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# Exposure to an environmentally representative mixture of polybrominated diphenyl ethers (PBDEs) alters zebrafish neuromuscular development

Alec McDermott, Cécilia Bernier, Vanessa Piché, Isabelle Plante [1], Shunmoogum A. Patten [1,*]

*INRS – Centre Armand Frappier Santé Biotechnologie, 531 Boulevard des Prairies, Laval, QC H7V 1B7, Canada*

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ABSTRACT

Polybrominated diphenyl ethers (PBDEs) are a prevalent group of brominated flame retardants (BFRs) added to several products such as electronics, plastics, and textiles to reduce their flammability. They are reported as endocrine disruptors and neurodevelopmental toxicants that can accumulate in human and wildlife tissues, thus making their ability to leach out of products into the environment a great cause for concern. In this study, zebrafish (*Danio rerio*) embryos and larvae were exposed to a wide concentration range (1.5, 15, 150 and 300 pM) of a PBDE mixture from one to six days post-fertilization (dpf). Hatching rates, mortality and general morphology were assessed during the exposure period. A delay in hatching was observed at the two highest PBDEs concentrations and mortality rate increased at 6 dpf. By 4 dpf, larvae exposed to 150 pM and 300 pM PBDEs developed an upcurved phenotype. Analysis of motor behavior at 6 dpf revealed that PBDE exposure acutely reduced locomotion. To further analyze these motor deficits, we assessed the neural network density and motor neuron and neuromuscular junctions (NMJ) development by immunostaining and imaging. Acetylated α-tubulin staining revealed a significant loss of neurons in a dose-dependent manner. Synaptic vesicle protein 2 (SV2) and α-bungarotoxin (α-BTX) staining revealed a similar pattern, with a significant loss of SV2 and nicotinic acetylcholine receptors, thus preventing the colocalization of presynaptic neurons with postsynaptic neurons. Consistent with these results, the presence of cleaved caspase-3 and acridine orange positive cells showed increased cell death in zebrafish larvae exposed to PBDEs. Our results suggest that exposure to PBDEs leads to deficits in the zebrafish neuromuscular system through neuron death, inducing morphological and motor deficiencies throughout their development. They provide valuable insight into the neurotoxic effects of PBDEs, further highlighting the relevance of the zebrafish model in toxicological studies.

## 1. Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants (BFRs) incorporated into consumer products to reduce flammability and slow the spread of fire, with significant use in North America (Hale et al., 2003; Segev et al., 2009; Watanabe and Sakai, 2003). PBDEs consist of a central diphenyl ether structure with varying degrees of bromination, resulting in 209 possible configurations (Rahman et al., 2001). Since PBDEs are primarily used as additives and do not form covalent bonds with the materials they are added to, they are prone to environmental release, notably BDE-209, found in high concentrations (Stapleton et al., 2005). Human exposure to PBDEs typically occurs through inhalation and ingestion of dust (Wu et al., 2007). Owing to their persistent nature and bioaccumulative properties, PBDEs are widespread in human tissues, including breast milk (Toms et al., 2009). Despite regulatory measures and bans in several countries, PBDEs remain prevalent in the environment.

Elevated levels of PBDEs found in the human placenta and breast milk raise significant concerns due to their potential adverse effects on development (Carrizo et al., 2007; Frederiksen et al., 2010). PBDEs have been linked to adverse health effects, including their ability to disrupt endocrine function and their neurotoxicant properties affecting neurodevelopment. Studies in rats have demonstrated that exposure to PBDE-99 and PBDE-209 during critical periods of brain development can lead to reduced locomotion and disruptions in the cholinergic system (Johansson et al., 2008; Viberg et al., 2005). Furthermore, research has shown that exposure to PBDE-47 and one of its metabolites, 6-OH-BDE-47, can result in reduced locomotion and altered exploratory

* Corresponding author.
*E-mail address:* kessen.patten@inrs.ca (S.A. Patten).
[1] Authors contributed equally.

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behavior in zebrafish larvae (Chou et al., 2010; Macaulay et al., 2015). The locomotion deficits may be attributed to PBDE-47's inhibition of proper axonal growth in primary motoneurons (Chen et al., 2012).

The effects of PBDE exposure have not gone unnoticed in the human population. Cohort studies have established a connection between elevated levels of PBDEs in breast milk and adverse neurodevelopmental outcomes in children. Specifically, children exposed to elevated PBDE levels through breast milk exhibited impaired attention at age 5, poorer fine motor coordination at ages 5 and 7, and reduced verbal IQ at age 7 (Eskenazi et al., 2013). Furthermore, increased PBDE levels in breast milk have been associated with a lower body mass index, as well as smaller chest and head circumference in children (Chao et al., 2007).

The zebrafish (*Danio rerio*) is widely regarded as a valuable model organism to study the impacts of environmental pollutants acting as endocrine disruptors, as well as for assessing their effects on neurodevelopment and neurotoxicity (Creton, 2009; Segner, 2009). This is because fundamental mechanisms and processes of neurodevelopment are highly conserved across vertebrate species (Lein et al., 2005). Zebrafish models are characterized by their simplicity, cost-effectiveness, and rapid development, making them an ideal platform for conducting neurodevelopmental and toxicological research.

PBDEs pose significant risks to neurodevelopmental health, particularly for infants and toddlers who are most exposed through the consumption of mothers' breast milk and household dust (Allen et al., 2008; Stapleton et al., 2008, 2005; Toms et al., 2009). While many studies have examined the effects of individual PBDE congeners or metabolites, actual exposure often involves a mixture of compounds. Hence, evaluating the combined toxicological effects of these mixtures provides a more accurate reflection of real-world scenarios (Hamid et al., 2021). Regulatory agencies also recognize the importance of assessing mixture effects in risk assessment (Altenburger et al., 2012), emphasizing the need for comprehensive studies that reflect actual exposure conditions.

We hypothesise that exposure to a mixture of PBDEs representative of those found in human breast milk will reduce larval locomotion by disrupting normal neurodevelopment. The aim of this study is to evaluate the impact of the mixture on the early life behavior and neurodevelopment in zebrafish. Our findings reveal that zebrafish larvae exposed to PBDEs mixture exhibit reduced locomotion, increased apoptosis and impaired formation of neuromuscular junctions (NMJs).

## 2. Materials and methods

### 2.1. Zebrafish maintenance

Wild-type *Danio rerio* (AB/TL strain) and transgenic line Tg (*Hb9*:GFP) were kept at 28 °C under a 12-h light/12-h dark cycle, following the protocol outlined in the zebrafish book (Westerfield, 1993). All zebrafish in this study were housed in groups. Twice-a-day, fish were fed a steady diet of Skretting® Gemma Micro starting at 5 dpf. Fish larvae were fed with Gemma Micro 75, juvenile fish were fed with Gemma Micro 150 and adult fish were fed with Gemma Micro 300. Embryos were incubated at 28.5 °C. This study followed protocols approved by the INRS Animal Care and Use committee and used facilities, which are fully accredited by Canadian Council for Animal Care (CCAC) and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures adhered to the guidelines established by the CCAC for zebrafish and other small, warm-water laboratory fish for feeding and maintenance.

### 2.2. Study design

The primary objective of this study was to assess the effects of a PBDEs mixture, that is representative of those found in human breast milk, on neurodevelopment and behavior using the zebrafish model. Embryos were exposed to a mixture of PBDEs at varying concentrations (1.5 pM (0.8225 ppm), 15 pM (8.225 ppm), 150 pM (82.25 ppm), and 300 pM (164.50 ppm)), starting from 4 h post-fertilization (hpf) until 6 days post-fertilization (dpf). The PBDE mixture, representing those found in human breast milk (Lenters et al., 2019; Meng et al., 2021; Müller et al., 2019) (71.32 % DE-71, 27.25 % BDE-47, 0.72 % BDE-209, 0.7 % DE-79), was dissolved in DMSO to a final concentration of 133 nM and stored at −80 °C. Embryos were collected from spawning adults housed in two mating tanks, each with 2–3 couples, overnight (O/N). Embryos from both tanks were pooled to form one experimental replicate (N = 1). Subsequently, batches of 20 embryos (n = 20) were exposed to PBDEs or vehicle (0.2 % DMSO) for a total of 3 replicates (N = 3). Sample size was determined based on previous experience with similar toxicological, behavioral, and imaging experiments. No data were excluded from the analyses. All samples used in the experiments were randomly selected. All morphological analyses, fluorescent imaging, and behavioral analyses were successfully replicated and representative results were shown. Synaptic puncta quantification was performed using an unbiased approach as described below. Blinding during analysis was impossible as the dorsal-curvature phenotype appeared in a dose-response manner. To achieve an unbiased analysis, image acquisition and analysis were randomly performed by co-authors.

### 2.3. Survival, hatching and gross morphology assessment

Survival and hatching rates were followed through time. Gross morphology was performed at 3 and 6 dpf. Gross morphology was evaluated under a bright field stereomicroscope (Zeiss Stemi 305) and images were taken with an iPhone 8 using a LabCam ® iPhone holder. Area of the head and eyes were normalized on body length using ImageJ. Head to Tail angle was also evaluated at 6 dpf. Imaging was performed on a subset of embryos from different clutches of the same breeding days (n = 10 per condition) and repeated for a total of 3 times (N=3).

### 2.4. Locomotion assessment

Larvae at 6 dpf were individually placed into wells of a 96-well plate filled with 200 µl of E3 media. They were then allowed to habituate in the Daniovision® recording chamber (Noldus) for 30 min in the dark. After 30 min in the dark, the light was activated for 2 h to assess the locomotion activity of the larvae. Analysis of the recorded behavior was conducted using EthoVision XT12 software (Noldus) to quantify the total swimming distance in the light phase.

### 2.5. Neuromuscular junction staining and analysis

3 and 6 dpf zebrafish larvae were euthanized in ice water and fixed in 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4 °C O/N on a rotator. Subsequently, the larvae were rinsed three times in a 0.1 % Tween-20 solution in PBS (PBST) for 15 min at room temperature. Larvae were then permeabilized in 1 mL of 1 mg/mL of collagenase (Sigma-Aldrich; C0130) in PBS for 1 h (3 dpf) and 2.5 h (6 dpf) at room temperature. Next, larvae were blocked (1 % bovine serum albumin, 1 % DMSO, 1 % Triton-X, 2 % normal goat serum, in PBS) for 1 h at room temperature. Then, acetylcholine receptors were stained using 1 µg/mL of tetramethylrhodamine-conjugated α-bungarotoxin (Thermofisher; T1175) in PBST for 30 min, then rinsed with PBST (3×15 min) and incubated at 4 °C O/N with a freshly prepared blocking solution containing the primary antibody for synaptic vesicle 2 (SV2) diluted 1:200 (Developmental Studies Hybridoma Bank; AB_2315387). The following day, larvae were rinsed and incubated with Alexa Fluor 488 secondary antibody (Invitrogen; A10680, 1:1000) in blocking solution for 4 h at room temperature. After rinsing, larvae were mounted laterally on a slide using fluoromount-G (Invitrogen; 00–4958–02) and imaged using a Nikon A1R+ confocal microscope (Nikon). Three to four larvae were imaged per replicate. Images were analyzed using ImageJ and the NMJ Analyser plugin (Singh et al., 2023). The plugin provides an unbiased and automated method for counting puncta per somite. It begins by

creating a z-projection of the images to help the user identify the region of interest. Each z-slice is then processed for contrast enhancement and punctum identification. If multiple puncta are detected in an area, pixel expansion is used to segment them. The algorithm counts the number of puncta per slice and tabulates the total number within the regions of interest. Additionally, co-localization is performed for each punctum. This method offers an unbiased count of puncta within a channel and when multiple channels are used, the number of co-localized puncta.

### 2.6. Primary motor axon analysis

Transgenic zebrafish larvae (Tg(*Hb9*:GFP)) which express GFP in dendrites, axons and soma of spinal motor neurons were used to evaluate the effects of PBDEs on axonal projections of motor neurons. Embryos were exposed to the PBDEs mixture from 4 hpf to 3 dpf (Vehicle, 15 pM, 150 pM, 300 pM). At endpoint, larvae were euthanized in ice water and fixed in PFA for 20 min before lateral mounting using fluoromount-G (Invitrogen; 00–4958–02). Finally, a total of 10 larvae per condition were imaged using a Nikon A1R+ confocal microscope (Nikon). Primary motor axons length was measured using ImageJ. The average length of three primary motor axons was normalized on the width of the larvae. Three to four larvae were imaged per replicate.

### 2.7. Neuronal network assessment

Acetylated α-tubulin staining was used to assess the neuronal network of 6 dpf larvae. Larvae were euthanized in ice water and fixed in Dent's fixation (80 % methanol and 20 % DMSO) O/N at 4 °C. They were then rehydrated in 75, 50 then 25 % methanol in PBST for 30 min each. After being washed 4 times in PBST for 30 min, samples were blocked and permeabilized as previously described. Then, they were incubated O/N at 4 °C with acetyl-tubulin monoclonal antibody (Sigma-Aldrich; T7451) diluted 1:500 in the blocking solution. Next, larvae were washed 3 ×30 min in PBST and incubated with secondary antibody Alexa 488 (Invitrogen; A32723) O/N at 4 °C. Finally, larvae were rinsed and mounted laterally. Imaging was done using a Nikon A1R+ confocal microscope (Nikon) and z-stacks were analyzed using ImageJ. The branching density of the neuronal network was analyzed with the Sholl Analysis from the Neuroanatomy plugin with a step size radius size of 0.025. Two randomly chosen somites per larva were analyzed. Three to four larvae were imaged per replicate.

### 2.8. Apoptosis analysis

#### 2.8.1. Cleaved-caspase 3 immunostaining

After treatment, larvae were euthanized in ice water and fixed in PFA (3 dpf and 6 dpf), washed, permeabilized and blocked as previously described. Larvae were incubated in Cleaved-Caspase 3 (Cell Signaling; 9661) antibody diluted 1:200 in blocking solution O/N at 4 °C. After washing the primary antibody for 3 ×30 min at room temperature, larvae were incubated in the secondary antibody Alexa 568 1:1000 (Invitrogen; A10042) at room temperature for 3 h. The secondary antibody was washed and nuclei were stained for 5 min with DAPI 1:1000 (Thermo Scientific; 62248). Fish were mounted as previously described and imaged using Nikon A1R+ confocal microscope. Images were analyzed using ImageJ were Cleaved-Caspase 3 fluorescence intensity was normalized to DAPI. Three to four larvae were imaged per replicate.

#### 2.8.2. Acridine orange staining

Following exposure, the PBDE mixture (3 dpf and 6 dpf) larvae were rinsed in E3 water. Larvae were then placed in Acridine Orange (Sigma Aldrich; 235474) diluted in E3 water to a final concentration of 5 mg/mL for 30 min at 28.5 °C. Larvae were then washed 3 times for 10 min with E3 medium. They were anesthetized with 0.02 % tricaine (Sigma Aldrich; A5040) and visualized with a stereomicroscope equipped with fluorescence (Zeiss Axio ZoomV.16). Z-projection of the larvae's tail was analyzed for fluorescence intensity using ImageJ.

### 2.9. Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). Statistical significance was assessed using One-way ANOVA followed by a Tukey post-hoc test for multiple comparisons, provided that the assumptions of normality and equal variance were met. In cases where these assumptions were not met, the Kruskal-Wallis test followed by the Conover test for multiple comparisons was employed as a non-parametric alternative. p-value $< 0.05$ was used to determine significance. All statistical analyses were conducted using R Studio.

## 3. Results

### 3.1. Exposure to PBDEs significantly affects zebrafish development, resulting morphological changes and in reduced locomotion

Zebrafish embryos and larvae were exposed to varying concentrations of a PBDEs mixture representative of those found in human breast milk (Lenters et al., 2019; Meng et al., 2021; Müller et al., 2019) from 4 h post-fertilization (hpf) for a total of 6 days. Survival rates were affected by higher PBDEs concentration exposure. After 4 days of exposure to 150 pM, we observed dead larvae (1 on day 4; and another larva on day 5), but overall survival rates were not significantly affected by 150 pM PDBE mixture compared to controls (Fig. 1A). 300 pM had the biggest effect on zebrafish larvae survival, where 43 ± 5 % of the population died at 6 days of exposure (Fig. 1A). We also evaluated the effects of the PBDEs mixture on hatching rates. Hatching rates at 2 dpf were significantly lower for the 150 pM and 300 pM groups compared to controls and other treated groups (Fig. 1D). At 3 dpf, while most fish were expected to have hatched, the only treated group with an incomplete hatch was the 150 pM group (Fig. 1D). Finally, the appearance of a dorsal curvature phenotype in the fish population was observed after 4 days of exposure at 150 pM (27 ± 4 %) and 300 pM (74 ± 3 %) concentrations (Fig. 1A). By the following day, 12 ± 2 % of fish exposed to 15 pM displayed the phenotype, with almost all fish exposed to 150 pM (95 ± 3 %) and 300 pM (100 ± 0 %) being affected (Fig. 1A).

Evaluation of gross morphology revealed significantly smaller eyes and heads in larvae exposed to 150 pM compared to other treatments and controls at 3 dpf, indicating disrupted zebrafish development (Fig. 1B, E, F). At 6 dpf, PBDEs caused a marked reduction in head (300 pM) and eye areas (15 and 300 pM) (Fig. 1C, G, H), suggesting that the reduced eyes and head area observed at 3 dpf after an exposure to 150 pM is maintained in time (Fig. 1C, G, H). These results suggest that PBDEs exposure causes neurodevelopmental defects.

Interestingly, the appearance of a dorsal curvature phenotype became evident at 4 dpf. While after 6 days of exposure, controls and 1.5 pM are relatively flat (around 180°), larvae exposed to 15 pM displayed a significantly lower angle compared to controls, and this effect was pronounced at 150 pM and 300 pM (Fig. 1C, I).

### 3.2. PBDEs exposure diminishes swimming and induces the loss of neuromuscular junctions

Morphological changes leading to developmental defects and delayed hatching led us to hypothesise that exposure to PBDEs would diminish locomotion in zebrafish larvae. 6 days of exposure to 15 pM of PBDEs caused a significant reduction in total distance swam in bright settings for 2 h. Moreover, 150 and 300 pM exposures almost totally immobilize the larvae (Fig. 2A). It is important to note that larvae were alive before and after the locomotion analysis.

Given the reduced hatching rates and the reduced locomotion following exposure to PBDEs in zebrafish larvae, we hypothesise a potential neuromuscular toxicity. We thus sought to elucidate the impact

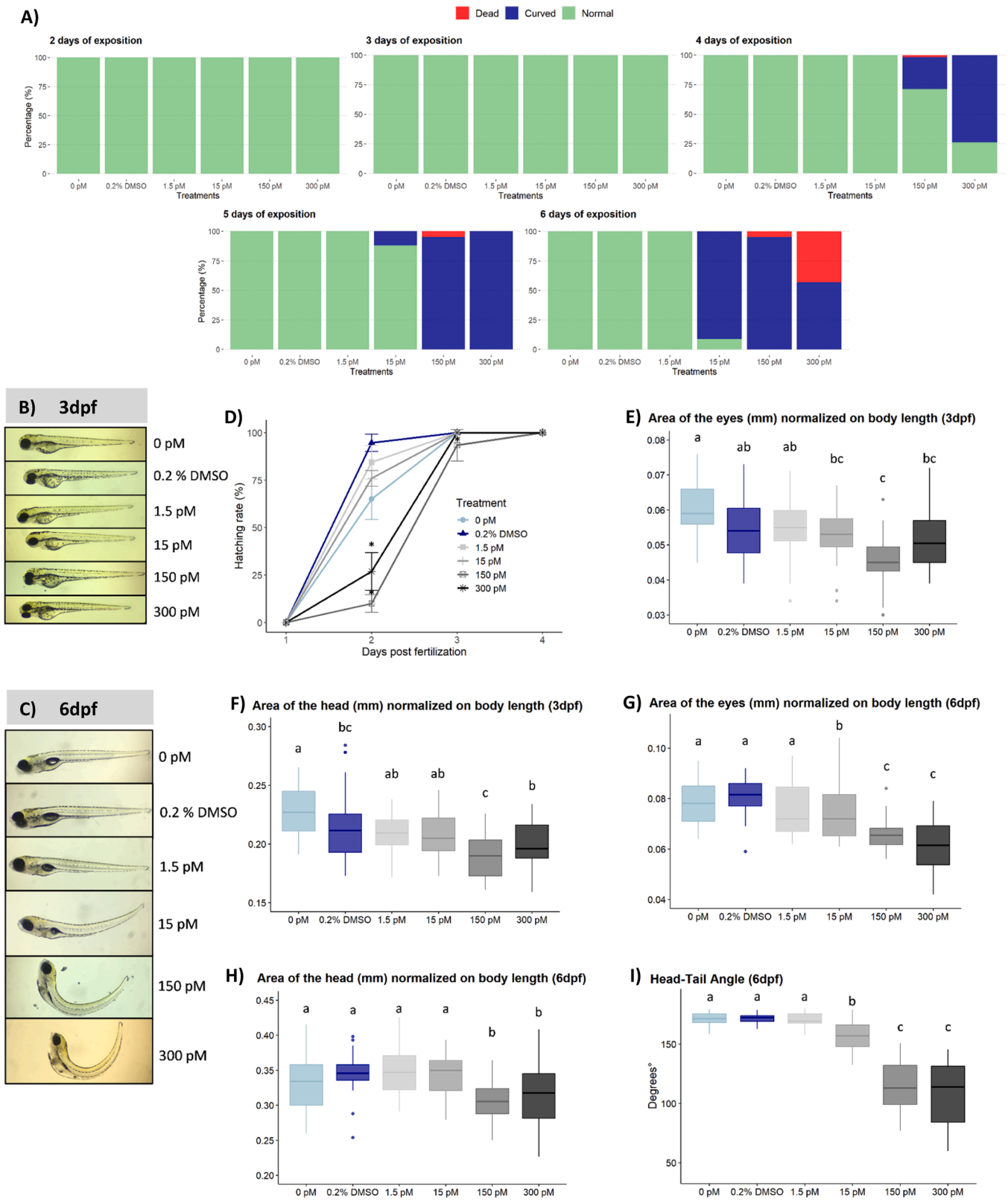


**Fig. 1.** Survival, hatching and morphological effects of PBDEs on zebrafish larvae. (A) Fish survival and morphology was followed for 6 days of exposition. (N=3, n=60). (B) Representation of zebrafish morphology after 3 days of PBDEs exposition. (C) Representation of zebrafish morphology after 6 days of PBDEs exposition. (D) Hatching rate of zebrafish larvae when exposed to 150 and 300 pM of PBDEs is diminish. (E-F) Zebrafish larvae area of the head and the eyes normalized on total fish length after 3 days of exposition to 150 pM of PBDEs is significantly reduced (N=3, n=10). (G-H) Zebrafish larvae area of the head and the eyes normalized on total fish length after 6 days of PBDEs exposition remains reduced in time (N=3, n=10). (I) Quantification of the dorsal curvature phenotype at 6dpf in degrees. Briefly, with the angle tool from ImageJ, a straight line was drawn from the mouth through the swim bladder, then angled up to the tip of the tail. ≤ 15 pM induces the phenotype (N=3, n=10). p-values were calculated with One Way ANOVA or Kruskal-Wallis and Variables are statistically indistinguishable if they share at least one letter. All graphs represent the means ± SEM.

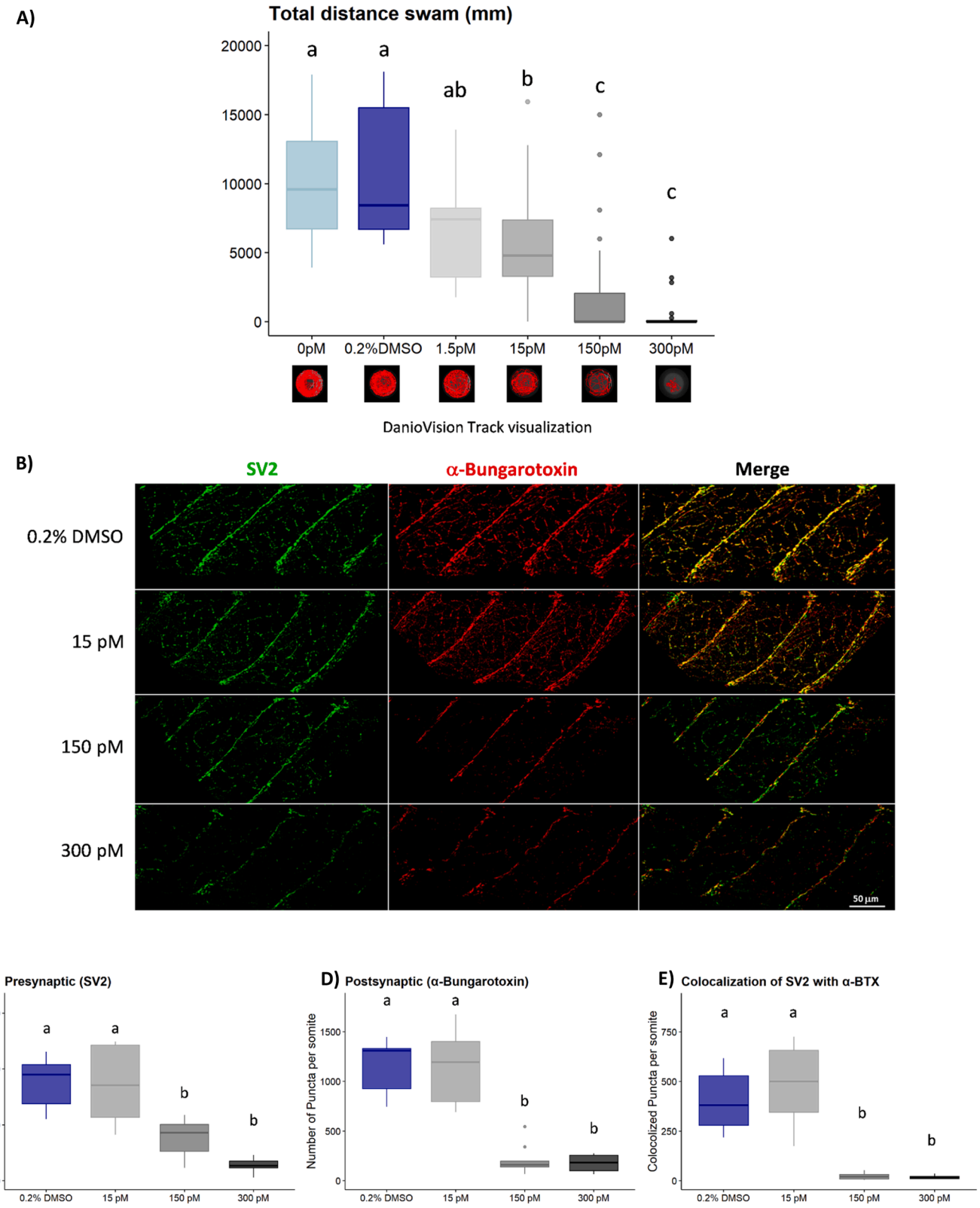


**Fig. 2.** PBDEs exposure diminishes locomotion at 6 dpf and alters NMJ. (A) Functional analysis of locomotion capabilities of larvae exposed to PBDEs for 6 days shows diminish locomotion ≤ 15 pM (N=3, n=60). (B) Z-projection of co-immunostaining of presynaptic neurons (Synaptic Vesicle 2 (SV2); green) and postsynaptic acetylcholine receptor (α-Bungarotoxin; red) after 6 days of PBDEs exposure. AChR was stained using tetramethylrhodamine conjugated α-bungarotoxin. (C-D) Quantitative analysis is showing diminish puncta of SV2 and α-BTX when exposed to 150 and 300 pM of PBDEs. (E) Colocalization analysis of SV2 puncta with α-BTX puncta is significantly reduced when exposed to 150 and 300 pM. p-values were calculated with One Way ANOVA or Kruskal-Wallis and Variables are statistically indistinguishable if they share at least one letter. All graphs represent the means ± SEM (N=3, n=10).

of long-term PBDEs exposure on the neuromuscular system. We assessed the effects of varying concentrations of the PBDEs mixture (vehicle, 15 pM, 150 pM, 300 pM) on NMJs integrity in zebrafish larvae at 6 dpf. Fish exposed to 150 pM and 300 pM PBDEs exhibited significantly reduced expression of the presynaptic marker, synaptic vesicle 2a (SV2), within the somites of zebrafish trunk muscles (Fig. 2B, C). Additionally, the muscle receptor, acetylcholine receptor, was also significantly decreased at 150 pM and 300 pM (Fig. 2B, D). The colocalization of presynaptic and postsynaptic puncta is necessary for proper muscle contraction and was significantly reduced at these concentrations (Fig. 2B, E).

Since PBDEs directly affected NMJ integrity, we sought to evaluate if PBDEs may impact proper neuron formation. To investigate the effects of PBDEs on neurons, we proceeded to immunolabel the acetylated α-tubulin present in neuron axons. Z-projections of total neurons within the larvae's tails revealed a reduced complexity of the network, indicated by decreased branching density (Fig. 3A, B). Exposure to PBDEs for 6 days led to nearly complete loss of neurons within fish somites at 150 pM and 300 pM, with a lesser, but still significant reduction observed at 15 pM (Fig. 3A, B).

We next sought to assess apoptosis in larvae treated with PBDEs using cleaved caspase 3 and acridine orange staining. Exposure to PBDEs resulted in elevated levels of cleaved caspase 3, particularly at 150 pM and 300 pM concentrations (Fig. 3C, D). Notably, the expression of cleaved caspase 3 was higher at 150 pM compared to 300 pM at 6 dpf, suggesting that cell death may occur earlier at higher doses (Fig. 3C, D). Similarly, we observed with acridine orange staining that 6 dpf zebrafish larvae treated with 150 and 300 pM of PBDEs exhibited a significant increase in the number of cells stained, suggesting an increase in apoptosis in these fish (Fig. S1C, D). Altogether these data suggest that prolonged exposures to PBDEs at 150 and 300 pM lead to cell death in the trunk region of the zebrafish, including in the spinal cord (Fig. S1C, arrows).

### 3.3. *PBDEs stunt the formation of NMJ, but does not affect primary motor neurons*

To comprehensively evaluate the effects of PBDEs not only as neurotoxicants but also as molecules capable of influencing neurodevelopment, we assessed various parameters at 3 dpf. Consistent with observations at 6 dpf, PBDEs (at 150 and 300 pM concentrations) inhibited the proper formation of NMJs at 3 dpf. This was evidenced by a significant reduction in the levels of SV2 and α-bungarotoxin puncta within the fish somites, thus affecting their colocalization (Fig. 4A-D).

To verify whether PBDEs impede NMJ formation, we evaluated apoptosis levels within the larvae's somites following 3 days of exposure to PBDEs. Our findings revealed a significant increase in cleaved caspase 3 levels at concentrations of 150 pM and 300 pM (Fig. 4E, F), indicating PBDEs-induced apoptosis. Once again, apoptosis was confirmed via acridine orange staining. An exposure to 150 and 300 pM for 3 days led to an increase of apoptosis within larvae's somites (Fig. S1A, B). We then evaluated the morphology of primary motor neurons that lead to NMJ. Interestingly, PBDEs exposure did not affect primary motor neurons at 3 dpf (Fig. 4G, H), suggesting a direct link between PBDEs effects on NMJ synapse disruption.

## 4. Discussion

Several studies have documented the potential adverse health effects of various congeners of PBDEs on developmental neurotoxicity (Chou et al., 2010; Lema et al., 2007; Macaulay et al., 2015). Notably, pre- and post-natal exposure to PBDEs has been associated with long-lasting behavioral defects, particularly affecting cognitive behavior and motor activity in rodents (Eriksson et al., 2002; Viberg, 2009). However, to our knowledge, our study is the first to elucidate the effects of a PBDEs mixture representative of those found in human breast milk on early neurodevelopment.

In this study, zebrafish larvae exposed to an environmentally representative mixture of PBDEs (71.32 % DE-71, 27.25 % BDE-47, 0.72 % BDE-209, 0.7 % DE-79) exhibited various developmental abnormalities, including morphological changes such as alterations in eye and head size and a dorsal curvature phenotype at later larval stages (4–6 dpf). Furthermore, PBDEs exposure led to inhibition of NMJ formation, incomplete neuronal network development, and increased cell death in larvae's somites. General morphological effects observed on spinal cord axis deformity is a phenotype highly observable when zebrafish larvae are exposed to different congeners of PBDEs (Lema et al., 2007; Usenko et al., 2011). Lema et al., reported that the cerebrospinal fluid (CSF) flow in the brain ventricles and central canal of the neural tube was significantly reduced in PBDE-47 treated fish, leading to dorsal curvature. PBDEs might interfere with the proper polarity of motile cilia of multi-ciliated ependymal cells, directly affecting the CSF flow (Boutin et al., 2014; Mirzadeh et al., 2010). Future studies are necessary to assess whether the PBDEs mixture may affect CSF flow and interfere with the proper polarity of motile cilia, leading to the dorsal curvature phenotype in zebrafish.

Reduced eye area observed may be attributed to increased apoptosis in the retina, as previously demonstrated (Dong et al., 2014). Head deformities have also been observed in both zebrafish and human cohorts exposed to PBDEs (Chao et al., 2007; Eskenazi et al., 2013; Macaulay et al., 2015). These gross morphological changes observed here in zebrafish population following PBDE exposure are consistent with literature, even with different types of exposure. Of particular concern is the observation that our PBDEs mixture induced similar effects at lower doses (pM) compared to higher doses of single congeners used in previous studies (nM and μM).

Our findings reveal a significant inhibition of neuromuscular junction formation at 3 dpf, persisting over time and resulting in impaired swimming capabilities. Intriguingly, despite these pronounced effects on NMJ development, the length and morphology of primary motoneurons remained unaffected by PBDEs exposure.

To date, limited information is available about the effects of PBDEs on NMJ development. However, previous research has demonstrated that exposure to the congener PBDE-47 can lead to a reduction in axonal growth of primary and secondary motor neurons during zebrafish developmental stages (Chen et al., 2012). Notably, significant reductions in axonal growth and motor behavioral defects were observed following exposure to higher concentrations of PBDE-47 (ranging from 1.25 to 20 μM for 6 hpf to 5 dpf) (Chen et al., 2012). Our study revealed that the mixture of PBDE did not alter primary motoneurons. In contrast, our exposure to the PBDEs mixture resulted in lethality at concentrations exceeding 300 pM, highlighting the potential synergistic effects of the molecules within the mixture compared to exposure to a single molecule. It is thus important to consider complex mixture exposures in toxicological studies, as interactions between different compounds may exacerbate toxicity or different adverse health problems beyond what would be expected from individual exposures. During development, synapses are initially formed in excess, and only the appropriate connections are consolidated. At the NMJ, competition between several motor nerve terminals occurs, resulting in the maturation of one axon while others are eliminated. Our results show a reduced branching density at 6 dpf as demonstrated by α-tubulin staining. Disruptive effects of PBDE exposure on neuron cytoskeleton regulation have been previously shown. Indeed, zebrafish larvae exposed to PBDE-71 (31, 68.7 and 227.6 μg/L) until 5 dpf exhibited decreased expression levels of α1-tubulin, mbp and sonic hedgehog (Chen et al., 2012). These sets of genes are important in cytoskeleton regulation, neuronal myelination, and neuron growth. Moreover, mice exposed to a single dose of PBDE-99 (12 mg/kg) on post-natal day 10 had significant alterations in the levels of proteins associated with cytoskeleton regulation, neuronal maturation, neurodegeneration, neuroplasticity, and synaptogenesis (Alm et al., 2008). The alteration of neuronal development via the cytoskeleton regulation can potentially lead to the lack of NMJs formation.

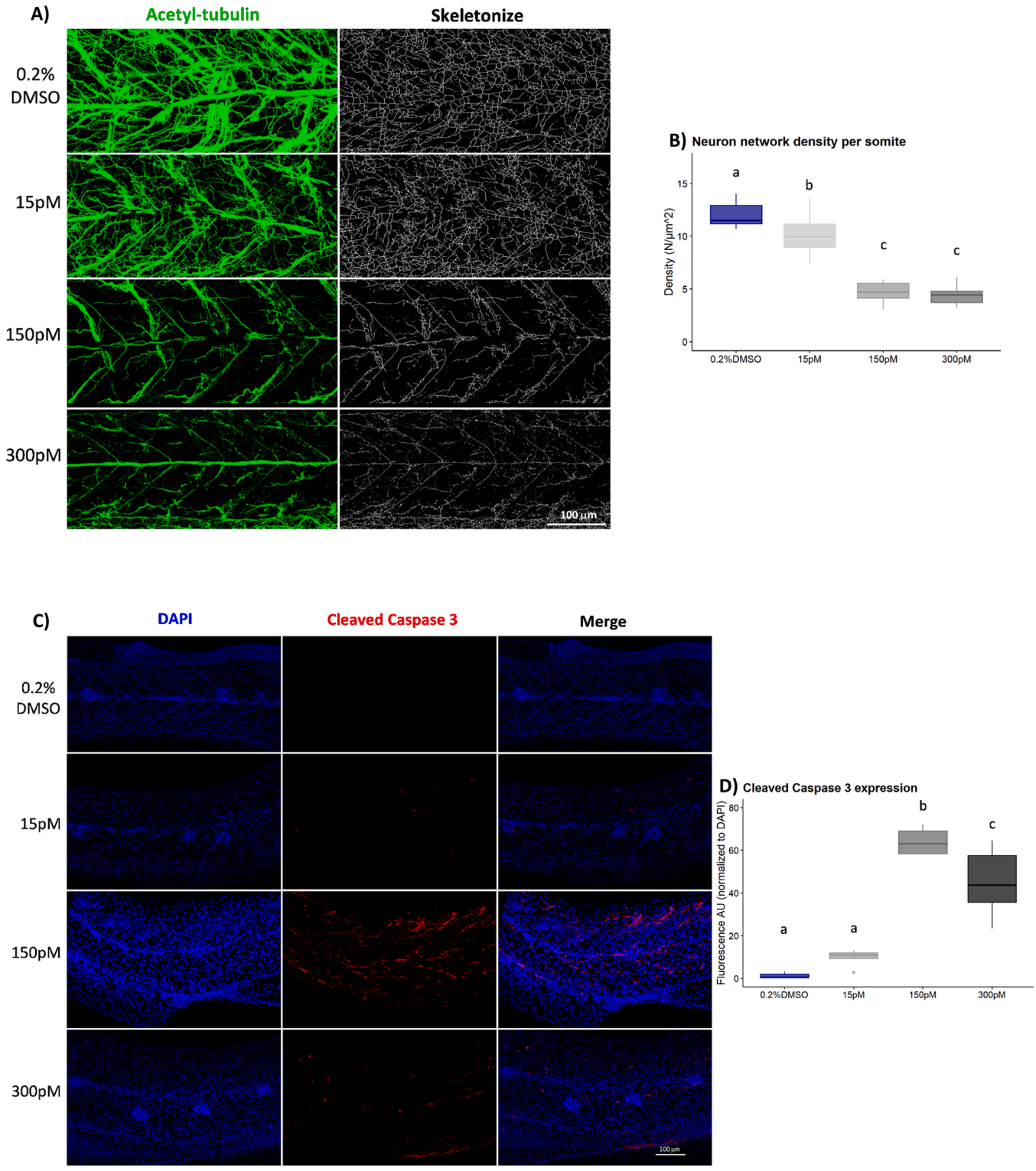


**Fig. 3.** PBDEs exposure reduces branching density of neuronal network and induces apoptosis. (A) Z-projection of the acetylated α-tubulin immunostaining and skeletonize version of the neuronal network used for analysis. (B) Neuronal network density within somites is decreased after 6 days of exposure to a mixture of 150 pM and 300 pM of PBDEs. (C) Z-projection of Cleaved Caspase 3 (red) and DAPI (blue) immunostaining showing apoptosis following PBDEs exposure for 6 days. (D) Quantitative analysis of Cleaved-caspase 3 fluorescence normalized to DAPI showing a significant increase at 150 and 300 pM of PBDEs after 6 days of exposure. p-values were calculated with One Way ANOVA or Kruskal-Wallis and Variables are statistically indistinguishable if they share at least one letter. All graphs represent the means ± SEM (N=3, n=10).

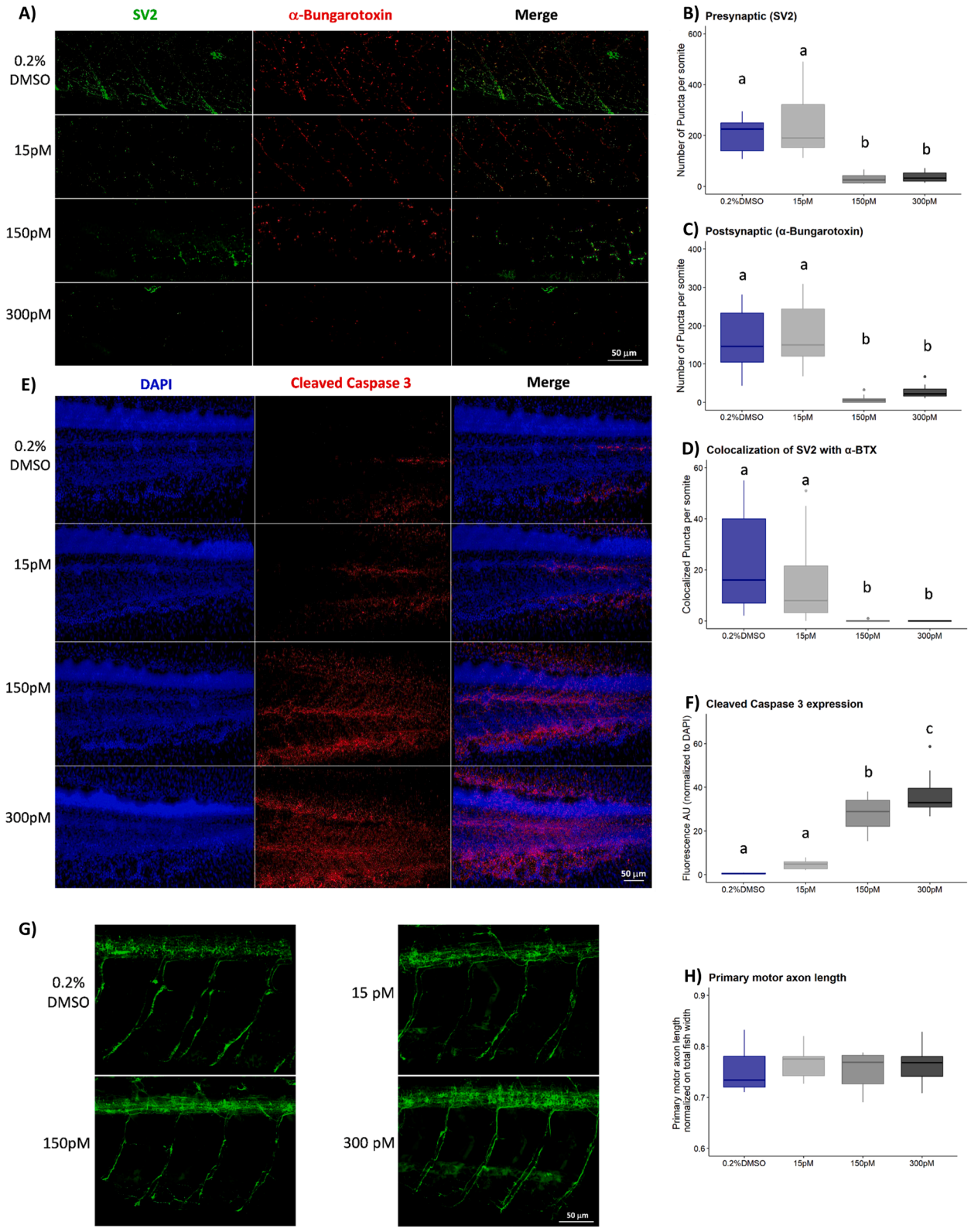


**Fig. 4.** PBDEs inhibits the formation of NMJ without affecting primary motor neurons at 3 dpf. (A) Z-projection of co-immunostaining of presynaptic neurons (Synaptic Vesicle 2 (SV2); green) and postsynaptic acetylcholine receptor (α-Bungarotoxin; red) after 3 days of PBDEs exposure. AChR was stained using tetramethylrhodamine-conjugated α-bungarotoxin. (B-C) Quantitative analysis is showing diminish puncta of SV2 and α-BTX when fish are exposed to 150 and 300 pM of PBDEs. (D) Co-localization analysis of SV2 puncta with α-BTX puncta is significantly reduced when exposed to 150 and 300 pM. (E) Z-projection of Cleaved Caspase 3 (red) and DAPI (blue) immunostaining showing apoptosis following PBDEs exposure for 3 days. (F) Quantitative analysis of Cleaved-caspase 3 fluorescence normalized to DAPI showing a significant increase at 150 and 300 pM of PBDEs after 3 days of exposure. (G) Z-projection of transgenic Hb9:GFP strain fish showing primary motor neurons. (H) Primary motor axon length is not affected by 3 days of exposure to PBDEs. p-values were calculated with One Way ANOVA or Kruskal-Wallis Variables are statistically indistinguishable if they share at least one letter (N=3, n=10). All graphs represent the means ± SEM.

In some studies, PBDE exposure led to a decreased level of acetylcholine (ACh). For instance, mice exposed to PBDE-209 via diet had elevated levels of Acetylcholinesterase activity in brain tissue leading to a reduction of ACh concentration (Liang et al., 2010). Similar results were obtained when exposing zebrafish larvae from 2 hpf to 5 dpf to PBDE-71 (Chen et al., 2012).

An alternative explanation for the lack of NMJs formation might be linked to diminish dendritic and neurite growth. Previous studies reported that PBDEs exposure can inhibit dendrite growth in primary cell cultures of rat cerebellum neurons, even at concentrations as low as 100 pM of PBDE-209 (Ibhazehiebo et al., 2011; Ibhazehiebo and Koibuchi, 2012; Xiong et al., 2012). Similarly, exposure to PBDE-47 and PBDE-49 (ranging from 20 pM to 2 μM) in primary cells from the rat hippocampus has been shown to delay neurite growth (Chen et al., 2017). Reduced dendritic and neurite growth can lead to the reduction of synaptogenesis at the NMJs.

Effects of PBDE on neurodevelopment and behavior has not gone unnoticed in the human population. A cohort study from Cincinnati (Ohio, USA) composed of 349 infants did not find any significant association between prenatal exposure to PBDEs and infant neurobehavior at 5 weeks of age (Vuong et al., 2016). On the other hand, the same cohort was evaluated later in life (age 3 and 5) and prenatal exposure to PBDEs was associated with cognitive deficits and motor development acting as a neurotoxicant (Chen et al., 2014). Additional cohort studies support these findings. For instance, a cohort study from Groningen (Netherlands) found that prenatal exposure to PBDEs had neurotoxicant effects and is correlated with worse fine manipulative abilities at age 5–6 (Roze et al., 2009). Moreover, a cohort of 232 Chinese infants shows no neurobehavioral impairments at the age of 1 but by age 2 gestational PBDE exposure correlates with lower language and social skills (Ding et al., 2015). Finally, in a cohort of Latino-American Californian families, an association between gestational PBDE exposure and impaired fine motor control in children age 5 (n=310) and 7 (n=323) was demonstrated. Most of these cohort studies found impairments in fine motor skills later in infant life (5–7 years of age) but not in early life (1–2 years of age). Research has shown that neurotoxicants causing an early neuron loss can lead to greater functional impairments later in life (Barone et al., 1995; Rodier et al., 1991).

In conclusion, our findings underscore the detrimental impact of PBDEs exposure on neurodevelopment, particularly through the disruption of NMJ formation, increased cell death, and deterioration of neuronal network complexity within the fish tail. These outcomes are consistent with disturbances in α-tubulin, neuron myelination and the growth of dendrites and neurites, all of which are vital for proper NMJ development. Given the potential long-term health risks posed by PBDEs through their impact on nervous system development, it is crucial to investigate further the mechanism by which PBDEs induce these adverse effects. Understanding these mechanisms will provide deeper insights into the pathogenesis of neurodevelopmental disorders and help identify potential intervention strategies to mitigate the harm caused by PBDEs. Furthermore, human studies have shown correlations between prenatal and breast milk PBDE exposure and negative impacts on neurodevelopment and motor skills, underscoring the importance of addressing this issue for public health.

Overall, our study contributes to a growing body of evidence highlighting the neurotoxic effects of PBDEs and underscores the importance of further research to elucidate the mechanisms underlying PBDEs-induced neurodevelopmental toxicity. These insights are crucial for informing regulatory measures and public health interventions to protect populations from the adverse effects of PBDEs, and possibly replacement molecules, exposure on neurodevelopment.

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## CRediT authorship contribution statement

**Cécilia Bernier:** Writing – review & editing, Investigation, Data curation. **Alec McDermott:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shunmoogum A. Patten:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Isabelle Plante:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Vanessa Piché:** Writing – review & editing, Investigation, Data curation.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Isabelle Plante reports financial support was provided by National Research Council Canada. Shunmoogum (Kessen) Patten reports financial support was provided by National Research Council Canada. Alec McDermott reports financial support was provided by Quebec Health Research Fund. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.neuro.2024.10.009.

## Data availability

Data will be made available on request.

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