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Effects of the Everyday Toxin, Titanium Dioxide, on Drosophila melanogaster Nervous System Development

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Effects of the Everyday Toxin, Titanium Dioxide, on Drosophila melanogaster Nervous System Development

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Experiment Type: Reverse transcriptase (RT) PCR
Feature: Titanium Dioxide
Gene: Neur: Neuralized (Neur) is an E3 ubiquitin ligase that plays a role in the endocytosis-dependent activation of ligands in the Notch signaling pathway. Neur also plays a role in Notch-independent epithelial morphogenesis.

1 Introduction

Titanium dioxide (TiO2) is a widely used compound found in everything from food packaging to sunscreens. When ingested, TiO2 is readily transported across membranes and efficiently stored within cells. Previous studies showed that exposure to TiO2 results in underdeveloped nervous systems. For our study, we attempted to answer the following question: Will exposing Drosophila melanogaster larvae to TiO2 affect expression of the Neur gene and development of the central nervous system? The Neur gene is crucial during the cell-determination stage of development as its encoded protein helps specify neuroblast development and aids in nervous system and sensory organ development. It was hypothesized that expression of the Neur gene would decrease in Drosophila larvae exposed to TiO2 and that nervous system development would be abnormal compared to control larvae.

To test this hypothesis, Drosophila larvae were randomly assigned to either a control group, which was cultured under ideal conditions, or a treatment group, which was exposed to a non-lethal concentration of TiO2. Following exposure, RNA extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR) was conducted to analyze expression of Neur. To quantify nervous system development, Drosophila larvae were subjected to a touch-response assay.

Because TiO2 likely interferes with neural development, it was predicted that Drosophila larvae would show decreased expression of Neur and that they would respond poorly to a mechanical touch-response assay.
2 Methods

Primer synthesis: Primers for Neur were designed using Flybase and Integrated DNA Technologies PrimerQuest. The sequences of the forward and reverse primers targeting Neur are as follows: ACTGCGGAAGGACACAATAC (Forward) and GGCCAAATCATGAGCAATGG (Reverse). The sequences of the control Gapdh primers are as follows: CGCCAAGAAGGTCATCATCTCTC (Forward) and CCTCGACCTTAGCCTTGATTTTC (Reverse).

Culturing Drosophila: Wild type Drosophila cultures were purchased from Carolina Biological and maintained in Formula 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, 25mM TiO2 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 6 days before being processed for RT-PCR and the touch response assay.

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 1000µL 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.

Touch Response Assay: Following six days of exposure to TiO2, the development of sensory and motor neuron responses of control and TiO2-exposed larvae was tested using a touch response assay. First, larvae were isolated from the culture media using forceps and
placed into 35mm petri dishes containing 1x PBS. Individual larva were then transferred to depression slides with a drop of 1x PBS and placed under a compound microscope for viewing (Nikon). An eyelash glued onto a wooden toothpick was used as the stimulus probe and was brushed along the side of each larva. A positive response was recorded when a larva bended its body in response to the probe while a negative response was recorded when a larva did not respond to the probe stimulus. The assay was performed using 10 larvae from each culture across four control and four experimental cultures (n = 40 larvae from each group).

3 Results

The touch stimulus assay showed that Titanium dioxide significantly affected the ability of Drosophila larvae to respond to external stimuli, indicating an underdeveloped nervous system (p = 0.000432). Meanwhile, there was no significant effect of Titanium dioxide on Neur RNA expression (p = 0.41). These results agreed with our hypothesis regarding the effects of Titanium dioxide on nervous system function; however, they disagreed with our hypothesis regarding Neur expression.

Future studies should focus on varying the concentrations of Titanium dioxide and employing a more quantitative measure of gene expression. Furthermore, assays looking at either sensory or motor neuron development should be used to determine how TiO2 is affected the ability of larvae to response to external stimuli.

4 Figures

4.1 Relative Expression of Neur in Control and Titanium dioxide-treated Drosophila Cultures

![Relative Expression of Neur in Control and Titanium dioxide-treated Drosophila Cultures](image)

The relative expression of Neur compared to the control gene Gapdh was measured using semi-quantitative RT-PCR across four control and four TiO2-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=0.41 for Neur and p=0.49 for Gapdh.

### 4.2 Touch Response Assay in Titanium dioxide-treated Drosophila cultures

![Bar graph showing percent positive response](image)

The average percent of Drosophila larvae that had a positive response to an external stimulus was measured across four control and four Titanium dioxide-treated cultures. The ability of TiO2 treated larvae to respond to touch stimuli was significantly reduced compared to control cultures (p=0.000432). Error bars represent the standard error of the means and the measure of statistical significance (p value) was determined using a two-tailed t-test assuming unequal variance.

### 5 Acknowledgements

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