DOES BISPHENOL-A, AN ESTROGEN-LIKE ENVIRONMENTAL TOXIN, DISRUPT EXPRESSION OF THE NEURONAL CHLORIDE EXPORTER PROTEIN DURING EARLY BRAIN DEVELOPMENT?

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Abstract

DOES BISPHENOL-A, AN ESTROGEN-LIKE ENVIRONMENTAL TOXIN, DISRUPT EXPRESSION OF THE NEURONAL CHLORIDE EXPORTER PROTEIN DURING EARLY BRAIN DEVELOPMENT?

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Bisphenol A (BPA), a manufactured compound found in consumer products, is known to adversely affect early brain development by disrupting normal estrogen signaling. Recently, BPA was reported to suppress expression of a gene encoding the neuron-specific chloride ion transporter, KCC2. Human and animal studies show suppressing KCC2 can cause neuronal and behavioral hyperactivity. Therefore, some adverse effects of BPA may be due to KCC2 suppression and consequent neuronal hyperactivity. This study aimed to determine whether BPA exposure during brain development alters KCC2 expression. A secondary purpose was to evaluate whether a new transgenic zebrafish line, KCC2:mCitrine, could be used to track changes in KCC2 expression in vivo by fluorescence imaging. Zebrafish embryos treated with 2.5µM BPA during days 0-5 post-fertilization were tested for (1) KCC2:mCitrine fluorescence brightness; (2) KCC2 gene expression using RT qPCR as an external validation for fluorescence; (3) Behavioral activity level, and; (4) estrogen signaling, as a BPA manipulation check. Results showed that BPA produced expected estrogenic effects in the developing brain and decreased KCC2 expression at specific developmental time points. However, BPA did not produce

expected behavioral hyperactivity. Lastly, RT-qPCR data were uninterpretable so fluorescence brightness could not be externally validated as a measure of gene expression. This pattern of results supports the conclusion that estrogenic effects of BPA can suppress KCC2 expression in developing zebrafish brain, consistent with previous research implicating BPA as a teratogen, and KCC2 expression as a mechanism -- and potential treatment target -- in human developmental disorders.

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Introduction

Each year, 1 in 54 people are diagnosed with autism spectrum disorder (Hyman et al., 2020). Epilepsy is also a common brain disorder, affecting about 0.05% of the population, or 1 in 200 people (Sander & Shorvon, 1996). Both of these neurological disorders, and others, are characterized by increased brain electrical activity, and the search for biological causes of neural hyperactivity has pointed toward dysfunctions in the γ-aminobutyric acid (GABA) neurotransmitter system, which is the main inhibitory neurotransmitter system in the brain. The following sections explain (a) how GABA's effect on neural activity depends on the regulation of chloride (Cl-) ions inside neurons; (b) how disruption of one key protein that controls neuronal Cl- levels, called KCC2, during early brain development, may lead to brain hyperactivity and neurological disorders; and (c) how a widespread industrial chemical, bisphenol-A (BPA), may disrupt KCC2 and increase the risk for disorders involving brain hyperactivity. Finally, I propose an experiment in zebrafish to test the potential of BPA to disrupt KCC2 expression during critical periods of brain development. The proposed experiment can be classified as a developmental neurotoxicology study and as part of an effort being made by multiple neuroscience researchers today to use animal models to assess the potential clinical relevance of KCC2 expression levels.

GABA effects depend on intracellular chloride concentration

The neurotransmitter, GABA, is generally thought of as an inhibitory

neurotransmitter because that is its function in most mature neurons. This fact about GABA is known to most people who understand that the action of common depressant drugs, such as alcohol and benzodiazepines, is to increase GABA effects. Such drugs inhibit the brain. However, in immature neurons, such as neurons in a fetal brain, the neurotransmitter, GABA, is excitatory. These varying effects of GABA signaling can be explained by the concentration of chloride ions (Cl-) in the neuron. GABA, acting through $GABA_A$ receptors ($GABA_AR$), serves to open Cl- ion channels in neuron membranes, but whether Cl- ions flow in or out of those channels depends on the baseline concentration of Cl- in the neurons: if baseline concentration is low, diffusion forces Cl- to flow in when channels open, and this inhibits neurons because the membrane voltage becomes more negative, but if baseline concentration starts high, Cl- ions will flow out, increasing net positive charge and electrically exciting the neuron. The key to understanding whether GABA is inhibitory or excitatory, therefore, is understanding the regulation of Cl- concentration in neurons.

Control of intracellular Cl- by Cl- transporters during development

There are two ion transporters that are constantly regulating intracellular Cl- in neurons, one that continuously pumps Cl- in and another that continuously pumps Cl- out. Both transporters are membrane-bound proteins comprised of twelve transmembrane domains that use cellular energy to actively transport ions. The Cl- importer protein is officially called the potassium-chloride cotransporter 1 (NKCC1). It is encoded by a gene called *SLC12a2* and is expressed in neurons and other tissues in the body (Ben-Ari,

2002). The Cl- exporter protein, called the potassium–chloride cotransporter 2 (KCC2), is encoded by the gene, *SLC12a5*, and is expressed only in neurons. These two proteins have antagonistic effects on intracellular Cl- concentration, so their relative level of activity determines net Cl- concentration in neurons. A major factor influencing the activity of either transporter is the amount of gene transcription (gene activation), which determines the number of copies of the protein that exist within the cell.

The major insight explaining why developing neurons switch from having GABA-induced excitation to GABA inhibition is that expression of the Cl- exporter gene is delayed relative to the Cl- importer (Barbato et al., 2010). Immature neurons predominantly express Cl- importers, leading to high intracellular Cl- concentration. When GABA signaling then occurs and opens Cl- diffusion channels, the diffusion force pushes Cl- out of the cell down its concentration gradient (Ben-Ari, 2002). The rapid outflow of negatively charged Cl- ions makes the neuron more electrically positive relative to the extracellular fluid, which is the definition of neural excitation. When excitation is sufficiently strong and the electrical charge in the neuron reaches a threshold of positive intracellular charge, many neurons generate action potentials, and in immature neurons GABA-induced Cl- efflux can be sufficient to cause action potentials. Action potentials are what conduct electrical signals long distances in the nervous system, such as from one brain area to another, so when GABA is excitatory due to high intracellular Cl- that does not affect just the neuron responding to GABA, because GABA-induced action potentials in those neurons are conducted to downstream connected brain areas. This state of heightened electrical excitability may be adaptive in immature neurons

because activity is important for brain development (Watanabe et al., 2019).

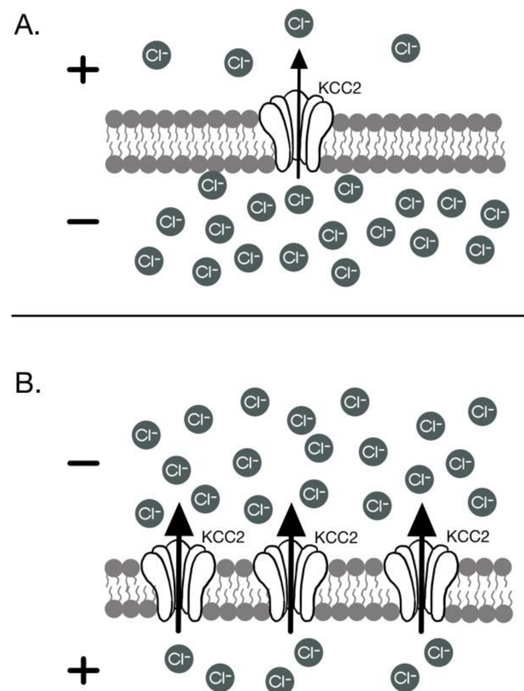
In later stages of development, neurons begin to increase expression of Cl- exporters (the KCC2 protein), and to decrease expression of Cl- importers (NKCC1), which together drive down intracellular Cl- concentration from about 20µM to about 4mM (Watanabe et al., 2019). This switches the direction of the diffusion force on Cl- so that GABA signaling induces Cl- inflow, which is inhibitory. These developmental dynamics in Cl- transporter expression (increased KCC2, decreased NKCC1) are a normal part of development, and is essential for GABA to transition from excitatory to inhibitory at the right time in development (Figure 1).

Disruption in these processes of Cl- regulation is linked to neurological symptoms (Cellot & Cherubini, 2014; Tang, 2020; Watanabe et al., 2019). For example, in human idiopathic epilepsy there is a downregulation of KCC2 (Duy et al., 2019). In mouse models, complete knockout of the KCC2 gene results in fatality immediately after birth due to respiratory complications and grievous motor dysfunctions, while less extreme deficiencies in KCC2 activity results in neural network hyperexcitability and neurobehavioral disorders including autism-like and seizure-like symptoms (Hübner et al 2001; Tang, 2020; Merner et al., 2015; Kaila et al., 2014). Increasing levels of KCC2, in contrast, can be an effective treatment for epileptic seizures and other disorders of neural hyperactivity including neuropathic pain, and Rett syndrome (Tang, 2020; Cellot & Cherubini, 2014). Rett syndrome is a neurodevelopmental disorder found in the autism spectrum disorder and these individuals suffer from seizures, intellectual disability (Tang et al., 2016), a decline in motor skills, and respiratory problems (Tang, 2020). In animal

models of Rett and autism, and in human patients, decreasing activity of the Cl- importer NKCC1 with the drug bumetanide (which has the same effect on cellular Cl- balance as increasing KCC2), can reduce autism and epilepsy symptoms (Holmes et al.,2015; Lemonnier et al., 2012). Together these findings underscore the harm of Cl- dysregulation and the potential therapeutic benefits of safe ways to control intracellular Cl- levels through Cl- transporters. This study focuses on KCC2 since it is a neuron-specific protein and therefore a reasonable suspect in distinctly neurological syndromes such as autism.

Figure 1

Ion flow in immature and mature neurons



Note. In Figure 1A: Immature neurons in the central nervous system predominantly express NKCC1, a Cl- importer, compared to KCC2, a Cl- exporter. When GABA signaling occurs, it leads to the opening of Cl- channels, leading to the diffusion of Cl- ions out of the cell down its concentration gradient. The efflux of negatively charged Cl- ions results in the neuron's intracellular space being more positive than the extracellular space. The outflow of Cl- causes the cell to depolarize and reach the threshold of excitation to send an action potential, resulting in a neural excitation. Figure 1B: Mature neurons in the central nervous system have an upregulation of KCC2 and a downregulation of NKCC1, a Cl- importer. When GABA signaling occurs, it leads to the

opening of Cl- channels, leading to an influx of negatively charged Cl- ions in the neuron. The influx of Cl- ion leads to the intracellular space being more negatively compared to the extracellular space, thus causing hyperpolarization.

Multiple functions of Cl- Exporter, KCC2, in Development

KCC2 is a protein that has multiple functions during development. In mice development, during the first two postnatal weeks, there is an increase of KCC2, a Cl- ion exporter in the hippocampus (Virtanen et al., 2021). The steep increase in KCC2 results in the GABA Cl- shift from excitatory to inhibitory but has other functions in developing neurons as well. Specifically, KCC2 is known to have an anti-apoptosis function, control aspects of neuron migration, and promote synapse formation (synaptogenesis) when neurons stop migrating and begin integrating into circuits. These alternative functions of KCC2 are briefly elaborated on below.

KCC2 has been shown to have an antiapoptotic role during prenatal development of the mouse neocortex (Virtanen et al., 2021). Apoptosis is the process of programmed cell death, which occurs in several phases during brain development as is a normal part of eliminating excess or damaged neurons. Mavrovic et al., 2020, created knockout KCC2 mice and observed a significant decrease in neurons after 2 days. This, along with supporting studies, was interpreted as suggesting that KCC2 normally protects neurons against elimination by apoptosis. KCC2 knockout in mice also altered the migration pattern of newly born neurons, suggesting that under normal conditions KCC2 upregulation is needed by neurons as a signal to stop migrating and begin forming

synapses (Virtanen et al., 2021). Lastly, KCC2 has a role in synaptogenesis. Researchers investigated whether a depletion or overexpression of KCC2 would have adverse effects on the formation of synapsis. In one study KCC2 knockdown was shown to alter the morphology of dendritic spines, which are post synapse structures that form on certain neurons (Virtanen et al., 2021).

Bisphenol A

Bisphenol A (BPA) is a ubiquitous organic compound, it has been found in consumer products such as water bottles, medical equipment, food containers (Liang et al., 2018; Yeo et al., 2013; Weber et al., 2015), thermal paper, baby bottles and dental sealants (Liang et al., 2018; Yeo et al., 2013; Xu et al., 2014). In addition, BPA was detected in meat products and canned food (Weber et al., 2015). In addition, BPA is known to be an endocrine disruptor (Xu et al., 2010, Kinch et al., 2015, Yang et al., 2018). BPA has the ability to mimic endogenous estrogen and bind to estrogen receptors (Yoon et al., 2014) as well as thyroid, androgen receptors, and estrogen-related receptors (Kinch et al., 2015). These are all nuclear receptors, meaning their effects are mediated by changes in gene expression, so the mechanism of BPA's effects, while largely still unknown, are also believed to involve changes in gene expression. Since estrogen is known to influence early development and BPA as a mimicking molecule, BPA itself would appear to have the potential to alter the development processes. In addition, estradiol is known to regulate Dickkopf 1 (Dkk1) which is known to activate and inhibit the Wnt signaling pathway. An increase in Dkk1 results in the inhibition of the Wnt

signaling pathway. However, estradiol is able to downregulate Dkk1 resulting in the activation of the Wnt signaling pathway and the transcription of β-catenin (Arevalo et al., 2015).

Since humans are constantly exposed to BPA it is essential to determine the adverse effects it has on neurodevelopment. The effects that BPA has on development are not completely understood. Researchers associated the exposure of BPA to increase dendritic spines and synapses (Yang et al., 2018; Xu et al., 2014). The result that BPA increases synapse formation seems to conflict with the finding that BPA exposure also decreases KCC2 expression in neurons (Yeo et al., 2013), because, as reviewed previously, KCC2 knockout in mice decreases (as opposed to increases) synapse formation (Virtanen et al., 2021). This discrepancy highlights the need for more research on BPA's neurodevelopmental effects.

In neurodevelopment is it important for the brain to develop normally to avoid neurological disorders. There are studies that indicate BPA exposure is harmful to fetal brains during development (Nishikawa et al., 2010). In one study, exposing mice to 100nM of BPA did not result in the elimination of the GABA Cl- shift. However, there was a delay in the GABA Cl- shift which resulted in a significant decrease in *KCC2/kcc2* mRNA expression (Yeo et al., 2013). Also, the exposure of BPA to pregnant mice resulted in a downregulation of *KCC2* mRNA in utero (Yeo et al., 2013). Moreover, BPA exposure can affect neuronal function and development by interfering with gene regulation. Another study found BPA exposure during fetal development may be harmful to the fetus. In the brain, BPA inhibits cell proliferation through inhibition of the Wnt/B-

catenin signaling pathway (Tiwari et al., 2015). In some cases, exposure to BPA has been associated with neurodevelopmental disorders (Murata & Kang, 2018).

During development, BPA has shown different effects on neurogenesis in the brain. In an *in vitro* experiment, the hippocampus was exposed to different concentrations of BPA. At 10-100mM of BPA, there was an increase in dendritic spines, spinogenesis, and synaptogenesis (Yang et al., 2018). In addition, Xu et al. 2014 found after 24 hours of exposing BPA, there was an increase in dendritic length, motility, the dendritic density of the hippocampus neuronal cultures, and an increase in N-methyl-D-aspartate (NMDA) receptors. BPA has been theorized to be associated with neuronal growth (Xu et al., 2014; Kinch et al., 2015). In addition, BPA is known to disrupt the wnt/ β-catenin pathway resulting in a significant decrease in neural stem cell proliferation and differentiation. In cultured neurons, BPA at doses lower than 10μM BPA enhances proliferation in the neural stem cells however doses higher than 100μM promote apoptosis. (Tiwari et al., 2015).

Zebrafish model organism

Zebrafish (*Danio rerio*) are commonly used as a model organism in biology research. These small tropic fish are used to study neurological disorders and human diseases. Zebrafish embryos facilitate research because they are translucent and develop rapidly. As zebrafish develop, the embryos are translucent which facilitates and enables non-invasive and detailed characterization of embryonic phenotypes (Santoriello & Zon, 2012). Besides being translucent and developing rapidly, zebrafish genes have high level

of functional conservation, having a 76% sequence overlap with the human genome (Howe et al., 2013). Consequently, zebrafish make a useful model organism for studying human diseases or neurological disorders since the zebrafish neuroanatomical and neurochemical pathways are similar to humans (Lucini et al., 2018). Lastly, zebrafish and humans have similar brain structures such as the medulla, cerebellum, and hypothalamus (Howe, 2013). In addition, the zebrafish pallium has a role in the control of sensory and motor autonomic function (Ganz et al., 2014). Furthermore, the pallium is homologous to the mammalian hippocampus, which is an area where seizures initiate (Podlasz et al., 2018). The mammalian hippocampus and the zebrafish pallium are rich in galanin protein, and a decrease in galanin protein have been found in mammals with epileptic seizure (Podlasz et al., 2018). Therefore, zebrafish can be used to research human neurological diseases (Ganz et al., 2014).

Bisphenol A has been shown to affect neurodevelopment in zebrafish. During hypothalamus development, zebrafish undergo neural differentiation between 18 hours post-fertilization (hpf) to 36 hpf and by 48 hpf most brain structures have developed (Kinch et al., 2015). BPA is known to promote neurogenesis in the hypothalamus. Kinch et al., 2015 exposed zebrafish larvae to 0.0068μM BPA from 0-5 dpf. Those that were exposed to BPA from 0-5 dpf showed significantly greater quantities of new neurons in the hypothalamus compared to the control group. In another experiment, a low dose exposure of BPA (0.01μM), in line with environmental exposure levels, led to learning deficits in adult zebrafish and hyperactivity in larval zebrafish (Saili et al., 2012). The fact that BPA affects zebrafish brain development and behavior suggests that zebrafish

can be useful in modeling the effects of BPA on these processes in humans. Specifically, studies in zebrafish could reveal whether any of these effects of BPA are mediated through effects on the Cl- exporter, KCC2.

Galanopoulou & Moshé 2003, investigated whether KCC2mRNA is regulated by sex hormones, which is relevant to BPA since it is an estrogen mimicking compound. The researchers analyzed substantia nigra reticulate (SNR) neurons in male and female rodents on postnatal day 15 (PN15). The results showed male PN15 rodents had an increase in KCC2mRNA expression after being exposed to testosterone and dihydrotestosterone (Galanopoulou & Moshé 2003). Interestingly, females also demonstrated a significant increase in KCC2mRNA expression after being exposed to testosterone. However, when male and female PN15 rodents were exposed to 17 β-Estradiol there was a downregulation of KCC2mRNA expression in SNR in males compared to females (Galanopoulou & Moshé 2003). The sex difference that occurred is hypothesized to be due to the sexual dimorphic $GABA_A$ receptor signing pathway (Galanopoulou & Moshé 2003). In this current study, larval zebrafish will be used to examine the effects of BPA on KCC2 expression. Since there is no major chromosomal sex-determining locus in the zebrafish genome, sex of larvae cannot be determined and these sex effects on the regulation of KCC2, while clearly important in rodents, will not be examined in the current study (Kossack & Draper, 2019).

Statement of the Problem

Bisphenol A (BPA) is a widespread toxin that may disrupt neurodevelopment processes normally controlled by estrogen. Potassium-chloride cotransporter 2 (KCC2) has an essential role in early development. KCC2 is a neuron-specific Cl- ion exporter that strongly influences the electrical excitability of neurons, with more KCC2 associated with more inhibition, and suppression of KCC2 associated with brain hyperexcitability and neurological disorders. Since estrogen has been shown to suppress KCC2 gene expression, it is possible that BPA can also suppress KCC2 and contributes to the risk for disorders of brain hyperexcitability including autism and epilepsy. Therefore, it is important to determine whether BPA affects KCC2 expression. The basic experimental strategy was to expose zebrafish embryos to BPA and measure KCC2 expression. KCC2 expression will be measured in two ways. One way is a novel transgenic zebrafish line (KKC2:mCitrine) in which a fluorescent protein (mCitrine) is expressed under the control of the KCC2 gene promotor, theoretically allowing KCC2 expression to be measured as fluorescence brightness using a fluorescence microscope. However, since that method of assessing KCC2 expression is not validated, reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) will be used as a secondary method of assessing KCC2 expression level.

In addition, BPA has been associated with anxiety-like behavior and epilepsy and this experiment also sought to examine locomotor activity. If BPA treated zebrafish embryos had a significant decrease in KCC2 expression then it would result in an

inhibitory/excitatory imbalance, leading to spontaneous locomotor activity (i.e. hyperactivity bursts). The spontaneous locomotor activity would be indicative of anxiety-like behavior. A behavioral assay was conducted to demonstrate whether BPA exposure during early development would result in spontaneous locomotor activity due to the decrease of KCC2.

A positive control was used in this experiment. Estrogen has been known to regulate several developmental processes by estrogen receptors (ERs), which regulate transcription factors that regulate CYP19a1b. BPA has been thought to have epileptogenic suppression of KCC2mRNA by its ability to weakly mimic estrogen. Therefore, by using exposing BPA to a transgenic CYP19a1b zebrafish line there would result in an increase in CYP19a1b.

Hypotheses

Hypothesis 1: Zebrafish embryos exposed to 2.5µM BPA from 8 hours post-fertilization (hpf) – 6 days post-fertilization (dpf) will significantly decrease KCC2-linked fluorescence brightness in neurons, especially in the pallium and the whole brain in KCC2:mCitrine zebrafish, compared to sibling control larvae raised in regular water.

Hypothesis 2: A secondary measurement, RT-qPCR, was used to validate transgenic fluorescence measurement. Zebrafish embryos exposed to 2.5µM BPA from 8hpf– 6dpf will show a decrease in KCC2mRNA expression compared to their sibling control.

Hypothesis 3: Zebrafish KCC2:mCitrine transgenic line embryos exposed to 2.5µM BPA from 8hpf – 6dpf will demonstrate significantly higher behavioral activity at 7dpf compared to their control siblings' larvae raised in water.

Hypothesis 4: Zebrafish embryos treated with 2.5µM BPA from 5hpf – 7dpf will increase CYP19a1b (brain aromatase) enzyme expression in neurons compared to their sibling control.

Rationale for Hypothesis 1-4. Previous studies have shown that this BPA exposure protocol in zebrafish (equivalent dose and exposure timing) affects the rate of neurogenesis in the hypothalamus and increases locomotor activity levels in larval

zebrafish. BPA has also been shown to directly suppress KCC2 expression in cultured neurons and rodent brains. Therefore, it is plausible that one of the effects of BPA in developing zebrafish brain is the suppression of KCC2 expression. Low levels of KCC2 expression is associated with neurological disorders in humans, so it is important to understand factors that could suppress KCC2. BPA is potentially one such factor. It is an industrial waste product present in the environment and in human bodies. Although toxic effects of BPA in humans has been of concern for many years, KCC2 suppression is a recent hypothesis for the mechanism of BPA toxicity. Our lab recently generated a transgenic zebrafish with fluorescence in neurons linked to KCC2 expression. This is a novel and unvalidated method for measuring dynamic changes in KCC2 expression during development. Therefore, a secondary method for measuring KCC2 expression is proposed, RT-qPCR, which measures KCC2mRNA copy and should be sensitive to any BPA-induced changes in expression level. This study is thus designed both to measure BPA effects on KCC2 expression and to validate our new transgenic fluorescence-based readout of KCC2 expression. Lastly, estrogen has been known to regulate several developmental processes by ERs, which regulate CYP19a1b. In addition, CYP19a1b has been known to be sensitive to estrogen levels.

Methods

Subjects

This study was conducted under the approval of the Institution Animal are and Use Committee (IACUC) protocol. Adult male and female KCC2:mCritrine zebrafish were placed in a tank for mating in order to collect embryos for the study.

Drug Dosage

The treatment solutions were dissolved in egg water and pH'd to ~7.2. There were two solutions, BPA at 2.5µM and control. Since BPA requires 0.01% of the solvent, dimethyl sulfate (DMSO), to go into aqueous solution, the control solution also contained 0.01% DMSO.

Experimental Design

Adult zebrafish KCC2:mCitrine females and males were removed from their home tanks and placed into a 1-liter mating tank. The mating tank contained a plastic divider separating female and male KCC2:mCitrine zebrafish. On the next day, the plastic divider was removed, and then I checked every 30-minute interval for fertilized eggs. All 8hpf fertilized eggs were removed and rinsed using a suction pipette and placed into petri dishes. Each petri dish contained 20mL of the treatment solution (2.5µM BPA

or 0.01% DMSO) and a maximum of 50 larvae. Afterward, the petri dish containing the larvae was placed in an incubator at 27 degrees Celsius, developing for 20 hours.

Every day the treatment solution was replaced, and dead embryos were removed and discarded using a suction pipette. The same embryos were measured repeatedly over time for a within subject analysis. Individual embryos were separated in separate wells of a 24well tissue culture plate and returned to the same well after each imaging point, allowing tracking of the same individual embryos over time. The imaging setting for the epifluorescence camera and confocal were kept constant across brains within each time point.

BPA effects on KCC2:mCitrine expression

KCC2:mCitrine embryos ($N = 48$) were randomly selected from the clutch. BPA (n = 24) and control (n = 24) zebrafish embryos were placed in two separate 24well tissue culture plates. Starting at 2dpf, fluorescence scans were taken to capture KCC2-linked mCitrine fluorescence from neurons. At 2dpf and 4dpf each embryo was placed on a coverslip for epifluorescence imaging. The embryos were positioned on the ventral side to capture a ventral view of the brain using the epifluorescence camera. The 2dpf embryos ($N = 48$) were not anesthetized because they were still and easy to manipulate their orientation to capture an image. At 3dpf, a control zebrafish embryo died so it was not used for imaging. At 4dpf, the control ($n = 23$) and BPA ($n = 24$) treated embryos had already hatched from their egg and had to be anesthetized to capture a still image. All

images captured at 2dpf and 4dpf were in 8bit grayscale with pixel brightness intensity ranging from 0-255.

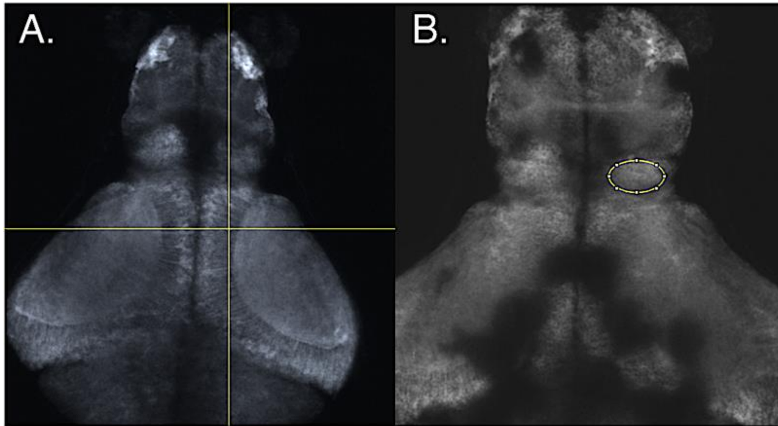
A BPA treated larvae died, so no data was obtained from the larvae. At 6dpf, the BPA (n = 23) and control (n = 23) larvae were anesthetized and embedded in agar for confocal imaging. Confocal images were captured in 16bit format with pixel brightness intensity ranging from 0-65,536. A series of 31 images were obtained through the dorsal-ventral axis of the brain, with 8µm steps between images. The start point of each depth series, at the dorsal surface of the brain, was determined by getting a clear view of the skin pigmentation on the zebrafish's dorsal surface. Individual embryos underwent ~20min of fluorescence imaging before being returned to their home dish of egg water in the incubator. After completing planned imaging time points, embryos were euthanized by rapid chilling in accordance with the IACUC-approved procedures.

The epifluorescence and confocal images were analyzed using ImageJ to determine the intensity of KCC2:mCitrine fluorescence. The confocal images captured at 16bit were transformed to 8bit to allow direct comparison to the 8bit epifluorescence images. Furthermore, slices 3-31 were used from the confocal images because they captured the whole brain. Then the images were cropped and rotated so that the sagittal midline lined up as straight as possible (Figure 2A). Then a montage (Figure 3) was made to aid in determining a region of interest (ROI) from which to quantify fluorescence brightness. After looking at the montage, it showed that the ROI should be around the pallium because it was free of pigment and visible in all specimens. An ROI was created and placed above the horizontal sulcus and most lateral on the right side to capture the

pallium (Figure 2B). The left pallium was used when the pigment was covering the right side of the pallium. In addition, another ROI was created to capture the whole zebrafish brain. ROI size and shape were kept consistent across brains. Lastly, attempts were made to adjust image brightness to normalize background brightness across specimens, but it was determined that this correction did not affect the outcome of statistical tests, so that effort was dropped and only data from uncorrected images are presented.

Figure 2

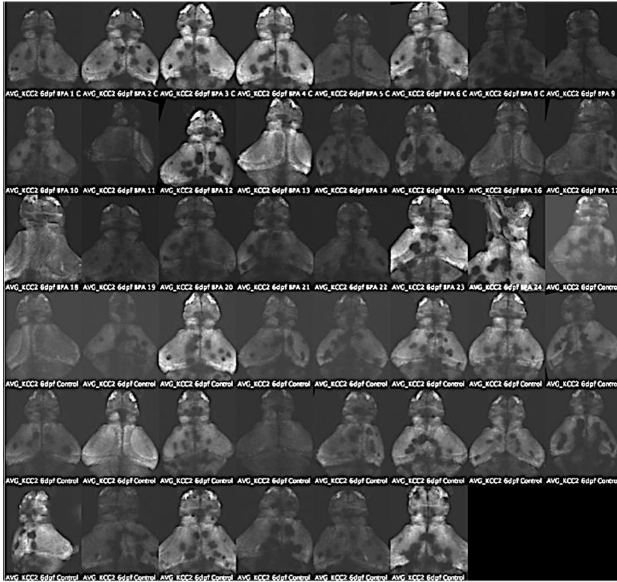
A whole brain confocal image at 6dpf zebrafish larva



Note. Each image is a projection of 29 images taken through the dorsal-ventral axis of the brain. Figure 2A demonstrates how the images that were obtained were cropped, rotated, and lined up. Figure 2B shows one example of an ROI that was created to capture the pallium.

Figure 3

A 6dpf zebrafish brain montage



Note. The confocal images were used to create a montage. The images were captures at 6dpf for BPA treated and control group.

RNA extraction and RT-qPCR

A secondary method for measuring KCC2 gene expression level was RT-qPCR, which measures KCC2mRNA copy number and was expected to be sensitive to any BPA-induced changes in expression level. Embryos were collected and treated as described above for fluorescence measurements. Following BPA or control treatment, embryos were euthanized by rapid chilling in ice, and processed in the RT-qPCR procedure, which is outlined in detail in the QuantiNova SYBR Green RT-PCR protocol. RT-qPCR determines the starting amount of mRNA of a target transcript (KCC2 in our

case) by measuring how many DNA amplification cycles (temperature cycles in which DNA is copied/amplified once each cycle) are required to reach a set amplification level, a measurement referred to as the Cq index. Two standard baseline zebrafish genes were used, elf-alpha and rpl13, which, as controls, were expected to have similar levels of amplification across conditions. In addition, primers were designed to target and amplify the KCC2b gene. In this experiment, the Quick-RNA Miniprep Kit was used for RNA extraction, and the QuantiNova SYBR Green RT-PCR kit for RT-qPCR.

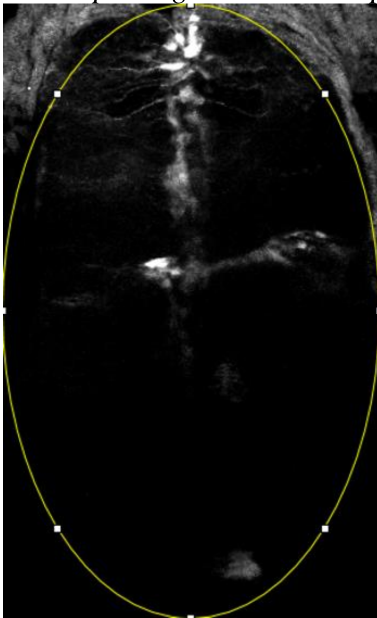
BPA effects on CYP 19 expression

CYP19a1b:GFP embryos ($N = 24$) were randomly selected for this positive control experiment. CYP19a1b:GFP embryos ($n = 12$) were collected and treated with BPA as described above for KCC2:mCitrine embryos used in fluorescence measurements. A BPA treated larvae died, so it was not used for imaging. At 7dpf, CYP19a1b larvae were anesthetized and embedded in agar for confocal imaging. The confocal setting was set at 16bit and the depth stack captured 31 slices at every 5µm moving dorsal to ventral. The start point was determined by getting a clear view of the skin pigmentation on the zebrafish's dorsal surface. Eleven BPA and twelve control zebrafish larvae underwent imaging. After all the images were collected, slices 4-31 were used because they captured most of the green fluorescent protein (GFP) expression from the brain. The slices were combined using the maximum brightness projection method to make a single image which was then cropped and rotated to normalize orientation across

brains. An ROI was created (seen in Figure 4) to capture the midline because that was where CYP19a1b:GFP was highly expressed.

Figure 4

ROI capturing CYP19a1b expression at 7dpf



Note. This is a maximum projection of 27 confocal images through the dorsal-ventral axis of the brain showing CYP19a1b:GFP fluorescence in the brains of 7dpf zebrafish larvae.

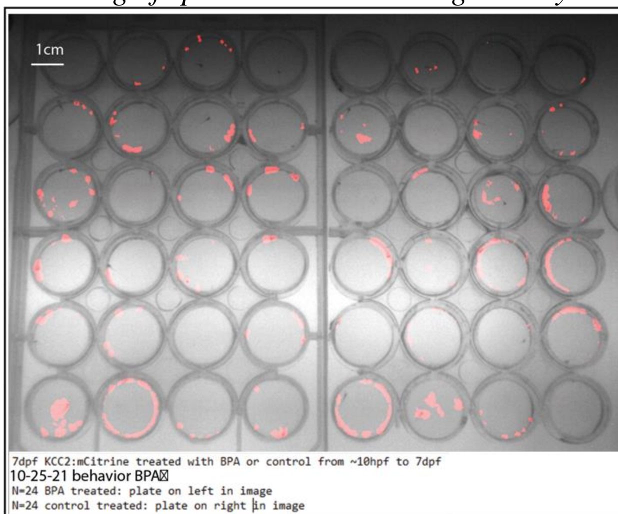
BPA effects on CYP 19 locomotor activity

At 7dpf, the BPA treated (n = 24) and control (n = 24) zebrafish were used in a behavioral assay to measure locomotor activity. In this locomotor behavioral assay, 24 BPA treated and 24 control zebrafish were placed in two 24 well plates (see Figure 5). The camera was positioned to capture both plates and two lightbulbs were placed on each

side for even illumination of the imaging field. DAQFactory was used to create a series of codes to control the camera. The recording block duration was set at 300sec (5mins), at 1 second per frame, then stop recording for 3300sec (55min). The series of codes was set to record for a total of 1,800 seconds (30 minutes) for a span of 6 hours. The goal was to capture a representative sample of behavioral activity by recording for 5 minutes per hour for 6 hours. After the completion of the 6 hours, the camera was programmed to stop recording.

Figure 5

Larvae exposed to BPA and the control solution were placed in individual wells for the recording of spontaneous swimming activity.



Note. Example of spontaneous swimming activity in 7-day old BPA-treated larvae (left plate) and control larvae (right plate). The image shows an overlay of a single unprocessed video frame in gray, and in red a projection of 300 video frames taken at a rate of 1 frame per second, spanning 5 minutes.

Results

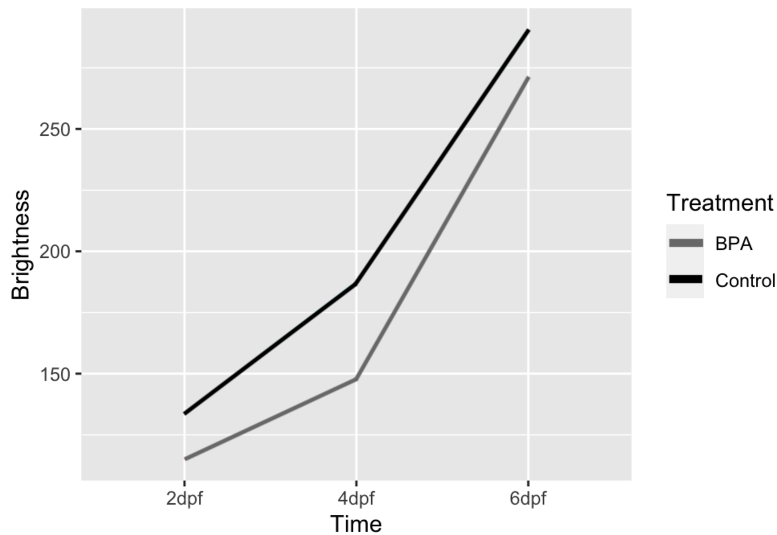
KCC2:mCitrine brightness in the pallium (Hypothesis 1)

This analysis attempted to examine whether there was a statistically significant difference in KCC2:mCitrine fluorescence intensity in the zebrafish pallium. A mixed model ANOVA was conducted to determine if there was a statistically significant difference in KCC2:mCitrine fluorescence brightness in the pallium of zebrafish embryos exposed to 2.5µM BPA versus their sibling control larvae raised in regular water with 0.01% DMSO over the course of 2, 4, and 6dpf. There was no violation in sphericity, and the results indicated there was a main effect for time (age), 2dpf (M = 124.39, SD = 34.47), 4dpf (M = 167.52, SD = 37.04), and 6dpf (M = 280.73, SD = 107.66), $F(2, 88) = 73.98$, $p < .001$, $ges = .49$. There was no main effect for BPA treatment (M = 177.90, SD = 102.13, and M = 203.85, SD = 86.09), for BPA and control groups, respectively, $F(1, 44) = 4.03$, $p = .05$, $ges = .04$. These main effects were not qualified by a significant interaction, $F(2, 88) = .39$, $p = .06$, $ges = .005$ (shown in Table 1). A series of paired t-tests were conducted and showed that at 2dpf, BPA (M = 115.10, SD = 34.74) treated embryos showed a significant difference in KCC2 brightness compared to 4dpf (M = 147.75, SD = 32.06), $t(22) = 4.1$, $p < .001$, $d = .98$. At 4dpf, BPA treated embryos showed significantly less KCC2 brightness expression compared to 6dpf (M = 270.86, SD = 126.09), $t(22) = 4.8$, $p < .001$, $d = 1.01$. When looking at the control group the paired t-tests showed a significant increase in KCC2 brightness from 2dpf (M = 133.69,

SD = 32.29) to 4dpf (*M* = 187.29, *SD* = 31.02), $t(22) = 5.2$, $p < .001$, $d = 1.08$, and from 4dpf to 6dpf (*M* = 290.60, *SD* = 87.22), $t(22) = 5.38$, $p < .001$, $d = 1.12$. In addition, a series of one-tail independent samples t-tests were conducted and demonstrated that at 2dpf BPA treated embryos (*M* = 115.10, *SD* = 34.74), had significantly less KCC2 expression than their control siblings (*M* = 133.69, *SD* = 32.29), $t(44) = 1.9$, $p = .03$, $d = 0.55$. Embryos at 4dpf treated with BPA (*M* = 147.75, *SD* = 32.06) had significantly less KCC2 brightness compared to the control (*M* = 187.29, *SD* = 31.01), $t(44) = 4.25$, $p < .001$, $d = 1.25$. However at 6dpf embryos exposed to 2.5µM BPA (*M* = 270.86, *SD* = 126.09) had no significant difference in KCC2 brightness compared to the control (*M* = 290.59, *SD* = 87.22), $t(44) = .62$, $p = .27$, $d = .18$ (Figure 6).

Figure 6

KCC2 Fluorescence Intensity in the Pallium



Note. The graph depicts that at 2dpf and 4dpf the control group had more KCC2:mCitrine fluorescence intensity in the pallium compared to the BPA treated larvae. However at 6dpf there was no significant difference in KCC2:mCitrine fluorescence intensity in the pallium between the control and BPA treated larvae. Lastly, there was a steep increase from 4dpf to 6dpf, consistent with the 'GABA switch' hypothesis of delayed upregulation of KCC2 expression.

Table 1.

KCC2 fluorescence intensity in the Pallium

Effect	***SS***	***df***	***MS***	***F***
Treatment	23242.4	1	23242.4	4.0
Time	599770.8	2	299885.4	73.9**
Treatment : Time	3189.4	2	1594.7	0.4

Note. ** p < .001. No sphericity adjustments were made. The table shows the main effects and age x treatment interaction of KCC2 fluorescence intensity in the pallium. There was no significant effect in treatment, however, there was a significant difference in time (age). These main effects were not qualified by a significant interaction.

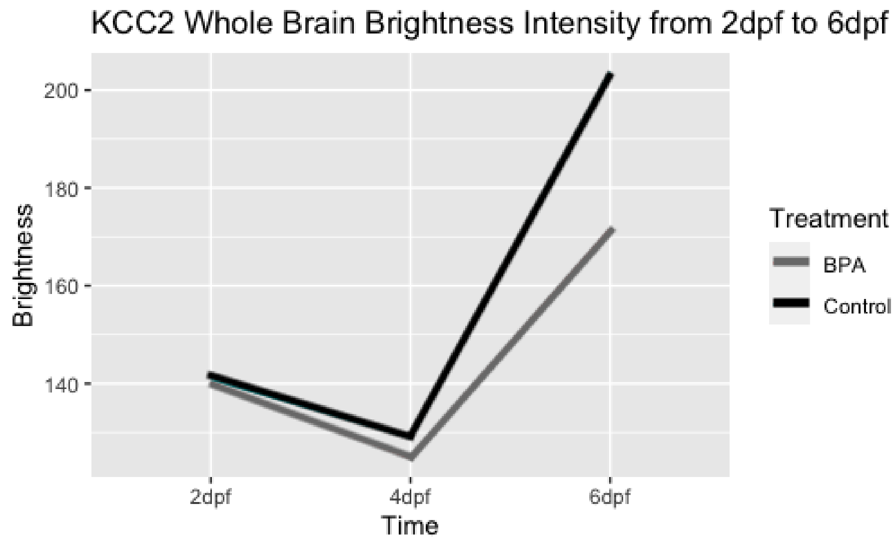
KCC2:mCitrine brightness for the whole brain (Hypothesis 1)

This second analysis used a mixed model ANOVA to examine whether KCC2:mCitrine fluorescence intensity measured across the entire brain was statistically different across ages or treatment conditions. A preliminary analysis showed there was no violation in sphericity. A mixed model ANOVA determined there were there was a statistically significant effect in treatment, BPA (M = 145.50, SD = 35.96) and control (M = 157.82, SD = 39.19), $F(1, 44) = 5.60$, $p = .02$, $ges = .05$. There was also a main effect in time (age), for 2dpf (M = 140.52, SD = 9.07), 4dpf (M = 126.88, SD = 10.99), and 6dpf (M = 187.57, SD = 45.99), $F(2, 88) = 80.40$, $p < .001$, $ges = .50$. These main effects were qualified by a statistically significant interactions, $F(2, 88) = 5.91$, $p = .003$, $ges = .07$ (shown in Table 2). A series of paired t-tests were conducted to look at KCC2:mCitrine brightness intensity for the whole brain over time for the control and BPA treated larvae.

A paired t-test showed at 2dpf, BPA (M = 140.19, SD = 9.00) treated embryos had significantly lower KCC2:mCitrine brightness compared to 4dpf (M = 124.80, SD = 11.55), $t(22) = 7.1$, $p < .001$, $d = 1.48$. When comparing 4dpf to 6dpf (M = 171.47, SD = 50.97), $t(22) = 3.1$, $p = .005$, $d = .86$ there was a significant increase in KCC2:mCitrine brightness intensity. When looking at KCC2:mCitrine brightness intensity of the whole brain over time in the control group a paired t-test showed that 2dpf (M = 140.84, SD = 9.33) had a significantly lower KCC2:mCitrine brightness compared to 4dpf (M = 128.96, SD = 10.22), $t(22) = 5.4$, $p < .001$, $d = 1.41$. Lastly, when comparing 4dpf to 6dpf (M = 203.66, SD = 34.46), $t(22) = 11.8$, $p < .001$, $d = 2.94$ there was a significant increase in brightness intensity. In addition, a series of one-tail independent samples t-tests were conducted to determine if there was a significant difference in the KCC2:mCitrine brightness for the whole brain in BPA treated embryos over time. A one-tail independent t-test at 2dpf demonstrated that BPA (M = 140.19, SD = 9.00) did not differ in KCC2 brightness intensity from the control (M = 140.84, SD = 9.33), $t(44) = .24$, $p = .41$, $d = .07$. At 4dpf there was no significant difference in KCC2 brightness intensity between BPA (M = 124.80, SD = 11.55) and control (M = 128.96, SD = 10.22), $t(44) = 1.29$, $p = .10$, $d = .38$. At 6dpf KCC2:mCitrine brightness was significantly lower in zebrafish larvae that were treated with BPA (M = 171.49, SD = 50.97) compared to the sibling controls (M = 203.66, SD = 34.46), $t(44) = 2.51$, $p = .008$, $d = .74$ (Figure 7).

Figure 7

KCC2:mCitrine Brightness for the Whole Brain from 2dpf to 6dpf



Note. A 2dpf and 4dpf there was not a significant difference in KCC2:mCitrine brightness intensity between the BPA treated zebrafish larvae and their sibling control. At 6dpf the zebrafish larvae control showed higher KCC2:mCitrine brightness intensity compared to the BPA treated larvae.

Table 2

KCC2 fluorescence intensity for the whole larvae brain

Effect	***SS***	***df***	***MS***	***F***
Treatment	5239.4	1	5239.4	5.6*
Time	93288.2	2	46644.1	80.4**
Treatment : Time	6860.3	2	3430.2	5.9*

Note. * $p < .05$, ** $p < .001$. No sphericity adjustments were made. The table shows the main effects and age x treatment interaction of KCC2 fluorescence intensity in the whole brain. The exposure of BPA did decrease KCC2:mCitrine brightness intensity compared to the BPA group over time. There was a significant main effect in treatment, time (age), and treatment x time (age) interaction.

RT- qPCR, quantifying KCC2mRNA (Hypothesis 2)

Two runs of the RT-qPCR experiment were conducted, on 2/26/2022 and 3/16/2022. Unfortunately, the data to date are equivocal. The primary measurement in RT-qPCR is called a cQ value, which is a measure of how much target cDNA has been amplified, which is dependent on the starting amount of mRNA in the sample from which the cDNA was made. cQ values, as a measure of the starting amount of target mRNA, is an estimate of the level of transcription of the gene of interest. I was primarily interested in the amount of KCC2b mRNA. However, cQ values for target genes must be interpreted relative to a baseline gene that is expected to have stable expression regardless of the experimental manipulation (in this case, BPA exposure). I used two standard baseline genes for zebrafish, *elf-alpha* and *rpl13*, which, as controls, were expected to show similar levels of amplification across conditions. On the first attempt,

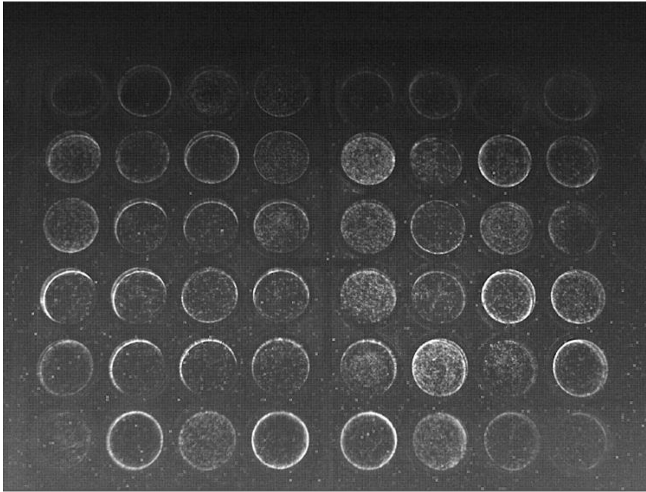
only 4 out of 80 reaction wells that were expected to show amplification did, meaning the PCR machine did not produce a cQ value for 76 samples. This included 4 baseline gene samples that were expected to amplify, strongly suggesting technical problems with the experiment. The suspected problem was an insufficient concentration of RNA in the original sample because nanodrop readings of RNA concentration showed marginal concentrations (below 90ng/ml) for some samples. In the second run, a different RNA extraction procedure was used, and higher RNA concentrations were recorded (all above 90ng/ml). However, no amplification was detected in any of the PCR reactions, suggesting a different technical problem. Therefore, no conclusions can be drawn from these RT-qPCR experiments.

BPA effects on locomotor activity (Hypothesis 3)

A stacked image capturing locomotor activity was obtained across 6 hours, with 5 minutes sampled per hour using a video rate of one frame per second (shown in Figure 8). A one-tailed Independent Samples T-test was conducted to determine if zebrafish embryos exposed to 2.5 µM BPA showed the expected increase in spontaneous locomotor activity compared to the control group. The analysis indicated BPA treated zebrafish (M = .36, SD = .18) did not significantly differ in locomotor activity compared to their control siblings raised in 0.01% DMSO (M = .42, SD = .22), t = .98, p = .16, d = .28 (Figure 9).

Figure 8

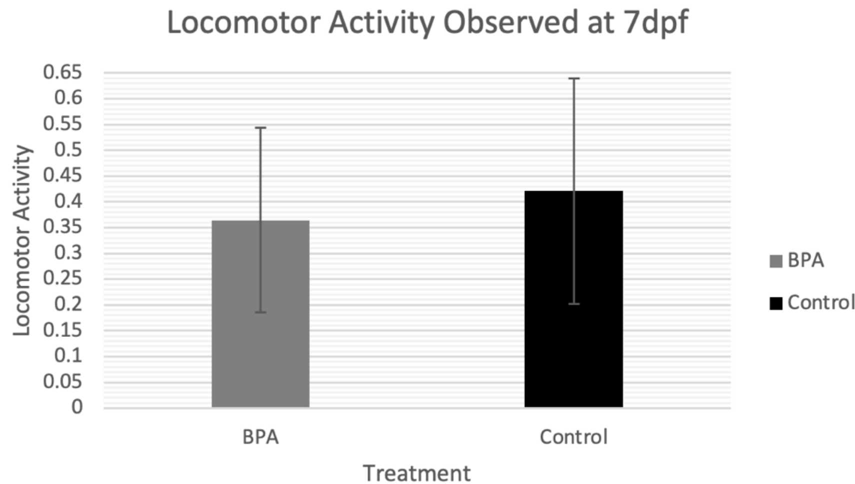
A Stack image capturing locomotor activity



Note. The stack image shows the locomotor activity across 6 hours, with 5 minutes sampled per hour using a video rate of one frame per second. Wells in the left 4 columns contain BPA-treated larvae and the right 4 columns are the controls.

Figure 9

Locomotor activity at 7dpf in BPA and control zebrafish larvae.



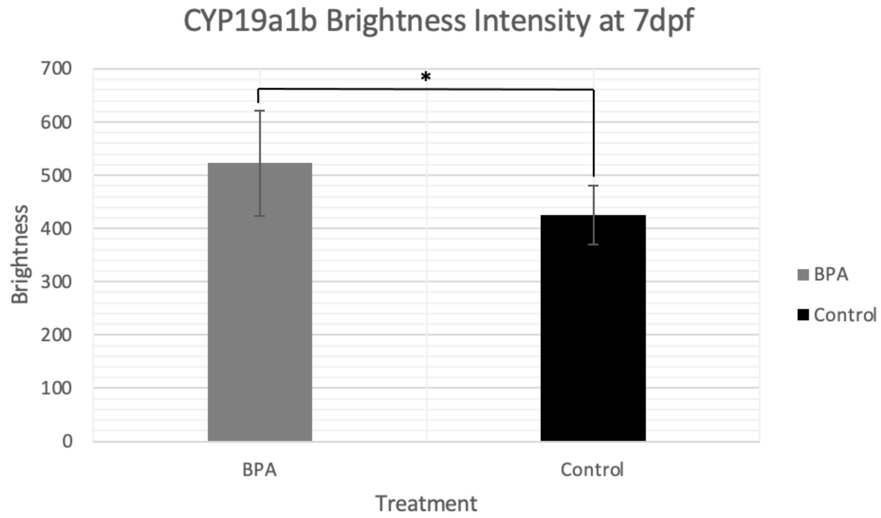
Note. Zebrafish larvae treated with BPA from 8hpf to 7dpf or with 0.01% DMSO (control group) had no difference in locomotor activity that is indicative of hyperactivity. Error bars show the standard deviation of the mean.

BPA effects on CYP 19 expression (Hypothesis 4)

A one-tailed independent t-test was conducted to determine if there was a statistically significant brightness intensity increase in CYP19a1b:GFP after BPA treatment compared to the control. Zebrafish embryos exposed to BPA ($M = 522.99$, $S = 99.09$) had a statistically significant higher CYP19a1b fluorescence intensity compared to their sibling control ($M = 425.62$, $S = 55.32$), $t = 2.94$, $p = .004$, $d = 1.23$. In other words, zebrafish exposed to 2.5µM BPA showed more fluorescence indicating more CYP19a1b compared to their sibling control raised in water (Figure 10).

Figure 10

CYP19a1b brightness intensity observed at 7dpf



Note. At 7dpf, there was higher CYP19a1b brightness indicating more CYP19a1b expression in zebrafish larvae treated with BPA as opposed to the control group.

Discussion

Bisphenol A is widely found in various customer products. BPA acts as a weak estrogen analog and therefore it is able to mimic estrogen's actions on receptors. Since estrogen influences early brain development, this study attempted to determine if exposure to BPA would downregulate KCC2 expression in zebrafish as shown previously in cell culture (Yeo et al., 2013). A downregulation in KCC2 has been associated with hyperexcitability that is seen in patients with autism spectrum disorder and epilepsy. These neurological disorders, and others, are characterized by increased synchronous electrical activity in the brain. Epilepsy affects 1 in 200 people and about 70% of them have no known cause (Sander & Shorvon, 1996; Fuller et al., 2018), raising the possibility that KCC2 dysregulation could explain some cases of idiopathic disease.

BPA effects on KCC2 expression during development (Hypothesis 1)

The pallium is a part of the zebrafish telencephalon. The pallium has been implicated to play a role in epilepsy due to the pallium having a role in the control of sensory and motor autonomic function (Ganz et al., 2014). A previous study found the *zfhx3* gene to be implicated in epilepsy and from 2dpf to 3dpf zebrafish have high amounts of *zfhx3* expressed in the pallium (Fuller et al., 2018). A downregulation in the *zfhx3* resulted in seizure sensitivity. In this current experiment, zebrafish embryos were exposed to 2.5µM BPA from 8hpf to 6dpf to determine if there was a decrease in KCC2 brightness which would indicate a downregulation in KCC2. The epifluorescence images

taken at 2dpf and 4dpf show there was a decrease in KCC2 brightness intensity compared to the control. However, at 6dpf the noticeable differences in KCC2 brightness intensity between the BPA treated and their sibling controls were gone. This indicates the pallium might be susceptible to BPA at an early stage of development as opposed to a later stage in development. The gradual increase in KCC2 fluorescence brightness seen from 2dpf to 6dpf in BPA and control groups is consistent with the GABA switch hypothesis of a delayed upregulation of KCC2 expression. A more noticeable steep increase in KCC2 fluorescence brightness was seen from 4dpf to 6dpf. These results are consistent with the literature. Zhang and colleagues found that in zebrafish retinal ganglion cells the GABA excitatory/inhibitory switch takes place at 2.5dpf (2010). The steep increase in KCC2 fluorescence was therefore likely a result of upregulation in KCC2. Although there were no noticeable differences at 6dpf between BPA and control, it indicates that BPA has an effect only during the early stages of development and pallial neurons are able to recover from BPA exposure.

BPA exposure was also shown to decrease KCC2:mCitrine fluorescence when analyzing the whole brain as opposed to just the pallium, although in that analysis the effect was seen only in larvae aged 6dpf. These results are in agreement with previous studies on the epigenetic suppression BPA has on KCC2. Exposure to BPA decreased KCC2mRNA and therefore the synthesis of KCC2 protein (Yeo et al., 2013). Furthermore, a decrease in KCC2 protein led to less Cl- ions being extruded out of the cell, resulting in a Cl- imbalance and ultimately to neuronal hyper-excitability.

Unfortunately, these results based on fluorescence measurements were not able to be validated by RT-qPCR due to technical failures discussed earlier.

RT- qPCR, quantifying KCC2 mRNA (Hypothesis 2)

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) is the gold standard for measuring gene expression (Lan et al., 2009), and I set out to use this method to test the external validity of our fluorescence brightness measures. In this study, RT-qPCR was used to determine the amount of KCC2b mRNA in the BPA treated zebrafish embryos and the control. On 2/26/2022 and 3/16/2022 two RT-qPCR experiments were conducted. Unfortunately, both data were uninterpretable. In the first RT-qPCR attempt, only 4 out of 80 reaction wells that were expected to show amplification did, meaning the PCR machine did not produce a cQ value for 76 samples. A possible technical issue was an insufficient concentration of RNA in the original sample. In the second attempt, a different RNA extraction kit was used, and it produced higher RNA concentrations. However, no amplification was detected in any of the PCR reactions, suggesting there was a different technical problem. Our team consulted with the PCR kit manufacturer, Qiagen, and they agreed to send us a new kit free of charge in case our previous failure was caused by defects in the kit materials. Therefore, at this time no results for the planned PCR experiments are available. Without the external validation from RT-qPCR, the fluorescence measures cannot be relied on. There are several external variables that affect reproducibility such as the thickness of the coverslip and how the zebrafish were mounted in agar.

BPA effects on locomotor activity (Hypothesis 3)

A downregulation in KCC2 has been associated with hyperexcitability in animal models and in patients with epilepsy related to KCC2 dysfunction (Duy et al., 2019; Hübner et al., 2001; Tang, 2020; Merner et al., 2015; Kaila et al., 2014). Seizures are characterized by an increased and synchronous electrical activity in the brain due to an excitatory/inhibitory imbalance. Therefore, a locomotor behavioral assay was conducted because a decrease in KCC2 was expected to lead to an increase in spontaneous locomotor activity in zebrafish larvae exposed to BPA. The locomotor behavioral assay demonstrated that BPA-treated larvae did not exhibit spontaneous locomotor activity compared to the control even though there was a significant downregulation in KCC2 expression in the entire brain. However, the pallium has a role in the control of sensory and motor autonomic function (Ganz et al., 2014). Since there was no difference in KCC2 fluorescence in pallium compared to the control, which indicated both groups had roughly the same about of KCC2 protein. Hence, there was no difference in BPA-treated larvae when looking at locomotor activity. However, due to the fact, that the RT-qPCR was deemed equivocal there is no way to determine if the apparent difference in KCC2 expression was valid. The result obtained from the locomotor assay was in agreement with the literature. In zebrafish, neurogenesis occurs between 16hpf to 36hpf, and Kinch et al., 2015 exposed 0.1µM BPA zebrafish before and during neurogenesis occurs. Kinch and colleagues found when zebrafish were treated with a low dose of BPA, they showed an increase in locomotor activity when exposed at 16-24hpf and 24-36hpf but not when

BPA was exposed before 10-16hpf (2015). In addition, the locomotor behavior was recorded at 30 frames per second for 5 minutes in a dark setting following a resting 20-minute period of exposure to light (Saili et al., 2012). An explanation for the results in this current study is since zebrafish were exposed to BPA from 8hpf to 6dpf they were able to recover and overcome the toxic exposure of BPA. A limitation was that locomotor activity was recorded at 1 frame per second, and a possibility is that zebrafish larvae have a quick locomotor behavior that cannot be detected at 1 frame per second.

BPA effects on CYP 19 expression (Hypothesis 4)

Adult zebrafish express high levels of CYP19a1b which is encoded by the *cyp19a1b* gene. CYP19a1b is also expressed in a developing zebrafish brain and is restricted to radial glial cells. In this current experiment, the effects of BPA on CYP19a1b served as a positive control to demonstrate that BPA does penetrate the blood-brain barrier, binds to estrogen receptors regulating gene expression, and has the ability to affect early development. The results showed BPA does affect CYP19a1b expression. Zebrafish embryos exposed to BPA from 5hpf to 7dpf showed higher levels of CYP19a1b compared to their sibling control raised in water. These results indicated that BPA does pass the blood-brain barrier and due to its estrogenic properties, it is able to upregulate CYP19a1b. The data is consistent with previous studies. Mouriec et al., 2009 showed that the *cyp19a1b* gene is sensitive to estrogen and showed that *cyp19a1b* expression is upregulated by estrogen. In addition, Kinch et al., 2015 confirmed that an

upregulation of CYP19a1b expression is attributed to the metabolism of androgen to estrogen which then activates estrogen receptor binding to *cyp19a1b* regulatory factors.

Study limitation and Future directions

There were several limitations in this series of experiments. First, sample size was limited due to time and difficulty involved in preparing larvae for fluorescence imaging and in performing the imaging. Second, the RT-qPCR methods did not work, and therefore the observed changes in fluorescence could not be directly validated as reflecting changes in KCC2 gene expression. Since our KCC2:mCitrine transgenic fluorescence marker zebrafish are a novel tool, it is a priority to validate these fluorescence measurements.

Aside from RT-qPCR, another validation approach is to test whether drugs known to increase KCC2 expression also increase KCC2:mCitrine fluorescence brightness in our zebrafish line. The neurohormone, brain derived neurotropic factors (BDNF) is known to regulate KCC2 expression. BDNF has an essential role in early neurodevelopment such as neuronal growth, differentiation, and has been implicated in plasticity and synaptic function (De Felice et al., 2014). BDNF binds to its receptor Tyrosine receptor kinase B (TrkB), which activates a series of signaling pathways that regulate KCC2 expression. In immature neurons, BDNF upregulates KCC2 expression through a series of signaling cascades. In immature neurons, the BDNF-TrkB, signaling MAP/ERK (MEK) and extracellular signal-regular kinase 1/2 (ERK1/2) signaling pathway to increase Erg 4 expression (Lee-Hotta et al., 2019). An increase in the transcription factor Erg 4 leads to

an increase in KCC2 expression and protein levels (Lee-Hotta et al., 2019). Therefore, if BDNF has the potential to upregulate KCC2:mCitrine expression. Interestingly, there is also evidence that BPA can inhibit *Bdnf* transcription (Kundakovic et al., 2014; Wang et al., 2016).

Conclusions

Downregulation of KCC2 during development can disrupt neuronal Cl- regulation and lead to neurological symptoms (Cellot & Cherubini, 2014; Tang, 2020; Watanabe et al., 2019). For instance, in humans, some cases of idiopathic epilepsy result from the KCC2 dysregulation (Duy et al., 2019). Previous studies have shown, in mouse models, that complete knockout of the KCC2 gene results in fatality immediately after birth due to respiratory complications and grievous motor dysfunctions, while less extreme deficiencies in KCC2 activity result in neural network hyperexcitability and neurobehavioral disorders including autism-like and seizure-like symptoms (Hübner et al 2001; Tang, 2020; Merner et al., 2015; Kaila et al., 2014). Therefore, KCC2 upregulation, perhaps through treatment with BDNF or other factors, has the potential of being a safe way to modulate intracellular Cl- levels for therapeutic effects. The fact that KCC2 is expressed only in neurons makes this approach more promising because specific upregulation of KCC2 would avoid off-target effects on other physiological systems. For many cases of autism and epilepsy, there is no known cause and limited treatment options. Therefore, research on KCC2 should continue to determine whether the extent to which this protein relates to the etiology of human neurological disorders and whether it is a good biological target for treatment.

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Appendix

A mixed model ANOVA to examine whether KCC2:mCitrine fluorescence intensity measured across the pallium and whole brain differed over time. The R code used to analyze the data are listed below.

A mixed model ANOVA – Pallium KCC2:mCitrine brightness

```
Pallium2_6dpf$Time<ordered(Pallium2_6dpf$Time, levels=c("2dpf",
"4dpf", "6dpf"))
levels(Pallium2_6dpf$Time)
Pallium2_6dpf$Treatment<-as.factor(Pallium2_6dpf$Treatment)
levels(Pallium2_6dpf$Treatment)
Pallium2_6dpf$Brightness<-as.numeric(Pallium2_6dpf$Brightness)
library(ez)
option(contrasts=c("contr.helmert","contr.poly"))
model2<-ezANOVA(data=Pallium2_6dpf, dv=.("Brightness"),
wid=.(ID), within = .(time), between = .(Treatment), type=3,
detail=TRUE
model2
```

A mixed model ANOVA – Whole brain KCC2:mCitrine brightness

```
WholeBrain2_6dpf$Time<ordered(WholeBrain2_6dpf $Time,
levels=c("2dpf", "4dpf", "6dpf"))
levels(WholeBrain2_6dpf$Time)
WholeBrain2_6dpf$Treatment<-as.factor(WholeBrain2_6dpf$Treatment)
levels(WholeBrain2_6dpf$Treatment)
WholeBrain2_6dpf$Brightness<-
as.numeric(WholeBrain2_6dpf$Brightness)
library(ez)
option(contrasts=c("contr.helmert","contr.poly"))
model1<-ezANOVA(data= WholeBrain2_6dpf, dv=.("Brightness"),
wid=.(ID), within = .(time), between = .(Treatment), type=3,
detail=TRUE
model1
```

A one-tailed independent samples t-test was conducted to determine if zebrafish embryos exposed to 2.5 µM BPA showed the expected increase in spontaneous locomotor activity compared to controls. The R code used to conduct the one-tailed independent samples t-test, effect size, standard deviation, and mean are listed below.

```
t.test(behavioral_KCC2$Locomotor ~ behavioral_KCC2$Treatment,
var.equal = TRUE, alternative = "less")
tapply(behavioral_KCC2$Locomotor, behavioral_KCC2$Treatment,
mean)
tapply(behavioral_KCC2$Locomotor, behavioral_KCC2$Treatment, sd)
lsr::cohensD(behavioral_KCC2$ Locomotor ~
behavioral_KCC2$Treatment)
```

A one-tailed independent t-test was conducted to determine if there was a statistically significant brightness intensity increase in CYP19a1b:GFP after BPA treatment compared to the control. The R code used to conduct the one-tailed independent samples t-test, effect size, standard deviation, and mean are listed below.

```
t.test(CYP19_data$Brightness ~ CYP19_data$Treatment, var.equal =
TRUE, alternative = "greater")
tapply(CYP19_data$Brightness, CYP19_data$Treatment, mean)
tapply(CYP19_data$Brightness, CYP19_data$Treatment, sd)
lsr::cohensD(CYP19_data$Brightness ~ CYP19_data$Treatment)
```