fish or the entire nervous system, but did not target specific neuronal population such as DA neurons.

In this study, we have developed a zebrafish model, which specifically labels the mitochondria of DA neurons by introducing a fusion protein that consists of the mitochondrial localizing signal (MLS) of Tom20 and the mCherry fluorescent protein. This fusion protein was placed under the control of the *cis*-regulatory elements of the *dopamine transporter (dat)* gene. This transgenic line allows the observation of mitochondria in real time, specifically in DA neurons, to better our understanding of the pathogenesis of PD following chemical neurotoxin exposure.

Materials and Methods

Animal maintenance

All experiments were performed according to the guidelines of the Canadian Council on Animal Care and were

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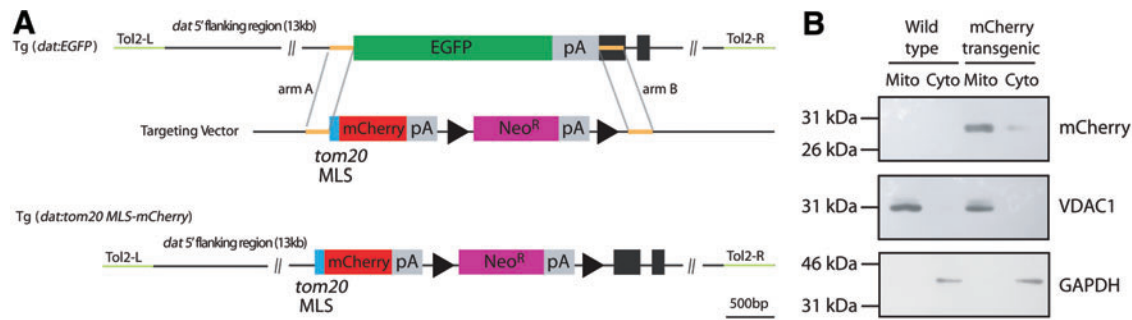


FIG. 1. Construct to produce Tg(*dat:tom20 MLS-mCherry*) zebrafish line. **(A)** The targeting vector was designed to contain two homologous arms, A and B (yellow). These arms allowed the replacement of the EGFP (green), previously used in the generation of the Tg(*dat:EGFP*) transgenic line, with a fusion protein between the mitochondrial localizing signal (MLS) of Tom20 (light blue) and mCherry (red). Tol2 left and right arms (light green) flanking the sequence were used to enhance transgenesis efficiency. A neomycin resistance cassette (Neo^R) was flanked by loxP sites (arrowheads). Polyadenylation signals (pA) are shown in gray. Exons of the *dat* gene are depicted by black boxes. The total size of the inserted DNA is ~30 kb and comprises ~13 kb of *dat* 5' flanking region and all *dat* exons, except for the last. **(B)** Western blotting demonstrates that the mCherry protein is localized to the mitochondria of transgenic fish. Mitochondria were isolated from Tg(*dat:tom20 MLS-mCherry*) and wild-type larvae at 5 days postfertilization (dpf). mCherry was abundantly detected in the mitochondrial fraction of transgenic larvae, but was not as prominent in the cytosolic fraction. The voltage-dependent anion selective channel protein 1 (VDAC1) was used as a mitochondrial marker, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a cytosolic marker. Color images available online at www.liebertpub.com/zeb

fusion protein targeted to the outer mitochondrial membrane by fusing mCherry with the MLS of zebrafish Tom20, a receptor located in the outer mitochondrial membrane.¹⁰

Primary transgenic fish were raised to adulthood (~4 months) and screened for germ line transmission of the transgene. We obtained seven independent transgenic lines. Of these, we selected a line that expressed strong mCherry fluorescence in several DA neuron clusters (Fig. 2A, B), including the subpallial region that is considered to be involved in movement control in zebrafish.¹¹

mCherry is properly targeted to the mitochondria in transgenic zebrafish

We isolated mitochondria from transgenic larvae at 5 dpf and compared the mCherry protein detected in the mitochondrial fraction to that in the cytosolic fraction by western blotting (Fig. 1B). A band representing mCherry is detected in the mitochondrial fraction from transgenic fish, while a low amount of mCherry is detected in the cytosolic fraction of transgenic fish, likely due to the transient transport of the Tom20 MLS-mCherry fusion protein to the mitochondria.

Colocalization of mCherry patterning with a dopaminergic neuron marker in transgenic zebrafish larvae

We examined the expression of mCherry in the transgenic Tg(*dat:tom20 MLS-mCherry*) zebrafish line. Fluorescence in this line was visible within the first 24 hpf, which agrees with prior observations of DA neuron development.¹¹ The Tg(*dat:tom20 MLS-mCherry*) transgenic line expressed mCherry in the olfactory bulb (OB), subpallium (SP), pretectum (Pr), diencephalic clusters 2 and 3 (DC2 and DC3), caudal hypothalamus (Hc), jaw, branchial arches (BA) (Fig. 2), retinal amacrine cells (RAC) (Fig. 3E''), and reticular astrocytes¹² of the optic nerve (data not shown). These cells of the jaw, branchial arches (BA), and reticular astrocytes of the optic nerve were seen in the Tg(*dat:EGFP*) line,⁸ as well

as a third transgenic line using the same genomic sequences from the *dat* locus.¹³ Global dorsal and lateral views of the overall mCherry expression in live larvae were taken at 3 dpf (Fig. 2A). In addition, two-photon confocal images were also taken from 2 to 9 dpf (Fig. 2B). Examining immunostained sections of larvae imaged under higher magnification using confocal microscopy revealed mCherry immunolabeling along axonal projections (Fig. 2C, arrowheads) and in the cytoplasm (Fig. 2C) of olfactory bulb DA neurons.

We colocalized the mCherry reporter by whole-mount immunostaining with the *dat* by whole-mount *in situ* hybridization (Fig. 3A–E'') and used a *th1* cRNA probe as a landmark for the identification of cells in the ventral diencephalon (Fig. 3F–G'') and data not shown). The analysis of individual z-stacks revealed colocalization between mCherry and *dat*-positive cells in the pretectum (Pr), locus coeruleus (LC), diencephalic clusters 2 and 3 (DC2 and DC3), olfactory bulb (OB), subpallium (SP), anterior preoptic area (POa), retinal amacrine cells (RAC), caudal hypothalamus (Hc), and preoptic area (PO) (Fig. 3A–E''). Using mCherry immunostaining and *th1* *in situ* hybridization, we observed colocalization within the Pr, SP, OB, POa, DC2, DC3, RAC, and PO, but not within DC4, DC5, DC6, and Hc (Fig. 3F–G'') and data not shown). These latter regions that have mCherry expression, but lack *th1* expression, could be explained by the more predominant *th2* expression in these regions.¹⁴ Moreover, using live imaging, we observed some mCherry expression in cells, which are unlikely to be dopaminergic. For instance, we saw colocalization between *dat*-driven mCherry and EGFP driven by regulatory elements of the *dlx5a/6a* locus, even though there is no evidence that *dlx* genes are expressed in dopaminergic neurons. The fate of these cells is currently under investigation.

MPTP neurotoxin treatment reduces mCherry expression in Tg(dat:tom20 MLS-mCherry) larvae

We exposed embryos of the Tg(*dat:tom20 MLS-mCherry*) line to MPTP. Starting at 2 dpf, ~30–50 embryos were

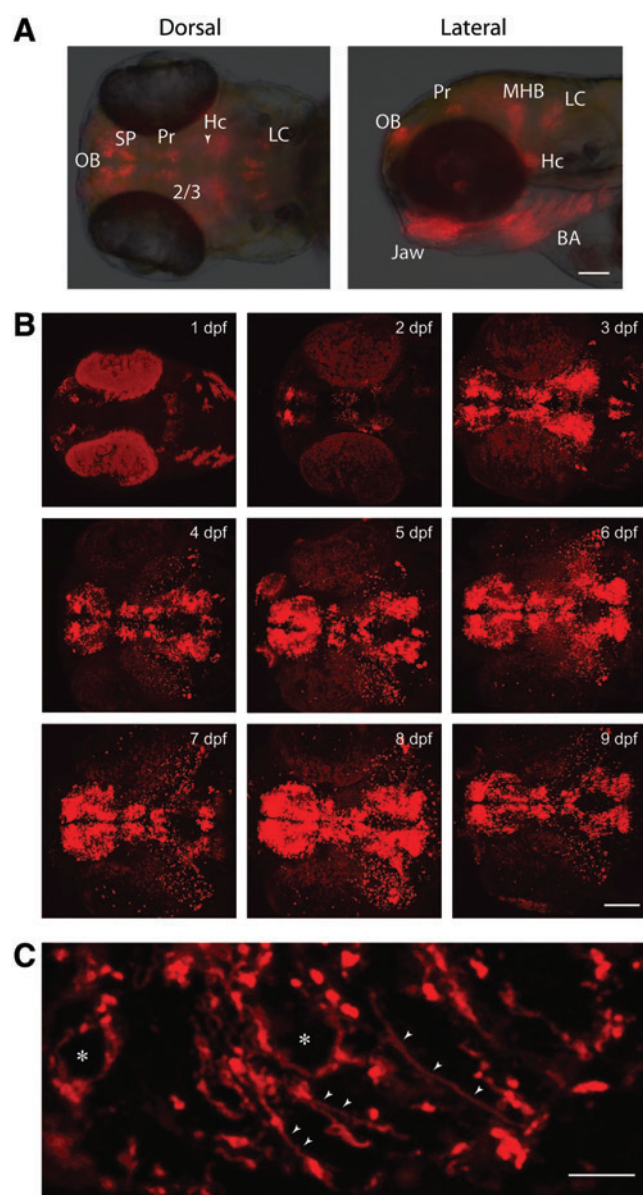


FIG. 2. Expression of mCherry in the *Tg(dat:tom20 MLS-mCherry)* fish. (A) Live dorsal and lateral composite fluorescent and bright field images of 3-day-old larvae taken with a dissecting microscope. The following clusters of cells express mCherry: olfactory bulb (OB), subpallium (SP), pretectum (Pr), diencephalic clusters 2 and 3 (DC2/3), caudal hypothalamus (Hc), midbrain hindbrain boundary (MHB), locus coeruleus (LC), and jaw and branchial arches (BA). Scale bar represents 50 μ m. (B) Two-photon confocal live images taken from 2 to 9 dpf. Scale bar represents 50 μ m. Images are dorsal facing up and anterior facing left. (C) Zoom in of mCherry-positive mitochondria in nigrostriatal dopaminergic (DA) neurons of the olfactory bulb from immunostained sections taken with 60 \times confocal objective. Arrowheads point to axonal projections and asterisks mark nuclei, which were visualized in the same field of view and focal plan under transmitted light (data not shown). Scale bar represents 5 μ m. Color images available online at www.liebertpub.com/zeb

treated with 1 mM MPTP for 72 h (Fig. 4A). A visible reduction of mCherry fluorescence in live MPTP-treated fish compared to untreated fish was observed at 120 hpf (Fig. 4B) and 144 hpf (Fig. 4C). We quantified the pixel intensity representing mCherry fluorescence in five separate regions containing DA neurons in both controls and MPTP-treated fish. At 120 hpf, a significant decrease in fluorescence was observed in the pretectum (29%, $p < 0.05$), olfactory bulb (33%, $p < 0.05$), subpallium (34%, $p < 0.05$), DA neuron clusters 2 and 3 (40%, $p < 0.05$), and caudal hypothalamus (Hc) (44%, $p < 0.05$). Moreover, the observed decrease in mCherry signal was more pronounced at 144 hpf in the pretectum (32%, $p < 0.05$), olfactory bulb (41%, $p < 0.01$), subpallium (55%, $p < 0.01$), DA neuron clusters 2 and 3 (57%, $p < 0.05$), and caudal hypothalamus (Hc) (62%, $p < 0.01$). Similar effects were observed when MPTP treatment was started at 3 dpf and continued for 48 h (data not shown). Overall, these results suggest that these DA neuron regions in the *Tg(dat:tom20 MLS-mCherry)* line are susceptible to the neurotoxin, MPTP, which targets mitochondria in DA neurons.

Discussion

We generated a transgenic zebrafish model for the study of mitochondria, specifically in DA neurons. The subpallial (SP) DA neurons, which are considered to be involved in movement control in zebrafish,¹¹ along with DA neurons present in the pretectum (Pr), locus coeruleus (LC), diencephalic clusters 2 and 3 (DC2 and DC3), olfactory bulb (OB), anterior preoptic area (POa), retinal amacrine cells (RAC), caudal hypothalamus (Hc), and preoptic area (PO), are labeled in our *Tg(dat:tom20 MLS-mCherry)* line. The pattern observed in the *Tg(dat:tom20 MLS-mCherry)* fish closely resembled the pattern of EGFP expression seen in the *Tg(dat:EGFP)* line. In comparison to the *Tg(dat:EGFP)* transgenic line,⁸ the mitochondria line labels all of the same DA neuron clusters with the exception of diencephalic clusters 4, 5, and 6. The reason for this difference is unclear, but could be attributable to subtle differences in the expression pattern caused by the site of transgene integration. Despite the lack of mCherry expression in diencephalic clusters 4, 5, and 6, the mCherry expression localized to the mitochondria of subpallial DA neurons is of particular relevance to the study of motor behavior in zebrafish. The use of this transgenic model could facilitate the study of how mitochondrial dynamics correlate with behavioral analysis following DA neuron perturbations.

Data from zebrafish MPTP experiments have shown that MPTP, at doses such as 0.1 mM, affects DA neurons, but may also have an effect on other neuron populations such as gamma aminobutyric acid (GABA) neurons¹⁵ and 5-HT cells, as previously summarized by Panula *et al.*¹⁶ In this study, we opted to use a higher dose of MPTP (1 mM) to facilitate live imaging. A trend toward a reduction in mCherry (by live confocal two-photon imaging) was observed in DA neurons of the *Tg(dat:tom20 MLS-mCherry)* fish at lower MPTP doses (i.e., 0.1, 0.042 mM), which fall into the ranges of MPTP concentrations previously used by others.^{17,18} However, this trend did not reach significance and was not as easily detected by live imaging.

Previous work has shown that mitochondria in the somites and pharyngeal arch are visible through confocal microscopy

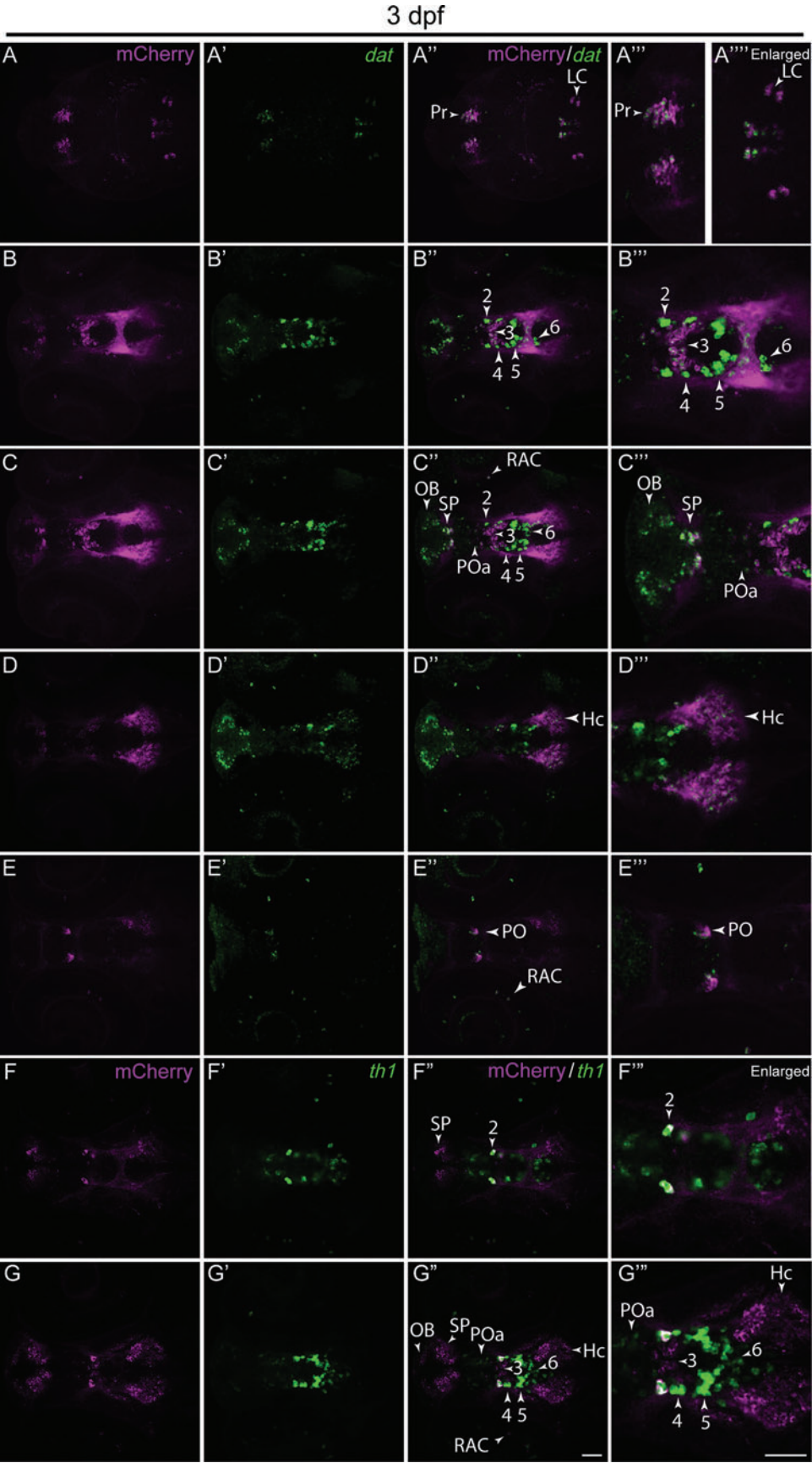
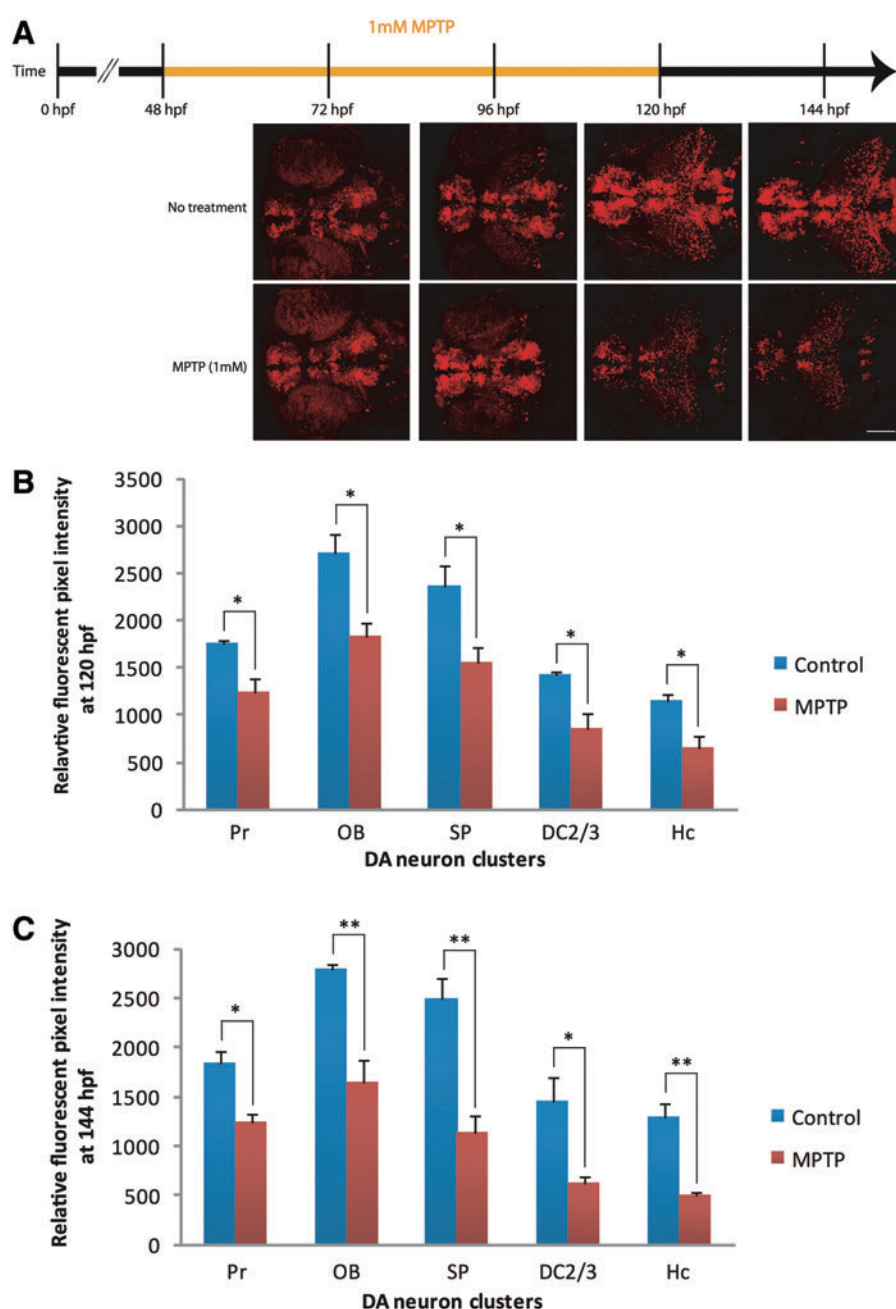


FIG. 3. Confocal z-stacks of immunolabeled mCherry colocalization with *dopamine transporter (dat)* (A–E''') and *tyrosine hydroxylase (th1)* (F–G''') cRNA probes in transgenic zebrafish at 3 dpf. mCherry-positive (magenta) and *dat*- or *th1*-(green) positive cells are labeled in individual 6.4- μ m-thick optical sections. DA neuron groups are labeled: pretectum (Pr), locus coeruleus (LC), diencephalic clusters 2 and 3 (DC2 and DC3), olfactory bulb (OB), subpallium (SP), anterior preoptic area (POa), retinal amacrine cells (RAC), caudal hypothalamus (Hc), and preoptic area (PO). Regions of mCherry expression that do not appear to colocalize with *dat* nor *th1* include DC4, DC5, and DC6. Images were taken with an upright LSM 510 confocal microscope equipped with a 20 \times air objective. Scale bar represents 50 μ m. Images are dorsal facing up and anterior facing left. Color images available online at www.liebertpub.com/zeb

FIG. 4. MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine) neurotoxin exposure in transgenic larvae. (A) 1 mM MPTP treatment started at 48 hours postfertilization (hpf) visibly diminished mCherry fluorescence at 120 and 144 hpf compared to control untreated larvae. The treatment regime is shown above with images corresponding to each time point below. Quantification of fluorescent pixel intensity at 120 hpf (B) and 144 hpf (C) revealed a significant reduction in mCherry-labeled DA neuron regions: pretectum (Pr), olfactory bulb (OB), subpallium (SP), diencephalic clusters 2 and 3 (DC2 and DC3), and caudal hypothalamus (Hc). Three representative embryos from a pool of 30–50 were used for quantification per treatment and per control group. Scale bar represents 50 μ m. Images are dorsal facing up and anterior facing left. *indicates $p < 0.05$ and **indicates $p < 0.01$. Color images available online at www.liebertpub.com/zeb



in transgenic zebrafish embryos expressing EGFP targeted to the mitochondria under the control of a ubiquitous promoter, which demonstrates that live imaging of mitochondrial fusion and fission events is possible *in vivo*.⁴ In addition, the transport and trafficking of mitochondria in zebrafish Rohon-Beard sensory nerve cells are visible in distal axon branches at the thin rim of the fin fold where the cell membrane is labeled with yellow fluorescent protein and the mitochondria are labeled with Cyan fluorescent protein.⁵ Another strategy, which has been used to visualize mitochondria in live zebrafish embryos, is the transient labeling of mitochondria by injection of a construct coding for a fusion protein between the MLS of the zebrafish cytochrome c oxidase subunit VIII and EGFP.⁶ Having the Tg(*dat:tom20 MLS-mCherry*) zebrafish model with fluorescent mitochondria in DA neurons

allows the direct examination of mitochondria exclusively in DA neurons.

Using Tg(*dat:tom20 MLS-mCherry*) embryos and larvae, we observed a significant reduction in mCherry fluorescence in the olfactory bulb, pretectum, subpallium, diencephalic clusters 2 and 3, and caudal hypothalamus following MPTP exposure. Once the active form of MPTP, MPP⁺ enters a DA neuron, MPP⁺ targets mitochondria and inhibits complex I of the electron transport chain leading to cellular energy depletion and reduction in DA cell markers. As a consequence, MPTP is used in research to model dopamine deficiency, since it causes a 30%–40% decrease in complex I activity in the substantia nigra pars compacta.¹⁹ McKinley *et al.* have previously shown that the mechanism of DA neuron toxicity induced by MPTP is conserved in zebrafish.¹⁸ Our findings

are in agreement with other MPTP studies in zebrafish, which show selectively perturbed DA neurons and result in behavioral deficits that mimic PD.^{8,17,18,20–23}

This transgenic line is an asset for future studies aiming to screen compounds or genes thought to be involved in mitochondrial dysfunction leading to DA neuron loss. Mitochondria could be compared from toxin-treated and untreated zebrafish or genetically impaired zebrafish, such as those induced by gene knockdown (e.g., RNAi, morpholinos) and targeted genomic deletions (e.g., ZFN, CRISPRs, TALENS). The ability to follow changes *in vivo* will allow easier tracking of differences in real time, over several time points, using the same fish. Alternative methods would involve immunostaining or *in situ* hybridization, which is time-consuming and requires more biological samples. A number of endpoints could be examined, such as mitochondrial fission and fusion (e.g., counts of elongated, intermediate, and fragmented mitochondria) and mitochondrial transport along the axon. In addition, cells from the Tg(*dat:tom20 MLS-mCherry*) zebrafish could be dissociated and mCherry-positive DA neurons could be collected by fluorescent activated cell sorting. Mitochondria from these mCherry-positive neurons could then be isolated and used to measure mitochondrial respiration.

We described the patterning of DA neurons in the Tg(*dat:tom20 MLS-mCherry*) line and observed a PD-relevant phenotype following exposure of these fish to MPTP. The amenability of zebrafish to transgenesis and drug exposure facilitated our observation of mitochondria within specific DA cell populations and allowed us to examine changes following neurotoxin exposure. Since dysfunctional mitochondria may contribute to DA neuron death and mitochondria are known to possess a dynamic morphology that is regulated by mitochondrial-shaping proteins,²⁴ our transgenic line can be used in future studies in the context of neurodegeneration and mitochondrial dynamics (fusion and fission), specifically in DA neurons *in vivo*.

Acknowledgments

We thank Dr. Philippe Vernier (Institut de Neurobiologie, Alfred Fessard, Gif-sur-Yvette, France) for the gift of the Tom20 plasmid. Gary Hatch for help with designing the *dat:tom20 MLS-mCherry* construct. We thank Vishal Saxena for assistance with fish husbandry. This work was funded by grants from the Natural Sciences and Engineering Research Council of Canada and by the Canadian Institutes of Health Research (grant no. MOP-14460 and 93803). S.N. and R.G. were supported by the Ottawa chapter of the Parkinson Society of Canada (grant no. 2012–15), an Ontario Graduate Scholarship, the University of Ottawa Department of Biology Scholarship, a Government of Ontario–Graduate Scholarship in Science and Technology and the Parkinson's Research Consortium–Toth Family Fellowship in Parkinson's Research. Funding sources had no involvement in the preparation of the article.

Disclosure Statement

No competing financial interests exist.

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