TEST OF BPA'S ESTROGENIC EFFECTS ON BRAIN AROMATASE EXPRESSION, NEURAL ACTIVITY, AND LOCOMOTIVE BEHAVIOR IN ZEBRAFISH LARVAE

By

Ryan Jordan McAuley

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Committee Membership

Dr. Ethan Gahtan, Committee Chair

Dr. Amanda Hahn, Committee Member

Dr. Bruce O'Gara, Committee Member

Dr. Christopher Aberson, Program Graduate Coordinator

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Abstract

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Ryan Jordan McAuley

Bisphenol A (BPA) is a well-known endocrine disrupting chemical that mimics the effects of estrogens. Aromatase B (Cyp19a1b) is a brain-specific enzyme that converts testosterone to estrogen and is highly upregulated in response to estrogen receptor activation localized to radial glial cells. During embryonic zebrafish development, there is a small window of time denoted by an increase in neurogenesis and estrogen receptor activity. Previous studies have demonstrated that a low dose BPA exposure (0.1µM) during this window causes hyperlocomotion in larval zebrafish, yet no further explanation for this behavior change has been described. The purpose of this study was to identify whether (0.1µM) BPA exposure during this developmental window could be influencing Ca^{2+} dynamics, and if this correlated to swim activity changes. Two transgenic zebrafish lines, Cyp19a1b:GFP and Elavl3:GCaMP, were used in order to measure changes caused by BPA exposure. Confocal microscopy imaging techniques quantified Cyp19a1b expression in radial glia and dynamic GCaMP expression in neurons over time but did not find significant effects between BPA-treated and control-

treated groups for either measurement. Furthermore, swim activity tests failed to replicate the difference in time spent swimming between BPA and control groups.

Acknowledgements

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Introduction

Endocrine disrupting chemicals (EDCs) are compounds that can mimic, antagonize, alter or otherwise interfere with endogenous steroid production, metabolism or action at receptor targets (Frye et al., 2012). Thousands of chemicals have been identified as having endocrine disrupting properties and are of both naturally-occurring or synthetic origin. They can originate from numerous sources and may enter the aquatic environment through wastewater, agricultural run-off, and groundwater discharge, leading to contamination of the ecosystem through bioaccumulation in plant and animal bodies (Caballero-Gallardo, Olivero-Verbel, & Freeman, 2016). Xenoestrogens are a class of EDC which appears to be especially persistent in the environment. They are natural or industrial compounds that specifically mimic endogenous estrogens or interfere with estrogen signaling pathways. Exposure through ingestion or contact can often result in detectable effects such as estrogen activation (EA) at low concentrations, particularly critical effects may occur during the fetal and postnatal developmental stages (Kerdivel, Habauzit, & Pakdel, 2013).

One such xenoestrogen which has garnered the attention of researchers is Bisphenol A (BPA). It is a high-volume production chemical present in polycarbonate plastics and epoxy resins which are used in many common products such as beverage/food containers, the interior lining of metal cans, and the ink for thermal paper receipts (Frye et al., 2012). BPA has been acknowledged as a high priority for health assessment risk due to widespread exposure in humans. 95% of American adults have

BPA present in their urine at biologically relevant levels (Calafat et al., 2005) and exposure is likely to occur due to it leaching from plastics at low concentrations, a process which can often be accelerated from common stressors such as sunlight, UV & microwave radiation, and moist heat (Yang, Yaniger, Jordan, Klein, & Bittner, 2011). Effects of BPA exposure in humans is not yet fully understood but it appears to be correlated with male & female infertility, precocious puberty, breast & prostate cancers, and metabolic disorders such as polycystic ovarian syndrome (Diamanti-Kandarakis et al., 2009).

Animal models are increasingly being used in recent years as tools further define BPA's actions on biological systems. Zebrafish (*Danio rerio*) as a model organism displays conservation of many aspects of the endocrine system when compared to other vertebrates, including humans (Löhr & Hammerschmidt, 2011). High reproducibility, short generation time, ease of care, transdermal absorption of drugs, transparency during embryonic development, and simplicity of behavioral responses are amongst the traits which make zebrafish ideal for researchers interested in questions about development and disease. Furthermore, the generation of transgenic zebrafish lines which express fluorescent proteins has proven to be an invaluable tool since zebrafish embryos are transparent during embryonic development, which makes *in vivo* imaging possible. Optogenetic research with transgenic zebrafish has been able to identify ER activity in response to both natural and synthetic estrogens by observing fluorescence in estrogenic tissues such as the liver, ovary, pituitary gland, and brain (Gorelick & Halpern, 2011).

Within the last 10 years, the generation of estrogenic-responsive transgenic zebrafish lines has led to more interest and progress in research describing the many effects of xenoestrogen exposure. Although research began by identifying effects on sexual development, mating behaviors, and general toxicity, only recently has research began to focus on effects directly exerted through interactions with the brain. The development of a transgenic Cyp19a1b:GFP zebrafish line which expresses green fluorescent protein in response to the brain aromatase Cyp19a1b promotor has become a useful tool for direct evaluation of xenoestrogens' EA and characterization of radial glia in the developing brain (Tong et al., 2009). In the past few years this line has been able to demonstrate that BPA induces fluorescence in embryos at environmentally relevant concentrations, and that exposure leads to changes in neuronal and glial proliferation as well as behavioral changes.

There is still much to be discovered about how these effects may be related. Replication of these results and further experiments designed to explain mechanisms by which the changes in neural development from BPA may lead to behavioral differences may both reinforce recent findings while helping fill in gaps in the literature.

Literature Review

Endocrine Disrupting Chemicals

The topic that this study wishes to investigate is the consequences of exposure to endocrine-disrupting chemicals during early brain development. Endocrine-disrupting chemicals (EDCs) are substances present in the environment, food, and consumer products which are implicated in interference with hormone biosynthesis, metabolism, homeostatic control, and reproduction (Diamanti-Kandrarakis et al., 2009).

Xenoestrogens. Xenoestrogens are a class of EDC that has been garnering attention from researchers for their ability to imitate estrogen. They can be either synthetic or natural molecules which have estrogenic activity (EA) when interacting with living organisms. Many synthetic xenoestrogens are present in the environment due to their use in a wide variety of industrial compounds. Thermoplastic resins created from polymerizing specific monomers and additives have been demonstrated to exhibit EA due to the physicochemical property of an insufficiently hindered phenol group which enables binding to estrogen receptors (Yang et al., 2011). Many types of polycarbonate (PC) plastic products are known to leach EA chemicals, and it has recently discovered that even PC-replacement products may leach chemicals with EA whether stressed or unstressed (Bittner, Yang, & Stoner, 2014). In mammals, chemicals which induce EA can produce many health-related problems such as obesity, early puberty in females,

reduced sperm counts, altered functions of reproductive organs, altered sex-specific behaviors, and increased rates of some breast, ovarian, testicular, and prostate cancers.

Bisphenol A (BPA). Bisphenol A, a xenoestrogen, is a key monomer used in the manufacturing of polycarbonate plastic or epoxy resins. Polycarbonate plastics are used to make a variety of products including water bottles and food containers. BPA has been shown to leach from these containers, especially when exposed to UV radiation, microwave radiation, and/or moist heat from boiling or dishwashing (Yang et al., 2011). BPA is also used as a color developer in carbonless copy paper and thermal point of sale receipt paper, and in these is present as a non-polymerized form more readily available for exposure (Hormann et al., 2014). BPA has been shown to be present in 94% of thermal receipt papers at concentrations up to 13.9mg/g, and thermal receipt papers contributed to over 98% of human exposure cases (Liao & Kannan, 2011). When BPA is ingested it does not linger in the body for long, it is conjugated to glucuronide and excreted by the kidneys, yet even while in BPA-G form it remains biologically active by increasing lipid accumulation and adipogenesis (Boucher, Boudreau, Ahmed, & Atlas, 2015). Urine samples of United States adults have shown that 95% of participants had BPA concentrations greater than 0.1μg/L, which indicates that human exposure to the chemical is widespread (Calafat et al., 2005). BPA has been acknowledged to interact with human estrogen receptors as an endocrine disruptor even at low doses, however its binding to ER and hormonal activity is extremely weak relative to estradiol (Takayanagi et al., 2006).

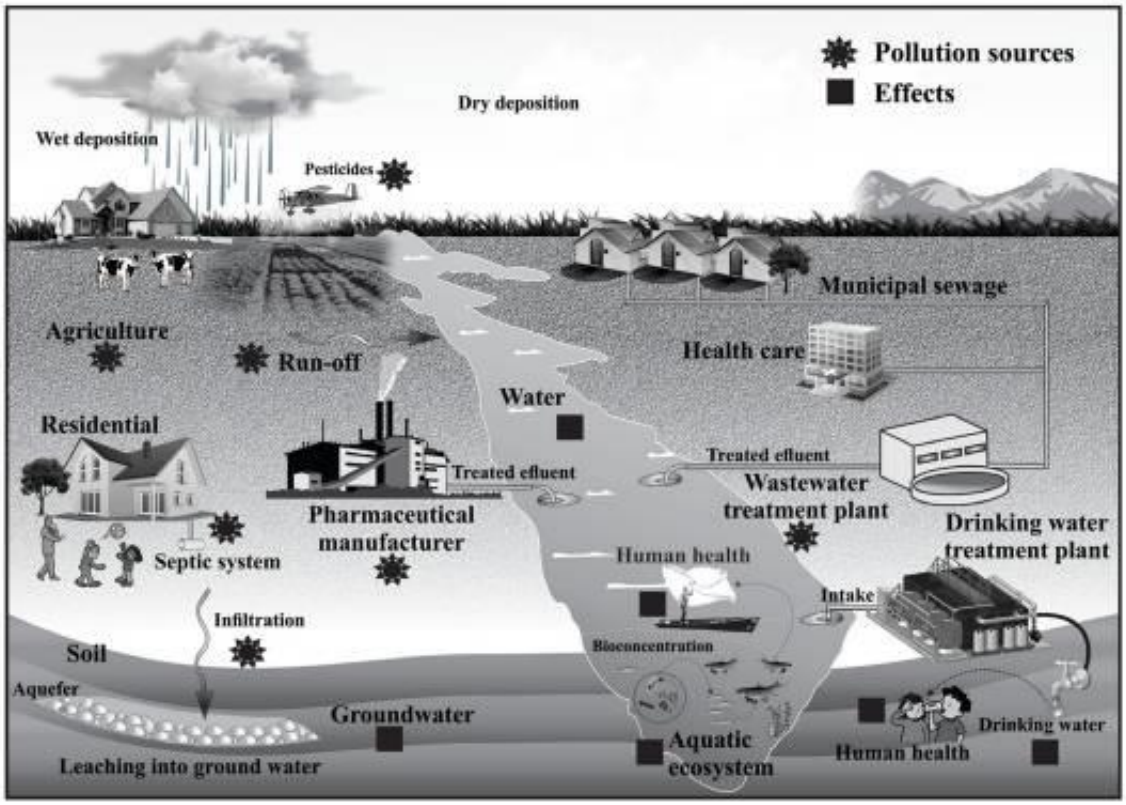

Figure 1. Sources of EDCs in environmental waters and human exposure.

Figure sourced from (Caballero-Gallardo, Olivero-Verbel, & Freeman, 2016).

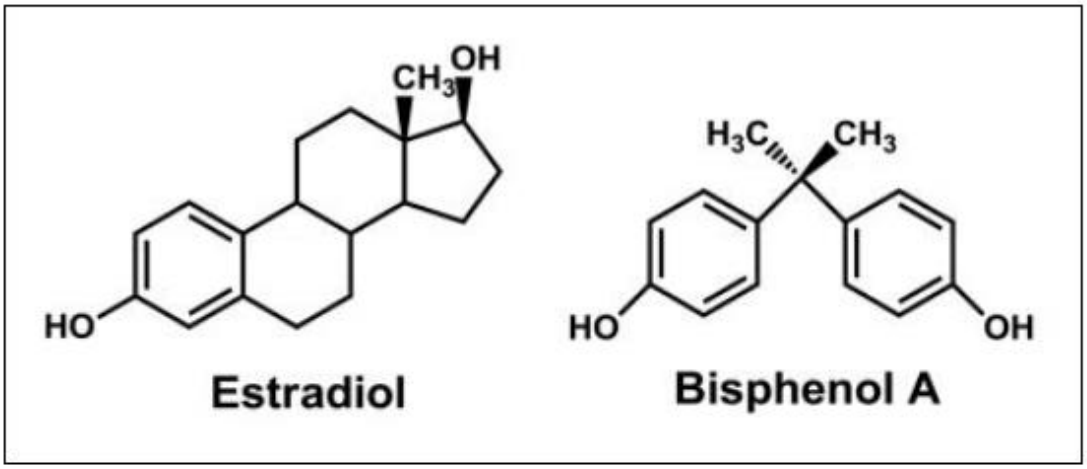

Figure 2. 2D Structures of Estradiol and Bisphenol A. Chemical structure images sourced from Baker and Hardiman (2014).

Zebrafish Model Organism

Vertebrate endocrine system. Research using zebrafish as a vertebrate model organism has been increasingly focused on questions relevant for human development and diseases. The hypothalamo-pituitary axis, which links the central nervous system, endocrine systems, and glands, has been shown to be highly conserved when comparing to zebrafish to humans (Löhr & Hammerschmidt, 2011). Optogenetic research with transgenic zebrafish has identified ER activity in response to both natural and synthetic estrogens by observing fluorescence in estrogenic tissues such as the liver, ovary, pituitary gland, and brain (Gorelick & Halpern, 2011).

Estrogen receptors. The zebrafish genome is known to code for three nuclear estrogen receptors Esr1, Esr2a, and Esr2b, which mediate the genomic response to estrogen signaling by acting as DNA-binding transcription factors. A fourth estrogen targeted receptor is the membrane localized G protein-coupled estrogen receptor (Gper) which induces activation of non-genomic responses. Importantly, the fetal expression of estrogen receptors during development is induced at 24 hours post fertilization (hpf), the most highly expressed of which appears to be Esr2a (Hao et al., 2013). Because estrogen receptors first become active at 24 hpf, this period is critical in the context of examining the effects of xenoestrogens on EA. BPA causes transactivation of all three nuclear estrogen receptors, but showed the highest selective affinity towards zfERα (Esr1) with an EC_{50} of 1μM and a relative estrogenic potency of 1.9×10^{-4} according to experiments conducted in vitro cell lines (Le Fol et al., 2017).

Biomarkers for estrogen activation (EA). Transcriptomic analysis of estrogen target genes during zebrafish embryonic development identified several genes which serve as biomarkers for EA. In particular, the brain-specific Cytochrome P450 aromatase (Cyp19a1b) gene serves as a potent indicator of EA. Fluorescence can be detected as early as 25-26hpf in Cyp19a1b:GFP transgenic fish treated with 10^{-8}M of estradiol (E2; Mouriec et al., 2009). The aromatase enzyme non-reversibly converts Testosterone into Estrogen, so overexpression of Cyp19a1b can lead to a positive feedback loop where more estrogen and subsequently more aromatase is created in the brain. Zebrafish plasma E2 concentrations increase after BPA exposure (Zhao et al., 2017) which can be attributed to increased aromatase expression. As the BPA dose increases, plasma testosterone concentrations decrease dramatically, leading to an elevated E2/T ratio as the pool of available testosterone is depleted (Zhao et al., 2017). Cyp19a1b mRNA expression has been found to be highly upregulated by estrogen in the brain at 2-4 days post fertilization (Hao et al., 2013). BPA exposure of 1μM in Cyp19a1b transgenic zebrafish for 4 days (2hpf-4dpf) has been shown to induce a 16-fold fluorescence intensity increase compared to DMSO control which has basal GFP fluorescence equivalent to water-only treatment (Cano-Nicolau et al., 2016).

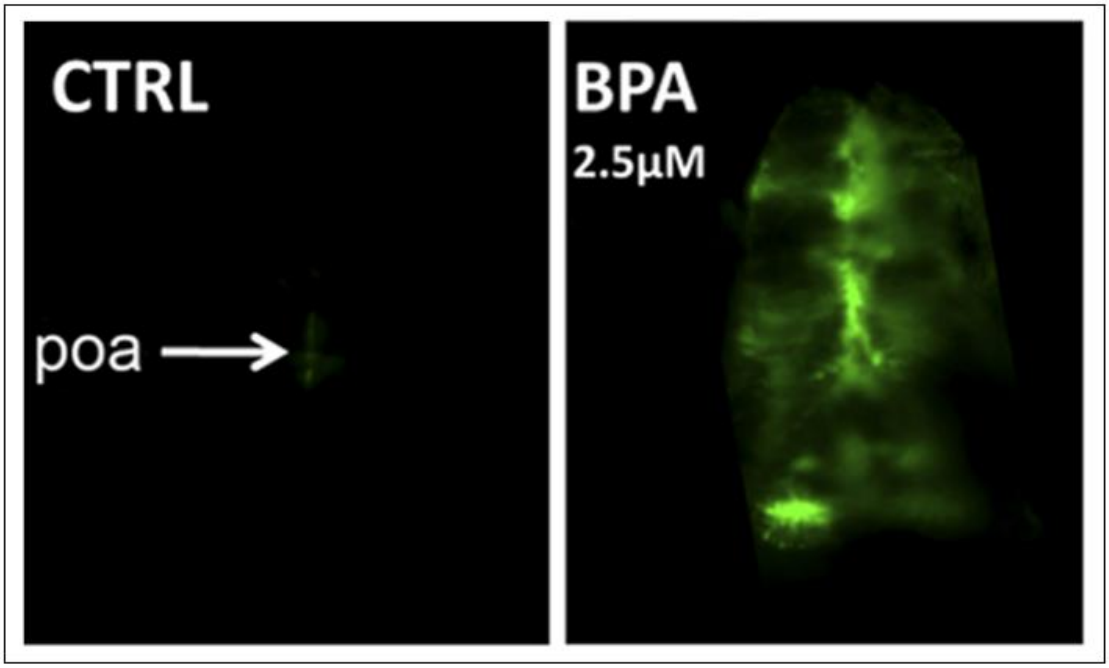

Figure 3. Fluorescence image of 5dpf Cyp19a1b:GFP zebrafish exposed to BPA. The left image shows a control fish which received no BPA treatment. The right image shows a fish raised in BPA 2.5µM solution from 0-5dpf. Figure sourced from Brion et al. (2012). Poa = preoptic area.

The relative estrogenic potency of BPA (compared to endogenous E2) has been reported to be 1.44×10^{-4} using the Cyp19a1b:GFP transgenic line, indicating a low binding affinity (Brion et al., 2012). The LC_{50} (Lethal Concentration for 50% of exposed animals) of BPA was measured in Cyp19a1b:GFP zebrafish and found to be $4.7e^{-5}$ mol/L (47μM), and GFP expression decreased at the highest concentrations which is consistent with the increase in mortality. Furthermore, the EC_{50} (Effective Concentration for 50% of maximal response) of BPA for GFP induction in this line was found to be $7.4e^{-6}$ mol/L (7.4μM; Petersen et al., 2013).

Radial Glial Cells

An interesting characteristic of Cyp19a1b is its exclusive expression in radial glial cells (RGC) due to the mandatory cooperation between ERs and a "glial"-specific factor (Gx) that binds upstream of the Cyp19a1b promotor (Le Page, Vosges, Servili, Brion, & Kah, 2011). The development of a transgenic Cyp19a1b:GFP zebrafish line which expresses green fluorescent protein in response to the brain aromatase Cyp19a1b promotor has become a useful tool for direct evaluation of xenoestrogens' EA and characterization of radial glia in the developing brain (Tong et al., 2009). Effect-directed analysis used in tandem with Cyp19a1b:GFP zebrafish embryos have recently shown success in detecting the presence of estrogenic compounds in fluvial sediment samples from the Czech river Bilina (Fetter et al., 2014).

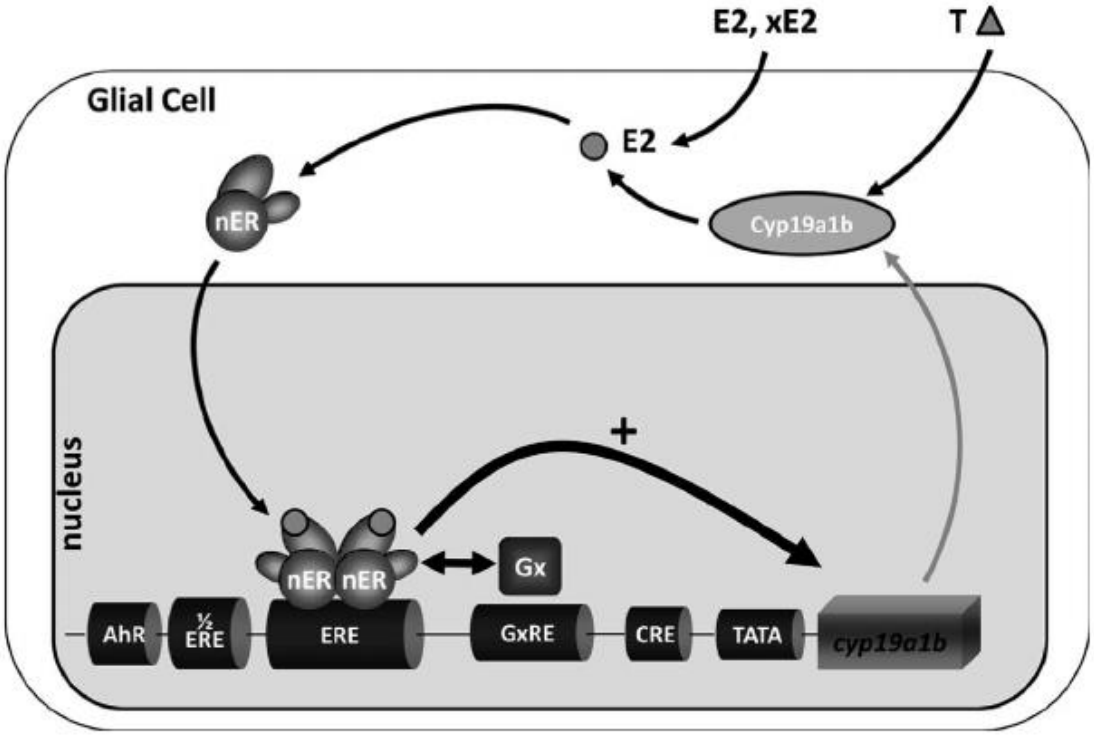

Figure 4. Mechanisms of Cyp19a1b upregulation by estrogens. Figure sourced from Le Page et al. (2011). "Mechanisms of cyp19a1b upregulation by estrogens (E2), xenoestrogens (xE2), or aromatizable androgens (testosterone, T) in the radial glial cells in the brain of fish. A mandatory cooperation between estrogen receptors and a "glial"-specific factor (Gx) that binds onto a GxRE sequence upstream from the ERE results in a high sensitivity of the cyp19a1b gene to E2 or xE2. As radial glial cells have aromatase activity, testosterone also upregulates aromatase B expression."

Fluorescence readings from a Cyp19a1b:EGFP (enhanced green fluorescent protein) line have shown that E2-treated embryos produced EGFP localized in the olfactory bulb, telencephalon, preoptic area, and mediobasal hypothalamus of the forebrain, which was first detected 3d after treatment and increased until 5dpf (Kim et al., 2009). Furthermore, whole-mount in situ hybridizations have shown that BPA (5μM) induces AroB overexpression in similar patterns to estrogen in the developing zebrafish brain and that the first 5dpf exposure window is the most sensitive, especially the 72-96hpf period (Chung, Genco, Megrelis, & Ruderman, 2011). Fluorescence does not appear to be induced in the hindbrain regions of zebrafish, which is consistent with a study on aromatase expression in the Japanese eel (a basal teleost) that found no cyp19a1 mRNA within the cerebellum or medulla oblongata, but did find expression in the telencephalon, preoptic area, and hypothalamus (Jeng et al., 2012). This indicates that the hindbrain can be a reference region to compare against the forebrain to examine localized effects of increased aromatase expression.

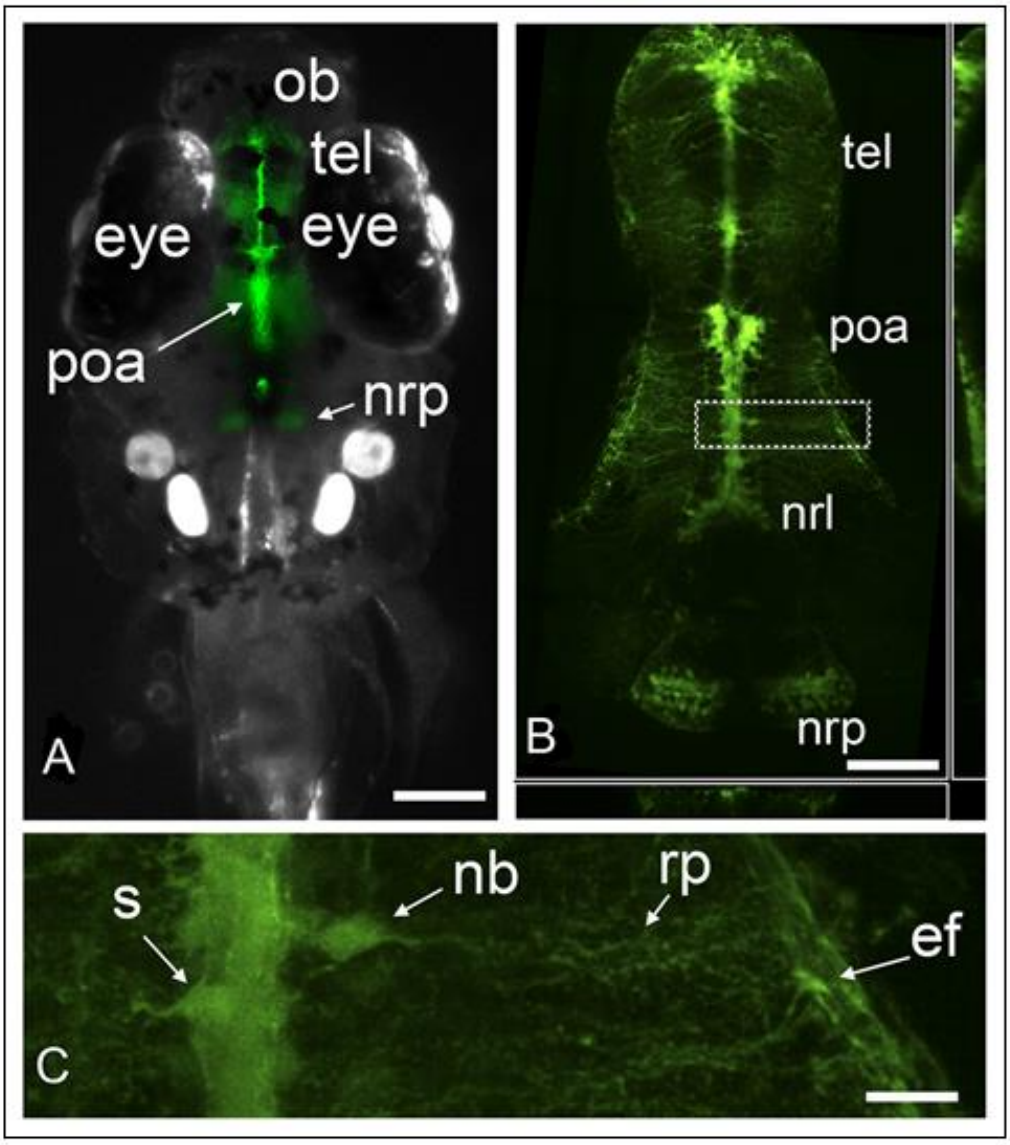

Figure 5. Fluorescence image of 8dpf Cyp19a1b:GFP zebrafish exposed to Estradiol. Larvae were raised in 10nM Estradiol solution from 0-5dpf. Figure sourced from Brion et al. (2012). A & B: Ob = Olfactory bulb, tel = Telencephalon, poa = Preoptic Area, nrp = Nucleus recessus posterioris of caudal hypothalamus, nrl = Nucleus recessus lateralis of caudal hypothalamus. C: S = Soma, nb = newborn cells, rp = radial processes, ef = end-feet.

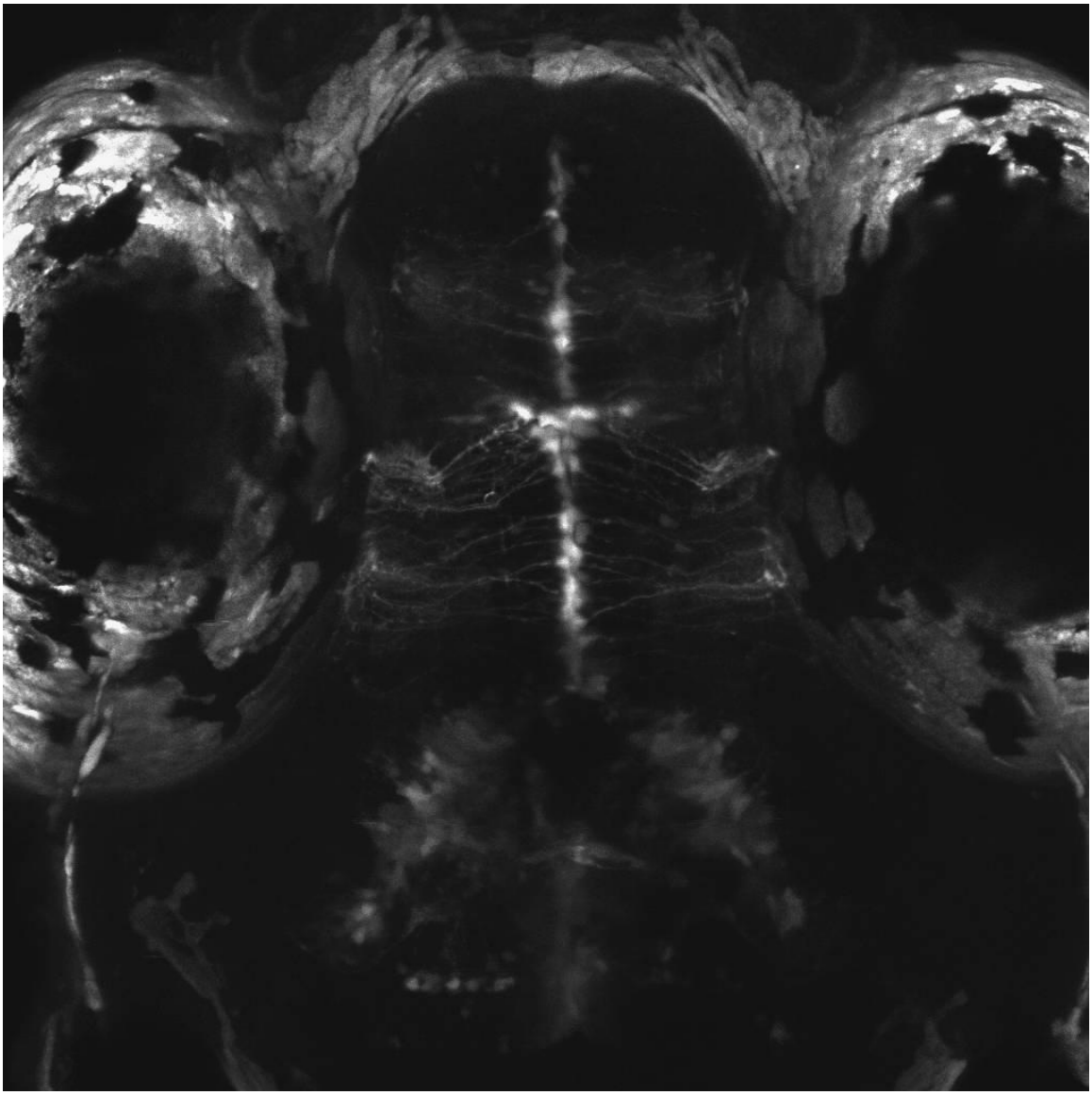

Figure 6. High-resolution fluorescence image of 6dpf Cyp19a1b:GFP zebrafish. Image taken in the HSU Graduate Psychology Research Lab. Results confirm that aromatase expression in radial glial cells follows the same patterns observed from Cyp19a1b:GFP zebrafish in research labs referenced for this thesis.

Progenitor cells. Zebrafish radial glial cells (RGC) act as progenitor cells which guide early brain development and persist into adulthood, whereas mammalian radial glia disappear shortly after birth (Schmidt, Strähle, & Scholpp, 2013). These progenitors are asymmetrically dividing stem cells which reside in the embryonic ventricular zone and generate neurons in addition to new radial glia. During development, they act as scaffolds for new neurons which use their long processes to migrate out of the ventricular zone (Schmidt et al., 2013).

Neurogenesis. Characterization of radial glia is of the utmost importance in the context of zebrafish embryonic neurogenesis. Proliferating cells in the developing hypothalamus express sex steroid receptors and undergo a period of neurogenesis from 16hpf-36hpf, representing a window of vulnerability to xenoestrogen exposure (Kinch, Ibhazehiebo, Jeon, Habibi, & Kurrasch, 2015). Kinch and colleagues were able to demonstrate through EdU pulse-labeling that very low dose exposure to BPA (0.0068μM) from 0-5dpf increased the number of newly born neurons in the hypothalamus by 180% at 24hpf. They also noted a 60% decrease in newly born neurons by 36hpf, which indicates that the precocious neurogenesis at 24hpf had exhausted a portion of the progenitor pool, and that the effects on neurogenesis were limited to the hypothalamus. It is worth noting that a higher dose of 100μg/L (~0.44μM) of BPA has been shown to increase hypothalamic gonadotropin-releasing hormone (GnRH3) neuron numbers at 25hpf (Qiu et al., 2016). Experiments using mice show that E2 treatment in the embryonic neocortex can rapidly promote neural proliferation (Martínez-Cerdeño, Noctor, & Kriegstein, 2006).

Recent research conducted by Pérez and colleagues demonstrated for the first time that a close relationship exists between 5-HT neurons and radial glial cells in the hypothalamus. Newborn cells generated by RGC in adult zebrafish can migrate centripetally and differentiate into 5-HT neurons. 5-HT cell bodies become tightly packed and localized close to the diencephalic ventricles or their recessi and contact cerebral spinal fluid-(CSF) by extending a short process toward the lumen of the ventricle. 5-HT cell bodies remain closely associated with RGC, where RGC form a continuous barrier along the ventricles through cytoplasmic processes which interconnect with one another, and the proximal 5-HT cells' processes cross this barrier through small pores into the ventricular lumen to contact CSF. Interestingly, addition of a selective irreversible inhibitor of tryptophan hydroxylase (Fenclonine), used to cause a decrease in 5-HT content, was shown to decrease cell proliferation in the caudal hypothalamus of adult zebrafish (Pérez et al., 2013). These discoveries bring into question how the disruption of RGCs by xenoestrogens might affect the proliferation of 5-HT neurons in the hypothalamus during early development.

A review paper on BPA's interaction with brain development and function notes that there are still very few results in the literature about its effects on pluripotent cells. They state that BPA has been demonstrated to disrupt neurogenesis by influencing neural stem cell proliferation and viability, modifying stem cell fate, and by promoting neural progenitor differentiation into neurons. It is suggested that since correct neuronal positioning relates to normal brain circuitry, BPA exposure's modifications of these processes may lead to abnormal brain connectivity in some areas (Negri-Cesi, 2015).

Adult proliferation and neurogenesis recapitulate some of the molecular and cellular mechanisms used during embryonic development of the CNS (Kaslin, Ganz, & Brand, 2008). Therefore, discoveries from studies on adult neurogenesis may still have important applications in the context of embryonic neurogenesis.

Estrogenic Effects on Neurons

Calcium activity. Rat studies demonstrate that estradiol (E2) enhances the depolarizing action of GABA in developing hypothalamic neurons by increasing the magnitude of the calcium transient with each depolarization, increasing the number of neurons that respond to $GABA_A$ receptor activation with a calcium transient, and extending the developmental duration of depolarizing GABA action (McCarthy, 2008). Currently there is difficulty establishing the functional significance of this effect because the only known way of enhancing GABA's depolarizing action is by E2 administration.

Gene expression and development. Embryonic zebrafish (3dpf) show highly comparable expression of genes and GABA cell patterns to mice at the neurogenic time window. At 3dpf zebrafish produce GABA in the subpallium, preoptic region, ventral and dorsal thalamus, and the hypothalamus (Mueller, Vernier, & Wullimann, 2005). Interestingly, most dopaminergic neurons also express GABAergic markers, and all catecholaminergic groups in zebrafish exhibit a dual transmitter phenotype of either a GABAergic or glutamatergic nature (Filippi, Mueller, & Driever, 2014). There is evidence that dopamine may upregulate Cyp19a1b mRNAs through D1 receptors, and stimulates RGC function through neuronal-glial interactions in teleost fish via the

D1R/cAMP/PKA/p-CREB pathway (Xing, McDonald, Da Fonte, Guiterrez-Villagomez, & Trudeau, 2015).

In early zebrafish development GABA acts as an excitatory neurotransmitter due to immature neurons initially expressing the NKCC1 transporter responsible for chloride uptake, but not the neuron-specific extrusive KCC2 transporter (expression first detected at approximately the time of hatching – 2dpf). Experiments where KCC2 was overexpressed before the onset of endogenous expression led to an early reversal of the chloride gradient, the proportion of spontaneously active neurons decreased and the number of silent neurons increased. KCC2 overexpression also led to perturbed development with multiple abnormal morphological features (overall smaller embryos, reduced head & eye size, pigmentless, increased tail curvature) and slow coils in response to touch. Reduced neurogenesis and diminished axon maturation were observed in both the brain and hindbrain, where significantly less newly differentiated neurons were found due to impaired birth of neurons (notably interneurons and motor neurons) without affecting the proliferation or death of progenitors (Reynolds et al., 2008). The results of this study display the importance of GABA's excitatory role during early neurogenesis and its guidance on morphological development of both the brain and body.

Zebrafish embryos exposed to BPA (50μM) at the prim-5 stage (24hpf) alters brain development by disturbing the process of brain regionalization, resulting in the development of abnormal rhombomeres, a restricted Mhb (Midbrain-hindbrain boundary), and a smaller midbrain structure (Tse, Yeung, Wan, & Wong, 2013). EE_2 exposure disrupts ontogeny of GnRH system by inducing an increased number of GnRH-

ir neurons and fibers, decrease the size of soma, and modify migration profile in a dose-dependent manner (Vosges et al., 2010).

BPA treatment (5mg/kg IP) on pregnant mice increased *in utero* Hoxa10 (homeobox gene that controls developmental organogenesis) expression and decreased cytosine-guanine methylation in the promotor and intron sequences of Hoxa10; this led to increased binding of ER-α to the Hoxa10 ERE both *in vitro* and *in vivo*, which in turn led to increased ERE-driven gene expression (Bromer, Zhou, M. Taylor, Doherty, & H. Taylor, 2010). This indicates that there is an additional epigenetic mechanism by which BPA can alter expression of estrogen-responsive genes and disrupt developmental programming.

Locomotor activity. There appears to be a dose-response relationship between BPA and swim activity. Swim hyperactivity has been defined as an abnormally fast swimming over an extended period, typically related to psychostimulant/convulsant action or anxiety-like behavior (Kalueff et al., 2013). BPA exposure of 0.1μM to larval zebrafish during the window of hypothalamic neurogenesis (16-36hpf) induces hyperactive behaviors (~300% increase in locomotor activity) at 5dpf (Kinch et al., 2015). A similar study had a 48-hour BPA exposure period begin at 8-10hpf and measured swim behavior at 5dpf; 0.01μM and 0.1μM doses resulted in larval hyperactivity whereas the higher 1μM and 10μM concentrations did not affect activity (Saili et al., 2012). However, an even higher dose of 15μM BPA from 6hpf-4dpf results in significantly decreased swimming behaviors measured at 5dpf (Wang et al., 2013).

Together, these results may indicate an inverse-U dose-response curve for BPA concentration and zebrafish swimming activity.

Brustein and colleagues demonstrated a relationship between 5-HT and locomotor activity. They note that locally restricted populations of serotonergic neurons and their projections appear in the hindbrain and spinal cord by 2dpf, and that 5-HT acts as a neuromodulator integrated into the locomotor network to affect swimming activity from 4dpf onwards. 5-HT was found to reduce intervals of inactivity (quiescence) but had little effect on the properties of activity periods (Brustein, Ghong, Holmqyist, & Drapeau, 2003). Further research clarifies that 5-HT modulates the quiescence period by increasing the amplitude of the chloride response by 40% when glycine is applied, this causes an increase in depolarization which is reflected behaviorally through doubling the number of swim episodes (Brustein & Drapeau, 2005).

Dopaminergic neurons within the zebrafish hypothalamus have recently been shown to mediate locomotor activity. Activation of Tyrosine Hydroxylase (TH)-positive neurons in the posterior recess through optogenetic transgene mechanisms resulted in an increased frequency of swimming and displacement compared to controls, suggesting either a role in direct activation or modulation of the threshold of motor response to sensory-evoked stimuli. Furthermore, ablation of these neurons reduced both the number of spontaneous swimming episodes and the time spent swimming, and restoration of TH neurons correlated with recovery of swimming deficits (McPherson et al., 2016).

Zebrafish Elavl3 (HuC Homologue)

Zebrafish Elavl3 is a HuC homologue which has been demonstrated to be one of the earliest markers for the identification of neuronal fate (Kim et al., 1996). Its expression is initiated in embryonic cells that are committed to developing into neurons, and it continues expression during all stages of neuronal development. Mutations can lead to a lethal phenotype during embryogenesis due to the degeneration of the CNS, indicating Elavl3 may be important for the development and maintenance of the nervous system (Park et al., 2000a).

A study looking to detail neurogenesis in the adult zebrafish brain used BrdU pulse-labeling experiments to detect proliferating cells in combination with HuC, Tyrosine hydroxylase (TH), and 5-HT immunostaining to detect newborn cells expressing distinct neuronal phenotypes. They found that proliferation zones existed throughout the entire anterior-posterior axis of the brain and that newborn cells move centrifugally into the surrounding mantle after differentiating into neurons. Importantly, they were able to identify TH-positive newborn neurons in multiple regions (mostly the preoptic area) as well as 5-HT-positive newborn neurons in the ventral periventricular hypothalamus (Grandel, Kaslin, Ganz, Wenzel, & Brand, 2006).

Development of the first stable line of HuC-GFP transgenic zebrafish which exhibit fluorescence in newly born neurons was done by Hae-Chul Park and colleagues (2000b). GFP fluorescence was found as early as 12hpf, and by 24hpf all early brain tracts and nuclei were recognized by anti-GFP labeling. They further demonstrated the

usefulness of the line as a tool for characterizing neurogenesis mutants by crossing HuC-GFP fish with the *mib* mutant zebrafish, which exhibits excessive early differentiating neurons, and found that neuronal hyperplasia was present due to there being much more intense GFP fluorescence in the double mutants compared to the HuC-GFP embryos.

Elavl3:GCaMP transgenic zebrafish line. Elavl3:GCaMP is another transgenic line utilizing the Elavl3 promotor which has a genetically encoded calcium indicator that fluoresces in the presence of calcium. The GFP in GCaMP is fused to Calmodulin, a protein which binds up to four Ca^{2+} ions, and undergoes a conformation change upon Ca^{2+} binding which results in bright fluorescence compared to the unbound state. This GCaMP line can be used to measure changes in spontaneous calcium dynamics within the brain caused by electrical activity.

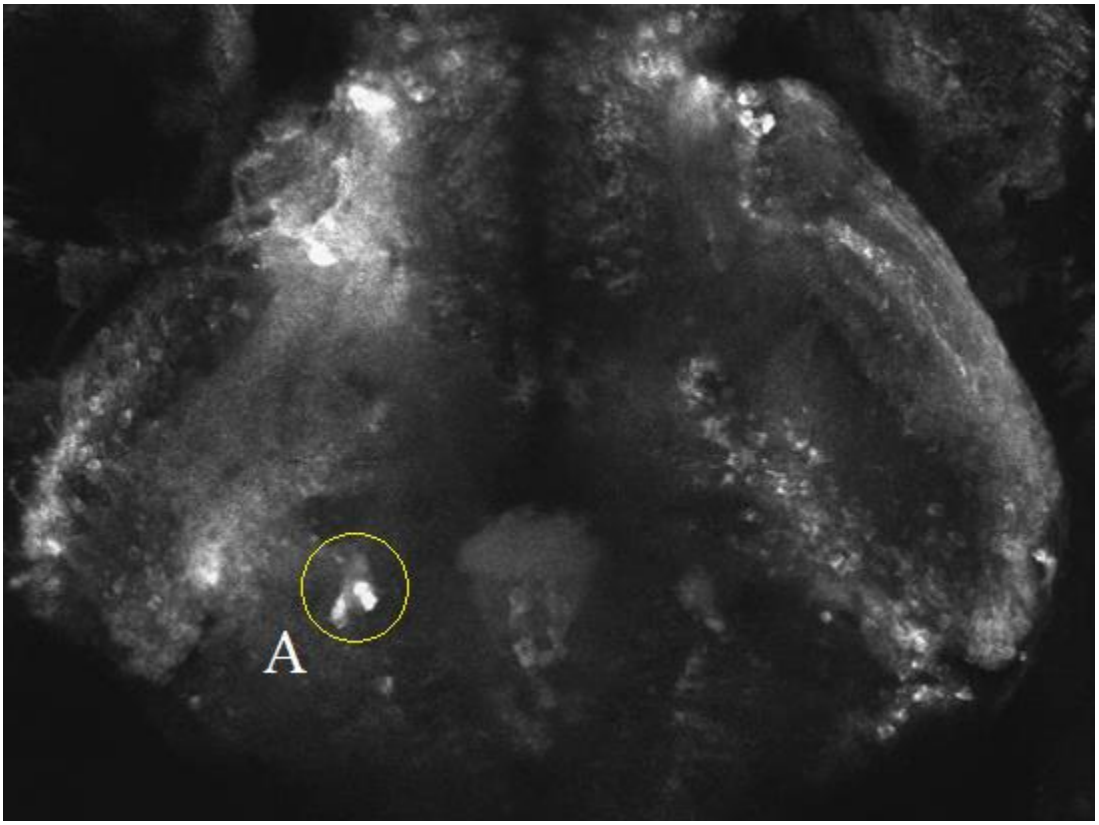

Figure 7. Fluorescence image stack of 6dpf Elavl3:GCaMP zebrafish hypothalamus. Region A = "DC7" dopaminergic cluster. Image taken in the HSU Graduate Psychology Research Lab. Results are consistent with Elavl3:GCaMP images provided by the Z Brain Atlas created by the Engert Lab at Harvard University.

Statement of the Problem

Until manufacturing processes adapt to use non-EDC components in plastic production, the problem of EDCs affecting organisms will persist. Substitute chemicals for plastic production which do not have EA properties have indeed been discovered, but lack of regulation and recycling of products containing EDCs means that they will an ever-present issue. Further research clarifying the effects of xenoestrogen exposure on early development is important because exposure is widespread and may have lasting consequences. BPA has been a focal point in the research of EDCs due to its EA ability at low doses, but only in the past decade has its effects on the brain begun to be investigated. Changes in brain development and behavior have been noted in zebrafish, but there remains the question of how calcium activity may be related to these changes. The current study seeks to replicate recent findings while exploring potential mechanisms by which the changes in neural activity might be responsible for later behavioral changes.

Research Hypotheses and Rationale

Hypothesis 1a. Cyp19a1b:GFP zebrafish larvae treated with 0.1μM BPA from 1dpf-5dpf will see a small increase in GFP expression when measured at 6dpf compared to the control group that does not receive treatment.

Hypothesis 1b. Cyp19a1b:GFP zebrafish larvae treated with 2.5μM BPA from 1dpf-5dpf will see a large increase in GFP expression compared to the control group that does not receive treatment.

Rationale. Replication is necessary to confirm BPA exposure causes Estrogen Activation (EA) which increases expression of Cyp19a1b within radial glial cells.

Hypothesis 2. Elavl3:GCaMP zebrafish larvae treated with 0.1μM BPA from 1dpf-5dpf will see an increase in spontaneous Ca^{2+} oscillations within the hypothalamus and medulla at 6dpf compared to the control group that does not receive treatment.

Rationale. EA enhances GABA's depolarizing action on hypothalamic neurons during early development by increasing calcium transit with each depolarization and decreasing threshold for depolarization. If these neurons also regulate motor behavior through a projection to the medulla then it may lead to more Ca^{2+} activity there as well.

Hypothesis 3. Elavl3:GCaMP zebrafish larvae treated with 0.1μM BPA from 1dpf-5dpf will see an increase in swimming activity due to decreased quiescence periods at 6dpf compared to the control group that does not receive treatment.

Rationale. EA leads to an overactive hypothalamus, a locomotion modulator, which translates into decreased periods of quiescence and therefore more swim episodes.

Method

Instruments

Microscope: Olympus Fluoview FV1000 with 20x .95NA water immersion lens.

Camera: Pixelink PLB-741 with IR illumination and visible light blocking filter.

Software programs: ImageJ for image analysis, R for statistical tests.

ImageJ plugins: Image Stabilizer (Li & Kang, 2008).

Subjects

Initial power analyses indicated that a total of 504 zebrafish larvae would be needed for all experimental comparisons and up to 30 adults will be used to generate these larvae. The Humboldt State University IACUC approved the use of animals for this research (protocol no. 17/18.P.34-A). It is worth noting that this study used significantly less specimens than indicated by a prior power analysis. Studies in this field conducted by leading research labs tend to use much smaller sample sizes in the range of as few as 6 subjects to as many as 20 subjects for any given measurement.

A total of $N = 245$ fish were examined across all experiments. For the Cyp19a1b:GFP experiment, 42 subjects were examined: 0.1μM vs control groups $n = 15$ each, 2.5μM vs control groups $n = 6$ each. For the Elavl3:GCaMP experiment, 57 subjects were examined: 0.1μM group $n = 28$, control group $n = 29$. For the Behavior

experiment, 146 subjects were examined: 0.1μM group *n* = 73, control group *n* = 73. All subjects were confirmed to be sufficiently healthy as indicated by inflated swim bladders.

Experimental Design

Experiment 1. Cyp19a1b:GFP zebrafish larvae (*n* = 42) were divided into two separate exposure protocols for fluorescence imaging to detect AroB expression in radial glia. The first protocol tested a dose of 0.1μM BPA (*n* = 15) against a control group (*n* = 15). The second protocol tested a dose of 2.5μM BPA (*n* = 6) against a control group (*n* = 6). The second protocol was added through an IACUC amendment as a validity check to test whether the chemical delivery method was effective. A small effect size was expected for the 0.1μM BPA group, where exposure results in a small but detectable increase in fluorescence due to increased AroB expression. A large effect size was expected for the 2.5μM BPA group, where exposure results in a large and easily detectable increase in fluorescence due to increased AroB expression.

Experiment 2. Elavl3:GCaMP zebrafish larvae (*n* = 57) were divided into two groups for Ca^{2+} activity recordings. A dose of 0.1μM BPA (*n* = 28) was compared against a control group (*n* = 29). Recordings of both the hypothalamus and medulla were taken within each fish at separate intervals. A medium effect size was expected where BPA exposure results in approximately two-fold the amount of dynamic Ca^{2+} oscillations within the hypothalamus and medulla.

Experiment 3. Zebrafish larvae (*n* = 146) were divided evenly into groups for spontaneous swim activity recordings. A dose of 0.1μM BPA (*n* = 73) was compared

against a control group (n = 73). A medium effect size was expected where BPA exposure results in approximately two-fold the amount of time spent swimming.

Procedure

Drug exposure. 10,000x stock solutions of 1mM and 25mM BPA [239658, Sigma-Aldrich] dissolved in DMSO (Dimethyl sulfoxide) were prepared and stored frozen. Exposure groups were raised in 60mm glass petri dishes (up to 30 larvae each) containing either 0.1μM BPA or 2.5μM BPA in embryo water (0.01% DMSO) for 5 days (1dpf-5dpf) before imaging on day 6. Control groups were raised in 60mm glass petri dishes containing an equal volume of DMSO solvent in embryo water with no BPA. The volume of embryo water solution in each dish was 20mL and half of this volume was replaced daily for both experimental and control groups. Dead or sick larvae were removed from petri dishes upon discovery when changing water. On day 6 the larvae were removed and transferred to clean embryo water for imaging and behavioral testing.

Embedding. Larvae were anesthetized for about 2 minutes using MS-222 to allow agar embedding, which lets experimenters position the larvae as needed on the microscope slide. 0.01% (w/v) MS-222 solution was be made fresh for each experiment by dissolving MS-222 and an equal weight of sodium bicarbonate (to maintain neutral pH) in embryo water. Larval fish were first transferred individually by plastic suction pipette from their home dishes to the MS-222 solution dish and watched closely for the onset of paralysis (usually within 30 seconds), and then pipetted onto a microscope slide for embedding.

To begin agar embedding, a drop of low melting temperature agar (1.3% w/v dissolved in embryo water at neutral pH) was placed to harden on a microscope cover glass, then a straight channel was cut into it with a razor blade. An anesthetized larva was pipetted into the agar channel with the dorsal side of the head touching the glass cover slip. A moistened Kimwipe was used to move the larvae into the correct position while absorbing excess water. Then, a drop of melted agar (< 32°C) was gently pipetted onto the positioned larva and allowed to harden, finishing the embedding process. Larvae were left embedded for at least 20 minutes on a slide warmer before imaging to acclimate them to the agar medium and allow the effect of MS-222 to wear off. After imaging was completed, larvae were de-embedded by gently dissecting agar away with a blunt tool underneath a dissecting microscope, then the freed larva is introduced to a clean petri dish containing fresh fish water and returned to the incubator.

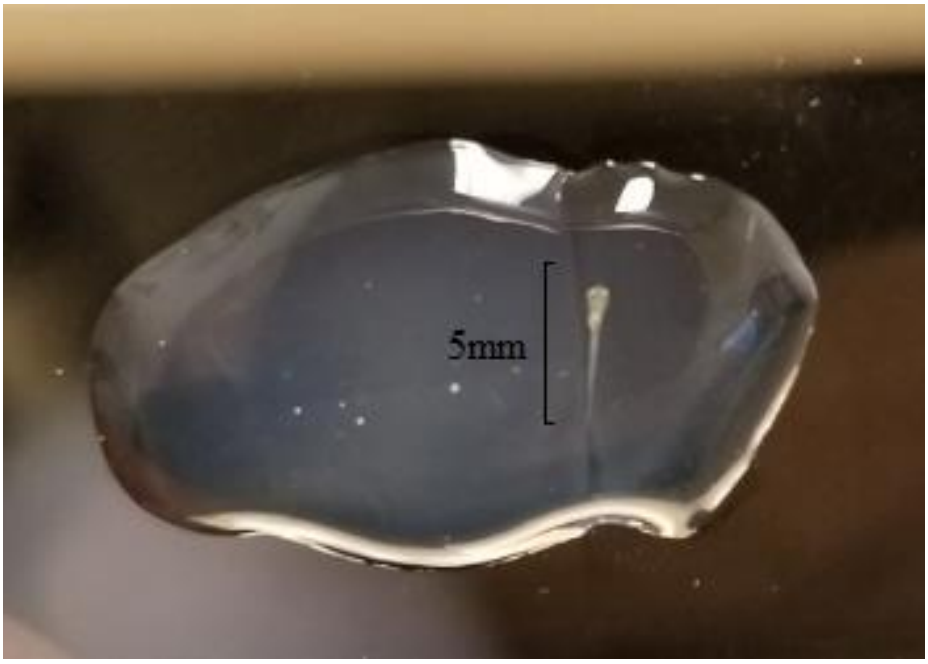

Figure 8. Zebrafish larva embedded in agar. Image taken in the HSU Graduate Psychology Research Lab. Larva is embedded in 1.3% agar solution with the dorsal side of the fish's head positioned against the glass slide.

Microscope imaging. An Olympus Fluoview FV1000 confocal laser scanning microscope was used to image both the Cyp19a1b:GFP and Elavl3:GCaMP larvae. The fluorescent confocal microscope illuminates cells with a narrow 488nm laser beam that scans across the imaging field. To avoid damaging any cells, exposure to the laser was limited to 30 minutes of intermittent scanning per larva with the minimum amount of laser power required to take adequate images. The standardized settings used to image each larva were as follows: 30% laser strength, HV = 536, Gain & Offset = 6, Zoom = 1.2x, 800x800 pixel image size, Capture rate = 2.0μs/pixel.

Cyp19a1b:GFP larvae were examined under the confocal microscope for the presence of GFP and adequate dorsal alignment before taking z-stack images. Z-stacks were standardized at 251 slices with a step size of 2.0μm, covering a 500μm depth from the top to bottom of the head in 10 minutes of scanning. The head was centered in the viewing frame so that the whole telencephalon and diencephalon was visible throughout the imaging process.

Elavl3:GCaMP larvae were examined under the confocal microscope for the presence of GCaMP and adequate dorsal alignment before taking time-lapse images. Time-lapses were capped at 3,000 images taken over a 10-minute period with an image capture interval set at 0.2s. The scanning area was cropped around the region of interest (ROI) to permit a scanning rate of 0.145s +/- 0.002s. This was done both to ensure that the areas being measured were the same size between subjects and that the capture rate was fast enough to detect activity changes. Recordings of the hypothalamus and medulla were taken in separate instances and were done on the same side of the brain.

Swim activity test. BPA- and control-treated larvae were transferred via plastic pipette individually into microplate wells containing 0.3mL of fish water. Fish were arranged into alternating BPA and control columns for easy labeling. A digital camera (Pixelink PLB-741) with a visible light-blocking, infrared-passing filter was placed above the wells to record the swimming activity for 10 minutes by taking still images at a rate of 5Hz. The inside of the recording chamber had an ambient temperature of 25-26°C. The first 5 minutes of recording was taken with the lights on inside the recording chamber to acclimate the fish to the environment. At the 5-minute mark, lights were switched off and a small visual indicator was automatically turned on to indicate the change. After recording, the larvae were removed by pipette and transferred into 60mm petri dishes with fresh fish water.

Data analysis procedures. Cyp19a1b:GFP expression analysis was performed in ImageJ using the depth-projection image stacks obtained from confocal imaging. For each subject a 16-bit "Z-project: Max" images was created out of a 50-image stack that ended at the bottom of the hypothalamus, then a Gaussian Blur filter with sigma value 1.0 was applied to decrease background noise. A standardized "Cyp19a1b ROI" was applied to the z-projection which measured the mean pixel intensity of the whole ventral hypothalamus and the midline of the preoptic area. A standardized "Reference ROI" was also applied inside one of the eyes to measure the baseline pixel intensity. Measurements were taken 5 times per subject to find the best fit, the highest "Cyp19a1b ROI" mean was kept and the lowest "Reference ROI" mean was kept for each subject. The true pixel

value caused by Cyp19a1b:GFP expression was calculated by subtracting the "Reference ROI" mean from the "Cyp19a1b ROI" mean within each subject.

Elavl3:GCaMP activity analysis was performed in ImageJ using the time-lapse image stacks obtained from confocal imaging. First, the hypothalamus and medulla time-lapse stacks needed to be stabilized due to the presence of movement artifacts. The Image Stabilizer plugin (Li & Kang, 2008) was used with the following settings: Translation, Max pyramids 1, Coefficient 1, Maximum iterations 200, Error Tolerance 0.0000001. Then, for each subject 32-bit "Z-project: Standard Deviation" images were created from both the hypothalamus and medulla stacks (3,000 images each). This method measures the standard deviation from the mean across the stack, assigning higher pixel values to pixels which have a high degree of change. This gives a measure of total calcium activity over time in a given area but does not capture any information about action potential frequency or neural circuitry. A standardized "GCaMP ROI" was drawn around the viewing area captured during the imaging process and the mean pixel value was calculated. A "Reference ROI" value was also taken outside the viewing area to act as a baseline in a similar way to the Cyp19a1b analysis. The true pixel value caused by calcium activity was taken by subtracting the "Reference ROI" mean from the "GCaMP ROI" mean.

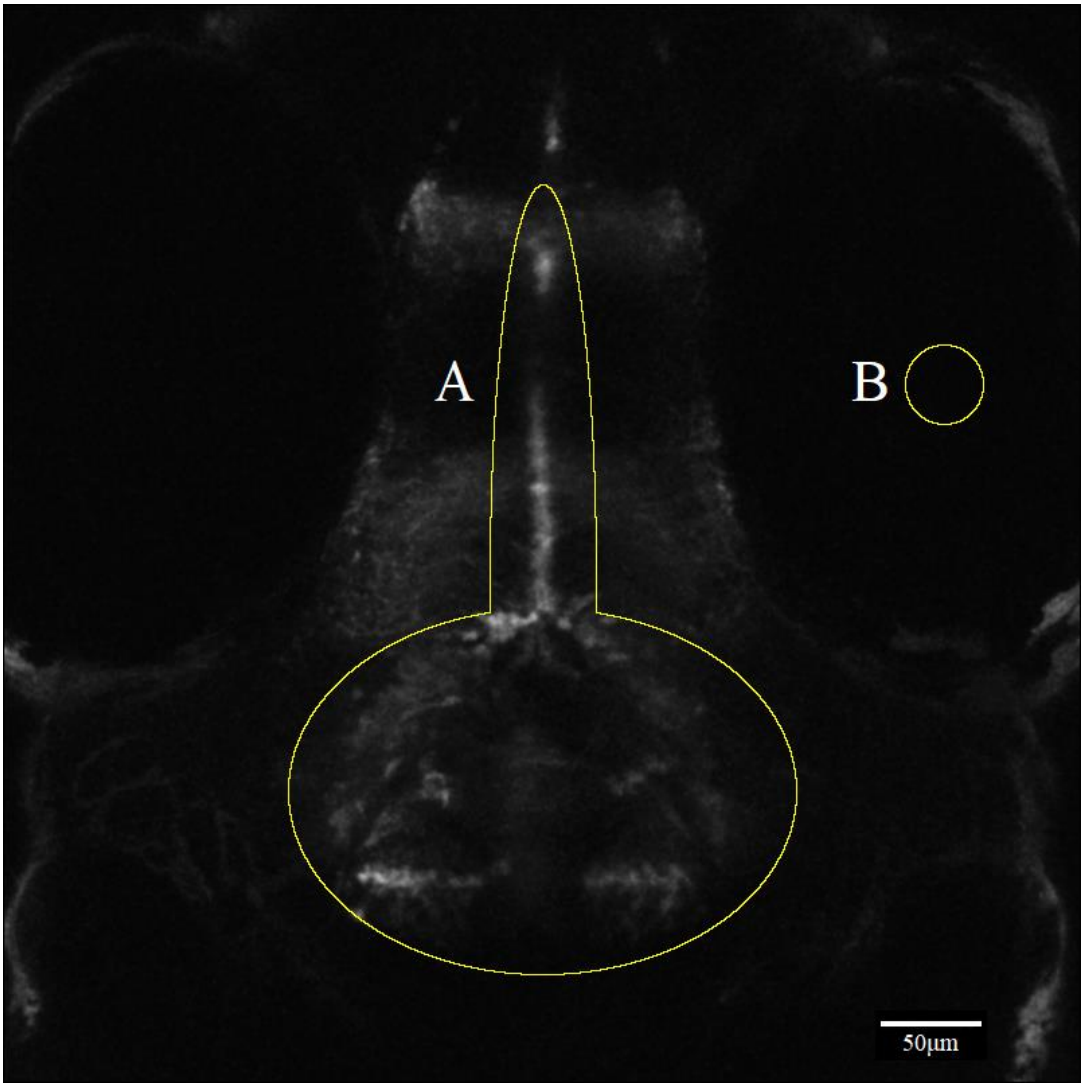

Figure 9. Example of Cyp19a1b:GFP ROI procedures in ImageJ. Region A = "Cyp19a1b ROI" measures pixel intensity due to GFP driven by Cyp19a1b expression. Region B = "Reference ROI" measures background value to infer baseline.

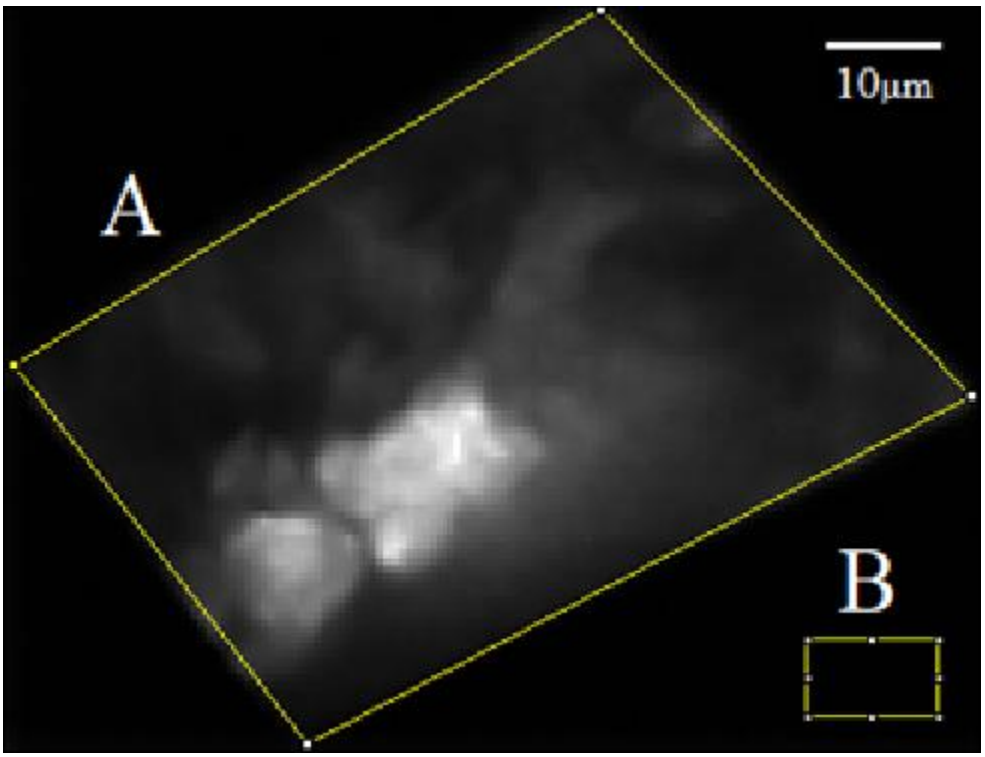

Figure 10. Example of Elavl3:GCaMP Hypothalamus ROI procedures in ImageJ. Region A = "GCaMP ROI" measures standard deviation pixel values caused by Ca^{2+} fluctuations. Region B = "Reference ROI" measures background value to infer baseline.

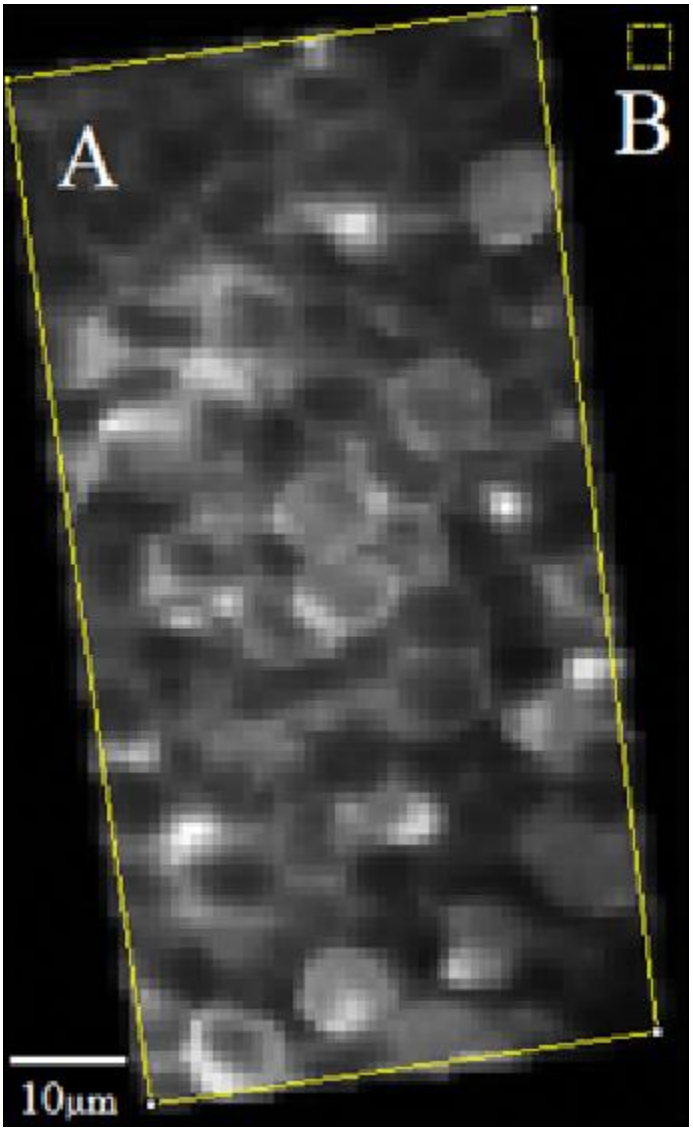

Figure 11. Example of Elavl3:GCaMP Medulla ROI procedures in ImageJ. Region A = "GCaMP ROI" measures standard deviation pixel values caused by Ca^{2+} fluctuations. Region B = "Reference ROI" measures background value to infer baseline.

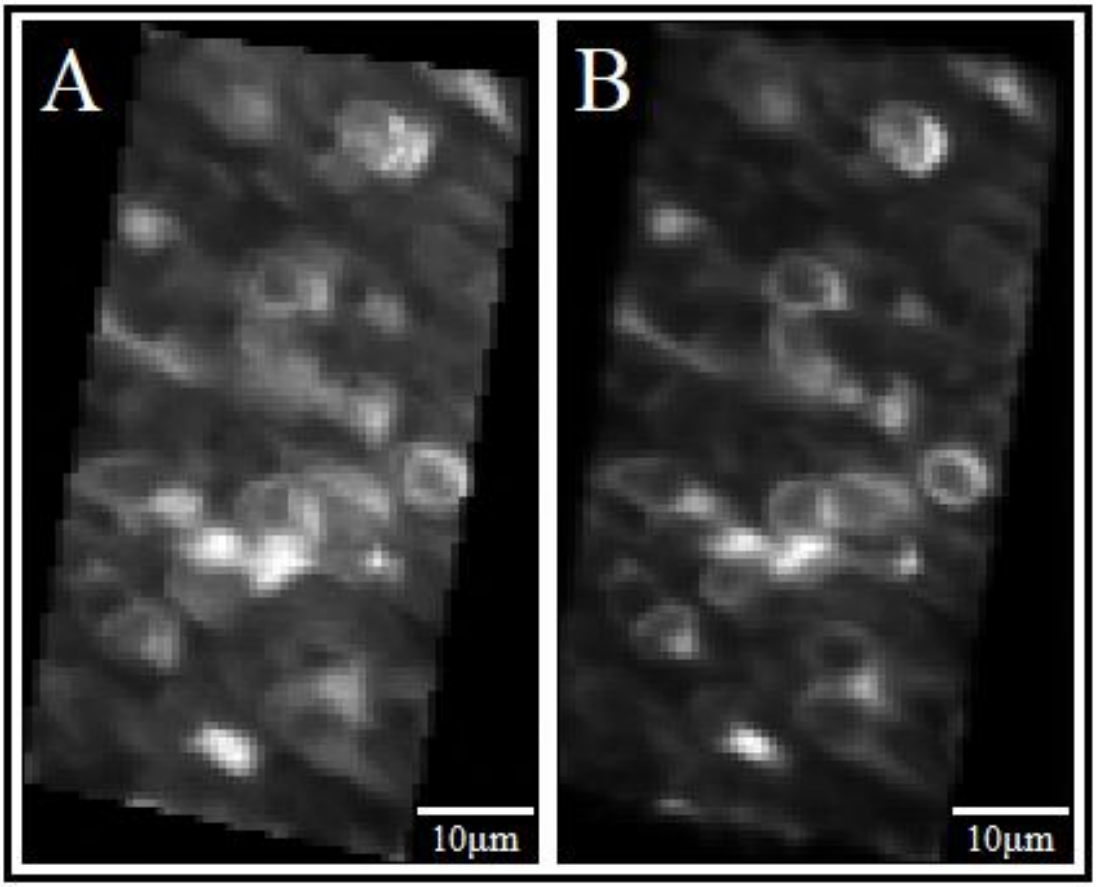

Figure 12. Example of "Image Stabilizer" procedure in ImageJ. A = Pre-stabilized image, which has visible blurring of cells due to movement. B = Post-stabilized image, which attempts to correct for movement artifacts and produces clearer cell boundaries. The "Image Stabilizer" procedure results in lower mean standard deviation pixel values, which would have otherwise been artificially inflated by movement artifacts.

Behavior video was analyzed in ImageJ to measure swimming activity. First, each frame of the video was subtracted from the previous frame, which resulted in a new video showing only the pixels whose brightness changed from frame to frame (every 200ms). The video was then thresholded (pixel value = 7) so bright pixels corresponding to the fish's location could be detected automatically by the Particle Analysis function of ImageJ. A ROI was then drawn around each well to assess whether the larvae had changed position from one frame to the next (minimum 4x4 pixel area), allowing calculation of percentage of time in motion. Percentage of time in motion was calculated for both the "Light On" and "Light Off" periods, then the values were converted into seconds of activity per minute of recording. In cases where movement could either not be detected throughout the entire period or camera errors occurred, an "NA" value was assigned so that the data was excluded from analysis.

Blinding procedures. Measures were taken to avoid potential experimenter bias before data analysis was performed in ImageJ. An independent party gave non-descriptive names to data files and created a key showing which files belonged to which group. The key was sealed until data analysis and statistics were completed by the blinded investigators. Upon completion the key was opened to reveal the identity of the BPA and Control groups.

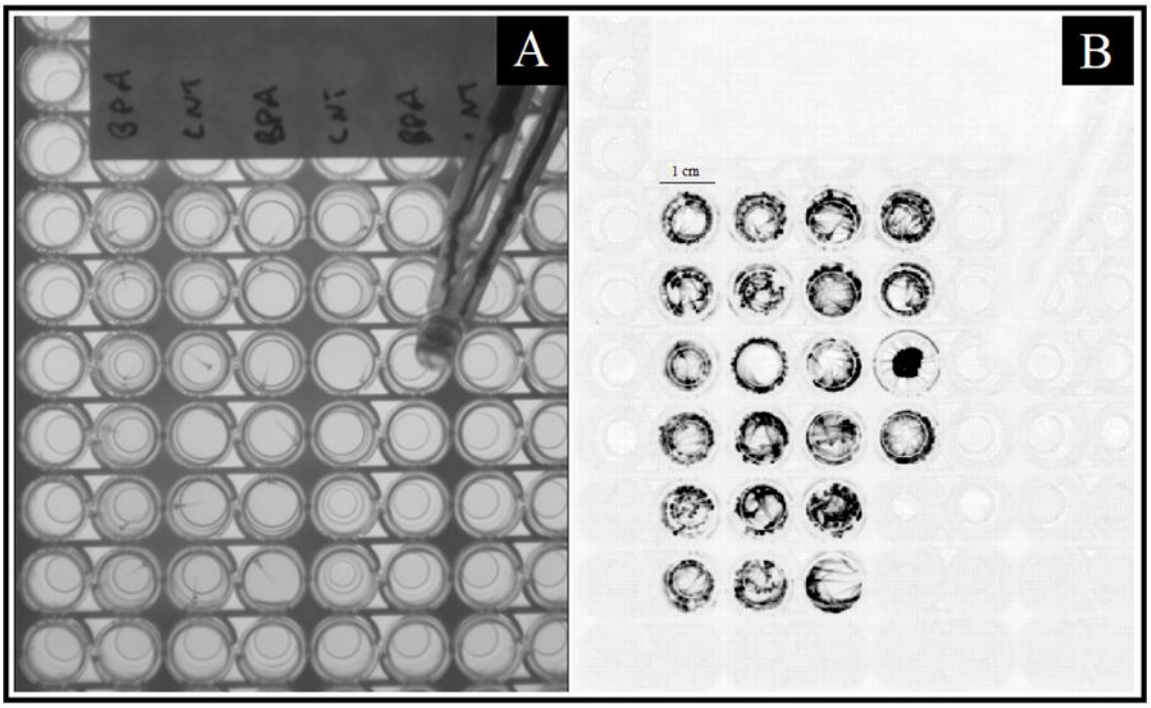

Figure 13. Example of behavioral analysis in ImageJ. Image A = Single unprocessed image. Image B = Standard deviation projection image of swimming activity over time. The overhead camera is easily able to track the movement of multiple fish in high detail.

Results

Statistical Assumptions

The primary assumptions for the statistical tests performed in this study were homogeneity of variance and normality of data. The criteria for homogeneity of variance was set as being less than a 4:1 ratio between groups. Normality of data was checked in R by calculating skew, kurtosis, and examining histogram charts. All datasets appeared to have a relatively normal distribution which did not require transformation. t-tests were performed using two-tails so that the significance threshold was more conservative.

Cyp19a1b:GFP Expression Analysis

0.1μM BPA vs control. Results did not find a significant difference in fluorescence between the 0.1μM BPA group (M = 61.473, SD = 21.829) and the corresponding control group (M = 60.266, SD = 15.064), $t(28) = 0.176$, $p = .861$, d = 0.064. The 95% CI around the difference between the means was [-12.820, 15.235]. The independent two sample t-test was calculated with the homogeneity of variance assumption being met (2.1:1).

2.5μM BPA vs control. Results showed a significant difference in fluorescence between the 2.5μM BPA group (M = 147.982, SD = 32.795) and the corresponding control group (M = 43.645, SD = 9.949), $t(5.9) = 7.457$, $p < .001$, $d = 4.306$. The 95% CI

around the difference between the means was [69.979, 138.695]. Welch's two sample t-test was used because the homogeneity of variance assumption was not met (10.9:1).

Post hoc analysis. An ANOVA performed on all groups found that the dose condition did have an overall significant influence on the amount of fluorescence detected, $F(3,38) = 34.76$, $p < 0.001$, partial $\eta^2 = 0.733$. Post hoc comparisons using the Tukey test indicated that the 2.5μM BPA group had a significantly higher mean than all other groups, but no other comparisons were found to be significant.

Table 1

Cyp19a1b:GFP Experiment Descriptive Statistics

Group	*n*	BPA conc.	*M*	*SD*	s^2
BPA 0.1μM	15	0.1μM	61.473	21.829	476.485
CTRL 0.1μM	15	0μM	60.266	15.064	226.931
BPA 2.5μM	6	2.5μM	147.982	32.795	1075.537
CTRL 2.5μM	6	0μM	43.645	9.949	98.976

Note. M units: Mean of pixel gray-values within ROI after removing background value.

Table 2

Cyp19a1b:GFP Multiple Comparisons of Means: Tukey Contrasts

Linear Hypotheses	*t*	*p*	*d*
BPA 2.5μM – CTRL 2.5μM	8.885	< 0.001*	4.306
BPA 2.5μM – CTRL 0.1μM	8.928	< 0.001*	3.437
BPA 2.5μM – BPA 0.1μM	8.805	< 0.001*	3.105
BPA 0.1μM – CTRL 2.5μM	1.815	0.277	1.051
CTRL 0.1μM – CTRL 2.5μM	1.692	0.337	1.302
BPA 0.1μM – CTRL 0.1μM	0.163	0.998	0.064

Note. *$p < .05$ for pairwise comparison.

Elavl3:GCaMP Activity Analysis

Hypothalamus activity. Results did not find a significant difference between the 0.1μM BPA group (M = 48.245, SD = 18.058) and the control group (M = 41.616, SD = 22.885), $t(55) = 1.213$, $p = .230$, $d = 0.321$. The 95% CI around the difference between the means was [-4.330, 17.607]. The independent two sample t-test was calculated with the homogeneity of variance assumption being met (1.6:1).

Medulla activity. Results did not find a significant difference between the 0.1μM BPA group (M = 179.290, SD = 58.562) and the control group (M = 165.409, SD = 81.705), $t(55)= 0.735$, $p = .466$, $d = 0.195$. The 95% CI around the difference between the means was [-23.971, 51.732]. The independent two sample t-test was calculated with the homogeneity of variance assumption being met (1.95:1).

Within-subject hypothalamus/medulla activity ratio. Results did not find a significant difference between the 0.1μM BPA group (M = 0.283, SD = 0.104) and the control group (M = 0.271, SD = 0.130), $t(55) = 0.403$, $p = .689$, $d = 0.107$. The 95% CI around the difference between the means was [-0.050, 0.075]. The independent two sample t-test was calculated with the homogeneity of variance assumption being met (1.55:1).

Table 3

Elavl3:GCaMP Experiment Descriptive Statistics

Measurement	Group	*n*	*M*	*SD*	s^2
Hypothalamus					
	BPA	28	48.254	18.058	326.105
	CTRL	29	41.616	22.885	523.728
Medulla					
	BPA	28	179.290	58.562	3429.526
	CTRL	29	165.409	81.705	6675.669
Hyp/Med Ratio					
	BPA	28	0.283	0.104	0.011
	CTRL	29	0.271	0.130	0.017

Note. *M* units: Mean of pixel gray-values within ROI after removing background value.

Spontaneous Swim Activity Analysis

Light-on swim activity. Results did not find a significant difference between the 0.1μM BPA group (M = 12.68, SD = 12.36) and the control group (M = 12.93, SD = 12.38), $t(118) = -0.11$, $p = .912$, $d = 0.02$. The 95% CI around the difference between the means was [-4.724, 4.225]. The independent two sample t-test was calculated with the homogeneity of variance assumption being met (0.99:1).

Light-off swim activity. Results did not find a significant difference between the 0.1μM BPA group (M = 29.66, SD = 9.16) and the control group (M = 29.61, SD = 7.51), $t(144) = 0.037$, $p = .970$, $d = 0.006$. The 95% CI around the difference between the means was [-2.689, 2.792]. The independent two sample t-test was calculated with the homogeneity of variance assumption being met (1.48:1).

Table 4

Spontaneous Swim Activity Descriptive Statistics

Light Period	Group	*n*	*M*	*SD*	s^2
On	BPA	62 (11 NA)	12.681	12.360	152.773
	CTRL	58 (15 NA)	12.930	12.378	153.205
Off	BPA	73	29.658	9.159	83.900
	CTRL	73	29.606	7.510	56.406

Note. *M* units: Seconds of movement per minute of recording. NA: Subject data excluded from analyses due to there being no detectable movement throughout the measurement period or due to the incidence of camera errors.

Discussion

Review of the Findings and Literature

The main goal of this study was to identify a potential mechanism by which a relatively low level of exposure (0.1μM) to BPA during development causes hyperlocomotion in larval zebrafish. Although previous studies documented relationships between BPA and swim behavior, the question remained what observable changes in brain activity resulted in swim activity differences. Some evidence suggested that the hypothalamus may modulate certain aspects of swim behavior in zebrafish (McPherson et al., 2016), and since the hypothalamus is a region rich in Cyp19a1b expression during larval neurogenesis (Kinch et al., 2015), I hypothesized that BPA may be causing neural hyperactivity within the hypothalamus due to estrogen stimulation and the neural hyperactivity producing a calcium transit (McCarthy, 2008). Two brain regions, the "DC7" region of the ventral hypothalamus and the anterior medulla, were selected to test this hypothesis in the Elavl3:GCaMP line. The study was unable to find any difference in calcium activity between 0.1μM BPA-treated and control-treated fish in these regions at 6dpf. To validate the findings of the main hypothesis, two other hypotheses were tested concurrently to replicate previous findings.

In the first replication, 6dpf Cyp19a1b:GFP larvae were used to test whether 0.1μM BPA caused a significant increase in Cyp19a1b expression as indicated by GFP fluorescence. Despite a reasonable sample size of 12 fish per group (10-15 per condition

in Brion et al., 2012), there were no detectable differences in fluorescence between BPA and control-treated fish at this dose. I was unable to find literature that confirmed an increase of fluorescence at doses below 1.0μM, the dose response curve generated by Brion et al. (2012) Fig. 3b did not indicate any increase at a 100nM (0.1μM) dose. Another study using real time RT-qPCR did find a 9-fold induction of Cyp19a1b transcripts at 0.1μM (Cano-Nicolau et al., 2016), which justifies the attempt to test for fluorescence change in the Cyp19a1b:GFP line at this dose. This leaves questions about differences between the sensitivity of *in vivo* and *in vitro* methods for testing gene expression of Cyp19a1b, which have been generally thought to be highly correlated. As a validity check, I also tested a higher dose of 2.5μM BPA which produced a 3.4-fold increase in fluorescence compared to the control group. Although significant, these results indicate a weaker effect compared to one study finding 16-fold GFP induction at 1.0μM (Cano-Nicolau et al., 2016) and another finding an EC_{50} of 3.3μM with a max fold induction of 11.5 (Brion et al., 2012). The method of *in vivo* fluorescence quantification used in this study was remarkably similar to those used in Cano-Nicolau et al. (2016) and Brion et al. (2012), using ImageJ software to measure the sum of gray-values of pixels within the region of interest while compensating for the background gray-value. One notable difference is that these studies used a 10x objective lens with a 134ms exposure time and maximal intensity, while the current study used a 20x .95NA water immersion lens that scanned the entire head at a lower intensity over a 10-minute period. Another potential difference is that the image analysis used in this study had an ROI surrounding the hypothalamus and midline of the telencephalon on a select subset of image slices,

which is likely different than what similar studies used. The inability of this study to find a difference in Cyp19a1b expression using confocal fluorescence microscopy at a 0.1μM BPA concentration may indicate that it is not an ideal method for quantification of estrogenic activity at doses below 1.0μM BPA, or simply that the methods need more refinement to detect differences at this lower dose. It also does not exclude the possibility that there was in fact an estrogenic effect at that dose that could have been detected by a more sensitive method such as qPCR. However, the absence of behavioral effects at that concentration builds confidence in the conclusion that there was no estrogenic effect at 0.1μM.

In the second replication, 6dpf zebrafish larvae from either transgenic line were used to test whether 0.1μM BPA increased spontaneous swim activity in both light and dark periods. Surprisingly, this study found no overall differences in swim activity despite a large sample size of 73 fish per group being used. I attempted to replicate methods used by two previous studies, Saili et al. (2012) and Kinch et al. (2015), which both found larval hyperactivity after 0.1μM BPA exposure, the latter of which had successfully replicated the results of the first. Both studies tested at 5dpf, housed larvae in a 96-well plate, and acclimated the fish to light for 20 minutes before recording locomotor activities during 5 minutes of darkness. They tracked locomotor activities at 30 frames per second with a 5-or-more pixel difference between frames being set as the threshold, and the duration of hyperactivity bursts being measured in seconds per minute for each larva. Each of these methodological aspects were very similar to how the methods of the current study was carried out. This study measured at 6dpf, housed larvae

in a 96-well plate, acclimated fish to light for 5-6 minutes before recording for 5 minutes of darkness, and used a 7-or-more pixel difference between frames as the threshold. To remain consistent with previous studies which calculated the duration of hyperactive bursts in seconds per minute, this study simply calculated the overall percentage of frames spent moving and then converted them into seconds of activity per minute. Small differences between previous studies and the current study were also noted. Previous studies used sample sizes of 5-10 per group (Kinch et al., 2015) and 42-47 per group (Saili et al., 2012), which are less than the sample size of 73 per group used in the current study. Also noteworthy is that the 30 frames per second framerate described in Saili et al. (2012) is faster than the 5 frames per second used in the current study. Another potentially significant difference is that Saili et al. (2012) maintained an ambient temperature of 28°C in their testing chamber, while the current study had a temperature in the range of 25-26°C. Despite these differences, they do not seem substantial enough to explain the failure to replicate the larval swim hyperlocomotion hypothesis. Kinch et al. (2015) found a 2.8-2.9-fold increase in swim activity in 0.1μM BPA exposed fish compared to controls, while Saili et al. (2012) found that 0.1μM BPA exposed fish had a movement duration of 24 seconds per minute compared to 16 seconds per minute in control fish (Fig. 3b). The current study's findings that the 0.1μM BPA and control groups both spent approximately 30 seconds per minute moving during the dark period is unexpected and raises questions about what may have caused this result. One notable difference between our results and those of Saili et al. (2012) is that our larvae were more active overall, particularly the untreated control larvae. Kinch et al. (2015) did not report

swimming activity in absolute units, only group differences, and it remains unknown how differences in baseline activity may affect the BPA response.

Limitations

One limitation of the current study relates to how neural activity was quantified. The calcium activity measurement of this study is unable to draw any information about action potential frequencies or the pattern of activity across different neurons. Taking the standard deviation projection over time gives a brightness value for each pixel, with brighter values being assigned to pixels that exhibited larger degrees of change over time. This is a logical way to measure total Ca^{2+} fluctuation over time, however, it is a novel method that has not yet been independently validated. One method to validate this method would be to compare GCaMP dynamics between control larvae and those treated with the anesthetic, MS-222, which blocks voltage gated sodium channels on neurons and is known to greatly inhibit GCaMP dynamics (Fosque et al., 2015).

Another consideration is the brain areas I selected to measure BPA-induced activation. In this study only two small subsections of the hypothalamus and medulla were selected to measure Ca^{2+} activity, however, other regions may have also been affected by the drug. The DC7 dopaminergic cluster of the ventral hypothalamus was selected because of dopamine's well-known involvement in locomotive behavior; while the anterior medulla was selected due to the prominence of descending motor neurons that exhibit calcium responses which initiate movement (Gahtan, Sankrithi, Campos, & O'Malley, 2002). Whether these two regions have an integrated neural circuit has not

been established, so the hypothesis that the DC7 cluster modulates anterior medulla activity may not have been well-founded. A subsequent review of the literature revealed research that shows dopaminergic clusters DC2, DC4, DC5, and DC6 within the hypothalamus have confirmed axonal projections into the hindbrain (Leng Tay, Ronneberger, Ryu, Nitschke, & Driever, 2011). DC7 was not amongst the clusters listed that project the hindbrain, and instead only had local projections to the surrounding hypothalamus and posterior recess. Furthermore, what I believed to be the DC7 cluster in the Elavl3:GCaMP line was not confirmed by anti-TH staining or by cross-comparison to our own DAT:eGFP line which produces eGFP in dopaminergic neurons; however, comparison to online anatomical resources appeared to support the identity of the structure being DC7. Although there was uncertainty surrounding the hypothalamic cluster selected for this study, the justifications for selecting this region were that it is a bright, distinct, and consistently identifiable structure in the ventral end of the hypothalamus. Future studies could use dopamine neuron-specific expression of GCaMP, for example by making GCaMP expression conditional on expression of the tyrosine hydroxylase enzyme, to be more certain of the dopaminergic identity of imaged cells.

In addition to the problems with the hypothalamic region selection, there were small discrepancies in the medulla region selections which are worth noting. The variability of pigmentation on the skin above the medulla posed the problem of selecting the same segment between subjects. As a result, there were small differences in the region selected on both the anterior-posterior and dorsal-ventral axis. This variability

may not have been reflected in the standard deviations of medulla measurements, which appeared similar to the standard deviations of the hypothalamus measurements. This problem could have been controlled for by raising larvae in 0.003% N-phenylthiourea (PTU) embryo water solution to inhibit the onset of pigment formation (melanogenesis), which was done in another study examining neuronal activity in the Elavl3:GCaMP line (Turrini et al., 2017).

The last important limitation of this study which should be mentioned is the removal of one of the originally proposed hypotheses. The literature review of this study includes a section on larval neurogenesis because I had intended to test whether there was an increase in neuron count in the hypothalamus at 6dpf due to the 0.1µM BPA exposure dose used in the other hypotheses. Both time constraints and the inability to implement defensible methods led to the abandoning of this hypothesis, which had not been central to the main goal of the study.

Directions for Future Research

This study is believed to be the first of its kind in using the Elavl3:GCaMP line to analyze Ca^{2+} activity in the larval zebrafish brain after BPA exposure. Due to the inconclusive results of this study, questions remain about whether BPA may affect Ca^{2+} activity, how varying doses may elicit different responses, and if Ca^{2+} dynamics can be correlated or otherwise causally connected to observed swim activity changes. There appear to be few literature search results for both the Elavl3:GCaMP zebrafish line as well as BPA's effect on swim behavior, so it could be that designing a study that

attempted to link early-stage research in these areas was overly ambitious in the context of a graduate student's thesis project. Although the project was not fruitful in terms of confirming hypotheses by finding significant results, there was progress made in laying out the theoretical and methodological groundwork for potential future studies seeking to test related concepts. A good direction for future research may be to further justify the theoretical relationship between BPA and Ca^{2+}, to narrow down the focus to specific brain regions that have been demonstrated to exhibit circuitry related to motor behavior, and lastly to attempt a range of doses that may elicit an effect on Ca^{2+} dynamics and swim activity in either direction rather than just one.

Conclusions, Summary or Recommendations

The pervasiveness of endocrine disrupting chemicals in modern society presents a continuous hazard to public health in both humans and wildlife alike. Xenoestrogens have been a focal point of research in this area due to their tendency to leech from plastics that humans commonly interact with. The effects of BPA exposure have been correlated to sexual dysfunction, fertility problems, obesity, type-2 diabetes, cardiovascular disease, neurobehavioral deficits in children, and many other deleterious effects in humans (Rochester, 2013). The perinatal and childhood development windows represent a critical period of vulnerability to the disrupting biological effects of exposure, which can result in adverse long-term effects. Zebrafish have become a lead model organism in toxicology research, with zebrafish embryo toxicity testing showing promise in the ability to accurately predict relative acute toxicity in mammals (Ducharme, Reif, Gustafsson, & Bondesson, 2015). Furthermore, the ability to develop transgenic lines that allow *in vivo* observation of specific biological responses to chemical exposures offers a distinct advantage over mammalian testing. The Cyp19a1b:GFP zebrafish line was developed to act as a sensitive tool which can measure estrogen-activating effects in the brain with remarkable specificity to neural progenitor cells. This line can be used to detail complex relationships between estrogenic activity in the brain and a wide variety of adverse effects both biological and behavioral, especially when used in conjunction with lines such as Elavl3:GCaMP or using other targeted methodologies.

Although the current study's attempt to draw connections between estrogenic activity, calcium dynamics, and behavior using transgenic zebrafish lines was unable to determine any relationships between them, the fact that it was able to be tried at all shows the remarkable potential that these lines offer to researchers.

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Appendix A: Cyp19a1b:GFP Experiment R Script

```
#Cyp19a1b:GFP dataset

> Cyp19_dataset <- read_excel("C:/Users/Ryan/Desktop/Cyp19
dataset.xlsx")
> View(Cyp19_dataset)
> Cyp19_dataset$Group<-factor(Cyp19_dataset$Group, levels = c(1:4))
> levels(Cyp19_dataset$Group)[1]<-"BPA 0.1"
> levels(Cyp19_dataset$Group)[2]<-"CTRL 0.1"
> levels(Cyp19_dataset$Group)[3]<-"BPA 2.5"
> levels(Cyp19_dataset$Group)[4]<-"CTRL 2.5"


#Descriptive Statistics

> summary(Cyp19_dataset)
Group       ROI mean        Baseline      Pixel Value
BPA 0.1 :15   Min.   :301.6   Min.   :254.2   Min.   : 24.82
CTRL 0.1:15   1st Qu.:442.5   1st Qu.:309.1   1st Qu.: 45.44
BPA 2.5 : 6   Median :570.8   Median :510.1   Median : 59.01
CTRL 2.5: 6   Mean   :522.0   Mean   :451.1   Mean   : 70.85
3rd Qu.:588.5   3rd Qu.:525.9   3rd Qu.: 82.79
Max.   :643.7   Max.   :559.2   Max.   :207.35

> tapply(Cyp19_dataset$`ROI mean`, Cyp19_dataset$Group, mean)
BPA 0.1 CTRL 0.1  BPA 2.5 CTRL 2.5
585.5105 582.0549 417.5735 317.5218

> tapply(Cyp19_dataset$`ROI mean`, Cyp19_dataset$Group, sd)
BPA 0.1 CTRL 0.1  BPA 2.5 CTRL 2.5
18.32439 25.69854 36.62011 24.62259

> tapply(Cyp19_dataset$`ROI mean`, Cyp19_dataset$Group, var)
BPA 0.1  CTRL 0.1   BPA 2.5  CTRL 2.5
335.7834  660.4150 1341.0324  606.2718

> tapply(Cyp19_dataset$Baseline, Cyp19_dataset$Group, mean)
BPA 0.1 CTRL 0.1  BPA 2.5 CTRL 2.5
524.0372 521.7891 269.5913 273.8767

> tapply(Cyp19_dataset$Baseline, Cyp19_dataset$Group, sd)
BPA 0.1 CTRL 0.1  BPA 2.5 CTRL 2.5
19.03797 15.57455 19.40222 26.33961

> tapply(Cyp19_dataset$Baseline, Cyp19_dataset$Group, var)
BPA 0.1 CTRL 0.1  BPA 2.5 CTRL 2.5
362.4443 242.5665 376.4461 693.7750
```

```
#Descriptives continued

> tapply(Cyp19_dataset$`Pixel Value`, Cyp19_dataset$Group, mean)
BPA 0.1  CTRL 0.1   BPA 2.5  CTRL 2.5
61.47333  60.26573 147.98217  43.64517

> tapply(Cyp19_dataset$`Pixel Value`, Cyp19_dataset$Group, sd)
BPA 0.1  CTRL 0.1   BPA 2.5  CTRL 2.5
21.828533 15.064232 32.795375  9.948652

> tapply(Cyp19_dataset$`Pixel Value`, Cyp19_dataset$Group, var)
BPA 0.1   CTRL 0.1    BPA 2.5   CTRL 2.5
476.48487  226.93108 1075.53664   98.97568

#Experiment 1 data subset & T-test

> Cyp19_Experiment_1<-Cyp19_dataset[(Cyp19_dataset$Group=="BPA
0.1"|Cyp19_dataset$Group=="CTRL 0.1"),]
> View(Cyp19_Experiment_1)

> t.test(Cyp19_Experiment_1$`Pixel Value`~Cyp19_Experiment_1$Group,
var.equal=TRUE)

Two Sample t-test

data:  Cyp19_Experiment_1$`Pixel Value` by Cyp19_Experiment_1$Group
t = 0.17634, df = 28, p-value = 0.8613
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  -12.81979  15.23499
sample estimates:
  mean in group BPA 0.1 mean in group CTRL 0.1
61.47333               60.26573

#Experiment 1 effect size

> library(lsr)
> Cyp19_Experiment_1$Group<-factor(Cyp19_Experiment_1$Group)
> levels(Cyp19_Experiment_1$Group)
[1] "BPA 0.1"  "CTRL 0.1"

> cohensD(Cyp19_Experiment_1$`Pixel Value`~Cyp19_Experiment_1$Group)
[1] 0.06439201

#Experiment 2 data subset & T-test

> Cyp19_Experiment_2<-Cyp19_dataset[(Cyp19_dataset$Group=="BPA
2.5"|Cyp19_dataset$Group=="CTRL 2.5"),]
> View(Cyp19_Experiment_2)
```

```
> t.test(Cyp19_Experiment_2$`Pixel Value`~Cyp19_Experiment_2$Group,
var.equal=FALSE)

Welch Two Sample t-test

data:  Cyp19_Experiment_2$`Pixel Value` by Cyp19_Experiment_2$Group
t = 7.4574, df = 5.9125, p-value = 0.0003211
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  69.97874 138.69526
sample estimates:
  mean in group BPA 2.5 mean in group CTRL 2.5
147.98217               43.64517

#Experiment 2 effect size

> library(lsr)
> Cyp19_Experiment_2$Group<-factor(Cyp19_Experiment_2$Group)
> levels(Cyp19_Experiment_2$Group)
[1] "BPA 2.5"  "CTRL 2.5"

> cohensD(Cyp19_Experiment_2$`Pixel Value`~Cyp19_Experiment_2$Group)
[1] 4.30551

#ANOVA & Tukey Test

> View(Cyp19_dataset_2_0)
> Cyp19_dataset_2_0$Group<-factor(Cyp19_dataset_2_0$Group, levels =
c(1:4))
> levels(Cyp19_dataset_2_0$Group)[1]<-"BPA 0.1"
> levels(Cyp19_dataset_2_0$Group)[2]<-"CTRL 0.1"
> levels(Cyp19_dataset_2_0$Group)[3]<-"BPA 2.5"
> levels(Cyp19_dataset_2_0$Group)[4]<-"CTRL 2.5"

> ANOVA<-aov(Pixel_Value~Group, Cyp19_dataset_2_0)
> summary(ANOVA)

            Df Sum Sq Mean Sq F value   Pr(>F)
Group        3  43136   14379   34.76 5.54e-11 ***
Residuals   38  15720     414
---
  Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> etaSquared(ANOVA)
eta.sq eta.sq.part
Group 0.732903    0.732903

> TUKEY<-glht(ANOVA,linfct=mcp(Group="Tukey"))
> summary(TUKEY)
```

```
Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts


Fit: aov(formula = Pixel_Value ~ Group, data = Cyp19_dataset_2_0)

Linear Hypotheses:
                          Estimate Std. Error t value      Pr(>|t|)
CTRL 0.1 - BPA 0.1 == 0    -1.208      7.427  -0.163    0.998
BPA 2.5 - BPA 0.1 == 0     86.509      9.825   8.805   <0.001 ***
CTRL 2.5 - BPA 0.1 == 0   -17.828      9.825  -1.815    0.277
BPA 2.5 - CTRL 0.1 == 0    87.716      9.825   8.928   <0.001 ***
CTRL 2.5 - CTRL 0.1 == 0  -16.621      9.825  -1.692    0.337
CTRL 2.5 - BPA 2.5 == 0  -104.337     11.743  -8.885   <0.001 ***
```

Appendix B: Elavl3:GCaMP Experiment R Script

```
#Elavl3:GCaMP dataset

> GCaMP_dataset <- read_excel("C:/Users/Ryan/Desktop/GCaMP
dataset.xlsx")
> View(GCaMP_dataset)
> GCaMP_dataset$Group<-factor(GCaMP_dataset$Group, levels = c(1:2))
> summary(GCaMP_dataset$Group)
1  2
28 29
> levels(GCaMP_dataset$Group)[1]<-"BPA"
> levels(GCaMP_dataset$Group)[2]<-"CTRL"
> summary(GCaMP_dataset$Group)
BPA CTRL
28   29

#Descriptive Statistics

> summary(GCaMP_dataset)
Group     Hypothalamus       Medulla          Hyp/Med
BPA :28   Min.   : 4.902   Min.   : 53.55   Min.   :0.06988
CTRL:29   1st Qu.:28.540   1st Qu.:113.17   1st Qu.:0.18584
Median :43.135   Median :173.01   Median :0.26288
Mean   :44.877   Mean   :172.23   Mean   :0.27704
3rd Qu.:58.140   3rd Qu.:209.78   3rd Qu.:0.34671
Max.   :86.859   Max.   :376.51   Max.   :0.56667

> tapply(GCaMP_dataset$Hypothalamus, GCaMP_dataset$Group, mean)
BPA     CTRL
48.25411 41.61552
> tapply(GCaMP_dataset$Hypothalamus, GCaMP_dataset$Group, sd)
BPA     CTRL
18.05838 22.88510
> tapply(GCaMP_dataset$Hypothalamus, GCaMP_dataset$Group, var)
BPA     CTRL
326.1049 523.7279

> tapply(GCaMP_dataset$Medulla, GCaMP_dataset$Group, mean)
BPA     CTRL
179.2896 165.4091
> tapply(GCaMP_dataset$Medulla, GCaMP_dataset$Group, sd)
BPA     CTRL
58.56215 81.70477
> tapply(GCaMP_dataset$Medulla, GCaMP_dataset$Group, var)
BPA     CTRL
3429.526 6675.669
```

```
> tapply(GCaMP_dataset$`Hyp/Med`, GCaMP_dataset$Group, mean)
BPA      CTRL
0.2834334 0.2708623
> tapply(GCaMP_dataset$`Hyp/Med`, GCaMP_dataset$Group, sd)
BPA      CTRL
0.1042215 0.1296929
> tapply(GCaMP_dataset$`Hyp/Med`, GCaMP_dataset$Group, var)
BPA       CTRL
0.01086213 0.01682025

#Independent Samples t-tests

> t.test(GCaMP_dataset$Hypothalamus~GCaMP_dataset$Group,
var.equal=TRUE)

Two Sample t-test

data:  GCaMP_dataset$Hypothalamus by GCaMP_dataset$Group
t = 1.213, df = 55, p-value = 0.2303
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  -4.329595 17.606775
sample estimates:
  mean in group BPA mean in group CTRL
48.25411           41.61552

> t.test(GCaMP_dataset$Medulla~GCaMP_dataset$Group, var.equal=TRUE)

Two Sample t-test

data:  GCaMP_dataset$Medulla by GCaMP_dataset$Group
t = 0.73489, df = 55, p-value = 0.4655
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  -23.97153  51.73246
sample estimates:
  mean in group BPA mean in group CTRL
179.2896           165.4091

> t.test(GCaMP_dataset$`Hyp/Med`~GCaMP_dataset$Group,
var.equal=TRUE)

Two Sample t-test

data:  GCaMP_dataset$`Hyp/Med` by GCaMP_dataset$Group
t = 0.40251, df = 55, p-value = 0.6889
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  -0.05001844  0.07516063
sample estimates:
```

```
  mean in group BPA mean in group CTRL
0.2834334          0.2708623

#Effect Sizes

> library(lsr)

> cohensD(GCaMP_dataset$Hypothalamus~GCaMP_dataset$Group)
[1] 0.3213719

> cohensD(GCaMP_dataset$Medulla~GCaMP_dataset$Group)
[1] 0.1947073

> cohensD(GCaMP_dataset$`Hyp/Med`~GCaMP_dataset$Group)
[1] 0.1066444
```

Appendix C: Behavior Experiment R Script

```
#Behavior dataset

> Behavior_dataset <- read_excel("C:/Users/Ryan/Desktop/Behavior
dataset.xlsx")
> View(Behavior_dataset)
> Behavior_dataset$Group<-factor(Behavior_dataset$Group, levels =
c(1:2))
> levels(Behavior_dataset$Group)[1]<-"BPA"
> levels(Behavior_dataset$Group)[2]<-"CTRL"

#Descriptive Statistics

> tapply(Behavior_dataset$`Light Off`, Behavior_dataset$Group, mean)
BPA     CTRL
29.65790 29.60635

> tapply(Behavior_dataset$`Light Off`, Behavior_dataset$Group, sd)
BPA     CTRL
9.159700 7.510379

> tapply(Behavior_dataset$`Light Off`, Behavior_dataset$Group, var)
BPA     CTRL
83.90010 56.40579

> summary(Behavior_dataset)
Group      Light Off         Light On
BPA :73   Min.   : 5.549   Min.   : 0.03976
CTRL:73   1st Qu.:23.047   1st Qu.: 1.10664
Median :28.636   Median : 8.89963
Mean   :29.632   Mean   :12.80158
3rd Qu.:35.606   3rd Qu.:21.86957
Max.   :55.915   Max.   :51.45969
                 NA's   :26

#Independent Samples T-test

> t.test(Behavior_dataset$`Light Off`~Behavior_dataset$Group,
var.equal=TRUE)

Two Sample t-test

data:  Behavior_dataset$`Light Off` by Behavior_dataset$Group
t = 0.037184, df = 144, p-value = 0.9704
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-2.688698  2.791797
```

```
sample estimates:
mean in group BPA mean in group CTRL
         29.65790           29.60635

#Effect Size

> library(lsr)
> cohensD(Behavior_dataset$`Light Off`~Behavior_dataset$Group)
[1] 0.006154658

#Descriptive Statistics continued
#Omitted NA values for 'Light On''

> Behavior_Omit_NA<-na.omit(Behavior_dataset[-2])
> View(Behavior_Omit_NA)

> summary(Behavior_Omit_NA)
Group       Light On
BPA :62   Min.   : 0.03976
CTRL:58   1st Qu.: 1.10664
Median : 8.89963
Mean   :12.80158
3rd Qu.:21.86957
Max.   :51.45969

> tapply(Behavior_Omit_NA$`Light On`, Behavior_Omit_NA$Group, mean)
BPA     CTRL
12.68109 12.93038
> tapply(Behavior_Omit_NA$`Light On`, Behavior_Omit_NA$Group, sd)
BPA     CTRL
12.36014 12.37762
> tapply(Behavior_Omit_NA$`Light On`, Behavior_Omit_NA$Group, var)
BPA     CTRL
152.7730 153.2054
> t.test(Behavior_Omit_NA$`Light On`~Behavior_Omit_NA$Group,
var.equal=TRUE)

#Independent Samples T-tests continued

> t.test(Behavior_Omit_NA$`Light On`~Behavior_Omit_NA$Group,
var.equal=TRUE)

Two Sample t-test

data:  Behavior_Omit_NA$`Light On` by Behavior_Omit_NA$Group
t = -0.11033, df = 118, p-value = 0.9123
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  -4.723597  4.225014
sample estimates:
```

```
  mean in group BPA mean in group CTRL
12.68109           12.93038

#Effect Size

> library(lsr)
> cohensD(Behavior_Omit_NA$`Light On`~Behavior_Omit_NA$Group)
[1] 0.02015522
```

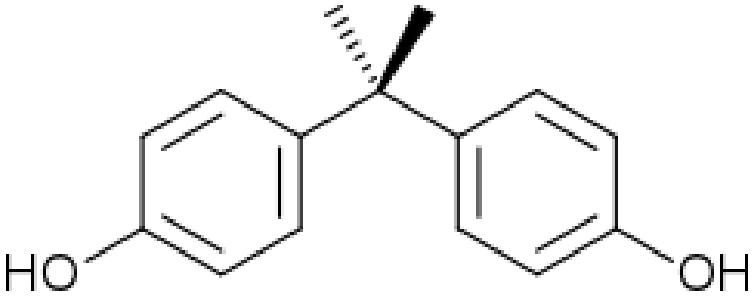
Appendix D: Chemical Data Table, Structures, and Safety Information

Chemical Data Table

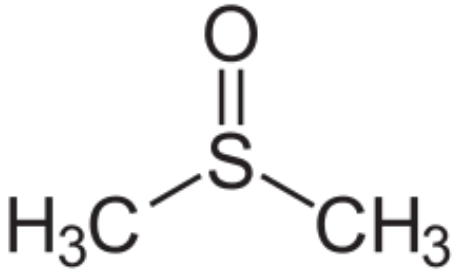
Chemical Name	Molar Mass (g/mol)	Melting point (°C)	Boiling point (°C)	Density (g/ cm^3)	Hazards
Bisphenol A (4,4'-isopropylidenediphenol)	228.291	158-159	220	1.20	Danger: Allergic skin reaction, serious eye damage, respiratory irritation, reproductive toxicity, flammable
Dimethyl Sulfoxide	78.13	19	189	1.1004	Warning: Skin irritation, eye irritation, respiratory irritation, flammable
Tricaine methanesulfonate	261.292	147-150	N/A	N/A	Warning: Skin irritation, eye irritation, respiratory irritation, anesthetic

2D Chemical Structures

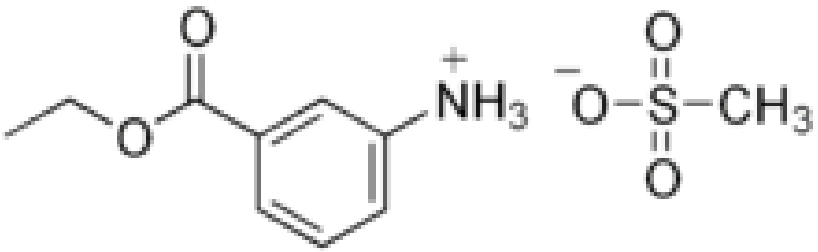
Bisphenol A.



Dimethyl Sulfoxide.



Tricaine methanesulfonate.



BPA Safety Information

Hazards.

Air & Water Reactions – The finely powdered resin is a significant dust explosion hazard. Insoluble in water.

Fire Hazard –. The chemical is combustible and may form explosive dust clouds. Static electricity can cause its dust to explode.

Health Hazard – Dusts irritating to upper respiratory passages; may cause sneezing.

Reactivity Profile – 4,4'-isopropylidenediphenol is incompatible with strong oxidizers. It is also incompatible with strong bases, acid chlorides and acid anhydrides.

Belongs to the Following Reactive Group(s) – Phenols and Cresols

First Aid.

EYES – Check if victim is wearing contact lenses and remove if present. Flush eyes with water or normal saline solution for 20-30 minutes while calling a hospital or poison control center, do not apply medication into eyes without specific instructions from a physician. IMMEDIATELY transport victim to hospital even if no symptoms are visible.

SKIN – IMMEDIATELY flood affected skin with water while removing and isolating all contaminated clothing. Gently wash all affected skin areas thoroughly with soap and water. If symptoms such as redness or irritation develop call a physician immediately.

INHALATION – IMMEDIATELY leave the contaminated area: take deep breaths of fresh air. If symptoms (such as wheezing, coughing, shortness of breath, or burning in the mouth, throat, or chest develop) call a physician and be prepared to transport the victim to a hospital. Provide proper respiratory protection to rescuers entering an unknown atmosphere. Whenever possible, Self-Contained Breathing Apparatus (SCBA) should be used; if not available, use a level of protection greater than or equal to that advised under Protective Clothing.

INGESTION – DO NOT INDUCE VOMITING. If the victim is conscious and not convulsing, give 1 or 2 glasses of water to dilute the chemical and IMMEDIATELY call a hospital or poison control center. Be prepared to transport the victim to a hospital if advised by a physician. If the victim is convulsing or unconscious, do not give anything by mouth, ensure that the victim's airway is open and lay the victim on his/her side with the head lower than the body. DO NOT INDUCE VOMITING. IMMEDIATELY transport the victim to a hospital.

Response Recommendations.

Isolation and Evacuation - As an immediate precautionary measure, isolate spill or leak area for at least 50 meters (150 feet) in all directions.

SPILL: Increase, in the downwind direction, as necessary, the isolation distance shown above.

FIRE: If tank, rail car or tank truck is involved in a fire, ISOLATE for 800 meters (1/2 mile) in all directions; also, consider initial evacuation for 800 meters (1/2 mile) in all directions.

Firefighting – Fire Extinguishing Agents: Foam, dry chemical, carbon dioxide

Non-fire Response – SMALL SPILLS AND LEAKAGE: Should a spill occur while you are handling this chemical, FIRST REMOVE ALL SOURCES OF IGNITION, then you should dampen the solid spill material with 60-70% ethanol and transfer the dampened material to a suitable container. Use absorbent paper dampened with 60-70% ethanol to pick up any remaining material. Seal the absorbent paper, and any of your clothes, which may be contaminated, in a vapor-tight plastic bag for eventual disposal. Solvent wash all contaminated surfaces with 60-70% ethanol followed by washing with a soap and water solution. Do not reenter the contaminated area until the Safety Officer (or other responsible person) has verified that the area has been properly cleaned.

STORAGE PRECAUTIONS: You should store this chemical under ambient conditions and keep it away from oxidizing materials.

Protective Clothing - Approved dust mask and clean, body-covering clothing sufficient to prevent excessive or repeated exposure to dust, fumes, or solutions. Safety glasses with side shields.

Safety information derived from CAMEO Chemicals:
https://cameochemicals.noaa.gov/chemical/8331