Octylphenol and UV-B Radiation Alter Larval Development and Hypothalamic Gene Expression in the Leopard Frog (*Rana pipiens*)

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We assessed octylphenol (OP), an estrogenic endocrine-disrupting chemical, and UV-B radiation, a known stressor in amphibian development, for their effects on hypothalamic gene expression and premetamorphic development in the leopard frog (*Rana pipiens*). Newly hatched tadpoles were exposed for 10 days to OP alone at two different dose levels; to subambient UV-B radiation alone; and to two combinations of OP and UV-B. Control animals were exposed to ethanol vehicle (0.01%) exposure, a subset of tadpoles from each treatment group was raised to metamorphosis to assess differences in body weight and time required for hindlimb emergence. Tadpoles from one of the OP/UV-B combination groups had greater body weight and earlier hindlimb emergence ($p < 0.05$), but neither OP nor UV-B alone produced significant changes in body weight or hindlimb emergence, indicating a potential mechanism of interaction between OP and UV-B. We hypothesized that the developing hypothalamus might be a potential environmental sensor for neurotoxicologic studies because of its role in the endocrine control of metamorphosis. We used a differential display strategy to identify candidate genes differentially expressed in the hypothalamic region of the exposed tadpoles. Homology cloning was performed to obtain *R. pipiens* glutamate decarboxylases—GAD65 and GAD67, enzymes involved in the synthesis of the neurotransmitter γ-aminobutyric acid (GABA). cDNA expression profiles revealed that OP and UV-B affected the levels of several candidate transcripts in tadpole (i.e., Nck, Ash, and phospholipase C γ-binding protein 4 and brain angiogenesis inhibitor-3) and metamorph (i.e., GAD67, cytochrome C oxidase, and brain angiogenesis inhibitor-2 and -3) brains. This study represents a novel approach in toxicology that combines physiologic and molecular end points and indicates that levels of OP commonly found in the environment and subambient levels of UV-B alter the expression of important hypothalamic genes and disrupt tadpole growth patterns. *Key words:* amphibians, angiogenesis, endocrine-disrupting chemicals, estradiol, frogs, GABA, hypothalamus, *Rana pipiens*. *Environ Health Perspect* 110:277–284 (2002). [Online 12 February 2002] *http://ehpnet1.niehs.nih.gov/docs/2002/110p277-284crump/abstract.html*

The presence of an increasing number of endocrine-disrupting chemicals (EDCs) in the environment may result in chronic exposure of humans and wildlife to low concentrations of contaminants including pesticides (e.g., dichlorodiphenyltrichloroethane, methoxychlor), polychlorinated biphenyls, synthetic steroids (e.g., ethinylestradiol), and some alkylphenolic compounds such as octylphenol (OP). Among wildlife species, the focus of research on EDC exposure has been on animals associated with wetlands or aquatic habitats receiving contaminated effluent and agricultural runoff, in which pollutant exposure has been linked to endocrine disruption (*1–4*). For example, developing alligator embryos collected from sites contamined with *p,p′*-dichlorodiphenyldichloroethene and 1,1′-(2,2-dichloroethylidene)bis[4-chlorobenzene] displayed altered sex determination, gonadal steroidogenesis, cliterophallus size, and endocrine or reproductive organ morphology (*1*). It has been hypothesized that exposure of amphibians to pollutants may disrupt endocrine systems controlling metamorphosis, stress response, and sexual development (*5–10*). Exposure to UV-B radiation (280–320 nm) has also been found to disrupt development in several amphibian species (*11–15*). The vulnerability of amphibian species to environmental stressors such as EDCs and UV-B may be associated with amphibian population declines that have been occurring worldwide since the 1960s (*16*).

In a report on environmental endocrine disruption, the U.S. Environmental Protection Agency suggested that amphibians might be good sentinel animal models for laboratory and field exposure studies (*17*). Several EDCs, including OP, can displace radiolabeled 17-β estradiol ($[^3H]E_2$) from the estrogen receptor (*18*), cause significant feminization at low concentrations (e.g., 10 nM OP), and stimulate vitellogenin (VTG) mRNA expression in cultured hepatocytes in *Xenopus* frogs (*9*). Reeder et al. (*6*) reported a weak association ($p = 0.07$) between the detection of the herbicide atrazine and the prevalence of intersex individuals of the cricket frog (*Acris crepitans*). These authors also found that sex ratios favored males in juvenile *A. crepitans* collected from sites contaminated by polychlorinated biphenyls and polychlorinated dibenzofurans. Nishimura et al. (*19*) raised *X. laevis* in water containing different concentrations of E_2 and observed malformations of the head and abdomen, suppressed organogenesis, and suppressed nervous system development at 10^{-5} M. Although the dose Nishimura et al. (*19*) used exceeds physiologic levels, the findings suggest that estrogen exposure can disrupt brain development. Accelerated metamorphosis was observed after the treatment of *Bufo bufo* and *B. americanus* tadpoles with E_2 (*20*) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (*21*). Cheek et al. (*7*) observed that the herbicide acetochlor, in the presence of exogenous thyroid hormone T_3, disrupted development by accelerating metamorphosis in *R. pipiens*.

Corticotropin-releasing hormone (CRH) plays a central role in amphibian metamorphosis by stimulating the pituitary-thyroid axis, resulting in elevated T_3 levels in the blood (*22–25*). This thyroid-regulating effect of CRH in amphibians is in addition to the well-characterized effects of CRH on adrenocorticotropin hormone (ACTH) release in vertebrates (*24*). The frog hypothalamus can be stimulated by biotic or abiotic factors in the larval habitat (e.g., pond desiccation) to release the neuropeptide CRH (*22*). Corticosteroids are also released after exposure to stress and can synergize with T_3 to accelerate metamorphosis (*22*). Inhibition of metamorphosis is mediated by hypothalamic neurosecretion of the tripeptide thyrotropin-releasing hormone, which controls the biosynthesis and release of the pituitary hormone prolactin (PRL) (*24,26*). PRL antagonizes metamorphosis and promotes larval growth leading to larger animals at metamorphosis (*24,26*). Thyroid status is easily altered by the addition of a variety of

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hormones or T_3 synthesis inhibitors to water (*23*), indicating that the hypothalamo–pituitary–thyroid axis is extremely vulnerable to endocrine disruption. Premature metamorphosis and/or abnormal growth patterns could alter fitness and survivorship in amphibians (*27*).

Alkylphenol polyethoxylates (APEOs) are a large group of nonionic surfactants in commercial production [approximately 250,000 tons produced per year (*28*)] that enter the aquatic environment predominantly via sewage treatment plants and pulp and paper mills (*3,29,30*). Upon discharge, APEOs are rapidly degraded to form relatively stable, hydrophobic metabolites; principally the alkylphenols nonylphenol (NP) and OP. OP and NP are estrogenic and stimulate VTG gene expression in trout hepatocytes, induce growth of several breast cancer cell lines (e.g., MCF-7 and ZR-75), inhibit testicular growth in rainbow trout, cause feminization in *Xenopus*, and competitively bind to both trout and mouse estrogen receptors (*9,29–31*). Blázquez et al. (*32*) found that 1 µM OP caused 50% mortality among immature male goldfish 20 days after exposure. There have been no reports on amphibian toxicity data for OP. Most evidence indicates that regardless of the species model, OP, at concentrations ranging from 0.1 µM to 10 µM, is a biologically active contaminant (*30–32*). Indeed, there is reason for concern because OP has been found at significant concentrations in fresh water (< 2.0 pM–2.5 nM), sediments (< 0.010–1.8 µg/g dry weight), and sewage treatment plant effluent (0.6–12 nM) and sludge (< 0.005–12.1 µg/g dry weight) in North America (*33,34*). Waterborne OP levels of approximately 63 nM have been reported in rivers and estuaries in the United Kingdom (*35*).

Previous studies have shown that UV-B radiation (280–320 nm) can disrupt normal development and hinder hatching success and larval survivorship of many amphibian species (*11,12*). Severe deformities including lordosis (curvature of the spine), bloating/distension, and abnormal development of the presumptive cornea were common pathologies associated with UV-B exposure (*13,14*). Ankley et al. (*15*) reported symmetrical hind-limb ectromelia and ectrodactyly after *R. pipiens* tadpoles were exposed to low levels (44 µW/cm^2) of UV-B. Again, metamorphic alterations of this nature in the wild would likely impair predator avoidance, reproductive success, and overall fitness. UV-B can also potentiate the toxicity of several pesticides and polycyclic aromatic hydrocarbons with respect to amphibian survivorship because of the generation of toxic photometabolites (*36,37*). Ahel et al. (*38*) determined the kinetics of OP photolysis in natural waters and calculated a half-life of approximately 10–15 hr. However, the photometabolites of OP were not identified. Bennie (*34*) noted that OP was relatively resistant to microbial degradation but that the final degradation products were water and carbon dioxide.

In the current study, we used sublethal, nonteratogenic levels of OP and UV-B to determine whether an interaction existed between these two stressors with respect to mRNA expression in the brain and effects on metamorphosis, specifically growth rate and hind-limb emergence (HLE). We focused on the tadpole diencephalon (preoptic/hypothalamic area) because the gene expression program for neural development of this brain region has been characterized (*23*). In addition, many aspects of hypothalamic function are modulated by sex steroids (*32*), so we hypothesized that the developing hypothalamus would be an effective and novel environmental sensor for neurotoxicologic studies.

Using a rapid molecular screening technique, we set out to isolate and identify candidate molecules in the hypothalamic region of developing leopard frog (*R. pipiens*) tadpoles that were differentially regulated by OP and UV-B, alone and in combination, as initially determined using differential display. We performed homology cloning to obtain *R. pipiens* glutamate decarboxylases GAD65 and GAD67, enzymes involved in the synthesis of the neurotransmitter γ-aminobutyric acid (GABA). We used a multiple-gene dot blot in semiquantitative reverse Northern hybridization experiments to determine the extent to which the expression patterns of candidate molecules were altered after treatment with OP and UV-B. We also studied the potential interaction between OP and UV-B on gene expression profiles.

Materials and Methods

Animals and rearing conditions. Two experiments were conducted with animals from different sources. For experiment 1 (30 January 1999) we used animals from an induced breeding event to isolate candidate differentially expressed molecules from tadpole brains. For experiment 2 (2 May 1999) we used animals from naturally fertilized eggs to isolate candidate differentially expressed molecules from metamorph brains. We also assessed growth and premetamorphic development.

Adult *R. pipiens* were purchased from a commercial supplier (Charles Sullivan & Co., Nashville, TN, USA) and housed in a flow-through aquarium with dry land refuge areas at 18°C. They were fed mealworms with Reptovit vitamin supplement (Terrafauna, Morris Plains, NJ, USA). For males and females, breeding was induced in mid-January 1999 by two injections of des-Gly^{10} [D-His (Bzl)^6]-luteinizing hormone-releasing hormone ethylamide (Sigma, St. Louis, MO, USA) 4 days apart at a dose rate of 50 ng/g in frog saline (0.6% NaCl). Fertilized eggs were maintained in polystyrene containers with aerated, filtered water (pH 7.0; dissolved oxygen 8.4–10 mg/L; 16.5–18°C) until stage 21 (*39*), after which they were distributed to the respective treatment exposures (experiment 1). In addition we collected two broods of naturally fertilized *R. pipiens* eggs on 29 April 1999 from a typical breeding pond on the campus of Trent University, Peterborough, Ontario (44°22' N, 78°17' W) and used them in experiment 2. All animal experimentation followed the standards and guidelines of the Canadian Council of Animal Care (*40*).

Conditions of exposure. We conducted a preliminary experiment to determine the 7-day LC_{50} (50% lethal concentration) for *R. pipiens* tadpoles. This was important so that subsequent exposures would be sublethal and environmentally relevant. We conducted experiments 1 and 2 under the same OP/UV-B regimes, and they lasted for 10 days. Fifteen tadpoles were reared in each 100 × 15 mm Pyrex petri dish (working volume 100 mL) and exposed in a controlled environmental chamber (18°C; 12:12 hr light:dark photoperiod) to two concentrations of the environmental estrogen OP (1 nM, 1 µM; groups OP_1 and OP_2, respectively), a subambient level of UV-B radiation (7 µW/cm^2; group UV), and their combinations, groups $UVOP_1$ and $UVOP_2$. In addition, an extra group receiving 0.01% ethanol served as a control. We prepared OP stock solutions (20.6 µg/mL, 20.6 ng/mL) by dissolving 99% pure technical grade 4-OP (Aldrich Chemical Co., Milwaukee, WI, USA) in 95% ethanol. We prepared the nominal concentrations by adding 100 µL of the appropriate OP stock solution to 1 L filtered water. Control and UV-B-only groups were treated with a 0.01% ethanol vehicle. The water was replaced every 48 hr during the study.

The supplemental lighting consisted of UV-B (UBL FS20T12/UVB-BP) and UV-A (combination of Cool White Sylvania F20T12 CW and NEC T10 Black Light Blue) lamps, as well as background laboratory lighting supplied by full spectrum bulbs (Verilux F40T12VLX; both from Sylvania, Mississauga, ON, Canada). Light panels (4 feet long) were positioned above the exposure containers to attain 7 µW/cm^2 UV-B. These are subambient levels because summer levels of ambient UV-B in eastern Ontario vary between 100 and 250 µW/cm^2 (*12,41*).

The UV lamps were on for 6 hr per day (1000–1600 hr), and UV measurements were carried out using an Oriel GOLDILUX UV meter with interchangeable UV-B and UV-A probes (ThermoOriel, Stratford, CT, USA).

The experiments were performed with four replicates per treatment. Each petri dish had 15 tadpoles that were fed Nutrafin fish flakes (Rolf C. Hagen, Montreal, Canada) daily *ad libitum* as of stage 24 [day 5 of the exposure (*39*)]. After the 10-day exposure, tadpoles from experiment 1 were anesthetized using MS222 (Sigma), and the diencephalon, which contains the developing preoptic/hypothalamic area, was rapidly dissected and frozen on dry ice for subsequent isolation of poly (A)$^+$RNA. To obtain sufficient tissue, we pooled diencephalons from 10 tadpoles in each of four independent replicates per treatment.

Postexposure developmental effects. After experiment 2 (12 May 1999), 20 tadpoles [stage 25 (*39*)], from a total of 60, were randomly selected from each of the 6 experimental groups and transferred into clean water in 4.5-L grow-out containers to monitor premetamorphic growth and development. The tadpoles were kept in a separate environmental chamber with no background UV-B under a 12:12 hr light:dark photoperiod supplied by full spectrum lamps (1–2 μW/cm^2 UV-A) and a constant temperature of 18 ± 1°C. To minimize density stress during tadpole development, we used two containers per group. Tadpoles were fed *ad libitum* daily, and overall health was monitored. Two months after the initial 10-day exposure (16 July 1999) stage, 29 tadpoles (*39*) were weighed to assess potential treatment-related differences in body weight. The onset of HLE, defined as stage 36 (*39*), of individual tadpoles was also documented daily. At metamorphic climax (tail resorption), hypothalami were rapidly dissected and frozen on dry ice for isolation of poly(A)$^+$RNA. We combined hypothalamic tissue from two tadpoles in each of four independent replicates per treatment group.

Differential display and transcript identification. The differential display methodology used in this study was similar to our previously reported strategy (*42*). Poly(A)$^+$RNA from diencephalons from all treatments of experiment 1 and metamorph hypothalami from all treatments of experiment 2 was isolated using the Straight A's mRNA isolation system (Novagen, Madison, WI, USA) and 100 ng was reverse transcribed using Superscript II (Gibco, Gaithersburg, MD, USA) to cDNA using one of three single 18-base arbitrary primers (primer A_3: 5′-AATCTAGAGCTCTCCTGG-3′; primer B_3: 5′-CATACACGCGTATACTGG-3′; primer C_3: 5′-CCATGCGCATGCATGAGA-3′). The resulting cDNAs were then amplified by polymerase chain reaction (PCR) in the presence of high specific-activity radiolabeled nucleotides ([^{32}P]dCTP; 10 mCi/mL) and the original primer used for cDNA first-strand synthesis. Amplification was carried out in a thermocycler with a single cycle of low-stringency amplification (94°C, 1 min/36°C, 5 min/72°C, 5 min) followed by 40 cycles with higher stringency [94°C, 1 min/54°C (for primers A_3 and B_3); 56°C (for primer C_3), 2 min/72°C, 2 min]. We separated and reamplified PCR products following Blázquez et al. (*42*). Of approximately 50 bands on the differential display gel, only those few transcripts that displayed an unequivocal presence/absence pattern in the various OP and UV-B treatments were considered differentially regulated. The differentially expressed transcripts were cloned into a pCR II-TOPO vector and transformed in *E. coli* competent cells using the TOPO TA Cloning Kit (Invitrogen, Groningen, The Netherlands). cDNA was sequenced by Canadian Molecular Research Services Inc (Ottawa, Ontario, Canada) using simultaneous bidirectional sequencing reactions with the DYEnamic Cycle Sequencing Kit (US79535) from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada) and IRD700 and IRD800 labeled vector primers (LiCor, Lincoln, NE, USA) flanking the DNA insertion site. The sequencing reactions were separated on a LiCor 4200L sequencer and analyzed with Sequencer 4.0 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequence results were compared to known sequences available in GenBank using the BLAST search accessible through the National Center for Biotechnology Information (*43*).

Reverse Northern multiple-gene dot blot. We used the reverse Northern technique, considered to be semiquantitative (*44,45*), to detect changes in the abundance of the mRNA transcripts isolated by differential display and homology cloning. Poly(A)$^+$RNA was purified from brain tissue collected during experiment 2 using the Straight A's mRNA isolation system (Novagen), and 200 ng was reverse transcribed following the methods of Parfett et al. (*44*), except that Superscript II (Gibco) was used in place of M-MLV reverse transcriptase. For this experiment, we used only brain tissues from the low level of OP (1 nM) and UV-B, alone and in combination, and the solvent control. We synthesized second-strand cDNA in the same salts/buffer mixture as the first-strand, and the 50 μL reaction mix contained 1.5 μL fresh deoxynucleotide triphosphates (10 mM), 1 μl bovine serum albumin (BSA; 0.1 μg/μL), 0.5 μL RNAse H (0.027 U/μL), and 0.5 μL *E. coli* DNA polymerase holoenzyme I (0.1 U/μL). The second-strand cDNA was incubated at 15°C for 1 hr, extracted with phenol, and precipitated with ethanol. Probe synthesis consisted of labeling 100 ng of double-stranded DNA with [^{32}P]dCTP using the Amersham Pharmacia random primer/T7 DNA polymerase oligo-labeling kit.

We chose a composite selection of seven candidate genes isolated by differential display from both tadpole and metamorph brains for the multiple-gene dot blot. Very few brain-specific molecules of any class have been cloned from the leopard frog. Thus the differential display strategy allowed for rapid isolation of candidate genes altered by UV-B and OP. In addition, the two isoforms of leopard frog glutamate decarboxylase [accession numbers AF202124 (GAD67); AF202125 (GAD65); Trudeau et al. (*46*)] and snapping turtle (*Chelydra serpentina*) β-actin (100% identical at the predicted amino acid level with *Xenopus* β-actin) were included, giving a total of 10 genes. To isolate approximately 30 μg of purified cDNA insert required for the dot blots, the Qiagen Plasmid Maxi Kit (Qiagen, Mississauga, ON, Canada) was used. cDNA inserts were digested with EcoRI (Gibco) and extracted from 1% agarose gels using the QIAquick gel extraction kit (Qiagen). Purified cDNA [300 ng/dot prepared in 240 μL 12 × standard sodium citrate (SSC)] of each of the 10 molecules was dotted, in triplicate, onto Hybond-N+ membranes (Amersham Pharmacia), producing a 10 × 3 gene array. Dot blots were made using a 96-well Schleicher and Schuell apparatus. Dot blots were hybridized (*44*) for 48 hr at 55°C with denatured, [^{32}P]-labeled double-stranded cDNA synthesized from poly(A)$^+$RNA from the various treatment groups to detect changes in the relative abundance of the 10 mRNA species.

After hybridization, membranes were sequentially washed for 30 min from low [2 × SSC/0.1% sodium dodecyl sulfate (SDS) at 55°C] to high stringency (0.1 × SSC/0.1% SDS at 55°C), and specific hybridization signals were visualized with the BioRad phosphor-imaging system and quantified using Quantity One software (BioRad Laboratories, Hercules, CA, USA). All data were normalized to β-actin, as it did not change with OP or UV-B treatments.

Statistical analysis of data. We used linear interpolation to estimate the LC_{50} value. The line between the two nearest concentrations that produced mortality levels above and below 50% was used in the calculation. We analyzed tadpole weight and age at HLE using a two-way analysis of variance (ANOVA). A post-hoc Fisher's least significant difference (LSD) multiple comparisons test was performed to identify

differences between treatment groups. The average of internal triplicates on one membrane was calculated per dot blot, and each treatment group had an independent replicate of three or four blots (n = 3 or 4) to ensure reproducibility. Gene expression data were not normal, so we used Friedman's test (a two-way ANOVA on the ranked data) to test for significant interactions between OP and UV-B with respect to mRNA expression (*47*). This was followed by Fisher's LSD test to determine differences between groups (*47*). For all statistical procedures, group means were considered different if $p < 0.05$. As indicated, gene expression data were nonparametric and therefore the median rather than the mean of each treatment group has been presented (*47*). We used Systat 7.0.1 software (SPSS Inc., Chicago, IL, USA) for all statistical procedures.

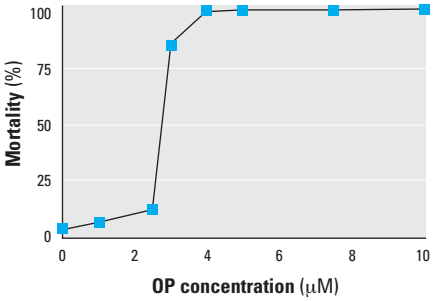

Figure 1. The dose–response curve for waterborne OP with respect to newly- hatched leopard frog (*R. pipiens*) tadpoles after exposure to eight different concentrations of OP.

Results

LC_{50} for OP

The calculated 7-day LC_{50} value for waterborne OP was 2.8 μM (Figure 1). Figure 1 shows that the toxic response was extremely abrupt: At 2.5 μM there was minimal mortality, and at 3 μM there was 84% mortality. The *R. pipiens* tadpoles exposed to 1 μM OP in this study displayed minimal (approximately 5%) mortality (Figure 1), and, subsequently, the levels of OP used were at or below 1 μM. No additional increases in mortality were observed in the 1 μM group between 7 and 10 days (data not shown). The ethanol-exposed control group displayed an extremely low mortality rate of approximately 2% (Figure 1).

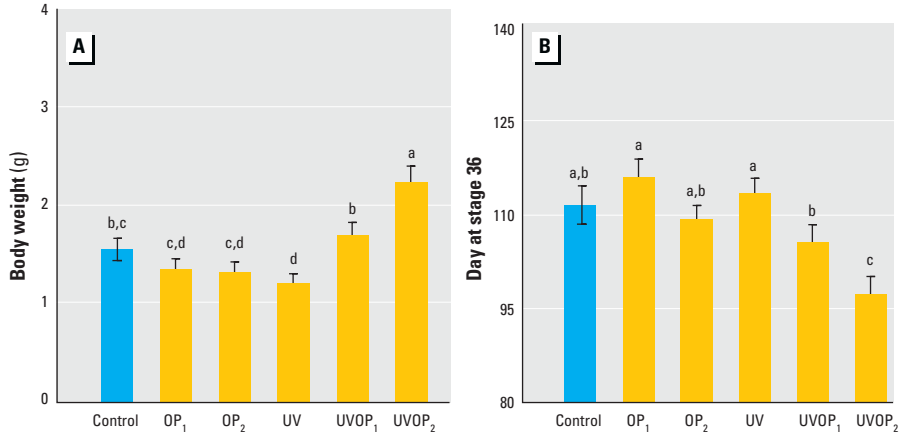

Figure 2. The effects of early exposure to OP (1 nM and 1 μM; groups OP_1 and OP_2, respectively) and UV-B radiation (7 μW/cm^2; group UV), and their combinations (groups $UVOP_1$ and $UVOP_2$), on premetamorphic parameters of *R. pipiens*. (*A*) Overall body weight (wet weight) of stage 29 (Gosner) tadpoles. (*B*) Day at stage 36 (Gosner; hind-limb toe development) as a function of treatment. Data are expressed as mean ± SEM. Different letters indicate statistical differences ($p < 0.05$).

Postexposure Developmental Effects

Tadpole body weight (wet weight) at stage 29 (*39*) changed according to early life-stage exposure. There was no change in weight in animals treated with OP alone (groups OP_1 and OP_2). However, tadpole weight in the UV group was significantly reduced. Moreover, tadpoles treated with a combination of 1 μM OP plus UV-B (group $UVOP_2$) were larger than tadpoles in all other treatments (Figure 2A). Treatments with either OP or UV-B (groups OP_1, OP_2, and UV) alone had no effects on the onset of HLE, stage 36 (*39*). However, tadpoles treated with 1 μM OP plus UV-B (group $UVOP_2$) displayed significant acceleration of HLE compared to all other treatments (Figure 2B).

Identification of Putative Treatment-Responsive Genes Using Differential Display

Tadpole diencephalon. The differential display strategy identified three candidate cDNA transcripts altered by OP, one transcript altered by UV-B, and two molecules that were unchanged based on a similar band intensity across all treatments (Table 1). The expression of a cDNA fragment (774 bp) approximately 66% identical to the 3′ end of human and rat plectin was potentially

Table 1. mRNA transcripts (with accession numbers) potentially altered by OP and UV-B in the hypothalamic tissue of leopard frog (*R. pipiens*) tadpoles and metamorphs as determined using differential display.

Stage, molecule (accession no.)	Functions	cDNA length	Primer	Percent identity[a]	Treatment effects
Tadpole					
Plectin (AF279869)	Mechanical strength for cytoskeleton; structural integrity	774 bp	C_3	66 (rat, X59601)	OP_1 ↑
NAP4 (AF279870)	Signal transduction; Growth factor action	798 bp	C_3	89 (human, PC4427)	OP_1 ↑
BAI2 (AF279868)	Development of glioblastoma	517 bp	A_3	59 (human, NP 001694)	OP_1 ↓ OP_2 ↓
Arcadlin (AF279872)	Ca2+ signaling; synaptic reorganization	403 bp	B_3	56 (rat, BAA82442)	UV ↓
Cytochrome C oxidase chain I (AF279871)	Mitochondrial respiratory protein; brain oxidative phosphorylation	676 bp	B_3	62 (alligator, AAD09982)	No change
NADH dehydrogenase subunit 4 (AF279867)	Glycolosis; cellular respiration	898 bp	A_3	54 (goldfish, BAA31247)	No change
Metamorph					
BAI3 (AF279866)	(see BAI2)	567 bp	A_3	90 (human, NP 001695)	No change
NADH dehydrogenase subunit 4 (AF279867)	Glycolosis; cellular respiration	896 bp	A_3	54 (goldfish, BAA31247)	All treatments ↑ except control

Treatments were OP_1 (1 nM), OP_2 (1 μM), and UV (7 mW/cm^2). ↑ = increased expression; ↓ = decreased expression.
[a]At the predicted amino acid level; in parentheses is the species and accession number of the GenBank sequence for the first BLAST search hit.

increased by 1 nM OP. Another transcript (798 bp) that revealed a potential increase in expression following 1 nM OP treatment was 89% identical to human Nck, Ash, and phospholipase C γ-binding protein 4 (NAP4). The expression of a 517-bp transcript was potentially decreased by both 1 nM and 1 µM OP and was 59% identical to human brain-specific angiogenesis inhibitor 2 (BAI2). A 403-bp transcript displayed a potential decrease of expression after UV-B treatment and was 56% identical to rat arcadlin. Finally, the expression of cytochrome C oxidase chain I, an important mitochondrial proton-pumping respiratory protein, and NADH dehydrogenase subunit 4, involved in glycolosis and cellular respiration, appeared to be unchanged across all treatments.

Metamorph hypothalamus. The expression of a 567-bp transcript that was 90% identical to human brain-specific angiogenesis inhibitor 3 (BAI3) was determined to be unchanged based on differential display (Table 1). Differential display also identified a 896-bp fragment in the metamorph hypothalamus that showed a potential increase in expression after exposure to 1 nM and 1 µM OP, alone and in combination with UV-B (groups OP_1, OP_2, $UVOP_1$, and $UVOP_2$, respectively). This molecule was 54% identical to goldfish NADH dehydrogenase subunit 4 (Table 1). Note that the expression of NADH dehydrogenase subunit 4 mRNA was unchanged in the tadpole brain, which suggests a stage- and treatment-specific shift in expression of this molecule.

Determination of Gene Expression Using Reverse Northern Blot Analysis

Tadpole diencephalon. Octylphenol (1 nM; group OP_1) induced a 3-fold increase in the expression of NAP4 mRNA (Figure 3A), confirming the initial differential display results (Table 1). All other treatments were without effects (Figure 3A). Although BAI3 mRNA was originally isolated from the hypothalamus of *R. pipiens* metamorphs, we found that its expression in the tadpole diencephalon was increased 5-fold in animals exposed to UV-B (Figure 3B). All other treatments were without effects (Figure 3B). In addition, we verified that the expression of cytochrome C oxidase chain I and NADH dehydrogenase subunit 4 was unchanged across the various treatment exposures (data not shown). The median values for plectin mRNA expression were not greater in the OP treatment and in fact were similar across all treatments (data not shown). This result, together with those of BAI2 and arcadlin, are inconsistent with the results obtained from the differential display (Table 1). Thus, the reverse Northern experiments confirmed the expression pattern of three of the six transcripts originally isolated from tadpole diencephalon using differential display. A similar level of confidence has been reported for differential display and reverse Northern blots in other vertebrate systems (*45*). In the tadpole diencephalon, the expressions of GAD65 and GAD67 were similar across all treatments (data not shown).

Metamorph hypothalamus. GAD67 mRNA levels were not affected by OP (1 nM; group OP_1) or UV-B alone. However, the expression of GAD67 mRNA was significantly decreased in the hypothalamus of metamorphs exposed to OP compared to those exposed to UV-B alone. Moreover, GAD67 expression was increased by approximately 2-fold in the hypothalamus of metamorphs coexposed to OP plus UV-B (group $UVOP_1$; Figure 4A). The expression pattern of BAI2 mRNA in the various treatment groups was similar to that observed for

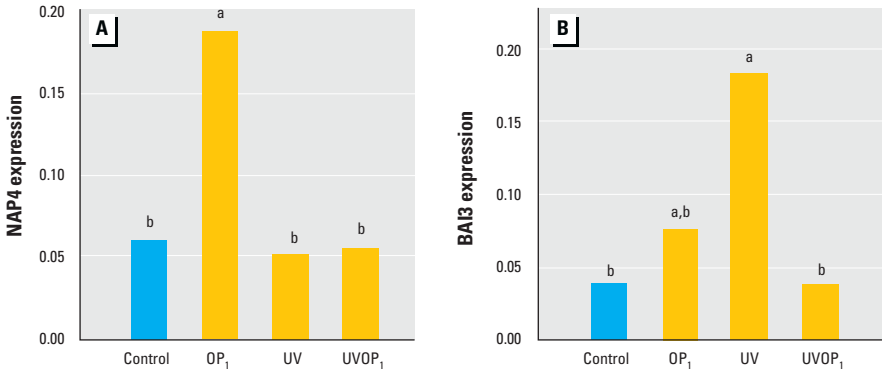

Figure 3. Relative mRNA expression of treatment-regulated molecules in the diencephalon of *R. pipiens* tadpoles after different treatments with OP (1 nM, group OP_1), UV-B radiation (7 µW/cm^2, group UV) and their combination (group $UVOP_1$). (*A*) NAP4; (*B*) BAI3. Median expression values have been normalized to β-actin; different letters indicate significant differences ($p < 0.05$).

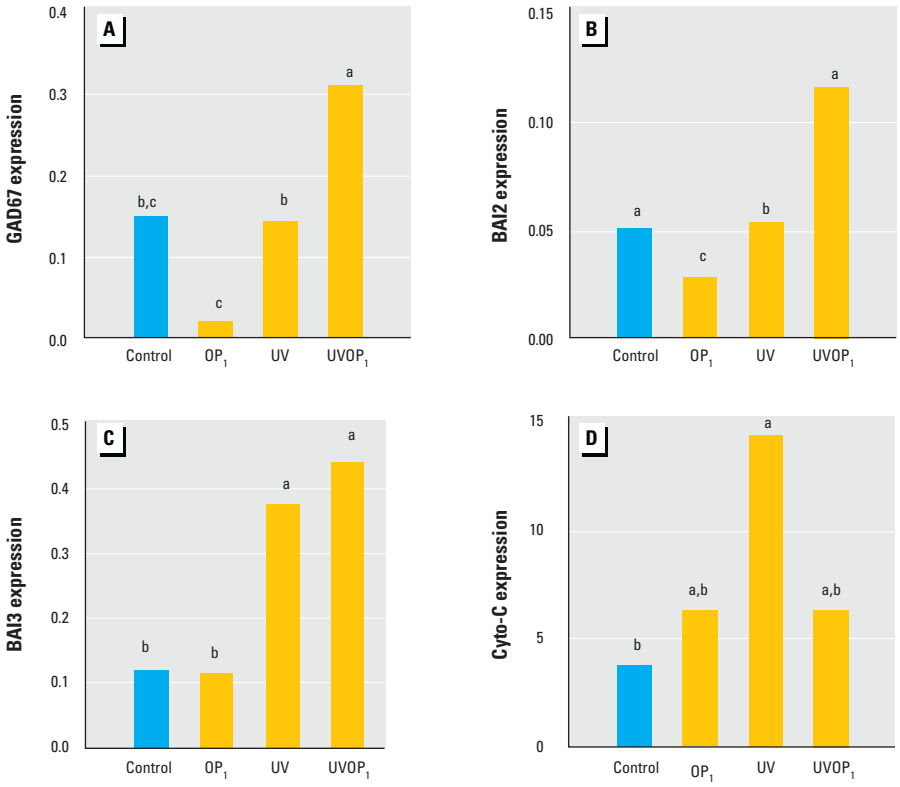

Figure 4. Relative mRNA expression of treatment-regulated molecules in the hypothalamus of *R. pipiens* metamorphs after different treatments with OP (1 nM, group OP_1), UV-B radiation (7 µW/cm^2, group UV), and their combination (group $UVOP_1$). (*A*) GAD67; (*B*) BAI2; (*C*) BAI3; (*D*) cyto-C (cytochrome C oxidase chain I). Median expression values have been normalized to β-actin; different letters indicate significant differences ($p < 0.05$).

GAD67 (Figure 4B). Early exposure to OP (group OP_1) resulted in a 2-fold decrease in BAI2 expression, whereas UV-B exposure alone did not affect BAI2 mRNA levels (Figure 4B). In contrast to OP and UV-B treatments alone, the combined OP plus UV-B exposure (group $UVOP_1$) significantly increased BAI2 mRNA expression in the metamorph hypothalamus (Figure 4B). BAI3 was originally determined by differential display to be similarly expressed in all treatment groups (Table 1). However, semiquantitative reverse Northern blots revealed that UV-B exposure increased BAI3 mRNA expression approximately 3-fold. Similarly, the expression of BAI3 was 4-fold higher in the hypothalamus of animals in group $UVOP_1$ than in the control or OP_1 group (Figure 4C).

As in the tadpole diencephalon (Figure 3B), BAI3 mRNA levels were increased by UV-B treatment in the metamorph hypothalamus (Figure 4C). As indicated previously, cytochrome C oxidase chain I mRNA was unchanged in the tadpole diencephalon. However, its expression in the metamorph hypothalamus was increased 3-fold in animals exposed to UV-B. In contrast, levels of cytochrome C oxidase chain I mRNA in the hypothalamus of animals treated with OP and OP plus UV-B (groups OP_1 and $UVOP_1$, respectively) were similar to that in controls (Figure 4D). NADH dehydrogenase subunit 4 expression was not regulated (data not shown) as predicted by differential display (Table 1). In addition, mRNA expression of GAD65, plectin, arcadlin, and NAP4 were similar across all treatments (data not shown).

Discussion

The present study shows that an early 10-day exposure of *R. pipiens* tadpoles to OP and UV-B radiation can disrupt normal premetamorphic development. Body weight before HLE was increased and the day at HLE (i.e., Gosner stage 36) was significantly accelerated by cotreatments of OP plus UV-B. Disruption of metamorphosis has been observed in other amphibian studies when certain toxins (acetochlor, TCDD) were combined with exogenous T_3 (*7,21*). Jung and Walker (*21*) found that 0.03 µg/L TCDD accelerated metamorphosis and that *B. americanus* tadpoles exposed to > 0.03 µg/L TCDD had a larger body mass at metamorphosis than vehicle-treated animals. Cheek et al. (*7*) reported that the herbicide acetochlor (10^{-8} M), in combination with T_3, accelerated forelimb emergence in *R. pipiens*. Contrary to the present study, they observed no alteration of tadpole growth or timing of HLE. Cheek et al. (*7*) proposed that acetochlor enhanced T_3 action, in the presence of exogenous T_3, to accelerate a specific metamorphic event, forelimb emergence. The authors further stated that acetochlor might have decreased the enzymatic activity of 5-deiodinase that inactivates T_4 to T_3 and T_3 to T_2, effectively increasing endogenous T_3 concentrations and indirectly accelerating metamorphosis.

The mechanisms involved in the changes in metamorphic parameters in this study are currently not well understood. The increase in weight and acceleration of HLE may be the result of a photochemical interaction between OP and UV-B because metamorphic alterations were observed only in this treatment group. The photointermediates of OP have not yet been identified; however, putative photochemical byproducts could conceivably affect the hypothalamo–pituitary–thyroid axis. Hypothalamic release of CRH and the downstream effects on T_3 concentrations could have been the target in this case, as exogenous T_3 was not required to observe the metamorphic alterations. Perhaps, as in the study by Cheek et al. (*7*), the combination of specific stressors accelerates specific metamorphic events. Regardless of the mechanism, physiologic end points such as metamorphosis provide promising indicators of disruption of the hypothalamo–pituitary–thyroid axis (*22,48*) by environmental contaminants.

Contrary to the laborious techniques of subtractive hybridization and/or library screening, our differential display strategy proved to be an effective method to isolate candidate genes rapidly from the leopard frog, a species whose genome is not characterized. We identified several functionally and structurally important molecules solely by cloning transcripts that displayed a presence/absence pattern in the differential display. These included plectin, an intermediate filament-binding protein that provides mechanical strength and structural integrity to the cytoskeleton (*49*); NAP4, implicated in coordinating various signaling pathways including growth factor and cell adhesion receptors (*50*); BAI2 and BAI3, membrane proteins that inhibit neovascularization and act as growth suppressors of glioblastoma (primary brain tumor development) (*51*); arcadlin, a novel cadherin molecule expressed at the synapses that may play an important role in activity-induced synaptic reorganization underlying long-term memory (*52*); cytochrome C oxidase chain I, an important mitochondrial proton-pumping respiratory protein; and NADH dehydrogenase subunit 4, involved in glycolosis and cellular respiration. The inclusion of the well-characterized neurotransmitter enzymes GAD65 and GAD67 allowed us to address known hypothalamic pathways in addition to the relatively unknown mechanisms of the candidate molecules.

The effects of OP on growth, metamorphosis, and hypothalamic gene expression observed in this study provide further evidence that OP is a biologically active contaminant at environmentally relevant levels. The 1-nM OP exposure level used in our study is 1,000 times lower than the concentration used in an *in vitro* culture study where OP (1 µM) induced PRL gene expression in rat pituitary cells (*53*). Additionally, the 1-nM OP exposure is approximately 2,800 times less than the calculated LC_{50} for *R. pipiens* tadpoles. The observed effects of UV-B on gene expression are in addition to the well-characterized effects of this stressor on several aspects of amphibian development and survival (*11–14*).

NAP4 expression was increased by 1 nM OP in the tadpole diencephalon, verifying the pattern observed in the initial differential display. NAP4 is a putative signaling molecule widely expressed in various tissues and during several developmental stages in mammals (*50*). It contains a complete Src homology region 2 (SH2) domain known to interact with the middle SH3 domain of Nck, an adaptor protein involved in tyrosine kinase-mediated signal transduction for both epidermal and vascular endothelial growth factors (*50,54*). In addition, Nck affects pathways leading to cellular mitogenesis and morphogenesis (*54*). During early tadpole development, the diencephalon undergoes substantial restructuring, and the regulation of intercellular communication is essential for proper development and survival (*23*). In this context, an increase in NAP4 expression after OP treatment could affect important aspects of development of the tadpole brain (e.g., tyrosine kinase-mediated growth factor signal transduction). However, deregulation of certain signal transduction pathways associated with cellular proliferation (e.g., Ras GTPases, extracellular signal-related kinase, ERK) can cause various abnormalities including cancer (*54*). Nck, and thus NAP4, appear to be functionally involved in Ras GTPases and ERK. Thus, a disruption of normal function caused by OP exposure could lead to similar consequences with respect to cellular proliferation in the hypothalamus.

The expression of BAI3 was increased in brain tissue of tadpoles and metamorphs exposed to UV-B and was additionally increased by the combination of OP plus UV-B in the metamorph hypothalamus. The expression of another member of the BAI family, BAI2, was decreased by OP treatment and was increased by the combination of OP plus UV-B in the metamorph hypothalamus. All members of the BAI family, composed

thus far of BAI1, BAI2, and BAI3, contain a seven-span transmembrane region with an extracellular motif recognized by the integrins and sequences corresponding to thrombospondin type I repeats that can inhibit experimental angiogenesis (*51*). A local balance between stimulators and inhibitors of neovascularization and vasoproliferation controls angiogenesis (*51,55*). Angiogenesis inhibitors block the signaling of cancer-cell inducers to surrounding blood vessels and inhibit the vasoproliferation necessary for the growth, persistence, and metastasis of solid tumors (*55*). The regulation of BAI-related molecules may be important for capillary formation and blood supply to the developing hypothalamus during metamorphosis.

NAP4 is also associated with signal transduction events involving inducers of vasoproliferation and has been shown to have an oncogenic role when overexpressed in fibroblasts (*54*). Although a decrease in expression of either BAI2 or BAI3 was not observed in the tadpole diencephalon, early exposure to OP did decrease BAI2 expression in the metamorph hypothalamus. NAP4 expression was significantly increased by OP in the tadpole diencephalon, which might stimulate signal transduction events that would lead to vasoproliferation and in metamorphs, the endogenous inhibitors of such vasoproliferation appear suppressed. Additionally, UV-B is a known carcinogen, and in both the tadpole diencephalon and metamorph hypothalamus BAI3 expression appears to be sensitive to UV-B treatment, producing a significant increase of expression. The increases in expression of both NAP4 and BAI3 after UV-B exposure could modulate vasoproliferation in the developing tadpole brain, perhaps contributing to disruption of neuroendocrine function.

GAD67 expression in the metamorph hypothalamus was altered after early exposure to OP and UV-B. Both GAD65 and GAD67 mediate the synthesis of GABA, but their distribution in the brain suggests divergent functional roles (*56*). GABA is the primary inhibitory neurotransmitter in the central nervous system and acts to induce membrane hyperpolarizations by binding to postsynaptic $GABA_A$ receptors and presynaptic $GABA_B$ receptors (*56*). GABA also acts as an important neurotrophic and neurodifferentiating signal molecule during early brain development (*57*). Interestingly, the gene expression pattern of BAI2 is similar to that of GAD67 in metamorph hypothalami, suggesting possible relationships between angiogenesis and GABA neurons during metamorphic development of the hypothalamus.

GABA is found throughout the hypothalamus and regulates most aspects of hypothalamic function (*58*). GABA has both stimulatory and inhibitory effects on thyrotropin (TSH) release in mammals (*58*). It has also been shown that GABA inhibits PRL release in both fish and mammals (*58,59*). In ovariectomized rats, GABA lowered plasma TSH via actions on the dopamine system. However, the inhibitory effects of GABA on TSH release were reduced in rats treated with E_2 and progesterone (*58*). On the other hand, GABAergic stimulation of TSH release in both males and ovariectomized female rats has also been reported (*58*). In amphibians, CRH stimulates pituitary TSH release, which in turn enhances thyroid production of T_3, the hormonal inducer of metamorphosis (*24,26*). PRL, on the other hand, antagonizes amphibian metamorphosis and promotes larval growth (*24,26*). Given that cotreatment with OP (1 nM) plus UV-B increases GAD67 mRNA levels and OP (1 µM) plus UV-B advances metamorphosis, there may be a GABAergic component to the control of TSH and/or PRL release in the leopard frog.

We found that the expression of cytochrome C oxidase chain I was increased approximately 3-fold in the metamorph hypothalamus as a result of early exposure to UV-B. This molecule has been shown to be T_3-regulated in the *Xenopus* gene expression program for neural development and thus is associated with changes in the diencephalon during metamorphosis (*23*). Cytochrome C oxidase is an important mitochondrial proton-pumping respiratory protein that catalyzes the transfer of electrons from cytochrome C to oxygen (*23*). The extensive brain restructuring involved in metamorphosis and the corresponding metabolic demands during cell proliferation and differentiation would likely require increased energy via the cytochrome C pathway. Denver et al. (*23*) proposed that the increased expression of cytochrome C oxidase subunit I in tadpoles might be correlated with changes in brain oxidative phosphorylation. The data in our study suggest that UV-B exposure alters the expression of this T_3-responsive molecule, indicating an alteration of a standard metabolic pathway in amphibian metamorphosis.

One major question remaining relates to the unexpected alterations of gene expression profiles when UV-B and OP were given together. For example, in the tadpole diencephalon, OP alone induces NAP4 expression, a response that is abolished in the presence of UV-B. Moreover, UV-B induced the expression of BAI3 in the tadpole diencephalon, and this effect was eliminated with cotreatment of OP. These rather complex modulated responses were also apparent in the metamorphs. Both GAD67 and BAI2 were reduced in OP-exposed animals, but not affected by UV-B; yet the cotreatments induced 2- to 3-fold increases in the expression of these molecules in the hypothalamus. Possible mechanisms for this interaction are speculative at best but likely involve complex gene interactions, perhaps as a result of altered metabolic pathways or signal transduction systems. It is clear from our data that UV-B increases cytochrome C gene expression in the metamorph hypothalamus, which could be reasonably expected to affect cytochrome C activity, perhaps affecting cellular metabolism and OP action. Moreover, interactions between signal transduction pathways are known to occur in neuroendocrine systems (*60,61*) and during embryonic development of the brain and spinal cord (*62,63*). Although we do not yet know which signaling pathways are involved in the UV-B and OP responses, we hypothesize that there may be an interaction in several downstream signaling mechanisms that could account for our observations. The molecular mechanisms involved should be explored in future work.

In summary, this study represents a novel technique in toxicology that combines physiologic and molecular end points for a species native to North America. The exact mechanism of action of OP and UV-B, alone or in combination, on the expression of the various candidate molecules is not well understood at this time. It is not possible, at present, to establish a cause-and-effect relationship between the observed changes in hypothalamic gene expression and metamorphosis. However, this study provides confirmation that stressors previously determined to affect amphibians can be assessed at a more subtle level and lends precedence for using molecular techniques to address toxicologic questions. This study is also among the first to attempt such work in the frog tadpole.

Recently, there has been an effort to use large-scale or whole-genome approaches to analyze the effects of drugs and toxicants. The standard Northern blot experiments have now been superseded by dot blots and cDNA microarrays, which can simultaneously determine expression patterns of many genes. The regulation of gene expression occurs primarily at the transcriptional level—hence the interest in developing techniques to measure differentially expressed genes via mRNA (*64*). It is important to recognize that changes at the mRNA level that were determined here using reverse Northern dot blots may not accurately predict the changes in the functional protein. In fact, there is a relatively poor correlation between gene and protein expression, and often the regulation of protein function is at the translational or post-translational level (*64*).

The functional analysis of protein expression would help determine the precise roles of these molecules in the context of amphibian hypothalamic development and metamorphosis. Nevertheless, early life exposure to a persistent EDC and a subambient level of UV-B radiation alters premetamorphic development and regulates the expression of hypothalamic genes important for signal transduction, angiogenesis, and neurotransmitter synthesis.

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