Environmental Estrogens Alter Early Development in *Xenopus laevis*

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A growing number of environmental toxicants found in pesticides, herbicides, and industrial solvents are believed to have deleterious effects on development by disrupting hormone-sensitive processes. We exposed *Xenopus laevis* embryos at early gastrula to the commonly encountered environmental estrogens nonylphenol, octylphenol, and methoxychlor, the antiandrogen, *p,p′*-DDE, or the synthetic androgen, 17α-methyltestosterone at concentrations ranging from 10 nM to 10 μM and examined them at tailbud stages (~48 hr of treatment). Exposure to the three environmental estrogens, as well as to the natural estrogen 17β-estradiol, increased mortality, induced morphologic deformations, increased apoptosis, and altered the deposition and differentiation of neural crest–derived melanocytes in tailbud stage embryos. Although neural crest–derived melanocytes were markedly altered in embryos treated with estrogenic toxicants, expression of the early neural crest maker *Xslug*, a factor that regulates both the induction and subsequent migration of neural crest cells, was not affected, suggesting that the disruption induced by these compounds with respect to melanocyte development may occur at later stages of their differentiation. Co-incubation of embryos with the pure antiestrogen ICI 182,780 blocked the ability of nonylphenol to induce abnormalities in body shape and in melanocyte differentiation but did not block the effects of methoxychlor. Our data indicate not only that acute exposure to these environmental estrogens induces deleterious effects on early vertebrate development but also that different environmental estrogens may alter the fate of a specific cell type via different mechanisms. Finally, our data suggest that the differentiation of neural crest–derived melanocytes may be particularly sensitive to the disruptive actions of these ubiquitous chemical contaminants. *Key words:* antiandrogens, apoptosis, embryogenesis, environmental toxicants, estrogens, melanocytes, neural crest, *Xenopus laevis*, *Xslug*. *Environ Health Perspect* 111:488–496 (2003). doi:10.1289/ehp.5500 Available via *http://dx.doi.org/* [Online 28 October 2002]

For nearly half a century, studies of both natural and laboratory vertebrate populations have suggested that exposure to a variety of environmental chemicals, including pesticides, herbicides, and industrial solvents, elicits deleterious effects during development by interfering with hormone-sensitive processes (Colborn et al. 1993). However, concern over the potentially harmful effects of these compounds has escalated within the past decade, spurred on by data demonstrating that more than 100,000 chemicals are now being produced on an industrial scale and several thousand new chemicals are introduced each year (Younes 1999). Some of the most prevalent and persistent of these compounds include the pesticide 1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane (DDT) and its major metabolite, 1,1-dichloro-2,2-*bis*(*p*-chlorophenyl) ethylene (*p,p′*-DDE; Kelce et al. 1998; Sohoni and Sumpter 1998); methoxychlor, an analog of DDT (Palanza et al. 1999); and nonylphenol and octylphenol, degradation products of the alkylphenol polyethoxylates (APEOs). These APEOs are widely used as nonionic surfactants in commercial production as well as in herbicides, pesticides, polystyrene plastics, and paints (Cooper and Kavlock 1997; Sonnenschein and Soto 1998; White et al. 1994). Although contamination by these environmental toxicants is often first evident in the water supply, most of these compounds are highly lipophilic and bioaccumulate in fatty tissue, thereby presenting potential developmental hazards for both aquatic and terrestrial species (Crews et al. 2000; Menditto and Turrio-Baldassarri 1999; Sonnenschein and Soto 1998).

The majority of environmental toxicants known to interfere with hormone signaling, including nonylphenol and octylphenol, are believed to exert their effects at nuclear estrogen receptors (ERα or ERβ; Mueller and Kim 1978; White et al. 1994). However, recently it has been shown that some compounds, including *p,p′*-DDE, are devoid of action at the ER, but block signaling mediated by the androgen receptor (Kelce et al. 1998; Kelce and Gray 1999; Sohoni and Sumpter 1998). Finally, some environmental estrogens, including the major metabolite of methoxychlor, 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane, have been shown not only to be active at the ER [for review, see Cummings (1997)] but also to elicit biologic actions as an antiandrogen via nuclear hormone-independent mechanisms (Ghosh et al. 1999; Ren et al. 1997; Waters et al. 2001).

There is a wealth of data on the effects of exposure of environmental toxicants believed to interfere with hormone-sensitive processes related to reproductive development and sexual differentiation (Crews et al. 2000; Gray 1992; Kelce and Gray 1999; Sharara et al. 1998), but few studies have examined the effects of early exposure to these environmental toxicants on other aspects of vertebrate development. Because of their rapid development and their aquatic nature, amphibians may be particularly sensitive and useful sentinels for studying the effects of environmental toxicants on early development (Blaustein et al. 1994; Kloas et al. 1999). In particular, the laboratory frog *Xenopus laevis* provides an excellent model system to assess the effects of early exposure to environmental toxicants because of its ability to generate embryos on a daily basis and because the molecular and organismal development of this vertebrate has been described extensively (for review, see Mayor et al. 1999; Spitzer and Ribera 1998; Weinstein and Hemmati-Brivanlou 1999). A recent study (Mann and Bidwell 2000) published during the course of the present experiments showed that chronic exposure (~96 hr) of *Xenopus* embryos to the nonylphenol polyethoxylate (NPEO), Teric GN8, at moderate concentrations (EC_{50} = 2.8–4.6 mg/L; ~5–8 μM assuming a standard oligomer length of 8) induced malformations in *Xenopus* tadpoles including cardiac edema, microphthalmia, and improper gut coiling in embryos examined at stage 46 (Nieuwkoop and Faber 1967) and increased mortality by stages 39–40 in embryos exposed to higher concentrations (6.0–10 mg/L; 10–17 μM).

The effects reported for exposure to this synthetic compound mirror those produced by the naturally occurring estrogen 17β estradiol) (E_2) (Nishimura et al. 1997), which is consistent with a common mechanism of action for NPEO and E_2. Specifically, Nishimura et al. (1997) demonstrated that exposure (beginning at stage 3) to 10 μM E_2 caused increases in mortality and increased incidence of malformations, including crooked vertebrae, swollen stomachs, small eyes and heads, and suppressed organogenesis

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of digestive organs and the nervous system. In this study no deleterious effects of E_2 exposure were evident before stage 27, but subsequent to this developmental stage both the incidence of malformations and mortality increased, and all embryos died by stage 42. Moreover, these investigators demonstrated that the ability of estrogens to induce these effects was limited to a specific critical period: No malformations were evident in embryos treated after stage 39. These studies indicate that exposure to estrogenic compounds at inappropriate and critical times during development induce aberrations in numerous organ systems, not solely those involved in reproductive function.

Normal development and remodeling of many organ systems requires programmed cell death, and this process plays a particularly prominent role in the development of neuronal lineages (for review, see Sastry and Rao 2000). In *Xenopus* there is an abrupt onset of programmed cell death, as indicated by the presence of apoptotic TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling)-stained cells. Apoptosis in *Xenopus* begins at gastrulation (stage 10.5) and continues throughout larval development (Hensey and Gautier 1998). The most appreciable staining was noted in the developing nervous system, with the highest levels of TUNEL staining observed in early neural plate–stage embryos (stage 16–17), followed by a decline during late neural plate stages, and a subsequent resurgence of apoptosis in later tadpole stages (stages 26–35; embryos not examined at later stages) (Hensey and Gautier 1998). Endogenous and environmental estrogens have been shown to alter apoptosis; however, most studies demonstrating effects of environmental estrogens have been performed on cell lines, and some of the studies that have been performed on intact embryos indicate the effects of these toxicants are limited to reproductive tissues (Weber et al. 2002).

In amphibians, as in mammals and birds, the developing neural plate gives rise to both the central nervous system and the neural crest. An extraordinary number of diverse cellular phenotypes including sensory neurons, sympathetic neurons, and Schwann cells in the peripheral nervous system, cartilage and bone in the head, smooth muscle, connective cells in the dorsal fin, and all nonretinal pigment cells, are derived from the neural crest (LeDouarin 1982). Neural crest–derived precursors arise at stage 15–16; migration begins at stage 19 and is completed by stage 40 (Epperlein and Löfberg 1993; Krotoski et al. 1988). Although neural crest derived precursor cells can be labeled experimentally to study the ultimate fate of these disparate cell types (Borchers et al. 2000; Carl et al. 1999; Collazo et al. 1993; Krotoski et al. 1988; LaBonne and Bronner-Fraser 2000), melanocytes, because of their pigmentation, have provided an excellent naturally occurring marker cell population for studies of neural crest development and differentiation in *Xenopus* (Epperlein et al. 1996; Krotoski et al. 1988). As with the development of other tissues, apoptosis has been shown to play a pivotal role in governing the normal acquisition of the differentiated phenotype for many neural crest derivatives (Graham et al. 1996; Wakamatsu et al. 1998).

Prospective neural crest can first be identified by the late gastrula stage (stage 12, ~13 hr after fertilization) by expression of mRNA encoding the zinc finger gene *Xslug* on the lateral edge of the neural plate (Mayor et al. 1995). *Xslug* expression is one of the earliest known responses to neural crest inducers in the dorsal marginal zone (Mayor et al. 1995), and its expression profile delineates premigratory and migratory neural crest. Specifically, *Xslug* mRNA is strongly expressed in the lateral neural folds in early neural plate (stage 14, ~16 hr) embryos, coalesces into patterns approximating the premigratory crest aggregates by stage 16 (~18 hr), is evident in migratory streams in the head and trunk in taibud stages (stages 22–26, ~24–30 hr), but diminishes drastically by midtadpole development (stage 34, ~44 hr) as migration is completed (Linker et al. 2000; Mayor et al. 1995). *Xslug* has been demonstrated to play a pivotal role in both the induction and the subsequent migration of neural crest cells in *Xenopus* (Carl et al. 1999; LaBonne and Bronner-Fraser 2000; Linker et al. 2000; Mayor et al. 1995) and has been postulated to regulate apoptosis (Hemavathy et al. 2000), a process essential for embryonic development and neural patterning for both the central nervous system and neural crest-derived structures (Graham et al. 1996; Sastry and Rao 2000; Wakamatsu et al. 1998).

In the present study, we treated *Xenopus* embryos beginning at stage 10.5, a time that corresponds to the onset of developmental apoptosis but is before the onset of neural induction, with toxicants known to act as environmental estrogens (nonylphenol, octylphenol), as antiandrogens (*p,p′*-DDE), or as mixed estrogenic/antiandrogenic agents (methoxychlor) and compared these results to embryos treated with saline, vehicle, the natural estrogen E_2, or the synthetic androgen 17α-methyltestosterone (17α-MeT). Tadpoles were examined at stage 37, a stage at the end of the critical period for the detrimental actions of E_2 (Nishimura et al. 1997) and when nervous system development has proceeded to the extent that animals manifest normal free-swimming motor patterns (van Mier et al. 1989). Here we report that exposure to compounds with estrogenic actions resulted in increased mortality, gross abnormalities in body shape, enhanced apoptosis, and significant aberrations in the differentiation of neural crest–derived melanocytes in tadpoles. The observed effects were both compound- and dose-dependent, and the actions of nonylphenol but not methoxychlor could be inhibited by the pure antiestrogen ICI-182,780 (Howell et al. 2000). Although the most marked changes induced by these estrogenic compounds were in the deposition and differentiation of neural crest–derived melanocytes, these effects were elicited in the absence of any marked change in the expression of the neural crest marker *Xslug*. Taken together, these data indicate that early exposure to estrogenic toxicants induces widespread abnormalities in a number of developing tissues. Moreover, although our results suggest that neural crest derivatives may be highly sensitive and excellent markers of the disruptive actions of environmental estrogens, these toxicants appear more likely to interfere with factors that regulate the later differentiation of neural crest cells rather than those that control their early induction.

Materials and Methods

Generation of embryos. We induced matings by injecting human chorionic gonadotropin (Sigma Chemical Company, St. Louis, MO) to adult pairs of *Xenopus laevis* (Henderson et al. 1984). We used albino frogs for *in situ* hybridization studies of *Xslug* mRNA expression and pigmented frogs for all other studies. Embryos were staged according to the normal table of Nieuwkoop and Faber (1967). Animal care procedures were approved by the Institutional Animal Care and Use Committee at Dartmouth and adhered to both the National Institutes of Health and the American Veterinary Medical Association guidelines.

Chemical compounds. Nonylphenol (4-nonylphenol; Aldrich Chemical Company, Milwaukee, WI), octylphenol [4-(*tert*-octyl)phenol; Aldrich], *p,p′*-DDE (Aldrich), methoxychlor (Aldrich), E_2 (Sigma), or 17α-MeT (Sigma) were dissolved in either 100% standard or 95% HPLC grade ethanol (EtOH; Aldrich) or 100% dimethylsulfoxide (DMSO; Fisher, Pittsburgh, PA), and embryos were exposed to a final vehicle concentration of 0.01% EtOH (no differences were noted between the standard and HPLC grade EtOH) or 0.004–0.01% DMSO. For each experiment separate batches of embryos were also exposed to 10% Holtfretter's solution (saline) or 0.01% EtOH or 0.01–0.004% DMSO in 10% Holtfretter's solution (vehicle). ICI 182,780 (Tocris Cookson Inc., Ellisville, MO) was dissolved in 100% DMSO and co-applied with 1 μM nonylphenol or 1 μM methoxychlor at a final concentration of

1 μM in 0.01–0.004% DMSO. One micromolar ICI 182,780 in the presence of 1 μM nonylphenol was estimated to block 999 of 1,000 available ERs, assuming nonylphenol and ICI 182,780 have equivalent access to ERs at equilibrium, that association rates do not vary over time, that [nonyphenol] and [ICI 182,780] >> [ER], and based on a relative binding affinity of ICI 182,780 to E_2 of 0.89 (Wakeling et al. 1991) and an IC_{50} of 30–50 μM for displacement of [^{3}H]E_2 by nonylphenol (Lutz and Kloas 1999; White et al. 1994).

We examined 20–30 embryos for each compound, at each concentration, and for each experimental paradigm. Treatments were initiated at stage 10.5 (11 hr). For all experiments except those examining *Xslug* mRNA expression, embryos were fixed at stage 37 (~49 hr) for 1 hr in MEMFA (100 mM MOPS, pH 7.4; 2 mM EGTA; 1 mM $MgSO_4$; 4% paraformaldehyde), dehydrated in methanol, and assessed for effects on body shape (abnormal dorsal curvature), body length, interocular distance, melanocyte differentiation, and apoptosis. For *in situ* hybridization analysis of *Xslug* mRNA expression, we treated embryos beginning at stage 10.5 and examined them at neural plate stages (stages 15–18, ~17–20 hr) or tailbud stage (stages 24–26, 26–29 hr). For all experiments we compared animals exposed to test compounds only with control and vehicle group animals harvested from the same mating.

Morphometric measurements. We made gross morphologic measurements of body length (rostral tip of the cement gland to the tip of the tail), interocular distance (between the medial edges of the eyes), and body shape (presence of abnormal dorsal curvature). During initial examination of morphology, it became apparent that melanocyte differentiation was appreciably altered in some groups of treated embryos, and so embryos were also assessed to determine if they displayed reduced numbers or an absence of melanocytes or if melanocytes lacked dendritic processes.

Assessment of programmed cell death. We processed fixed embryos for TUNEL staining as previously described (Hensey and Gautier 1998). Briefly, embryos were first rehydrated in PBT [phosphate-buffered saline (PBS) + 20% Tween-20] and washed in PBS (2 × 15 min), followed by incubation in 0.5 μM digoxigenin-dUTP (Roche, Indianapolis, IN) and 150 U/mL TdT (terminal deoxynucleotidyl transferase; Invitrogen, Carlsbad, CA) overnight. The next day, the reaction was terminated in PBS/1 mM EDTA, at 65°C, followed by washes (6 × 1 hr) in PBS. The embryos were then blocked (1 hr) in 20% goat serum, incubated overnight in an antidigoxigenin antibody coupled to alkaline phosphatase (1:2,000) (Roche), washed extensively in PBS (at least 6 × 1 hr) and overnight in PBS, followed by a chromogenic nitro blue tetrazolium chloride (NBT) reaction [NBT/BCIP (5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt) tablets, Roche] for 10–30 min, until color was visible. The reaction was stopped by MEMFA. Embryos were dehydrated in methanol (2 × 5 min) and cleared in a 2:1 solution of benzyl benzoate/benzyl alcohol. Embryos were mounted on slides with Permount. Apoptotic cell bodies were counted manually by visualization of the TUNEL-positive cells using 100× magnification. TUNEL staining has been reported previously to vary between different batches of embryos (Hensey and Gautier 1998), and we also noted in the present experiments that the numbers of TUNEL-stained cells varied from mating to mating in saline-treated embryos. Because of this inherent variability in the absence of added compounds, determinations of effects of environmental contaminants on the numbers of apoptotic cells were made for three batches of embryos at 100 nM concentrations of compounds, five batches for 500 nM, and two batches for 1 μM.

Neural crest derivatives arise from the neural plate and migrate to their final destinations *a*) along cranial pathways; *b*) through the dorsal fin and on both the lateral and medial aspects of the trunk somites; *c*) in a pathway that circumnavigates the tail; and *d*) along an enteric pathway into the ventral fin (Mayor et al. 1999). Thus, we assessed TUNEL-stained cells in cranial, trunk, enteric, and tail regions of each embroyo.

Nonisotopic whole-mount* in situ *hybridization. Embryos were treated beginning at stage 10.5 with methoxychlor or nonylphenol (1, 5, or 10 μM). Because *Xslug* mRNA expression subsides to minimal levels by stage 37, we examined embryos at neural plate stages (stages 15–18) or tailbud stage (~stage 24). These two stages correspond to epochs when there is a highly distinctive pattern of expression of *Xslug* mRNA that also coincides with active periods of neural crest migration, a process that extends until stage 40 in *Xenopus* (Krotoski et al. 1988; Linker et al. 2000; Mayor et al. 1995). We prepared digoxigenin-UTP-labeled sense and antisense probes from a plasmid containing the *Xslug* cDNA (Mayor et al. 1995; graciously provided by M. Bronner-Fraser, California Institute of Technology) according to the manufacturer's instructions (DIG RNA SP6/T7 labeling kit, Roche). Nonisotopic *in situ* hybridization was carried out with minor modifications according to Harland (1991). Fixed embryos were rehydrated with 95% MeOH/H_2O, 70% MeOH/1× PBS, 30% MeOH/1× PBS, washed 3 times for 10 min each with 0.1% Tween-20/1× PBS, treated for 15 min with 7.5 μg/mL proteinase K, rinsed 2 times with 0.1 M triethanolamine (TEA; pH 7.8) and 2.5 μL acetic anhydride/mL TEA added twice for 5 min each to the second rinse. Embryos were washed again with 0.1% Tween-20/1× PBS, refixed for 20 min in 4% paraformaldehyde/1× PBS, washed again, and incubated with hybridization buffer (50% formamide, 5× SSC (sodium chloride sodium citrate), 1 mg/mL *Torula* RNA, 100 μg/mL heparin, 1× Denhardt's, 0.1% Tween-20, 0.1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid), 5 mM EDTA) for 10 min at 60°C. Embryos were prehybridized in hybridization buffer for 6 hr at 60°C; probe was added at a concentration of 1 μg/mL and hybridization carried out overnight at 60°C. Posthybridization washes were carried out as follows: 3 × 20 min 2 × SSC/0.1% Tween-20 at 60°C; 30 min 2 × SSC/20 μg/mL RNAseA/10 U/mL RNAseT_1 at 37°C; 10 min 2 × SSC/0.1% Tween-20 at 60°C, 2 × for 10 min in 0.2 × SSC/0.1% Tween-20 at 60°C, and 2 × for 10 min in maleic acid buffer (MAB: 0.1 M maleic acid, 0.15 M NaCl).

To visualize hybridization, we replaced MAB with MAB/2% BMB (10% blocking buffer; Roche) for 15 min, followed by subsequent replacement with this solution for 1 hr. A 1:1500 dilution of antidigoxygenin-AP Fab fragments (Roche) was then added, and the embryos were incubated overnight at 4°C and the reaction was allowed to proceed according to the kit manufacturer's instructions. When NBT/BCIP color reaction was completed, the embryos were fixed overnight in MEMFA, cleared and dehydrated in serial dilutions of ethanol.

Statistical analysis. For all experimental conditions, embryos and tadpoles were examined by investigators who were blind to the experimental conditions (two or more investigators for many experiments). Values given are means ± SEM or percentages. As noted above, we compared experimental data only to the vehicle-alone group from embryos obtained from the same mating and maintained under

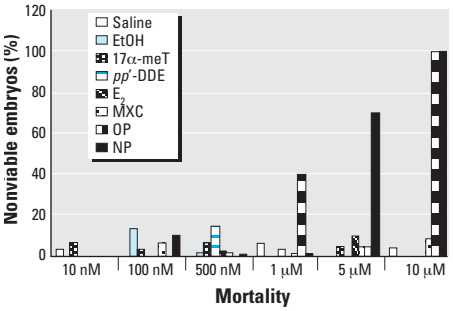

Figure 1. Cumulative data indicating the percentage of dead embryos when assessed 48 hr after treatment (when control and vehicle alone-treated embryos reached stage 37) after having been maintained in 10% Holtfretter's solution (saline), vehicle alone (EtOH), 17α-MeT, *p,p′*-DDE, E_2, methoxychlor (MXC), octylphenol (OP), or nonylphenol (NP) at concentrations between 10 nM and 10 μM.

identical experimental conditions in parallel. Multivariate analysis of variance (MANOVA) was performed for assessments of body length, interocular distances, and numbers of apoptotic (TUNEL-stained) cells using the general linear model procedures and the Duncan's multiple range test of SAS (SAS Institute 1990). Percentages of embryos displaying abnormal body shape, melanocyte abnormalities, and percentage of viable embryos were assessed using the chi-square test. An alpha value of $p < 0.05$ was established as significant. All data designated as significant met or exceeded this criterion.

Results

Gross morphologic changes and increased mortality. Mortality for embryos exposed at stage 10.5 to saline, vehicle, 17α-MeT, or any of the test concentrations of E_2, *p,p′-DDE,* or methoxychlor and examined at stage 37 was 0–13% (Figure 1), and none of the embryos treated with any of the concentrations of *p,p′-DDE* or methoxychlor appeared moribund. In contrast, exposure to micromolar concentrations of nonylphenol or octylphenol significantly increased mortality, and in the presence of the highest concentration (10 µM) of these compounds, no embryos examined at stage 37 were alive (Figure 1). It should be noted that although embryos treated with 10 µM E_2 were alive, they were moribund and would have been unlikely to have survived another day had they not been fixed at stage 37. In addition, in contrast to tadpoles maintained in saline, vehicle, or nonestrogenic compounds who would swim normally when touched on the head, tadpoles that had been treated with micromolar concentrations of estrogenic compounds did not display normal swimming patterns but twitched spastically when touched. Although embryos maintained in 10 µM concentrations of estrogenic compounds were viable at earlier time points (< 30 hr), because of the drastic decrease in viability at later times, data for 10 µM concentrations were not included in subsequent analyses of tadpoles assessed at stage 37.

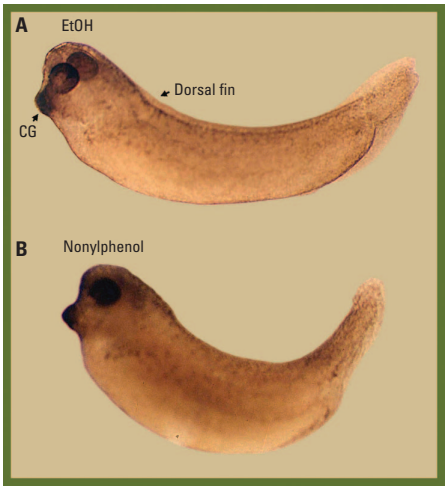

Figure 2. Representative examples of (*A*) an embryo maintained in vehicle alone (EtOH) and (*B*) one maintained in saline containing 1 µM nonylphenol beginning at stage 10.5 for 48 hr (~stage 37 for control embryos). Crooked backs, kinked tails, poorly developed somites and dorsal fins, and "chicken-beaked" cement glands (CG) were often evident in embryos treated with micromolar concentrations of the estrogenic compounds, E_2, octylphenol, or nonylphenol.

Abnormalities that included malformed cement glands (chicken beaks) pronounced dorsal flexure, poorly developed somites, swollen guts, or sloughing of epidermal cells (Figure 2) were evident in embryos exposed to 5 µM concentrations of estrogenic compounds (E_2, methoxychlor, octylphenol, and nonylphenol). Measurements of body length and interocular distance were made for all treatment groups at concentrations of compounds from 10 nM–5 µM. The F-approximation of Wilks' lambda for results of MANOVA indicated a significant effect of the chemical [$F(14,1816) = 20.41$, $p < 0.0001$], a significant effect of concentration [$F(8,1816) = 161.34$, $p < 0.0001$], and a significant effect for chemical × concentration [$F(56,1816) = 15.34$, $p < 0.0001$]. The most dramatic effects were observed with exposure to the alkylphenolic compounds nonylphenol and octylphenol, which caused significant decreases in body length at concentrations as low as 500 nM (Figure 3A). In contrast to the effects of nonylphenol and octylphenol, exposure to 5 µM *p,p′*-DDE, methoxychlor, E_2, or 17α-MeT did not significantly stunt body length. Assessments of body shape were also made by determining the percentage of embryos that displayed abnormal dorsal flexure or crooked vertebrae. As with measurements of body length, the most dramatic effects on this parameter were induced by exposure to nonylphenol, and significantly greater numbers of deformed embryos were observed at concentrations as low as 500 nM nonylphenol compared to saline- or vehicle-treated embryos (Figure 3B). Surprisingly, while interocular distance was also significantly shorter in embryos exposed to 500 nM nonylphenol compared to saline- or vehicle-treated animals, interocular distances were not significantly different from those of saline or vehicle-treated animals at the higher concentrations of 1 or 5 µM nonylphenol (Figure 3C).

Melanocyte differentiation. During the course of evaluating changes in overall morphology, we noted significant differences in the numbers and the patterning of neural crest-derived melanocytes in embryos treated with 100 nM–5 µM concentrations of nonylphenol, methoxychlor, or E_2 (Figure 4A–C) and, to a lesser extent, with octylphenol (data not shown). Exposure to these estrogenic compounds not only diminished the number of melanocytes per embryo but also altered the appearance of dendritic processes of these pigmented cells in comparison to saline- or vehicle-treated embryos (Figures 4A,B and 5A,B). Moreover, in many embryos treated with estrogenic compounds, pigment cells were completely absent from the dorsal fin (Figure 5B), and in some cases the dorsal fin itself (which is also a neural crest–derived structure) was absent.

The most striking differences in melanocyte differentiation were elicited with exposure to methoxychlor, which caused a dramatic blunting of dendritic processes so that melanocytes resembled "dalmatian" spots (Figures 4B and 5B). These alterations in melanocyte patterning were specific for compounds with estrogenic effects (nonylphenol

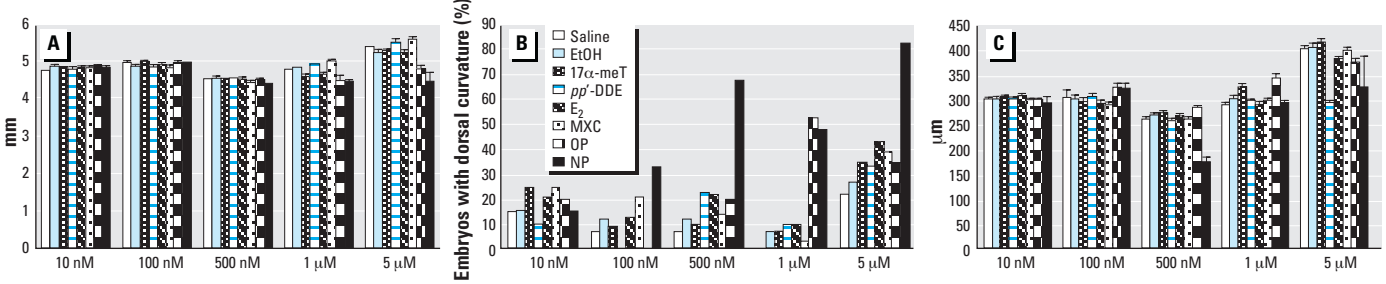

Figure 3. Cumulative data demonstrating measurements of (*A*) body length, (*B*) body shape, and (*C*) interocular distances in embryos maintained for approximately 48 hr (beginning at stage 10.5 through stage 37) in control saline or saline supplemented with vehicle alone (EtOH), 17α-MeT, *p,p′*-DDE, E_2, methoxychlor (MXC), octylphenol (OP), or nonylphenol (NP) at concentrations ranging from 10 nM to 10 µM. We measured 20–30 embryos from a given mating for each experimental condition. Error bars are + SEM as defined in "Material and Methods."

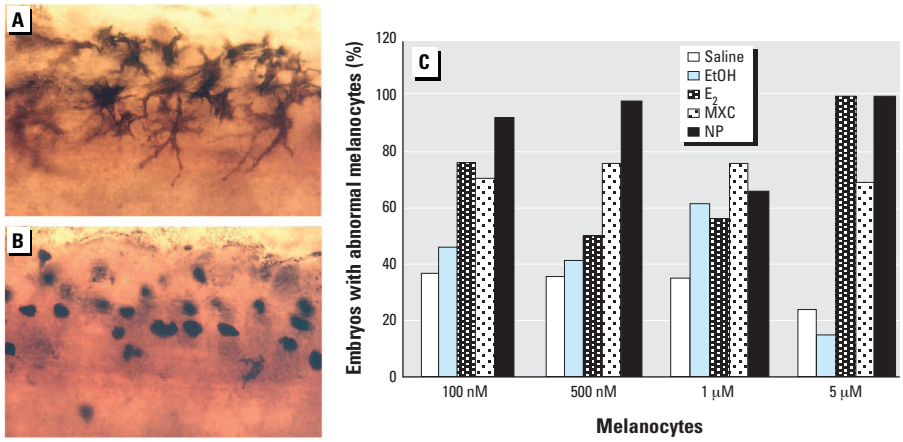

Figure 4. Representative photomicrographs of melanocytes in (*A*) vehicle alone (EtOH) and (*B*) methoxychlor-treated (1 μM) embryos. (*C*) Cumulative data indicating that significant aberrations in melanocyte differentiation were evident with exposure to estrogenic compounds at concentrations as low as 100 nM.

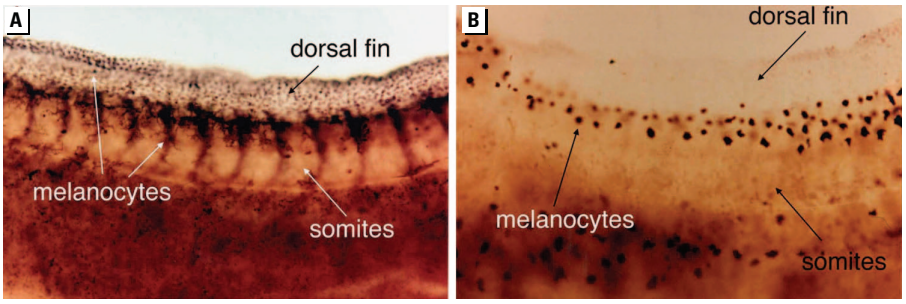

Figure 5. Representative photomicrographs of the dorsal aspect of the trunk of two stage 37 tadpoles. (*A*) Embryo maintained in saline demonstrating the presence of the dorsal fin populated by small melanocytes and larger dendritic melanocytes along the surface of the spinal cord. (*B*) Embryo treated with 1 μM methoxychlor demonstrating a thin, poorly developed dorsal fin devoid of melanocytes, spotty melanocytes atop the spinal cord, crooked spine, and poorly defined somites. (Both embryos had also been processed for TUNEL staining.) Magnification 100×.

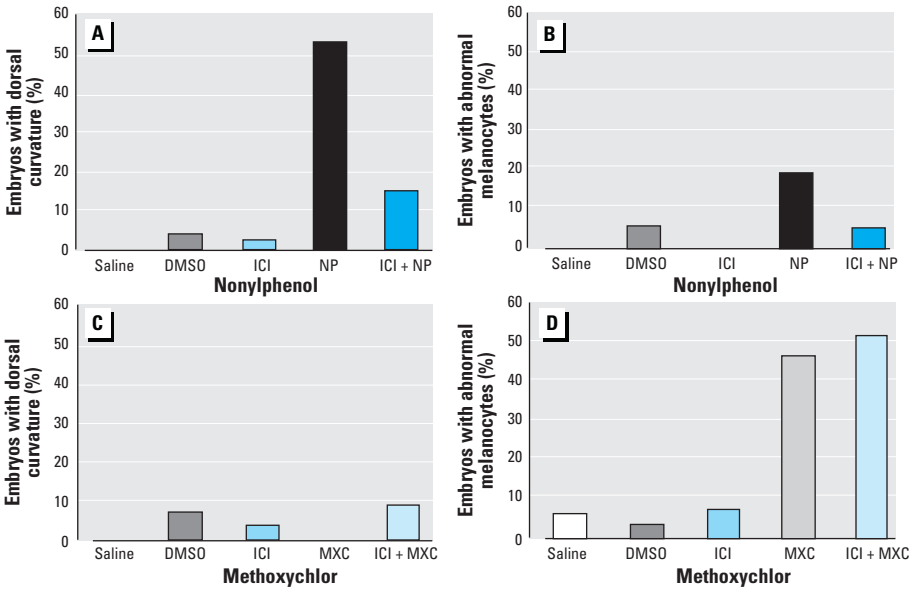

Figure 6. Cumulative data demonstrating that coincubation of embryos with 1 μM ICI 182,780 significantly inhibited the effects of 1 μM nonylphenol in (*A*) inducing abnormal dorsal curvature and (*B*) abnormal melanocyte differentiation. Vehicle alone (0.01–0.004% DMSO) and ICI 182,780 alone were without significant effect. (*C*) Methoxychlor (1 μM), either alone or in conjunction with 1 μM ICI 182,780 had no effect on body shape (percentage embryos with dorsal curvature). (*D*) Methoxychlor (1 μM) induced significant increases in the percentage of embryos with abnormal melanocytes, which was not inhibited by 1 μM ICI 182,780.

and octylphenol, E_2, and methoxychlor). Neither the antiandrogenic compound *p,p′*-DDE or the synthetic androgen 17α-MeT induced significant changes in melanocyte deposition or differentiation (data not shown). In contrast to the changes in morphology that were only evident for E_2 and methoxychlor at the highest (5 μM) concentration, all estrogenic compounds (except methoxychlor) tested induced significant abnormalities in melanocyte differentiation at concentrations as low as 100 nM (significant differences were observed for methoxychlor at 500 nM). Taken together, these results suggest that neural crest–derived melanocytes may serve as highly sensitive indicators of exposure to estrogenic contaminants.

Effects of a ER antagonist on the action of nonylphenol and methoxychlor. To determine whether the effects of estrogenic compounds required ER signaling, embryos were exposed to 1 μM nonylphenol or 1 μM methoxychlor in the presence or absence of 1 μM of the pure antiestrogen ICI 182,780 and assessed for changes in body shape and melanocyte differentiation. Co-incubation with ICI 182,780 significantly decreased but did not completely abolish the abnormalities in body shape and melanocyte differentiation induced by nonylphenol (Figure 6A). As shown in previous experiments (Figure 3B), 1 μM methoxychlor did not induce significant changes in body shape (Figure 6C). However, in contrast to the inhibition by ICI 182,780 of the abnormalities in melanocyte differentiation elicited by exposure to nonylphenol, ICI 182,780 did not interfere with the ability of 1 μM methoxychlor to induce melanocyte abnormalities (Figure 6D).

Number of TUNEL-stained cells. To determine if exposure to estrogenic compounds alters the extent of apoptosis that occurs during development, we assayed *Xenopus* embryos exposed to nonylphenol, octylphenol, methoxychlor, or E_2 at early gastrula stages for the number of TUNEL-stained cells in cranial, trunk, tail, and enteric regions (Figure 7). Patterns of TUNEL-stained cells were similar to that previously reported (Hensey and Gautier, 1998). The heaviest concentration of labeled cells was observed in the developing neural structures, but stained cells were also highly expressed in the dorsal fin and, although fewer in number, were also evident in the enteric regions. Exposure to all the estrogenic compounds at 1 and 5 μM caused a significant increase in the number of TUNEL-stained cells in all four regions compared with saline- or vehicle-treated embryos. As with assays of morphology, the most marked effects were elicited by exposure to nonylphenol. Nonylphenol induced the largest increases in the numbers in TUNEL-stained cells at any given concentration. Moreover, significant

increases in the numbers of TUNEL-stained cells over saline- or vehicle-treated embryos were evident at concentrations as low as 100 nM (Figure 7). For all compounds tested the most marked increases in the numbers of TUNEL-stained cells were observed in the cranial region (Figure 7A). Paradoxically, as with effects on body length, 500-nM concentrations of all compounds tested were without appreciable effect in this region (Figure 7A). Significant changes in the numbers of apoptotic cells were not observed in embryos treated with the antiandrogenic compound *p,p′*-DDE, or the synthetic androgen 17α-MeT (data not shown).

Expression of* Xslug *mRNA. To determine if the abnormalities in neural crest–derived melanocytes and increased apoptosis in neural regions could be attributed, at least in part, to changes induced in the expression of the neural crest-specific transcription factor *Xslug*, nonisotopic *in situ* hybridizations with a digoxigenin-labeled riboprobe directed against *Xslug* cDNA (Mayor et al. 1995) were made for embryos exposed to 1, 5, or 10 µM nonylphenol or 1 or 10 µM methoxychlor beginning at stage 10.5 and examined at neural plate stages (stages 15–18) or tailbud stages (~stage 24). We compared exposed embryos to embryos maintained in saline or vehicle alone. No staining was evident when sense probes were used (data not shown). In neural plate stage embryos (stages 15–18) hybridized with antisense probes, staining was observed along the neural fold regions corresponding to premigratory cranial neural crest for control, vehicle alone (Figure 8A), nonylphenol (Figure 8C), and methoxychlor (data not shown) treatments. For tailbud stage embryos (~stage 24), staining was observed in the branchial arches, as well as along the dorsal midline in control and vehicle-alone conditions (Figure 8B) and in embryos exposed to nonylphenol (Figure 8D) or methoxychlor (data not shown). The staining patterns at both stages were comparable to those described previously (Linker et al. 2000; Mayor et al. 1995), and no appreciable differences were observed either in the pattern of staining or in the qualitative intensity of staining in embryos exposed to either nonylphenol or methoxychlor, even at the higher concentrations of 5 or 10 µM (data not shown), versus untreated embryos.

Discussion

Exposure of *Xenopus* embryos during the period when neural crest- and neural plate-derived structures develop (stages 10.5–37) to micromolar concentrations of the environmental estrogens nonylphenol, octylphenol, and methoxychlor induced significant gross morphologic defects, including crooked vertebrae, swollen guts, poorly defined somites, and sloughing of epidermal cells, and in the case of nonylphenol and octylphenol, increased mortality by stage 37. These results are in good agreement with those of Nishimura et al. (1997) and Mann and Bidwell (2000), who reported comparable effects of limited exposure on early larval development in *Xenopus* to micromolar concentrations of E_2 and NPEO, respectively. Specifically, Nishimura et al. reported that viability drops to 0% by stage 42 for embryos maintained in 10 µM E_2, and Mann and Bidwell reported that mortality occurred consistently by stages 39–40 for embryos maintained in 6–10 mg/L Teric GN8. In contrast to the deficits induced by exposure to these estrogenic compounds, no significant effects were elicited by exposure to the anti-androgenic contaminant *p,p′*-DDE, or 17α-MeT, a synthetic androgen that is not aromatized to E_2 (Quincey and Gray, 1967).

Although micromolar exposure to environmental estrogens induced pleomorphic effects on a number of organ systems in this study, the most marked and sensitive changes we observed were in the differentiation of neural crest–derived melanocytes. These pigmented cells, which provide an excellent naturally occurring marker cell population for studies of neural crest development and differentiation in *Xenopus* (Epperlein et al. 1996; Krotoski et al. 1988), are normally abundantly expressed in epidermal and dermal layers, as well as in perineural and perivascular tissues of pigmented *Xenopus* tadpoles. Exposure to 100 nM–5 µM concentrations of nonylphenol, octylphenol, E_2, or methoxychlor induced significant changes in the number, shape, and location of melanocytes. In embryos exposed to these estrogenic compounds, melanocyte numbers were often

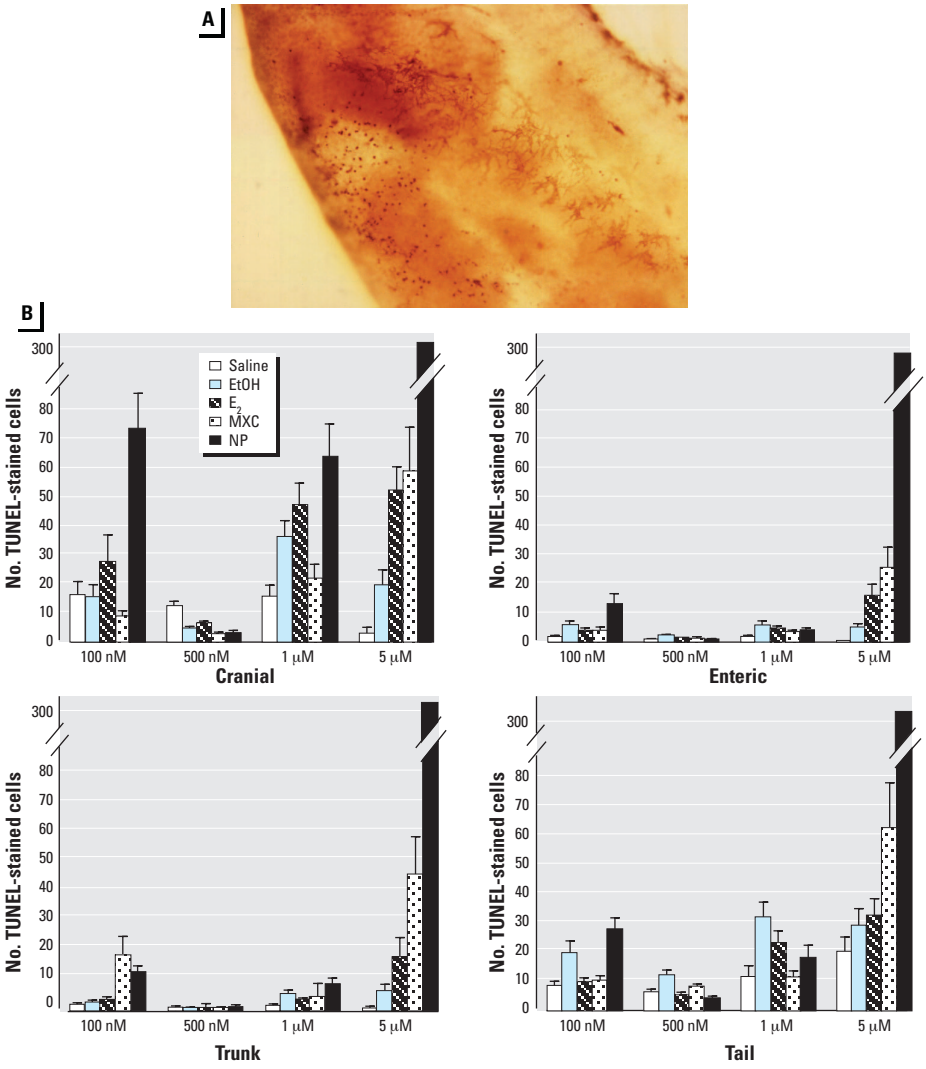

Figure 7. (*A*) Representative photomicrograph of the ventral and caudal portion of a stage 37 embryo treated with 1 µM nonylphenol demonstrating the presence of TUNEL-stained cells in the gut. (*B*) Cumulative data indicating that micromolar concentrations of estrogenic compounds significantly increased the number of TUNEL-stained cells. For embryos treated with 5 µM nonylphenol, accurate counts of the number of TUNEL-stained cells could not be made beyond 300. Error bars are + SEM.

significantly decreased, they were often found in ectopic locations (e.g., in the ventral gut), and they displayed altered morphology, the most prevalent abnormality being blunted or absent dendritic processes. Melanocytes reside in close apposition to sensory nerve endings and are known to produce a number of neuropeptides and neurotransmitters (for review, see Tsatmali et al. 2002). This biochemical and physical arrangement has led others to speculate that melanocytes may act as local stress sensors that are key intermediaries in transmitting information from the environment to the central nervous system [for discussion, see Tsatmali et al. (2002)].

Melanocyte differentiation was altered by exposure to a number of chemically distinct environmental compounds with estrogenic activity, a result consistent with the observation that E_2 alters melanogenesis in and decreases the number of human melanocytes *in vitro* (McLeod et al. 1994). While the pure antiestrogen ICI 182,780 was able to inhibit the effects of nonylphenol on melanocyte differentiation (and on body shape), this ER antagonist was not able to inhibit the effects of methoxychlor on melanocyte differentiation. These data suggest that although both nonylphenol and methoxychlor have significant effects on melanocyte differentiation, they may do so via different signaling pathways. Specifically, a majority of the effects of nonylphenol appear to require ER activation, and it has been shown that ER mRNA is expressed in *Xenopus* throughout the time period studied here (stages 10.5–37) (Nishimura et al. 1997). In contrast, classical signaling through the ER does not appear to be required for the effects of methoxychlor on these pigment cells. The ER-independent action of methoxychlor on melanocyte differentiation is consistent with previous reports that indicate this toxicant has ER-independent effects in a number of other cell types (Ghosh et al. 1999; Ren et al. 1997; Waters et al. 2001).

Exposure to high nanomolar to micromolar concentrations of estrogenic contaminants also significantly increased apoptosis in developing tadpoles. As with all other assays made in this study, the largest increases in TUNEL-positive cells were observed upon treatment with nonylphenol. Increases in the numbers of TUNEL-stained cells induced by nonylphenol were most striking in the cranial part of the embryo and in regions corresponding to the developing nervous system. It is noteworthy that the number of apoptotic cells observed with 500 nM concentrations of nonylphenol were markedly lower than those observed with either 100 nM or 1 and 5 µM concentrations. This loss of effect at 500 nM was also observed for measurements of mortality, interocular distance, and body length with nonylphenol, but was not observed in assays of body shape or melanocyte abnormalities. Previous studies have suggested that there may be a U-shaped dose–response relationship with respect to effects of nonylphenol on the development of mammalian reproductive structures, but these results are controversial (for discussion, see Safe et al. 2001).

Previous studies have demonstrated that environmental estrogens alter apoptosis in breast cancer cell lines (Diel et al. 2002; Ren et al. 1997) and in cells of reproductive tissues (Hughes et al. 2000; Weber et al. 2002), but few studies have examined the effects of these environmental contaminants on apoptosis in whole embryos and in nonreproductive tissues. Apoptotic cells were observed within regions that could correspond to migratory pathways for neural crest cells not only in the head but also in the dorsal fin and enteric regions, but it was not possible by TUNEL staining to identify these cells as neural crest derivatives. Future studies in which TUNEL staining is assessed in conjunction with approaches that selectively label neural crest cells (Borchers et al. 2000; Carl et al. 1999; Krotoski et al. 1988; LaBonne and Bronner-Fraser 2000) or studies in which the effects of these environmental estrogens on neural crest cells maintained in culture will be needed to establish if the observed changes in apoptosis are specific for neural crest-derived cells.

The transcription factor Xslug is not only essential for proper development of the neural crest, but overexpression of Xslug leads to overproduction of melanocytes (LaBonne and Bronner-Fraser 1998), and this factor has been postulated to regulate apoptosis (Hemavathy et al. 2000). We hypothesized that the actions of different estrogenic compounds might converge to alter the expression of *Xslug* and thus lead to aberrant melanocyte differentiation and altered apoptosis. However, we found no appreciable differences in the pattern or qualitative intensity of *Xslug* mRNA expression in embryos treated with 1–10 µM nonylphenol or methoxychlor versus saline- or vehicle-treated embryos. Moreover, because *Xslug* expression was not appreciably altered by these compounds, it is unlikely that they have significant actions on proneural genes, such as bone morphogenic

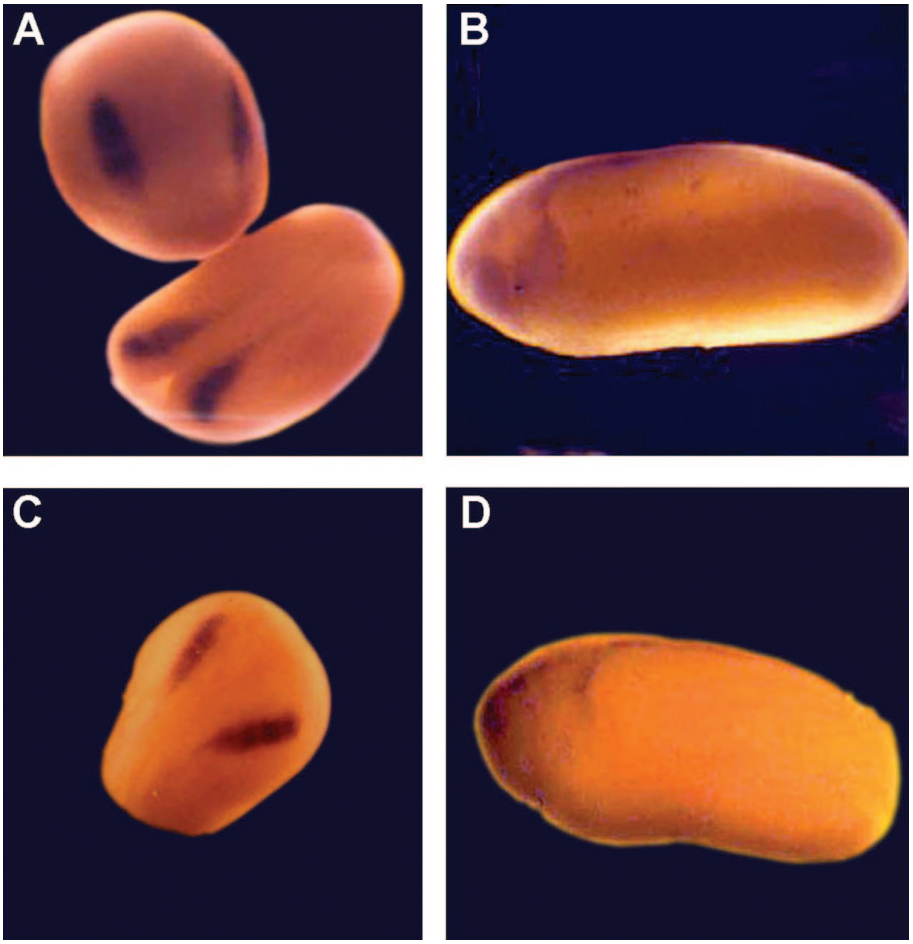

Figure 8. Representative embryos maintained in vehicle alone (*A,B*) or in 1 µM nonylphenol (*C,D*) at either neural plate stages (stages 15–18: *A,C*) or tailbud stages (stages 24–26: *B,D*) demonstrating the expression of *Xslug* mRNA. Magnification 25×.

proteins or Wnts, that are upstream of *Xslug* (Mayor et al. 1999). More likely possibilities are that inappropriate exposure to estrogenic compounds alters the deposition of extracellular matrix molecules or signaling molecules such as the neurotrophins, which are crucial for the survival and differentiation of neural crest-derived structures (Chao and Hempstead 1995; Fariñas 1999; Lewin and Barde 1996). In particular, nerve growth factor (NGF) and neurotrophin 3 have been shown to enhance dendricity and promote migration of melanocytes (Sieber-Blum and Zhang 1997; Yaar et al. 1994). Moreover, estrogens regulate the expression of both neurotrophic factors and their cognate receptors, as well as downstream signaling from those via ER-dependent and ER-independent mechanisms (Jezierski et al. 2001; Miranda et al. 1994; Sohrabji et al. 1995; Toran-Allerand 1996) and to induce significant effects on neuronal apoptosis in the developing mammalian nervous system (Wade et al. 1999). Early exposure to inappropriately high levels of estrogenic compounds in *Xenopus* embryos may alter the expression of neurotrophins and or their receptors and thus interfere with neurotrophin-mediated development of neural crest–derived melanocytes and normal programmed cell death. Consistent with this hypothesis, preliminary data from our laboratory indicate that nonylphenol blocks the ability of NGF to elicit enhanced neurite outgrowth from neural crest–derived *Xenopus* Rohon-Beard neurons in dissociated cell culture (Bevan CL. Unpublished data).

The data presented here indicate that brief exposure to nonylphenol induces significant and detrimental effects on *Xenopus* development at concentrations ranging from 100 nM to 10 μM. Although concentrations of nonylphenol and its ethoxylates assessed in river water samples ranged from undetectable to ~5 nM (Naylor et al. 1992), concentrations of 100 nM–1 μM have been measured in primary effluent from sewage treatment plants (Giger et al. 1987), and concentrations higher than 10 μM have been detected in river sediment samples (Naylor et al. 1992). Thus aquatic organisms may be exposed to micromolar concentrations of compounds in the environment. In addition, because many environmental contaminants, including the APEOs, bioaccumulate (Ekelund et al. 1990) and organisms are rarely, if ever, exposed to a single compound (Crews et al. 2000; Kavlock 1999), it seems likely that the concentrations of environmental estrogens found in this study to elicit abnormalities in the development of *Xenopus* in the laboratory will also be relevant to organisms in the natural environment. Future studies will be needed to assess if the estrogenic compounds that when given individually, disrupted early development in *Xenopus* have synergistic effects when present as a mixture of lower individual concentrations of multiple toxicants. In addition, the experiments performed in this study have only tested the effects of a limited exposure to these compounds during a brief period of early development, clearly a paradigm that does not mirror environmental conditions where organisms may be exposed throughout life. Future experiments will also need to be made to determine if chronic exposure to lower concentrations of environmental estrogens elicit detrimental effects, and if chronic exposure to these compounds alters the differentiation of neural crest-derived structures such as the sensory and sympathetic ganglia, which arise at later developmental stages.

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