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Parkinson's disease linked neurotoxins induce apoptotic neurodegeneration in zebrafish

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Introduction

Parkinson's disease is the second most common neurodegenerative disease and presents as a chronic progressive movement disorder. This disease is caused by the death of dopamine neurons in a region of the brain called the *substantia nigra*. In the majority of cases, Parkinson's disease presents as an idiopathic disease, and more than 85% of patients have no known genetic association [1].

Rotenone and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) are two neurotoxins that have strongly been associated with an increased chance of developing Parkinson's disease. Rotenone is a commercial insecticide that displays strong oxidative properties. While farming life has been associated to an increased risk of developing Parkinson's disease, rotenone is suspected to play a role in dopaminergic neuron degeneration [2]. MPTP is a secondary product of desmethyprodine (an opioid drug) synthesis. The implication of MPTP as a cause for Parkinson's disease was discovered after drug users ingested an illicitly synthesized opioid preparation of and developed movement disorders similar to Parkinson's disease [3].

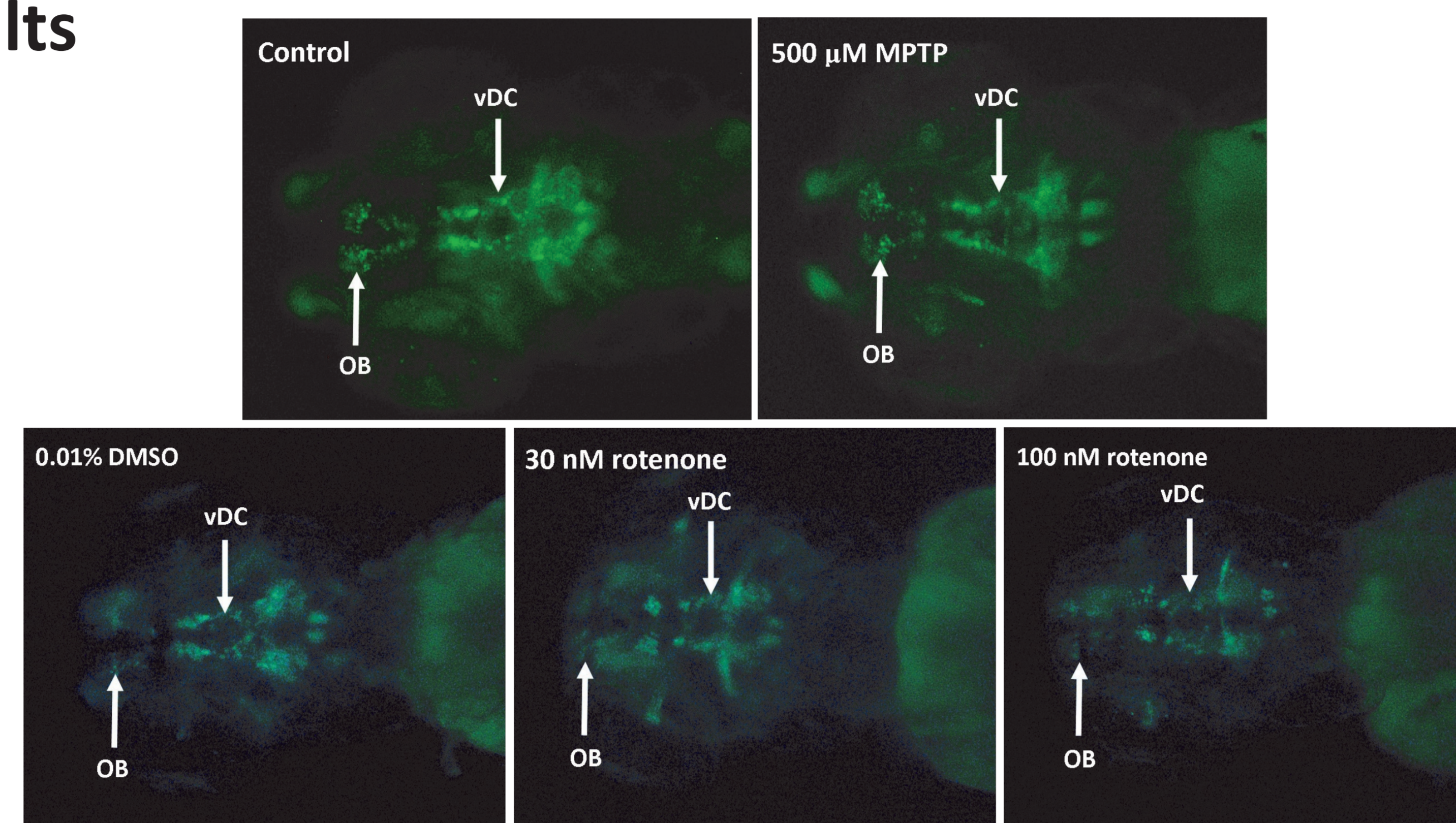
The recent increase in Parkinson's cases calls for investigation of the environmental factors associated to the development of the disease. Therefore, this study aims to establish a causative relationship between neurotoxin exposure and dopaminergic neurodegeneration using the zebrafish (*Danio rerio*) as a live animal model. It seeks to quantify the degree of impact of these two neurotoxins on dopaminergic neuron loss and to identify the mechanism of death of the affected neurons. In addition, this study may lead to develop a reliable model of dopamine neuron loss in zebrafish to study Parkinson's disease.

- [1] Kitada T *et al.* 2012. Considerations regarding the etiology and future treatment of autosomal recessive versus idiopathic Parkinson disease. *Curr Treat Options Neurol.* 14(3):230-240.
- [2] Hatcher J *et al.* 2008. Parkinson's disease and pesticides: a toxicological perspective. *Trends in Pharmacological Sciences* 29(6): 322-329.
- [3] Fahn, S. 1996. The Case of the Frozen Addicts: How the solution of an extraordinary medical mystery spawned a revolution in the understanding and treatment of Parkinson's disease. *The New England Journal of Medicine* 335 (26):2002-2003.

Methodology

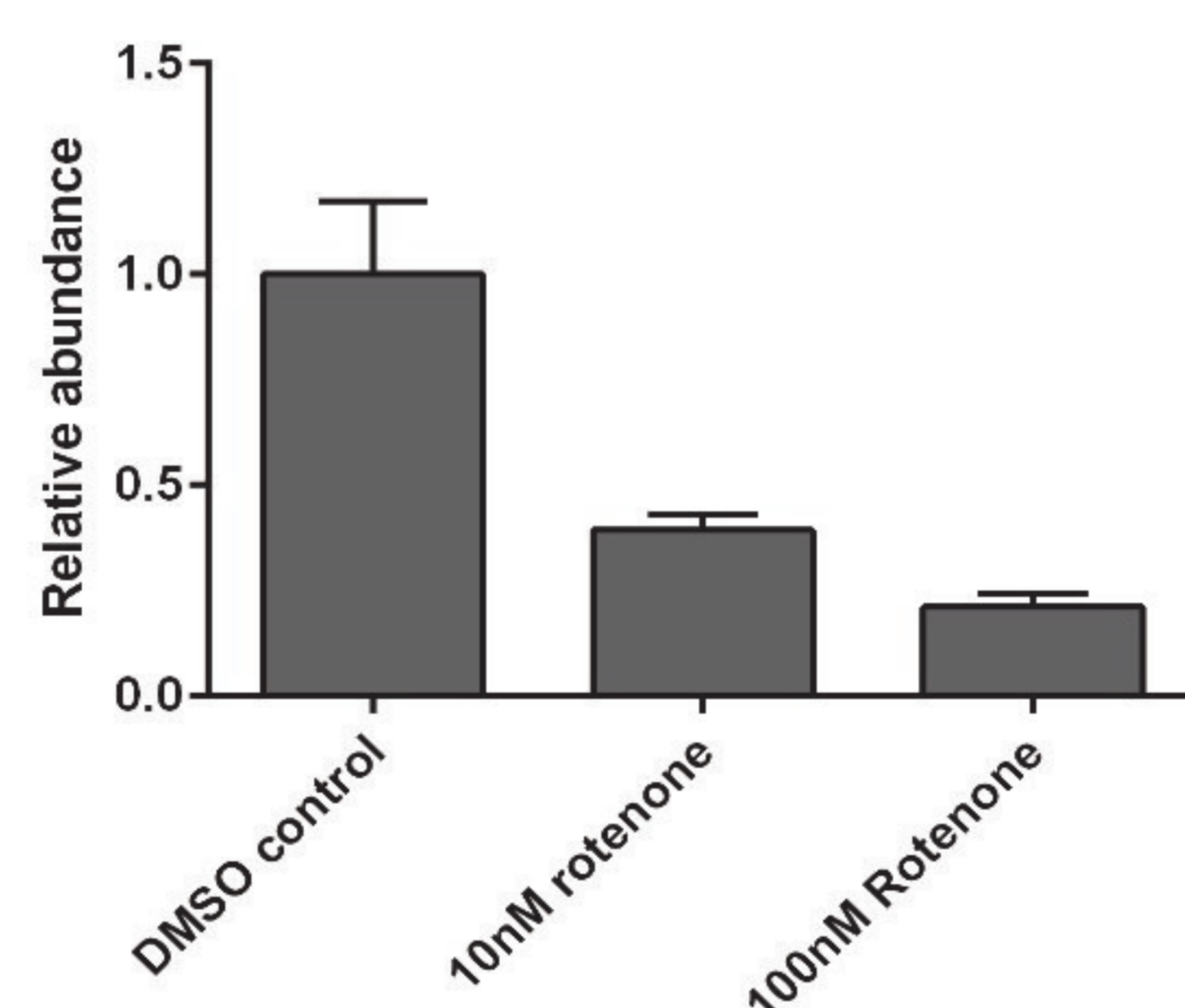
Transgenic zebrafish expressing the green fluorescent protein (GFP) under the dopamine transporter cis-regulatory elements (*dat:eGFP* embryos; Xi *et al.*, 2011) permitted the mapping of the dopaminergic system in the zebrafish brain. Fluorescence microscopy allowed visual characterization of the impact of the neurotoxins on the dopaminergic system of the fish. To confirm our observations, real-time quantitative reverse transcription PCR was used to determine the levels of dopamine receptor D2b (*drd2b*) and dopamine transporter (*dat*) transcript in 5 days post-fertilization zebrafish homogenate. The expression of these two genes should be characteristic of dopaminergic neurons, as they code for protein that play a role in dopamine signalling and reuptake. In order to explore the mechanism of neuron death following neurotoxin exposure, caspase-9 whole-mount immunostaining was performed on 3 days post-fertilization fish. Caspase-9 is an initiator caspase that plays an essential role in apoptosis signalling.

Results



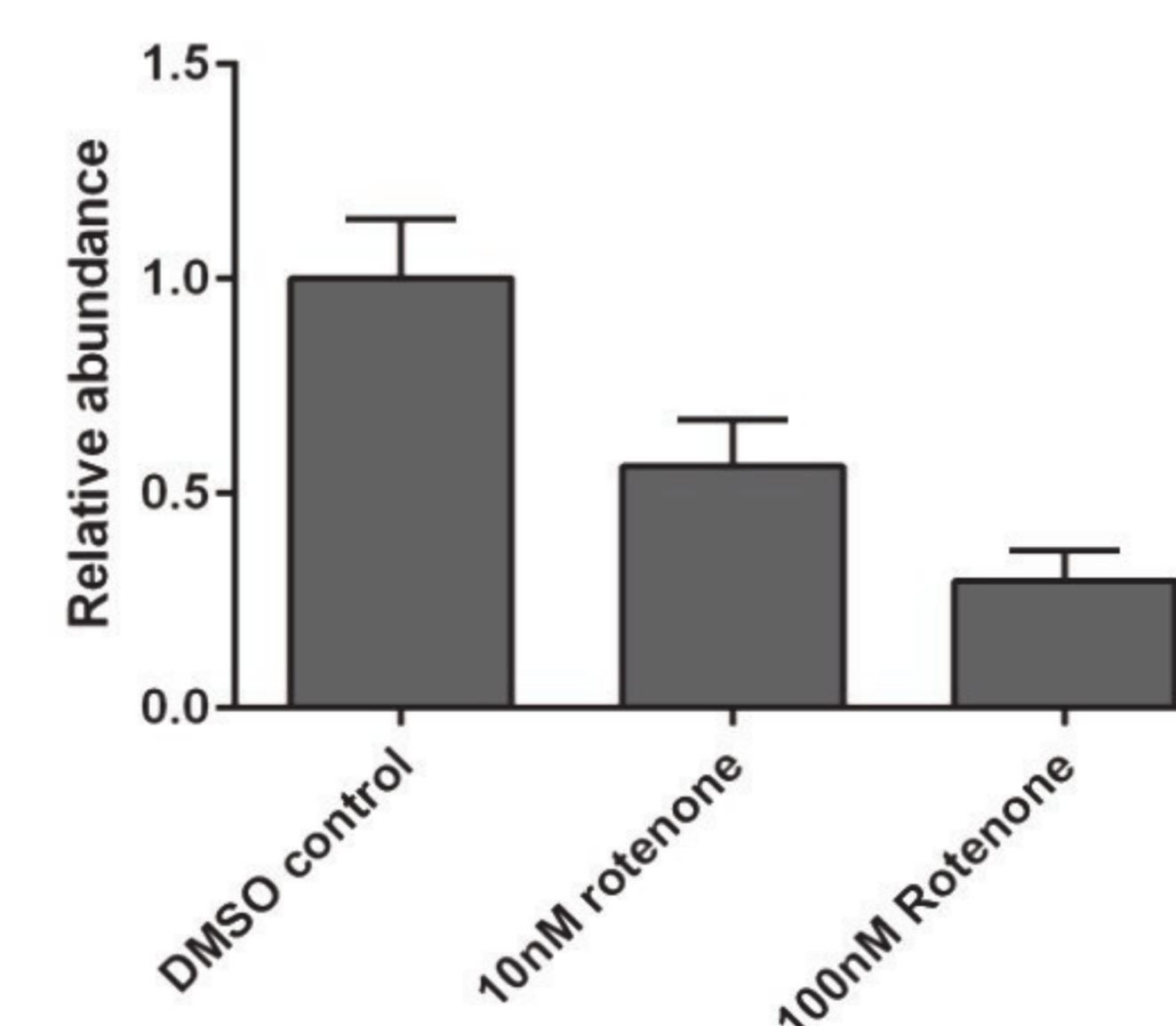
MPTP and rotenone induce a dose-dependent chemical ablation of the dopaminergic neurons in the ventral diencephalon (vDC) and olfactory bulb (OB) of zebrafish embryos.

Transgenic zebrafish embryos expressing eGFP under the dopamine transporter cis-regulatory elements were exposed to various concentrations of MPTP and rotenone at 24 hours post-fertilization. The rotenone exposed embryos shown were imaged and photographed at 5 days post-fertilization and the MPTP exposed embryos were imaged and photographed at 7 days post-fertilization with a Nikon DXM 1200C camera mounted on a Nikon SMZ 1500 dissecting microscope to examine for changes in green fluorescence patterning. Green fluorescence was markedly reduced in the ventral diencephalon and olfactory bulb regions of the embryos following exposure to a 500 micromolar concentration of MPTP. Green fluorescence patterning in the ventral diencephalon was reduced and disrupted with increasing concentrations of rotenone.



Relative expression of *dat* transcript in 5 days post-fertilization zebrafish homogenate.

RT-qPCR performed on zebrafish larvae homogenate demonstrates a dose-dependent reduction in expression of the dopamine transporter (*dat*) gene in fish exposed to rotenone. The error bars represent the standard error of the mean.

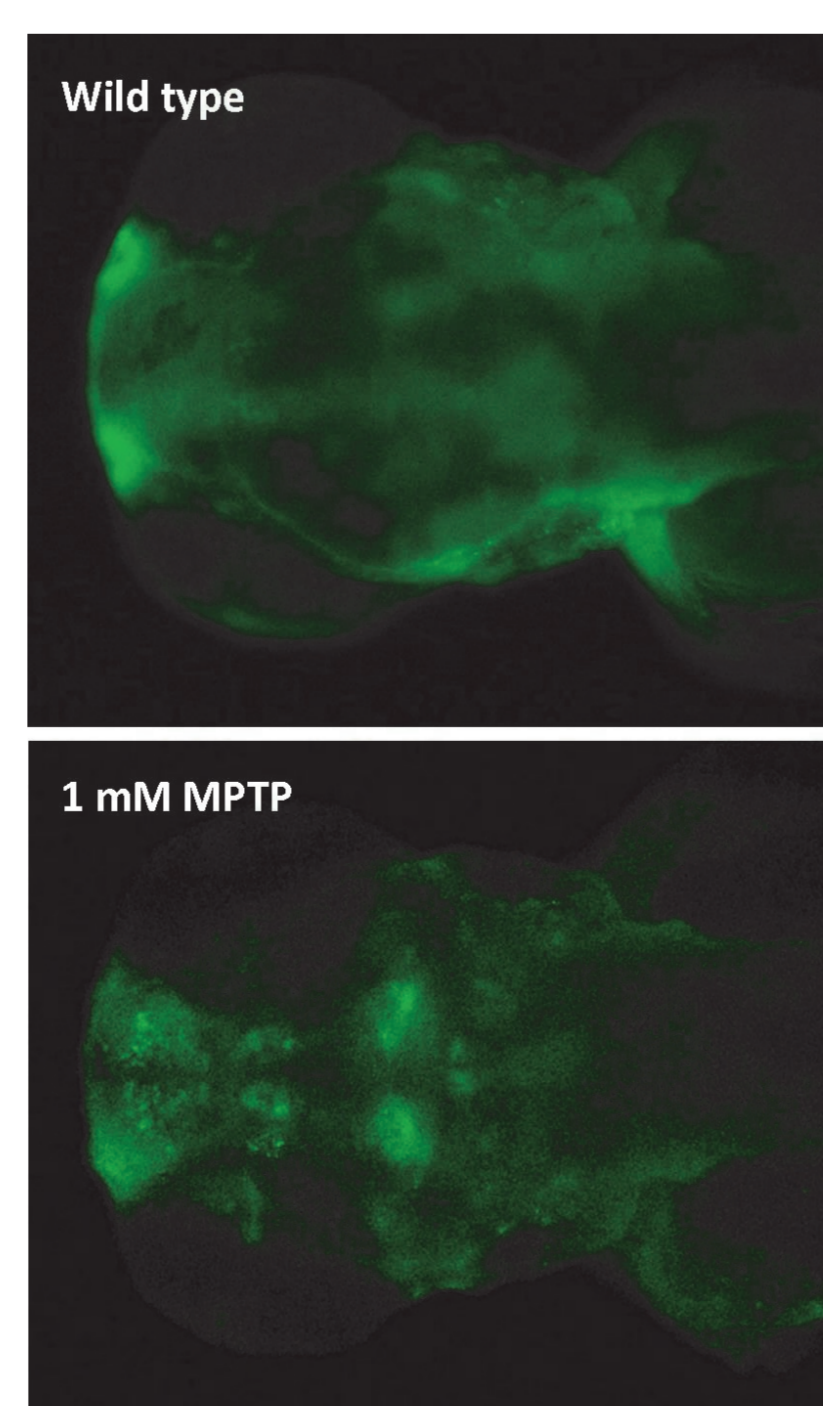


Relative expression of *drd2b* transcript in 5 days post-fertilization zebrafish homogenate.

RT-qPCR performed on zebrafish larvae homogenate demonstrates a dose-dependant reduction in expression of the dopamine receptor D2b (*drd2b*) gene in fish exposed to rotenone. The error bars represent the standard error of the mean.

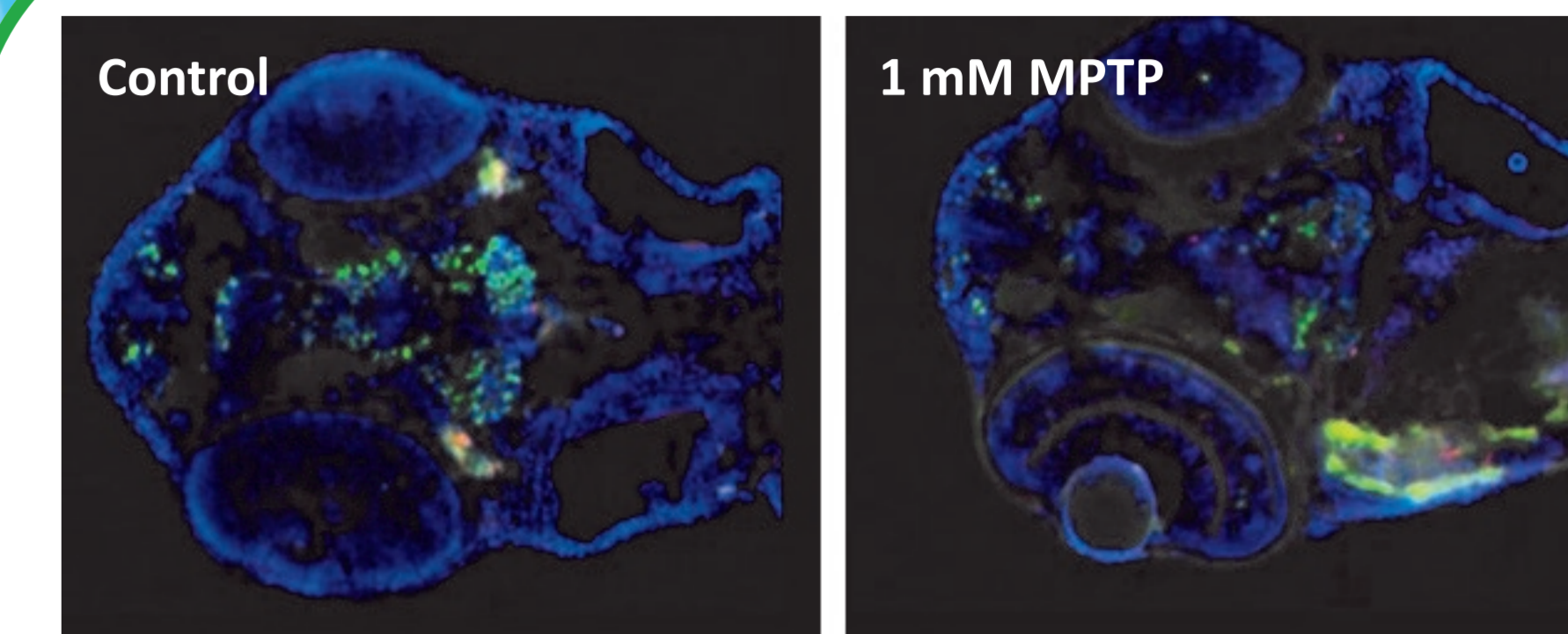
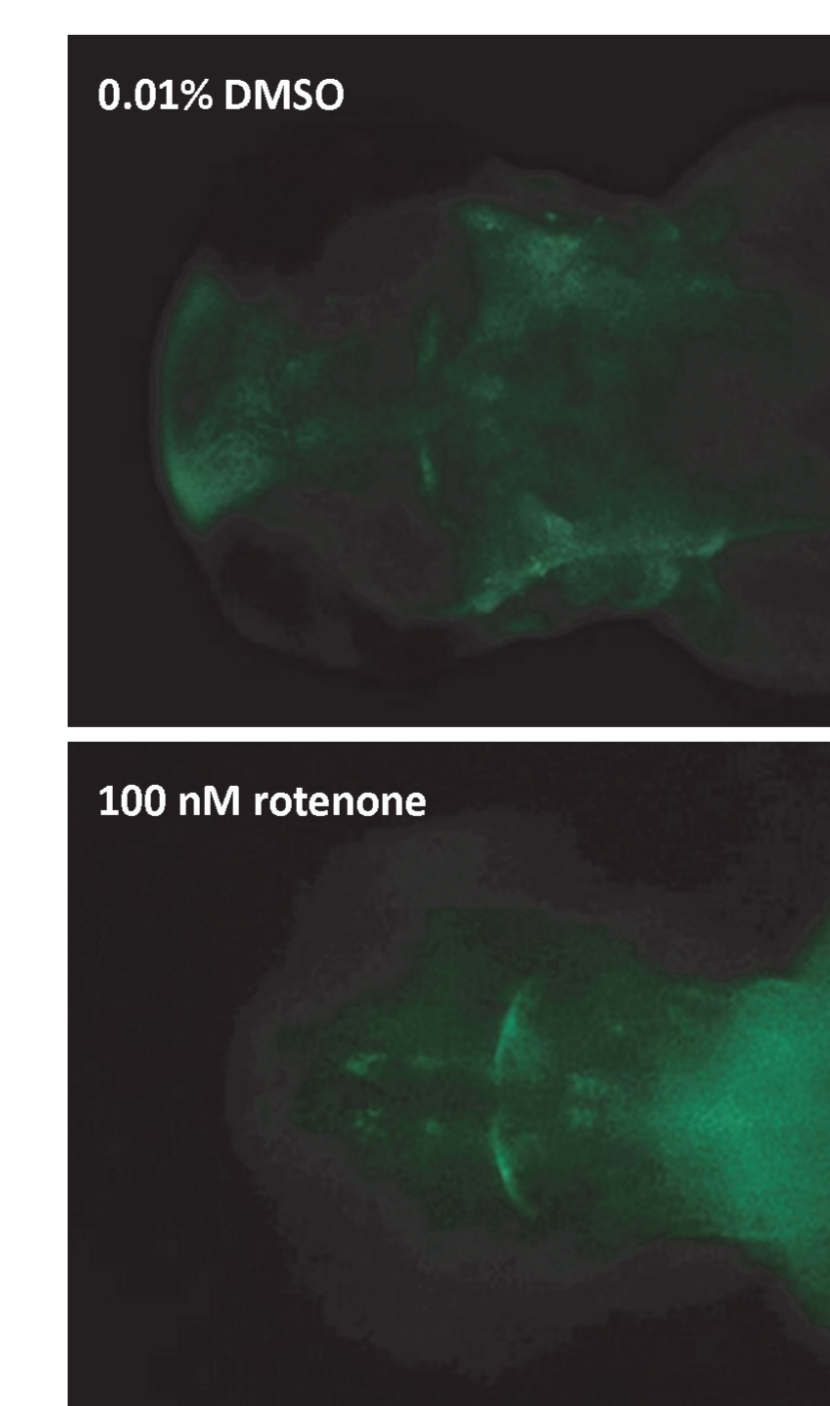
MPTP exposure increases caspase-9 activity in the brain.

Whole mount immunostains using anti-caspase-9 antibodies were performed on 3 days post-fertilization zebrafish embryos exposed to a 1 millimolar concentration of MPTP. Fish exposed to the neurotoxin demonstrate increased caspase-9 activity in multiple areas of the brain, including the olfactory bulb and ventral diencephalon.



Rotenone exposure increases caspase-9 activity in the brain.

Whole mount immunostains using anti-caspase-9 antibodies were performed on 3 days post-fertilization zebrafish embryos exposed to a 100 nanomolar concentration of rotenone. Fish exposed to the neurotoxin demonstrate increased caspase-9 activity in some areas of the brain, particularly in the ventral diencephalon region.



Double immunofluorescence on dorsal sections of *dat:eGFP* 7 days post-fertilization embryos.

DAPI staining of cell nuclei appears in the blue channel and anti-GFP staining appears in the green channel. Images were captured separately and superimposed. Comparable sections will be needed for GFP positive cell counting.

Conclusions

The zebrafish is a promising model for the study of environmentally induced Parkinson's disease. Rotenone and MPTP neurotoxicity appears to cause pharmacological disruption of the midbrain dopaminergic neurons in a dose-dependent manner. Treated embryos demonstrate a significantly reduced amount of dopaminergic cells in the midbrain in comparison to the untreated embryos. Dopaminergic disruption in the ventral diencephalon of the treated zebrafish is particularly interesting, as such region is believed to comprise structures that share homology with the human substantia nigra.

The lower levels of *dat* and *drd2b* transcript in treated zebrafish homogenate demonstrate that zebrafish exposed to rotenone and MPTP exhibit dopaminergic system alterations at both cellular and molecular levels. The caspase-9 staining pattern observed in the treated embryos shows great similarity with the green fluorescence neuronal patterning of the transgenic *dat:eGFP* embryos, indicating that dopaminergic neurons seem to be targeted. While the MPTP treated zebrafish tend to demonstrate increased caspase-9 activity in multiple regions of the brain, rotenone seems to increase caspase-9 activity specifically in the ventral diencephalon. Caspase-9 staining patterns suggest that both neurotoxins induce neurodegeneration by apoptotic cell death.

These data provide a foundation for the study of environmentally induced Parkinson's disease, and yield an interesting insight to characterize the mechanisms of neurotoxin induced neurodegeneration.

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