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Brandon Adair  
*Carroll College, badair@carroll.edu*

Tamra E. Jones  
*Carroll College, tjones@carroll.edu*

Stefanie Otto-Hitt  
*Carroll College, sotto-hitt@carroll.edu*

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Manganese Toxicity in the Dopamine Synthesis Pathway in Drosophila melanogaster

Brandon Adair, Tamra Jones, and Stefanie Otto-Hitt, PhD
Carroll College
July 19, 2018

Experiment Type: Reverse transcriptase (RT) PCR
Feature: Manganese Toxicity
Gene: ple: Pale (ple) is a tyrosine hydroxylase that functions in the first and rate-limiting step of dopamine synthesis

1 Introduction

Manganese is prevalent in Montana, and Manganese toxicity symptomatically resembles Parkinson’s disease. The goal of this project was to determine whether exposure to high levels of Manganese affects the Dopamine synthesis pathway. For our experiment, we chose to measure the expression of the ple gene in Drosophila melanogaster that were exposed to Manganese during development. The ple gene codes for Tyrosine Hydroxylase, an enzyme that functions in the first rate-limiting step of Dopamine synthesis. We hypothesized that an abundance of Manganese would result in decreased expression of ple in larvae and observable motor function deficits among adult flies.

To carry out the experiment, flies were randomly assigned either to a control group, cultured under ideal conditions, or a treatment group, cultured in the presence of 0.1mM Manganese Chloride. The experimental group was exposed to Manganese Chloride for 72 hours, after which RNA extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR) was conducted to compare ple expression. Furthermore, flies were randomly selected to develop into adulthood, after which an assay was performed to compare and quantify motor function in both the control and experimental groups.

Because of the oxidative stress Manganese places on the Dopamine synthesis pathway it was predicted that the expression of ple in the experimental groups would be reduced and the developed motor function would be significantly compromised due to the deficiency of Dopamine.
2 Methods

Primer synthesis: Primers for ple were designed using Flybase and Integrated DNA Technologies PrimerQuest. The sequences of the forward and reverse primers targeting ple are as follows: AGCCGACCAAGAACGATTAC (Forward) and TGGTCATCAGATGGTTGCAG (Reverse). The sequences of the control Gapdh primers are as follows: CGCCAAGAAGGTCATCATCTC (Forward) and CCTCGACCTTAGCCTTGATTTT (Reverse).

Culturing Drosophila: Wild type Drosophila cultures were purchased from Carolina Biological and maintained in Formulæ 4-24 Instant Drosophila Medium in culture vials as per the manufacturers protocol (Carolina Biological). Briefly, equal volumes of water and dry media were added to the culture tubes along with several grains of bakers yeast (Red Star Yeast). The cultures were incubated at room temperature and were sub-cultured every two weeks. The anesthetizing of the flies was accomplished using CO2 FlyBeds (Azer Scientific) and The Flowbuddy CO2 regulator (Flystuff.com). For the experiment, an equal volume of 0.1M Manganese Chloride was added to the dry Formula 4-54 media and water was added to the control cultures. The adult flies were applied to the culture vessels for 24hrs before being removed and the resulting larvae were maintained for 72hrs before being processed for RT-PCR.

RNA extraction: RNA was extracted from control and experimental Drosophila larvae after 72hrs using Qiagens RNeasy Mini Kit as per the manufacturer’s instructions. To prepare for RNA extraction, the larvae were transferred, using forceps, into 35mm petri dishes filled with 1x PBS (ThermoFisherScientific). The larvae were then placed in pre-chilled Eppendorf tubes and incubated in the freezer for 10 minutes. After freezing, a 1000uL micropipette tip was used to grind the larvae for 1 minute. Following the addition of Buffer RLT, the larval tissue was gently passed through an 18 gauge-needled syringe 10 times followed by a 25 gauge-needled syringe 10 times. During RNA extraction, the samples were subjected to on-column DNase treatment using an RNase-free DNase kit (Qiagen).

RT-PCR: cDNA was synthesized using the RevertAid RT kit (ThermoScientific) and following the manufacturer’s protocol. PCR was performed using GoTaq Green PCR Master Mix (Promega) using the manufacturer’s protocol. PCR amplification of Gapdh cDNA was used as a positive control for the Drosophila RT-PCRs while the RevertAid Gapdh was used as a positive control for the entire RT-PCR experiment as the reagents for this sample were provided in the RevertAid kit. The No Template Control (NTC) reactions contained all reagents for the RT-PCR reaction, except nuclease-free water was added in place of RNA template.

Gel electrophoresis: The RT-PCR reactions were electrophoresed on a 1% agarose gel (BioRad) in 1xTBE (VWR). The ImageJ gel analysis program was used to determine the relative intensities of the PCR products for semi-quantitative analysis.

Negative Geotaxis Assay: After the control and Manganese Chloride-treated larvae developed into adults, 30 flies were selected at random from each group with 10 randomly
assigned to one of three empty fly culture vials. Flies were tapped to the bottom of the vial and then allowed to climb. A performance index was calculated by measuring the percentage of flies in each vial that could climb above 8-cm by 10 seconds following the tap. This was reproduced ten times for each vial, giving n=60 for control and treatment groups.

3 Results

The expression of ple across four control and four Manganese Chloride-treated groups was found not to be statistically significant (Figure 1, p = 0.71). However, the negative geotaxis assay showed a statistically significant decrease in motor function in 0.1 mM Manganese Chloride treated Drosophila melanogaster when compared to controls (Figure 2, p = 9.77E-6). These combined results suggest that Parkinsons-like symptoms in Manganism patients are likely not linked to defects in the enzyme Tyrosine Hydroxylase.

Future experiments should focus on the second step in the Dopamine biosynthesis pathway, where L-DOPA is converted into Dopamine via the enzyme Aromatic L-amino acid Decarboxylase, which is encoded by the Dmel/Ddc gene. Analysis of Dmel/Ddc gene expression could strengthen our current finding that deficits in the Dopamine biosynthesis pathway are not the cause of similar symptoms between Manganism and Parkinsons patients. Furthermore, a more quantitative measure should be performed to better determine whether Manganese Chloride affects expression of the ple and Dmel/Ddc genes.

4 Figures

4.1 Relative Expression of ple in Control and MnCl2-treated Drosophila Cultures

The relative expression of ple compared to the control gene Gapdh was measured using semi-quantitative RT-PCR across four control and four MnCl2-treated Drosophila cultures. The error bars represent the standard error of the means for each condition. A student’s t-test was performed to determine the significance of changes in gene expression between the
control and experimental cultures, with \( p = 0.7147 \) for ple and \( p = 0.7133 \) for Gapdh.

### 4.2 Negative Geotaxis Assay of Control and MnCl$_2$-treated Drosophila

The distribution of performance index percentages for both control and MnCl$_2$-treated Drosophila was found to be significantly different (\( p = 9.77 \times 10^{-6} \)) following 72hrs of MnCl$_2$ treatment.

### 5 Acknowledgements

We would like to thank our faculty advisor, Dr. Stefanie Otto-Hitt for her guidance throughout this process.