Assessment of Estrogenic Endocrine-Disturbing Chemical Actions in the Brain Using in Vivo Somatic Gene Transfer

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Estrogenic endocrine-disrupting chemicals abnormally stimulate vitellogenin gene expression and production in the liver of many male aquatic vertebrates. However, very few studies demonstrate the effects of estrogenic pollutants on brain function. We have used polyethyleneiminemediated in vivo somatic gene transfer to induce an estrogen response element–thymidine kinase–luciferase (ERE-TK-LUC) construct into the brain. To determine if waterborne estrogenic chemicals modulate gene transcription in the brain, we injected the estrogen-sensitive construct into the brains of Nieuwkoop-Faber stage 54 Xenopus laevis tadpoles. Both ethinylestradiol (EE2; \( p < 0.002 \)) and bisphenol A (BPA; \( p < 0.03 \)) increased luciferase activity by 1.9- and 1.5-fold, respectively. In contrast, low physiologic levels of 17β-estradiol had no effect (\( p > 0.05 \)). The mixed agonist/antagonist tamoxifen was estrogenic in vivo and increased (\( p < 0.003 \)) luciferase activity in the tadpole brain by 2.3-fold. There have been no previous reports of somatic gene transfer to the fish brain; therefore, it was necessary to optimize injection and transfection conditions for the adult goldfish (Carassius auratus). Following third brain ventricle injection of cytomegalovirus (CMV)-green fluorescent protein or CMV-LUC gene constructs, we established that cells in the telencephalon and optic tectum are transfected. Optimal transfections were achieved with 1 µg DNA complexed with 18 nmol 22 kDa polyethyleneimine 4 days after brain injections. Exposure to EE2 increased brain luciferase activity by 2-fold in males (\( p < 0.05 \)) but not in females. Activation of an ERE-dependent luciferase reporter gene in both tadpole and fish indicates that waterborne estrogens can directly modulate transcription of estrogen-responsive genes in the brain. We provide a method adaptable to aquatic organisms to study the direct regulation of estrogen-responsive genes in vivo. Key words: bisphenol A, brain, estrogen response element, ethinylestradiol, goldfish, somatic gene transfer, Xenopus laevis. Environ Health Perspect 113:329–334 (2005). doi:10.1289/ehp.7418 available via http://dx.doi.org/[Online 2 December 2004]

In both female and male vertebrates, estrogens affect many aspects of development, growth, sexual differentiation, and reproductive behavior. Estrogens also exert positive and negative feedback effects on the hypothalamopituitary axis to regulate the secretion of gonadotropic hormones, which in turn induce development of secondary sex characteristics in fish (Arcand-Hoy and Benson 1998; Bogi et al. 2002; Mackenzie et al. 2003). The diversity of structure and origin of the multitude of compounds currently known to bind to estrogen receptors (ER-α and ER-β) makes it difficult to predict activities in vivo in vertebrate animals (Sanchez et al. 2002; Segner et al. 2003; Yoon et al. 2001). Large-scale screening for estrogenic activities by traditional physiologic and toxicologic methods is time-consuming and costly. A variety of effective in vitro ER binding assays and estrogen-responsive reporter systems in bacterial, yeast, and vertebrate cell systems have defined much of our understanding of estrogen and EDC actions (Ackermann et al. 2002; Matthews et al. 2002; Metivier et al. 2001, 2003; Petit et al. 1997; Zacharewski 1997). However, results derived in vitro for ER binding, hepatocyte vitellogenin induction, or ER reporter gene assays often do not always accurately reflect results obtained in vivo (Andersen et al. 1999; Segner et al. 2003). When E2 or estrogenic mimics bind to ERs, receptor dimerization and recruitment of transcriptional modulators are initiated, and the
hormone–receptor complex binds to the estrogen response element (ERE) and subsequently regulates transcription in an ordered and cyclic manner (Metivier et al. 2003; Robinson-Rechavi et al. 2003). Some of the discrepancies between in vitro assays and in vivo physiologic experiments may reflect the observations that ERα and ERβ differ dramatically in tissue and cellular distributions, biologic function (Abraham et al. 2004; Hess 2003; Korach et al. 2003), and their affinities for estrogenic chemicals (Le Guevel and Pakdel 2001; Yoon et al. 2001). Moreover, the likelihood that the availability of transcriptional modulators of the ERs in vitro and in vivo is similar is highly unlikely (Graham et al. 2000), and thus, in vitro models cannot mimic the complexities of whole animal systems with respect to estrogen-dependent processes and responses to EDCs.

To begin to overcome some of the challenges of in vivo assessment of EDC modulation of gene transcription, we have validated polyethylenimine (PEI)-mediated somatic gene transfer (Lemkine and Demeneix 2001; Ouatas et al. 1998) to introduce an estrogen gene transfer (Ouatas et al. 1998) and subsequently returned to the larval brain. In all cases, we report nominal water concentrations of estrogenic chemicals. In experiment 1, tadpoles were preexposed for 48 hr to 0.5 nM EE2, 5 nM E2, 50 nM BPA (bisphenol A methylacrylate; Sigma, St. Louis, MO, USA), or ethanol vehicle (0.4 mL in 4 L water in 10-L glass tanks; 20–22°C). In experiment 2, tadpoles were similarly preexposed to 200 nM tamoxifen (TAM; Sigma), a mixed ER antagonist/agonist. After the preexposure period, tadpoles were injected with ERE-TK-LUC (200 ng in 1 µL) complexed with 6 equivalents (eq) of 22 kDa polyethylenimine (PEI; Euromedex, Souffelweyersheim, France) in a 5% glucose solution into the brain as previously described (Ouatas et al. 1998) and returned to clean water freshly treated with estrogenic chemicals and exposed a further 48 hr. Animals were then sacrificed and whole brains dissected for determination of total luciferase activities.

Development of a somatic gene transfer method for the goldfish brain. All fish were purchased from a local supplier (Paris, France) and maintained at 20–22°C. Adult male and female goldfish were used to optimize in vivo transfer methods and to determine if waterborne estrogenic chemicals activate ERE-TK-LUC injected into the larval brain. In all cases, we report nominal water concentrations of estrogenic chemicals. In experiment 1, tadpoles were preexposed for 48 hr to 0.5 nM EE2, 5 nM E2, 50 nM BPA (bisphenol A methylacrylate; Sigma, St. Louis, MO, USA), or ethanol vehicle (0.4 mL in 4 L water in 10-L glass tanks; 20–22°C). In experiment 2, tadpoles were similarly preexposed to 200 nM tamoxifen (TAM; Sigma), a mixed ER antagonist/agonist. After the preexposure period, tadpoles were injected with ERE-TK-LUC (200 ng in 1 µL) complexed with 6 equivalents (eq) of 22 kDa polyethylenimine (PEI; Euromedex, Souffelweyersheim, France) in a 5% glucose solution into the brain as previously described (Ouatas et al. 1998) and returned to clean water freshly treated with estrogenic chemicals and exposed a further 48 hr. Animals were then sacrificed and whole brains dissected for determination of total luciferase activities.

Materials and Methods

Plasmid constructs. We used a consensus ERE with a minimal thymidine kinase promoter driving firefly luciferase activity (ERE-TK-LUC) as described previously (Metivier et al. 2001). This ERE reporter system is well characterized in vitro in several cell lines (Ackermann et al. 2002; Metivier et al. 2001) and responds to both zebrafish (Menuet et al. 2002) and goldfish ER-α and ER-β subtypes (Marlatt V, Trudeau VL, Moon TW, unpublished data). cytomegalovirus (CMV)-luciferase (CMV-LUC) and CMV-green fluorescent protein (CMV-GFP) were from Vical Inc. (San Diego, CA, USA) and Invitrogen (Carlsbad, CA, USA), respectively.

Luciferase activity. Brains from luciferase-transfected X. laevis tadpoles or goldfish were dissected and frozen in liquid nitrogen and stored at −80°C until assayed for luciferase activity [relative light units (RLUs)] according to the manufacturer’s instructions (Promega, Charbonnieres, France). Frozen brains were sonicated in ice-cold luciferase lysis buffer (200 µL for tadpoles, 500 µL for goldfish) and then centrifuged 10 min at 12,000g (4°C) to precipitate nonsoluble particles and proteins. Twenty microliters of the supernatant was mixed by vortexing with 100 µL luciferase substrate and counted immediately (10 sec) using a single-well luminometer as previously reported (Ouatas et al. 1998).

Assessment of ERE-TK-LUC activity in the brains of X. laevis tadpoles. Previous data have demonstrated that somatic gene transfer is an effective method to study thyroid hormone (TH) responses in the X. laevis tadpole (Ouatas et al. 1998). To avoid possible TH–E2 interactions in the brain (Dellovode et al. 1999), we used Nieuwenkoop-Faber (NF) stage 54 X. laevis tadpoles (Nieuwenkoop and Faber 1967) in which TH synthesis was inhibited by 1 g/L sodium perchlorate to determine whether waterborne estrogenic chemicals activate ERE-TK-LUC injected into the larval brain. In all cases, we report nominal water concentrations of estrogenic chemicals. In experiment 1, tadpoles were preexposed for 48 hr to 0.5 nM EE2, 5 nM E2, 50 nM BPA (bisphenol A methylacrylate; Sigma, St. Louis, MO, USA), or ethanol vehicle (0.4 mL in 4 L water in 10-L glass tanks; 20–22°C). In experiment 2, tadpoles were similarly preexposed to 200 nM tamoxifen (TAM; Sigma), a mixed ER antagonist/agonist. After the preexposure period, tadpoles were injected with ERE-TK-LUC (200 ng in 1 µL) complexed with 6 equivalents (eq) of 22 kDa polyethylenimine (PEI; Euromedex, Souffelweyersheim, France) in a 5% glucose solution into the brain as previously described (Ouatas et al. 1998) and returned to clean water freshly treated with estrogenic chemicals and exposed a further 48 hr. Animals were then sacrificed and whole brains dissected for determination of total luciferase activities.

Effects of estrogenic chemicals on ERE-TK-LUC in goldfish brain. For this experiment, we used small goldfish of both sexes (in 50–70 L glass tanks). Because these animals were in the early stages of seasonal gonadal redevelopment and could not be...
distinguished by external secondary sex characteristics, they were randomly assigned to each of the treatment groups. To determine whether waterborne estrogenic chemicals activate ERE-TK-LUC injected into the adult brain, groups of animals were preexposed for 48 hr to 10 nM E2, 10 nM EE2, or ethanol vehicle (0.1 mL/L water). After the preexposure period, ERE-TK-LUC was injected as described above, and the fish were returned to water freshly treated with estrogenic chemicals and exposed a further 48 hr, at which time the water was changed again. The injected ERE-TK-LUC (1 µg DNA in 4 µL) was complexed with 6 eq of 22 kDa PEI in a 5% glucose solution. Whole brains were dissected at 96 hr after injection. Injections, exposures, and dissections were randomized over 3 days. At the time of dissection, body weights and sex of the animals were recorded.

**Statistical analysis.** The levels of luciferase activity (RLU) per whole X. laevis tadpole brain are expressed relative to mean expression levels per experiment (i.e., for the corrected RLU the mean equals 1). Goldfish injected with the ERE-TK-LUC construct varied in size (3–10 g), and therefore an additional correction was made based on milligrams of brain tissue. Activities are expressed relative to mean expression weights and sex of the animals were recorded.

**Results**

**Effects of estrogenic chemicals on ERE-TK-LUC activity in the brains of X. laevis tadpoles.** Figure 1A shows the effects of exposure to E2 (5 nM), EE2 (0.5 nM), and BPA (50 nM) on luciferase expression in the brains of ERE-TK-LUC–injected tadpoles. In this experiment the average activity (1 corrected RLU unit) represents approximately 73,000 RLU/brain. All data are expressed relative to this average value. Statistical analysis (ANOVA or Student’s t-test) as appropriate (SigmaStat, version 2.03; SPSS Inc., Chicago, IL, USA). The effects of the various treatments on luciferase activity in the brains of ERE-TK-LUC–injected tadpoles; data are presented as mean ± SEM. *p < 0.03, **p < 0.002, and #p < 0.003 compared with ethanol controls.

Figure 1. Effects of estrogenic chemicals on ERE-TK-LUC activity in the brains of perchlorate-treated NF stage 54 X. laevis tadpoles. (A) Expression of GFP in the telencephalon (TEL) and optic tectum (OT) of freshly dissected intact brain. Note the high expression around the brain third ventricle (V3); bar = 100 µm. (B) Sagittal section (25 µm) through the telencephalon of a goldfish showing a neuron extending dorsolaterally. The cell body (not easily visualized) is toward the top left corner; bar = 5 µm.

Time-course analysis (Figure 3B) was performed. The highest luciferase expression was 96 hr after brain injection.

**Effects of estrogenic chemicals on ERE-TK-LUC in goldfish brain.** After having established a method for injection of DNA into the goldfish brain (Figures 2 and 3), we examined the effects of E2, EE2, and BPA in small female and male goldfish. Figure 4 shows the effects of exposure to E2 (10 nM), EE2 (10 nM), and BPA (100 nM) on luciferase expression in the brains of ERE-TK-LUC–injected females and males. In this experiment the average activity (1 corrected unit) represents approximately 15,000 RLU/mg protein. All data are expressed relative to this average value. The effects of the various treatments on luciferase activity were dependent on the sex of the fish (two-way ANOVA: sex × treatment, p < 0.019). Basal luciferase activity was similar in control females and males (p > 0.05). In males treated with E2, mean levels were approximately 1.5-fold higher than in controls; however, this change was not statistically significant (p > 0.05). Additionally, E2 did not affect (p > 0.05) luciferase activity in females. In contrast, waterborne EE2 induced a 2-fold increase (p < 0.05) in luciferase activity in the male brain but had no effect in females (p > 0.05). Moreover, BPA did not affect (p > 0.05) luciferase activity in either sex.
Discussion
Our results indicate that waterborne estrogenic chemicals can modulate brain activity in aquatic vertebrates. Using somatic gene transfer into the brains of tadpoles and adult fish, we showed that estrogenic chemicals activate expression of an introduced ERE-TK-LUC construct. This required adaptation of somatic gene transfer methods previously used in X. laevis (Ouatas et al. 1998) and mice (Guissouma et al. 1998) to study TH-driven gene expression and in Xenopus tropicalis (Rowe et al. 2002) to analyze apoptosis during metamorphosis. To our knowledge, PEI-mediated somatic gene transfer into the fish brain has not been previously reported. Optimal transfections were achieved with 1 µg DNA complexed with 18 nmol 22 kDa PEI 4 days after brain injections. However, longer time periods were not analyzed, and it is possible that expression in the adult goldfish brain would increase after 96 hr.

The potent estrogen from female contraceptives, EE2, and the natural estrogen E2 are found at picomolar to nanomolar concentrations in both European and North American sewage effluents and surface waters (Kolpin et al. 2002; Metcalfe et al. 2003; Ternes et al. 1999). We showed that short-term exposure to 0.5 nM EE2 in tadpoles and 10 nM EE2 in male goldfish increased the activity of a known estrogen-responsive reporter gene construct by approximately 2-fold. In contrast, female goldfish were not responsive to 10 nM waterborne EE2. The plasticizing agent BPA and the mixed ER antagonist/agonist TAM were, however, similar to those obtained with PEI-mediated transfection of hypothalamic neurons of neonatal mice with the same CMV-LUC construct (Guissouma et al. 1998). Our results showed that cells in the adult goldfish forebrain and optic tectum are transfectable in vivo. Autoradiographic (Kim et al. 1978), immunocytochemical (Navas et al. 1995), and in situ hybridization (Menuet et al. 2002) studies showed that both ER-α and ER-β are expressed in the telencephalon and hypothalamus and especially in the preoptic area of fish. Using reverse-transcriptase polymerase chain reaction (RT-PCR), Choi and Habibi (2003) also detected both ERs in goldfish brain. Our results showed that in vivo transfection in the goldfish telencephalon can be used to study the regulation of ERE-driven expression by an estrogenic pollutant.

In both animal models, there was a relatively high basal luciferase activity in controls. This is likely due to two interacting factors: high in vivo activity of the minimal thyminid kinase promoter and effects of endogenous neuroestrogen on basal expression of the ERE-TK-LUC gene construct. In X. laevis tadpoles, estrogen production in the brain has not been studied, but at NF stage 54, whole-body E2 levels are easily detectable despite having declined relative to very high levels in early stages of development (Bogi et al. 2002). Male and female gonads are distinguishable by gross morphologic characteristics at NF stage 56 (Bogi et al. 2002). Therefore, it is likely that our tadpoles were producing endogenous estrogen. Relatively high basal ERE-TK-LUC activity at this stage of tadpole development suggests that ERs are active and/or that endogenous E2 is being produced and delivered to the transfected cells. The goldfish brain has a remarkable capacity to produce E2 from testosterone because of very high aromatase activity (Callard et al. 2001; Pasmanik and Callard 1988). The dose of E2 we used is within the physiologic range and thus would be unlikely to raise brain E2 above endogenous brain E2 concentrations, especially in females. It is known that EE2 is more potent that E2 in several assay systems using the same ERE-TK-LUC reporter gene (Ackermann et al. 2002; Le Guevel and Pakdel 2001). In female goldfish, 10 nM EE2 did not affect luciferase expression, similar to what was observed with E2. This is in contrast to males where EE2 induced a 2-fold increase in activity. We have previously observed marked sex differences in goldfish neuroendocrine responses to sex steroids (Bosma et al. 2001). For example, whereas testosterone inhibited the expression of glucocorticoid dehydrogenases (GADD65 and GADD67) in the telencephalon of sexually mature males, it was without effect in females (Lariviere K, Trudeau VL, unpublished data).

Our results indicate that short-term exposure to environmentally relevant water levels of BPA (50 nM, ~18 µg/L) can activate the ERE-TK-LUC construct in the tadpole brain. In contrast to effects in fish (Metcalfe et al. 2001; Staples et al. 1998), the effects of BPA in amphibians are not well studied. Kloas et al. (1999), using a static renewal exposure protocol, reported that BPA has estrogenic activity at 2.3 µg/L (~6.3 nM) and induces female-biased sex reversal in X. laevis. In a second study, the same researchers found that 100 nM BPA induced female-biased sex reversal in X. laevis (Levy et al. 2004). However, in a flow-through exposure system (Pickford et al. 2003), there were no observable effects of a range of BPA concentrations (0.83–497 µg/L; ~2.3–141 µM) on larval growth, development, or sexual differentiation of X. laevis tadpoles. High, nonenvironmental

![Figure 3](image-url) Optimization of PEI-based gene transfer in the goldfish brain. (A) Comparison of the efficiencies of 22 kDa linear PEI used at different ratios of PEI amines to DNA anions. Animals were injected with CMV-LUC DNA (1 µg in 4 µL) complexed with 0 (n = 9), 3 (n = 10), 6 (n = 10), and 9 (n = 10) eq of PEI; brains were dissected at 48 hr postinjection; and luciferase activity (RLU/mg protein × 10⁻⁴; mean ± SEM) was determined.

![Figure 4](image-url) Effects of estrogenic chemicals on ERE-TK-LUC activity in the brains of male and female goldfish preexposed for 48 hr to E2 (10 nM; n = 14 males and 14 females), EE2 (10 nM; n = 8 males and 8 females), or ethanol vehicle (0.1 mL/L water; n = 8 males and 16 females). Data are presented as mean ± SEM. *p < 0.05 compared with the male control values.
levels of BPA (10–25 µM; 3,644–9,110 µg/L) have both teratogenic and antimitochondrial actions in *X. laevis* (Iwamuro et al. 2003), suggesting interference with the thyroid system. It is difficult at present to reconcile the different conclusions concerning the estrogenicity of BPA in frogs. However, given that BPA is continually being added to aquatic ecosystems through industrial and sewage effluent discharges and activates a known ERE–reporter gene construct in tadpole brain, it is a contaminant of environmental concern.

Activation of an ERE-dependent luciferase reporter gene in both tadpole and fish indicates that waterborne estrogens can directly modulate transcription of estrogen-responsive genes in the brain. Previous work from our laboratory demonstrated that environmentally relevant levels of the estrogenic pollutant octylphenol modulates the expression of multiple hypothalamic genes in frog tadpole brains (Crump et al. 2002) and in hatching snapping turtles (Trudeau et al. 2002). In the latter study, differential display PCR was used, and it is not known if the affected transcripts were directly or indirectly regulated by 4-α-octylphenol or E2. As quantified in these latter studies using reverse Northern blotting, changes in several hypothalamic mRNAs induced by waterborne estrogenic chemicals appear to be tissue specific. The ERE-dependent reporter gene assay, however, has the distinct advantage of being applicable to other tissues or species because the technique is based on the transcriptional activation properties, and tissue distributions. Biol Reprod 66(8):1881–1882.

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