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A rapid throughput approach identifies cognitive deficits in adult zebrafish from developmental exposure to polybrominated flame retardants

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Abstract

A substantial body of evidence has correlated the human body burdens of some polybrominated diphenyl ether (PBDE) flame retardants with cognitive and other behavioral deficits. Adult zebrafish exhibit testable learning and memory, making them an increasingly attractive model for neurotoxicology. Our goal was to develop a rapid throughput means of identifying the cognitive impact of developmental exposure to flame retardants in the zebrafish model. We exposed embryos from 6 hours post fertilization to 5 days post fertilization to either PBDE 47 (0.1 μ M), PBDE 99 (0.1 μ M) or PBDE 153 (0.1 μ M), vehicle (0.1% DMSO), or embryo medium (EM). The larvae were grown to adulthood and evaluated for the rate at which they learned an active-avoidance response in an automated shuttle box array. Zebrafish developmentally exposed to PBDE 47 learned the active avoidance paradigm significantly faster than the 0.1% DMSO control fish ($P < 0.0001$), but exhibited significantly poorer performance when retested suggestive of impaired memory retention or altered neuromotor activity. Learning in the PBDE 153 group was not significantly different from the DMSO group. Developmental exposure to 0.1% DMSO impaired adult active avoidance learning relative to the sham group ($n = 39$; $P < 0.0001$). PBDE 99 prevented the DMSO effect, yielding a learning rate not significantly different from the sham group ($n = 36$; $P > 0.9$). Our results underscore the importance of vehicle choice in accurately assessing chemical effects on behavior. Active avoidance response in zebrafish is an effective model of learning that, combined with automated shuttle box testing, will provide a highly efficient platform for evaluating persistent neurotoxic hazard from many chemicals.

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Conflicts of interest None

1. Introduction

Polybrominated diphenyl ether (PBDE) flame retardants entered the marketplace in the 1960's and found widespread application in textiles, electrical and electronic components, foams for automobile and airplane seats, wire insulation, and plastics for printed circuit boards and for the casings of televisions and personal computers. Being lipophilic and hydrophobic, they accumulate in aquatic and terrestrial food webs (Stapleton et al., 2003, Voorspoels et al., 2007). Since 2001, exposure to PBDEs has been associated with human developmental neurotoxicity (Eriksson et al., 2001). Motor, cognitive, and behavioral performance in 6-year-old Dutch children was correlated with maternal serum levels of PBDEs measured in the 35th week of pregnancy (Roze et al., 2009). PBDE concentrations in blood from umbilical cords have been associated with neurodevelopmental effects in children from 1 to 6 years old (Herbstman et al., 2010). High levels of PBDE congeners (BDE 47, 99, 100, 153, and 209) in human blood have been associated with reduced cognitive ability, reduced motor function, and alterations in levels of both thyroid stimulating hormone and thyroid hormone FT3 (Kicinski et al., 2012).

A small number of animal studies have indicated that developmental exposure to PBDEs produces long-lasting behavioral impacts, particularly to motor activity and cognitive functions (Costa et al., 2008). Exposure of neonatal mice and rats to PBDEs -47, -99, -153, -183, -203, -206, -209 caused hyperactivity and poorer performance in learning and memory tests (Eriksson et al., 2001, Eriksson et al., 2002, Viberg et al., 2002, 2003a, 2004, 2007, Viberg et al., 2003b, Viberg et al., 2006).

The translatability of flame retardant neurotoxic effects from animal models to humans highlights an opportunity to use a lower vertebrate model of learning to more rapidly assess neurotoxic potential of alternative flame retardants. The zebrafish is highly prolific and shares a highly conserved anatomy and physiology with higher vertebrates, while having low maintenance costs. Several paradigms have been developed to measure complex behaviors in zebrafish (Gerlai, 2012) and there are paradigms showing active avoidance responses in zebrafish (Morin et al., 2013, Rawashdeh et al., 2007, Xu et al., 2007). Active avoidance conditioning is a technique often used in psychopharmacology studies in rodents. The naïve animal has to learn to actively shuttle, at each trial, from one side to the other of a shuttle box to avoid a mild electrical shock. We report here that a rapid throughput approach to active avoidance learning is feasible using zebrafish. We built and automated the simultaneous operation of an array of 14 shuttle boxes and developed a testing paradigm to compare the effects of PBDEs 47, 99 and 153 on active avoidance learning. Our results demonstrate the utility of zebrafish cognition as an endpoint in larger scale chemical screening.

2. Materials and Methods

2.1 Zebrafish husbandry

Embryonic zebrafish were obtained from a Tropical 5D strain of zebrafish (*Danio rerio*) reared in the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University. Adults were kept at standard laboratory conditions of 28°C on a 14-h light/10-h dark

photoperiod in fish water (FW) consisting of reverse osmosis water supplemented with a commercially available salt (Instant Ocean®) to create a salinity of 600 microsiemens. Sodium bicarbonate was added as needed to adjust the pH to 7.4. Zebrafish were group-spawned, and embryos were collected and staged as described by Kimmel et al (Kimmel et al., 1995).

2.2 Chemical exposures

For static exposure of zebrafish to PBDEs, 1000× (100 µM) stock solutions of PBDE 47 (2,2',4,4'-tetrabromodiphenyl ether), PBDE 99 (2,2',4,4',5-pentabromodiphenyl ether) and PBDE 153 (2,2',4,4',5,5'-hexabromodiphenyl ether) were made from neat preparations from AccuStandard (www.accustandard.com) by dissolution in DMSO. A 1:1000 dilution in embryo medium (EM) produced a final (exposure) PBDE concentration of 0.1 µM and 0.1% DMSO. Embryos were enzymatically dechorionated at 4 hours post fertilization (hpf) (Mandrell et al., 2012) and exposed to the PBDE, 0.1% DMSO vehicle or EM from 6 to 120 hpf in 96 well plates. All treatments except EM were co-arranged on a single plate, 24 wells per treatment, and 4 duplicate plates were run. The EM treatment was run as a single, separate plate. Embryos were placed one per well into 100 µl of each test solution.

2.3 Shuttle box design

A detailed design overview with in depth description of the hardware and shuttle box software control is presented in Supplemental Materials. Briefly, the shuttle box hardware (Figure 1) consisted of an opaque shuttle box constructed of 5 mm black acrylic with an outside length of 200 mm, width of 100 mm and inside depth of 90 mm. A central divider with a 10 mm gap between the floor of the box and the bottom of the divider allowed the fish to change chambers (escape shock) while shuttling past light beam detectors. Water depth in the shuttle box was approximately 3.5 cm such that the bottom 5 mm of the central divider was immersed. The conditioned stimulus (CS) was generated by LED light bars at each box end (Figure 2) which housed three high intensity surface-mount RGB, switchable LEDs (www.blinkm.thingm.com). The unconditioned stimulus (US), mild electric shock, was provided from the potential established between stainless steel plates covering each end of the shuttle box (long axis). This enabled the entire box to be shocked when a fish was presented the US and avoided having to separately control shocking of each side of the box. Two Keyence (www.keyence.com) FS-N11P industrial thru-beam sensor/detector sets with their beams pointed across the container were mounted externally to the shuttle box and configured to detect fish shuttling from one side of the container to the other. Two sensors allowed tracking the direction the fish shuttled, thereby monitoring the fish's location without the need for video imaging. The shuttle box was controlled by an Arduino UNO R3 (www.arduino.cc) micro-controller and a custom circuit board for LED (CS) control, beam break monitoring and US shock control. For US shock voltage, the system used a pulse-width modulation (PWM) controller to vary the amount of power. The input voltage was fixed at 5.0 volts, while the duty cycle of the PWM signal determined the apparent voltage across the box. The range of values for the shock voltage to the PWM controller were 0.1 – 50.0. An empirical determination of the controller “shock voltage” settings determined that a setting of 30.0 (apparent voltage of 3.0 volts) sufficiently stimulated wildtype 5D zebrafish to escape their current location. Shocks could be applied at millisecond intervals and for

durations specified in milliseconds. The Arduino serial over USB outputs were connected via a USB hub to the host PC allowing at least 7 shuttle boxes per host, and the host PC to send & receive commands and data to/from the Arduino controller.

2.4 Shuttle box experiments

A detailed explanation of the shuttle box experimental configurations, data collection and output are presented in Supplemental Data. The experimental paradigm was based on that of Xu et al. (Xu et al., 2007) and modified to optimize the automation features of our design. All of the zebrafish tested were from the same mass spawn (from approximately 1100 breeding adults) generated on August 14, 2012. At the time of the testing reported here, the fish were 25 weeks old. Each adult zebrafish was subjected to a series of 50 consecutive trials of active avoidance conditioning (Train phase), followed by a 1 hour quiescent period, then a second series of 50 trials (Recall phase). The structure of a trial series is diagrammed in Figure 2. Briefly, a 10 minute Acclimation period was allowed after first introduction to the shuttle box and placement of the box's opaque lid. Thereafter, a trial consisted of a 12 second Avoidance period during which the side the fish was on at the end of the Acclimation period became the side to escape from (dim green light and shocked) and the side opposite was the non-shocked chamber (no light). The non-shocked chamber was always the dark side of the box. At the end of the 12 second Avoidance period, a 12 second Shock (escape) period began: if a fish never shuttled to the dark side of the chamber the entire box was shocked (3V, 500 ms duration, 1 s intervals) for the full 12 s. If a fish escaped (shuttled to the dark side) the shock was terminated immediately. Any return to the CS side during the 12 second avoidance period triggered reinstatement of the US (shock). Thus, total trial time was always 24 s while Avoidance and Shock periods were dependent on the fish's decisions. Each trial was followed by a 12 s inter trial interval (ITI) where the shuttle box displayed the non-shocked (dark) condition on both sides. The non-shocked side was always the side opposite the fish's location at the end of the last ITI. A humane 'Fault Out' limitation was encoded in the shuttle box control such that either 8 consecutive trials of a fish never shuttling to the non-shocked side would automatically halt the fish's testing. After 8 consecutive trials during which a shock was never delivered, the task was considered mastered and further testing of the fish was stopped.

2.5 Data analysis

All statistical analysis was performed using code developed in R version 3.0.1 ((R Development Core Team, 2010); www.R-project.org) and run in RStudio (www.rstudio.com). Data were recorded using custom software where Shocked (the cumulative time per trial that the fish was actually being shocked, in seconds; see Supplemental Materials) was recorded for each trial, and a total of 50 trials were conducted for Train and Recall. All output files (230) were processed and merged into one file in R for this experiment. For each treatment, the data were fit using group linear regression models and both slopes and intercepts for each testing phase were identified. A group linear regression using LOESS smoothing (Cleveland, 1979) for treatment : Train/Recall phase pair-analysis allowed observance of variance and reduced the sensitivity to outliers that occurred by evaluating on a per treatment: fish basis. Individual fish linear regressions would not provide a means of down-weighting outliers (observations outside the mean and

its bounds at every trial), but only an indication that they were present. Because outliers were a frequent occurrence at every shuttle box trial when the performance of all fish in a treatment was considered (Figure 5), we could not legitimately exclude them from any of our data without biasing analysis of the fish:trial interval. Our use of a group linear regression with LOESS smoothing, by down-weighting outliers, minimized data skewing. To determine within a treatment if there were differences in regression lines, an analysis of variance (AOV) was conducted. Once significant differences between Train and Recall phases were identified, a comparison of treatments for each phase was achieved by running an AOV with a Tukey's Honest Significant Differences (HSD) test for each pairwise comparison. For the Shock Shuttles parameter, an ordered Tukey's HSD test was used over the trial period for Train or Recall. Evaluation of the lower range value of the mean comparison identified significance between treatments (higher positive values represented larger mean differences). Figures were generated using R packages: reshape2 (Wickham, 2007) and ggplot2 (Wickham, 2009).

3. Results

The effect on survival from developmental exposure of zebrafish embryos to 1 μ M PBDE 47, 99 and 153, and the number of resulting adults tested for active avoidance learning is reported in Table 1. The 0.1 μ M PBDE exposures in 0.1% DMSO did not result in any malformations in surviving larvae by 5 days post fertilization (dpf) (Truong et al., 2011), as was desirable for ensuing behavior experiments that required unfettered swim performance. The PBDE exposures did not contribute to mortality above that caused by the 0.1% DMSO vehicle though we note that 0.1% DMSO was associated with significantly higher mortality than seen in non-exposed (embryo medium) embryos. PBDE 99 significantly mitigated the mortality associated with vehicle exposure.

3.1 Variances of active avoidance learning parameters

The shuttle box software tracked and computed a variety of fish performance parameters during each trial for putative measures of avoidance learning (see Supplemental Materials). Figure 3 reports the variances in each parameter based on the data from all experimental animals. The 'Shocked' output parameter was the cumulative amount of time per trial that a fish was shocked, i.e., on the unconditioned stimulus (US) side, when the trial was in the shock period. In addition to being an intuitive measure of active avoidance learning, the 'Shocked' parameter also displayed consistently low variance across treatments. The 'Shock Shuttles' parameter was the cumulative number of times per trial that a fish returned to the 'shocked' side after the initial shuttle to the non-shocked side, thus, initiating another shock. This parameter showed high variance but was important in distinguishing learning effects from memory effects. The 'Accept' and 'Reject' parameters, i.e., cumulative time per trial spent on the non-shocked (dark) or shocked (lighted, CS) side, respectively during the Shock period, and the 'Shock' parameter, i.e., total amount of time a shuttle box spent in Shock period, per trial, also exhibited low variance across the treatments. The 'Time To A Side' parameter, i.e., number of seconds per trial that a fish took to shuttle to the non-shocked side of the box, while an intuitive metric of learning, was too variable across treatments for

statistical comparison. Each of the other parameters described were suitable metrics of active avoidance learning, but for brevity we focused mainly on the 'Shocked' parameter.

3.2 Incidences of fish that learned to always or never avoid the unconditioned stimulus

The software control of the shuttle box trials was configured such that fish that accumulated zero shocked time over 8 consecutive trials were automatically halted from further testing and were *learners*. Similarly, fish that never shuttled to the non-shocked side for 8 consecutive trials were halted from further testing as a humane endpoint and were *non-learners*. The frequency of fish in these groups by treatment is summarized in Table 2. There were few fish in either the learner or non-learner groups as most learned to shuttle to the non-shocked side, but not without the need for impetus shocks. In the EM treatment 27.1% of fish learned to avoid the shock entirely while the next best performance was 19.4% in the PBDE 99 group. The PBDE 47 group had 13.2% learners. The DMSO and PBDE 153 treatments had the lowest percentage of learners, 7.7% and 6.9%, respectively. Most of these learner designations occurred in the Recall (second set of 50 trials) phase. The percentages of non-learners in each treatment did not correlate with the pattern seen in the learner's percentages and the non-learner designation occurred only in the Train (first set of 50 trials) phase. Because so few fish learned to completely avoid the shock, learning rate was also measured by a treatment group's ability to minimize the shocked duration (Shocked parameter).

3.2 Effect of developmental exposure to PBDE on active avoidance learning relative to the sham control

The effect on active avoidance learning in adults from developmental exposure to PBDE 47, 99 or 153 is summarized in Figure 4. The baseline for adult learning of our paradigm in the Tropical 5D zebrafish strain is represented by the sham, embryo medium group. Trial number was regressed against the amount of time that a fish spent being shocked during each trial for both the Train (50 trials) and Recall (50 trials) phase for each treatment (Figure 5). The y-intercept values from the linear regressions in Figure 5 appear on the Y-axis of Figure 4. The dot sizes in Figure 4 represent the slope of the regression line in the Train and Recall phases. Thus the y-intercept reported the treatment group's initial performance at the start of each phase and the slope reported the treatment group's improvement, or rate of learning to avoid shocking, during each phase. Note that all groups had a significant negative slope in the Train phase regression indicating that measurable learning was occurring in the first 50 trials. Also note from the plots in Figure 5 that fish from the EM and PBDE 99 treatments began the Recall phase (after 1 hr quiescent period) at the same level of shock avoidance performance with which it ended the Train phase trials. The 0.1%DMSO, PBDE 47 and 153 groups resumed the Recall phase having regressed to the performance of approximately trial 30 of the Train phase.

For all treatment groups the Recall phase showed a highly significant ($P < 0.0001$) reduction in the average time spent receiving shocks relative to the treatment's Train phase performance. For the EM and 99 groups and, to a lesser degree, the 47 group, most of the improvement in active avoidance learning occurred in the Train Phase (steeper negative slope, larger dot) with little additional learning occurring in the Recall phase. However, in

the DMSO, 47 and 153 groups learning in both phases was similar. None of the treatments blocked active avoidance learning but treatment markedly influenced the rate of active avoidance learning. Groupwise statistical comparisons of active avoidance learning rates (regression slopes by Tukey's Honest Significance Difference test) are summarized in Table 3. Fish in the EM and the PBDE 99 groups exhibited, by far, the highest rates of learning. The PBDE 99 group showed no significant difference from the EM group in its learning rate in either phase, most of the learning occurred in the Train phase and thus, both groups had the largest spread between mean y-intercepts.

3.4 PBDE effects relative to the DMSO vehicle

It was important to scale the PBDE responses relative to the learning in the 0.1% DMSO group, typically our only negative control for high throughput chemical screening. The PBDE 47 fish improved significantly faster (steeper negative slope, Figure 4 and Table 3) than the 0.1% DMSO group in both phases of the experiment. However, their initial Shocked duration was longer and the deterioration in performance between phases (Figure 5) meant that the PBDE 47 fish, on average, could never achieve the same level of shock avoidance that the DMSO fish did. This suggested that developmental PBDE 47 exposure may have fostered hyperactivity or impacted memory. We refined the PDE 47 analysis by examining the output parameter 'Shocked Shuttles' (section 3.1) for all treatments. The PBDE 47 –exposed fish shuttled back to the CS (shocked) side during the Shock period consistently and significantly more often at each trial (Table 4) than the other treatment groups.

The PBDE 99 fish learned considerably faster than the DMSO group and faster than the PBDE 47 group (Figure 4), but did not exhibit deterioration in performance between phases (Figure 5). Active avoidance learning by the PBDE 153 fish was not significantly different (Table 3) from the 0.1%DMSO learning rate (negative slope) or in degree of performance deterioration between phases.

4. Discussion

4.1 Shuttle box array throughput and performance

An array of 14 automated shuttle boxes enabled us to assay the active avoidance learning of 5 treatment groups totaling 230 adult zebrafish, in 5 days. Developmental exposure to DMSO and PBDEs 47 and 153 significantly slowed active avoidance learning. We believe the throughput achieved with this platform is unprecedented for adult learning assays in a vertebrate model.

We did not anticipate that the 0.1% DMSO vehicle would significantly impair active avoidance learning relative to the EM sham group. Almost no data on the developmental neurotoxicity of DMSO are available, but Chen et al (Chen et al., 2011) reported that 0.1% DMSO caused zebrafish larvae to exhibit hyperactivity and less complicated swimming paths, and suggested that extra caution is warranted in the interpretation of developmental behavior results when using a DMSO vehicle. Our adult learning results support a

cautionary use of DMSO. An evaluation of several organic solvent alternatives for use with the zebrafish model has recently been reported (Maes et al., 2012).

PBDE 99 was associated with modest but significant mitigation of the 0.1% DMSO effect on survival at 5 and 30 dpf (Table 1.) PBDE 99 appeared to block the negative learning effects of 0.1% DMSO, an effect that was not anticipated. But at least some of this effect was due to the fish in the PBDE 99 group having had, on average, worse performance at the beginning of training than the fish in the DMSO group, while both groups achieved similar shock avoidance by the end of the Test phase. The data do not associate developmental PBDE 99 exposure with cognitive deficits in adult zebrafish. This contrasts with the learning and memory deficits observed in adult mice from developmental exposure to PBDE 99 (Eriksson et al., 2001). Further characterization of PBDE 99 effects in an alternative vehicle, and confirmation that our results were not artifacts due to compound contamination, are necessary.

At first view, a potential doubling of the throughput of the array could be achieved by omitting the Recall phase (second 50 trial session). For example, the EM and PBDE 99 Train phase avoidance regression slopes were nearly double that of the Recall phase slopes, indicating that most of the learning occurred in the Train phase, apparently diminishing the utility of the Recall phase. However, the Recall phase revealed that the EM and PBDE 99 groups ended the Train phase and began the Recall Phase at the same performance level, but that the DMSO, 47 and 153 group performances were deteriorated between phases. This important effect would have been missed without a multipartite approach.

The deterioration effect was especially strong in the PBDE 47 fish which never achieved the final shock avoidance times that the control groups did, i.e., they did not ultimately learn or retain the avoidance conditioning as well as the control fish, even though their performance in the Train phase would suggest they were learning. The PBDE 47 fish also shuttled back to the shocked (US) side during the shock period on average twice as often, at every trial, as fish in the EM and DMSO control groups did. Together, these performance aspects would suggest that developmental PBDE 47 exposure resulted in adult hyperactivity or memory deficits in adult zebrafish. Hyperactivity and decreased thigmotaxis in adult mice and rats are known effects of developmental exposure to PBDE 47 (Eriksson et al., 2001, Gee et al., 2008, Suvorov et al., 2009). Spatial learning and memory deficit in adult rats and mice are also known effects of developmental exposure to PBDE 47 (Koenig et al., 2012, Ta et al., 2011, Yan et al., 2012).

4.2 Shuttle box array design rationale

A variety of approaches to measuring conditioned learning in fish have appeared in the literature in the last several years. These have included apparatus such as plus and T-mazes (Sison et al., 2011, Swain et al., 2004, Vignet et al., 2013) shuttleboxes of varying design (Morin et al., 2013, Rawashdeh et al., 2007, Xu et al., 2007) and single chambers (Karnik et al., 2012). The recent literature contains reports of successful conditioning using stimuli as simple as lighting changes, digitally generated light patterns or water depth (Ng et al., 2012, Valente et al., 2012), or more complex stimuli such as conspecific imagery (Gerlai, 2012, Karnik et al., 2012, Sison et al., 2011), olfactory stimuli (Morin et al., 2013) and food

reinforcement (Colwill et al., 2005, Sison et al., 2010). To facilitate automation of our active avoidance paradigm it was critical that we opted for the simplest conditioned stimulus (light changes) with mild shock reinforcement and the omission of video tracking.

4.3 Performance comparison to other fish shuttle box studies

The shuttle box controls we developed (see Supplemental Materials for full description) are flexible enough that a wide range of parameters for this CS (light)-US (shock) combination can be easily configured within a single software window. The active avoidance paradigm we reported achieved statistical robustness, and hence confidence in the relative rates of learning among the treatments. However, we acknowledge an important discrepancy with previous studies: While our use of the *learner* criterion (conditioning to the point of shock avoidance without return to the US side) was insufficient for our analysis due to < 30% of the fish in any treatment meeting the criterion, a similar criterion was met by 60–80% of untreated adult zebrafish in two previous studies (Rawashdeh et al., 2007, Xu et al., 2007, Xu et al., 2012). Xu et al. initially obtained the 60% metric in pet store zebrafish of unknown lineage and Rawashdeh et al. obtained a >80% metric in the common AB strain. This would strongly suggest that the parameters controlling behavior in our active avoidance task still require significant improvements. While genetic background may account for some of the discrepancy, it is more likely that the differences were methodological. For instance, our use of a 12 s inter trial interval (ITI) was similar to that of Xu et al. (Xu et al., 2007, Xu et al., 2012), but Rawashdeh et al. used no ITI while Yliff et al. (Yliff et al., 2008) reported that an 80 s ITI was optimal in Nile tilapia and goldfish when the US shocks were discontinuous (pulsed), but that a 20 s ITI was optimal with continuous shock. The strength of the shock stimulus that we used was based on these same previous reports and adopted by us because a 3 – 5 volt application to the shuttle box elicited a visible escape response from untreated adult zebrafish. However, the voltages range, under our water conductance parameters, may have been inadequate to condition adult zebrafish to not challenge the shock. Voltage and pulse pattern should be further investigated as a means toward more robust conditioning.

The pattern of shuttling between chambers in the present study is another important difference from previous studies. Our automated paradigm always set the non-shocked side to be opposite the fish's location at the end of the initial Acclimation period and thereafter at the end of the ITI. Thus, with our use of a 12 s ITI for which both sides of the shuttle box were dark, the fish experienced 12 s of free swim and, therefore, no pattern of CS-US side switching was established. The highest learning paradigm (>80%) reported by Rawashdeh et al. (Rawashdeh et al., 2007) used no ITI and, after each trial, the compartments containing the US (shock stimulus) and CS (light-stimulus) were switched, thus establishing a regular back and forth pattern throughout trialing. One might reasonably expect that an A-B pattern of CS-US side switching would enhance the efficiency of active avoidance conditioning relative to a similar paradigm without the spatial pattern. The other previously cited shuttle box paradigms for zebrafish, tilapia and goldfish did not specify the pattern of shock versus no-shock presentations.

4.4 Conclusions

Assessing adult neurotoxicity associated with low level developmental exposure is an important frontier in toxicology. Zebrafish is an excellent model in which to develop the throughput necessary to query developmentally persistent neurotoxicity using large scale chemical screens. To that end we emphasized a rapid throughput approach for assessing active avoidance learning and evaluated our design with several polybrominated flame retardants with known cognitive effects in mammals. Learning rate, as opposed to a somewhat arbitrary learning criterion, was a robust metric and obviated the need for lengthy empirical development of a learning paradigm or assiduous replication of previously reported paradigms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Our goal was to develop a rapid throughput means of identifying the cognitive impact of developmental exposure to flame retardants in the zebrafish model.

Zebrafish developmentally exposed to PBDE 47 learned the active avoidance paradigm significantly faster than the 0.1% DMSO control fish.

Zebrafish developmentally exposed to PBDE 47 exhibited a worsening performance upon retest suggesting poorer memory retention or altered motoric effects.

0.1% DMSO impaired adult active avoidance learning relative to the sham group.

PBDE 99 prevented/inhibited/blocked the adverse effects of DMSO.

The zebrafish is an effective model of cognitive learning that, combined with automated shuttle box testing, will provide a highly efficient platform for evaluating persistent neurotoxic hazard from many chemicals.

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Figure 2.

Timing of the different periods of an active avoidance trial series. The Avoidance period could last up to 12 seconds before the Shock period automatically started. The first shuttle to the non-shocked (escape) side also triggered the beginning of the Shock phase, though a fish could only be shocked if it remained on or re-entered the shocked (US) side.

Figure 3.

Variance in six parameters of active avoidance performance. The Shocked parameter (cumulative time per trial spent receiving shocks) was focused on due to its low variance and intuitive measure of active avoidance. The Shock Shuttles parameter (number of shuttles at each trial during the shock period) was subsequently used as a primary metric of forgetting or altered motor activity.

Figure 4.

Summary of active avoidance learning in adult zebrafish developmentally exposed to PBDE flame retardant. Dot size represents the negative slope value (learning rate) from the regression analyses of Shocked duration versus trial number. Position on the y-axis is the y-intercept (initial shocked duration at the start of each phase) from the regression analyses. The large spread between phases for the EM and PBDE 99 groups indicated that little additional learning for those groups occurred in the Recall phase and that there was minimal degradation in performance between phases. The opposite was true for the other treatments, especially for PBDE 47.

Figure 5. Active avoidance learning represented as Shocked duration at each trial for each fish in a treatment. The regression lines indicate the rate of learning in each phase of the experiment. The Train (red) and Recall ('Test' line on graph) phases were separated by a 1 hour quiescent period where both sides of the shuttle box were dark.

Table 1

Impact of developmental PBDE exposure on zebrafish survival, morphology and final number of adults used in assessment of active avoidance learning.

Chemical exposure	Initial N	5 dpf survival N	Morphology	30 dpf survival N	Active avoidance tested N ^a
Embryo medium	96	95/99%	Normal	94/98%	59
0.1% DMSO	96	82/85.4%	Normal	61/63.5%	39
PBDE 47 [*]	96	81/84.3%	Normal	56/58.3%	38
PBDE 99 [*]	96	89/92.7%	Normal	69/71.8%	36
PBDE 153 [*]	96	81/84.3%	Normal	58/60.4%	58

^{*} PBDEs were each at a 0.1μM final concentration in 0.1% DMSO.

^a Adult fish were 25 weeks old at the time of shuttle box tests.

Table 2

Incidence of adult zebrafish that learned to *always* avoid the shock (learners) or that *never* avoided the shock (non-learners).

Treatment	N	Learners ^a			Non-learners ^b		
		Total	Train ^c	Recall ^c	Total	Train ^d	Recall ^d
EM	59	16 (27.1%)	5	11	3 (5.1%)	3	0
0.1% DMSO	39	3 (7.7%)	1	2	1 (2.6%)	1	0
PBDE 47	38	5 (13.2%)	3	2	3 (7.9%)	3	0
PBDE 99	36	7 (19.4%)	0	7	5 (13.9%)	5	0
PBDE 153	58	4 (6.9%)	1	3	1 (1.7%)	1	0

^aTotal number of fish (and percent of N) from treatment that avoided receiving a shock without returning to the unconditioned stimulus for 8 consecutive trials.

^bTotal number of fish (and percent of N) from each treatment that never escaped the entire 12 second shock period for 8 consecutive trials at which point testing was automatically aborted.

^cIndicates in which phase the 8th consecutive successful trial occurred.

^dIndicates in which phase the 8th consecutive failed trial occurred.

Table 3

Groupwise statistical comparisons of active avoidance learning rates.

Comparison	Train phase P-value ^a	Recall phase P-value
EM to 0.1% DMSO	0.60281	1.00E-06
EM to PBDE 47	1.00E-06	1.00E-06
EM to PBDE 99	0.18682	0.99873
EM to PBDE 153	0.02576	1.00E-06
0.1% DMSO to PBDE 47	1.00E-06	0.00126
0.1% DMSO to PBDE 99	0.95223	1.00E-06
0.1% DMSO to PBDE 153	0.74049	0.98683
PBDE 47 to PBDE 99	0.00011	1.00E-06
PBDE 47 to PBDE 153	7.00E-05	0.00215
PBDE 99 to PBDE 153	0.99448	1.00E-06

^a P-value from Tukey's Honest Significant Difference test of pairwise comparisons of individual ANOVAs for the regressions in Figure 5.

Table 4

Comparison of the mean number of shock period shuttles in each treatment to those in the 0.1% DMSO control group.

Comparison	Train phase mean difference ^a	Train phase P- value ^b	Recall phase mean difference	Recall phase P-value
0.1% DMSO to EM	0.30768	0.83449	0.44927	0.25654
0.1% DMSO to PBDE 47	7.45172	1.00E-06	4.63178	1.00E-06
0.1% DMSO to PBDE 99	0.89718	0.04792	0.19842	0.93165
0.1% DMSO to PBDE 153	1.42576	1.00E-05	0.54345	0.09259

^aThe mean number of shuttles in the 0.1% DMSO groups was 5.4 ± 0.2 in the Train phase and 3.0 ± 0.2 in the Recall phase.

^bP-value from Tukey's Honest Significant Difference test of pairwise comparisons of individual ANOVAs.