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Zebrafish as a Model for Developmental Neurotoxicity Testing

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BACKGROUND: To establish zebrafish as a developmental toxicity model, we used 7 well-characterized compounds to examine several parameters of neurotoxicity during development. **METHODS:** Embryos were exposed by semistatic immersion from 6 hrs postfertilization (hpf). Teratogenicity was assessed using a modified method previously developed by Phylonix. Dying cells in the brain were assessed by acridine orange staining (these cells are likely to be apoptotic). Motor neurons were assessed by antiacetylated tubulin staining and catecholaminergic neurons were visualized by antityrosine hydroxylase staining. **RESULTS:** Atrazine, dichlorodiphenyltrichloroethane (DDT), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were primarily teratogenic and not specifically neurotoxic. 2,4-dichlorophenoxyacetic acid (2,4-D), dieldrin, and nonylphenol showed specific neurotoxicity; dieldrin and nonylphenol were specifically toxic to catecholaminergic neurons. Malathion, although not teratogenic, showed some nonspecific toxicity. **CONCLUSIONS:** Teratogenicity measured in 96-hpf zebrafish is predictive of mammalian teratogenicity and is useful in determining whether a compound causes specific neurotoxicity or general developmental toxicity. Induction of apoptosis or necrosis is an indicator of neurotoxicity. An effect on motor neurons in the caudal third of the embryo correlates with expected defects in motility. Overall, our results showed a strong correlation with mammalian data and suggest that zebrafish is a predictive animal model for neurotoxicity screening. *Birth Defects Research (Part A) 76:553–567, 2006.* Published 2006 Wiley-Liss, Inc.†

Key words: zebrafish; developmental neurotoxicity; teratogenicity

INTRODUCTION

Every year, billions of pounds of toxic chemicals are released by industrial facilities and by agricultural practices, much of which ends up in the air or ground water; 3 of 4 of these chemicals are known or suspected neurotoxicants (Schettler et al., 2000). Pesticides are of particular concern because they are designed to be neurotoxic to insects and can also have deleterious effects on other species. Currently, in the United States, 85,000 industrial chemicals are manufactured every year and an additional 2000–3000 new chemicals are registered; yet for over 70% of these chemicals, there are little or no toxicity data (Claudio et al., 2000).

The developing brain is more sensitive to chemical toxicants than the adult brain, and exposure during development has been implicated in neurological diseases and mental retardation (Anderson et al., 2000). Because of these concerns, a number of legislative and regulatory measures have been taken to strengthen requirements for developmental toxicity testing (Kimmel and Makris, 2001). Although these guidelines have now existed for several years, very few chemicals currently released into the environment have been assayed for neurotoxicity or developmental neurotoxicity. It is clear that, although regulatory agencies perceive an urgent need for neurotoxicity testing, there is a significant lack of data and an enormous backlog of chemicals that require testing (Claudio et al., 2000). A zebrafish model for developmental neurotoxicity can be used to rapidly screen environmental contaminants for potential neurotoxicity prior to extensive mammalian evaluation. Although the through-

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put using zebrafish is lower than can be achieved using cell-based assays, the information obtained is more relevant to vertebrates, and compared with rodents and other animal models, zebrafish assays can completed in days rather than weeks or months.

Zebrafish are exceptionally well suited for developmental studies that combine cellular, molecular, and genetic approaches. Because the embryo is transparent and develops rapidly, development of specific neurons and axon tracts can be visualized in live embryos using differential interference contrast (DIC) microscopy or by injecting live dyes (Kuwada and Bernhardt, 1990; Chitnis and Dawid, 1999). Specific types of neurons can be visualized in whole fixed embryos by immunohistochemistry or in situ hybridization (Chandrasekhar et al., 1997; Moens and Fritz, 1999). Motoneuron activity can be monitored in vivo by calcium imaging and patch clamp recording (Drapeau et al., 2002). Function of individual neurons can be elucidated by specific neural lesion using toxic dye injection (Gahtan and O'Malley, 2001) or laser ablation (Fetcho and Liu, 1998). In addition, mutants with characteristic morphological phenotypes or behaviors have been isolated from large-scale screens that have been useful in understanding early central nervous system (CNS) patterning (Jiang et al., 1996; Schier et al., 1996).

Historically, zebrafish have been used in a variety of applications to assess environmental toxicity. Zebrafish assays have been used to directly monitor water, soil, and wastewater quality for ecotoxicity studies (Ruoppa and Nakari, 1988). Zebrafish have also been used to assess risks, to predict toxicity associated with petroleum products and byproducts, and to evaluate working conditions in the petrochemical and mining industries (Vitozzi and De Angelis, 1991; Lele and Krone, 1996). In addition, the effects of pesticides, polychlorinated biphenyls (PCBs), dioxin, and some of their derivatives have been extensively studied in zebrafish embryos (Burkhardt-Holm et al., 1999; Wiegand et al., 2000, 2001; Andersson et al., 2001; Andreasen et al., 2002). In Europe, zebrafish are a widely accepted model for ecotoxicity studies; a number of recent guidelines from the Organization of Economic Cooperation and Development (OECD, Guidelines 203, 210, and 212) (OECD, 1992a, 1992b, 1998) recommend zebrafish as a model for aquatic toxicity testing.

In this report, we developed several zebrafish bioassays that comprise a comprehensive screening protocol for testing environmental neurotoxicants. These assays focus primarily on endpoints that can be quantified and can be used to assess a broad range of potential neurological effects. Specifically, we assessed 7 environmental toxicants on the following elements of embryonic development: 1) brain-specific cell death (apoptosis), 2) axon tracts in the brain and caudal third of the embryo (the tail region of the embryo responsible for movement of the animal), 3) specific toxicity on motor neuron development in the caudal third of the embryo, 4) catecholaminergic neurons in the brain, and 5) general developmental toxicity (teratogenicity).

ASSAYS

The zebrafish develops rapidly; gastrulation begins about 6 hrs postfertilization (hpf) and embryogenesis is essentially complete by 96 hpf, as most organs are formed and functioning by this time. Neurogenesis begins around the onset gastrulation (Eisen, 1991; Jiang et al., 1996); also at this time it is possible to distinguish fertilized from unfertilized embryos, which generally do not progress much further than the 4 to 8-cell stage and degenerate by 6 hpf. We therefore removed unfertilized embryos and began chemical exposure at 6 hpf. Neurogenesis and axon path-finding are maximal during the first 48 hpf (Kuwada and Bernhardt, 1990; Morin-Kensicki and Eisen, 1997); we therefore assessed axon tracts by α-tubulin staining at 48 hpf. Further differentiation of neurons in the CNS continues for several hours, for example, by following expression of the tyrosine hydroxylase and dopamine transporter genes, Holzschuh et al. (2001) showed that catecholaminergic neurons begin to differentiate at 18 hpf, dopaminergic neurons begin at 24 hpf, and by 96 hpf all of the clusters of catecholaminergic neurons found in the adult can be identified. Therefore, we assessed cell death in brain and catecholaminergic neurons at 96 hpf.

Teratogenicity

Because a compound can display neurotoxicity as a secondary effect on general development, we evaluated developmental toxicity by visually scoring various developmental parameters at either 48 or 96 hpf, the 2 developmental endpoints assessed in the neuronal assays. The teratogenic index (TI) is determined by generating dose-response curves for mortality and developmental malformation. The dose at which 50% mortality is observed (LC_{50}), is compared to the dose at which 50% developmental malformation occurs (EC_{50}); the TI is defined as the ratio of LC_{50}/EC_{50}. Because the neurotoxicity assays were carried out at 48 and 96 hpf, we calculated the LC_{50} and TI at each of these time points. The results of the neurotoxicity assays can be interpreted relative to the teratogenicity of a compound to ascertain whether the effect is specific or general. In addition, using the TI, compounds can be ranked with respect to their relative teratogenicity. Our previous results using a similar scoring system showed that teratogenicity measured in zebrafish correlates well with results in mammals (Zhang et al., 2003), indicating the predictive value of zebrafish for assessing developmental toxicity.

Brain-Specific Cell Death

Apoptosis, or programmed cell death, is a common cellular response to exposure to toxic chemicals (reviewed in Corcoran et al., 1994). Apoptosis is induced in a number of mammalian cell lines, including neuronal cell lines (Ahmadi et al., 2003; Caughlan et al., 2004), and in mouse embryos by exposure to pesticides (Greenlee et al., 2004), and organophosphate pesticides induce apoptosis in cultured rat cortical cells (Kim et al., 2004). In whole animals, acridine orange (AO) accumulates in acidic granules that are formed primarily in cells undergoing apoptosis (Darzynkiewicz et al., 1992). It has been shown that AO stains apoptotic and not necrotic cells in live *Drosophila* embryos (Abrams et al., 1993) and AO is a stain commonly used to identify apoptotic cells in whole zebrafish, for example (Furutani-Seiki et al., 1996; Hill et al., 2003; Barrallo-Gimeno et al., 2004; Guo et al., 2004; Parng et al., 2004; Parng, 2005). Therefore, we assessed whether exposure to each of the 7 environmental toxi-

cants resulted in increased AO staining in the brain of live zebrafish embryos; analogous to the findings in *Drosophila*, cells stained by AO in the live zebrafish are likely to be apoptotic.

Visual Assessment of Axon Tracts in the Brain and Caudal Third of the Embryo (Tail Region)

Antiacetylated tubulin (α-AT) reacts generally with all axon tracts allowing observation of tract and commissure patterns throughout the embryo (Wilson et al., 2002). The characteristic pattern of axon tracts in the 48-hpf brain was examined for alterations, as an indication of region of the brain that might be affected by compound exposure.

Quantification of Motor Neurons in 5 Segments of the Caudal Embryo

The caudal two-thirds of the embryo contains the region of the fish responsible for movement; this part of the embryos is composed of segments containing notochord, muscle, and nerve fibers. Each segment has a simple repeating pattern of axon tracts, and the ventral segment contains only motor neurons. Axons were visualized using fluorescently-labeled α-AT, and the fluorescent signal contained in the ventral portion of 5 adjacent segments at 48 hpf was photometrically quantified.

Quantification of the Number of Catecholaminergic Neurons

Tyrosine hydroxylase (TH) catalyzes the first step in the catecholamine biosynthetic pathway. α-TH labels all catecholaminergic neurons, including dopaminergic, noradrenergic, and adrenergic neurons. At 96 hpf, there are several TH-positive cells in 4 clusters in the forebrain (anterior, A) and midbrain (posterior, P); the dopaminergic neurons are contained primarily within the posterior clusters (Holzschuh et al., 2001); cells within these clusters were quantified visually by counting.

Compounds

The compounds chosen for assessment are known or suspected neurotoxicants in mammals. Developmental exposure to certain organochlorine pesticides has been associated with the developmental of Parkinson's-like symptoms in mice, including tremors, rigidity, slow neuromuscular response, and writhing (Giasson and Lee, 2000). These symptoms are thought to be due, in part, to a loss of dopamine-producing neurons. Pesticides associated with this phenomenon include dichlorodiphenyltrichloroethane (DDT), dieldrin, and 2,4-dichlorophenoxyacetic acid (2,4-D). In addition, a wide variety of naturally occurring and man-made chemicals, including phytoestrogens, pesticides (atrazine), polychlorinated biphenyls, dioxins (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]), and synthetic estrogens (nonylphenol), have been shown to interfere with the endocrine system of vertebrates after release into the environment (Colborn et al., 1993; Kavlock and Ankley, 1996) and exposure to endocrine disrupters is associated with developmental neurotoxicity (Ferguson et al., 2000). TCDD is teratogenic in mice and was used as a positive control for developmental toxicity. The organophosphate pesticide malathion was used as a potential negative control for neurotoxicity since studies have shown no evidence of neurotoxicity in mammals, although neurotoxicity of this compound in zebrafish had not previously been investigated. Because the assays are performed after a short period of exposure, much higher concentrations of compounds were used in our experiments than are typically found in the environment.

MATERIALS AND METHODS

Generation of Embryos

Embryos were generated by natural pairwise mating, as described in the Zebrafish Handbook (Westerfield, 1993). A total of 4–5 pairs were set up for each mating and, on average, 100–150 embryos per pair were generated. Embryos were maintained in fish water (5 gm of Instant Ocean Salt [Marineland, Moorpark, CA] in 25 liters of distilled water) at 28°C.

Compound Exposure

Embryos were distributed into 24-well microtiter plates, 30 embryos per well, in 3 ml of zebrafish water and exposed by semistatic immersion from 6 hpf until the specific assay endpoint (96 hpf for brain AO staining, 48 hpf for antiacetylated tubulin staining, 96 hpf for antityrosine hydroxylase staining, and 48 and 96 hpf for LC_{50} and teratogenicity assessment). A total of 3 μl of 1000× compound stock was added directly to 3.0 ml of embryo water. To make 1000× stocks, compounds were dissolved and diluted in dimethylsulfoxide (DMSO; Sigma-Aldrich Chemical Company, St. Louis, MO). The compound stocks were then diluted directly in embryo water to a final concentration of 0.1% DMSO. For example, to generate a final compound concentration of 100 μM, 3 μl of a 100 mM stock was added to the embryos in 3 ml of fish water. Compounds were refreshed daily. Embryos exposed to 0.1% DMSO were used as controls. Chemicals used were DDT (Sigma-Aldrich), 2,4-D (Sigma-Aldrich), atrazine (Sigma-Aldrich), dieldrin (Sigma-Aldrich), malathion (Sigma-Aldrich), nonylphenol (Sigma-Aldrich), and TCDD (Cerilliant, Round Rock, TX).

Lethality Curves and Calculation of LC_{50}

The LC_{50} for each compound was first determined by exposing zebrafish to a wide range of compound concentrations and measuring lethality every 24 hr until 96 hpf. A total of 30 zebrafish were scored per concentration and LC_{50} was estimated at 48 and 96 hpf for each compound. We first tested 5 concentrations, 1 log apart, beginning with 0.01, 0.1, 1, 10, and 100 μM. Higher or lower concentrations of compounds were tested as needed to generate lethality concentration curves. In 3 cases, to reach the desired concentration, it was necessary to add a higher volume of compound stock, resulting in a 0.2% (for 1 mM DDT and 20 μM TCDD) or 0.5% (for 50 μM TCDD) final DMSO concentration. In each case, zebrafish exposed to the corresponding concentration of DMSO alone were used as controls. Tests of trend in percent lethality by concentration were calculated using the Cochran Armitage χ^2 test. The LC_{50} values were then estimated using probit models. In the cases of DDT and TCDD at

48 hpf, the lethality measured was too low to estimate LC_{50}.

Developmental Malformation Scoring, Generation of EC_{50}, and Estimation of Teratogenicity

We used a modified scoring system based on previous studies from our company (Zhang et al., 2003). Compound-exposed and control zebrafish were scored for several developmental endpoints (5 zebrafish were scored at each concentration), and the number of positive scores was totaled and divided by the total number of parameters to determine the developmental malformation index (DMI) for that concentration.

The following developmental endpoints were scored at 48 and 96 hpf:

- Heart rate: Because the heart rate is sensitive to temperature, MESAB (0.5 mM 3-aminobenzoic acid ethyl ester, 2 mM Na_2HPO_4) and methylcellulose, the heart rate was counted first. The ventricular beat rate of each zebrafish was measured for 15 sec for each animal using a stopwatch and counter, and multiplied by 4 to obtain beats per min.
- Circulation: Movement of blood cells through the heart and major vessels and the rate and pattern of blood flow was visually examined. Abnormal circulation includes slow or fast circulation and defects in circulatory pattern (e.g., circulation only in the head or trunk).
- Number of red blood cells (48 hpf only): By 48 hpf, zebrafish red blood cells (RBCs) can be seen circulating in transparent blood vessels. A significant decrease in the number of RBCs in circulation was scored as abnormal.
- Edema: Edema is visualized as an accumulation of fluid in the interstitial space surrounding an organ or tissue. We assessed the presence of heart and trunk edema, which can reflect circulatory or excretory problems, respectively.
- Hemorrhage: Hemorrhage may be caused by blood vessel malformation or rupture of tissues or organs after compound exposure. In zebrafish, hemorrhage is visualized as a pool of blood clustered in an area outside of the normal circulation. The presence of a hemorrhage anywhere in the body was considered a positive.
- Ventricle swelling (48 hpf only): The third brain ventricle is visible at 48 hpf. Accumulation of fluid in this ventricle was scored as a positive.
- Brain necrosis: Normal brain tissue is transparent; necrosis results in cloudy, white, or brown areas. The appearance of 1 or more necrotic areas in the brain was scored as a positive.
- Jaw formation (96 hpf only): The jaw develops from the 7 pharyngeal arches and by day 5 has a characteristic shape. Malformations of the jaw are commonly associated with problems in bone formation or neural crest migration. An abnormal jaw was scored as a positive.
- Caudal embryo morphology: Abnormalities scored as positive include bent or misshapen caudal region of the embryo; such defects reflect possible problems in development of muscle or the nervous system.
- Motility: To assess potential neuronal and muscular disorders, motility was examined. A dissecting needle was used to touch the caudal region of the embryo. In wild type zebrafish, this touch normally elicits a rapid and brief swim response; a lack of response indicates a defect in neuromuscular transmission and was scored as a positive.

After obtaining a DMI for each concentration, the EC_{50} was estimated using the same statistical analysis described to determine the LC_{50}. The TI (defined as LC_{50}/EC_{50}) was calculated for each compound; a ratio of >1 was considered teratogenic.

Antibody Staining

For antiacetylated tubulin (α-AT; Sigma-Aldrich) staining, embryos were harvested at 48 hpf, fixed in Dent's fixative (4:1 methanol:DMSO) at 4°C overnight, dehydrated stepwise into methanol, and stored at −20°C. Whole-mount antibody labeling was performed using standard methods, as described in Westerfield (1993). Secondary antibody was anti-mouse IgG conjugated to Alexa-flour 546 (Invitrogen, Carlsbad, CA). A similar staining procedure was used for antityrosine hydroxylase staining (α-TH; Immunostar, Hudson, WI), except that embryos were fixed in 4% paraformaldehyde at 4°C overnight, and the secondary antibody was HRP-conjugated anti-mouse IgG + IgM (Jackson Immunoresearch, West Grove, PA) (Chen et al., 2001; Wullimann and Rink, 2001; Rink and Wullimann, 2002).

AO Staining

AO staining in the brain was assessed as a general, quantifiable indicator of neurotoxicity at 96 hpf. Compound-exposed and control embryos were immersed in 1.5 μg/ml AO (acridinium chloride hemi-[zinc chloride]; Sigma-Aldrich) in fish water at 28°C for 60 min (Furutani-Seiki et al., 1996). AO was removed by washing in fish water 3 times, 5 min each. Zebrafish were anesthetized with MESAB, and mounted in methylcellulose in a depression slide for microscopic observation and photography.

Microscopy and Image Capture

All fluorescence microscopy studies were performed using a Zeiss M2Bio fluorescence microscope equipped with rhodamine and FITC filter sets and a chilled CCD camera (Axiocam MRM). Images were captured using Axiovision software Rel 4.0 (Carl Zeiss Microimaging, Thornwood, NY). Visible light microscopy was performed using a Zeiss Stemi-2000-C stereo microscope or a Zeiss AxioStar Plus (for α-TH stained zebrafish) and photographed using a Spot Insight digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were processed using Adobe PhotoShop 6.0 (Adobe, San Jose, CA) to generate figures (for analysis and quantification, no PhotoShop manipulation was used; see below).

Quantification of Neural Effects and AO Staining

The fluorescent or colorimetric signal was quantified using Scion Image (Scion Corporation, Frederick, MD) using a modified threshold macro that calculates the

Table 1
Developmental Scoring Index Parameters: Examples from Data for 2,4-D

		Concentration (μM)	
		200 (48 hpf)	75 (96 hpf)
Parameter			
Heart rate (bpm)	Measure	110	130
>2 SD from control	Yes (+)	(−)	(+)
Circulation	Absent (+)	(+)	(+)
Number of RBCs	<50% wt (+)	(+)	NA
Heart edema	Present (+)	(+)	(+)
Trunk edema	Present (+)	(−)	(−)
Hemorrhage	Present (+)	(−)	(−)
Ventricle swelling	Present (+)	(−)	NA
Brain necrosis	Present (+)	(−)	(−)
Jaw malformation	Present (+)	NA	(+)
Abnormal caudal region	Present (+)	(+)	(−)
Motility	Absent (+)	(+)	(+)
Length (μM)	Measure	146	175
>2 SD from control	Yes (+)	(−)	(−)
Total positive		5/11	5/10

wt, wild type; bpm, beats per min; NA, not applicable.

average pixel density for the entire image and thresholds based on an empirically-derived multiplier of the SD of the background of the image. For example, for quantification of motor neurons in the caudal embryo by α-AT staining, an image of 6 segments was used for photometric analysis. To this image, a threshold tolerance of 1.75 SD above background was applied. After applying the threshold, the total area (TA) of the signal was measured for each image. Ten images were analyzed for each compound concentration. To compare effects between compounds, a signal index (SI) was generated for each compound relative to the control:

$$\mathrm{SI} = \frac{\mathrm{TA\ compound} - \mathrm{TA\ control}}{\mathrm{TA\ control}} \times 100\%$$

Relative SIs were then graphed and, for AO staining results, dose-response curves were generated using Microsoft Excel (Microsoft, Redmond, WA).

Statistics

In the case where more than 2 data sets were compared (e.g., dose-response), ANOVA was first used to detect significant differences between datasets. Differences were then evaluated for significance using Dunnett's *t* test. In the cases where a single, sublethal test concentration was compared to a control, Student's *t* test was performed to compare the data (only pairwise comparisons were made). In the case where the data were dichotomous (e.g., estimation of LC_{50} and EC_{50}), a χ^2 test was used to determine trends. ANOVA, χ^2 tests for trends, and probit analyses were performed by DM-STAT (Malden, MA), an independent biostatistics consulting firm.

RESULTS

Lethality and Teratogenicity

For the 7 compounds tested, dose-response curves for lethality and developmental defects were generated to determine teratogenicity. Teratogenicity is an assessment of general developmental toxicity and can be used to determine if the neurotoxic effects seen after compound exposure are specific to the nervous system, or are due to general toxicity. Teratogenicity of a compound is determined by comparison of LC_{50} to EC_{50}: the TI = LC_{50}/EC_{50}.

Parameters used in our zebrafish assay to determine EC_{50} are described in Materials and Methods and listed in Table 1; an example of 1 scored zebrafish exposed to 2,4-D is shown for each time point. A DMI was calculated for each concentration: the number of positives/total number of parameters (e.g., at 48 hpf, 11 parameters × 5 zebrafish = 55 parameters total; and a score of 5 positives would yield a DMI of 5/55 = 9%). The DMI were graphed against concentration to generate dose-response curves and the EC_{50} (concentration that causes 50% of the total measured developmental defects) was calculated as described in Materials and Methods.

At 48 hpf, Malathion and TCDD were the most teratogenic (Table 2, Figure 1), with TIs of 3 and much greater than 1, respectively (based on the LC_{50} curve, TCDD is likely to have a TI much greater than 1, striped bar in Fig. 1). Dieldrin was somewhat teratogenic with a TI of 1.8, and nonylphenol was slightly teratogenic with at a TI of 1.2. Because of solubility limitations, LC_{50} and EC_{50} could not be determined for DDT; however, it is likely that DDT is teratogenic at 48 hpf, since some developmental defects were seen at concentrations as low as 100 μM, whereas no lethality was seen up to 1 mM. At 96 hpf, Atrazine (TI = 2.8), DDT (TI = 3.5), and TCDD (TI = 15.3) were the most teratogenic; TCDD was the most teratogenic of the 7 compounds. 2, 4-D was somewhat teratogenic, with a TI of 1.8.

Significant Developmental Phenotypes

Due to the transparency of the zebrafish embryo, many parameters of development can be visually observed. Table 3 summarizes the major phenotypes observed for each compound at 48 and 96 hpf. The lowest concentration at which the listed phenotypes were observed is given. This information suggests target organs for compound toxicity and allows qualitative comparison to mammalian observations.

Quantification of Toxicity-Induced AO Staining in the Brain

Cell death (apoptosis) in the brain was assessed in live compound-exposed and control animals by AO staining

Table 2
Teratogenic Indices

	48 hpf			96 hpf		
	LC_{50} (μM)	EC_{50} (μM)	TI	LC_{50} (μM)	EC_{50} (μM)	TI
2,4-D	152	222	0.68	132	73	1.8
Atrazine	517	~797	~0.52	1255	440	2.8
DDT	≫1000	≫1000	ND	95	27	3.5
Dieldrin	504	281	1.8	21	200	0.1
Malathion	78	26	3	2.3	14.5	0.16
Nonylphenol	4.8	4	1.2	2.7	3.3	0.8
TCDD	≫100	~100	≫1	0.026	0.0017	15.3

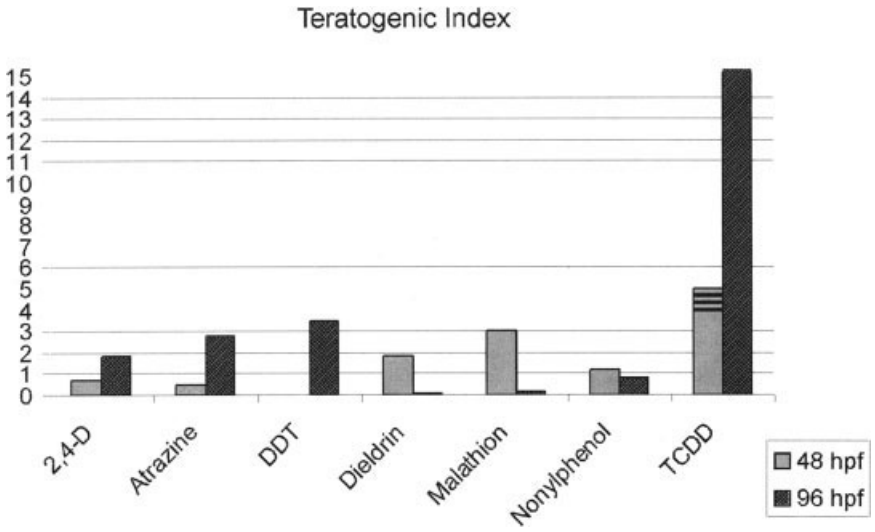

Figure 1. Comparative teratogenicity of the 7 compounds at 48 and 96 hpf. At 48 hpf, dieldrin, malathion, and TCDD were the most teratogenic; the TI of TCDD is estimated to be much greater than 1 (striped bar; DDT is also a likely teratogen at 48 hpf, see text.) At 96 hpf, atrazine was slightly teratogenic, and DTT and TCDD were the most teratogenic.

at 96 hpf and quantified by image analysis (Figs. 2 and 3; Table 4). DDT and dieldrin caused a significant increase in AO staining in the brain at both concentrations tested. 2,4-D, atrazine, and malathion caused significant increases in AO staining only at the highest concentration tested (Fig. 3). Neither nonylphenol nor TCDD (data not shown) had a significant effect on AO staining at either concentration; however, TCDD caused necrosis in the brain at the concentrations tested as evaluated by visual inspection (see below).

Visual Assessment of Axon Tracts in the Brain

Axon tracts in the brain and caudal embryo at 48 hpf were visually assessed by staining with an antiacetylated tubulin-specific antibody (α-AT) followed by a fluorescently-labeled secondary antibody. In the brain, the pattern and size of axon tracts can be used as an indicator of affected brain regions. Exposure to 100 μM dieldrin caused a reduction of the optic tectum (OT) and reduced projections to the posterior commissure (Fig. 4). A total of 20 μM TCDD generally decreased axons in all areas of the brain; this general phenotype may be due to a general effect on development (Fig. 4; see below). 2,4-D (200 μM), atrazine (500 μM), and malathion (10 μM) slightly reduced axon projections to the optic tectum (data not shown; see below). Exposure to DDT (up to 1 mM) or nonylphenol (up to 3 μM) did not affect the α-AT pattern in the head.

Axon Tracts in the Caudal Embryo: Visual Assessment of Pattern and Quantification of Motor Neuron Tracts

Each segment in the caudal embryo has a simple repeating pattern of axon tracts; the ventral side of each segment contains caudal primary motor neuron axons (capa) (Fig. 5). The effect of each compound was visually assessed to determine whether the compound caused a decrease in the number, truncation, or disorganization of axon projections (Table 5). Motor neuron axon tracts in the caudal region of zebrafish exposed to 2,4-D are thin and truncated (Fig. 6). Atrazine and dieldrin caused disorganized axons, but no decrease in axon-associated signal (see below). DDT exposure resulted in an increased number of axons that ran outside of the normal axon tracts. Interestingly, in addition to increased axon signal, DDT caused an increase in motor neuron activity

Table 3
Summary of Significant Visible Developmental Phenotypes*

	48 hpf	96 hpf
2,4-D	200 μM[a]: heart edema, hemorrhage, short body	50 μM: slow heart rate, decreased motility
Atrazine	500 μM[a]: slow heart rate, bent caudal embryo, swollen ICM, decreased motility	200 μM: slow heart rate, heart and trunk edema, underdeveloped jaw, short
DDT	100 μM: increased motility (agitation), hemorrhage (rare at 1 mM)	10 μM: slow heart rate, increased motility
Dieldrin	20 μM: slow heart rate, decreased motility, short caudal embryo	1 μM: slow heart rate, tremor
Malathion	10 μM: slow heart rate, swollen ICM, short condensed caudal embryo	1 μM: short body (slight)
Nonylphenol	5 μM[a]: slow heart rate, decreased motility, short body	1 μM: slow heart rate
TCDD	0.1 μM: heart edema, decreased motility, brain necrosis, short body, rare hemorrhage	0.005 μM: slow heart rate, heart edema, underdeveloped jaw, decreased motility, brain necrosis, short body

*Concentrations shown are the lowest detectable concentrations for the described phenotypes.
[a]Concentrations near or above the LC_{50}.

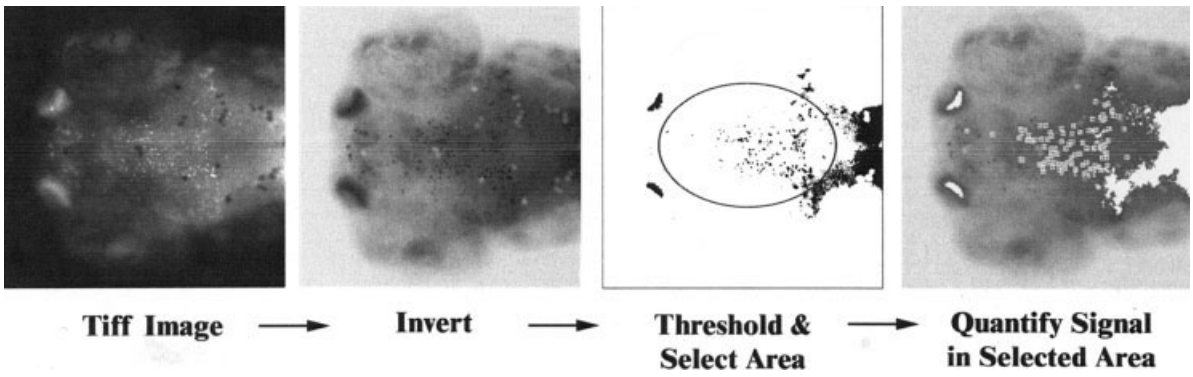

Figure 2. Quantification of apoptosis in the brain by AO staining followed by image analysis. Dorsal views of stained embryos were photographed, inverted and analyzed using Scion Image (Scion Corporation). The signal was determined by application of a signal threshold macro as described in Materials and Methods and the total area (in pixels) of particles in the brain of selected size was calculated.

(Table 3). Zebrafish exposed to malathion had slightly thin capa, whereas the capa in zebrafish exposed to TCDD were thin and truncated. Nonylphenol did not appear to affect the axon tracts in the caudal embryo.

Quantification of Motor Neuron Tracts in 5 Segments of the Caudal Embryo

We quantified the fluorescent signal from α-AT–stained capa in the ventral aspect of 6 segments in the caudal region of the embryo (Figs. 5 and 6; Table 6). Compounds significantly affecting fluorescent signal were 2,4-D, DDT, and TCDD. 2,4-D and TCDD caused a significant loss of signal, whereas DDT increased signal.

Quantification of the Number of Catecholaminergic Neurons

We counted the number of α-TH–positive cells in 4 bilaterally symmetric compartments of the brain at 96 hpf (Fig. 7). The number of cells in the anterior (A) or posterior (P) compartments of 10 zebrafish was averaged for control or exposed zebrafish (Table 7). To compare the effect of different compounds, SIs (see Materials and Methods) were calculated.

All 7 compounds significantly decreased the number of anterior cells. DDT, dieldrin, and nonylphenol also significantly decreased the number of posterior cells. Of the 7 compounds tested, nonylphenol and dieldrin had the greatest effect on both anterior and posterior cells,

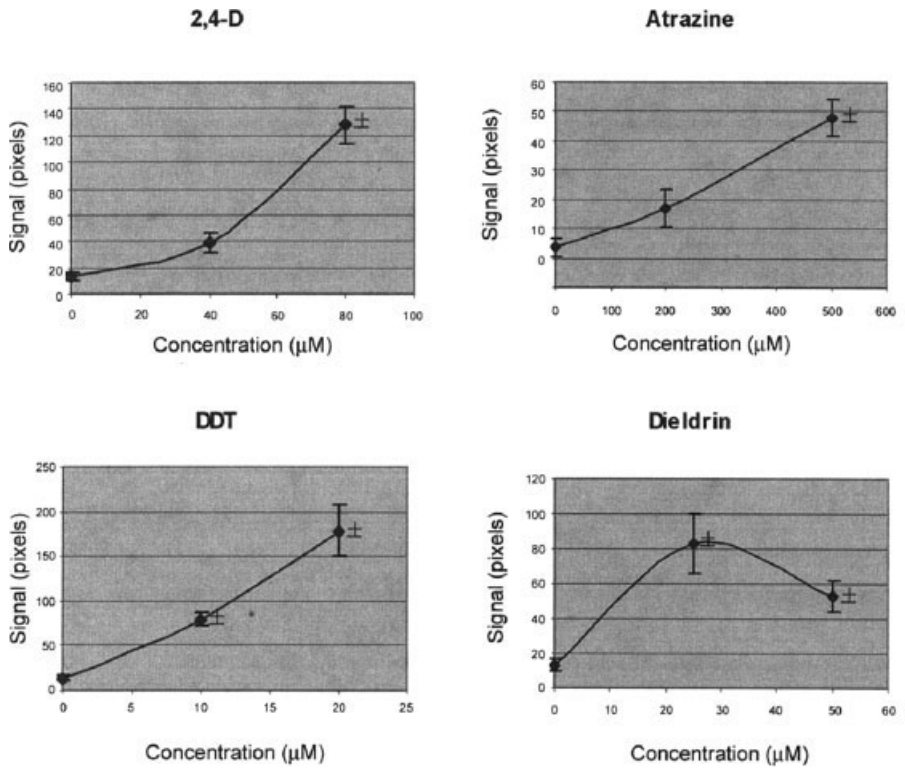

Figure 3. Concentration-response curves for AO staining in the brain at 96 hpf. Cell death (apoptosis) was measured by AO staining and quantified by image analysis. Total signal in the area of interest was quantified and averaged for each concentration. 2,4-D, atrazine, DDT, dieldrin, and malathion significantly increased AO staining in the brain at 96 hpf. Standard error bars are shown, and data points that are significantly different ($P < .5$) from control by the Dunnett's t test are demarcated with ±.

Table 4
Quantification of AO Staining in the Brain in 96-hpf Zebrafish

2,4-D			Atrazine			DDT		
Concentration	Area[a]	SE[a]	Concentration	Area[a]	SE[a]	Concentration	Area[a]	SE[a]
80 μM	128[b]	14	500 μM	58[b]	6.4	20 μM	179[b]	28
40 μM	39	7.6	200 μM	27	6.4	10 μM	79 [b]	7.9
0 μM	13.5	3.2	0 μM	13.5	3.2	0 μM	13.5	3.2
Dieldrin			Malathion			Nonylphenol		
Concentration	Area[a]	SE[a]	Concentration	Area[a]	SE[a]	Concentration	Area[a]	SE[a]
50 μM[c]	53[b]	9.4	5 μM[c]	46[b]	8.3	2 μM	55	18
25 μM[c]	83[b]	17.3	2.5 μM[c]	5.4	2.5	1 μM	54	13
0 μM	13.5	3.2	0 μM	13.5	3.2	0 μM	13.5	3.2

[a]Area and SE were divided by 10 for ease of graphing.
[b]Comparisons significant at the 0.05 level by Dunnett's *t* test.
[c]Concentrations near or above LC_{50}.

whereas atrazine had the least effect on cells in both compartments.

Comparison of Zebrafish Results with Results in Mammals

The zebrafish data are summarized and compared with mammalian data compiled from the literature in Tables 8 and 9. Some endpoints that are easily accessible in zebrafish, for example total cell death in the brain or visual assessment of axon tracts in the brain, are not feasible in mammals. Information on toxicity in mammals is primarily on behavior or motor activity rather than on the motor neurons themselves; however, some correlations can be made between motor activity in mammals and the direct effects seen on motor neurons or catecholaminergic neurons in zebrafish (Table 9). During assessment of teratogenicity, zebrafish were scored for a basic behavior relating to motor activity (touch response) and, in the case of zebrafish exposed to dieldrin, tremors were noted; these behaviors were compared directly with the mammalian data. Compared with mammalian data, teratogenicity assessed in zebrafish at 96 hpf was particularly similar (Table 8). In addition, many of the developmental phenotypes seen in zebrafish are quite similar to mammalian phenotypes (Table 9).

DISCUSSION

Assessment of Teratogenicity to Determine Whether Compound Toxicity is General or Specific Neurotoxicity

A teratogen is defined as a compound that causes developmental defects at concentrations that are lower than concentrations that cause lethality. This ratio can change depending on the developmental stage at which the compound demonstrates its particular toxic effect on development. For example, atrazine was not teratogenic when assessed at 48 hpf, yet was teratogenic when assessed at

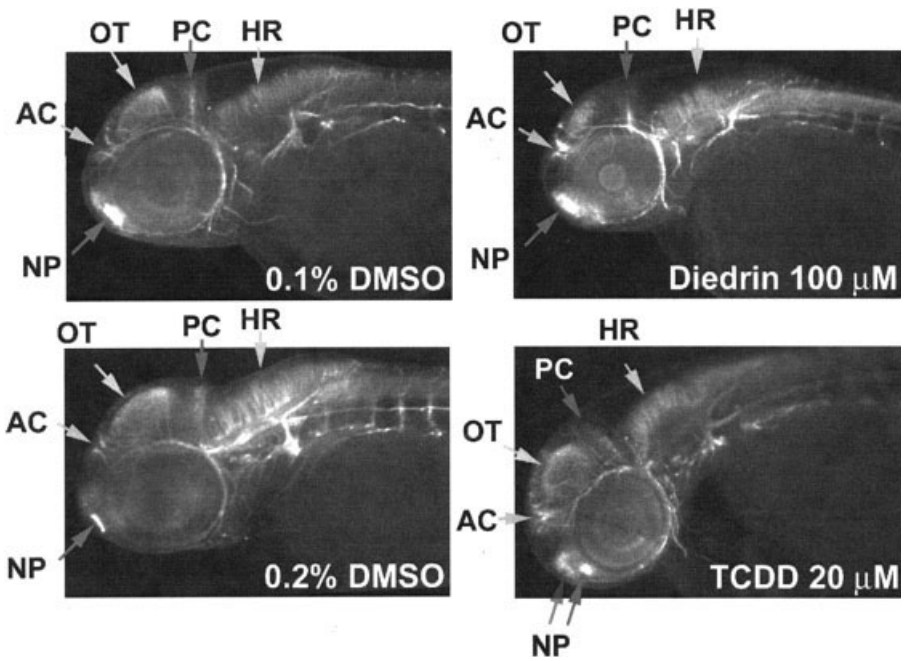

Figure 4. Visual assessment of axon tracts in the brain of control and compound-exposed embryos at 48 hpf. AC, anterior commissure (arrow); HR, hindbrain region (arrow); NP, nasal placode (arrow); OT, optic tectum (arrow); PC, posterior commissure (arrow). Anterior is left and dorsal is up.

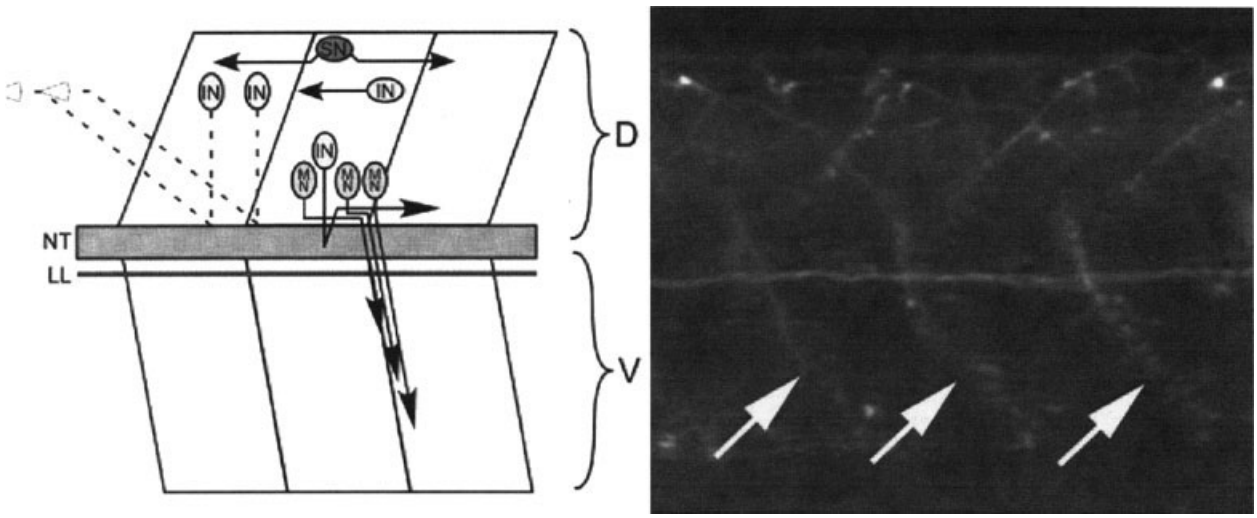

Figure 5. Visual assessment of axon tracts in the caudal embryo. Diagram on the left shows that the dorsal segment (D) contains sensory neurons (SN), interneurons (IN), and motor neurons (MN). Right panel shows axon tracts in a fixed 48 hpf zebrafish stained with an α-AT antibody. Motor neuron tracts are visible ventrally (V) between each segment (white arrows in photograph). LL, lateral line (sensory); NT, neural tube. Dorsal is up, rostral to the left. Diagram after Kuwada and Bernhardt (1990).

96 hpf, suggesting that atrazine is toxic to later developmental events (those that occur between 48 and 96 hpf). Conversely, both DDT and TCDD are teratogenic when assessed at both developmental stages; the developmental effects of these compounds are evident from early stages. It is therefore necessary to establish both the LC_{50} and teratogenicity at each developmental stage of interest. We assessed LC_{50} and teratogenicity at 48 and 96 hpf, the developmental stage endpoints for the neurotoxicity assays, so that we could determine whether the neurotoxic effects seen were due to general developmental toxicity or were specific to the nervous system. If the compound is both neurotoxic and a teratogen, it is likely that the neurotoxicity is a nonspecific effect caused by general developmental toxicity; if, however, a compound is neurotoxic and not teratogenic, it is likely that it is a neurotoxin.

Comparison of Teratogenicity Assessed in Zebrafish with Teratogenicity Assessed in Mammals

As expected, the teratogenicity of the 7 compounds tested varied depending on the stage of assessment; teratogenicity determined at 96 hpf in zebrafish is more similar to teratogenicity reported in mammals than teratogenicity determined at 48 hpf (it should be noted that estimates of teratogenicity in mammals also vary by species). Teratogenicity in mammals is generally assessed at relatively late developmental stages, which could account for the high correlation with zebrafish results at 96 hpf.

Neurotoxicity

2,4,-D. 2,4-D affects motor neuron function in mammals and in adult rainbow trout; sublethal exposure to 2,4-D inhibited swimming, feeding, and predator escape behaviors (Little et al., 1990). Consistent with these findings, in zebrafish embryos, 2,4-D caused a significant increase in brain cell death (apoptosis), disrupted motor neuron growth at high concentrations, and decreased motility even at moderate concentrations. As in mammals, 2,4-D was only slightly teratogenic in zebrafish; therefore, the neurotoxicity observed is likely to be specific.

Atrazine. Atrazine affects motor neuron activity and dopamine production in mammals; however, studies in fish species have focused on detoxification and estrogenic and developmental effects and there is little information regarding neurotoxicity in fish (Wiegand et al., 2000; Bringolf et al., 2004; Spano et al., 2004). In zebrafish, atrazine caused a significant increase in brain cell death (apo-

Table 5
Visual Analysis of α-AT Staining in the Axons of the Brain and Caudal Region at 48 hpf

Compound	Concentration	Axons in brain	Axons in caudal region
2,4-D	200 μM[a]	Slightly reduced optic tectum (OT)	Short, thin
Atrazine	500 μM[a]	Slightly reduced optic OT	Disorganized with multiple projections
DDT	1 mM	Normal	Capa normal but with extra-tract axons
Dieldrin	100 μM	Reduced OT and posterior commissure (PT)	Distal projections disorganized
Malathion	10 μM	Slightly reduced OT	Capa slightly thin
Nonylphenol	2 μM	Normal	Capa normal
TCDD	20 μM	Reduced OT, PT, and hindbrain region (HR)	Capa thin, truncated

[a]Concentrations near or above LC_{50}.

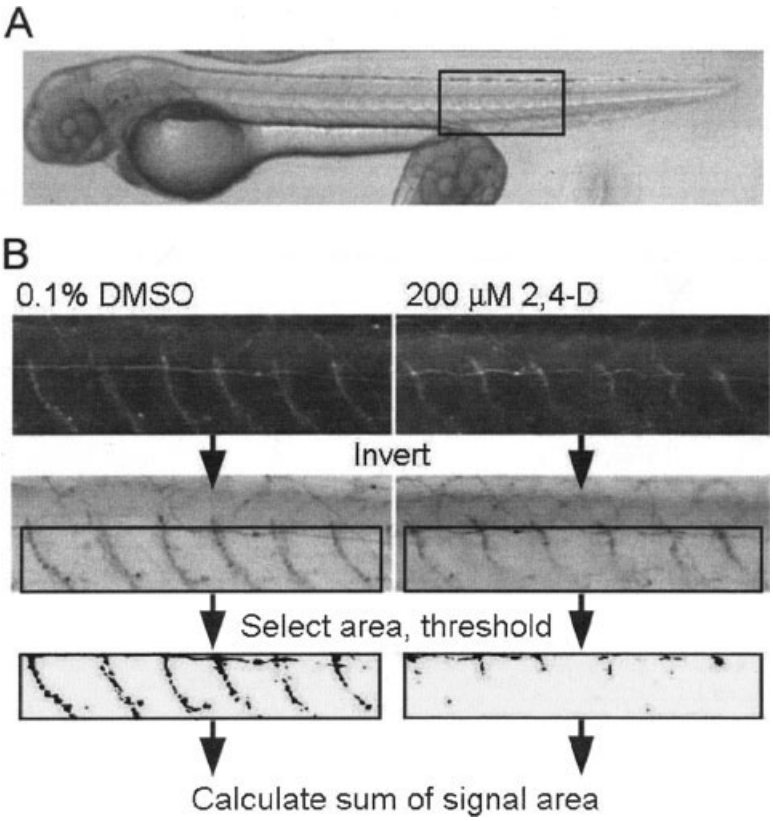

Figure 6. Axons in the caudal embryo of control and compound-exposed embryos at 48 hpf were quantified by image analysis following α-AT staining. **A:** Live 48 hpf zebrafish: Boxed area in photograph shows region measured. **B:** Top photographs show images of the caudal regions of fixed and stained 48 hpf zebrafish, containing 5 segments and the 6 bordering caudal primary motor neurons axons (arrows). To quantify α-AT signal in the 6 axon tracts, the images were inverted and thresholded as described in Materials and Methods, and the total signal in pixels was calculated using Scion Image software.

ptosis) at the highest concentration tested, but had little effect on axon tracts or catecholaminergic neurons. Although atrazine is not teratogenic in mice, there is evidence that it causes general disruption of development in medaka and zebrafish (Wiegand et al., 2001; Ishaque et al., 2004). In our studies, atrazine was teratogenic in zebrafish at 96 hpf; therefore, the neurotoxicity evidenced at 96 hpf is likely to be due to general developmental toxicity.

DDT. Neurotoxicity has been associated with DDT exposure in mammals; however, there are little data regarding neurotoxicity of DDT in fish. Studies of effects of DDT in fish have focused on accumulation in agriculturally significant species and on the estrogenic effects (Brucker-Davis, 1998; Zaroogian et al., 2001; Leanos-Castaneda et al., 2002; Papoulias et al., 2003; Kuhl et al., 2005). In zebrafish, DDT caused significant cell death (apoptosis) in the brain, increased motility, and stimulated extra motor neuron axons in the caudal embryo; these findings correlate with the observations in mammals that DDT exposure causes excitability, tremors, and hyperactivity. In zebrafish, DDT caused loss of catecholaminergic neurons, including the posterior group made up primarily of dopaminergic neurons, which correlates with known toxicity to dopaminergic neurons in vitro. However, similar to effects seen in mice, after continuous exposure from 6 hpf, DDT is teratogenic at both 48 and 96 hpf in zebrafish; therefore, it is likely that the neurotoxicity caused by DDT is due to more general developmental toxicity.

Dieldrin. Dieldrin is a nervous system stimulant and causes tremors in mammals; however, like DDT, little is known about neurotoxicity in fish, and studies in fish have focused primarily on bioaccumulation, estrogenic activity (dieldrin appears to have little estrogenic activity in fish), and liver toxicity (Pedrajas et al., 1996; Leanos-Castaneda et al., 2002; Tollefsen et al., 2002). In zebrafish, dieldrin caused cell death (apoptosis) in the brain, disorganized motor neuron axons, tremors, and a loss of catecholaminergic neurons, including dopaminergic neurons.

Table 6
Effects on Motor Neuron Signal in the Caudal Region of 48-hpf Zebrafish by α-AT Staining

	0.1% DMSO	200 μM[a] 2,4-D	500 μM[a] atrazine	1 mM DDT	100 μM dieldrin	10 μM malathion	2 μM nonylphenol	20 μM TCDD
Average	7438	4064	6290	10797	6766	7639	7479	3882
SE	670	371	705	655	611	529	666	586
t test		0.00074	0.25	0.002	0.99	0.82	0.95	0.001
SI		−45	−15	+45	−9	+2.7	+0.55	−48

[a]Concentrations near or above LC_{50}.

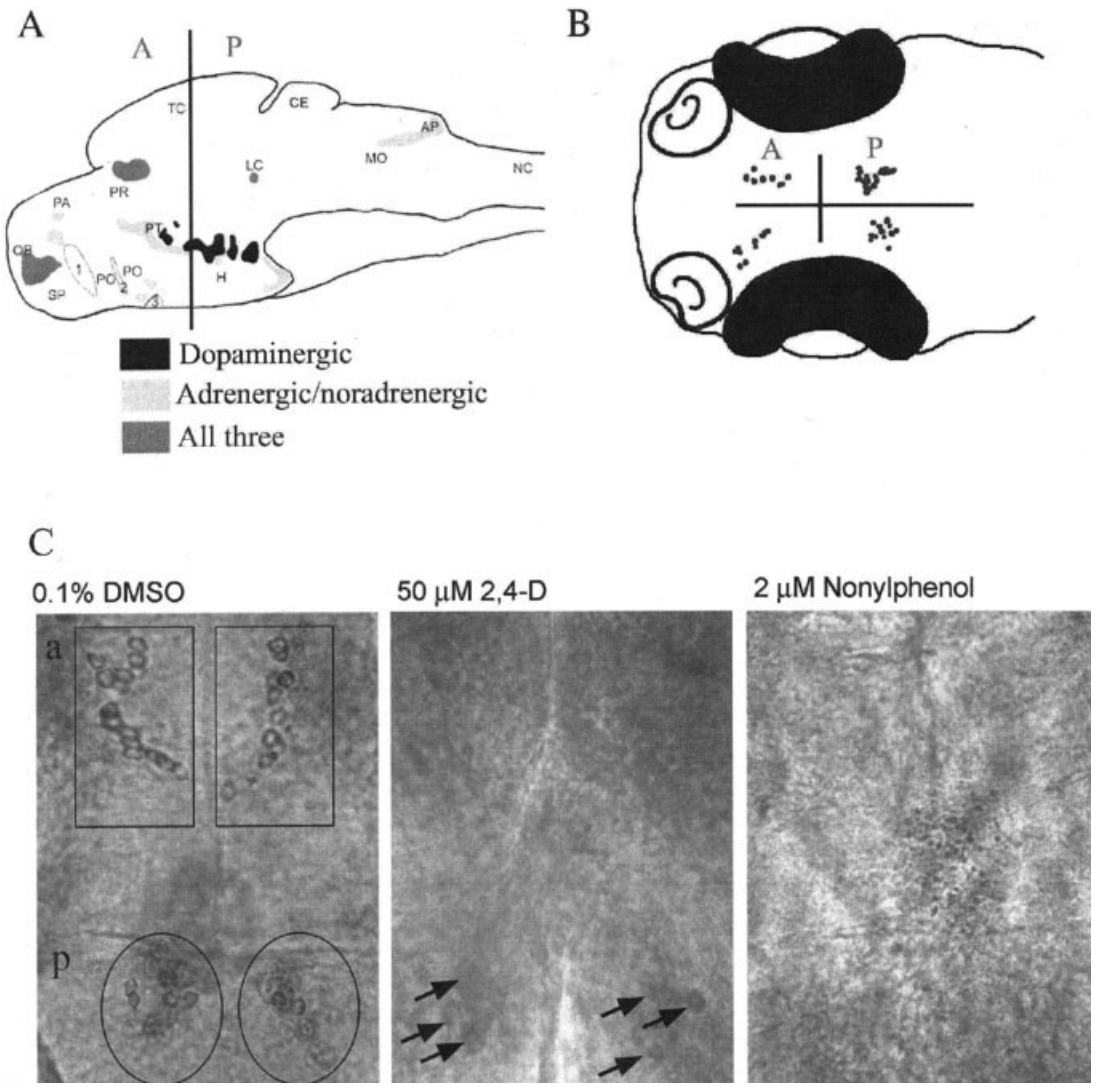

Figure 7. Effect of compounds on catecholaminergic neurons in the zebrafish brain at 96 hpf. **A:** Diagrammatic representation of the distribution of catecholaminergic neurons based on differential staining with various markers (from Holzschuh et al., 2001). **B:** Traced from a dorsal view photo of α TH-stained control embryo showing the 4 clusters of positive cells, divided into pairs of anterior (A) and posterior (P) groups. Comparison of the 2 diagrams suggests that A clusters correspond to several clusters of adrenergic/noradrenergic neurons and 2 large clusters of all 3 types of neurons, and P clusters correspond primarily to dopaminergic neurons. **C:** Catecholaminergic neurons in the brain at 96 hpf visualized by α-TH staining. Staining in control (0.1% DMSO) zebrafish is in 4 bilaterally symmetric clusters of neurons: 2 anterior (boxes) and 2 posterior groups (ovals). Cells in the anterior groups are missing and reduced in the posterior group of zebrafish exposed to 2,4-D (arrows); cells in both A and P groups are missing in nonylphenol-exposed zebrafish. AP, area postrema; CE, corpus cerebellum; H, hypothalamus; LC, locus coeruleus; MO, medulla oblongata; OB, olfactory bulb; PA, pallium; PO, preoptic area; PR, pretectal area; PT, posterior tuberculum; SP, subpallium; TC, tectum; 1, anterior commissures; 2, 2nd ventricle; 3, optic chiasma.

Dieldrin is known to inhibit acetylcholinesterase in mammals, resulting in tremors, and is toxic to dopaminergic neurons in vitro. Dieldrin is not teratogenic in mammals and is only slightly teratogenic in zebrafish at 48 hpf; therefore, it is likely that dieldrin is a specific neurotoxin, especially with regard to the later phenotypes of brain cell death and loss of catecholaminergic neurons.

Malathion. Although malathion is not known to cause developmental toxicity in mammals, acute toxicity in the adult rat includes decreased motor activity and reduced touch response. At concentrations similar to those tested here, Malathion has been shown to cause oxidative stress and significant developmental toxicity in catfish and zebrafish (Lien et al., 1997; Nguyen and Janssen, 2002; Galloway and Handy, 2003; Cook et al., 2005; Rosety et al., 2005). In our experiments, malathion caused cell death (apoptosis) in the brain and loss of catecholaminergic neurons at concentrations above LC_{50}; these phenotypes are therefore likely due to general toxicity. Exposure to lower concentrations of Malathion at 48 hpf decreased axon projections to the optic tectum and posterior commissure in the brain; however, we observed teratogenicity when assessed at 48 hpf and these phenotypes

Table 7
Compound Effects on Anterior and Posterior Compartments of Catecholaminergic Neurons in the Brains of 96-hpf Zebrafish

	DMSO		50 μM 2,4-D		500 μM atrazine		20 μM DDT	
	A	P	A	P	A	P	A	P
Average	4.35	11.5	1.1	8.15	1.3	9.65	0.6	4.2
SE	0.8	1.2	0.49	1.3	0.33	1.3	0.28	1
t test			0.0016	0.088	0.0018	0.376	0.0002	9.00E–05
SI			−0.75	−0.28	−0.7	−0.15	−0.86	−0.63
	50 μM[a] dieldrin		2.5 μM[a] malathion		2 μM nonylphenol			
	A	P	A	P	A	P		
Average	0.6	1.5	0.4	7.7	0.1	0.45		
SE	0.18	0.39	0.15	1	0.069	0.29		
t test	7.60E–05	1.00E–07	1.00E–04	0.031	4.00E–05	2.00E–08		
SI	−0.93	−0.87	−0.91	−0.32	−0.98	−0.96		

[a]Concentrations near or above LC_{50}.
A, anterior; P, posterior.

are likely to be due to developmental toxicity. Therefore, malathion is not likely to cause specific neurotoxicity.

Nonylphenol. Nonylphenol does not cause neurotoxicity in mammals; likewise, it has no effect on motor neurons in the brain or in the caudal region of the zebrafish, nor did it cause cell death (apoptosis) in the brain. Nonylphenol did cause a significant loss of catecholaminergic neurons in zebrafish; however, there is no evidence in the literature of a similar effect in mammals. Nonylphenol is not teratogenic in either mammals or other fish species (Schwaiger et al., 2000; Balch and Metcalfe, 2006) and no teratogenicity was seen in zebrafish assessed at either 48 or 96 hpf. Nonylphenol has been shown to have estrogenic activity in mammals and other fish species (Schwaiger et al., 2002; Li and Wang, 2005; Meucci and Arukwe, 2005), and although some endocrine disruptors can cause neurotoxicity, there is little evidence in the literature for neurotoxicity caused by nonylphenol. The results from our zebrafish studies indicate that nonylphenol causes specific toxicity to catecholaminergic neurons but causes little or no other neurotoxicity.

TCDD. Although when compared with other fish, zebrafish embryos are relatively insensitive to TCDD (Henry et al., 1997; Teraoka et al., 2002), zebrafish exhibit signs of toxicity in response to TCDD exposure that are common to many fish species, including pericardial edema, slow circulation, growth retardation, and toxicity to vascular endothelial cells (Guiney et al., 1997; Henry et al., 1997; Cantrell et al., 1998; Dong et al., 2001, 2002; Teraoka et al., 2002). TCDD is also highly teratogenic in mammals and causes several similar developmental phenotypes, including cardiovascular problems, impairment of function of the cerebellum, and liver toxicity. In our experiments, TCDD was highly teratogenic at both 48 and 96 hpf, although, as previously reported (Henry et al., 1997; Teraoka et al., 2002), early stages of embryogenesis are only affected by very high concentrations of TCDD (reflected in the much higher LC_{50} and EC_{50} values at 48 hpf). We report major developmental phenotypes similar to those seen in other reports, including cardiovascular defects (decreased heart rate, edema), decreased motility, and short body length.

Both necrosis and apoptosis in the brain have been observed in fish embryos exposed to TCDD (Spitsbergen et al., 1990; Henry et al., 1997; Dong et al., 2001, 2002; Hill et al., 2003; Dong et al., 2004). At environmentally relevant concentrations (100–500 ppt; 0.312–1.56 nM), TCDD causes a reduction in overall brain volume (30% reduction at 168 hpf) with associated apoptosis, likely due to decreased expression of neurogenin and sonic hedgehog (Hill et al., 2003). However, it has been shown in fish that the primary target of TCDD toxicity is vascular endothelial cells, and it is thought that neurotoxicity is secondary to vascular toxicity (Guiney et al., 1997; Cantrell et al., 1998; Dong et al., 2002). In our experiments, TCDD exposure reduced the number of axon tracts throughout the brain, and significantly reduced fluorescent signal of axon tracts in the caudal region of the embryo. Even at the lowest TCDD levels tested, necrosis was observed in the brain, which obscured the results of assays for cell death and catecholaminergic neurons. The concentrations used in our experiments (e.g., 5 nM at 96 hpf) were slightly higher than those in reports noting TCDD-related brain apoptosis in zebrafish (e.g., 1.5 nM at 60 hpf), which may explain why we observed necrosis rather than AO-positive cells in the brain, which are likely to be apoptotic. Because TCDD is highly teratogenic in both zebrafish and mammals, any effects on the nervous system are likely to be secondary to general developmental toxicity, and therefore we would not consider TCDD to be a specific neurotoxicant.

Table 8
Teratogenicity in Mammals Compared with 96-hpf Zebrafish

Compound	Mammals	96-hpf zebrafish
2,4,-D	No (rats, rabbits); Slight (mouse)	Slight
Atrazine	No (mouse)	Yes
DDT	Yes (mouse)	Yes
Dieldrin	No	No
Malathion	No (rat, rabbit)	No
Nonylphenol	No (rat)	No
TCDD	Highly (rat, mouse, hamster)	Highly

Table 9
Comparison of Zebrafish and Mammalian Developmental Neurotoxicity

	Developmental and neurotoxicity phenotypes	
Compound	Mammalian	Zebrafish
2, 4-D	Acute (rat): transient coordination problems Decreased motor activity Chronic (rat): retinal degeneration Chick embryo: hypomyelination Parkinson's like phenotypes: tremor, rigidity, slow movement In mice: embryotoxic, slightly teratogenic In rats: not teratogenic In rabbits: not teratogenic References for mammalian data: (Charles et al., 2001; Mattson et al., 2001; Mori de Moro et al., 1993; USDHHS, 1993; USEPA, 1993)	Increased cell death in brain at high concentrations Slightly reduced axon projections into optic tectum at high concentrations Disruption of motor neuron axon growth at high concentrations Decreased motility Loss of catecholaminergic and probable loss of dopaminergic neurons Slightly teratogenic
Atrazine	Decreased electrical activity of cells in the rat cerebellum (the part of the brain concerned with motor function, muscle tone, and balance) Alters production of dopamine and norepinephrine Not teratogenic (mice) Damage to liver and heart EXTOXNET, (Cox, 2001; Podda et al., 1997; Taylor, 2002)	Increased cell death in brain Causes disorganized motor neuron axon growth Slight loss of catecholaminergic neurons Teratogenic at 96 hpf Circulatory problems: decreased heart rate, edema
DDT	Acute (human, mouse): nausea, irritability, malaise and excitability, tremors Chronic (mouse): liver, kidney, nervous and immune system toxicity; tremors, hyperactivity in older animals Alters ion channel function Dopaminergic neuron toxicity in vitro Teratogenic (mouse) impaired learning performance in maze tests, abnormal caudal embryo development EXTOXNET, (Bloomquist, 1996; Leung et al., 2003; USEPA, 1998)	Increased cell death in brain Increased random motion, motility Induced extra motor neuron axons in the caudal embryo Loss of catecholaminergic and probable loss of dopaminergic neurons Teratogenic at 96 hpf
Dieldrin	Not teratogenic Chronic (mouse, rat): liver damage Acute (mouse, rat): central nervous system stimulant Inhibits acetylcholinesterase, causing tremors Dopaminergic neuron toxicity in vitro (Jorgenson, 2001); IPCS INCHEM, http://www.inchem.org/; Pesticide Action Network, http://www.pesticideinfo.org	Teratogenic at 48 hpf, not at 96 hpf Increased cell death in brain Tremor Disorganized motor neurons in the caudal embryo Loss of catecholaminergic and probable loss of dopaminergic neurons
Malathion	No developmental or reproductive toxicity in rat or rabbit Acute (rat): decreased motor activity; exaggerated touch response at moderate doses, reduced touch response at high doses, decreased plasma and erythrocyte cholinesterase activity Chronic (rat): inhibition of brain, erythrocyte and plasma cholinesterase ATSDR, 2000	Teratogenic at 48 hpf, not at 96 hpf Short body Caused decreased axons in optic tectum and posterior commissure in brain, decreased motor neurons in the caudal embryo Decreased catecholaminergic neurons in forebrain
Nonylphenol	Not teratogenic (rat) No neurotoxicity observed Acute: nontoxic in rat Chronic: liver toxicity in rat, beagle Slightly estrogenic in vitro and in vivo (Bakke, 2003; Yokota et al., 2001)	Not teratogenic Did not increase cell death in brain No effect on axon growth Loss of catecholaminergic and probable loss of dopaminergic neurons
TCDD	Potent teratogen (rat, mouse, hamster) Acute (mouse, guinea pig, rat): weight loss, thymic atrophy, cardiovascular problems, liver toxicity Chronic (rat, mouse): anemia, carcinogenicity Affects GABAergic neurons in preoptic area (mouse) Affects cerebellum in mice TOXNET, (Hays et al., 2002; Olson et al., 1990; Williamson et al., 2005)	Highly teratogenic Circulatory problems No increased AO staining (apoptosis; necrosis noted) Effect on axons: normal for developmentally delayed embryo No effect on catecholaminergic neurons

FUTURE DIRECTIONS

Our results suggest that the zebrafish is a good model with which to initially assess compound effects on developmental neurotoxicity. To further establish zebrafish as a predictive model, additional compounds must be assessed using this system. In the absence of necrosis, AO staining for apoptosis in the brain is a good indicator of more specific neurotoxicity and the assay is simple and fast. Necrosis is easily seen by visual inspection, and a screen that combines a quick preliminary assessment for necrosis followed by AO staining would provide a relatively high-throughput whole-animal assay to identify compounds that are potentially neurotoxic. Positive compounds from this initial assessment would then be subjected to the specific assays described. It is also essential to include an assessment of teratogenicity to discriminate between specific neurotoxicity and general developmental toxicity. The correlation between zebrafish and mammalian teratogenicity data was significant, especially at 96 hpf; this correlation is significant, considering that teratogenicity often differs even among mammals. If results with additional compounds support our preliminary data, a battery of zebrafish assays similar to those described here would provide a comprehensive, relatively rapid and inexpensive method to identify potential neurotoxicants.

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