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Full Length Article

Toxicant induced behavioural aberrations in larval zebrafish are dependent on minor methodological alterations

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ABSTRACT

Alterations in zebrafish motility are used to identify neurotoxic compounds, but few have reported how methodology may affect results. To investigate this, we exposed embryos to bisphenol A (BPA) or tetrabromobisphenol A (TBBPA) before assessing larval motility. Embryos were maintained on a day/night cycle (DN) or in constant darkness, were reared in 96 or 24 well plates (BPA only), and behavioural tests were carried out at 96, 100, or 118 (BPA only) hours post fertilisation (hpf). We found that the prior photo-regime, larval age, and/or arena size influence behavioural outcomes in response to toxicant exposure. For example, methodology determined whether 10 μM BPA induced hyperactivity, hypoactivity, or had no behavioural effect. Furthermore, the minimum effect concentration was not consistent between different methodologies. Finally, we observed a mechanism previously used to explain hyperactivity following BPA exposure does not appear to explain the hypoactivity observed following minor alterations in methodology. Therefore, we demonstrate how methodology can have notable implications on dose responses and behavioural outcomes in larval zebrafish motility following identical chemical exposures. As such, our results have significant consequences for human and environmental risk assessment.

1. Introduction

The zebrafish (*Danio rerio*) is an established vertebrate model in developmental biology and is becoming increasingly popular as a tool for identifying neurotoxic compounds. In particular, a growing number of studies use larval zebrafish motility during light/dark cycles to test new and existing compounds for neurotoxicity (i.e. Jarema et al., 2015; Noyes et al., 2015). Importantly, the results from such tests share a general agreement with those of other vertebrate models and cell lines (Ali et al., 2012; Irons et al., 2010; Rubinstein, 2006; Ton et al., 2006), thereby demonstrating the potential for zebrafish larvae in toxicity screening. However, compared to rodent models, zebrafish behavioural research is still in its infancy and there is little standardisation between studies on larval motility. This is a concern, as little is known about how alterations in methodology may influence toxicity testing.

The most common test when using larval zebrafish is to assess motility during alternating periods of light and dark at approximately 120 h post fertilisation (hpf). Here, one expects control larvae to show freeze behaviour during periods of bright light, but a sharp increase in motility following the onset of darkness (Jarema et al., 2015). However, activity levels are known to be influenced by the timing of the experiment. For example, larvae are typically reported to become more active in the light with increasing age (i.e. Esch et al., 2012) and the level of activity during the dark period is also known to change throughout the day (MacPhail et al., 2009). The reasons behind these observations are unclear, but the larvae are growing rapidly and the brain is continuously maturing during this life period (Wullimann and Knipp, 2000). To date, little information exists as to the extent of these changes in basal activity on toxicant responses.

There are several examples within the literature of inconsistent dose and/or behavioural responses with the same compound. For example, the brominated flame retardant tetrabromobisphenol A (TBBPA) was recently reported to reduce larval motility, from 64 nM (Noyes et al., 2015) up to 5 μM (Chen et al., 2016), whereas the plasticiser bisphenol A (BPA) has been found to induce hyperactivity with peak activity at 100 nM (Kinch et al., 2015; Saili et al., 2012). In contrast, Jarema et al.

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(2015) reported no behavioural effects of TBBPA following developmental exposure at concentrations between 1.2–3.8 μM. Similarly, Wang et al. (2013) found BPA induced hypoactivity between 1 and 15 μM, whereas Saili et al. (2012) found no behavioural effects at 1 or 10 μM. These studies all used minor alterations in methodology in comparison to one another, which included differences in larval age and the time of testing, as well as water temperature, photo-periods before and during testing, strain, toxicant purity, dosing regimen (single vs multiple), and the number of light cycles during the test, to name but a few. Of particular note, Noyes et al. (2015) and Saili et al. (2012) reared their embryos/larvae in constant darkness prior to behavioural testing (R. Tanguay, personal communication), whereas Jarema et al. (2015) and Wang et al. (2013) used a day/night (DN) cycle during rearing. This is of particular interest, as photoperiods play an important role in fish developmental biology and the development of circadian rhythms (Hurd and Cahill, 2002), but the effect on toxicity testing remains unknown.

Initially, our objective was to investigate whether larval age during day five (*i.e.* 96, 100, and 118 hpf) or the photo-regime during rearing, continuous darkness *vs.* a DN cycle, could influence the behavioural effects on larval zebrafish motility exposed to different doses of either BPA or TBBPA. Leading on from this, we explored whether a mechanism previously identified to explain hyperactivity in larval zebrafish exposed to BPA, whereby BPA acted via androgen receptors to induce aromatase B expression (Kinch et al., 2015), could explain the hypoactivity we observed when using an alternative methodology. In addition, we also assessed whether rearing larvae in constant darkness could influence the level of anxiety-like behaviour compared to larvae reared on a DN cycle, and whether the arena size could influence behavioural results in response to BPA exposure.

2. Methods

2.1. Chemicals

Stock solutions of TBBPA (97% purity, Sigma Aldrich), BPA (> 99% purity, Sigma Aldrich), fluvestrant (ICI, > 98% purity, Sigma Aldrich), flutamide (FLU, Sigma Aldrich), fadrozole hydrochloride (FAD, ≥98% purity, Sigma Aldrich), and 17α-ethinyl estradiol (EE2, Sigma Aldrich) were prepared in dimethyl sulfoxide (DMSO, high performance liquid chromatography grade, Sigma Aldrich). The final concentration of DMSO in all test concentrations of TBBPA, BPA, ICI, FLU, FAD, EE2, and the solvent control, was 0.01%.

2.2. Fish husbandry

The study was performed at the Section for Experimental Biomedicine at The Norwegian University of Life Sciences, Oslo, Norway. The unit is licensed by the Norwegian Animal Research Authority (NARA) (www.mattilsynet.no) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (www.aaalac.org). The study was carried out under the regulations approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) following Norwegian laws and regulations controlling experiments and procedures on live animals in Norway.

AB wild-type zebrafish were maintained at 28 °C under a 14:10 light/dark photoperiod at the Norwegian University of Life Sciences (NMBU), Oslo, Norway. Adult care and breeding were conducted in accordance with the protocols under the IACUC. Fish were fed three times per day, twice with artemia and once with dry feed (Special Diet Services, UK). To generate embryos, adults were placed in spawning tanks in the afternoon, the fish were spawned following the onset of light (08:00) the next day, and the embryos collected (09:00) and maintained in embryo media (60 μg/ml Instant Ocean® sea salts) until the time of exposure.

2.3. Chemical exposure

Fertilised embryos were transferred by pipette into clear polystyrene 96-well plates (Nunc™ MicroWell™) and continuously exposed under static conditions to TBBPA, BPA, or the solvent control from 6 to 7 hpf until the time of testing (i.e. the larvae were tested with the chemical present in the media). One embryo was placed into each individual well. All behavioural experiments were repeated six times.

For the initial comparison of larval age and rearing photo-regime, five nominal concentrations of BPA ranging from 1 nM to 10 μM and a solvent control were equally distributed across two 96 well plates (n = 8/concentration/96 well plate. Total n = 96/group in total). For TBBPA, five nominal concentrations, ranging from 150 pM to 1.5 μM, and a solvent control were equally distributed across one 96 well plate (n = 16/concentration. Total n = 96/group). The highest concentrations of both TBBPA and BPA were below those found to be teratogenic (< 5% of the embryos from any one group were dead and/or deformed).

For EE2, one nominal concentration of 10 nM and a solvent control were equally distributed across a 96 well plate (n = 12/concentration. Total n = 72/group). We selected this dose of EE2 as it induces aromatase B overexpression in larval zebrafish (Brion et al., 2012; Chung et al., 2011).

For mechanistic studies, larvae where co-exposed to BPA and either ICI (1 μM), FLU (6.18 μM), or FAD (1 μM). Here, embryos were distributed over one 96 well plate (n = 8/treatment. Total n = 48/group).

To assess thigmotaxis, larvae were distributed in 24 well plates and embryos were exposed from 6 to 7 hpf until the time of testing. Only one treatment (10 μM and 100 nM BPA for the DN cycle and constant darkness, respectively), plus the solvent control were used per plate (n = 12/concentration/replicate. Total n = 72/group).

When 24 well plates were used, 1 ml of exposure media was added to each well, whereas only 200 μl of media was used for each well in 96-well plates. For all exposures, embryos were reared in an incubator at 28 °C. The light cycle within the incubator was 14:10 light/dark. Where complete darkness was required, the embryos were reared in the same incubator as the embryos on a DN cycle, but the well plate was wrapped in aluminium foil.

2.4. Larval behaviour

Behavioural tests were conducted using a ViewPoint® Zebrabox and the accompanying video tracking software (ViewPoint Life Sciences, Lyon, France). A difference of ≥ 5 pixels between each consecutive frame (25 frames per second) was set as the threshold for the detection of movement. Behavioural testing was undertaken at three time points for BPA, 96, 100, and 118 hpf, two time points for TBBPA, 96 and 100 hpf, and one time point for EE2, 96 hpf. These corresponded to tests beginning 90 min (09:00), 330 min (13:00), and immediately after the cessation of light in the incubator (07:30), for 96, 100, and 118 hpf, respectively. For each time point, different larvae were used to assess behaviour (i.e. no individual larvae was tested more than once). Larval behaviour, including the cumulative distance travelled and the time spent active per minute, were simultaneously measured for all larvae on a plate during a light-dark-light cycle that lasted for a total of 40 min and consisted of 20 min of light, 10 min of darkness, and a final 10 min of light. The light level was set to 100% (7.45 Klux, TES 1337 light meter) on the ViewPoint software during the lighted periods, and 0% (0 lx) during the dark periods when infrared light is used to track larval activity. When using 24 well plates, the arena was split into two zones, a centre zone and an outer zone, to assess thigmotaxis as detailed in Schnorr et al. (2012). Previous work has demonstrated that 5 dpf larvae treated with anxiolytic compounds are more active in the inner zone, whereas anxiogenic compounds increase the amount of movement in the outer zone (Schnorr et al., 2012; Richendrfer et al., 2012). After the