ESTROGEN DISRUPTION OF HYPOTHALAMIC NEURAL ACTIVITY

By

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Abstract

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The brain is highly dependent on the endocrine system for proper neurodevelopment, as it plays a key role in many biological processes. Bisphenol A is a chemical found in plastics that has the potential to mimic the effects of Estrogen in the body, at least weakly. People interact with plastic that contains BPA regularly, and people are at risk for exposure even before being born. The abundance of BPA, along with other exogenous estrogens, makes examining the relationship between early exposure and changes in brain activity imperative. The current study aims to establish a relationship between disrupted estrogen function and brain activity. Because estrogen receptors are abundantly found in several hypothalamic nuclei, the goal is to specifically examine whether BPA exposure leads to hyperactivity in the hypothalamus, behavioral hyperactivity, and to investigate the impacts on aromatase activity.

Acknowledgements

I would first and foremost like to acknowledge and thank Dr. Gahtan for his unwavering support during this long process. Every time I felt discouraged you never failed to urge me towards the finish line, I could not have been paired with a better advisor. This project would not have been possible without the help of our undergraduate research assistants in the lab. I thank you all for the time and energy you poured into this project. I would also like to thank my committee for supporting the development of my project. Advice given by Dr. Singleton has been a great help in maintaining my focus throughout my last year at Humboldt State University. Finally, I wish to thank my mother, family, friends, and partner for their support and encouragement throughout my study.

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Literature Review

Estrogen Serves Many Functions

The endocrine system is complex and involved in the regulation of many important processes that occur in the body, and produces over twenty hormones. There are three major classes of hormones; amino acid-derived, peptide, and lipid-derived. Lipid-derived hormones include the steroid hormones; testosterone, androgen, and estrogen. Lipid hormones are derived from cholesterol and are very similar to it in structure. The lipid hormone estrogen, typically referred to as the "female hormone," is a main focus of this study because the chemical I am studying, Bisphenol A, mimics the effects of estrogen in the body.

Estrogens, produced by both males and females, are a part of the "sex hormone" group of steroid hormones, along with androgens and progestogens according to Nelson and Bulun (2001). Estrogens play important roles in maintaining homeostasis that allows for normal cell functionality. Estrogens have microbiological impact, but their impact can also be observed on a larger scale. One example of this being the hormone regulation of bone metabolism in both males and females (Khosla, Oursler, & Monroe, 2012). While there are many hormones involved in maintaining homeostasis, the main focus here is Estradiol, which is the most potent estrogen in the human body.

Estrogen is referred to as the female hormone because of its role in controlling the menstrual cycle in women. Estrogen is crucial in developing sexual maturity and sexual dimorphism (structures and characteristics that differ between males and females)

according to Fuentes and Silveyra (2019). Estrogen is produced mainly in the ovaries in females, and in smaller amounts by the adrenal gland and testes in males, and circulates throughout the body because lipid hormones can freely cross cell membranes. Velarde (2013) found that estrogen-mediated sexual dimorphism is not limited to anatomical differences, and is thought to contribute to many behavioral and physiological differences between women and men, such as differences in aggressive behavior and the longer average lifespan of women.

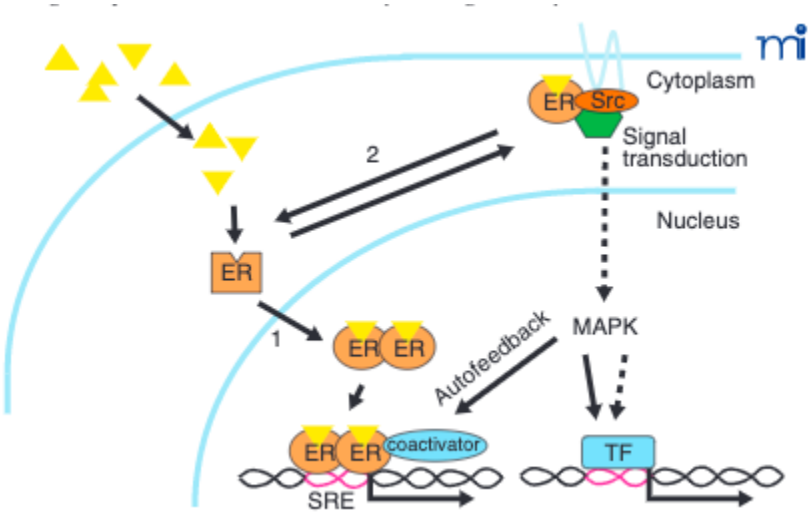
Estrogen Receptors Influence Gene Transcription

As previously stated, Estradiol, being lipophilic, circulates throughout the body and any cell that expresses an estrogen receptor will respond to circulating estrogens. Estrogen receptors (ER) are cytoplasmic, meaning they are freely floating inside cells in their unbound (unactivated) state. Once estrogen binds, the ER becomes activated and moves (translocates) to the cell nucleus where it acts as a transcription factor. The activated ER/estrogen complex recognizes specific DNA sequences in the genome and binds to those sequences to activate or suppress the transcription (expression) of neighboring genes. Hundreds or even thousands of genes are regulated in this way by estrogen receptor signaling (Fuentes & Silveyra, 2019; Ikeda, Horie-Inoue, and Inoue, 2015; Edwards & Boonyaratanakornkit, 2003). There are two subtypes of the ER, called alpha and beta. While these proteins are similar in structure, their distribution in body differs, for example with ER-beta being the dominant subtype in the brain, and research

also indicates a difference in the transcriptional actions of the two ER subtypes (Brinton, 2004).

Figure 1.

Model of classical and non genomic actions of the estrogen receptor



Notes . The figure displays how Estrogen receptors regulate transcriptional factors both directly and indirectly, from "Rapid Extranuclear Signaling by the Estrogen Receptor (ER): MNAR Couples ER and Src to the MAP Kinase Signaling Pathway" by D. Edwards & V. Boonyaratanakornkit, 2003, Molecular interventions, doi: 10.1124/mi.3.1.12. (1) indicates indirect action (2) is indirect. The receptors in the nucleus bind directly to DNA (1) or regulate gene expression by indirect binding of other transcription factors to the DNA (2) (Velarde, 2013).

Estrogen's effects in the brain

Multiple lines of evidence show that estrogen can strongly affect brain function and behavior. One clear indication of this is that ERs are expressed in neurons in multiple brain areas, including the hypothalamus, prefrontal cortex, and hippocampus (Liu and Shi, 2015). It is therefore predictable that estrogen would alter gene transcription in neurons, and this has been confirmed in many studies. Humphreys, Ziegler, and Nardulli for example found that after treating 14 week old mice with Estradiol (E2) for 7 days, which resulted in an approximate 25 pg/ml level of physiological circulating E2, found that E2 treatment increased expression of some genes and decreased expression of other genes (2014). Specifically E2 increased expression of genes involved in neurite extension, but decreased the expression of oligodendrocyte associated genes. Estrogen alters gene expression through both classic and non classic pathways as shown in Figure 1. E2 caused rapid firing the MAP Kinase pathway in the brains of experimental mice, a finding which has been shown to have an important role in neuroplasticity and survival (Humphreys, Ziegler, & Nardulli, 2014). ERs in neurons may also influence neuron function independently of their gene regulatory effects because ERs are also found in dendrites where they appear to influence synaptic physiology. Mcewen and colleagues (2001) found suggestions in recent research that indicates genomic and non-genomic actions assist in synaptic formation.

Estrogen signaling in the brain has also been directly shown to influence behavior. Estrogen administration influences reproductive and aggression behaviors in

rodents through its actions on hypothalamic neurons and modulates learning and memory through its actions on hippocampal neurons (Loredo, Landeros, & Trainor, 2014; Daniel & Bohacek, 2010; Chen et al., 2017).

In addition to these effects of estrogen on brain physiology and behavior in mature animals, estrogen also plays a major role in brain development. Estrogen dramatically influences the growth of neurons during early brain development, as reflected by extreme sexual dimorphism of certain brain areas such as the sexually dimorphic nucleus of the hypothalamus which can be up to seven times larger in males than in females (McCarthy, 2008). Estrogen does this by affecting the normal developmental process of apoptosis (programmed cell death). Males and females have equivalent numbers of neurons in this brain area in early stages of development, but only females undergo rapid apoptosis in the sexually dimorphic nucleus following birth (the time immediately following birth is considered a sensitive period for sexual differentiation of the brain). In contrast apoptosis in males is likely blocked by testosterone. Forger (2010) found that the binding of testosterone, which leads to the activation of androgen and estrogen receptors, results in changes in the expression of proteins involved in cell death. Multiple mechanisms account for sexual differentiation of the brain. Other research shows that estradiol specifically promotes proliferation of neural cells. Zhang and colleagues (2017) conducted a study testing the effects of exogenous estradiol treatment on differentiation. The results indicated that female rats treated with E2 showed an increase in new cells in the hippocampus, and the ventromedial hypothalamus.

In addition to its effects on brain cell proliferation during development, which can result in large-scale anatomical differences in brain structure between sexes, estrogen can also influence the physiology of neurons by altering expression of genes that encode proteins that influence the electrical excitability of neurons. For example, prenatal administration of estrogen to rats downregulates expression of the chloride exporter protein, KCC2, which is a critical regulator of neuronal excitability (Galanopoulou and Moshe, 2013). This finding fits with lower levels of neuronal KCC2 expression found in females. KCC2 functions to pump chloride ions out of neurons, maintaining a low intracellular chloride concentration, which ensures that the neurotransmitter GABA, which opens chloride channels, will produce an inward, inhibitory flow of chloride ions across the membrane. An estrogen-induced decrease in KCC2 expression would therefore be expected to increase neural activity by switching GABA effects from inhibitory to excitatory. Estrogen does not appear to modulate KCC2 expression in mature neurons in the same way (Nakamura, 2004) highlighting the particular importance of estrogen during development.

Bisphenol A, an Estrogen Disrupting Chemical Affecting Brain Development

Considering estrogen's potent effects on brain function and development there is concern about the health effects of exposure to exogenous estrogens, from estrogen or estrogen-like chemicals that are introduced from outside of the body. Estrogen Disrupting Compounds (EDC's), are chemicals that can enter the body and mimic or block the

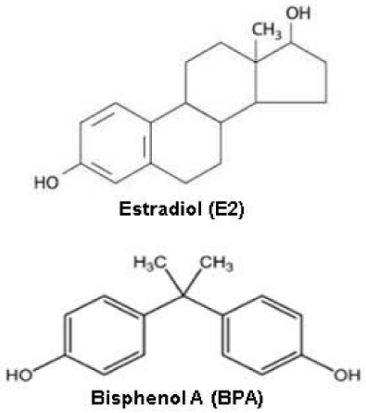
effects of endogenous estrogens. Such disruption in regular hormone exposure, especially during early development, can cause a wide array of issues (Menuet et al., 2002).

Bisphenol A (BPA) is an industrial chemical used in plastic production that is widespread in the environment and known to have the ability to disrupt estrogen function (McCarthy, 2008). Specifically, BPA is known to activate ERs in neurons and other tissues in a way that mimics the effects of natural estrogen, potentially increasing estrogen signaling beyond baseline levels (Chen et al., 2017). Animal models have shown detrimental effects of early BPA exposure on brain structure and behavior, particularly effects that parallel the normal functions of estrogen on neural proliferation and behavior. For example, Wenhui and colleagues (2016) exposed embryonic and larval zebrafish to Bisphenol A and Bisphenol B to examine the effects it has on neuroendocrine function. Both BPA and BPS were shown to increase the number of gonadotropin releasing hormone expressing neurons (GnRH), which are cells in the brain that control the release of reproductive hormones from the pituitary gland, in the hypothalamus at 25 hours post fertilization. There was also an increase in reproductive neuroendocrine-related gene expression 25 hours post fertilization (Wenhui et al., 2016). Follow up research by Kinch and colleagues replicated these effects of BPA increasing neural proliferation in zebrafish and extended this to show an effect of embryonic BPA exposure on behavior in zebrafish as well, and effects of very low BPA doses. In that study, zebrafish exposed to a low concentration of BPA (0.1uM) in line with real environmental exposure levels, or to higher concentrations (1 μM) during early neurogenesis between 16 and 36 hours post-fertilization, exhibited locomotor hyperactivity and increased neurogenesis in the

hypothalamus (but not in the thalamus, tectum, and hindbrain) when tested at 5 days post fertilization (Kinch et al., 2015). These findings from basic research studies underscore the threat of teratogenic effects of BPA in humans, and are more alarming in the light of evidence that children in the US have higher levels of BPA in their body tissues than adults (Calafat et. al, 2008).

Figure 2.

The structures of endogenous estrogens and BPA



Notes. The chemical structures of endogenous Estradiol and Bisphenol A (BPA), taken from "The effects of early low dose exposures to the Environmental Estrogen Bisphenol A on the Development of Childhood Asthma" by Terumi Midoro-Horiuti and Randall M Goldblum, 2017, *Arch Asthma Allergy Immunology*, 1: 015-027. DOI: 10.29328/journal.haard.1001003. 2017. BPA is an exogenous estrogen and a weak mimicker of the hormone, but still has action when binded to estrogen receptors.

Statement of the Problem

This study is inspired by previous evidence that early developmental exposure to BPA can increase the rate of neurogenesis in the hypothalamus and lead to behavioral hyperactivity. More specifically, the current research tests one hypothesis implied by Kinch and colleagues' 2015 finding that BPA increases neurogenesis and causes behavioral hyperactivity in zebrafish. Therefore, I reasoned that increased neural activity could mediate the relationship between greater neurogenesis and increased behavioral activity, in other words, that more neurons would result in more neural activity and in turn more behavior. To test this hypothesis, I replicated the zebrafish BPA exposure procedure described by Kinch et al (2015), and measured neural activity using a method called calcium imaging in which neurons are genetically modified to express a calcium-sensitive fluorescent protein. Because calcium levels are correlated with neuron activity, changes in fluorescence brightness, measured with time lapse microscope imaging, serve as an index of neural activity. We measured neural activity in the hypothalamus because that is where BPA-induced neurogenesis was previously observed, but our calcium imaging method allowed activity in other brain areas to be measured simultaneously. As a positive control, we confirmed estrogenic activity of our BPA treatment by measuring BPA effects on aromatase-b, an enzyme whose expression is highly dependent on estrogen signaling.

This study follows up on Kinch et al (2015) along with a previous study conducted by McAuley & Gahtan which found that there was no hyperactivity in the

hypothalamus as indicated by the results. The results are likely attributed to the very low dose of BPA (0.1uM) that was used. Most importantly, this experiment failed to show an effect of BPA on aromatase-b expression, suggesting that the dose used was not effective. My experiments used a higher dose of BPA (2.5uM) and confirmed that our BPA treatment protocol increased aromatase-b expression before conducting calcium imaging experiments to measure BPA effects on neural activity.

Hypotheses

Hypothesis 1.

Exposure of zebrafish embryos to 2.5 μM BPA from 0-5 days post fertilization will activate estrogen receptors in the brain as shown by an increase in expression of the estrogen-responsive gene, Cyp19a1b (also called Aromatase-b), in BPA treated versus control zebrafish. Aromatase-b expression will be measured by an fluorescence brightness from radial glial cells in the brains of Cyp19a1b:GFP transgenic zebrafish, a line in which a fluorescent marker protein (GFP) is expressed under the control of the estrogen-responsive gene, Cyp19a1b (aromatase-b). In these zebrafish, aromatase-b fluorescence is limited to forebrain radial glia cells, which are neural precursor cells that give birth to new neurons.

Rationale.

This is a replication of Kinch et al (2015) but necessary to confirm that our BPA treatment method is having the expected estrogenic effect in the brain. This experiment constitutes a positive control.

Hypothesis 2.

Exposure of zebrafish embryos to a 2.5 μM BPA will increase neural activity in the hypothalamus as measured by fluorescence calcium imaging in elavl3:GCaMP6s zebrafish larvae.

Rationale.

BPA exposure was previously shown to increase the rate of neurogenesis hypothalamic in zebrafish larvae. This hypothesis is based on the reasoning that if there are more functional neurons, there will be more neural activity, and therefore greater GCaMP dynamics in BPA-treated versus control zebrafish. GCaMP dynamics will be measured as fluorescence brightness changes, which indicate changing levels of calcium inside neurons and therefore neural activity. To measure this, fluorescence brightness in the hypothalamus will be imaged at a rate of 1 image per second for 10 minutes in control and treated larvae, and the standard deviation of fluorescence brightness will be calculated as a measure of change in fluorescence (ie, neural activity dynamics) over time.

Methods

This work uses existing data that was collected by Dr. Gahtan in March 2020 because the lab was closed because of the COVID 19 pandemic. My work is thus a secondary data analysis. The following section presents methods that were used to acquire the data so readers understand what was measured and why, and then presents methods on the data analysis work that is the core project of my thesis. Finally, it must be noted that not all planned experiments were completed due to the campus closure, resulting in lower than planned sample sizes, but data were obtained for both experiments aims.

Facilities and Instruments

All experiments were performed in the behavioral neuroscience lab in BSS room 122 at Humboldt State University. The main instruments used were an Olympus FV1000 confocal microscope system for fluorescence imaging; Nikon SZM1500 stereo zoom microscope for sample preparation; Pixelink PL-A741 machine vision camera for behavioral recordings; Labjack U9 input-out interface and DacFactory software for experiment control of cameras and sensory stimuli; ImageJ for quantitative image analysis; Microsoft Excel and R for data management and statistical analyses.

Subjects.

Zebrafish larvae used for this study were generated from available breeding stocks in the BSS 122 zebrafish facility. All experiments were reviewed and approved by the Humboldt State University IACUC (protocol #1718.P.34).

Measuring BPA Effects on Brain Estrogen Signaling (Experiment 1)

To be certain that our BPA exposure procedure had the expected estrogenic effects, a replication experiment based on (Kinch et al., 2015) was done to show that BPA increased expression of the estrogen-activated enzyme, brain aromatase, which is encoded by the gene, Cyp19a1b. Cyp19a1b:GFP transgenic zebrafish express fluorescence under the control of the CYP19a1b gene, resulting in fluorescence in a small subset of brain cells in the developing brain called radial glia. Fluorescence brightness from these radial glial cells was measured as an indicator of estrogen signaling activity in the brain. If BPA increased estrogen signaling, there would be more brain aromatase enzyme expression, and therefore more fluorescence protein being made and a brighter fluorescent signal.

Zebrafish were incubated in BPA (2.5uM) or control solution from age 16 hours until testing on day 4, 5 or 6 post fertilization. Cyp19a1b:GFP fluorescence brightness was measured in each larvae individually by these steps: (a) transferring to an anesthetic solution (0.01% weight/volume of MS-222) to temporarily immobilize it; (b) embedding horizontally in agarose gel to stabilize and protect it during imaging; (c) imaging to

center the brain in the XY dimensions; (d) imaging to save a series of 24 pictures spanning the depth of the brain at 10um steps, capturing the entire brain volume in which CYP19a1b expression occurs. The precise same embedding and imaging parameters were used for all samples within each experiment so brightness would be directly comparable across individual larvae. A power analysis conducted to determine the appropriate sample size for Experiment 1. With an alpha = .05 and power = 0.80, the projected sample size needed was $N = 2$.

Measuring BPA effects on neural activity (Experiment 2)

Neural activity was measured as a time lapse recording of fluorescence brightness from neurons throughout the brain that expressed a genetically encoded calcium indicator, called GCaMP6s, under the control of a pan-neuronal gene promoter, called Elav13. Groups of BPA and control larvae were formed from individual clutches of Elavl3:GCaMP6s zebrafish embryos (they were all siblings, minimizing individual genetic differences), with the BPA group receiving the same incubation treatment (2.5uM solution in egg water from age 16 hours to 5 days) described previously for measuring BPA estrogenic effects on brain aromatase. All larvae were tested on day 5 post fertilization. Both spontaneous and sensory-evoked neural activity was measured in each larva. The basic steps and key parameters for these measurements were: (a) The larva was briefly anesthetized (~10sec immersion in 0.01% MS-222) to allow positioning during agar embedding; (b) it was then rinsed and embedded on its side on a microscope slide for imaging in the sagittal plane; (c) the slide was placed into a water perfusion

chamber on the heated microscope stage to keep the embedded larva warm and oxygenated during imaging; (d) The fluorescent image of the brain was focused on the hypothalamus because that was the structure of most interest, but several other identifiable brain structures anterior, posterior, and dorsal to the hypothalamus were also routinely present in the field of view, including the thalamus, tectum, cerebellum, medulla, tegmentum, and habenula; (e) After at least 20 minutes of habituation to the imaging environment a time lapse series of images of spontaneous neural activity was taken at 1 image per second for 10 minutes, resulting in a 600 frame video that was saved for later analysis. In most cases frequent spontaneous brightness changes from neurons throughout the brain could be observed; (f) immediately after the 10min spontaneous activity recording a single, 60 second time lapse series was recorded (with the same field of view imaged at the same 1Hz frame rate) that included a dark flash stimulus (the dimming of an ambient white light LED that had illuminated the larva through the recording session) from second 30 through 60. This stimulus reliably evokes a neural response and, in freely swimming larvae, a behavioral escape response, and was used to test the potential of BPA to alter evoked brain responses; (g) larvae were then removed from the imaging platform, de-embedded from agar, placed into a petri dish containing normal fish water, and returned to standard maintenance care. The power analysis conducted to determine the appropriate sample size for Experiment 2, also with an alpha = .05 and power = 0.80, projected a needed sample size of $N = 78$.

Data analysis for BPA effects on brain estrogen signaling

The measure for BPA effects on brain estrogen signaling is the relative brightness of fluorescence from radial glia cells in the brain that express CYP19:GFP. Data is comprised of a series of images through the depth of the brain capturing a 3D volume of the brain in which these radial glia cells occur. Two measures will be used to quantify fluorescence brightness, the first is the direct brightness value of pixels in 8-bit grayscale format (which can range from 0-255), the second is the percentage of the image area occupied by fluorescence pixels. As with image acquisition parameters, image analysis parameters will be kept consistent across the brain so the measurements are directly comparable. Image processing will be done using the program, ImageJ, according to the following steps:

a. convert the image series to 8-bit format
b. Crop each image according to reliable anatomical landmarks so that the same brain volume is quantified in each
c. Create a maximum projection of each image series resulting in a single image of the same size in which each pixel value is the maximum value of that pixel position across the input parent series
d. The maximum projection image will then be thresholded into a binary image format, meaning that a pixel brightness value will be selected below which all pixels will be coded 0 and above which all pixels will be coded 1. The threshold

will be set to include only those pixels that are part of fluorescent radial glia cell population, and will be the same for all brains analyzed.

e. For direct brightness measurement, the average brightness of all pixels within the thresholded area will be measured for brightness in 8 bit format, resulting in a single brightness measurement per brain
f. For area percentage measurement the fraction of all pixels within the thresholded area relative to the entire image area will be measured, resulting in a single measurement (area fraction) per brain
g. To determine if BPA increased Cyp19a1b brightness, separate, one-tailed, independent-samples t-tests, comparing average brightness or area fraction between BPA and control brains, will be performed.

Data analysis for BPA effects on brain activity

The measure for BPA effects on neural activity is the brightness dynamics (change over time) of fluorescence from neurons expressing the GCaMP6s calcium indicator. The data take the form of a series of images over time (time lapse), and GCaMP brightness will be measured on each image to track activity over time. My main hypothesis about BPA effects on neural activity is about activity in the hypothalamus since a previous study showed effects of BPA on the hypothalamus in zebrafish, but nearly all neurons in the Elavl3:GCaMP6s zebrafish larvae used express GCaMP6s, so the brain images will have to be divided into separate areas of analysis based on identifiable brain areas. Both spontaneous and evoked neural activity were measured, and

would be analyzed differently. To measure spontaneous neural activity, first a standard deviation projection was made across all frames of the neural activity video, and then average brightness of the resulting image was measured to capture the amount of change in neural activity over time. Evoked neural activity will be measured as the percentage of brightness changes from before to after the visual stimulus. The image processing will be done using the ImageJ program following these basic steps:

a. The 500-frame spontaneous activity time series will be projected into a single averaged image to reduce image noise and highlight anatomy, creating an anatomy reference image
b. Boundaries will be drawn manually around identifiable brain areas in the anatomy reference image and saved as a 'regions of interest' file in ImageJ. Determination of brain area boundaries will be based on the z-brain atlas available through the Zebrafish Brain Browser website (http://vis.arc.vt.edu/projects/zbb/). This atlas can display zebrafish larva brain anatomy in the context of the same genetic expression driver (Elval3) used in our images of Elavl3:GCaMP zebrafish which greatly facilitates matching of views between the atlas and our data. After previewing these images I anticipate being able to identify the following 6 brain regions in all recorded brains: hypothalamus, thalamus, tectum, cerebellum, medulla, tegmentum.
c. Brain area regions of interest will be applied to the spontaneous and evoked activity time series images, and the Stack Measure feature in ImageJ will be used to measure brightness on each frame of the time series video separately for each

brain region. These brightness values will be exported to Microsoft Excel or R for further analysis.

d. BPA and control larvae will be compared on the standard deviation of brightness values for spontaneous neural activity, and percent brightness increase for evoked activity. Because there is an a priori hypothesis that the hypothalamus will show increased activity after BPA, the measures will be compared for the hypothalamus using 1-tailed, independent samples t tests. For analysis of other brain areas, a 2X6 multifactorial ANOVA will be done with treatment (BPA or control) as a between subjects independent variable, brain area (6 brain regions listed above) as a within subjects independent variable, and either standard deviation of brightness or percent brightness increase at the dependent variable.

Results

Estrogenic Effects of BPA Analysis

Results found that BPA treatment significantly increased cyp19a1b:GFP fluorescence area in two separate experiments, consistent with an estrogenic signaling effect of BPA (Figure 3). Incubation in 5uM BPA from 16 hours to 4 days post fertilization increased fluorescence area by 45% in the control group (M = 1.38, SD = 1.15) and the BPA group (M = 6.35, SD = 2.50), t(5.63)=-4.03, p= 0.004, d = -2.551.The 95% CI around the difference between the means was [-8.02, -1.903]. Incubation in 2.5uM BPA from 16hours to 5 days post fertilization increased fluorescence area by 51% in the control group (M = 1.31, SD = .69) as well as the BPA group (M = 6.74, SD = 3.75), t(4.23) = 3.179, p = .015, d = -2.010. The 95% CI around the difference between the means was [-10.051, -.802]. Fluorescence brightness was numerically higher in the BPA group in both experiments but not statistically different, suggesting BPA primarily increased the number of Cyp19a1b-expressing cells.

Table 1

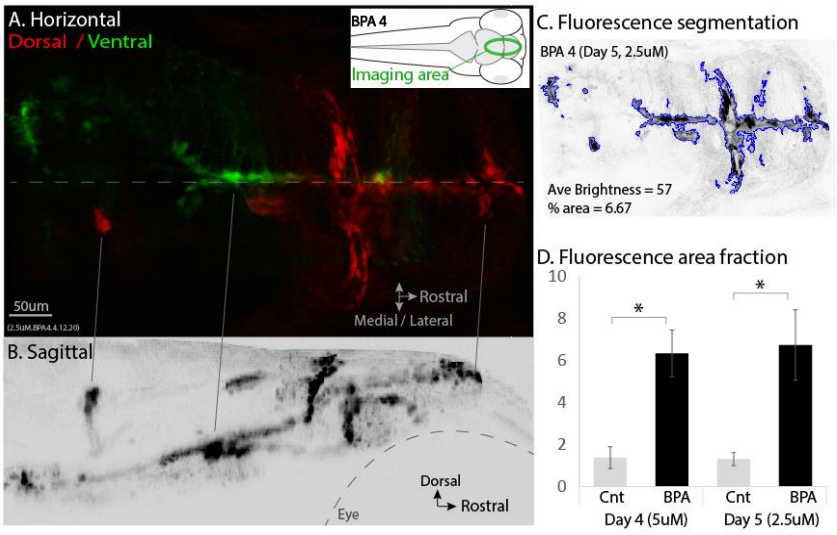
BPA Effects on Brain Estrogen Signaling Descriptive Statistics

Group	*n*	*M*	*SD*
BPA 5 μM	5	6.35	2.50
CTRL	5	1.38	1.15
BPA 2.5μM	5	6.74	3.75
CTRL	5	1.31	.69

Note. M units: Mean of Cyp19a1b:GFP fluorescence area.

Figure 3.

Estrogenic Signaling Effect of BPA



Notes. Cyp19a1b:GFP fluorescence brightness was imaged with a confocal microscope (Olympus FV1000 using YFP filters and a 10x 0.35NA or 20x 0.95NA objective), in each larva individually, by (a) transferring to an anesthetic solution (0.01% weight/volume of MS-222) to temporarily immobilize it; (b) embedding horizontally in 1.2% agarose gel to stabilize and protect it during imaging; (c) centering the field of view on Cyp19a1b-expressing cells in the forebrain; (d) acquiring a series of 24 images across the depth range, at 10um steps, to capture the entire brain volume in which CYP19a1b expression occurs.

BPA Effects on Spontaneous Neural Activity Analysis

Results did not find significance difference in CYP19:GFP brightness in the medulla between the 2.5 μM BPA group (*M* = 392.878, *SD* = 136.249) and the control group (*M* = 399.563, *SD* = 133.150), *t*(17.306) = .118, *p* = .454, *d* = .049. The 95% CI around the difference between the means was [-112.643, 126.012]. The Welch's t-test was calculated with the homogeneity of variance assumption not being met.

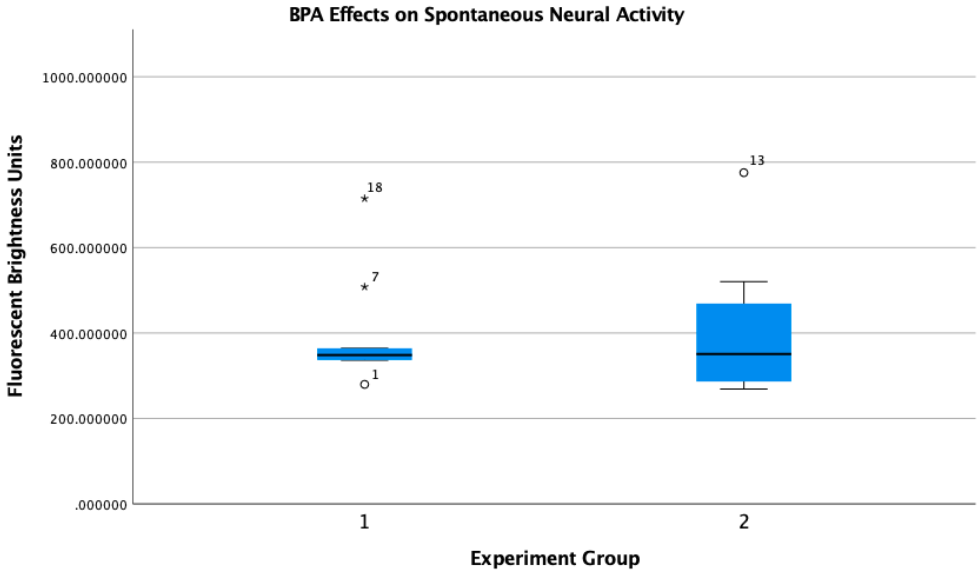
Table 2

GCaMP Spontaneous Neural Activity Descriptive Statistics

Group	*n*	*M*	*SD*
BPA 2.5μM	15	392.878	136.249
CTRL	9	399.563	133.150

Note. M units: Mean fluorescent brightness

Figure 4.

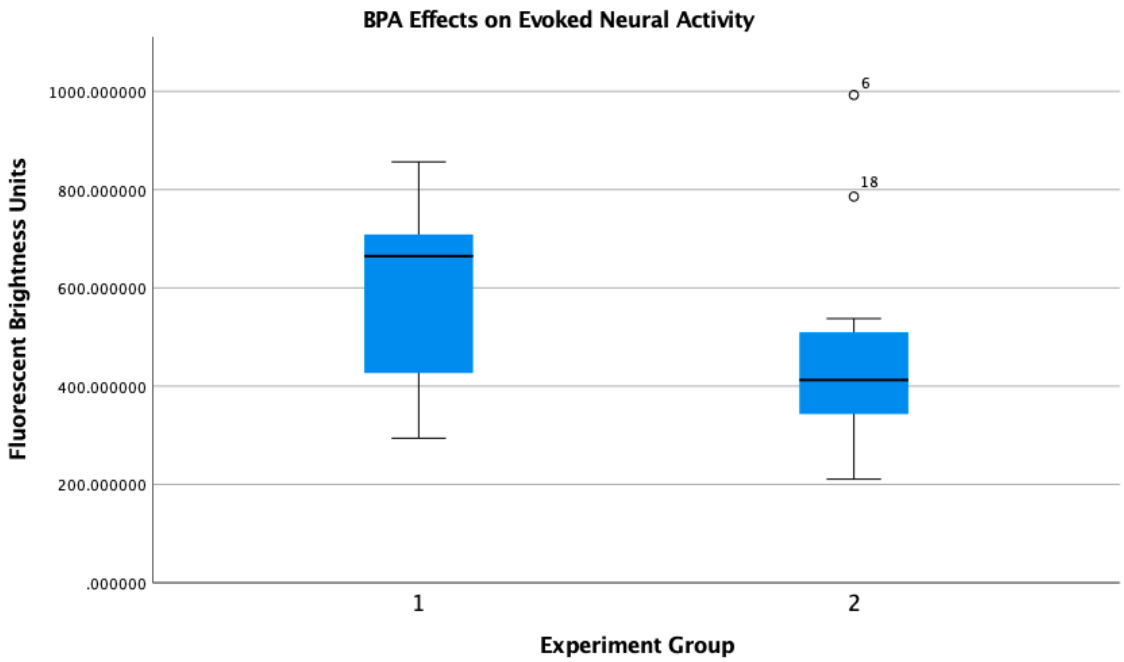


Notes. Spontaneous neural activity in the control GCaMP zebrafish (group 1) versus BPA treated GCaMP zebrafish (group 2) over a 500 second period.

BPA Effects on Evoked Neural Activity Analysis

Results did not find significance difference in darkflash-induced GCaMP brightness in the hypothalamus between the 2.5 μM BPA group (M = 461.532, SD = 208.856) and the control group (M = 586.313, SD = 186.095), t(18.669) = 1.495, p = .076, d = .622. The 95% CI around the difference between the means was [-.50.089, 299.612]. The Welch's t-test was calculated with the homogeneity of variance assumption not being met.

Figure 5.



Notes. Evoked neural activity in control GCaMP zebrafish (group 1) versus BPA treated GCaMP zebrafish (group 2) over a 60 second period.

Table 3

GCaMP Evoked Neural Activity Descriptive Statistics

Group	*n*	*M*	*SD*
BPA 2.5µM	14	461.532	208.856
CTRL	9	586.313	186.095

Note. M units: Mean fluorescent brightness

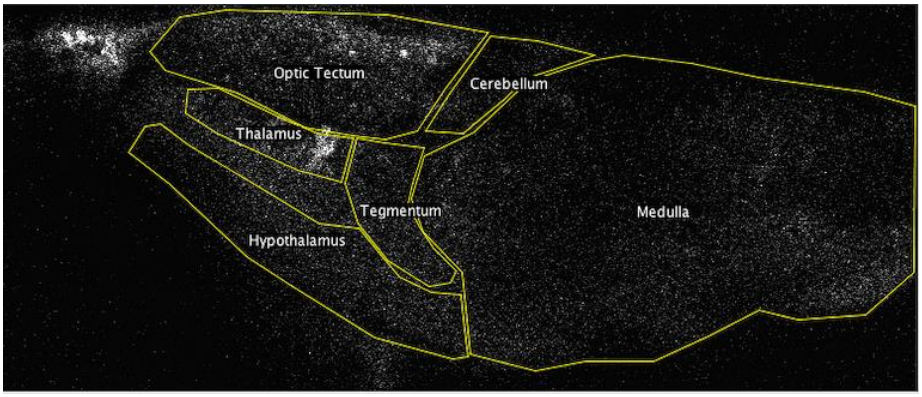
Discussion

Review of Findings and Literature

The aim of the study was to establish a relationship between disrupted estrogen function and brain activity. The goal here mainly was to examine whether BPA exposure led to hyperactivity in the hypothalamus, behavioral hyperactivity, and to investigate the impacts on aromatase activity. Once we established that BPA was inducing an estrogenic effect in our first experiment, the medulla was used for analysis in the spontaneous activity experiment because it was the most consistent structure to identify as shown in Figure 4 and also because of its involvement in the motor system. The hypothalamus was an area of interest because estrogen receptors are abundantly found in several hypothalamic nuclei. Previous studies have shown detrimental effects of early BPA exposure on brain structure and behavior, particularly effects that parallel the normal functions of estrogen on neural proliferation and behavior (Wenhui et al.,2016). The study was unsuccessful in finding a difference in brightness between control and BPA treated zebrafish brains in both the spontaneous and evoked activity experiments conducted.

Figure 6.

Spontaneous Brain Activity



Notes. Displayed is a projected image of spontaneous brain activity observed in one of the zebrafish in the the 2.5 μM BPA group. The medulla is one of the larger structures and was used for analysis in the spontaneous activity experiment because it was the most consistent structure to identify.

The previous study conducted in Dr. Gahtan's lab by former graduate student Ryan McCauley suggested that a 2.5μM dosage of BPA would gain a greater estrogenic effect than the lower dosages he had used in his original experiment. McCauley did later run an experiment using a 2.5μM dosage of BPA, which we chose to replicate. While this did have an estrogenic effect in our brain aromatase experiment, it did not affect neural activity. It's possible that other methods of data quantification possibly would have proved to be more useful. Specifically, BPA could have potentially affected the frequency of recorded neural activity. However, a frequency-based analysis, which might

have been accomplished through a type of Fourier analysis, was not practical for me to try to undertake independently.

The methods used in the current study were a full replication of the McCauley study, using the same housing and imaging protocol, the current study just excluded testing of lower doses of BPA. Although an effect was not found using the confocal microscope it is possible that using other methods to detect an estrogenic effect could have indicated if there actually was one at a 2.5μM dosage of BPA. Also, studies such as Kinch et al. (2015) showed that there was larval activity at doses lower than a 2.5μM BPA. This indicates that abandoning the current study's behavioral assays could have taken away from evidence of estrogenic effect as well.

Limitations

One of the main limitations of the current study is how we decided to analyze the data. We decided to quantify fluorescence brightness over time, as it seemed the most obvious way to visualize the effect BPA was having on brain regions. There were other measurements, such as the percentage of the image area occupied by fluorescence pixels, that may have provided other insight into the neural activity we were interested in. Again, this was a follow up study so the hypothalamus and medulla were of particular interest. Another limitation here is that selecting only the medulla and hypothalamus for analysis rules out that BPA could have induced calcium activity in other areas of the brain.

A major issue in the current study was the medulla being used to observe spontaneous neural activity in the medulla instead of the hypothalamus. Unfortunately the

hypothalamus was not reliably visible in all of the images, which is why we chose a region that was more consistently imaged. While it is thought that there are estrogen receptors all over the brain most research indicates that membrane-associated ERs are most abundantly observed in the prefrontal cortex, dorsal striatum, nucleus accumbens, and hippocampus; also known as areas known to be involved in learning and memory (Almey, Milner, & Brake, 2015). Although the medulla did not initially inspire our hypotheses we did still expect to see an uptake of neural activity in this region, which the results did not indicate.

Another limitation of the study was the limited and unequal sample sizes of the two groups compared to one another. Initially the goal was to have equal groups and to also have a larger number of subjects, but due to physical presence in the lab being limited data collection was limited. If data from more subjects was collected it maybe would have impacted the results in a different way. The initial experimental design also included a behavioral assay which would have looked into how BPA affected the tail movement of the zebrafish we were studying. Unfortunately this hypothesis had to be abandoned because the months-long Covid-19 campus shutdown occurred during my window of opportunity to conduct the experiments.

Directions for Future Research

This study, along with McCauley's, are two of the few that have examined how BPA impacts brain activity. While we were unsuccessful in finding an effect that is possibly due to how the data was quantified and the limitations on sample size. The

zebrafish animal model provides amazing ways to visualize neural activity at the cellular level in intact animals, so conducting this study using this model should not be abandoned. Future studies using the same basic approach but with greater statistical power and parallel behavioral measures of BPA effects would still be useful in clarifying the relationship between BPA's estrogenic actions and neural activity. Finding a way to more consistently image all areas of the brain would also allow for better more in depth analysis.

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