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RESEARCH ARTICLE

Effects of 4-methylbenzylidene camphor (4-MBC) on neuronal and muscular development in zebrafish (*Danio rerio*) embryos

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Abstract The negative effects of overexposure to ultraviolet (UV) radiation in humans, including sunburn and light-induced cellular injury, are of increasing public concern. 4-Methylbenzylidene camphor (4-MBC), an organic chemical UV filter, is an active ingredient in sunscreen products. To date, little information is available about its neurotoxicity during early vertebrate development. Zebrafish embryos were exposed to various concentrations of 4-MBC in embryo medium for 3 days. In this study, a high concentration of 4-MBC, which is not being expected at the current environmental concentrations in the environment, was used for the purpose of phenotypic screening. Embryos exposed to 15 μM of 4-MBC displayed abnormal axial curvature and exhibited impaired motility. Exposure effects were found to be greatest during the segmentation period, when somite formation and innervation occur. Immunostaining of the muscle and axon markers F59, znp1, and zn5 revealed that 4-MBC exposure leads to a disorganized pattern of slow muscle fibers and axon pathfinding errors during the innervation of both primary and secondary motor neurons. Our results also showed reduction in AChE activity upon 4-MBC exposure both in vivo in the embryos (15 μM) and in vitro in mammalian Neuro-2A cells (0.1 μM), providing a possible mechanism for 4-MBC-induced muscular and neuronal defects. Taken together, our results have shown that 4-MBC is a teratogen and influences muscular and neuronal development, which may result in developmental defects.

Keywords 4-Methylbenzylidene camphor · Zebrafish embryos · Somite · Neuron · Acetylcholinesterase · Toxicity

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Introduction

Ultraviolet (UV) filters are active ingredients used in sunscreens as well as in many other personal care products to protect human skin from the negative effects of solar UV radiation (Schlumpf et al. 2004). Because of their high photostability and lipophilicity, UV filters tend to bioaccumulate in aquatic organisms and humans. In humans, dermal absorption is the major route of UV filter exposure (Hagedorn-Leweke and Lippold 1995). A previous study identified six UV filters in two fish species sampled in Meerfelder Maar lake in Germany (Nagtegaal et al. 1997), suggesting that UV filter used by humans can also result in the occurrence in the food chain (Soto and Sonnenschein 2005).

4-Methylbenzylidene camphor (1,7,7-trimethyl-3-[(4-methylphenyl)methylene]-bicyclo[2.2.1]heptan-2-one; 4-MBC), an organic camphor derivative, is a commonly used organic chemical UV filter in sunscreens. 4-MBC dissipates absorbed radiation as heat by photo-induced geometrical

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isomerization (*cis-trans* photoisomerization). 4-MBC is likely to accumulate in the human body due to its high lipophilicity (log K_{ow} = 5.1, Balmer et al. 2005), and it was reported that 4-MBC concentration in plasma increased significantly in humans after daily repetitive application (Janjua et al. 2004). There is also a wealth of knowledge on 4-MBC toxicity in relation to estrogenic endocrine disruption (Schlumpf et al. 2001; Lichtensteiger et al. 2002; Durrer et al. 2005; Klann et al. 2005; Hofkamp et al. 2008). In addition, the risk of estrogen-mediated cancer associated with 4-MBC exposure has also been described. For example, proliferation of MCF-7 breast cancer cells is shown to be elevated by 4-MBC (Schlumpf et al. 2001, 2004). However, little information is known about other adverse effects of 4-MBC, especially effects on early vertebrate development.

4-MBC is a terpene ketone, a group which includes well-documented acetylcholinesterase (AChE) inhibitors. An in vitro study has shown that essential oils (monoterpenes) extracted from *Melissa officinalis* and *Rosmarinus officinalis* caused AChE inhibition (Howes et al. 2003). In addition, stronger AChE inhibition has been reported for terpene ketones in comparison with other terpenes, like terpene alcohols and terpene hydrocarbons (Miyazawa et al. 1997). The chemical properties and usage of 4-MBC thus indicate that it is important to study its AChE inhibitory activity and potential interactions between AChE inhibition and 4-MBC-induced developmental defects.

In this study, zebrafish (*Danio rerio*) embryos were used as a model to study the teratogenicity of 4-MBC during early vertebrate development and to elucidate the underlying mechanisms involved. Zebrafish have been highlighted by the National Institutes of Health (NIH) as an effective model organism for developmental toxicity studies (Mirkes et al. 2003). Zebrafish embryos have several advantages, including their small size, well-known developmental patterns, and their capacity to rapidly absorb low-molecular-weight compounds, diluted in the surrounding media, through their skin and gills (Langheinrich 2003; Zon and Peterson 2005). To investigate the potential health risks of environmental pollutants, knowledge on the degree of exposure is required, as well as on dose–response relationships and the mode of action of the substance.

In this work, we studied the morphological defects and abnormal behavior led by 4-MBC exposure. We also investigated the effects of 4-MBC on muscular and neuronal development using immunostaining of different muscle/neuron biomarkers. We have also demonstrated the 4-MBC-induced inhibitory effect of acetylcholinesterase (AChE) via enzymatic activity staining. We aimed to study the embryonic toxicity of 4-MBC, and our data suggested that 4-MBC functions as an AChE inhibitor to cause impairment in early vertebrate development.

Materials and methods

Zebrafish maintenance and embryo collection

Wild-type zebrafish (*D. rerio*) were cultured, and embryos were collected as described previously (Cheng et al. 2000). Embryos were collected during the first hour of the light period in the 14:10-h light/dark cycle. All embryos were collected and incubated in embryo medium (reverse osmosis filtered water with Instant Ocean dissolved at 60 mg/ml) at 28.5 °C until chemical treatments. Healthy and normally developing embryos were selected under a stereo-microscope at 4 h post-fertilization (hpf), the sphere stage of the blastula period. All embryos were handled in accordance with the license for the control of experiments on animals approved by the Department of Health of the Government of the Hong Kong SAR (Ref (11–8) in DH/HA&P/8/2/5 Pt.1).

Chemical exposures

In order to determine lethal and effective concentrations (LC_{50} and EC_{50}, respectively), zebrafish embryos were exposed to a concentration series of 4-MBC (HPLC >98 %, US Pharmacopeia, Rockville, MD, USA) at 0, 1, 5, 10, 13.75, 17.5, 21.25, 25, and 50 μM. DMSO (Sigma, St. Louis, MO, USA) was used as a carrier solvent for 4-MBC in embryo medium. The final concentration of DMSO in the treatment medium was 0.1 % (*v*/*v*), a concentration which was shown to cause no observable developmental defects to zebrafish embryos (Hallare et al. 2004; Chen et al. 2011). Exposure began after the embryo selection from approximately 4 hpf. Embryos were kept in 60-mm glass Petri dishes. Triplicates consisting of 20 embryos per dish were incubated with 10 ml of the control, solvent control, or 4-MBC solutions. 1-Phenyl-2-thiourea (PTU) (Sigma) was applied to each dish at 0.003 % (*w*/*v*) to suppress the expression of pigment (Westerfield 1993). The embryos were exposed to 4-MBC until 72 hpf. The results were plotted, and LC_{50} and EC_{50} at 72 hpf were determined. For further studies of developmental effects, a concentration of 15 μM was used for treatment.

It has been reported that the process of segmentation during somitogenesis is important in teleost trunk development (Chow and Cheng 2003). In order to investigate the critical period of exposure which caused the altered axial curvature phenotype, embryos were exposed to 4-MBC at 15 μM during the gastrulation period only (4–10.5 hpf), the segmentation period only (10.5–24 hpf), or both periods (4–24 hpf). Effects of 4-MBC exposure after the somitogenesis were also investigated (24–72 hpf). Embryos were then transferred to clean embryo medium and maintained after until 7 days post-fertilization (dpf) to investigate whether 4-MBC-induced morphological effects were irreversible.

Chemical analysis

The solvents used for chemical analysis and as the mobile phase in the instrumental analysis were Milli-Q water (Millipore, Bedford, MA, USA), HPLC-grade methanol (Sigma-Aldrich, St. Louis, MO, USA), HPLC-grade ethyl acetate (Duksan, Gyeonggi-do, Korea), and GC-grade *n*-hexane (Tedia, Fairfield, OH, USA). Embryo medium samples were extracted by solid-phase extraction (SPE) using 60-mg HLB cartridges purchased from Waters (Milford, MA, USA). Each cartridge was preconditioned successively with 6 mL *n*-hexane, 6 mL of 50:50 *v*/*v* methanol/ethyl acetate (MeOH/EA), and 9 mL of Milli-Q water. After loading the water samples and washing, the extraction cartridges were dried under a vacuum for 10 min. The target compound was eluted from the cartridges using 3 × 3 mL of *n*-hexane. Sample extracts were blown to dryness under a gentle stream of nitrogen, and the final volume was adjusted to 0.5 mL with methanol.

Twenty embryos were collected in 1.5-mL centrifuge tubes and stored at −80 °C until analysis. One hundred-microliter Milli-Q water was used to homogenize embryos, and 900 μL Milli-Q water was used to rinse the tubes after homogenization. Each homogenized extract was transferred to a glass tube, and 2 mL of *n*-hexane was added to extract the target compound through vortex extraction for 1 min followed by centrifugation for 10 min at 3000 rpm. The vortex extraction was repeated three times for each sample. The supernatant (approximately 6 mL in total) was transferred to another glass tube and blown to dryness under a gentle stream of nitrogen. Final volume was adjusted to 0.5 mL with methanol and transferred to amber sample vials before instrumental analysis. All sample extracts were analyzed by a high-performance liquid chromatography-electrospray ionization-tandem mass spectrometer (HPLC-ESI-MS/MS) composed of an Agilent HP1100 LC (Agilent, Palo Alto, CA, USA) interfaced with an AB SCIEX API 2000 triple quadrupole tandem MS equipped with a TurboIonSpray source operated in both negative and positive modes (AB SCIEX, Framingham, MA, USA). A 10 μL aliquot of extract was injected onto an XBridge™ C_{18} column (Waters Corporation, 5 μm, 2.1 mm × i.d. 50-mm length) equipped with a guard column at a flow rate of 0.3 mL min^{-1} using pure Milli-Q water (A) and pure methanol (B) in a gradient elution (0 min, 5 % B; 15 min, 100 % B; 20 min, 100 % B; 20.1 min, 5 % B; 30 min 5 % B). Analytes were determined by ESI-MS/MS in positive mode by multiple reaction monitoring (MRM). TurboIonSpray source and MS/MS parameters were as follows: curtain gas (CUR), 15 psi; collision gas (CAD), 5 psi; ion spray voltage, 4000 V; temperature, 500 °C; ion source gas 1 (GS1), 60 psi; and ion source gas 2 (GS2), 70 psi. The linear response range of the HPLC-MS/MS instrument was investigated with standards at seven different concentrations ranging from 12.5 to 400 μg/L. Within this range, the system provided linear response plots (peak area versus standard concentration) with linearity (R^2) at 0.998. 4-MBC was identified by comparing retention time and the ratio of the two selected precursor-product ion transitions with that of the calibration curve. Recovery experiments for water and fish were determined by spiking a known concentration (20 ng) of 4-MBC prior to extraction in triplicate and comparing the results with calibration curve. Bioconcentration factor (BCF) was used to investigate the partitioning behavior of 4-MBC to the embryos. It was calculated by dividing the average measured concentration in the embryo medium to the average measured concentration in zebrafish tissue at each time point.

Motion capture

In order to investigate whether motility was affected in 4-MBC-treated embryos, the swimming activity of the untreated control embryos and 4-MBC-treated embryos was recorded at 72 hpf, a time when they should be able to swim freely. The swimming activity was recorded every 2 min; three embryos were recorded each time, and the observation was repeated five times. The motion and movement of the embryos were observed and recorded for 10 min by digital videomicroscopy using a Leica M205 FA fluorescent stereomicroscope equipped with an AG 1200s (PCO, Germany) high-speed camera and LAS AF software.

Tactile response

To investigate the behavioral consequences of altered axial curvature resulting from 4-MBC exposure, the response to tactile stimulation of untreated control embryos and 4-MBC-treated embryos was recorded. Five representative embryos were selected from the control and treatment groups, and live images were recorded five times at intervals of 60 ms. Embryos at 72 hpf were gently touched on the head with a probe (blunt end of a glass needle) controlled by a robotic manipulator. Embryos that swam away after one or two stimulations were scored as responders. All others were scored as non-responders. Selected examples were recorded by digital videomicroscopy using a Leica M205 FA fluorescent stereomicroscope equipped with an AG 1200s (PCO, Germany) high-speed camera and LAS AF software.

Whole-mount immunohistochemistry

The impairment of swimming activity by 4-MBC-exposure led us to study the effects of 4-MBC on slow muscle development. A previous study showed that slow locomotory activity is powered by slow muscle (Altringham and Ellerby 1999). Slow muscle fiber organization was examined by whole-mount immunostaining with F59 antibody, and the number of embryo showing observable fiber disorganization

was scored. Neuronal innervation is an important step during somitogenesis. The effect of 4-MBC on the innervation of somites by motor neurons was also studied. Motor neuron projection was examined with the antibody znp1, which labels the extending axons of all primary motor neurons. The primary motor axon CaP (caudal) is normally projected dorsally and can be detected in the anterior half of the somite. The secondary motor neurons develop slightly later (Zhang et al. 2001). Their axons extend to the ventral myotome in the same way as CaP. The expression pattern was revealed by immunostaining with zn5 antibody. The number of embryos showing axon growth defect and the axon length was scored and measured after the staining. Embryos were collected at 28, 48, and 72 hpf, washed three times in phosphate-buffered saline with 1 % Triton X-100 (PBT), and fixed in 4 % paraformaldehyde (PFA) buffer at 4 °C overnight. Whole-mount immunostaining was performed according to the method described by Cheng et al. (2000). Culture supernatants against the following markers were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, IA, USA): zn12 (1:250; Metcalfe et al. 1990), F59 (1:50; Crow and Stockdale 1986), and znp1 (1:200; Trevarrow et al. 1990) and from the University of Oregon Monoclonal Antibody Centre: Zn5 (1:100; Fashena and Westerfield 1999). The secondary antibody was Alexa 488-linked goat anti-mouse (Molecular Probes Inc, Eugene, OR, USA) used at a dilution of 1:200. Images from whole-mount-stained embryos were collected using an upright compound microscope (Olympus, BX61) equipped with disc spinning unit (DSU) system and acquired by Electron Magnifying (EM) CCD camera (QImaging, MGi plus).

AChE activity detection

Detection of AChE activity in fixed embryos was determined as described by Karnovsky and Roots (1964)). Embryos at 24 hpf ($n = 10$) were fixed overnight with 4 % PFA. Fixed embryos were then incubated in 60 mM sodium acetate buffer (5 mM sodium citrate, 4.7 mM $CuSO_4$, 0.5 mM $K_3(Fe(CN)_6)$, 1.7 mM acetylthiocholine iodide, pH 6.4) for 3 to 4 h and washed extensively with PBT before the observation under an Olympus SZX12 stereomicroscope connected to a color digital cooled Olympus charge-coupled device (3CCD) camera.

Neuro-2a cell culture and 4-MBC incubation

The Neuro-2a mouse neuroblastoma cell line (ATCC, CCL131; ATCC, Manassas, VA) was cultured in 75-cm^2 tissue culture flasks at 37 °C in 5 % CO_2 using RPMI 1640 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10 % heat-inactivated fetal bovine serum (BD Biosciences, San Jose, CA, USA), 2 g/L Na_2CO_3, and antibiotic solution (50 units/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml Fungizone®; Gibco Life Technologies). Cells were seeded onto a 96-well plate and allowed to attach for 24 h. Cells were then incubated with 4-MBC or carbaryl (a known AChE inhibitor) (HPLC 99.7 %, Riedel-de Haën) at concentrations of 0, 0.1, 1, 10, and 100 μM for 45 min. Upon completion of exposure, the medium was removed and the harvested cells were tested for AChE activity using Ellman's method (Ellman et al. 1961). Cells were incubated with Ellman reagent for 60 min. The AChE activity of each treatment was represented by % absorbance read at 412 nm on a spectrometer relative to that of controls. Absorbance was corrected for blank and non-specific reduction of the chromogen.

Statistical analysis

Dose–response curves for 4-MBC were plotted using GraphPad Prism (version 2.0, GraphPad Software Inc., San Diego, CA) and are presented as mean ± SD. Significant differences between the control 4-MBC-exposed embryos were determined by one-way ANOVA with Dunnett's multiple comparison test performed using SPSS (version 19, SPSS Inc., Chicago, IL).

Results

Mortality and effects of 4-MBC on early developing zebrafish embryos

Zebrafish embryos were selected and exposed to different concentrations of 4-MBC (0, 1, 5, 10, 13.75, 17.5, 21.25, 25, and 50 μM) at 4 hpf and were scored for mortality and trunk malformation after 72 h of exposure. The results indicated that mortality increased with increasing 4-MBC concentration in a dose-dependent manner. Below 10 μM, mortality was less than 10 %. There was a sharp increase in mortality from 15 to 25 μM, and all embryos died at concentrations greater than 25 μM. The LC_{50} at 72 hpf was determined to be 19.82 μM (Fig. 1).

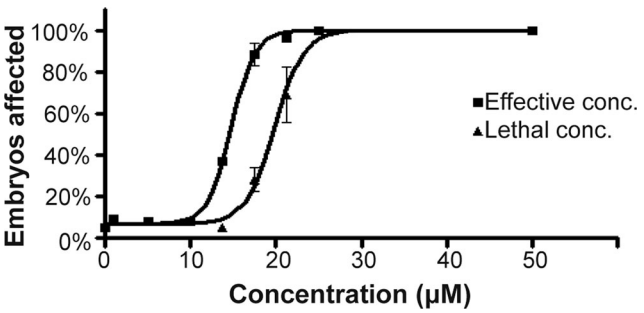

Fig. 1 Dose–response curves for embryos exposed to 4-MBC from 4 to 72 hpf. The altered axial curvature was investigated and scored ($n = 20$). The LC_{50} of 4-MBC was 19.82 μM and the EC_{50} was 14.5 μM

The overall percentage of 4-MBC-exposed embryos with altered axial curvature at 72 hpf increased with 4-MBC concentration (Fig. 1). 4-MBC-exposed embryos presented trunk malformation characterized by a distorted body axis with a curled tail as the most prominent deformity (Fig. 2a–f), whereas untreated control embryos exhibited a straight trunk. Treated embryos also showed other defects like heart edema and delayed development. In this study, the trunk malformation phenotype is specifically referred as altered axial curvature, which was considered to the major observed morphological defect. Embryos were affected beginning at 10 μM. The percentage increased sharply, and all embryos were affected at about 22 μM. The EC_{50} was determined to be 14.5 μM. Based on these results, a concentration of 15 μM was selected for subsequent experiments.

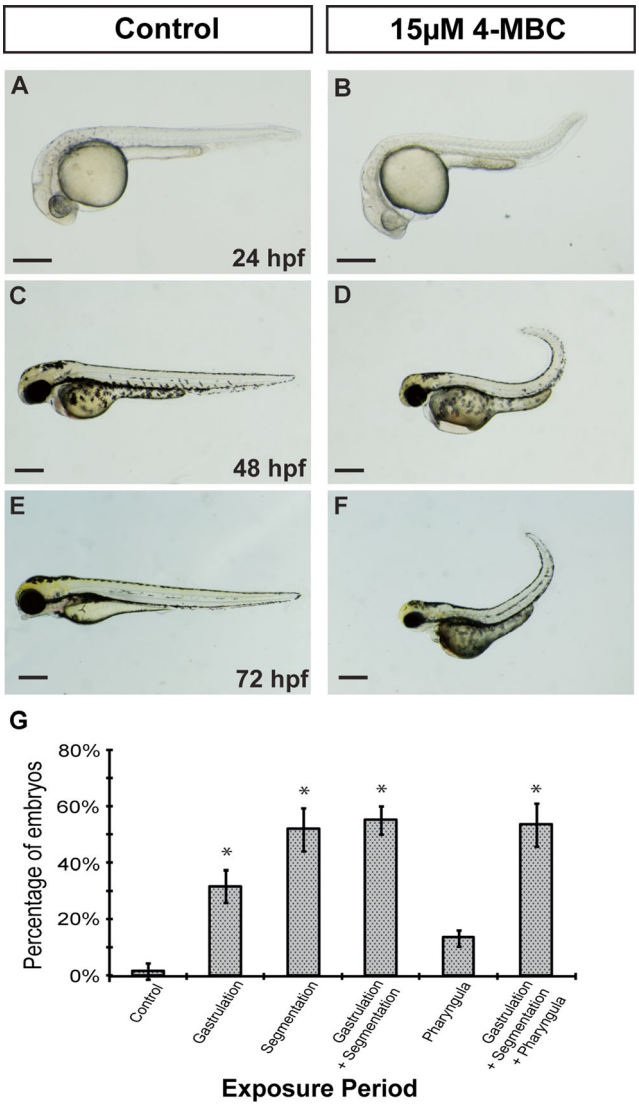

Fig. 2 Morphological defects upon 4-MBC exposure in zebrafish embryos. Lateral views of control (**a**, **c**, **e**) and 4-MBC-exposed (**b**, **d**, **f**) embryos at 24, 48, and 72 hpf, respectively. *Scale bar* = 0.4 mm. **g** Zebrafish embryos ($n = 20$) were exposed to 15 μM 4-MBC during different developmental periods. Data are mean standard error of the mean (*vertical bars*). *$p < 0.01$, ANOVA

Exposure to 4-MBC during gastrulation and segmentation

Exposure to 4-MBC during segmentation was found to be critical to the induction of altered axial curvature (Fig. 2g). Altered axial curvature was found in 31.67 ± 6.24 % of embryos ($n = 20$) that were exposed to 4-MBC only during gastrulation (4–10 hpf). 4-MBC exposure during the segmentation period (10–24 hpf) caused this trunk defect at a much higher percentage (51.7 ± 6.3 %). The highest incidence of altered axial curvature was induced by 4-MBC exposure during both periods (4–24 hpf), with 55.0 ± 7.1 % of embryos affected. The incidence of altered axial curvature was significantly increased ($n = 60$, $p < 0.01$) by 4-MBC exposure in these three treatments when compared to the controls where only 1.7 ± 2.9 % of embryos displayed trunk defects. Exposure after somitogenesis did not exert any significant effects on embryonic development. Prolonged exposure to 4-MBC after segmentation (24–72 hpf) did not influence the malformation incidence when compared to the exposure during gastrulation and segmentation. It should be noted that none of the 4-MBC-induced altered axial curvature could be rescued by placing the embryos in clean embryo medium for 1 week after 4-MBC exposure.

Concentrations of 4-MBC in exposure medium and zebrafish tissue

The analytical methods for measuring 4-MBC in the embryo medium and embryo tissue provided good accuracy and precision as determined by the average recovery and relative standard derivation (RSD). Spiked water and fish samples had an average recovery of 79 % ± 3.5 and 92 % ± 6.6, respectively. The target compound was below detection limit in all control groups.

The bioconcentration of 4-MBC in zebrafish embryos tissue increased from 24 to 72 hpf and decreased slightly at 96 hpf, with the maximum concentrations measured at 72 hpf (Fig. 3); these levels were 67–174 times higher than the quantified exposure concentrations. The log BCF values calculated in the present ranged from 2.4 to 3.3.

Effects of 4-MBC on locomotory activity and tactile stimulation

Wild-type zebrafish embryos showed normal touch response as indicated by effective swimming movements of the tail, which can be used as a simple assay for sensorimotor integration (Stehr et al. 2006). At 72 hpf, untreated control embryos were touched on the head and were found to respond to tactile stimulation as demonstrated by movement of the fins and tail

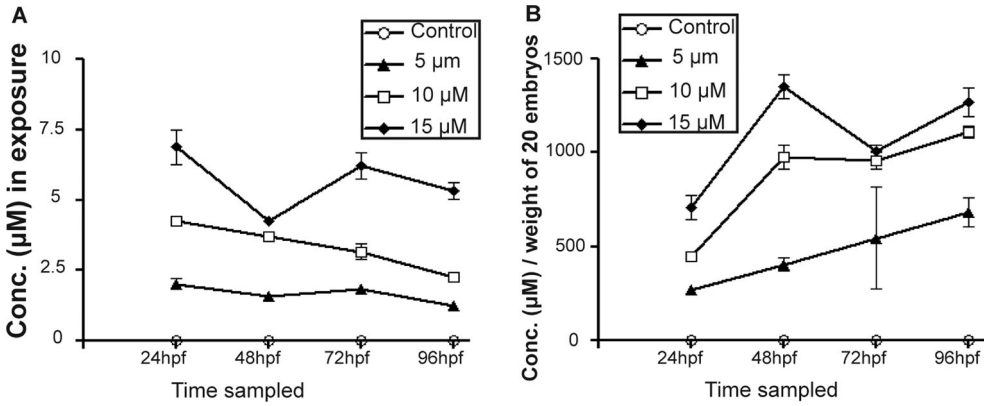

Fig. 3 The average concentration represented as the mean ± SD ($n = 3$) in **a** exposure medium and **b** zebrafish embryos over the course of a 4-day exposure to 4-MBC

(Fig. 4a–h). In contrast, 4-MBC-treated (15 μM) embryos with altered axial curvature showed no escape response to the touch stimulation.

Embryos exposed to 4-MBC were found completely paralyzed (Fig. 5g–l). Untreated embryos showed normal axial curvature and swam rapidly, while all 4-MBC-exposed (15 μM) embryos showed no swimming activity and only 51.6 ± 8.5 % of the 20 embryos (triplicates) displayed fin paddling motions.

Effects of 4-MBC on slow muscle development

Lateral views of stained embryos showed that the slow muscle fiber pattern was disrupted in 4-MBC-exposed embryos and that the muscle fibers appeared frequently thinner. The number of embryos showing observable fiber organization abnormalities after 4-MBC exposure was found significantly increased when compared to the controls ($n = 20$, $p < 0.01$) (Fig. 6b).

Effects of 4-MBC on motor axon extension

At 24 hpf, no differences were observed between control and 4-MBC-exposed embryos, except that shorter axons were detected in 35.8 ± 6.3 % of embryos showing altered axial curvature in the 4-MBC-exposed embryos ($n = 20$) (Figs. 6c and 4d). At 48 hpf, CaP extended in a wide arc in control embryos, while 10.7 ± 5.8 % of 4-MBC-exposed embryos had CaP axons that followed a more linear ventral trajectory (Figs. 6e and 4f). Thus, the axons covered a narrower area of the somite as compared with the control embryos. Moreover, a marked reduction of axon extension length of CaP was observed in 4-MBC-exposed embryos ($n = 20, p < 0.05$) (Figs. 6e and 4f).

In 4-MBC-exposed embryos, secondary motor neuron axonal growth was altered in a similar way as that of the primary motor axon, covering a narrower area of the somite when compared with the control embryos ($n = 20$, $p < 0.01$) (Fig. 6g–j). In addition, ectopic branching of the axon was observed in 41.7 ± 16.1 % of 4-MBC-exposed embryos (Fig. 6g, h).

Effects of 4-MBC on AChE inhibition

AChE activity in the trunk regions of control and 4-MBC-exposed embryos at 24 hpf was examined by AChE staining. Brown signals indicating AChE activity were observed in the trunk region of both control and 4-MBC-exposed embryos

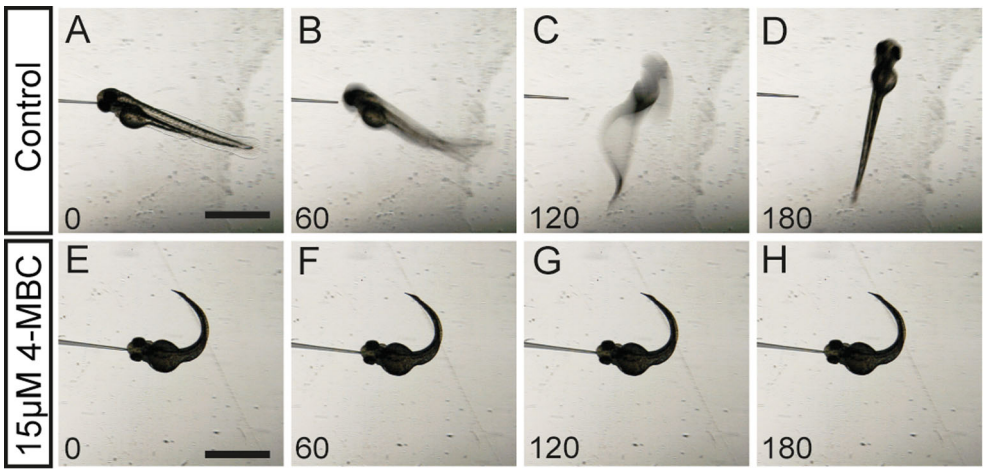

Fig. 4 Abnormal response to a mechanical stimulus (touch) following 4-MBC exposure ($n = 5$). **a**–**d** Sequential frames from a digital video recording of a control embryo at 72 hpf responding to touch with two alternating flicks of the tail. **e**–**h** The characteristic swimming response was absent in 4-MBC-treated embryos. Elapsed time (ms) is indicated in the *lower left corner*. *Scale bar* = 0.5 mm

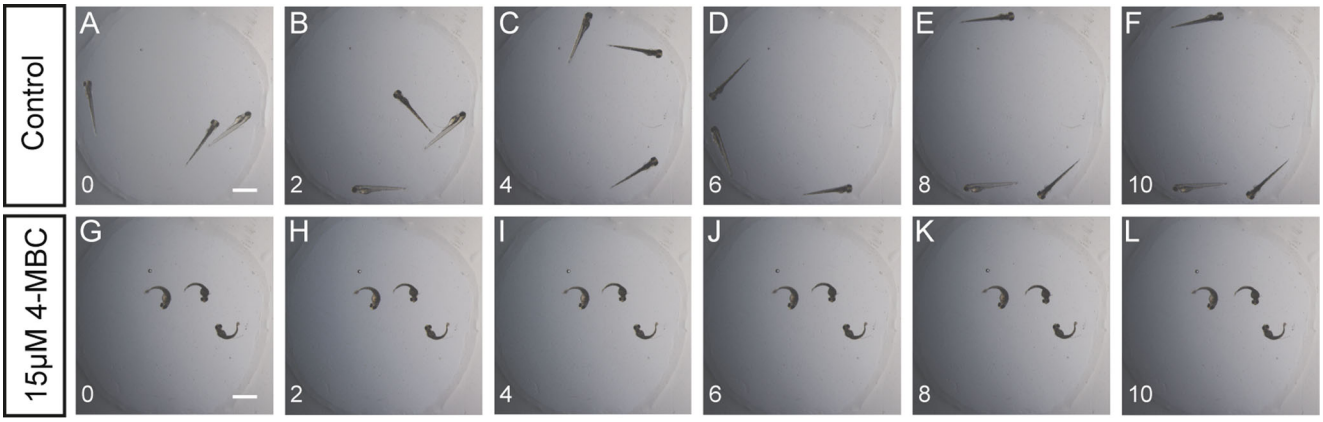

Fig. 5 Swimming behavior in embryos captured in a 10-min video ($n = 5$). **a**–**f** Control embryos swam freely throughout the 10 min. **g**–**l** Immotility was observed in the 4-MBC-treated embryos. Elapsed time (min) is indicated in the *lower left corner. Scale bar* = 0.5 mm

($n = 20$) (Fig. 7a, b), but a much lighter color was detected in 4-MBC-exposed embryos, which implies that AChE inhibition occurred.

Dose-dependent AChE inhibition in a neuroblastoma cell line

To further confirm the AChE inhibitory effect on neuronal cells, 4-MBC exposure was performed in the Neuro-2a cell line. Consistent with the in vivo study, 4-MBC-induced AChE inhibition was observed in a dose-dependent manner, ranging from 33.3 % reduction at 1 μM up to 41.9 % at 100 μM (Fig. 7c). A trend of increased apoptosis and decreased proliferation was also observed, but the effects were marginal. Significant inhibition ($p < 0.01$) was found at the highest concentration (10 μM). In parallel, a full dose–response relationship was obtained for carbaryl, which is an insecticide designed to inhibit AChE (Gruber and Munn 1998), from 0 to 100 μM after 45 min of exposure (Fig. 7c). These results demonstrated that 4-MBC exerts an inhibitory effect on AChE similar to that of other common cholinesterase inhibitors but to a lesser extent. These results support the in vivo observations of developmental toxicity caused by 4-MBC.

Discussion

This study demonstrated the adverse effects caused by 4-MBC on the muscular and neuronal development and the behavior in zebrafish embryos. The LC_{50} and EC_{50} of 4-MBC in zebrafish embryos at 72 hpf were determined to be 19.82 μM (5.04 mg/L) and 14.5 μM (3.69 mg/L), respectively. Although these concentrations are greater than the levels previously detected in environmental waters (surface water, 2–1140 ng/L; wastewater, 600–6500 ng/L) (Balmer et al. 2005; Rodil et al. 2009), they are of the same order of magnitude as the levels reported for a German lake where recreational activities are the major source of 4-MBC (1140 ± 50 ng/L) (Rodil et al. 2009). It must be emphasized that our study did not seek to evaluate toxicological responses at environmental realistic concentrations but aimed to investigate the developmental defects in teleost embryos exposed to 4-MBC. Since zebrafish embryos generally have a high tolerance to chemical toxicity, such as heavy metals and dioxins (Henry et al. 1997), high concentrations of 4-MBC were used for the purpose of phenotypic screening. Consistent with our predictions, the EC_{50} of 4-MBC on zebrafish embryos (3.69 mg/L) was found to be higher than that of the other aquatic organisms, such as *Oncorhynchus mykiss* (0.415 mg/L; Kunz et al. 2006) and *Potamopyrgus antipodarum* (1.71 mg/L; Schmitt et al. 2008). In the present study, the highest BCF of 4-MBC was calculated to be 1355.6 ± 111.5. The accumulation of 4-MBC by the fish could be due to its high octanol-water partition coefficient (log K_{ow}) of 5.8 (Giokas et al. 2007). Similar to other lipophilic contaminants, bioaccumulation of 4-MBC might increase with prolonged exposure time and trophic level (Law et al. 2003). Due to the possibility that long-living predator fish can have a high body burden of 4-MBC, it is not surprised that developing fish embryos may also be exposed to high levels of 4-MBC through maternal transfer in lipid-rich yolk reverses. Further research is warranted to address this knowledge gap.

Little information is known about the effects of 4-MBC on developmental processes in vertebrates. In this study, we demonstrated that 4-MBC induced abnormal axial curvature, impaired tactile response, and immotility in zebrafish embryos. Impaired motility first appeared at the end of the first day of development, and complete paralysis occurred by 72 hpf. It was also noted that 4-MBC exposure resulted in permanent morphological defects. The impaired motility of the 4-MBC-exposed embryos could not be rescued by replacing the 4-MBC medium with water for 1 week after the exposure. These abnormal phenotypes and behavioral defects suggested that 4-MBC might cause adverse effects on somitogenesis

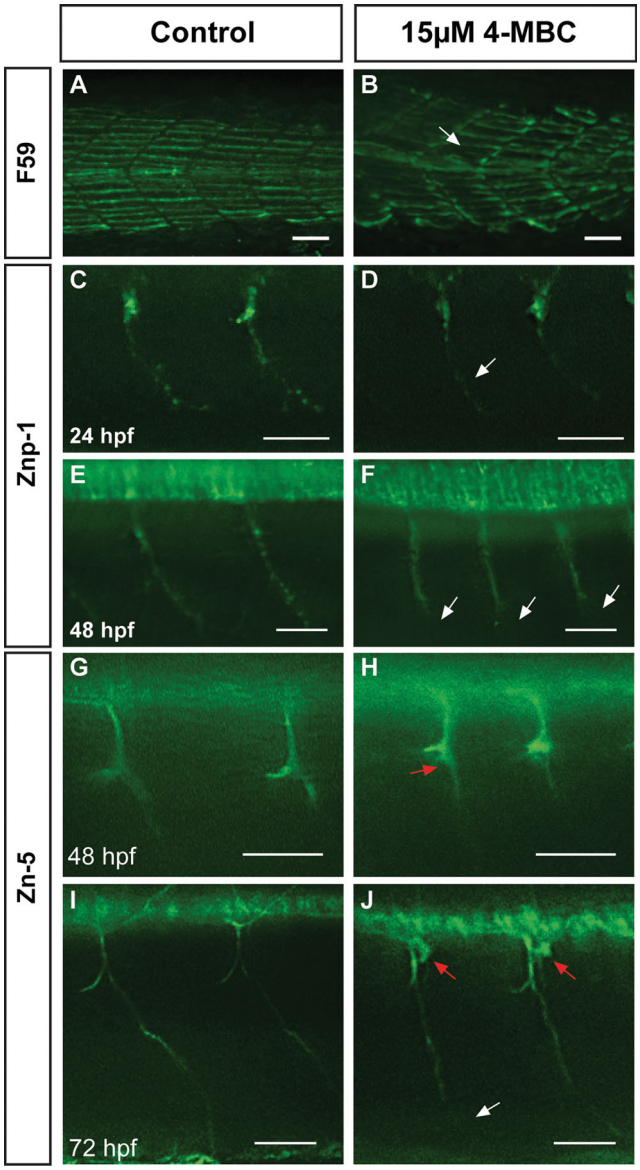

Fig. 6 Immunostaining of muscle and motor axon. **a**, **b** Immunostaining with F59 antibody in control and 4-MBC-exposed embryos at 24 hpf showing disruption of the expression pattern of slow muscle myosin in 4-MBC-exposed embryos ($n = 20$). The muscle fiber pattern is disorganized (*arrowed*). **c**–**f** Immunostaining with znp-1 antibody labeling the motor axon extension in embryos at 24 and 48 hpf. Axons of the CaP motor axon appear thinner and shorter in 4-MBC-exposed embryos ($n = 20$) (*white arrowed*). CaP axons form a shallower arc in 4-MBC-exposed embryos at 48 hpf (**c**, **d**). **g**–**j** Immunostaining of secondary motor axon extension in embryos at 48 and 72 hpf. Staining with zn5 antibody revealed the effect of 4-MBC on secondary motor axon extension. Ectopic branching was found in 4-MBC-exposed embryos ($n = 20$) (*red arrowed*). Axons formed a shallower arc covering a narrower area of the somite in 4-MBC-exposed embryos at 72 hpf (*white arrowed*). Lateral views with the anterior to the *left* and dorsum up. *Scale bar* = 30 μm

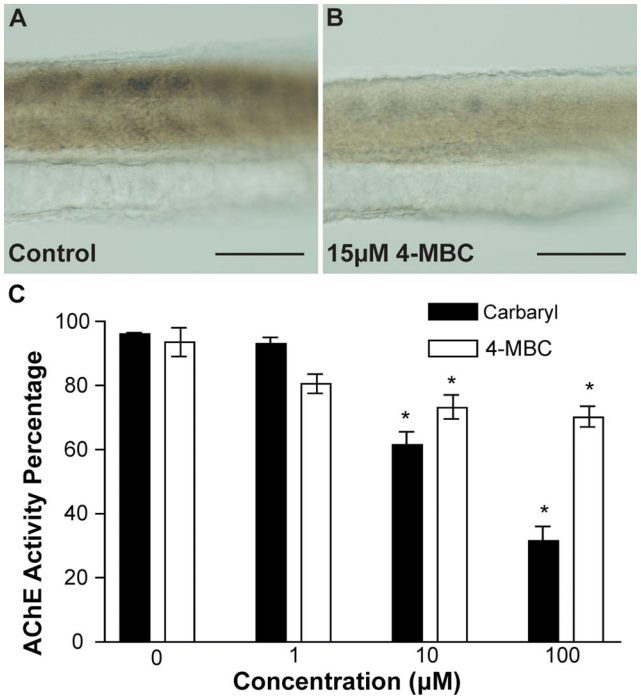

Fig. 7 4-MBC inhibited acetylcholinesterase activity. **a**, **b** AChE enzymatic activity in zebrafish embryos at 24 hpf ($n = 20$). Embryos were exposed to 15 μM of 4-MBC from 4 to 24 hpf. The *brown color* indicates the presence of AChE activity, with a *darker color* indicating higher enzymatic activity. Lateral views with the anterior to the *left*. *Scale bar* = 200 μm. **c** AChE enzymatic activity in the Neuro-2a mouse neuroblastoma cell line ($n = 3$) exposed to 4-MBC or carbaryl for 45 min. Both 4-MBC and carbaryl inhibited AChE activity in a concentration-dependent manner. *$p < 0.01$

during vertebrate development (Chow and Cheng 2003). Examination of 4-MBC effects during specific developmental windows of somitogenesis showed that the segmentation period, when the formation of somites occurs, was the most sensitive exposure period to 4-MBC (Fig. 2g).

Embryos exposed to 15 μM 4-MBC showed altered somites with distorted chevron shape. Myotomes, the muscle derived during somitogenesis, play an important role in zebrafish axial development. Previous studies have shown that myotomes can be target tissues for toxic chemicals such as cadmium and cypermethrin (Cheng et al. 2000; Anwar 2004). The abnormal axial curvature caused by 4-MBC exposure led us to investigate its effect on axial muscle development. Our data revealed that the pattern of slow muscle cells was clearly disrupted and the fiber organization was affected. Impaired innervation during somitogenesis, such as a marked reduction of CaP extension and alteration of its extension trajectory, was observed in 4-MBC-exposed embryos. This abnormal axonal pattern was consistent with those observed in AChE mutants (Behra et al. 2002) and suggested that the 4-MBC-induced axon pathfinding defect was caused by AChE inhibition. An in vitro study showed that AChE was required to promote neurite growth from chick nerve cells (Layer et al. 1993). Overexpression of human AChE also enhanced the growth rate of *Xenopus* motor neurons (Sternfeld et al. 1998). Our data also showed ectopic branching of secondary

motor neurons in 4-MBC-exposed embryos. This secondary axon pathfinding effect could also be explained by AChE inhibition. It has been reported that inhibition of AChE leads to nicotinic acetylcholine receptor (nAChR) inactivation and subsequently disrupts calcium homeostasis in the axon growth cone. As a result, the axons target peripheral tissues in an unorganized manner (Gomez and Spitzer 1999).

Zebrafish AChE mutants show impaired motility at the end of the first day of development and are completely paralyzed after 3 days. The mutants also show defects in primary motoneuron axon pathfinding, muscle development, and early death of Rohon-Beard sensory neurons (Behra et al. 2002). Together with our preliminary studies showing that embryos exposed to 49.7 μM (10 μg/ml) carbaryl, a well-known AChE inhibitor, show similar defects caused by 4-MBC, including notochord vacuolation failure, altered axial curvature, shortened tails, and abnormal locomotion (data not shown), these studies suggest that 4-MBC may act as an AChE inhibitor. As expected, AChE enzymatic activity was reduced in 4-MBC-exposed embryos (Fig. 7a, b). To further confirm this finding, 4-MBC exposure was performed in Neuro-2a cells and a dose-dependent reduction of AChE activity was observed (Fig. 7c). However, the maximum AChE inhibition by 4-MBC was only half that of carbaryl, another well-known AChE inhibitor. This difference is likely due to the structural properties of the two chemicals; the carbamoyl nitrogen and its associated substituents, which 4-MBC lacks, have been reported to be an important moiety for AChE inhibition (Roy et al. 2008). The lack of this moiety thus is likely to result in weaker binding of 4-MBC to AChE and, thus, a smaller inhibitory effect.

The inhibition of AChE by 4-MBC could explain the developmental defects observed in the present study. The physiological role of AChE is to hydrolyze acetylcholine (ACh), thus terminating neural transmission at cholinergic synapses. In addition to its "classical" role in terminating synaptic transmission, AChE is believed to play "non-classical" roles that may be involved in vertebrate early development, as AChE appears long before synapses are functional. AChE is expressed in all primary motoneurons, interneurons, and sensory before axonal growth during zebrafish embryonic development (Hanneman et al. 1988a, b; Wilson et al. 1990; Ross et al. 1992). Previous studies also showed that AChE is involved in neuronal differentiation and development (Coleman and Taylor 1996). AChE inhibition has been suggested to lead to the accumulation of ACh and saturation of nAChRs, leading to persistent activation of the receptor and eventually resulting in inactivation (Behra et al. 2002). Another study using a pharmacological approach reported that the inhibition of nAChRs by α-bungarotoxin completely abolished cytosolic calcium transients in slow muscle cells of 24 hpf zebrafish embryos, which lead to disruption in myofibril organization (Brennan et al. 2005).

Many studies have reported that 4-MBC exerts estrogenic effects and acts as an endocrine disruptor in adult vertebrates. The present study provides some insights into the AChE inhibitory effect of 4-MBC, showing that 4-MBC affects organisms through non-estrogenic pathways. Our data provide evidence that 4-MBC is a teratogen and has a potential to influence muscular and neuronal development and subsequent function. Further knowledge of the pharmacokinetics of 4-MBC is needed to predict its effects in humans and in other organisms, particularly with respect to its potential teratogenicity.

Conclusion

In summary, this study demonstrated the utility of zebrafish embryos as a model to assess the developmental toxicity of 4-MBC. We found that 4-MBC exposure caused abnormal axial curvature and exhibited impaired motility. Both our in vivo and in vitro studies showed that 4-MBC has acetylcholinesterase inhibitory effect, which thereby affects normal slow muscle development and axon pathfinding. Considering that the effective level of 4-MBC to cause this developmental toxicity was orders of magnitude greater than those detected in environmental waters, more work is needed to examine whether similar developmental impacts might occur in native species under long-term exposure to lower 4-MBC concentrations.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

References

Altringham JD, Ellerby DJ (1999) Fish swimming: patterns in muscle function. J Exp Biol 202:3397–3403

Anwar K (2004) Toxic effects of cypermethrin on the development of muscle in chick embryo of *Gallus domesticus*. Int J Agric Biol 6:2

Balmer ME, Buser HR, Müller MD, Poiger T (2005) Occurrence of some organic chemical UV filters in wastewater, in surface waters, and in fish from Swiss lakes. Environ Sci Tech 39:953–962

Behra M, Cousin X, Bertrand C, Vonesch JL, Biellmann D, Chatonnet A, Strahle U (2002) Acetylcholinesterase is required for neuronal and muscular development in the zebrafish embryo. Nat Neurosci 5:111

Brennan C, Mangoli M, Dyer CEF, Ashworth R (2005) Acetylcholine and calcium signaling regulates muscle fibre formation in the zebrafish embryo. J Cell Sci 118:5181–5190

Chen TH, Wang YH, Wu YH (2011) Developmental exposures to ethanol or dimethylsulfoxide at low concentrations alter locomotor activity in larval zebrafish: implications for behavioral toxicity bioassays. Aquat Toxicol 102:162–166

Cheng SH, Wai AWK, So CH, Wu RSS (2000) Cellular and molecular basis of cadmium-induced deformities in zebrafish embryos. Environ Toxicol Chem 19(12):3024–3031

Chow ESL, Cheng SH (2003) Cadmium affects muscle type development and axon growth in zebrafish embryonic somitogenesis. Toxicol Sci 73:149–159

Coleman BA, Taylor P (1996) Regulation of acetylcholinesterase expression during neuronal differentiation. J Biol Chem 271:4410–4416

Crow MT, Stockdale FE (1986) The developmental program of fast myosin heavy chain expression in avian skeletal muscles. Dev Biol 118:333–342

Durrer S, Maerkel K, Schlumpf M, Lichtensteiger W (2005) Estrogen target gene regulation and coactivator expression in rat uterus after developmental exposure to the ultraviolet filter 4-methylbenzylidene camphor. Endocrinology 146:2130–2139

Ellman GL, Courtney KD, Andes V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharm 7:88–95

Fashena D, Westerfield M (1999) Secondary motoneuron axons localize DM-GRASP on their fasciculated segments. J Comp Neurol 406: 415–424

Giokas DL, Salvador A, Chisvert A (2007) UV filters: from sunscreens to human body and the environment. Trends Anal Chem 26:360–374

Gomez TM, Spitzer NC (1999) *In vivo* regulation of axon extension and pathfinding by growth-cone calcium transients. Nature 6717:350–355

Gruber SJ, Munn MD (1998) Organophosphate and carbonate insecticide in agricultural waters and cholinesterase (ChE) inhibition in Common carp (*Cyprinus carpio*). Archit Environ Control Toxicol 35:391–396

Hagedorn-Leweke U, Lippold BC (1995) Absorption of sunscreens and other compounds through human skin in vivo: derivation of a method to predict maximum fluxes. Pharm Res 12:1354–1360

Hallare AV, Köhler HR, Triebskorn R (2004) Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. Chemosphere 56(7):659–666

Hanneman E, Trevarrow B, Metcalfe WK, Kimmel CB, Westerfield M (1988a) Segmental pattern of development of the hindbrain and spinal cord of the zebrafish embryo. Development 103(1):49–58

Hanneman E, Trevarrow B, Metcalde WK, Kimmel CB, Westerfield M (1988b) Segmental pattern of development of the hindbrain and spinal cord of the zebrafish. Development 103(1):49–58

Henry TR, Spitsbergen JM, Hornung MW, Abnet CC, Peterson RE (1997) Early life stage toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish (*Danio rerio*). Toxicol Appl Pharmacol 142:56–68

Hofkamp L, Bradley S, Tresguerres J, Lichtensteiger W, Schlumpf M, Timms B (2008) Region-specific growth effects in the developing rat prostate following fetal exposure to estrogenic ultraviolet filters. Environ Health Perspect 116(7):867–872

Howes MR, Perry NSL, Houghton PJ (2003) Plants with traditional uses and activities, relevant to the management of Alzheimer's disease and other cognitive disorders. Phytother Res 17:1–18

Janjua NR, Mogensen B, Andersson AM, Petersen JH, Henriksen M, Skakkebaek NE, Wulf HC (2004) Systemic absorption of the sunscreens benzophenone-3, octylmethoxycinnamate, and 3-(4-methyl-benzylidene) camphor after whole-body topical application and reproductive hormone levels in humans. J Invest Dermatol 123(1):57–61

Karnovsky MJ, Roots L (1964) A direct colouring thiocholine method for cholinesterase. J Histochem Cytochem 12:219–221

Klann A, Levy G, Lutz I (2005) Estrogen-like effects of ultraviolet screen 3-(4methylbenzylidene)-camphor (Eusolex 6300) on cell proliferation and gene induction in mammalian and amphibian cells. Environ Res 97:274–281

Kunz PY, Galicia HF, Fent K (2006) Comparison of in vitro and in vivo estrogenic activity of UV filters in fish. Toxicol Sci 90:349–361

Langheinrich U (2003) Zebrafish: a new model on the pharmaceutical catwalk. Bioessays 25:904–912

Law RJ, Alaee M, Allchin CR, Boon JP, Lebeuf M, Lepom P, Stern GA (2003) Levels and trends of polybrominated diphenylethers and other brominated flame retardants in wildlife. Environ Int 29:757–770

Layer PG, Weikert T, Alber R (1993) Cholinesterases regulate neurite growth of chick nerve cells in vitro by means of a non-enzymatic mechanism. Cell Tissue Res 273:219–226

Lichtensteiger W, Ceccatelli R, Conscience M, Cotton B, Durrer S, Faass O, Fleischmann I, Ma R, Maerkel K, Schlumpf M (2002) Newly arising endocrine disruptors: UV screens and PBDE. Reprod Toxicol 16:397–398

Metcalfe W, Myers P, Trevarrow B, Bass M, Kimmel C (1990) Primary neurons that express the L2/HNK-1 carbohydrate during early development in the zebrafish. Development 110:491–504

Mirkes P, McClure M, Heindel J, Sander M (2003) Developmental toxicology in the 21st century: multidisciplinary approaches using model organisms and genomics. Birth Defects Res Part Clin Mol Teratol 67:21–34

Miyazawa M, Watanabe H, Kameoka H (1997) Inhibition of acetylcholinesterase activity by monoterpenoids with a *p*-menthane skeleton. J Agric Food Chem 45:677–679

Nagtegaal M, Ternes TA, Baumann W, Nagel R (1997) UV-Filtersubstanzen in Wasser und Fischen. UWSF-Z Umweltchem Ökotox 9:79–86

Rodil R, Schrader S, Moeder M (2009) Non-porous membrane-assisted liquid–liquid extraction of UV filter compounds from water samples. J Chromatogr A 1216:4887–4894

Ross LS, Parrett T, Easter SS Jr (1992) Axonogenesis and morphogenesis in the embryonic zebrafish brain. J Neurosci 12:467–482

Roy KK, Dixit A, Saxena AK (2008) An investigation of structurally diverse carbamates for acetylcholinesterase (AChE) inhibition using 3D-QSAR analysis. J Mol Graph Model 27(2):197–208

Schlumpf M, Cotton B, Conscience M, Haller V, Steinmann B, Lichtensteiger W (2001) *In vitro* and *in vivo* estrogenicity of UV screens. Environ Health Perspect 109:239–244

Schlumpf M, Schmid P, Durrer S, Conscience M, Maerkel K, Henseler M, Gruetter M, Herzog I, Reolon S, Ceccatelli R, Faass O, Stutz E, Jarry H, Wuttke W, Lichtensteiger W (2004) Endocrine activity and developmental toxicity of cosmetic UV filters—an update. Toxicology 205:113–122

Schmitt C, Oetken M, Dittberner O, Wagner M, Oehlmann J (2008) Endocrine modulation and toxic effects of two commonly use UV screens on the aquatic invertebrates Potamopyrgus antipodarum and Lumbriculus variegates. Environ Pollut 152:322–329

Soto AM, Sonnenschein C (2005) Shining a light on sunscreens. Endocrinology 146:2127–2129

Stehr CM, Linbo TL, Incardona JP, Scholz NL (2006) The developmental neurotoxicity of fipronil: notochord degeneration and locomotor defects in zebrafish embryos and larvae. Toxicol Sci 92(1):270–278

Sternfeld M, Ming G, Song H, Sela K, Timberg R, Poo M, Soreq H (1998) Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable C termini. J Neurosci 18(4):1240–1249

Trevarrow B, Marks DL, Kimmel CB (1990) Organization of hindbrain segments in the zebrafish embryo. Neuron 4:669–679

Westerfield M (1993) A guide for the laboratory use of zebrafish *Danio* (*Brachydanio*) *rerio*. University of Oregon Press, Eugene, OR, USA

Wilson SW, Ross LS, Parrett T, Easter SS Jr (1990) The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, *Brachydanio rerio*. Development 108: 121–145

Zhang J, Malayaman S, Davis C, Granato M (2001) A dual role for the zebrafish *unplugged* gene in motor axon pathfinding and pharyngeal development. Dev Biol 240(2):560–573

Zon LI, Peterson RT (2005) *In vivo* drug discovery in the zebrafish. Nat Rev Drug Discov 4:35–44