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The Effects of Bisphenol A (BPA) on the Neural Development of the Xenopus laevis Tadpole

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Background
History of BPA:

Bisphenol A (BPA) is a chemical component of polycarbonate containers and bottles. It is also a component of coatings used in cans to protect food from direct contact with metal. Before it was used as an additive to plastics, BPA was used as a synthetic estrogen until it was found to be ineffective when compared to other synthetic estrogens. It was not until 1988 that a regulatory standard for this chemical was established. (Vogel 2008). The Federal Food and Drug Administration (FDA) concluded the estrogenic properties of BPA to be negligible. In 1993, endocrinologists of Stanford University, discovered BPA leaching from the polycarbonate flasks. What was first thought to be an endogenous estrogen, when searching for said estrogen in yeast, was actually found to be BPA. This discovery was a leading cause of BPA regaining the attention of many researchers. (Vogel 2008).

Most recently, BPA has been found to also affect the developing nervous system. For example, in mice, the prenatal exposure of BPA was found to cause hyperactivity disorder, a central nervous system malfunction (Nakamura et al., 2006), and in early development of Xenopus tadpole embryos, BPA induces microcephaly (Sone et al., 2004). Finally, BPA was found to cause eye dysplasia via inhibiting the function of gamma secretase. The Pratt lab is currently studying the role of this secretase in the development of neural circuits, and our preliminary data indicate that gamma secretase is essential for the proper development and function of the tadpole visual system (Liu et al., unpublished data).
We hypothesized, therefore, that exposure to BPA disrupts normal neural circuit development. We tested this hypothesis by carrying out a set of behavioral, imaging, electrophysiological-based experiments to characterize how BPA effects neural circuit form and function. For this, we used the Xenopus laevis tadpole retinotectal projection as our model system.

*The Xenopus tadpole model system:*

The retinotectal projection consists of the retinal ganglion cells (RGCs) in the eye, which project their axons to the brain where they synapse directly onto tectal neurons in the optic tectum (Figure 1). This is a classic model system to study neural development. The tadpole is transparent, so that the RGC axons can be imaged non-invasively and in vivo from when they exit the eye to when they form synapses onto the tectal neurons on the optic tectum. Also, because their brain is situated extremely dorsally, electrophysiological recordings from tectal cells can be readily carried out. For my project, we carried out a well-established set of experiments to test how BPA impacts the development and function of the retinotectal projection.

*Figure 1: The retinotectal projection.* Overhead view of a tadpole showing the retinal ganglion cell axons (drawn in red) projecting to the brain, crossing the midline at the optic chiasm and synapsing directly onto tectal neurons of the contralateral tectum.
Research Design

Xenopus tadpoles are obtained from natural matings of in-house adult breeder frogs. Matings are set up on a weekly basis to maintain a steady supply of tadpoles at the developmental stages that were the focus of this project (between stage 20, approximately 1-day post fertilization, to stage 49, approximately 3 weeks post fertilization). Tadpoles are reared in Steinberg’s solution, at 22 degrees C, and on a 12:12 light:dark cycle (Nieuwkoop and Faber). All of the methods described in this project have been approved by the University of Wyoming Institutional Animal Care and Use Committee (IACUC).

Determining sub-lethal concentrations of BPA

The focus was on the effects of (BPA) on the development of the visual system of the Xenopus laevis tadpole. We began by exposing groups of 10 tadpoles to 0.15µM, 1.5µM, 10µM, 15µM, 20µM, and 30µM concentrations of BPA, stages 12 to 24 post fertilization. They were reared in BPA for two weeks. After multiple groups were tested, it was concluded that concentrations at 20µM of BPA and higher caused morphological affects that made it virtually impossible to test the tadpoles. These morphological effects included abnormalities of the head, abdominal, tail, and gastro-intestinal regions were observed. Loss of pigmentation and difficulty swimming were also observed at the highest concentration. Therefore, we focused on the lower concentrations: 0.15, 1.5, 10, and 15µM.
Having established the sub-lethal concentrations of BPA, a series of experiments were carried out to characterize the effect of these low, sub-lethal concentrations of BPA on the development of the tadpole visual system, at the behavioral, circuit, and cell levels.

**Characterizing the effect of BPA at the behavioral level:**

To study the effect of BPA on visually guided behaviors, we used an established “moving dot” assay that tests visual avoidance, a behavior that requires a properly formed and functioning retinotectal circuit (Dong et al, 2009). To simulate objects a tadpole might encounter in its natural habitat, a clear container filled to a depth of one centimeter with Steinberg’s solution is placed over a flat computer screen, and the screen plays a continuous, video loop of randomly placed, moving dots for the tadpoles to avoid. We established that control tadpoles avoid 0.4cm black moving dots that are projected onto the floor of their tank with an approximate success rate of 40%. For this experiment, groups of tadpoles were placed in the container. The tadpoles were recorded for 3 minutes, at 6, 7, and 8 days post fertilization. This was done for the control tadpoles, and tadpoles that had been reared in the presence of 0.15, 1.5, 10, and 15µM concentrations of BPA. If a tadpole encountered a dot, and avoided (dodged) the dot – characterized by an abrupt change

**Figure 2:** The top picture is a tadpole encountering a dot, and avoiding the dot (abrupt change in speed and direction). The bottom picture is a tadpole failing to dodge the dot.
in swimming trajectory and an obvious acceleration in swimming speed – it was considered a successful avoidance behavior. If the tadpole did not dodge, it was considered a failure (Figure 2). To be counted as a failure to dodge, the tadpoles head must overlap with the dot in the Z-plane. For this study, a total of 5 encounters per tadpole, were scored as either a “successful dodge” or a “failure to dodge”. An average score was generated for each tadpole by averaging the 5 encounters and was expressed as a probability of a successful dodge. For example, a score of 1.0 indicates that the particular tadpole successfully dodged an approaching dot 5/5 times. The results were then averaged for each concentration group and compared to the success rate of the control group.

**Characterizing the effect of BPA at the circuit level**

We studied the effect of BPA at the circuit level by recording responses of tectal neurons to various intensities of light projected onto the retina. For this, one end of an optic fiber is placed adjacent to the tadpole’s eye, and at the other end of the optic fiber is an LED connected to a variable resistor. This allows different intensities of light to be projected onto the retina as responses are recorded from the

**Figure 3:** *Left, BPA treated and Right, control RGC axons, illuminated with Di-I, terminating in the optic tectum. Notice how the BPA axons are not as evenly spaced as they terminate in the tectum.*
tectal neurons. Proper retinotectal circuit function also requires that retinal ganglion cell axons be guided to their proper targets, the neuron of the contralateral optic tectum. The guidance of retinal ganglion axons from when they leave the eye to when they terminate at the optic tectum can be tracked by injecting a lipophilic dye (Di-I) into the eye of the tadpole. The dye is then taken up by RGC’s, enabling the visualization and imaging of the entire projection, including their termination in the optic tectum using a Zeiss 710 Confocal Microscope (Figure 3). The imaged axons of BPA tadpoles were compared to the imaged axons of control tadpoles.

**Characterizing the effect of BPA at the single cell level.**

To characterize the effect of BPA at the single cell level, whole cell electrophysiological recordings were carried out. These recordings allowed us to quantify many aspects of synaptic and intrinsic properties, which determine the neuron’s input and output functions, respectively. Intrinsic properties were quantified by measuring intrinsic excitability (the ease in which a neuron can fire action potentials). This was measured by injecting various amounts of current into the cell body and by counting the number of action potentials that are fired in

![Figure 4: Electrophysiological properties are characterized by carrying out a set of whole cell recordings. Intrinsic excitability is characterized by assessing the ability of this neuron to fire action potentials in response to a 250 msec. current injection. Shown here is an example of action potentials recorded from a control neuron.](image-url)
response (Figure 4). We also measured synaptic properties by recording spontaneous excitatory postsynaptic currents (sEPSCs). All electrophysiological recordings were carried out using an electrophysiology rig, which consists of a high powered up-right Zeiss microscope in combination with an amplifier in series with an analog to digital converter (Axon Instrument).

**Characterizing the effect of BPA using evoked responses.**

The final test we used to quantify the effect of BPA was the evoked response test. In this test, a whole brain prep was done on a 15µM tadpole and a control tadpole. Then, 5 tectal neurons were tested for each tadpole using a glass micropipette connected to the labs electrophysiological rig (as in the “single cell level” tests). We evoked retinal ganglion cell (RGC) responses of the control and 15µM tadpoles, in order to observe the amplitude of the responses (Figure 5). This test is similar to the “single cell level” test in its process, however, the evoked responses are, as the name implies “evoked,” specific to the RGCs of the retinotectal circuit. These responses can give information on whether or not the retinotectal circuit has actually been affected by BPA.
Results

At the behavioral level

Multiple groups of control tadpoles, with a sample size (n) of 42, were tested. Multiple groups of 0.15, 1.5, 10, and 15µM BPA exposed tadpoles, with an n of 13, 21, 28, and 20 respectively, were tested. The average success rate of the control was 40.48% (standard error of the mean = 0.0364). The average success rate of the 0.15µM BPA exposed tadpoles was 35.38% (standard error of the mean = 0.0722). The average success rate of the 1.5µM exposed tadpoles was 35.22% (standard error of the mean = 0.0702). The average success rate of the 10µM exposed tadpoles was 24.64% (standard error of the mean = 0.0410). The average success rate of the 15µM exposed tadpoles was 20.00% (standard error of the mean = 0.0435). These results can be seen in figure 6. These data suggest a dose responsive effect of BPA on visual guided behaviors.

At the circuit level

Like normal retinal ganglion cell axons, BPA exposed Xenopus laevis tadpole retinal ganglia axons travel from the eye to the contralateral optic tectum. However, the BPA-exposed axons appear “uncoiled” when compared to control tadpoles (Figure 3).
At the single cell level

Control tadpoles, with an n of 10, were tested and found to have an average synaptic amplitudes of -4.94 picoAmperes (pA) and an average frequency of 6.33 events/s. 15µM BPA exposed tadpoles, were tested and found to have an average synaptic amplitude of -5.4048 and an average frequency of 1.5817 events. See figure 7. Only the effect on the frequency of synaptic events was statistically significant.

Evoked Responses

A control tadpole, with an n of 5 cells, had an average amplitude of 104.31 pA. A 15µM BPA tadpole, with an n of 5 cells, had an average amplitude of 70.99 pA. These results were not significant.
Discussion

At the behavioral level there was not a significant difference between control tadpoles and the tadpoles of the 0.15 and 1.5 µM BPA concentrations, however, there was not only a significant, but a substantial differences between the success rates of control tadpoles compared to the 10µM and 15µM BPA exposed tadpoles. Overall, a trend can be seen from the control tadpole all the way to the 15µM BPA concentration exposed tadpoles.

At the single cell level, the decrease in frequency of post synaptic currents in 15µM BPA exposed tadpoles usually means there are less synapses, however, “usually” must be emphasized.

The morphological differences between the retinal ganglia axons of a control tadpole and a BPA exposed tadpole, coupled with the decrease of post synaptic currents can begin to explain the decrease in the success rate at the behavioral level. In order to explain all these results, the evoked response test was used to determine whether or not there is an actual difference in the retinotectal circuit of the tadpoles exposed to BPA.

As explained in the results section, there was not a significant difference in the average amplitudes of the evoked responses between the control tadpole and the 15µM exposed BPA tadpole. This creates an interesting story, for it is obvious that BPA has an affect on tadpoles, however, we are still yet unable to explain this difference, in fact,
it could be as simple as BPA has altered their fear response and they are unafraid of the moving dots. However, the n of the evoked response tests is low, and testing was only completed on one tadpole of control vs. one tadpole of the 15µM concentration. Therefore, we will continue to rear tadpoles in our determined concentrations and perform evoked response tests to increase our sample size. Ultimately this work will be submitted for publication at the Journal of Neurotoxicology.
Works Cited


Updated from Mark Merlino’s INBRE Fellowship Proposal and Final Update