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Assessing CaMPARI as new approach methodology for evaluating neurotoxicity

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Highlights

- New approach methodologies (NAM) can facilitate screening to identify potential neurotoxic exposures.
- Calcium-modulated photoactivatable ratiometric integrator (CaMPARI) is a NAM that can be used to examine how toxicants affect brain activity in zebrafish.
- CaMPARI experiments reveal that brain activity profiles and behavioral responses are not always concordant.
- The integration of multiple assays is essential for identifying the neural correlates of toxicant-induced brain dysfunction.

Abstract

Developmental exposure to environmental toxicants has been linked to the onset of neurological disorders and diseases. Despite substantial advances in the field of neurotoxicology, there remain significant knowledge gaps in our understanding of cellular targets and molecular mechanisms that mediate the neurotoxicological endpoints associated with exposure to both legacy contaminants and emerging contaminants of concern. Zebrafish are a powerful neurotoxicological model given their high degree sequence conservation with humans and the similarities they share with mammals in micro- and macro-level brain structures. Many zebrafish studies have effectively utilized behavioral assays to predict the neurotoxic potential of different compounds, but behavioral phenotypes are rarely able to predict the brain structures, cell types, or mechanisms affected by chemical exposures. Calcium-modulated photoactivatable ratiometric integrator

(CaMPARI), a recently developed genetically-encoded calcium indicator, undergoes a permanent green to red switch in the presence of elevated intracellular Ca^{2+} concentrations and 405-nm light, which allows for a “snapshot” of brain activity in freely-swimming larvae. To determine whether behavioral results are predictive of patterns of neuronal activity, we assessed the effects of three common neurotoxicants, ethanol, 2,2’,3,5’,6-pentachlorobiphenyl (PCB 95), and monoethylhexyl phthalate (MEHP), on both brain activity and behavior by combining the behavioral light/dark assay with CaMPARI imaging. We demonstrate that brain activity profiles and behavioral phenotypes are not always concordant and, therefore, behavior alone is not sufficient to understand how toxicant exposure affects neural development and network dynamics. We conclude that pairing behavioral assays with functional neuroimaging tools such as CaMPARI provides a more comprehensive understanding of the neurotoxic endpoints of compounds while still offering a relatively high throughput approach to toxicity testing.

Keywords
CaMPARI; neurotoxicity; zebrafish; behavior

Introduction

The developing central nervous system is vulnerable to changes in its chemical environment, including exposure to environmental toxicants and drugs (Costa et al., 2004). Developmental exposure to toxicants has been linked to the onset of various neurological and neuropsychiatric disorders in

humans, including attention deficit hyperactivity disorder, autism spectrum disorder, and Parkinson's disease (Engel and Wolff, 2013; Lein, 2015; Lyall et al., 2017; Mandy and Lai, 2016; Mattson et al, 2011; Miodovnik et al, 2011; Pessah et al., 2019; Rice and Barone, 2000; Tchounwou and Litvan, 2016). Despite the important contribution of environmental exposures to the etiology of neurological disorders and disease, our ability to identify the specific mechanisms by which toxic chemicals increase risk for disease and the neurological pathways by which exposure to toxicants leads to dysfunction are still comparatively poorly understood. This is, in part, due to the challenges associated with using conventional *in vitro* and *in vivo* systems to evaluate neurotoxicity. While *in vitro* assays are useful for evaluating preliminary toxicity, they rarely predict *in vivo* results as they are far removed from the complex physiology of the organism. *In vivo* mammalian studies allow us to better understand how exposure to toxicants impacts behavior and underlying neural mechanisms, but they are expensive, time-consuming and are typically low-throughput. Zebrafish are an increasingly popular vertebrate toxicology model due to their small size, high fecundity, and rapid, easily observable development (Busch et al., 2011; Nishimura et al., 2016; Sipes et al., 2011; Tal et al., 2020; Teraoka et al., 2003). In addition, approximately 70% of human genes have at least one zebrafish orthologue and zebrafish share many similarities in macro- and micro-level brain structures with humans (Howe et al., 2013; Kalueff et al.,

2014), making them an effective animal model for studying brain development and disorders.

Given these advantages, the zebrafish model has been increasingly used as a toxicological model with many of the current studies for assessing neurotoxicity relying heavily on behavioral assays (Garcia et al., 2016; Kalueff et al., 2013; Miller et al., 2018). While these assays offer a high throughput approach of determining how exposure to potentially neurotoxic compounds can functionally affect the nervous system, behavioral phenotypes are rarely able to accurately predict the specific brain regions or cell types affected by exposure or the mechanisms of neurotoxicity. Our ability to use behavioral assays to understand the impact of toxicant exposures on the development and function of the nervous system is complicated by the observation that different modes of action can converge on the same behavioral phenotype and neurotoxicants with the same mechanism of action do not always elicit the same behavioral response (Leuthold et al., 2019). Therefore, additional, complementary, approaches are needed to identify the brain regions and cell types affected by neurotoxicant exposures.

Functional neuroimaging studies provide researchers with the ability to identify changes in neuronal activity caused by neurotoxicant exposures. By pairing behavioral assays with neuroimaging assays, researchers can provide

a more complete understanding of how neurotoxic exposures produce functional changes in the nervous system. Existing imaging assays include functional magnetic resonance imaging (fMRI), PET, post hoc staining for immediate early genes (IEGs), calcium dyes, and genetically encoded calcium (Ca^{2+}) indicators (GECIs) (Chatterjee et al., 2015; Guzowski et al., 2005; Marciano et al., 2022; Winter et al, 2021). fMRI and PET are powerful techniques that can be used to investigate molecular and functional brain alterations, but they suffer from low throughput and high cost barriers to access the instrumentation (Marciano et al., 2022; Winter et al, 2021). IEGs are transcribed rapidly after a neuron is stimulated and activity can be detected by *in situ* hybridization or immunohistochemistry (Chatterjee et al., 2015; Guzowski et al., 2005). While this method benefits from the ability to perform measurements following naturalistic behavior, it suffers from low temporal resolution and is an indirect correlate of neural activity (Fields et al. 1997; Sheng et al., 1993). Cells can be bathes in calcium-sensitive dyes and calcium-sensitive dyes can be injected into tissues of interest, which allows for the monitoring of cellular calcium dynamics using microscopy. However, these dyes are generally less specific than GECIs because they are not under the control of a cell-type specific promoter (Russell, 2011). GECIs are engineered constructs that allow for the visualization of changes in cellular calcium and can be used in both *in vitro* and *in vivo* systems to monitor neural activity (Lin and Schnitzer, 2017). GECIs work through calcium-dependent modulation of fluorescence signals caused by conformational

changes within the GECI that are triggered by transient changes in intracellular calcium concentrations. However, due to the rapid temporal dynamics of neuronal firing, *in vivo* imaging of GECIs is limited to a small population of cells and, in mammals, requires a cranial window. Calcium-modulated photoactivatable ratiometric integrator (CaMPARI), a recently developed GECI, combines the genetic targeting of direct reporters of neural activity with the ability to permanently mark active neurons during short, user-defined windows of time (Fosque et al., 2015). CaMPARI undergoes a permanent green to red switch in the presence of elevated intracellular Ca^{2+} concentrations and 405-nm light. This allows for an "activity snapshot" of brain regions of interest in freely-swimming larvae. Given these advantages, we sought to assess the utility of CaMPARI as a neurotoxicological tool using developing zebrafish.

2. Materials and Methods

2.1 Zebrafish Husbandry

Zebrafish (*Danio rerio*) maintenance and experiments adhered to the National Institute of Health's "Guide for the Care and Use of Laboratory Animals" and were approved by the Brown University Institutional Animal Care and Use Committee. Zebrafish colonies were maintained in an aquatic housing system (Aquaneering Inc., San Diego, CA) at a temperature of 28.5 ± 2°C, and filtration, purification, automatic pH (7.0 - 7.5) and conductivity stabilization (600 - 800 μs), and ultraviolet disinfection were maintained.

Adult and larval zebrafish were housed under a 14 hour: 10 hour light-dark cycle as previously described by Westerfield (2000). The facility underwent routine pathogen monitoring including the use of semi-annual polymerase chain reaction (qPCR) panels to detect pathogens.

Adult zebrafish were separated by sex with a transparent partition and placed into 1.7L sloped spawning tanks (Techniplast, USA) 15-18 hours prior to breeding. Two hours after the onset of the light cycle, the partition was removed and zebrafish were allowed to spawn for 1 hour. Embryos were collected in approximately 30 mL of fresh egg water (60 mg/l Instant Ocean Sea Salts; Aquarium Systems, Mentor, OH) and placed into 100 mm non-treated culture petri dishes (CytoOne, Cat. No. CC7672-3394) until the time of toxicant exposure. After exposure, embryos were maintained in an incubator (Powers Scientific Inc., Pipersville, PA) at 28.5 ± 1°C with the same light cycle until 5 days post fertilization (dpf).

2.2 Toxicant Exposures

Stock solutions. All zebrafish utilized in this study were *Tg(elavl3:CaMPARI)* in an AB genetic background. Exposure concentrations for each toxicant were chosen based on previous studies that demonstrated behavioral phenotypes following toxicant exposure (Haigis et al., 2022; Tal et al., 2012; Yaghoobi et al., 2022). 2,2',3,5',6-pentachlorobiphenyl (PCB 95) (Accustandard, Cat. No. C-095N-75MG) stock solutions were prepared by dissolving PCB 95 in 100% DMSO. Stocks were diluted to 10 µM and 30 µM (0.0033 mg/mL and 0.0098

mg/mL) in 0.01% DMSO. Monoethylhexyl phthalate (MEHP) (Accustandard, Cat. No. ALR-138N) stock solutions were prepared by dissolving MEHP in 100% DMSO. Stocks were diluted to 0.2 mg/mL (0.01% DMSO) and 2.0 mg/mL (0.01% DMSO) in egg water. Ethanol was diluted to either 0.5% or 1.0% v/v (3.95 mg/mL or 7.89 mg/mL, respectively) by dissolving absolute ethanol (Fisher, Cat. No. BP2818-500) in egg water.

Exposures. Adult zebrafish were spawned for 1 hour. Embryos were collected and screened for fertilization at 4 hours post fertilization (hpf). Fertilized embryos were plated at a density of 3 per well in 24-well plates (Padilla et al., 2011). Prior to exposure, PCB 95 (10 μM, 30 μM), MEHP (0.2 mg/mL, 2.0 mg/mL), or ethanol (0.5%, 1.0%) were diluted in egg water to the appropriate final concentrations. Egg water containing 0.01% DMSO was used as a vehicle control for the PCB 95 and MEHP exposures, and egg water was used as a vehicle control for the ethanol exposures. Embryos were exposed to 2 mL of toxicant solution or vehicle control in 24 well plates at a density of 3 embryos per well. Plates were covered with parafilm to limit evaporative loss and placed in an incubator (28.5 ± 1°C) for rearing. Embryos were manually dechorionated at 24 hpf and replated at a density of 3 embryos per well in a 24-well plate. PCB 95-exposed embryos were either acutely exposed 20 minutes prior to experiments or chronically exposed from 4-120 hpf in a non-renewed solution. MEHP-exposed embryos were chronically exposed from 4-120 hpf with the solution being replenished every 24 hours with fresh MEHP. Ethanol-exposed embryos were statically exposed

from 4-24 hpf. Within each toxicant group, chemical exposures for control and experimental groups occurred concurrently and at least three technical replicates were performed on different days with different clutches of embryos. *Tg(elavl3:CaMPARI)* larval zebrafish were placed into individual wells in 24-well plates approximately 18-24 hours prior to behavioral assays or CaMPARI imaging studies (ThermoFisher, Cat. No. 144530). Wells contained either egg water or 0.01% DMSO in egg water (for acute exposures and vehicle controls) or exposure solution (for chronic exposures). Zebrafish continue to be reared in an incubator (28.5 ± 1°C) until imaging or behavioral assays were performed.

2.3 Neurotoxicity behavioral response

Behavioral assays were performed at 120 hpf. At least three technical replicates were performed with approximately 10 larvae per replicate to achieve an “n” of approximately 30 per group. Each replicate was performed with a different clutch of embryos and on a different day. Larvae were placed into the DanioVision Observation Chamber (Noldus, Wageningen, The Netherlands) and subjected to alternating light/dark cycles. Briefly, zebrafish were allowed to acclimate during a 15-minute dark cycle. Tracking began with a 5-minute light cycle (Light 1) followed by: a 5-minute dark cycle (Dark1), a 5-minute light cycle (Light 2), and a 15-minute dark cycle (Dark 2). The entire experiment lasted a total of 45 minutes. All experiments were performed during the same two-hour period that began within two hours of normal light cycle onset.

2.4 CaMPARI photoconversion and image acquisition

The CaMPARI assay was performed at 120 hpf. Three technical replicates were performed with approximately 6 larvae per group per replicate to achieve an “n” of approximately 20 per group. Each replicate was performed with a different clutch of embryos and on a different day. Individual larvae were placed into a modified 1-well dish (15 mm diameter) containing either treatment solution, egg water, or 0.1% DMSO depending on the exposure paradigm. The dish was placed onto a constructed pedestal, to decrease the distance to the light source and increase the intensity of light, inside a DanioVision Observation Chamber (Noldus, Wageningen, The Netherlands) that was adapted with optogenetics components (Prizmatix, Southland, MI). Free-swimming larvae were exposed to a 405 nm (135mW/cm^2) wavelength light for 1 minute immediately prior to imaging. Live zebrafish larvae were then anesthetized in 0.02% tricaine (MS-222) and mounted in 2% low-melting agarose (Fisher Scientific, Cat. No. BP160-100) in 35 mm glass bottom microwell dishes (MatTek, Part No. P35G-1.5-14-C). Confocal z-stacks were acquired using a Zeiss LSM 880 confocal microscope and 10x objective lens with a numerical aperture of 0.45. Image parameters were a line averaging of 4, an image size of 1024 x 1024 pixels, a step size of 3.49 μm, and pinhole of 0.75 AU for the red channel and 0.94 AU for the green channel. Imaging parameters were set during the first image acquisition for each replicate and maintained for all subsequent images. A single image took between 10 and 11 minutes to acquire. ImageJ was used to quantify

fluorescence in the whole brain (WB) and in four brain regions: the forebrain (FB), optic tectum (OT), cerebellum (CB), and hindbrain (HB).

2.5 Removing Surface Autofluorescence with SurfCut

Using ImageJ, confocal z-stack files were converted into 8-bit TIFFs and the red and green channels of each file were separated. Individual channels for each file were opened with the ImageJ macro SurfCut by following the tool's user guide (Erguvan et al., 2019). Each file was calibrated individually to account for subtle variations in head shape, skin thickness, and pigmentation pattern. The macro was set to remove only skin autofluorescence while maintaining the rest of the signal within the z-stack. The edited files were exported as maximum intensity projections.

2.6 Statistical analysis

To determine if the measured behavioral differences between toxicant-exposed and vehicle control larvae were significant, we employed both Kruskall-Wallis (KW) tests and Bonferroni-corrected pairwise Kolmogorov-Smirnof (B-KS) tests. The KW tests were used as an initial scan for stochastic dominance within the comparison sets to determine if a significant effect from the control had been observed, while the B-KS tests were used to determine which toxicant-exposed groups differed from the control. Note, the reported p-values of the corrected tests are much smaller than the traditional 95%-99% cutoff, due to the correction accommodating the increased number of tests with the same samples. While restrictive, we

justify this approach due to the need to avoid Type I errors in reporting our measured effects, thus increasing the confidence in our reported findings.

3. Results

3.1 Behavioral responses in toxicant-exposed fish

To evaluate the utility of CaMPARI as a neurotoxicological tool, three established and common neurotoxicants, PCB 95, ethanol, and MEHP, were chosen for this study. All three toxicants were previously shown to produce functional changes in the nervous system. Specifically, the selected neurotoxicants were shown to alter locomotor activity in the light/dark assay, a common early stage life behavioral assay used to assess the behavioral responses of freely-swimming zebrafish during alternating periods of light and dark (Haigis et al., 2022; Tal et al., 2012; Yaghoobi et al., 2022) (Fig. 1a).

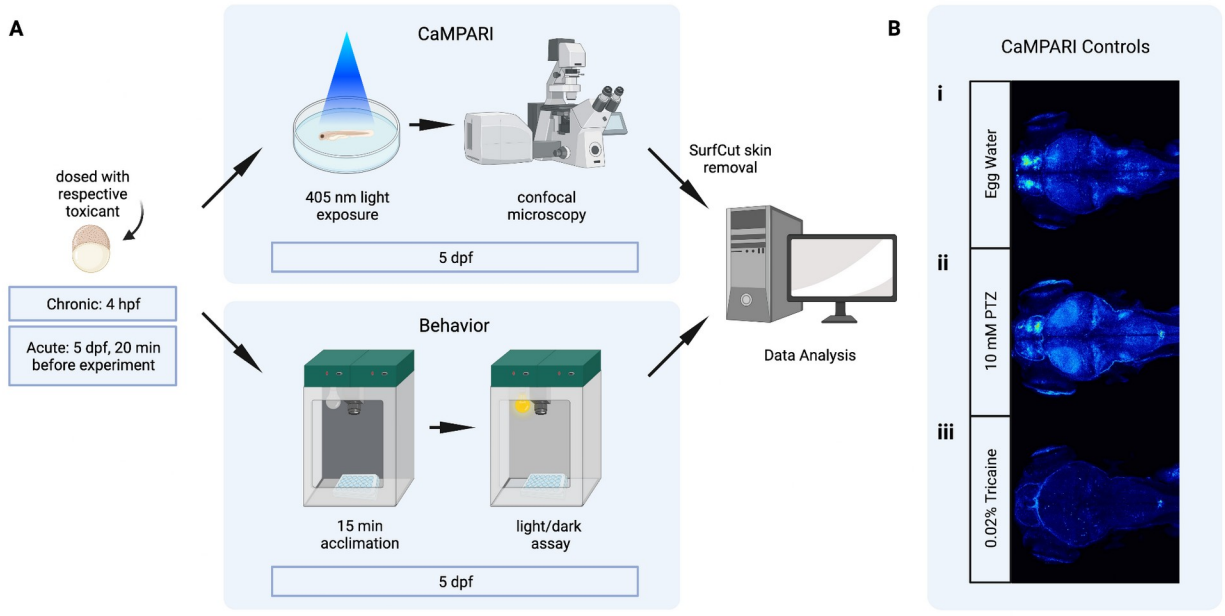

Figure 1: Overview of experimental methods and CaMPARI controls. **(A)** Schematic illustrating the overall experimental approach. **(B)** Heat map of the photoconverted channel

of 5 dpf larvae expressing neuron-specific CaMPARI (*Tg(elavl3:CaMPARI)*) exposed to **(i)** egg water, **(ii)** pentylenetetrazole (PTZ), and **(iii)** tricaine. Low to high intracellular calcium is depicted by a blue (low calcium) to white (high calcium) spectrum. Figure created with BioRender.com.

Previous reports using the light/dark assay demonstrate that zebrafish are generally more active during the dark cycles and remain relatively still during periods of light (MacPhail et al., 2009). Given that all three toxicants were reported to alter behavior in the light/dark assay, we sought to use CaMPARI as a tool to determine if behavioral responses were predictive of altered patterns of brain activity.

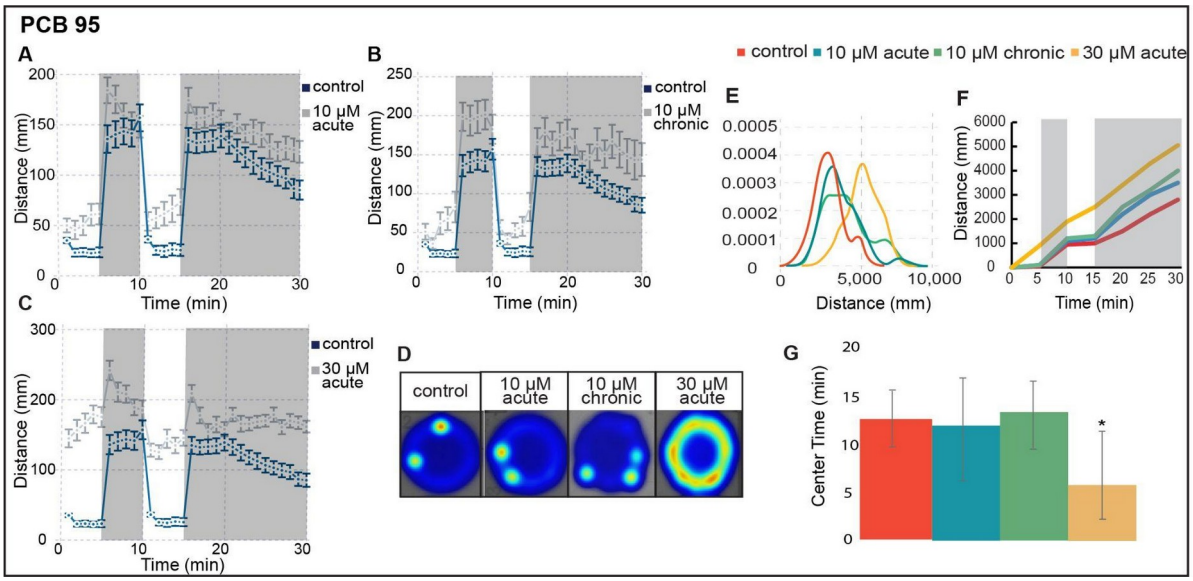

Figure 2: Behavior of PCB 95-Exposed Larvae. (A-C) Distance traveled per minute averaged across the population for **(A)** 10 µM acute, **(B)** 10 µM chronic, and **(C)** 30 µM acute PCB95-exposed larvae. (n=26-30 per group over 3 technical replicates). **(D)** Representative heat map depicting the location of larvae for the duration of the light/dark

transition assay. Low to high time spent in a given area is indicated by a blue to red spectrum. **(E)** Distribution of distance traveled by individual larvae in each exposure group. **(F)** Cumulative distance traveled versus time of PCB 95-exposed zebrafish in the light/dark transition assay. The light gray box overlays indicate the dark cycles, and the regions without boxes indicate the light cycle. The speed can be determined by the slope of the line. **(G)** Time spent in the center of the well for the duration of the light/dark transition assay.

PCB 95-exposed larvae were more active than control fish. A *post hoc* B-KS test revealed that zebrafish exposed acutely to 30 μM PCB 95 traveled a greater overall distance compared to control fish ($p < 0.01$) as well as greater distances in both the light and dark cycles ($p < 0.01$ and $p < 0.01$, respectively) (Fig. 2c, 2e, and 2f). The 30 μM acutely exposed group traveled significantly faster than the control group ($p < 0.01$) (Fig. 2f). The 30 μM acutely exposed group also lost their sensitivity to the light stimulus, as seen by constant swimming throughout the light cycles (Fig. 2c and 2f). The 10 μM acute and 10 μM chronic exposures did not affect the light cycle behavior (Fig. 2a, 2b, and 2c). While statistical analysis was performed using population means, we sought to also analyze how individual fish behavior contributed to the means. To do this, we plotted the distance traveled by each individual fish as a distribution to detect more subtle changes across toxicant exposures (Fig. 2e). While the 10 μM acute and 10 μM chronic exposures did not result in statistically significant differences in behavior, we see that the distribution of individual behavior trended towards longer distances and faster speeds when compared to controls (Fig. 2e).

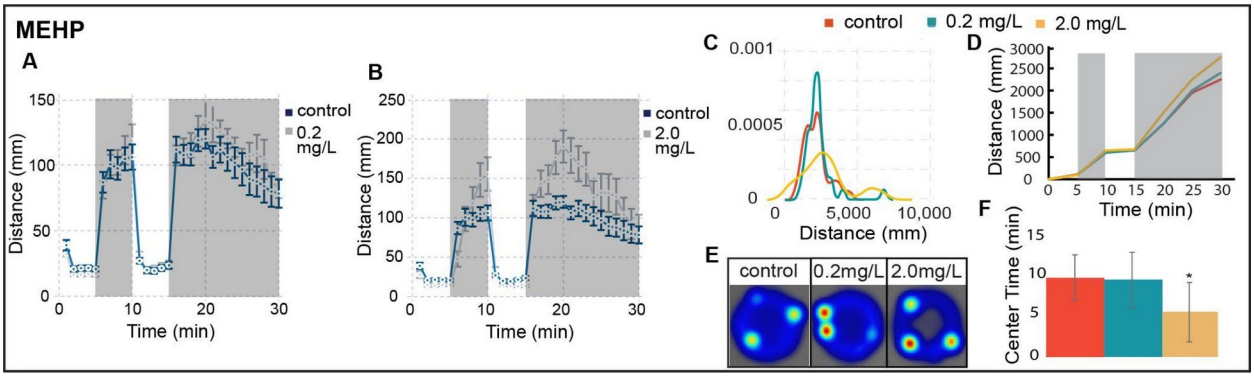

Figure 3: Behavior of MEHP-Exposed Larvae. (A-B) Distance traveled per minute averaged across the population for **(A)** 0.2 mg/L and **(B)** 2.0 mg/L MEHP-exposed larvae. (n=30-34 per group over 3 technical replicates). **(C)** Distribution of distance traveled by individual larvae in each exposure group. **(D)** Cumulative distance traveled versus time of MEHP-exposed zebrafish in the light/dark transition assay. The light gray box overlays indicate the dark cycles, and the regions without boxes indicate the light cycle. The speed can be determined by the slope of the line. **(E)** Representative heat map depicting the location of larvae for the duration of the light/dark transition assay. Low to high time spent in a given area is indicated by a blue to red spectrum. **(F)** Time spent in the center of the well for the duration of the light/dark transition assay.

Exposure to 2.0 mg/L MEHP resulted in a statistically significant decrease in activity during the light cycle compared to control ($p < 0.02$). All MEHP exposures showed normal freezing in the light cycles followed by increased movement in the dark cycles, indicating that MEHP did not affect their sensitivity to the light/dark stimulus. We also observed subtle changes in

total distance traveled in the distribution of individual behavior for both 0.2 mg/L and 2.0 mg/L despite the population means not reaching statistical significance.

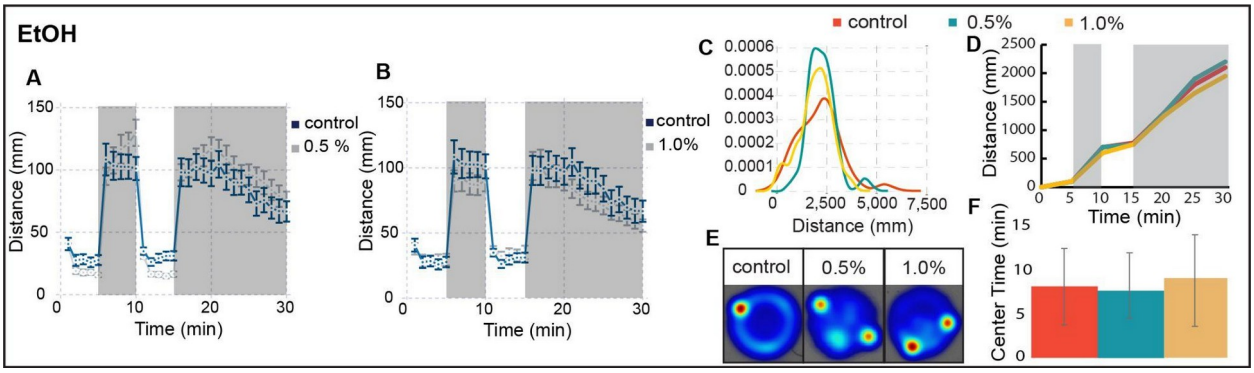

Figure 4: Behavior of Ethanol-Exposed Larvae. (A-B) Distance traveled per minute averaged across the population for **(A)** 0.5% and **(B)** 1.0% ethanol-exposed larvae. (n=29-36 per group over 3 technical replicates). **(C)** Distribution of distance traveled by individual larvae in each exposure group. **(D)** Cumulative distance traveled versus time of ethanol-exposed zebrafish in the light/dark transition assay. The light gray box overlays indicate the dark cycles, and the regions without boxes indicate the light cycle. The speed can be determined by the slope of the line. **(E)** Representative heat map depicting the location of larvae for the duration of the light/dark transition assay. Low to high time spent in a given area is indicated by a blue to red spectrum. **(F)** Time spent in the center of the well for the duration of the light/dark transition assay.

Exposure to ethanol did not significantly affect the overall distance traveled or distance traveled in light or dark cycles at either concentration tested. Similarly, the speed at which the ethanol-exposed fish traveled was not significantly affected compared to the control. All ethanol exposures also showed normal freezing in the light cycles and increased movement in the dark cycles, indicating that ethanol exposure did not affect normal sensitivity

to the light/dark stimulus. Ethanol exposure also lacked changes in total distance traveled as a function of individual behavior, as we see minimal shifting of the distribution curve for either the 0.5% ethanol or 1.0% ethanol groups.

In addition to distance traveled and speed, we assessed zebrafish preference for the center of the well versus the periphery. This assessment is similar to the mammalian open field test where center-avoidance is indicative of anxiety-like behavior, while willingness to cross the center is indicative of reduced anxiety-like behavior (Ahmad et al., 2013; Colwill and Creton, 2011; Godwin et al., 2012; Richendrfer et al., 2012a, 2012b). Zebrafish exposed to 2.0 mg/L MEHP and acutely to 30 μM PCB 95 spent significantly less time in the center of the wells during the light/dark behavior assay compared to control fish ($p < 0.01$ and $p < 0.01$, respectively). Exposure to ethanol did not significantly affect the time spent in the center of the well at either dose tested.

3.2 CaMPARI Imaging Analysis

CaMPARI undergoes an irreversible green-to-red switch upon irradiation with 405-nm light, but only when calcium levels are high and simultaneous binding of free intracellular calcium occurs (Fosque et al., 2015). As a result, inactive neurons remain green at the time of photoconversion, while active neurons convert to red. By comparing the ratio of red cells to green cells, we

were able to compare the active neuron populations across larvae (Fosque et al., 2015). Using the transgenic zebrafish line *Tg(elavl3:CaMPARI),* which drives the CaMPARI construct in all differentiated neurons, we were able to visualize the impact of toxicant exposure on neural activity at a fixed point in time. Freely-swimming zebrafish were subjected to 405-nm light for 1 minute to photoconvert the CaMPARI construct (Fig. 1a). To confirm photoconversion and CaMPARI imaging pipelines, we used 10 mM pentylenetetrazole (PTZ) and 0.02% tricaine-s solutions as positive and negative controls, respectively. Indeed, exposure to 10 mM PTZ, a $GABA_A$ receptor antagonist previously shown to induce neuronal hyperactivity and seizures in zebrafish (Mussulini et al., 2013), led to an increase in neuronal activity, while tricaine, an anesthetic agent (Stoskopf and Posner, 2008), led to a decrease in activity (Fig. 1b(ii), and 1b(iii)).

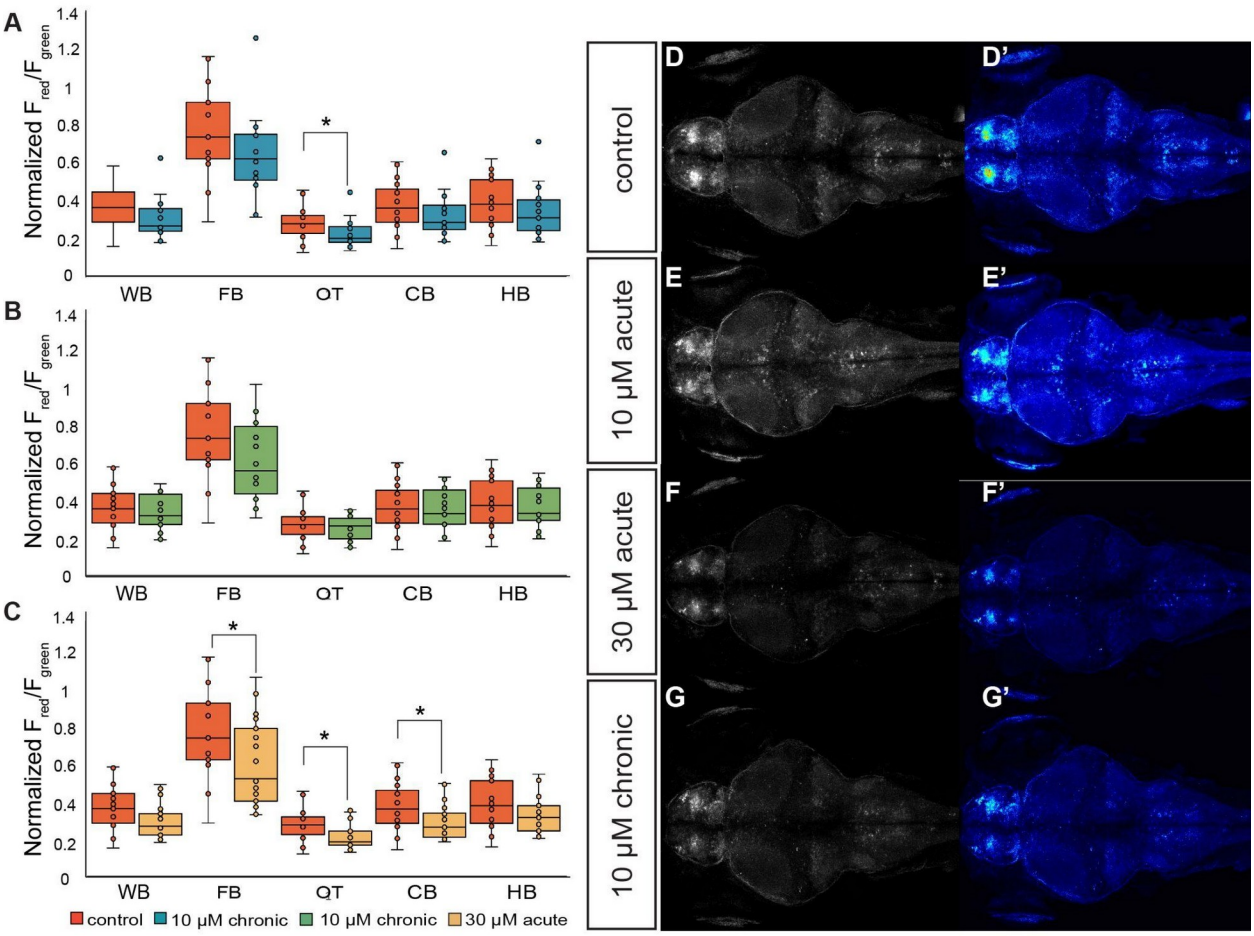

Figure 5: Neuronal Activity of PCB 95-Exposed larvae. (A-C) Quantification of neuronal activity (as observed in F-H) using the ratio of fluorescent intensity in the red vs green channel (F_{Red}/F_{Green}) for **(A)** 10 µM acute, **(B)** 10 µM chronic, and **(C)** 30 µM acute PCB 95 exposure (n=16-21 per group over 3 technical replicates). **(D-G)** Photoconverted channel of 5 dpf larvae expressing neuron-specific CaMPARI (*Tgelavl3:CaMPARI*) exposed to **(D)** control, **(E)** 10 µM acute, **(F)** 10 µM chronic, and **(G)** 30 µM acute PCB95. **(D'-G')** Heat map of the red, photoconverted channel shown in G-J. Low to high intracellular calcium is depicted by a blue to white spectrum. Micrographs of 5 dpf larvae exposed to **(D')** control, **(E')** 10 µM acute, **(F')** 10 µM chronic, and **(G')** 30 µM acute PCB 95.

Exposure to PCB 95 at different concentrations and durations resulted in differential effects on neuronal activity. Acute exposure to 10 μM PCB 95 resulted in decreased neuronal activity in all regions of the brain with a significant decrease in neuronal activity in the optic tectum ($p < 0.04$) (Fig. 5a, 5e, and 5e'). Acute exposure to 30 μM PCB 95 also resulted in a decrease in neuronal activity in all brain regions with significant decreases in the forebrain ($p < 0.05$), optic tectum ($p < 0.02$), and cerebellum ($p < 0.03$) (Fig. 5c, 5f, and 5f'). Chronic exposure to PCB 95 did not significantly alter neuronal activity, but we observed a similar, albeit not statistically significant, decrease in forebrain activity (Fig. 5b, 5g, and 5g').

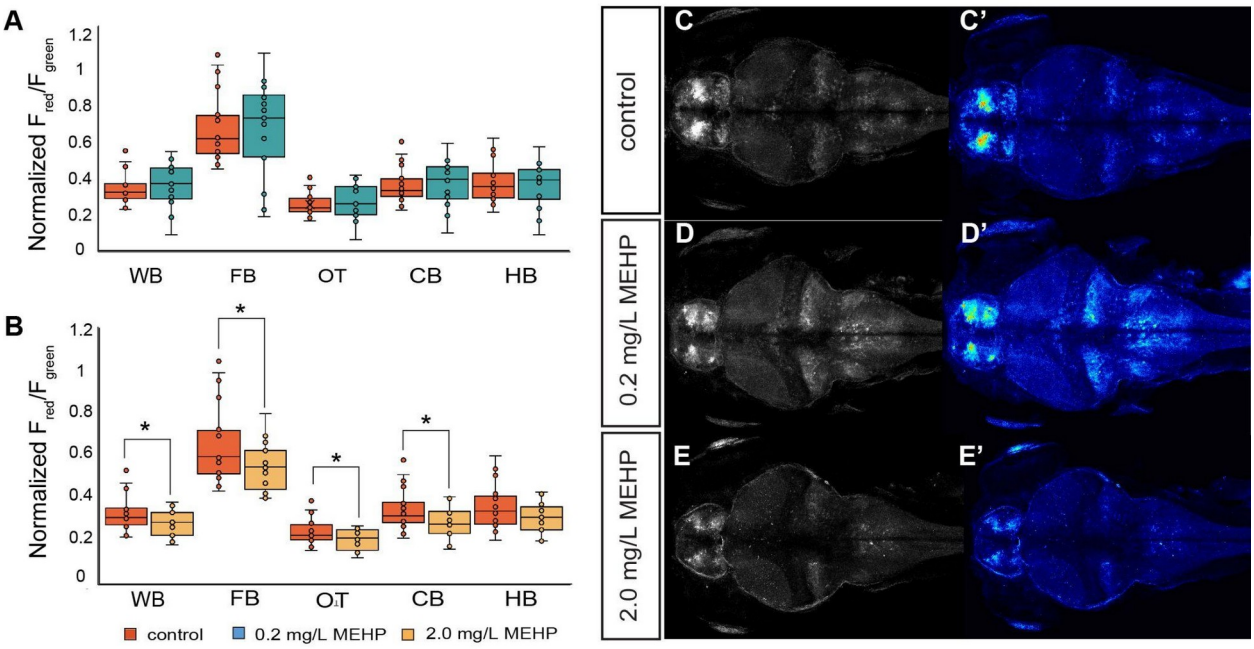

Figure 6: Neuronal Activity of MEHP-Exposed larvae. (A-B) Quantification of neuronal activity (as observed in F-H) using the ratio of fluorescent intensity in the red vs green channel (F_{Red}/F_{Green}) for **(A)** 0.2 mg/L MEHP exposure and **(B)** 2.0 mg/L MEHP exposure

(n=15-20 per group over 3 technical replicates). **(C-E)** Photoconverted channel of 5 dpf larvae expressing neuron-specific CaMPARI (*Tgelavl3:CaMPARI*) exposed to **(C)** control, **(D)** 0.2 mg/L MEHP, **(E)** and 2.0 mg/L MEHP. **(C'-E')** Heat map of the red, photoconverted channel shown in F-H. Low to high intracellular calcium is depicted by a blue to white spectrum. Micrographs of 5 dpf larvae exposed to **(C')** control, **(D')** 0.2 mg/L MEHP, and **(E')** 2.0 mg/L MEHP.

Chronic exposure to 2.0 mg/L MEHP resulted in a significant decrease in neuronal calcium activity in the forebrain ($p < 0.04$), optic tectum ($p < 0.05$), cerebellum ($p < 0.04$), and whole brain ($p < 0.05$) (Fig. 6b, 6e, and 6e'). Exposure to 0.2 mg/L MEHP showed an opposing trend of increases in activity throughout the brain, but none of the regions that were examined exhibited a statistically significant difference compared to controls (Fig. 6a, 6d, and 6d').

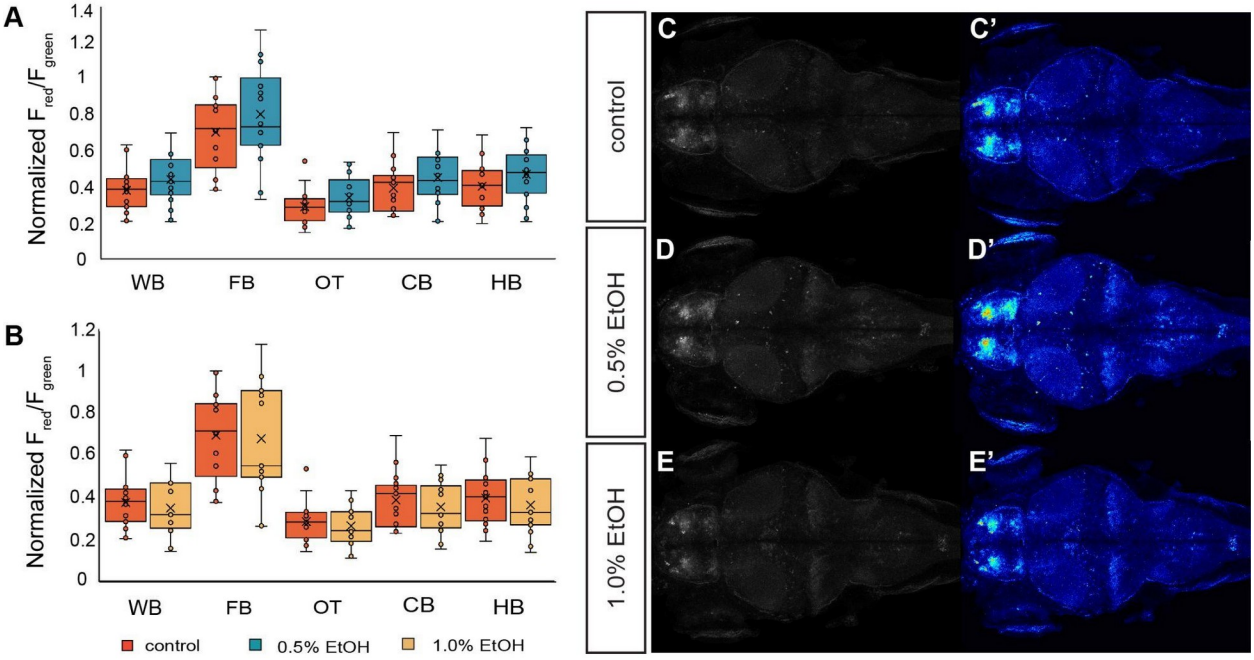

Figure 7: Neuronal Activity of Ethanol-Exposed Larvae. (A-B) Quantification of neuronal activity (as observed in C-E) using the ratio of fluorescent intensity in the red vs green channel (F_{Red}/F_{Green}) for **(A)** 0.5% ethanol exposure and **(B)** 1.0% ethanol exposure (n=15-18 per group over 3 technical replicates). **(C-E)** Photoconverted channel of 5 dpf larvae expressing neuron-specific CaMPARI (*Tgelavl3:CaMPARI*) exposed to **(C)** control, **(D)** 0.5% ethanol, **(E)** and 1.0% ethanol. **(C'-E')** Heat map of the red, photoconverted channel shown in F-H. Low to high intracellular calcium is depicted by a blue to white spectrum. Micrographs of 5 dpf larvae exposed to **(C')** control, **(D')** 0.5% ethanol, and **(E')** 1.0% ethanol.

Exposure to 0.5% ethanol showed a trend of increasing neuronal activity across all brain regions, but none of the regions tested exhibited a statistically significant difference compared to control (Fig. 7a, 7d, and 7d'). Exposure to 1.0% ethanol did not significantly alter neuronal activity across the regions examined (Fig. 7b, 7e, and 7e').

Discussion

To determine the utility of CaMPARI as a developmental neurotoxicological tool in larval zebrafish, we used a stable transgenic line expressing CaMPARI in differentiated neurons to assess the impact of three neurotoxicants of interest, PCB 95, ethanol, and MEHP, on brain activity. All three neurotoxicants were previously shown to alter locomotor activity in the light dark assay, but have different modes of action that have been elucidated to varying degrees (Haigis et al., 2022; Tal et al., 2012; Yaghoobi et al., 2022). Polychlorinated biphenyls (PCBs) are a class of 209 structurally similar

compounds that, despite their worldwide ban in the early 2000s, continue to pose a significant risk to the development of the human brain (Klocke et al., 2019). PCB 95 is a non-dioxin-like (NDL) PCB congener that has been shown to promote dendritic growth in primary rat and mouse hippocampal and cortical neurons and alter intracellular Ca^{2+} dynamics in primary rat hippocampal neurons via sensitization of ryanodine receptors (RyRs) (Keil et al., 2018; Pessah et al., 2010, 2019; Wayman et al., 2012a, 2012b). A recent study by Yaghoobi et al. (2022) found that chronic exposure to PCB 95 led to continuous swimming during light cycles in a light/dark assay, indicating that PCB 95- exposed larvae do not exhibit the freezing response typically induced by a light stimulus (Yaghoobi et al., 2022). Despite the evidence that PCB 95 is a developmental neurotoxicant, the specific neural underpinnings of the functional changes observed in larval zebrafish remain unclear.

Consistent with previous reports, larvae exposed to PCB 95 in this study generally exhibited an increase in locomotor behavior. The 30 μM acute exposure exhibited a similar loss of sensitivity to a light stimulus that was previously reported in zebrafish larvae chronically exposed to 10 μM PCB 95 (Yaghoobi et al., 2022). However, in this study, a chronic exposure to 10 μM PCB 95 did not significantly increase in the distanced travel by exposed larvae during either the light or the dark phase. These findings are not consistent with the results reported in Yaghoobi et al. (2022), which demonstrated 5 dpf larvae traveled statistically more in light cycles and

statistically less in dark cycles. While the light cycle behavior shows a similar trend towards an increase in activity, the dark cycle behavior is trending in the opposite direction. These inconsistencies could be explained by differences in experimental design between these two studies, including differences in the genetic backgrounds, well sizes, and exposure paradigms. Differences in genetic background have been previously shown to impact behavioral results (Abozaid et al., 2020). The previous study used 5D fish whereas this study used zebrafish with an AB genetic background. Additionally, the previous report dechorionated embryos at 4 hpf and plated larvae into 96-well plates, while this study dechorionated embryos at 24 hpf and plated into 24-well plates. Variation in well size has been shown to alter swimming behavior with significant differences found between swimming distance and pattern in 96-well plates versus 24-well plates (Padilla et al., 2011).

Despite the robust, hyperactive behavioral response, the CaMPARI neuronal activity analysis revealed that acute exposure to 10 μM and 30 μM PCB95 resulted in a significant decrease in neuronal activity across several brain regions. PCB 95 is known to sensitize RyR receptors, resulting in a transient rise in intracellular Ca^{2+} (Wong et al., 2001). However, this rise in intracellular Ca^{2+} has been shown to occur through the depletion of endoplasmic reticulum Ca^{2+} stores (Wong et al., 2001). We propose that upon acute exposure to high concentrations of PCB 95, intracellular Ca^{2+}

concentrations initially increase but are then followed by a quick depletion of intracellular stores, resulting in a significant decrease in neuronal Ca^{2+}. However, the chronically-exposed model exhibited no significant change in neuronal activity compared to controls. Due to the deleterious consequences of continuously depleting neuronal calcium stores, we reason that there must be additional compensatory mechanisms that reestablish baseline neuronal calcium homeostasis over time. In addition to hyperactivity and decreased neuronal activity, larvae acutely exposed to 30 μM PCB 95 also spent significantly less time in the center of the wells compared to control during the light/dark assay, which could indicate anxiety-like behavior. This behavior was also absent in the chronically-PCB 95 exposed group. Additional compensatory mechanisms, or possibly those involved in reestablishing neuronal calcium homeostasis, could also be involved in reducing PCB 95-induced, anxiety-related behavior.

MEHP is the primary metabolite of diethylhexyl phthalate (DEHP), which is a phthalate used to make common consumer products, including shower curtain liners, furniture, garden hoses, and medical devices. Exposure to DEHP has been shown to cause adverse effects on male reproductive tract development and recent studies have shown that prenatal exposure to DEHP negatively impacts cognitive, psychomotor, and behavioral development in children (Zhang et al., 2019). In mice, prenatal DEHP exposure leads to elevated anxiety-like behavior and impaired recognition memory through an increase in oxidative stress and inflammation in the hippocampus (Barakat

et al., 2018). In zebrafish, DEHP exposure has been shown to cause altered locomotor activity in light/dark assays (Haigis et al., 2022; Tran et al., 2021). Fewer studies have probed the effects of MEHP on behavioral and neurological development in zebrafish, with one study reporting a decrease in locomotor activity in the light cycle of a light/dark assay (Lu et al., 2021).

In this study, MEHP-exposure resulted in subtle behavioral responses with a significant decrease in the total distance traveled in the light cycle for the 2.0 mg/L exposed group, but no significant difference in total distance traveled in the dark cycle or overall distance traveled compared to control. The observed decrease in activity in the light cycle is in agreement with previously reported results demonstrating a reduced locomotion following MEHP exposure (Lu et al., 2021). Additionally, the 2.0 mg/L exposed group spent significantly less time in the center of the wells during the light/dark assay compared to control, which is indicative of anxiety-like behavior. The 0.2 mg/L exposed group exhibited no significant differences in total distance traveled across light or dark cycles or in time spent in the center of the wells compared to control. CaMPARI results showed a significant decrease in neuronal activity in the 2.0 mg/L exposed group in the whole brain, forebrain, optic tectum, and cerebellum, which is concordant with the behavioral results. To the best of our knowledge, this is the first report of developmental MEHP exposure altering neuronal activity in larval zebrafish. Recent studies have reported that MEHP alters neural excitability and

synaptic plasticity of projection neurons in *Drosophila* by inhibiting sodium and calcium channel activity, as well as significantly reduces the peak current densities of sodium and calcium channels in rat CA3 hippocampal neurons (Liu et al., 2021; Lu et al., 2022). Given the significant reduction in intracellular Ca^{2+} concentrations throughout the brain in the 2.0 mg/L exposed group, we hypothesize that observed MEHP-induced changes in neural activity in zebrafish could occur through the same mechanisms that leads to ion channel disruption in other model organisms.

Ethanol is a widely studied neurotoxicant that is known to cause significant behavioral and physiological impairments. Human embryonic exposure to ethanol can lead to fetal alcohol spectrum disorders (FASD) that manifest clinically as physical, behavioral, and cognitive deficiencies (Barr and Streissguth, 2001; Roozen et al., 2016). Ethanol has been shown to disrupt neural crest development, producing distinctive craniofacial malformations across species including humans and zebrafish, indicating that the same molecular mechanism(s) may be affected in both species (Arenzana et al., 2006; Bilotta et al., 2002; Caravan et al., 2004; Chmielewski et al., 1997; Matsui et al., 2006; Tenkova et al., 2003). Ethanol is thought to induce apoptosis of neural crest progenitors by an initial significant rise in intracellular calcium that originates from G-protein-coupled signaling (Debelak-Kragtorp et al., 2003; Garic-Stankovic et al., 2005). Ethanol also alters the function of voltage-gated calcium channels in developing neurons

(Mah et al., 2011). Chronic ethanol exposure was shown to reduce depolarization-induced Ca^{2+} responses in axonal growth cones while also up-regulating L-type channel expression (Mah et al., 2011).

In our studies, ethanol exposure resulted in no significant differences in behavior, center-avoidance, or neuronal activity at either concentration tested. The behavior results are in opposition to a previous report that showed ethanol exposure from 4-24 hpf resulted in hyperactivity at 5 dpf in a light/dark transition assay (Tal et al., 2012). Other studies that have reported effects of developmental exposure to ethanol have assessed behavior at time points ranging from 6 dpf to 9 dpf, and results from these studies vary. One study reported that at 6 dpf ethanol-exposed zebrafish exhibited significantly hypoactive behavior (Cadena et al., 2020). A different study assessed behavior from 7-9 dpf and reported differential effects depending on strain and exposure paradigm, with ethanol-exposed larvae of the AB strain generally displaying a greater level of hyperactivity compared to those of the TL strain (Abozaid et al., 2020). In addition to the difference in the developmental time point assessed and genetic strain of the zebrafish, another area of variability between these studies that could contribute to conflicting results is the difference in well size for behavioral testing. Across the aforementioned studies, one used a 96-well plate, two used 24-well plates (including this study), and one used 3.5 cm petri dishes. Previous research has shown that well size alters swimming behavior (Padilla et al, 2011). These differences represent important variations in experimental

design that make comparisons and integration of datasets across laboratories challenging and further emphasize the need for a community-wide standardization of methodology to increase the rigor and reproducibility of zebrafish neurotoxicity assays across laboratories.

Behavioral assays like the light/dark assay have been widely used to predict neurotoxicity of compounds in animal models, including zebrafish. However, the observed behavioral and neuronal toxic effects of PCB 95, MEHP, and ethanol demonstrate that behavior is not sufficient to predict how toxicant exposure may affect neural development and network dynamics. In particular, PCB 95 exposure led to discordant brain activity profiles and behavioral phenotypes, in which acute exposure to 30 μM PCB 95 led to robust behavioral hyperactivity but significantly less neuronal activity in three brain regions. Together these findings demonstrate that while behavioral assays offer a high throughput screen for general toxicity, they are unable to predict the ways in which exposure to different compounds elicits specific behavioral responses. While CaMPARI shows promise as a useful tool for evaluating neurotoxicity in zebrafish, it is also important to note there are limitations to the approach. One methodological limitation we uncovered was that the pigmentation in the PTU-treated larval zebrafish is not fully suppressed at 5 dpf and that pigmentation auto-fluoresced in the red channel, which partially masked our signal. While this could be avoided using genetic mutants that lack pigment, pigment is used by Noldus software

to track larval behavior. Therefore, to perform behavioral assay and CaMPARI image analysis using the same genetic background, we digitally removed the skin layer overlying the brain using the surfcut plugin in ImageJ. Additionally, while we were able to detect toxicant-induced changes in brain activity, CaMPARI may not be sensitive enough to detect smaller changes in brain activity that may result from lower dose exposures or from exposure to compounds that have a less severe impact on intracellular calcium concentrations. Furthermore, it is important to note that CaMPARI does not capture the temporal dynamics of neural activity and, therefore, is inherently unable to identify all of the ways in which neural networks may be affected by chemical exposures. When attempting to comprehensively understand the impact of chemicals on neural activity and function, additional complementary approaches to CaMPARI should be considered, including electrophysiological recordings and *in vivo* imaging of cellular populations of interest using temporally sensitive genetically encoded calcium indicators such as GCaMP. Despite these limitations, we found that CaMPARI is a useful tool that can help determine whether compounds are acting directly on the central nervous systems and is able to help identify brain regions mediating neural dysfunction following toxicant exposures.

Conclusion

CaMPARI is a useful neurotoxicological tool that can be used to understand how brain activity changes in response to toxicant exposure. While

behavioral assays offer a higher throughput way of predicting neuroactive/toxic endpoints, we found that behavioral activity is not always predictive of changes in neuronal activity. Therefore, behavioral assays alone are not sufficient to understand, at a systems level, how toxicant exposure affects neural development and network dynamics. Although neuroimaging assays like CaMPARI are not as high-throughput as behavioral assays, we demonstrated that pairing behavioral assays with CaMPARI provides a more comprehensive understanding of the neurotoxic endpoints of compounds while still offering relatively high throughput on the order of minutes per larva. Future studies aimed at further improving throughput via the use of automated confocal microscopy could further promote pairing behavioral assays with neuroimaging techniques like CaMPARI in developmental toxicology studies. In this study CaMPARI was driven by the pan-neuronal promoter *elavl3* to visualize all neuronal populations. Future studies probing the activity of specific populations of neurons and glia will be useful for uncovering the cellular targets of different neurotoxicants and are important for gaining additional mechanistic insight into how different chemical compounds affect the functioning and health of the nervous system.

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