

# Octylphenol (OP) Alters the Expression of Members of the Amyloid Protein Family in the Hypothalamus of the Snapping Turtle, *Chelydra serpentina serpentina*

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The gonadal estrogen estradiol-17 $\beta$  (E<sub>2</sub>) is important for developing and regulating hypothalamic function and many aspects of reproduction in vertebrates. Pollutants such as octylphenol (OP) that mimic the actions of estrogens are therefore candidate endocrine-disrupting chemicals. We used a differential display strategy (RNA-arbitrarily primed polymerase chain reaction) to isolate partial cDNA sequences of neurotransmitter, developmental, and disease-related genes that may be regulated by OP or E<sub>2</sub> in the snapping turtle (*Chelydra serpentina serpentina*) hypothalamus. Hatchling and year-old male snapping turtles were exposed to a 10 ng/mL nominal concentration of waterborne OP or E<sub>2</sub> for 17 days. One transcript [421 base pairs (bp)] regulated by OP and E<sub>2</sub> was 93% identical to human APLP-2. APLP-2 and the amyloid precursor protein (APP) regulate neuronal differentiation and are also implicated in the genesis of Alzheimer disease in humans. Northern blot analysis determined that the turtle hypothalamus contains a single APLP-2 transcript of 3.75 kb in length. Exposure to OP upregulated hypothalamic APLP-2 mRNA levels 2-fold ( $p < 0.05$ ) in month-old and yearling turtles. E<sub>2</sub> did not affect APLP-2 mRNA levels in hatchlings but stimulated a 2-fold increase ( $p < 0.05$ ) in APLP-2 mRNA levels in yearling males. The protein  $\beta$ -amyloid, a selectively processed peptide derived from APP, is also involved in neuronal differentiation, and accumulation of this neurotoxic peptide causes neuronal degeneration in the brains of patients with Alzheimer disease. Therefore, we also sought to determine the effects of estrogens on the expression of  $\beta$ -amyloid. Using homology cloning based on known sequences, we isolated a cDNA fragment (474 bp) from turtle brain with 88% identity to human APP. Northern blot analysis determined that a single 3.5-kb transcript was expressed in the turtle hypothalamus. Waterborne OP also increased the expression of hypothalamic APP after 35 days of exposure. Our results indicate that low levels of OP are bioactive and can alter the expression of APLP-2 and APP. Because members of the APP gene family are involved in neuronal development, we hypothesize that OP exposure may disrupt hypothalamic development in young turtles. **Key words:** alkylphenol polyethoxylates, Alzheimer disease, brain, estrogen, turtles. *Environ Health Perspect* 110:269–275 (2002). [Online 12 February 2002] <http://ehpnet1.niehs.nih.gov/docs/2002/110p269-275trudeau/abstract.html>

It is increasingly apparent that several common pollutants have profound effects on embryonic development, reproduction, and growth of aquatic animals because they mimic or suppress the actions of the sex steroid estradiol (E<sub>2</sub>). Of particular concern are the alkylphenol-polyethoxylates (APEOs), a large group of nonionic surfactants in commercial production (approximately 250,000 tons produced per year) that enter the aquatic environment mainly from sewage treatment and pulp and paper mill effluents (1–4). Upon discharge, APEOs are rapidly degraded to form relatively stable, hydrophobic metabolites, principally the alkylphenols nonylphenol (NP) and octylphenol (OP). These estrogenic metabolites competitively bind to both trout and mouse estrogen receptors, stimulate vitellogenin production in trout hepatocytes, inhibit testicular growth in trout, stimulate prolactin gene expression in rat pituitary cells, and induce growth of MCF-7 and ZR-75 breast cancer cell lines (1,5–8). Moreover, Blázquez et al. (8) found that

0.2  $\mu$ g/mL waterborne OP caused 50% mortality among immature male goldfish 20 days after exposure, but immature females were unaffected. Therefore, there is considerable evidence that indicates that OP over a range of concentrations (0.02–2  $\mu$ g/mL) is a biologically active contaminant. Indeed, there is reason for concern because OP has been found at significant concentrations in fresh water (< 0.2  $\mu$ g/mL–0.5 ng/mL), sediments (< 0.010–1.8  $\mu$ g/g dry weight), sewage treatment effluent (0.1–2.5 ng/mL), and sludge (< 0.005–12.1  $\mu$ g/g dry weight) in North America (3,4,9). Waterborne OP levels of approximately 12 ng/mL have been reported in UK rivers and estuaries (10).

Despite evidence that OP disrupts vitellogenin production in several fish species, there have been few investigations on higher-level aquatic tetrapods such as frogs and turtles. OP has, however, been shown to stimulate vitellogenin production (11) and cause feminization in *Xenopus* (12), suggesting that it is also estrogenic in frogs. There have been no reports on the effects of OP in

reptiles. Turtles may represent a unique model in which to study the effects of estrogenic pollutants such as OP. We have chosen to use the common snapping turtle. Its life history is predominantly aquatic and it spends a considerable amount of time buried in the muddy bottoms of lakes and streams. The highest levels of NP and OP in Great Lakes regions are found in sediment samples (9). Moreover, it is well established that sexual differentiation in reptiles such as turtles and alligators is labile and extremely sensitive to the effects of estradiol, as determined in extensive laboratory experimentation (13–16) and also in field studies in Florida, where DDT and its metabolites and many other common pollutants have profound effects on reproductive function (17). Developmental abnormalities in embryos and hatchling snapping turtles in polluted areas of the Great Lakes–St. Lawrence River Basin have been reported (18). Demasculinization of adult male snapping turtles was observed in three Lake Ontario sites (19), suggesting that wild turtle populations are being exposed to xenoestrogens.

We set out to identify novel gene targets responding to estrogenic compounds found in the aquatic environment. Extensive research has shown that estrogens have tremendous importance for nervous system function, including hypothalamic development and induction of sexually dimorphic patterns of neurotransmitter synthesis (20–22). In all vertebrates, estrogen receptors are highly expressed in the hypothalamus.

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The hypothalamus is the feedback control center regulating the release of pituitary hormones such as growth hormone and gonadotropins that respectively regulate growth and reproduction. Although the hypothalamus is the main target for central estrogen action, few attempts have been made to determine whether estrogenic pollutants affect gene expression in this part of the brain. MacLusky et al. (23) have shown that halogenated arylhydrocarbons, some of which have estrogenic or antiestrogenic activity, may disrupt central nervous system development in rats. OP has been shown to induce persistent estrus without disrupting ovulation. The observation that OP did not block ovulation led Blake and Ashiru (24) to conclude that OP had no major effect on neuronal mechanisms controlling ovulation. Moreover, OP stimulates estrogen-dependent uterine growth in prepubertal rats but apparently has no influence on prenatal sexual differentiation of the rat brain (25). Therefore, both research groups have implied that OP is not neuroactive, although they did not thoroughly test this possibility. Here we used a differential display polymerase chain reaction (PCR) strategy to identify genes in the snapping turtle hypothalamus that may be regulated by estrogen or OP. We demonstrate that an environmentally relevant dose of OP alters the expression of members of the amyloid protein family. These proteins have roles in neuronal development in rodents and in the etiology of Alzheimer disease in humans.

## Materials and Methods

**Animals and in vivo exposures to estradiol ( $E_2$ ) and OP.** Common snapping turtle (*Chelydra serpentina serpentina*) eggs were collected in early June from Algonquin Park, Ontario, and incubated at 26°C throughout development; at this temperature the turtles hatch in mid- to late August. Sexual differentiation in this species is temperature dependent, and previous studies have shown that 26°C is the male-producing temperature (26). The turtles were housed in 28 × 17 × 13 cm rat cages containing 1 L water. Three turtles were kept in one cage and were separated by moveable partitions and were fed chopped chicken hearts every Tuesday and Thursday. Every Monday and Friday, the water and chemicals were renewed. For the injection experiment,  $E_2$  and OP were dissolved in peanut oil that had been purified by charcoal-dextran to remove any steroids or organic contaminants. For the initial differential display screen, yearling turtles were injected subcutaneously with a physiologic-dose of estradiol (20 ng/g;  $n = 16$ ) or a low dose of OP (20 ng/g;  $n = 16$ ) or the peanut oil vehicle (5  $\mu$ L/g body weight;

$n = 16$ ). The injection site was near the neck between the shell and the scapula of the right forelimb. Twenty-four hours after injection, the animals were killed by rapid decapitation, the cranium cut open, and the brain removed and placed in a petri dish on ice. The hypothalami were dissected using a dissection microscope and fine scissors, placed in sterile 15-mL tubes, and rapidly frozen on dry ice for subsequent isolation of total RNA or poly(A)<sup>+</sup> RNA. To obtain sufficient RNA for a single sample, it was necessary to pool hypothalami from three or four turtles. For exposures in the water,  $E_2$  and OP were first dissolved in a 1:1 acetone and 99% ethanol mixture. Two hundred microliters of the acetone/ethanol mixture containing either 50  $\mu$ g/ $\mu$ L  $E_2$ , 50  $\mu$ g/ $\mu$ L OP, or no chemicals (control) were added to 1 L of water, vigorously shaken, and the resultant solution (10 ng/mL nominal concentration) placed in the turtle cages. In 1998, 50 yearling (10–30 g) and 80 one-month-old turtles (6–15 g) were randomly exposed to the nominal 10 ng/mL concentration of  $E_2$  or OP or the vehicle only in water for 17 days before tissue collection. In 2000, yearling turtles (30–45 g) were exposed to 10 ng/mL OP ( $n = 18$ ) or vehicle ( $n = 18$ ) for 35 days. For the environmental exposure experiments, hypothalamic dissections followed the protocol described above.

**RNA arbitrarily primed PCR (RAP-PCR) differential display strategy.** To obtain candidate hypothalamic genes regulated by  $E_2$  or OP, we used a RAP-PCR strategy similar to that reported previously by our laboratory (27). The differential display strategy is used as a rapid method to obtain cDNA fragments that can be sequenced and identified. The technique is especially useful in obtaining these cDNAs from a species such as the snapping turtle because its genome has not been characterized. Subsequent to obtaining the sequences, the expression of the genes can be studied in detail. We extracted total RNA from hypothalami using the Trizol reagent (Gibco/BRL, Burlington, ON, Canada) following the manufacturer's protocol. We isolated poly (A)<sup>+</sup> RNA from total RNA using a commercial kit (Oligotex mRNA mini kit; Qiagen, Mississauga, ON, Canada). For cDNA synthesis, 100 ng of poly (A)<sup>+</sup> RNA was reverse transcribed by SuperScript II Rnase H-Reverse Transcriptase (200U/ $\mu$ L; Gibco/BRL) and a single arbitrary primer (5'-AATCTAGAGCTCTCCTGG-3'). We PCR-amplified 1  $\mu$ L of the RT reaction with [ $\alpha$ -<sup>32</sup>P]dATP and the same arbitrary primer used in the cDNA synthesis. We performed the PCR reaction in a Hybaid Touchdown thermal cyclor (Hybaid/Life Sciences International, Ashford, UK) using

the following program: 94°C for 5 min to denature, 36°C for 5 min for low stringency annealing, and 72°C for 5 min for extension. This was followed by 40 high-stringency cycles: 94°C for 1 min, 54°C for 2 min, and 72°C for 2 min. PCR products were loaded onto a 4% acrylamide/7 M urea sequencing gel and electrophoresed at 1,500 V until the xylene cyanol dye had reached the bottom of the gel. The gels were dried under vacuum at 80°C for 2 hr and exposed to X-ray film for 2–3 hr at –80°C. We cut out differential display DNA bands from the gel. The DNA was extracted in TE buffer, ligated into a pCR2.1 vector, and transformed in *E. coli* competent cells using the TA-cloning kit (Invitrogen, Groningen, The Netherlands). Positive white colonies were selected and cultured overnight at 37°C in 15 mL medium, and plasmids were isolated with the Plasmid mini kit (Qiagen). Cloned inserts were sequenced by Molecular Research Services Inc. (Ottawa, ON, Canada). Nucleotide sequences obtained from the differential display experiments were submitted to BLAST for comparison to known sequences in GenBank accessible through the National Center for Biotechnology Information website (28).

**RT-PCR.** One transcript obtained by RAP-PCR was the snapping turtle homolog of amyloid-like precursor protein-2 (APLP-2). The APLP-2 protein and major amyloid precursor protein (APP) have similar roles and may affect neuronal differentiation and postnatal development in mice (29,30). We first studied the effects of chemical exposure on APLP-2 mRNA levels, and in a subsequent experiment we also studied the effects of OP on APP. To obtain the 3' end of the turtle APP cDNA, primers were designed based on the conserved region of known APP sequences of Torpedo ray, pufferfish, and mouse, which are all available from GenBank. Primers used for APP were 5'-GGACTBACACACGACCGTTCTG-3' and 5'-GCATCTACTTGTGGTACTGCACAGC-3'. We also wanted to clone turtle  $\beta$ -actin to use as a control probe for RNA loading. The  $\beta$ -actin primers used were based on conserved regions in the mouse sequence and were 5'-TTTGATGTCACG-CACGATTTCC-3' and 5'-TGTGATG-TGGGAATGGGTACG-3'. One hundred nanograms of hypothalamic poly (A)<sup>+</sup> RNA was reverse transcribed into cDNA using oligo(dT) 12–18 primer (0.5 mg/mL) and SuperScript II RNase H-Reverse Transcriptase (Gibco/BRL). We PCR-amplified 2  $\mu$ L cDNA (30 cycles) in the presence of either APP primers with 58°C annealing temperature or  $\beta$ -actin primers with 48°C annealing temperature. Amplified products of the expected size were ligated

into a pCR2.1 vector, cloned, sequenced, and identified as described above.

**Northern blotting.** Total RNA and protein was extracted from hypothalami using the Trizol reagent (Gibco/BRL). Total RNA (12 µg) was fractionated on 1% agarose/formaldehyde gels, transferred onto Hybond-N+ nylon membrane (Amersham, Québec, Canada) by vacuum blotting, and UV-crosslinked for 5 min. Membranes were prehybridized in Rapid-Hyb buffer (Amersham) at 65°C for 30 min and hybridized for 4 hr at 65°C with APP, APLP2, β-actin, or S7 cDNA probes labeled with [ $\alpha^{32}$ ]dCTP using a random priming kit (Amersham). Membranes were washed with 2 × standard sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at room temperature for 30 min and then with 0.2 × SSC, 0.1% SDS at 65°C for 1 hr before autoradiography. Radioactive signals were detected with the BioRad phosphor-imaging system and quantified using Quantity One software (BioRad Laboratories, Hercules, CA, USA). Initially we normalized the data for APLP-2 expression two ways. One fragment isolated from the differential display coded for ribosomal protein S7 and was not regulated by E<sub>2</sub> or OP. We normalized APLP-2 mRNA levels to both the S7 and β-actin signals. The use of β-actin as an internal control is often criticized, and we wanted to ensure that its expression did not change with E<sub>2</sub> or OP treatments.

**Western blotting.** After RNA and protein extraction, the protein pellets were washed with 0.3 M guanidine HCl in 95% ethanol and were redissolved in a solution containing 10 M urea and 50 mM DTT. Protein samples were electrophoretically separated by SDS-PAGE (10% acrylamide/0.35% bis-acrylamide; 100 V for 2 hr). The resolved proteins were transferred at 100 V for 90 min to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA) in transfer buffer [24.8 mM Tris, 192 mM glycine, 20% (V/V) methanol and 0.038% SDS, pH 9.2] using the BioRad Trans-Blot cell apparatus. The membrane was blocked with 5% skim milk in TBS-T (Tris-buffered saline with 0.5%

Tween 20) for 1 hr, and then incubated with antimouse APLP2 antibody D211 (31) 1:2,500 dilution in 5% skim milk, TBS-T) or anti-mouse neuron-specific β-tubulin monoclonal antibody Tuj1 (32) (1:25 dilution in 5% skim milk, TBS-T) at room temperature for 3 hr. The blots were washed for 5 × 5 min with TBS-T and then incubated with secondary antibody HRP-linked protein A (Sigma, St. Louis, MO, USA) for 1 hr in 1:3,000 dilution in 5% skim milk TBS-T. After extensive washing, ECL detection reagents (Amersham) were used to detect the immunoreactive protein. The chemiluminescence signals were captured using the Bio-Rad Chemi-Doc system and quantified using Quantity One software. All data were normalized to the β-tubulin signal because its expression did not change with E<sub>2</sub> or OP treatments. We also attempted to detect APP proteins in the turtle hypothalamic extracts. Monoclonal antibodies 4G8 and 6E10 against human APP fragments (Senetek PLC, Napa, CA, USA) did not cross-react, so we are not able to determine the levels of APP products in the turtle brain at this time.

**Statistical analyses.** We analyzed the effects of 17-day exposure to E<sub>2</sub> and OP on APLP-2 mRNA and protein levels using the one-way analysis of variance followed by Scheffé's test. We analyzed the effects of 35-day exposure to OP on APLP-2 and APP levels using a *t*-test. Both statistical tests were performed using the GB-Stat software for Windows (Dynamic Microsystems, Inc., Silver Spring, MD, USA).

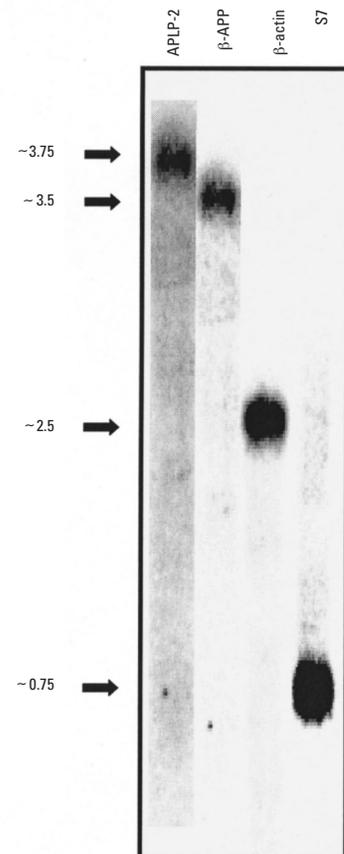
## Results

**RAP-PCR and gene cloning.** We used the RAP-PCR differential display strategy to identify candidate E<sub>2</sub> or OP-regulated transcripts in the snapping turtle hypothalamus. We decided to first treat animals by injection to have precise control of the amount of E<sub>2</sub> or OP received by each animal. As well, we chose to look for transcripts that may be regulated within 24 hr of exposure. A series of transcripts appeared to be regulated by either E<sub>2</sub> or OP. Sequence analysis of the RAP-PCR cDNA fragments revealed that a highly

divergent set of genes may be regulated (Table 1). Two novel nonhomologous transcripts, which we have named octylphenol-reponsive gene-1 (OPRG-1) and OPRG-2, did not have significant similarities to any Genbank entries and were upregulated by OP injection as determined using RAP-PCR. Cytochrome C oxidase subunit 1 appeared to be upregulated by E<sub>2</sub> injection. In contrast, a sequence with low identity to a novel homeobox gene ANF-1 and a second a transcript with similarities to a human brain dynein β chain, the KIAA0944 protein, both appeared to be downregulated by E<sub>2</sub>. Another transcript was a fragment of the turtle γ-amino-butyric acid (GABA) transporter-3 (GAT-3) homolog, which was downregulated by injection of both E<sub>2</sub> and OP. Of particular interest was the observation that an APLP-2-related product, having, respectively, 95% and 93% identity at the amino acid level with rat and human APLP-2, was downregulated by E<sub>2</sub> injection. Given the high similarity of turtle APLP-2 to human APLP-2 and that APLP-2 is implicated in neuronal differentiation (31) and the etiology of Alzheimer disease in

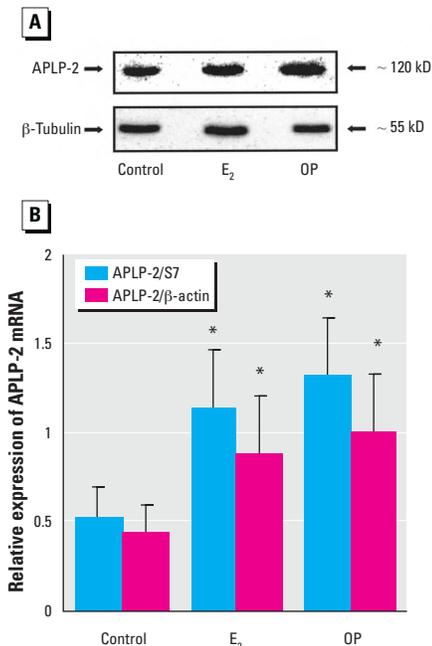
**Table 1.** Genes regulated by 10 ng/g E<sub>2</sub> or OP 24 hr after subcutaneous injection in the yearling snapping turtle hypothalamus as determined using the RNA-arbitrarily primed PCR (RAP-PCR) differential display strategy.

| RAP-PCR results                        | Transcript obtained (accession no.)              | Fragment length (bp) | Percent amino acid identity (species, accession no.) |
|--|--|----------------------|--|
| Upregulated by OP                      | Octylphenol-responsive gene-1 (AF469178)         | 560                  | Novel, no known similarities                         |
|  | OPRG-2 (AF469179)                                | 560                  | Novel, no known similarities                         |
| Upregulated by E <sub>2</sub>          | Cytochrome C oxidase subunit-1 (AF469183)        | 348                  | 72 (goldfish, D1032194)                              |
|  | Putative dynein chain                            | 422                  | 84 CAC21651.1  |
|  | APLP-2 (AF469181)                                | 422                  | 95 (rat, P15943)                                     |
| Downregulated by E <sub>2</sub> and OP | GABA Transporter-3 (AF469182)                    | 321                  | 91 (human, P48066)                                   |
| Not affected by E <sub>2</sub> or OP   | Ribosomal protein S7 (AF469180)                  | 482                  | 98 (human, NP001002)                                 |
|  | H <sup>+</sup> -ATPase subunit (VATB) (AF469184) | 366                  | 81 (chicken, V61724.1*)                              |
|  | PEX11-alpha (AF469186)                           | 368                  | 55 (human, NP003838.1)                               |

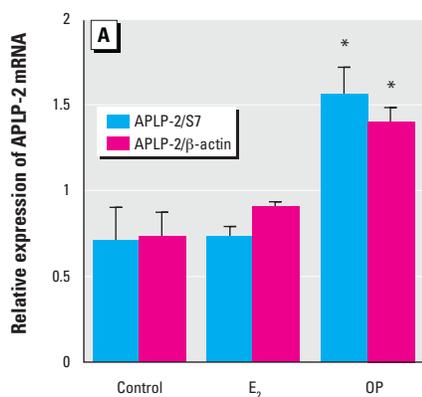


**Figure 1.** Northern blot showing sizes (kilobases) of APLP-2, β-APP, β-actin, and S7 transcripts. Twelve micrograms total hypothalamic RNA was loaded and membranes probed separately for the four transcripts.

aging individuals (33), we decided to characterize further the effects of environmental exposure to both E<sub>2</sub> and OP. Additionally, the expression of three of the transcripts we sequenced did not change after 4-OP or E<sub>2</sub> injection (Table 1). One transcript coding for the ribosomal protein S7 was



**Figure 2.** (A) An example of a Western blot showing the effects of 17 days of exposure to waterborne E<sub>2</sub> and OP on APLP-2 protein levels in yearling snapping turtle hypothalamus. Also shown are the levels of  $\beta$ -tubulin. (B) The effects of 17 days of exposure to waterborne E<sub>2</sub> and OP on APLP-2 mRNA levels in yearling snapping turtle hypothalamus. APLP-2 mRNA levels were expressed relative to both  $\beta$ -actin and ribosomal protein S7. Data are the means  $\pm$  SEM ( $n = 3$  pools of hypothalamic tissue from 3–4 turtles per pool). \* $p < 0.05$  versus control.



subsequently studied. We compared the expression of APLP-2 relative to both S7 and  $\beta$ -actin. This was necessary to confirm that S7 was not regulated and that  $\beta$ -actin was an appropriate control for RNA loading on Northern blot gels.

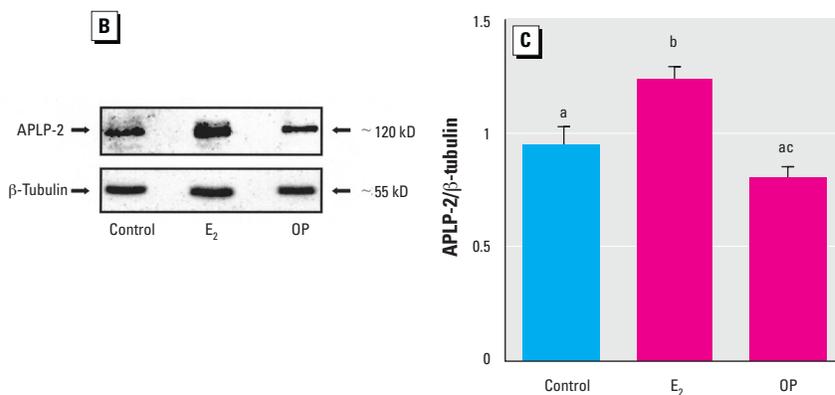
Using RT-PCR, we also cloned cDNA fragments of the turtle homologs of APP (474 bp) and  $\beta$ -actin (514 bp). The turtle  $\beta$ -APP fragment was 88% identical to human APP, whereas the turtle  $\beta$ -actin fragment was 100% identical to human  $\beta$ -actin at the predicted amino acid level.

*The effects of 17-day exposures to waterborne E<sub>2</sub> and OP.* Yearling turtles were exposed for 17 days to 10 ng/mL waterborne E<sub>2</sub> or OP. Single transcripts for APLP-2 (~3.75 kb), APP (~3.5 kb),  $\beta$ -actin (~2.1 kb), and S7 (~0.75 kb) were visualized using Northern blots (Figure 1). S7 and  $\beta$ -actin levels did not change relative to each other following exposures (data not shown). This was important to establish because it confirmed our RAP-PCR results for S7 and validated the use of  $\beta$ -actin as a control for RNA loading. We then expressed APLP-2 levels relative to both control transcripts. Regardless of whether APLP-2 levels were expressed relative to the S7 or  $\beta$ -actin, both E<sub>2</sub> and OP increased APLP-2 expression approximately 2-fold (Figure 2A). The effect of the 17-day exposure to waterborne E<sub>2</sub> to increase APLP-2 mRNA levels is in contrast to the original RAP-PCR experiment, where a single intraperitoneal injection of E<sub>2</sub> decreased APLP-2 gene expression after 24 hr (Table 1). Western blot analysis for the APLP-2 protein in the yearling hypothalamus indicated the presence of a single band of an approximate molecular weight of 120 kD (Figure 2B). The  $\beta$ -tubulin protein was highly expressed in the hypothalamus, and a

single band of about 55 kD was detected with the Tuj1 antibody. We measured the levels of APLP-2 protein in two independent hypothalamic samples of yearling male turtles exposed to E<sub>2</sub> or OP. In the hypothalamus of yearling turtles, E<sub>2</sub> increased APLP-2 protein levels by an average of about 28% compared to control values. Similarly, APLP-2 protein levels were about 46% higher in OP-treated animals compared to control values.

In a separate experiment, month-old hatchling male turtles were also exposed to 10 ng/mL OP and E<sub>2</sub>. In contrast to the results in the older males, the month-old animals did not respond to E<sub>2</sub> with an increase in hypothalamic APLP-2 mRNA. However, OP again effectively increased hypothalamic APLP-2 mRNA levels approximately 2-fold (Figure 3A). As with the yearling turtles, identical results were obtained regardless of whether we expressed APLP-2 mRNA levels relative to S7 or  $\beta$ -actin. Western blot analysis for the APLP-2 protein in the hypothalamus of month-old turtles indicated the presence of a single 120-kD band, as in the yearling hypothalamus (Figure 3B). In the hypothalamus of month-old turtles, E<sub>2</sub> increased ( $p < 0.05$ ) APLP-2 protein levels by about 30% compared to control values. In contrast, APLP-2 protein levels were similar ( $p > 0.05$ ) in OP-exposed and control animals (Figure 3C).

*Effects of 35-day exposures to waterborne OP.* A preliminary study (data not shown) suggested that hypothalamic APP mRNA levels may also be increased by 17-day exposures to waterborne OP. This increase, however, was not statistically significant. We reasoned that perhaps a longer exposure may be needed to alter APP mRNA expression. Therefore, animals were exposed for 35 days to 10 ng/mL OP and APLP-2 and  $\beta$ -APP



**Figure 3.** (A) The effects of 17 days of exposure to waterborne E<sub>2</sub> and OP on APLP-2 mRNA levels in month-old snapping turtle hypothalamus. APLP-2 mRNA levels were expressed relative to both  $\beta$ -actin and ribosomal protein S7. Data are the means  $\pm$  SEM ( $n = 4$  pools of hypothalamic tissue from 3–4 turtles per pool). (B) An example of a Western blot showing the effects of 17 days of exposure to waterborne E<sub>2</sub> and OP on APLP-2 protein levels in month-old snapping turtle hypothalamus. Also shown are the levels of  $\beta$ -tubulin. (C) The effects of 17 days of exposure to waterborne E<sub>2</sub> and OP on APLP-2 protein levels in month-old snapping turtle hypothalamus. APLP-2 levels were expressed relative to  $\beta$ -tubulin. Data are the means  $\pm$  SEM ( $n = 4$  pools of hypothalamic tissue from 3–4 turtles per pool). Means with different superscripted letters are significantly different ( $p < 0.05$ ).

\* $p < 0.05$  versus control.

mRNA levels determined by Northern blot. In contrast to the 17-day exposure, hypothalamic APLP-2 mRNA levels were not statistically different ( $p > 0.05$ ) in control animals compared to those exposed to OP for 35 days (Figure 4A). APLP-2 protein levels were also similar in the two groups. However, 35 days of exposure to OP increased APP mRNA levels by 42% ( $p < 0.05$ ), confirming our hypothesis that a longer period of exposure was needed to alter APP expression (Figure 4B).

## Discussion

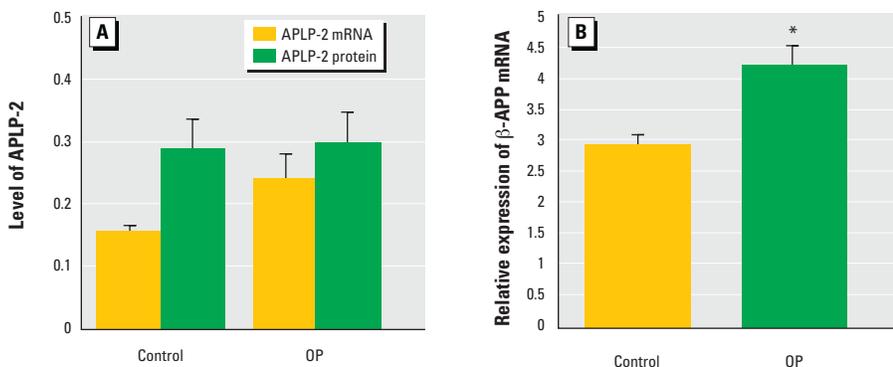
We demonstrated for the first time that  $E_2$  and OP affect gene expression in the snapping turtle hypothalamus. OP exposure for 17–35 days upregulated the expression of APLP-2 and APP genes in the amyloid precursor protein molecular family having important roles in brain development. While it has been shown in several systems that OP at a range of environmental and pharmacologic dose is biologically active, previous studies have examined effects only on peripheral organ systems. For example, it is well known that OP and related alkylphenolic metabolites induced an abnormal production of vitellogenin in male fish. This hepatic phosphoprotein is normally secreted to the blood and subsequently incorporated into egg yolk in the ovaries of female fish and other nonmammalian vertebrates (2). By using a differential display strategy, the study presented here showed that OP can affect the central nervous system as well. Because the hypothalamus is a major site for estrogen action in the brain and because it is the integrative control center of reproduction, growth, metabolism, and osmoregulation, a pollutant affecting hypothalamic function may disrupt one or several of these physiologic processes.

The major goal of our strategy was to identify novel candidate targets for OP or  $E_2$  action, and our focus has been on one of the RAP-PCR products, APLP-2, which is a member of a family of highly homologous amyloid precursor-like proteins, including APP and APLP-1. We cloned APP from the turtle hypothalamus using a degenerate PCR primer strategy. We focused on APLP-2 and APP because they are integral membrane glycoproteins that have important roles in neuronal development. APLPs undergo unusual post-translational processing to produce secretable bioactive fragments. In the case of APP, the C-terminal fragment  $A\beta$ , of approximately 4 kD, can have both neurotrophic or neurodegenerative properties, depending on  $\alpha$ - and  $\beta$ -secretase-mediated post-translational processing and the resultant peptide fragment length. Whereas the 40 amino acid  $A\beta$  fragment is not neurotoxic, C-terminal extension of the peptide by two or three amino acids produces the amyloidogenic peptides  $A\beta$ -42 and  $A\beta$ -43, which form neurodegenerative plaques in the brains of Alzheimer disease and Down syndrome patients (34,35). Although processing of the C-terminal of APLP-2 does not yield the  $A\beta$  peptide, the expression of APLP-2 in brains of Alzheimer disease patients is nevertheless increased in a subset of neuritic plaques in the neocortex and hippocampal formation that do not contain  $A\beta$  peptides (33). The predicted amino acid sequence of the turtle  $A\beta$  fragment is 100% identical with human and other mammalian forms. It is 89% identical to *Xenopus*  $A\beta$  and 80–85% identical with known fish  $A\beta$  fragments. This high similarity of the  $A\beta$  region of APP in the major vertebrate classes emphasizes the importance of this bioactive peptide for nervous system function.

As demonstrated mainly by *in vitro* cell culture experiments, APP modulates neuronal

excitability, synaptic plasticity, neurite outgrowth, synaptogenesis, and cell survival (36). It has been suggested that APLP-2 may also be involved in similar processes—for example, axogenesis, neuronal pathfinding, or synaptogenesis (37). To examine whether APLP-2 and APP serve similar roles *in vivo*, single and double gene knockout (KO) mice have been generated. Mice with targeted disruption of the APP gene have 15–20% reduced body weight, decreased locomotor activity, and some degree of hippocampal gliosis (37). The APLP-2 KO mouse has a very mild phenotype, suggesting that highly homologous members of the APLP family can functionally substitute for each other. Results from more recent experiments where combined gene knockouts for APP, APLP-1, and APLP-2 were generated confirm a high level of functional redundancy in the amyloid family (30). Double KO mice that do not express either APP or APLP-2 exhibit significantly impaired development (29). Approximately 80% of these animals die in the first week after birth, suggesting important roles for these proteins in early postnatal development. Despite normal testicular and ovarian development, these animals mate poorly. Moreover, given that recombinant human APP and APLP-2 have similar neurotrophic actions on chick sympathetic neurons (38), it was important for us to evaluate the effects of OP on both APP and APLP-2.

In our preliminary RAP-PCR assay, we found that APLP-2 gene expression was altered 24 hr after injection of  $E_2$ . We therefore considered that APLP-2 was a likely estrogen-responsive transcript that may also respond to OP. We designed experiments to determine the effects of waterborne OP on APLP-2 and APP mRNA levels in the snapping turtle hypothalamus. We chose an environmentally relevant nominal concentration of 10 ng/mL OP. This represents an intermediate dose between the range of concentrations found in water and contaminated sediments in the Great Lakes–St. Lawrence basin and is similar to levels found in some UK water systems. In yearling males, both  $E_2$  and OP increased the expression of APLP-2, suggesting that OP has estrogenlike effects. On the other hand, in month-old animals,  $E_2$  was without effect, yet OP exposure for 17 days consistently increased hypothalamic APLP-2 mRNA levels approximately 2-fold. Thus,  $E_2$  and OP had similar effects on APLP-2 mRNA levels in yearlings, whereas  $E_2$  was less active in the younger animals. This suggests that there may be developmental differences in the APLP-2 mRNA response to  $E_2$ . Studies on the sex-reversing effects of estrogens in turtles (13,39) and in other animals where steroid treatments modify sex determination (8) also



**Figure 4.** (A) The effects of 35 days of exposure to waterborne OP on APLP-2 mRNA and APLP-2 protein levels in month-old snapping turtle hypothalamus. APLP-2 mRNA levels were expressed relative to  $\beta$ -actin, and APLP-2 protein levels were expressed relative to  $\beta$ -tubulin. Data are the means  $\pm$  SEM ( $n = 4$  pools of hypothalamic tissue from 3–4 turtles per pool). (B) The effects of 35 days of exposure to waterborne OP on APP mRNA levels in month-old snapping turtle hypothalamus. APP levels were expressed relative to  $\beta$ -actin. Data are the means  $\pm$  SEM ( $n = 4$  pools of hypothalamic tissue from 3–4 turtles per pool).

\* $p < 0.05$  versus control.

indicate that there are steroid-dependent and steroid-insensitive periods in development.

Using a specific antibody to APLP-2, we were able to detect a single 120-kD APLP-2 gene product in the snapping turtle hypothalamus. In the yearlings, both E<sub>2</sub> and OP increased APLP-2 protein levels in parallel with increased APLP-2 mRNA levels. In month-old males, E<sub>2</sub> induced the production of the APLP-2 protein, whereas OP was without effect. This is in contrast to the effects that E<sub>2</sub> and OP had on APLP-2 mRNA levels, suggesting that these substances may have different effects on transcription versus translation of the APLP-2 gene. For example, OP increased APLP-2 mRNA levels but did not affect protein levels, suggesting different sensitivities of the transcription and translation processes to OP. Differential effectiveness of OP to mimic the effects of E<sub>2</sub> have been reported in other systems (7). For example, E<sub>2</sub> had approximately 1,000-fold higher affinity for the trout estrogen receptor, and E<sub>2</sub> was also 1,000-fold more active at inducing vitellogenin production in trout hepatocytes *in vitro*. On the other hand, E<sub>2</sub> was only 100-fold more active than OP in stimulating human MCF-7 breast cancer cell proliferation. Moreover, mutational analysis of the mouse estrogen receptor- $\alpha$  indicated that OP activated transcription *in vitro* by a similar mechanism and to a similar level as E<sub>2</sub> (7).

We isolated several other differentially expressed cDNAs from the snapping turtle hypothalamus (Table 1). We isolated two unidentified transcripts (OPRG1 and OPRG2) potentially regulated by OP. These were cDNA fragments without significant homologies to any GenBank entries and likely represent novel sequences. We also isolated the turtle homolog of cytochrome C oxidase subunit 1, suggesting that E<sub>2</sub> injection may alter hypothalamic metabolism by modulating this important mitochondrial proton-pumping respiratory protein. A sequence with low similarity to a novel homeobox gene ANF-1 in the chicken was also found. Moreover, a transcript with similarities to a human brain dynein  $\beta$  chain, the KIAA0944 protein, may also be regulated by E<sub>2</sub>. Dyneins are major proteins associated with microtubules and vesicular movement. Additionally, a GABA transporter-3 (GAT-3) homolog was downregulated by injection of both E<sub>2</sub> and OP. GABA is the major inhibitory neurotransmitter required for normal development of the embryonic hypothalamus and it plays an important role in reproduction (40,41). GAT-3 removes GABA from the synaptic cleft, and thus rapidly attenuates neurotransmission (42). We predict that changes in turtle GAT-3 following OP would alter many aspects of

neuroendocrine function given the importance of GABA in the control of the release of all the major pituitary hormones (41,43). When using the differential display strategy, it is essential to demonstrate that not all transcripts are altered by a particular treatment. Indeed, not all transcripts we isolated were affected by injection of E<sub>2</sub> and OP. As mentioned previously, ribosomal protein S7 was not regulated, nor was peroxisomal biogenesis factor 11A (PEX11- $\alpha$ ), which is important for peroxisome abundance.

In addition to the results on amyloid proteins, it appears that E<sub>2</sub> and OP may have effects on several uncharacterized systems, metabolic pathways, structural proteins, and GABAergic neurotransmission. There may also be important differences in the effects of E<sub>2</sub> and OP. This was evident in the results for both APLP-2 mRNA and protein. Moreover, the transcripts isolated from hypothalami of the E<sub>2</sub>- and OP-treated animals in the initial RAP-PCR screening were not identical. These results suggest that OP interacts with a different estrogen receptor than does E<sub>2</sub>, or that OP action to alter gene expression in the central nervous system may not be exclusively estrogenic. Nevertheless, altered gene expression patterns in the developing hypothalamus of the snapping turtle demonstrate that OP is bioactive in reptiles. We hypothesize that upsets in early posthatching development of the hypothalamus in turtles or other vertebrates after chronic, low-level exposure to OP or related xenobiotics may have longer-lasting effects.

A major question not addressed by our study concerns the consequences of OP exposure to the health and survival of young turtles. It is tempting to speculate that long-term exposure to OP or other estrogenic compounds may disrupt the normal expression of amyloid proteins, leading to upsets in the functioning of hypothalamic neurons, possibly neurodegeneration. However, we have not yet assessed such possibilities, nor is the role of amyloid proteins in the control hypothalamic function well established. Such experiments will require longer-term studies and the development of antibodies that recognize turtle APP, in addition to that which we have shown for APLP-2 expression. Nevertheless, overexpression of human APP in transgenic mice leads to many of the neuropathologic hallmarks of Alzheimer disease (44). In the case of hatchling snapping turtles, treatments for up to 35 days did not affect body weights or food consumption (data not shown). Given that turtles grow very slowly, we hypothesized that a longer period of observation may reveal effects on growth. In a preliminary study by Brooks et al. (45), animals exposed for 17 days were allowed to grow for 6 months. OP-exposed

animals were approximately 25% larger ( $p < 0.05$ ) than control animals. We do not know how alterations in hypothalamic APLP-2 and APP gene expression relate to changes in growth; however, recent work with double APP/APLP-2 KO mice demonstrate that a lack of these proteins reduces postnatal growth (29). Together, these data suggest that amyloid proteins may have a role in the hypothalamic control of growth.

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