THE UNIVERSITY OF ALABAMA IN HUNTSVILLE | COLLEGE OF SCIENCE

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# Optimizing a Method for Investigating the Role of p75$^{NTR}$ in Neurodegeneration Associated with Parkinson's Disease

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## Introduction

Parkinson's Disease (PD)is a progressive neurodegenerative condition characterized by tremors, rigidity, and bradykinesia, among other symptoms. The degeneration of dopaminergic (DA) neurons in a region of the brain known as the Substantia Nigra pars compacta (SNpc) is associated with PD. The DA neurons of the SNpc have axonal projections that reach another region known as the striatum (Dauer & Przedborski, 2003). In PD, the DA neurons within the striatum undergo oxidative stress and mitochondrial dysfunction, resulting in the breakdown of the axonal projections in the Nigrostriatal pathway, eventually leading to the death of DA neurons (Chang & Chen, 2020). While PD is a heavily researched condition, the cellular mechanisms responsible have yet to be fully elucidated. Key to the development of future treatments and prevention of PD is a better understanding of the cellular signaling that leads to PD.

P75$^{NTR}$ protein is a transmembrane protein that has been shown to interact with co-factors in the brain, resulting in either pro-death or pro-survival signaling in the cell (Ali et al., 2024). Cell culture studies have shown that P75$^{NTR}$ plays a role in the regulation of apoptosis in DA neurons in cell culture studies (Kraemer et al., 2021); however, P75$^{NTR}$ 's role in vivo has yet to be confirmed. This project aimed to optimize procedures to investigate the role of P75$^{NTR}$ in the death of DA neurons in vivo. PD mouse models were produced using stereotaxic surgery techniques and verified for efficacy using immunohistochemistry and stereology. Immunofluorescence staining was also used to test the efficacy of a sortilin antibody.

# Methods

Immunohistochemistry

Brain tissue samples were collected one-week post-op, cryosectioned, and alternating sections were stained for tyrosine hydroxylase (TH). A new TH antibody was used for staining; therefore, experiments were conducted to optimize the antibody concentrations. 50 µm and 15 µm sections of the SNpc and striatum were collected, permeabilized in 0.3% PBS Triton, blocked in 10% normal goat serum (NGS) in PBS, and incubated overnight at 4°C in Tyrosine Hydroxylase (TH) antibody (E2L6M) at a dilution of 1:400 and 1:200 in 10% NGS in PBS. On the second day, sections were incubated in 3% hydrogen peroxide in water, except for some SNpc sections, which went without hydrogen peroxide. The sections were then incubated in a biotinylated secondary antibody solution and sequentially incubated in ABC reagent with avidin coupled with a biotin-conjugated HRP. Sections were then incubated in DAB substrate for exactly 10 minutes. Stained nigra sections were assessed by stereology via the optical fractionator method using Stereologer by SRC Biosciences.

Immunostaining

50 µm sections of the hippocampus and Substantia Nigra were collected from a wild type mouse. The sections were permeabilized in 0.3% PBS Triton for 20 minutes and blocked in 10% NGS in 0.1% PBS Triton for 1.5 hours. For TH staining, sections were incubated overnight in 1:400 dilution of anti-TH (E2L6M) before incubating in 568 Alexa Fluor-conjugated secondary antibody. For sortilin staining, the sections were incubated in anti-sortilin antibody (MAB3154) overnight at various concentrations: 1:200, 1:500, 1:1000, and a condition without primary antibody. Sections of the hippocampus were stained at similar concentrations as a positive control. The sections were then incubated in Alexa Fluor 488+ conjugated secondary antibody. The sections were imaged using confocal microscopy one-day post-stain.

Stereotaxic Surgery

C57Bl6 wild type mice were administered 6-hydroxydopamine (6 OHDA), a neurotoxin that induces oxidative stress, by stereotaxic injection. Thirty minutes before the procedure, the mice were given an intraperitoneal injection of 10 µg/Kg desipramine. The desipramine is a norepinephrine uptake inhibitor that prevents the uptake of 6-OHDA by adrenergic neurons, making the injection more specific to DA neurons. Using a Kopf stereotaxic frame and 10 µL Hamilton syringe, the striatum was injected with 2 µL 6-OHDA. The mice were then euthanized 1-week post-op, and brain samples were fixed in 4% PFA for 17 hours.

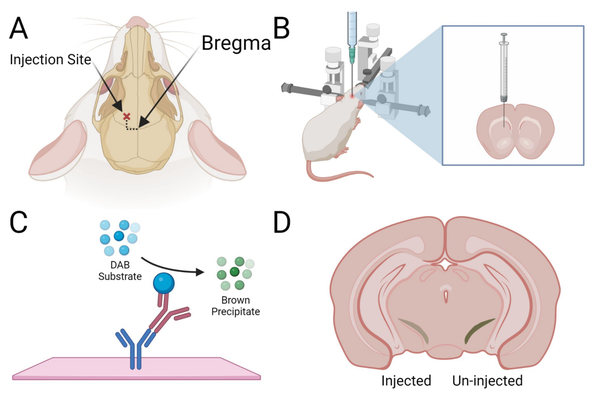


Figure 1. Stereotaxic surgery process. A) The injection site was identified using bregma as a landmark. B) The stereotaxic frame was used to position the mouse to allow for precise injection. C) DAB IHC utilizes a secondary antibody with a coupled peroxidase reacts with the DAB substrate to form a brown precipitate. D) The brown precipitate formed in a section can be viewed on brightfield microscopy.

## Results

DAB Staining Optimization

Optimization of the IHC protocol first began with the concentration of primary antibody (E2l6M) to use. 15µm and 50 µm sections were stained at 1:200 and 1:400 concentrations of primary antibody (Figure 2A). The optimal staining concentration was shown to be region-dependent, with sections of the striatum having the desired prominence of the staining region with the 15 µm sections with a dilution of 1:200 and the SNpc sections at 50 µm sections at 1:400 primary antibody dilution. However, the problem with the thicker sections was the warped tissue morphology after blocking it with hydrogen peroxide. Hydrogen peroxide is commonly used in DAB staining to block endogenous peroxidases and to remove background noise. Another test was performed to determine if the hydrogen peroxide was necessary, and the results indicated that blocking endogenous peroxidases was unnecessary.

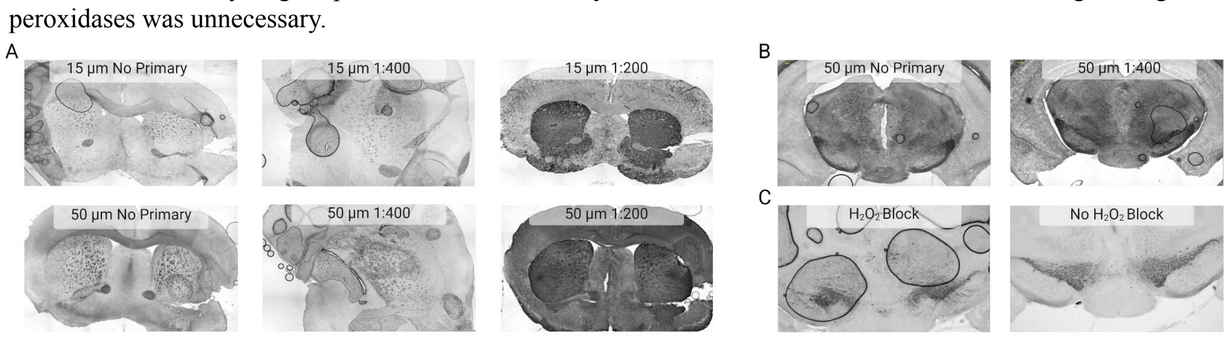


Figure 2. Brain tissue sections of IHC optimization A) 15 µm and 50 µm sections stained with 1:200 and 1:400 anti-TH antibody B) 50 µm sections of SNpc stained with 1:400 anti-TH antibody C) 50 µm sections of SNpc stained with 1:400 anti-TH, one with the Hydrogen peroxide block and one without.

Sortilin Antibody for Staining

A previous study indicated that sortilin is expressed in the SNpc. However, other research groups have not yet replicated this finding, so we sought to confirm sortilin expression in nigral DA neurons. Sections of the SNpc were stained for sortilin as green and TH as red. A range of concentrations of the anti-sortilin antibody MAB3154 was used to test the efficacy further. There was little evidence of sortilin expression seen in the SNpc. Sections of the hippocampus were also stained with similar conditions, which served as the positive control as sortilin has already been shown to be expressed in the hippocampus, and there was also no signal indicating the presence of sortilin. These results suggest that the anti-sortilin antibody was not suitable for staining.

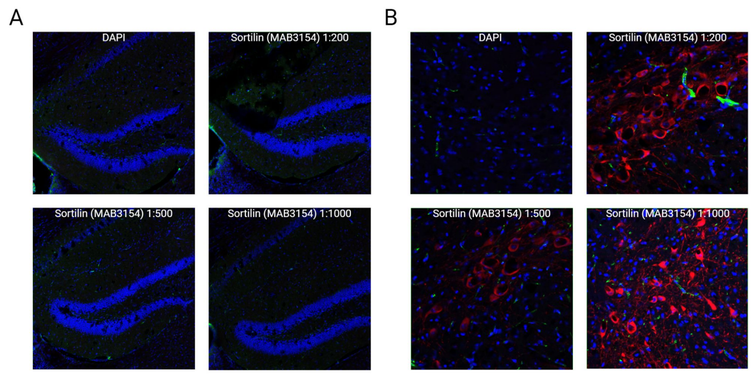


Figure 3. Immunostained tissue. Blue is the nuclei, red is TH, and green is sortilin
A) tissue samples of the hippocampus B) Tissue samples of the Nigra

Efficacy of Stereotaxic Surgery

Stereology was performed to verify the efficacy of the intrastriatal 6-OHDA injection in promoting dopaminergic neurodegeneration. Brains were collected one-week post-op and cryosectioned after fixing. Every fifth 15 µm section of the striatum and every 50 µm section of the SNpc was collected. Every other slice of the SNpc was DAB stained and analyzed using Stereologer. DA neuron estimates for the injected side were lower than the contralateral un-injected side (Figure 4b). The results suggest that the stereotaxic surgery procedure successfully produced a PD mouse model.

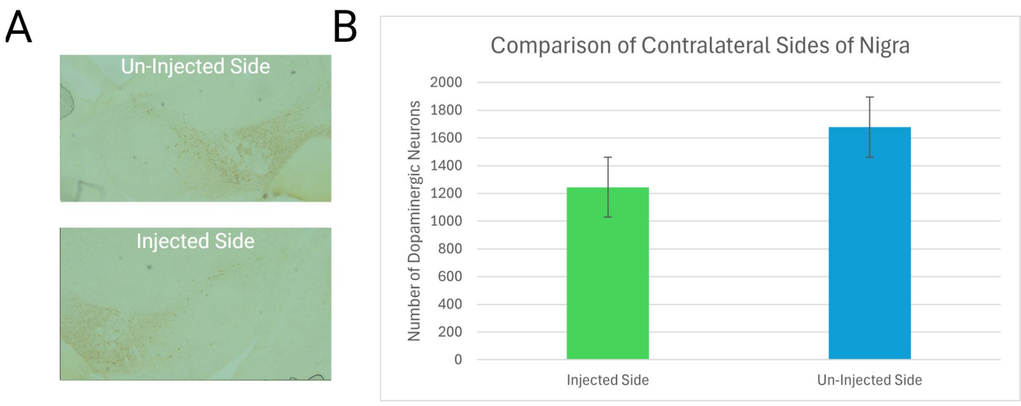


Figure 4. Verification of the efficacy of the stereotaxic procedure. A) Comparison of the un-injected side of the SNpc to the injected Side. B) Quantitative comparison of the contralateral sides of the injected mouse brains (n=3).

## References

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