# EFFECTS OF BISPHENOL A EXPOSURE

# ON CENTRAL NERVOUS SYSTEM DEVLOPMENT

An Undergraduate Research Scholars Thesis

by

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Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Louise Abbott

May 2017

Major: Biomedical Science

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# ABSTRACT

Effects of Bisphenol A Exposure on Central Nervous System Development

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Bisphenol A (BPA), a common environmental toxicant and endocrine disrupting chemical, is becoming increasingly important due to its potential carcinogenicity. The aim of this research project was to study the effects of BPA exposure on the developing central nervous system. Recent studies have characterized the effects of BPA exposure on reproductive and cardiac systems. However, the neurobiological deficits of BPA exposure are largely unknown. Using zebrafish as a model organism, embryos were raised beginning 6 hours post fertilization (hpf) in medium containing varying concentrations of BPA from 1μM to 25μM. Between 6 hpf and 72 hpf, length, movement, hatching, and mortality were measured. Once the embryos matured to 72 hpf, they were euthanized, fixed, embedded in paraffin, and sectioned for immunohistochemistry procedures to study the expression patterns of myosin, PCNA (a marker of cellular proliferation), and caspase-3 (a marker of apoptosis). Additionally, this study aimed to investigate whether BPA exposure interfered with dopamine pathways by examining the expression patterns of tyrosine hydroxylase, using immunohistochemistry procedures and gene expression analyses. These data are potentially relevant to human exposure to BPA, to elucidate its potential negative effects on neurological development and health.

# DEDICATION

I would like to dedicate this paper to my parents for their continual encouragement to do my best work. I would not be where I am today without their unconditional love and support.

## ACKNOWLEDGEMENTS

I would like to thank my professors, Dr. Abbott and Dr. Villalobos, without whom I could not have completed this project. Their mentorship and support have been instrumental throughout my time at Texas A&M. I am thankful to have been able to learn much from them over the last 3 years.

I also want to extend my gratitude to Dr. Liheng Shi and the Ko Lab for their patience and willingness to help with all of the gene expression protocols, as well as Jennifer Dong and the Riley Lab for their assistance with the zebrafish breeding and embryo collection.

Finally, thanks to the undergraduates in the Abbott Lab- Kristin, Daniel, Lauren, Megan, Heather, and Xioayan- for assisting with all of the data collection.

## NOMENCLATURE

| | |
|---|---|
| BPA | Bisphenol A |
| HPF | Hours Post Fertilization |
| ZFEs | Zebrafish Embryos |
| PCNA | Proliferating Cell Nuclear Antigen |
| DMSO | Dimethyl Sulfoxide |
| DAB | Diaminobenzene |
| RT-qPCR | Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction |
| TH | Tyrosine Hydroxylase |

# CHAPTER I

# INTRODUCTION

Although Bisphenol A (BPA) is a prevalent environmental toxicant, little is known about its adverse effects on the body. BPA is found as a component in many plastics and resins, and can seep into foods and beverages from the lining of their containers (Bauer, 2016). In the past, it was difficult to study the direct effects of BPA exposure on vertebrates due to their long developmental periods, cost and space limitations, and the internal nature of their fertilization and development, but the emergence of zebrafish as a model organism to study translational neuroscience and central nervous system function (Stewart et al., 2014) has bridged that gap. Multiple advantages of using zebrafish include high physiological and genetic homology to mammals, external fertilization and rapid development, transparency of embryos and larvae, and cost- and space-effectiveness (Stewart et al., 2014).

BPA is most commonly known as an endocrine disrupting chemical. Recent studies in zebrafish have determined that BPA exposure alters early dorsoventral patterning, segmentation, and brain development in early embryogenesis (Tse et al., 2013). BPA early-life exposure toxicity has proven to cause cardiac edema, cranio-facial abnormalities, swimbladder inflation failure, and poor tactile response in zebrafish as well (Lam et al., 2011). In male zebrafish, its effects on the reproductive system include increased cell apoptosis in the testis, changes in the mitochondria and endoplasmic reticulum in Sertoli and Leydig cells, and elevated activated caspase-3 levels in the cytoplasm, which indicates increased rates of cellular apoptosis (Liu et al., 2013). A study examining human children identified several behavior deficits, such as anxiety, depression, and hyperactivity, corresponding with higher BPA levels in the urine

(Harley et al., 2013). These data suggest that the neural circuitry may be altered by BPA exposure.

Studies have shown that dysregulation of many functional processes including emotion, locomotion, learning, and memory, can lead to various neurological disorders such as schizophrenia and Parkinson's disease (Boehmler, 2006). Since BPA exposure is known to affect locomotion and emotion, it may also play a role in these neurological disorders. Researchers have identified hyperactive dopamine signaling at D2 receptors as a possible cause of schizophrenia since it increases both the positive and negative symptoms associated with the disease. Additionally, amphetamines and cocaine stimulate schizophrenia-like symptoms by affecting dopamine pathways. Schizophrenia can be treated with antipsychotics that lower dopamine levels. Conversely, Parkinson's is a disease that involves nerve damage to the substantial nigra, which decreases dopamine levels. Symptoms of Parkinson's include slow movement and anxiety, which are also documented effects of BPA exposure. Therefore, it is important to examine the effects of BPA exposure on dopamine signaling. By using live embryo, immunohistochemistry, and gene expression analysis procedures, this study aimed to examine the expression patterns of tyrosine hydroxylase following BPA exposure.

# CHAPTER II

# METHODS

**BPA exposure in zebrafish embryos**

Adult zebrafish were bred in the Biology Department, under the guidance of Jennifer Dong. At 2 hours post fertilization (hpf), the zebrafish embryos (ZFEs) were collected and brought to Dr. Abbott's lab for BPA exposure and incubation at 28.5°C. The ZFEs were transferred into 24-well plates, with 4-5 ZFEs per well, and immersed in 2 mL of embryo medium. At 6 hpf, the embryo medium was removed and replaced with embryo medium containing 1μM, 5 μM, 10 μM, and 25μM BPA. Because BPA is water insoluble, it was dissolved in DMSO to make a working stock solution, and then diluted in embryo medium to the specified concentrations. Due to the use of DMSO to dissolve the BPA, we created two control groups: control (no BPA and no DMSO) and DMSO control.

**Live embryo procedures**

The timeline of live ZFE exposure can be seen in Fig. 1 below. The first data collection point was at 24 hpf, at which time spontaneous movement activity of the ZFEs was recorded. Spontaneous movement was measured by counting the number of times the ZFEs moved during a 1-minute interval ($n = 30$ ZFEs per exposure group). At 30 hpf, the number of alive *vs*. dead and hatched *vs*. unhatched ZFEs were recorded. Similarly, alive *vs*. dead and hatched *vs*. unhatched counts were recorded at 54 hpf. The last live embryo data collection time point was 72 hpf, when elicited movement activity, alive *vs*. dead, and hatched *vs*. unhatched measurements were all recorded. Elicited movement was measured by gently touching the tails of the ZFEs and

recording how far they moved (n = 10 ZFEs per exposure group). Following the conclusion of the live embryo studies at 72 hpf, ZFEs were euthanized with MS-222 and immersion-fixed in 10% neutral buffered formalin. At this point, total length of a subset of the fixed ZFEs was measured (n = 20 ZFEs per exposure group). Then, the ZFEs were either embedded in paraffin and sectioned for immunohistochemistry or immersed in RNAlater® and frozen for gene expression assays.

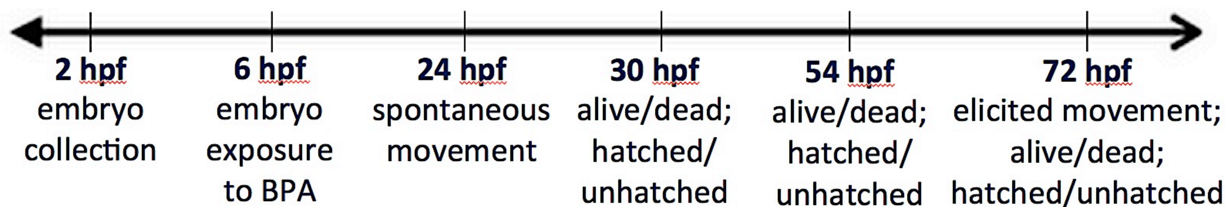


Fig. 1. Live ZFE Exposure Timeline

**Immunohistochemistry**

Zebrafish embryo sections that were embedded in paraffin were cut in the Veterinary Integrative Biosciences Histology Laboratory and sectioned onto slides. The slides were deparaffinized and blocked in normal horse serum to minimize non-specific binding of antibody. ZFE sections then were probed with primary antibody for the following proteins of interest: myosin (a protein expressed in muscle), proliferating cell nuclear antigen (PCNA– a protein expressed in cells undergoing cell division), activated caspase-3 (a protein expressed in cells dying via apoptosis), and tyrosine hydroxylase (TH– an enzyme involved in the production of dopamine). Next, the slides were incubated with a secondary antibody, biotinylated-horse anti mouse serum, and stained using diaminobenzine (DAB). The slides were viewed with a light microscope and staining intensity was analyzed using the NIS-Elements software. Mean gray values were calculated using the Image J program. Statistical analysis was performed by

ANOVA, followed by Tukey and Student-Newman-Keuls tests to determine whether differences in protein expression were statistically significant among the various BPA concentrations.

## Gene expression analysis

*RNA extraction and cDNA synthesis*

RNA was extracted from whole ZFEs using TRIzol® reagent, and RNA cleanup was performed using the Qiagen® RNEasy Mini Kit. Then, nanospectrophotometry analysis was used to determine the RNA concentration in each sample. cDNA was synthesized using the Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR. The protocol was based off Peterson and Freeman's article in the Journal of Visualized Experiments (Peterson and Freeman, 2009).

*Quantitative real-time reverse transcriptase PCR*

The SsoAdvanced™ Universal SYBR® Green Supermix was used to perform quantitative real-time reverse transcriptase PCR (RT-qPCR). First, a standard curve was run in triplicate for each gene to determine that the optimal volume of cDNA needed for each reaction was 2 μL. Then, the mastermix, forward and reverse primers, water, and cDNA from each sample were combined, and the assay was run in triplicate for each gene- TH1, TH2, and β-actin (a housekeeping gene used as a reference control). The primer sequences used were as follows: *TH1*: forward, GACGGAAGATGATCGGAGACA; reverse, CCGCCATGTTCCGATTTCT. *TH2*: forward, CTCCAGAAGAGAATGCCACATG; reverse, ACGTTCACTCTCCAGCTGAGTG. *β-actin*: forward, CGAGCAGGAGATGGGAACC; reverse, CAACGGAAACGCTCATTGC. Primer sequences were purchased from Bioneer Corporation and modeled after those used in a different study by Chen et al., 2012. The

following cycling parameters were used, which were based on the same paper by Chen et al., 2012: 95°C for 30 s and 45 cycles of the following pattern, 95°C for 10 s and 62°C for 45 s. SYBR Green was used to monitor fluorescence changes after every cycle. Resulting data were calculated by the comparative method using Ct values of β-actin as the reference control.

# CHAPTER III

# RESULTS

### Spontaneous and elicited movement following BPA exposure

To study the effects of BPA exposure on the developing central nervous system, we first examined changes in movement. Since ZFEs were still in their chorion and unhatched at 24 hpf, spontaneous movement was tested. ZFEs exposed to 10 μM and 25 μM BPA exhibited a significant decrease in spontaneous movement ($p<0.01$) when compared to both the control and DMSO control groups. The 10 μM BPA-exposed group experienced a 0.30-fold decrease while the 25 μM BPA-exposed group experienced a 0.46-fold decrease in spontaneous movement (Fig. 2).

At 72 hpf, hatched ZFEs were tested for possible BPA effects on elicited movement. As seen with the spontaneous movement, ZFEs that were exposed to 10 μM and 25 μM BPA exhibited a significant decrease in elicited movement ($p<0.05$ and $p<0.01$). The 10 μM BPA-exposed group displayed a 0.31-fold decrease, while the 25 μM BPA-exposed group displayed a 0.82-fold decrease (Fig. 3). These data are consistent with the qualitative observations of increasing inactivity following exposure to increasing levels of BPA.

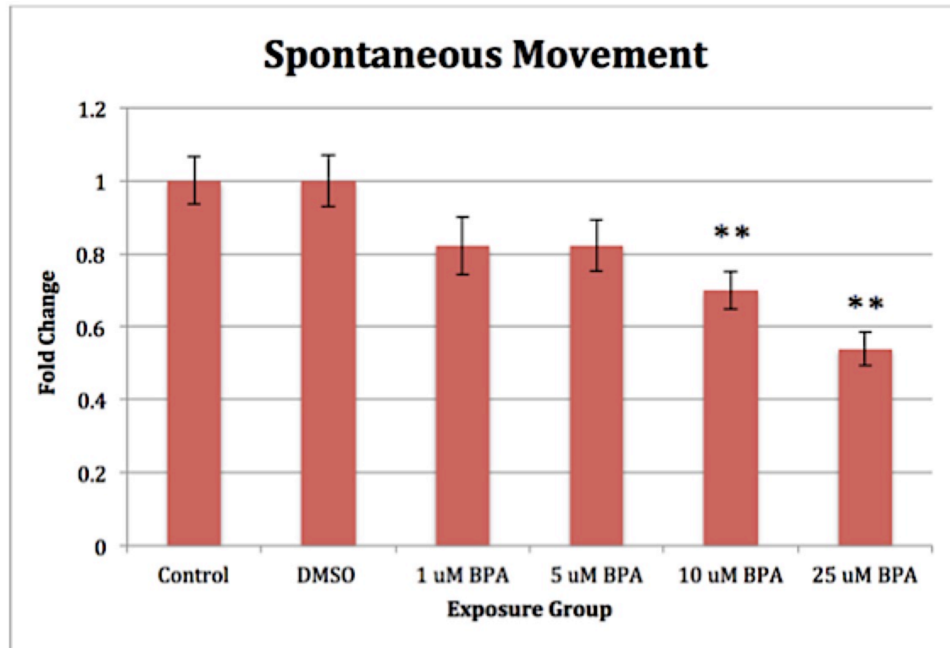


Fig. 2. BPA Induced Changes in Spontaneous Movement in ZFEs 24 hpf

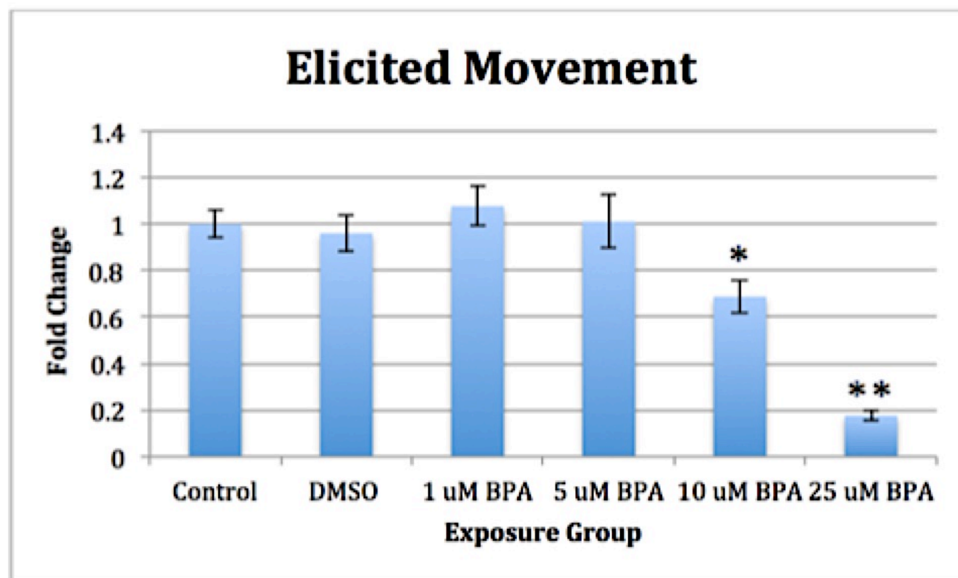


Fig. 3. BPA Induced Changes in Elicited Movement in ZFEs 72 hpf

**Total overall length of ZFEs**

Following live embryo procedures, the lengths of ZFEs were recorded at 72 hpf to determine whether total overall length was correlated with BPA exposure. BPA exposure resulted in more ZFEs with curved tails. However, BPA exposure did not significantly affect the total overall length of ZFEs except for those in the 25 μM BPA group (Fig. 4). The 25 μM BPA exposed group showed a 0.06-fold decrease in overall length ($p<0.01$) as compared to control ZFEs and a 0.04-fold decrease in overall length ($p<0.01$) when compared to the DMSO control group. Although ZFEs in the DMSO control group had slightly shorter overall lengths, the difference between the control and DMSO control groups was not statistically significant. This helps to confirm that any changes observed in ZFE length were due to the BPA exposure rather than DMSO exposure.

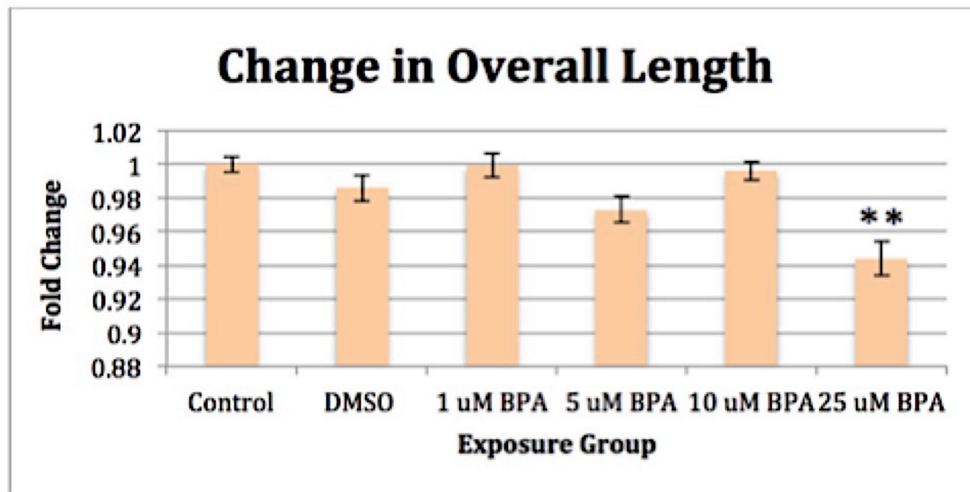


Fig. 4. BPA Induced Changes in Overall Length in ZFEs 72 hpf

**Myosin and PCNA expression in BPA-exposed ZFEs**

*Myosin*

Myosin is a motor protein that is best known for its role in muscle contraction and motility. After observing a decrease in both spontaneous and elicited movement due to BPA exposure, we examined the effects of BPA on myosin. To assess the myosin expression pattern in ZFE tail regions, immunohistochemistry was performed on 72 hpf sections. The mean gray value of multiple areas along the tail muscle fibers was recorded and averaged. Higher levels of myosin staining were evident in control ZFEs (Fig. 5A) compared to the 25 μM BPA-exposed ZFEs (Fig. 5B). The 5 μM, 10 μM, and 25 μM BPA-exposed ZFEs all showed significantly decreased levels of myosin expression ($p<0.01$, $p<0.01$, and $p<0.05$). However, the 10 μM BPA exposure group showed the largest reduction in myosin protein expression compared to the control and DMSO control groups (Fig. 6).

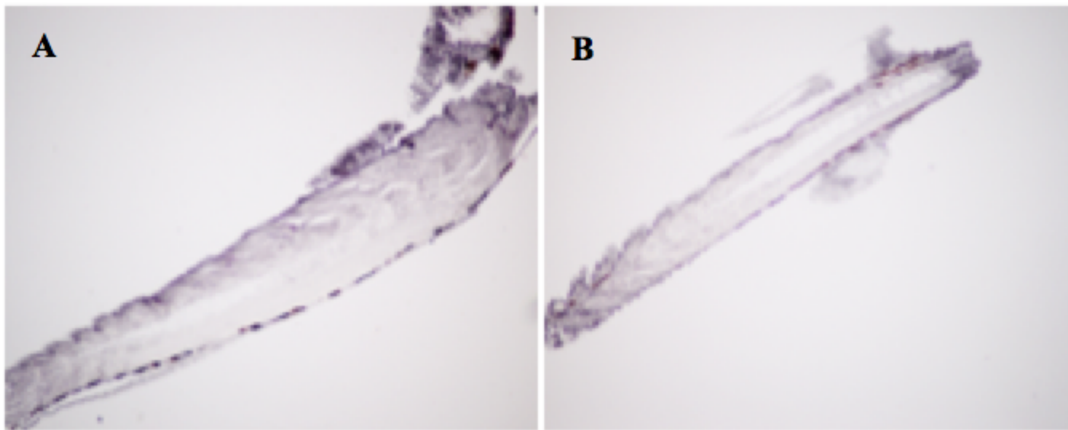


Fig. 5. Myosin Expression in (A) Control ZFEs and (B) 25 μM BPA Exposed ZFEs at 72 hpf

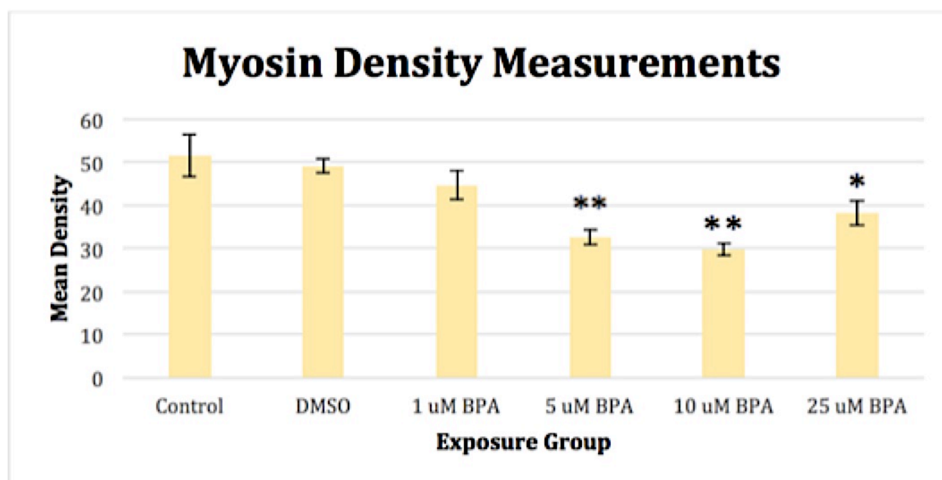


Fig. 6. BPA Induced Changes in Density of Myosin Staining in ZFEs 72 hpf

*PCNA*

PCNA is a protein essential for DNA replication in eukaryotic cells. Since BPA exposure has already been shown to increase apoptosis in the testis and cytoplasm of the male reproductive system in zebrafish, we investigated whether BPA exposure conversely decreases the number of proliferating cells. We used immunohistochemistry to assess the expression pattern of PCNA in ZFE brain regions, using sagittal sections of 72 hpf ZFEs. The mean gray value of the entire brain region up to the level of the beginning of the yolk sac was recorded. Levels of PCNA expression in control ZFEs (Fig. 7A) were higher than levels of PCNA expression in 25 μM BPA-exposed ZFEs (Fig. 7B). BPA exposure significantly reduced PCNA expression in 1 μM, 5 μM, 10 μM, and 25 μM BPA groups ($p<0.05$) when compared to the control and DMSO control groups (Fig. 8).

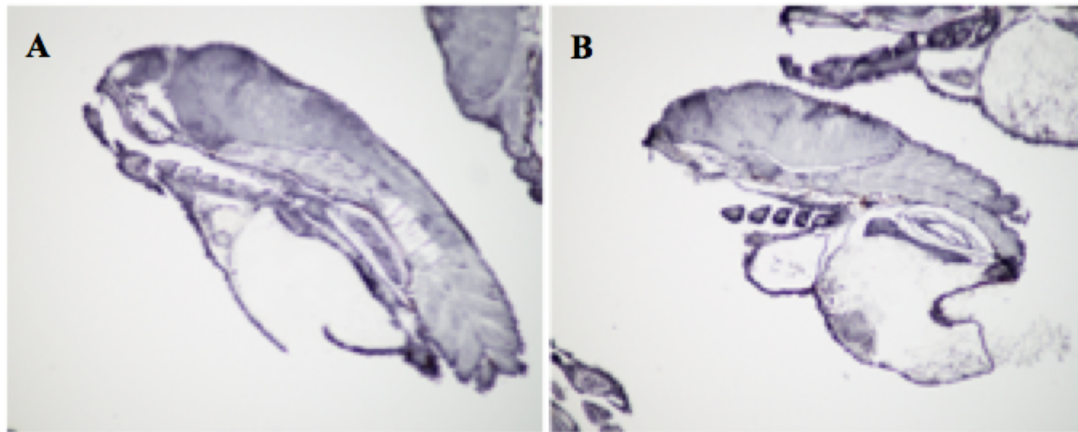


Fig. 7. PCNA Expression in (A) Control ZFEs and (B) 25 μM BPA Exposed ZFEs at 72 hpf

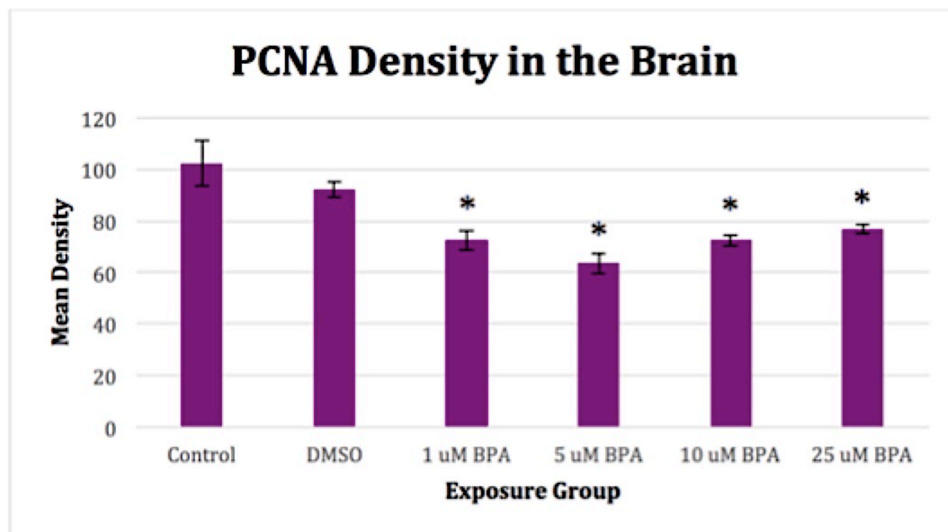


Fig. 8. BPA Induced Changes in Density of PCNA Staining in ZFEs 72 hpf

**TH1 and TH2 gene expression levels**

BPA exposure decreased spontaneous and elicited movement at an organismal level, as well as decreased myosin protein expression. Since dopamine pathways play a critical role in

motor control, we hypothesized that BPA exposure would also affect dopamine signaling. TH1 and TH2 are genes that code for tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis. Tyrosine hydroxylase converts tyrosine into L-DOPA, which is a precursor to dopamine. Using RT-qPCR, changes in TH1 and TH2 gene expression were measured and normalized to β-actin gene expression. We observed an apparent increase in TH1 gene expression and decrease in TH2 gene expression following BPA exposure. However, the standard error was too large for the results to be statistically significant. Therefore, the expression patterns of TH1 and TH2 were not significantly altered by BPA exposure (Fig. 9).

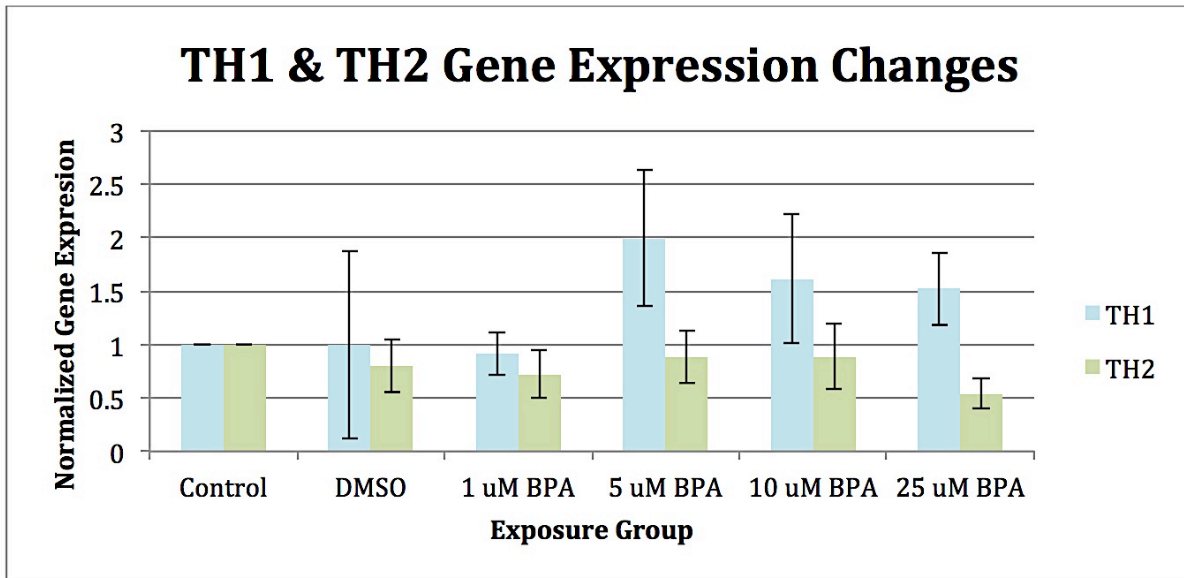


Fig. 9. Normalized TH1 and TH2 Gene Expression in ZFEs following BPA Exposure

# CHAPTER IV

# CONCLUSION

We examined dose-dependent effects of BPA exposure on the developing central nervous system using zebrafish as our model animal. On the organismal level, decreases in spontaneous movement, elicited movement, and total overall length of ZFEs were observed. These changes prompted investigations into the effects of BPA exposure on various proteins. Levels of myosin expression in ZFE tail regions were significantly decreased by exposure to BPA at 5 μM, 10 μM, and 25 μM. Additionally, PCNA expression in ZFE brain regions were significantly reduced in all BPA-exposed groups: 1 μM, 5 μM, 10 μM, and 25 μM. PCNA is a protein that is expressed in dividing cells. Reduced expression of PCNA is suggestive that fewer cells are dividing in BPA-exposed ZFEs compared to control ZFEs. It is possible that developing brain cells are not dividing as fast or that cells could be dying in excessive numbers compared to the normal cell death rate that is expected in the developing ZFE brain. Further experimentation is ongoing to determine whether BPA exposure also affects levels of activated-caspase-3, a marker of cellular apoptosis, in ZFE brain regions.

Changes in movement and myosin expression due to BPA exposure led us to explore the possibility that BPA exposure interferes with dopamine signaling, as dopamine pathways are integral to proper motor functions (Seidler et al., 2010). Gene expression analyses of TH1 and TH2 levels indicated that BPA exposure does not significantly alter the levels of tyrosine hydroxylase coding genes. These data suggest that BPA exposure may not be involved in epigenetic changes to DNA, but may play a role in altering protein expression. Current

immunohistochemistry testing is underway to determine whether tyrosine hydroxylase protein expression is affected.

Future directions include studying the effects of BPA exposure on other movement-related pathways to determine how myosin expression and motor functions are inhibited. Furthermore, it will be important to identify alternative genes involved in the development and protection of the dopaminergic system to elucidate the effects of BPA exposure on movement and catecholamines in fetal central nervous systems. It also would be important to examine the effects of BPA on developing central nervous systems following maternal, paternal, and *in utero* exposure to the toxicant.

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