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Gene expression profiling in the neuroendocrine brain of male goldfish (*Carassius auratus*) exposed to 17α-ethinylestradiol

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Martyniuk CJ, Xiong H, Crump K, Chiu S, Sardana R, Nadler A, Gerrie ER, Xia X, Trudeau VL. Gene expression profiling in the neuroendocrine brain of male goldfish (*Carassius auratus*) exposed to 17α-ethinylestradiol. *Physiol Genomics* 27: 328–336, 2006. First published September 5, 2006; doi:10.1152/physiolgenomics.00090.2006.—17-α Ethinylestradiol (EE2), a pharmaceutical estrogen, is detectable in water systems worldwide. Although studies report on the effects of xenoestrogens in tissues such as liver and gonad, few studies to date have investigated the effects of EE2 in the vertebrate brain at a large scale. The purpose of this study was to develop a goldfish brain-enriched cDNA array and use this in conjunction with a mixed tissue carp microarray to study the genomic response to EE2 in the brain. Gonad-intact male goldfish were exposed to nominal concentrations of 0.1 nM (29.6 ng/l) and 1.0 nM (296 ng/l) EE2 for 15 days. Male goldfish treated with the higher dose of EE2 had significantly smaller gonads compared with controls. Males also had a significantly reduced level of circulating testosterone (T) and 17β-estradiol (E2) in both treatment groups. Candidate genes identified by microarray analysis fall into functional categories that include neuropeptides, cell metabolism, and transcription/translation factors. Differentially expressed genes verified by real-time RT-PCR included brain aromatase, secretogranin-III, and interferon-related developmental regulator 1. Our results suggest that the expression of genes in the sexually mature adult brain appears to be resistant to low EE2 exposure but is affected significantly at higher doses of EE2. This study demonstrates that microarray technology is a useful tool to study the effects of endocrine disrupting chemicals on neuroendocrine function and suggest that exposure to EE2 may have significant effects on localized E2 synthesis in the brain by affecting transcription of brain aromatase.

microarray; hypothalamus; aromatase; endocrine disruption

THERE HAS BEEN CONSIDERABLE attention in recent years to the detrimental effects of endocrine disrupting chemicals (EDCs) and pharmaceuticals on wildlife populations (52). The contraceptive estrogen, 17α-ethinylestradiol (EE2), is a pharmaceutical of concern because it is constantly being introduced into the environment (i.e., pseudopersistant). EE2 is detectable in the final effluent of some municipal wastewater treatment plants at concentrations approaching 40 ng/l (~0.2 nM) (44, 56), and a recent study of 139 US rivers reported maximum concentrations of 830 ng/l (~2.8 nM) for EE2 at the point source of sewage effluent (21). High levels of EE2 (>1,000 ng/l) have been shown to induce severe morphological deformities in fish such as skeletal abnormalities (e.g., lordosis and craniofacial) and soft tissue swelling (6). At lower concentrations, EE2 disrupts sex determination at ~15 ng/l (1) and reduces successful reproduction in fish at ~100 ng/l, thereby reducing the lifetime reproductive fitness of an individual (22). It has also been reported that the potency of EE2 in vivo in fish may be up to 30-fold higher than the natural estrogens, 17β-estradiol (E2) and estrone (E1) (47). Thus, EE2 has the potential to disrupt reproductive processes in fish at relatively low concentrations.

In vitro and in vivo assays are now widely used to detect the effects of EDC exposure in aquatic animals. The yolk precursor protein vitellogenin (Vtg) in the liver is commonly used as a sensitive and robust in vivo biomarker for estrogenic exposure in male fish (38, 43). However, data collected from multiple endpoints should be evaluated in order for environmental monitoring programs to be reliable, sensitive, and robust in detecting EDC exposure. Gene expression profiling offers an advantage over more traditional endpoints by screening large numbers of genes simultaneously, and gene arrays for teleost fish have recently been used to study the genomic response in cold acclimation (15, 19), hypoxia (48), and pharmaceuticals of concern, such as chlorpromazine (55). Most data available for the effects of estrogenic compounds on the transcriptome in fish have largely considered a limited number of genes in peripheral tissues such as liver (7, 24). To our knowledge, no studies report on the effects of waterborne EE2 in the brain of a teleost fish.

Here we describe our strategy to construct a goldfish brain-derived cDNA array to study neuroendocrine function in the goldfish. Our goldfish brain cDNA array was produced using suppressive subtractive hybridization and contains ~1,200 partial gene fragments. In addition, we have printed our goldfish cDNAs along with genes isolated from a mixed tissue carp (*Cyprinus carpio* L.) microarray (15). The common carp is a cyprinid fish that is closely related to the goldfish, diverging less than 10 million years ago (58). Rise and colleagues (37) constructed an expressed sequence tag (EST) library derived from a number of salmonid species and tissues and investigated the applicability of using cross-species hybridizations. The authors found that ~76% of ESTs that were aligned between Atlantic salmon (*Salmo salar*) and rainbow trout (*Onchorhynchus mykiss*), two salmonids that diverged from a common ancestor ~8–20 million years ago, had >90% identity at the nucleotide level. Therefore, a cross-hybridization approach is useful for studies involving closely related fish species.

To date, there is limited data on the impact of environmental pharmaceuticals on normal neuroendocrine function in nontarget aquatic organisms. However, this is important considering that coordination of the hypothalamic-pituitary-gonadal axis is critical to sexual differentiation and normal reproductive function. We investigated the effects of waterborne EE2 in the

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neuroendocrine brain of male goldfish. The effects of EE2 were studied because of its high potency, pseudopersistance, and its presence in many water systems. We chose two nominal concentrations of 0.1 nM (29.6 ng/l) and 1.0 nM (296 ng/l) EE2. The low dose was similar to environmental concentrations of EE2, and the higher dose was used to represent the highest amount reported in some river systems in the US (21). We studied the response of the transcriptome to EE2 in the hypothalamus using our goldfish-carp microarray because this tissue is high in brain aromatase (cyp19b) activity in teleosts (33), suggesting that the teleost brain is sensitive to E2 and potentially to xenoestrogens. The expression of a subset of genes was also investigated in the telencephalon because this region of the brain contains the preoptic area in fish and plays a significant role in the central nervous system (CNS) control of reproduction.

MATERIALS AND METHODS

Experimental design and EE2 exposure. Common adult male goldfish (Aleong's International, Mississauga, Ontario, Canada) were purchased in October 2004. Goldfish ranged between 11 and 40 g for absolute body weight. Fish were acclimatized over several months to 18°C under a natural photoperiod and maintained on standard flaked goldfish food. Goldfish were anesthetized using 3-aminobenzoic acid ethyl ester for all handling and dissection procedures. Care was taken to standardize all handling, injections, and sample protocols. In March 2005, gonad-intact male goldfish were separated into 15 fish per 70-liter tank and exposed to nominal concentrations of 0.1 nM EE2 and 1.0 nM EE2 for 15 days. EE2 was dissolved in 95% ethanol and added to treatment tanks every 3 days (static incubation; final ethanol concentration was <0.001% volume of tank). All control tanks received an equal amount of ethanol throughout the experiment. At the end of every third day, fish were removed from tanks and placed into new tanks with a fresh dose of EE2. After treatment, a subset of goldfish was weighed, and a blood sample taken by puncture of the caudal vasculature via a 25-gauge needle attached to a 1-ml syringe. Serum was collected by spinning the blood at 7,500 rpm at 4°C for 15 min. Serum was stored at −20°C until used for the radioimmunoassay (RIA). Gonad weight was also recorded for the aforementioned subset of fish. The hypothalamus and telencephalon for all fish were rapidly dissected, pooled (2–3 tissues/tube) and stored at −80°C until total RNA extraction.

Testosterone (T) and E2 RIA. Steroids were extracted and measured following McMaster et al. (30). Percent cross reaction of antigen with the steroid of interest was >99% (Medicorp, QC, Canada). Unpaired Student's *t*-test followed by Dunn's multiple-comparisons test was used to test for significant differences between the control and the treatment group.

Subtracted brain goldfish cDNA library. To produce our brain enriched cDNA array, we extracted total RNA from the hypothalamus, telencephalon, and muscle of 10 (5 males and 5 females) goldfish using the RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada) following the manufacturer's protocol. mRNA was isolated from total RNA using Oligotex mRNA Mini Kit (Qiagen). To generate a subtracted library for genes expressed in the hypothalamus and telencephalon, PCR-select cDNA Subtraction kit (BD Clonetech, Mississauga, Ontario, Canada) was used. Hypothalamus and telencephalon mRNA were used as the "tester" and muscle mRNA as the "driver". mRNA (2 μg) from tester and driver was heated with 10 mM cDNA synthesis primer (final volume of 5 μl) to 70°C for 2 min and then cooled on ice for 2 min. Each reaction mixture was made up to 10 μl by adding 2 μl 5× first-strand buffer, 1 μl dNTP (10 mM each), 1 μl sterile water, and 1 μl avian myeloblastosis virus reverse transcriptase. The reaction mixtures were incubated at 42°C for 1.5 h in an air incubator. Following first-strand synthesis, second-strand synthesis was performed by adding 48.4 μl sterile water, 16 μl 5× second-strand buffer, 1.6 μl dNTP (10 mM), and 4 μl 20× second-strand enzyme cocktail. Reactions were incubated at 16°C for 2 h. The double-stranded cDNA was blunted by the addition of 2 μl T4 DNA polymerase, followed by incubation at 16°C for 30 min before the reaction was terminated by the addition of 4 μl 20× EDTA/glycogen mix. Double-stranded cDNA was purified by phenol-chloroform extraction. Double-stranded cDNA from the tester and driver were separately digested with *Rsa*I at 37°C for 1.5 h. The digested fragments were purified by phenol-chloroform extraction. cDNA fragments from the tester were linked to *adaptor 1* and *adaptor 2* by T4 ligase. For the first hybridization, cDNA from tester with *adaptor 1* and *adaptor 2* was mixed with driver cDNA separately and hybridized at 68°C for ~8 h. Additional fresh driver was added directly to the pooled mix of the two previous hybridizations and incubated at 68°C for 20 h for the second hybridization.

Differentially expressed cDNA were selectively amplified during the following two PCR reactions. In the first amplification, only double-stranded cDNA with different adaptor sequences on each end are amplified with PCR primer (5′-CTAATACGACTCACTAT-AGGGC-3′). In the second amplification, nested PCR was used to further reduce background and enrich for differentially expressed sequences with nested primers (5′-TCGAGCGGCCGCCCGGGC-AGGT-3′ and 5′-AGCGTGGTCGCGGCCGAGGT-3′). The nested PCR cDNA mix was further incubated at 72°C for 1 h with additional dATP Taq DNA polymerase to ensure that most of the cDNA fragments contained A overhangs. Approximately 100 ng of PCR cDNA were ligated into 50 ng of pCRII-TOPO vector and transformed in *Escherichia coli* competent cells using the TA-cloning kit (Invitrogen Life Technologies, Carlsbad, CA).

Goldfish brain cDNA array. Approximately 1,100 positive white colonies were selected at random and cultured overnight at 37°C in 3 ml of Luria-Bertani medium for storage. In addition to the random selection of clones from the library, we targeted ~30 genes for the array, including several genes involved in neurotransmitter function. We also obtained an additional 20 gene fragments from collaborators, such as genes coding for receptors and neuropeptides. To amplify cDNA fragments, 4 μl of template was added to a 100-μl reaction containing 1× PCR buffer, 1.5 mM MgCl, 150 μM dNTPs, 300 μM forward primer (5′-CACGCAGTTGTAAAACGAC-3′) and reverse primer (5′-GGATAACAATTTCACACAGG-3′), and 2.5 units of Taq polymerase (Invitrogen). PCR cycles consisted of a denaturation step at 95°C for 15 s, followed by an annealing step at 52°C for 30 s, and an extension step at 72°C for 45 s for 38 cycles. Amplified PCR products were purified using Millipore PCR clean-up kit or MicroSpin columns (S-300HR; Amersham Pharmacia Biotech, Piscataway, NJ) and resuspended in a final concentration of 3× SSC in 50 μl of DNase/RNase-free H_2O in 96-well plates. Final concentrations of purified PCR product ranged between 50 and 80 ng/μl. Vectors containing single gene fragment were sent to Canadian Molecular Research Services (Ottawa, Ontario, Canada) for sequencing. Nucleotide sequences were compared with known sequences in GenBank in the National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) database. Goldfish clones were sent to the University of Liverpool Microarray Facility, UK (http://www.liv.ac.uk/lmf/root/Liverpool-Microarray-Facility/index.htm) for printing with 8,000+ carp genes. All genes were printed in duplicate onto poly-L-lysine-coated glass sides. Our cDNA microarray also included the Stratagene SpotReport Alien Array Validation System (Stratagene, La Jolla, CA). Goldfish clones have been annotated and sequence information has been deposited in NCBI GenBank (dbEST). More information about the goldfish clone database can be found on www.auratus.ca.

Microarray hybridization and scanning. For microarray hybridizations, total RNA was extracted using TRIzol Reagent (Invitrogen) as per the manufacturer's protocol. Total RNA was resuspended in 30 μl of RNase-free water and quantified using a GeneQuant spectropho-

tometer. We pooled total RNA from ~30 fish into a reference control RNA pool. Three separate pools of RNA from treated fish were hybridized to the printed microarrays, and a fourth hybridization was a replicate dye-reversal of one of the three RNA pooled samples. This was done with hypothalamic RNA from both treatments and resulted in a total of eight microarrays (four arrays/EE2 dose). We used the Genisphere Array 900MPX cDNA microarray labeling kit (Genisphere, Hatfield, PA) for all microarray hybridizations. This indirect labeling kit uses Cyanine 3 (Cy3) and 5 (Cy5) as the fluorescent dyes. The complete hybridization protocol is found at (http://www.genisphere.com/pdf/array900mpx_protocol_v06–22-04.pdf). We used 2 μg total RNA for the first strand synthesis. The 2× formamide-based hybridization buffer was used for the microarray prehybridization step and the 2× SDS-based hybridization buffer was used for the light capture reaction (3DNA hybridization step).

Microarrays were scanned at full-speed 10-μm resolution on the ScanArray 5000 XL system (Packard Biosciences/Perkin-Elmer) using both red and blue lasers. Images were obtained with ScanArray Express software using automatic calibration sensitivity varying photomultiplier tube (PMT) gain (PMT starting at 65% for Cy5 and 70% for Cy3) with fixed laser power at 80% and the target intensity set for 90%. Microarray images were opened using QuantArray (Packard Biosciences/Perkin-Elmer) and raw signal intensity values obtained for duplicate spots of genes. Raw intensity values for all microarray data and microarray platform information have been deposited to the NCBI Gene Expression Omnibus database (platform accession no. GPL3735; series accession no. GSE4868).

Data normalization and identification of differentially expressed genes. The array quality filter test (AQF) (40) was first applied to check the raw data. The AQF values of all slides were less than the threshold of 0.5. Spots that had been manually flagged due to poor hybridization and spots in which the estimated fluorescence intensity was below or equal to the estimated background signal intensity in either channel were removed before further analysis. Several normalization strategies, including intensity-dependent Lowess normalization, within-tip-group Lowess normalization and global normalization, were compared (55a) through examining normalization effects on M/A plots where M is log ratio and A is overall signal intensity from both two channels. The intensity-dependent Lowess normalization with span 0.4 was chosen for normalization because there was no significant variance between the print tips. Box plots of the Lowess $\log_2$ ratios for each of the four slides were centered at zero and had similar spreads (Fig. 1), therefore no further normalization procedure between slides was carried out. Significance analysis of microarray (SAM) method was performed to assess the significance of differential expression of the genes (54). This technique computes a statistic for each gene and measures the strength of the relationship between gene expression and the response variable. Repeated permutations of the data determine whether the expression of a specific gene was significantly different between test groups. The criterion for significance was $q < 5\%$, which is based on the false detection rate (FDR). FDR is the percentage of significant genes identified by chance, while the q value is the minimum false discovery rate at which the gene is significant ($q < 5\%$). The q value is an adjusted *P* value, and it is designed for the FDR analysis using SAM.

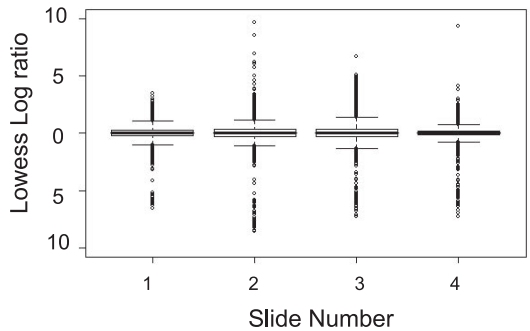

Fig. 1. Box-plot displaying the log ratio for microarray replicates in the hypothalamus (1.0 nM group) after Lowess normalization.

Real-time RT-PCR. All primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Invitrogen. Primers of 18–22 base pairs (bp) with optimal annealing temperature between 58 and 62°C were designed to amplify sequences of 100–250 bp within the gene region printed on the array. Primers were initially tested using goldfish whole brain cDNA, and the resultant amplicons were cloned and sequenced to confirm specificity.

Real-time RT-PCR analysis of gene expression was carried out on first-strand cDNA derived from DNase-treated RNA samples from control and treatment groups. We also tested whether genes identified as being regulated by microarray analysis in the hypothalamus were also regulated in the telencephalon. Each PCR reaction contained the following final concentrations; ~25 ng first-strand cDNA template, 1× QPCR buffer, 3.5 mM $MgCl_2$, 100–150 nM gene-specific primer (depending on the primer set used), 0.25× SYBR green (Invitrogen), 200 μM dNTPs, 1.25 units HotStarTaq (Invitrogen), and 100 nM carboxy-X-rhodamine reference dye, in a 25-μl reaction volume. The primer sets used in this study are reported in Table 1.

The thermal cycling parameters were an initial 1 cycle of Taq activation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 58–60°C for 5 s (depending on the primer set used), 72°C for 30 s, and a detection step at 80°C for 8 s. Dilutions (1:10 to 1:31,250) of cDNA from each sample were used to construct a relative standard curve for each primer set. After the reaction was complete, a dissociation curve was produced starting at 55°C (+1°C/30 s) to 95°C. Real-time RT-PCR was assayed on an MX4000 Multiplex Quantitative PCR system (Stratagene), and the accumulation of PCR product was measured in real time as the increase in SYBR green fluorescence. Data were analyzed using the MX4000 software package. Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using the amplified PCR product as a template and were used to calculate mRNA copy number in each sample.

We also chose four candidate control genes with differing biological functions [β-actin, β-2 microglobulin, ribosomal 18 subunit, and elongation factor 1 (EF-1)] as possible controls to normalize our expression data. We used geNORM software (http://medgen.ugent.be/~jvdesomp/genorm/) to determine the most stable housekeeping gene. To further evaluate the sensitivity of our microarray, we chose three additional genes as negative controls to investigate with real-time RT-PCR that *1*) were not identified by our microarray analysis as being regulated by EE2 and *2*) show seasonal variation and sexual dimorphism in mRNA expression. These included glutamic acid decarboxylase (GAD) 65 and 67 (5, 23) and the neuropeptide isotocin (32). We did this to further demonstrate and increase our confidence in the ability of our microarrays to identify regulated transcripts. This is rarely done in microarray analysis. GAD is the enzyme that converts glutamate into GABA, a neurotransmitter that stimulates luteinizing hormone (LH) release in fish (51). Isotocin is highly expressed in the teleost brain and is involved in reproductive behavior in fish (14). An unpaired Student's *t*-test on transformed data followed by Dunn's multiple-comparisons test was used to determine whether there were significant differences in expression between the control and treatment group. When data could not be normalized, a Mann-Whitney *U*-test was used.

RESULTS

Gonadosomatic index (GSI) of EE2-treated male goldfish. Body weight in control and treatment groups did not significantly differ from each other and ranged from 11 to 40 g for

Table 1. *Primers for real-time RT-PCR*

Gene	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)
elongation factor-I	GAT TGT TGC TGG TGG TGT TG	GCA GGG TTG TAG CCG ATT T
β-actin	CTG GGA TGA TAT GGA GAA GA	CCA GTA GTA CGA CCT GAA GC
B2 microglobin	GCC CTG TTC TGT GTG CTG TA	AAG GTG ACG CTC TTG GTG AG
ribosomal 18s	AAA CGG CTA CCA CAT CCA AG	CAC CAG ATT TGC CCT CCA
brain aromatase	TGC TGA CAT AAG GGC AAT GA	GGA AGT AAA ATG GGT TGT GGA T
secretogranin III	TTT GCG CTT ATA GAG GAG AGG	TTA CAG CAA GGA CAA CCA CAA
IFDR-1	GCT CCA CAC AAA CGC TCT C	GTC TCC CCA GCA GCA ATC
GAD 65	GGA TAC GTG CCG TTC TTT GT	CTC GAC TCC ATT CAG CTT CC
GAD 67	CCA AAG GCA TGT CTG TAG CA	CCC TTC TGT TTG GCA TCA AT
isotocin	ATC TTG GCT ACT GGC AGG TT	GTA TCT GCT GTG GTG AAG GT
ependymin	TGA GCG GAA CAA TGA AAG TG	TCA GAC TCG TGA GTG GCA TC

IFDR, interferon-related developmental regulator; GAD, glulamic acid decarboxylase.

both control and treatment fish. GSI is defined as (gonad weight/body weight) × 100%. Male goldfish treated with 1.0 nM EE2 had significantly smaller gonads than goldfish treated with 0.1 nM EE2 ($P < 0.01$) and ethanol-treated controls ($P < 0.02$) (Fig. 2). There was no significant difference in GSI between control goldfish and the 0.1 nM EE2 treatment group.

Serum T and E2 levels. Male goldfish exposed to both levels of EE2 had significantly reduced levels of circulating T compared with control animals (Fig. 3*A*) ($P < 0.02$). Mean T (±SE) levels in control goldfish were 1,600 ± 580 pg/ml and ranged between 800 to 3,300 pg/ml ($n = 5$). In males exposed to 0.1 nM EE2, mean T was 550 ± 140 pg/ml and ranged from 210 to 770 pg/ml. In males exposed to 1.0 nM EE2, mean T was 390 ± 60 pg/ml and ranged from 210 to 480 ng/l.

Male goldfish exposed to both levels of EE2 had significantly reduced levels of circulating E2 compared with control animals (Fig. 3*B*) ($P < 0.01$). E2 was detected at very low levels in the blood of all males sampled. Mean (±SE) E2 levels in control goldfish were 210 ± 54 pg/ml E2 and ranged between 33 and 460 pg/ml ($n = 7$). In males exposed to 0.1 nM EE2, mean E2 was 5 ± 2 pg/ml and ranged from not detectable to 14 pg/ml. All samples in the high EE2 treatment were below the detection limit of the assay and were assigned the lowest detectable concentration value of the assay based on the standard curve and efficiency of our E2 assay. Thus, we were not able to determine the absolute pg/ml concentration of circulating E2 in treatment males. However, there was a dramatic and significant reduction in treatment groups compared with controls.

Microarray and real-time RT-PCR expression data. The goldfish brain-derived portion of the current microarray contains ~5% receptor-related genes [e.g., estrogen receptors (ERs), GABA receptor subunits, dopamine receptors], 15% structural proteins (e.g., ependymin, tubulin), 5% metabolic-related, 10% neuropeptide or neurotransmitter-related [.g., gonadotropin-releasing hormone (GnRH), transporters, isotocin], 2–3% enzymes (e.g., TH, GADs), and 31% cell cycling/transcription factors. Approximately one-third of the goldfish genes have not been characterized. We are continuing to update sequences on the microarray and investigating the function of ESTs using bioinformatic techniques (see http://dambe.bio.uottawa.ca/goldminer.asp for more information on goldfish sequences). Additional brain-derived genes are being added to the goldfish-carp microarray for future experiments using both goldfish and carp.

In this first experiment, our strategy was to be strict with our microarray data analysis and focus on genes important for neuroendocrine and reproductive function. Putative regulated transcripts identified by microarray analysis are listed in Table 2. At a nominal dose of 0.1 nM EE2, we did not detect significant gene targets (q > 5%). However, we do list genes that showed the lowest q values (i.e., approaching q = 5%) with our analysis for a comparison to the 1.0 nM treatment. Candidate genes identified in the low EE2 treatment group were all reduced after EE2 exposure. Some of these genes, for example, ceruloplasmin, sp1 transcription factor, and interleukin 6 signal transducer are known to be responsive to estrogens. Transcripts that are identified and showed a significant

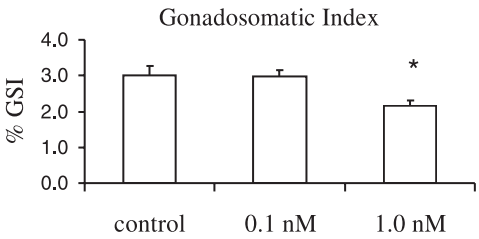

Fig. 2. Mean (± SE) gonadosomatic index (GSI) in control and treated (0.1 and 1.0 nM) goldfish. *Significant difference $P < 0.05$; unpaired Student's *t*-test compared with controls ($n = 9$–10).

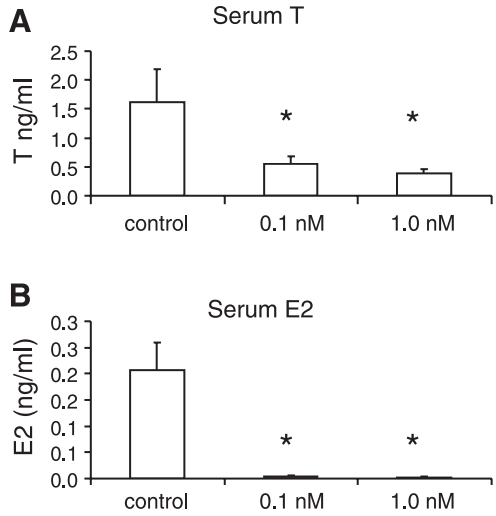

Fig. 3. Mean (±SE) serum concentration of T ($n = 5$) (*A*) and 17β-estradiol (E2, $n = 7$) (*B*) in control and treated (0.1 and 1.0 nM) goldfish. *Significant difference ($P < 0.05$) compared with controls.

Table 2. *List of candidate genes identified by microarray analysis in the 0.1 nM EE2 group and the 1.0 nM EE2 group*

Gene	Fold Change	Accession # (nucleotide BLAST)
0.1 nM EE2 treatment group		
Downregulated genes		
spI transcription factor	−2	BC067713.1
similar to Stathmin (phosphoprotein p19) (pp19) (oncoprotein 18) (Op18)*	−1.93	NM_001017850.1
mitochondrion-hypothetical 18K protein	−1.79	AY714387.1
histone 2, H2, like	−1.6	BC059463.1
amyloid beta precursor protein binding protein I	−1.57	BC055513.1
aldolase A fructose-bisphosphate	−1.57	AY394966.1
similar to NEFA-interacting nuclear protein NIP30*	−1.54	BC056511.1
prophenoloxidase activating factor*	−1.53	BX842643.1
hypothetical protein PC000284.00.0*	−1.48	CR318588.10
hypothetical protein LOC496821*	−1.48	AC108830.13
nucleobindin 2a	−1.47	BC046077.1
secretogranin III	−1.45	BC059577.1
hypothetical protein*	−1.44	BX548249.13
similar to zinc finger protein 262 (LOC559580)	−1.44	XM_682936.1
poly(rC) binding protein 2	−1.4	AY398394.1
ceruloplasmin	−1.39	BC064000.1
similar to interleukin 6 signal transducer (LOC558568)	−1.36	XM_681798.1
1.0 nM EE2 treatment group		
Upregulated genes		
GAPDH	2.25	XM_679205.1
aromatase†	1.96	AB009335.1
fibrinogen, gamma polypeptide	1.82	BC045868.1
fibrinogen, B beta polypeptide	1.77	BC066629.1
similar to apolipoprotein (A-I)	1.66	XM_696149.1
similar to apolipoprotein C-I precursor	1.65	XM_694143.1
protein tyrosine phosphatase, receptor-type, F interacting protein, binding protein 2	1.62	BC055935.1
dopamine DI/beta receptor†	1.59	AJ005433.1
splicing factor, arginine/serine-rich 5	1.5	BC063235.1
Early growth response protein I (Krox-24 protein) nerve growth factor*	1.45	BX470252.10
troponin T3b, skeletal, fast	1.39	BC065452.1
similar to 1-aminocyclo propane-1-carboxylate synthase*	1.37	BX321900.10
MD18 GAPDH mRNA	1.37	AY818346.1
similar to ribosome binding protein I	1.34	XM_684962.1
ependymin II	1.34	J04986.1
nucleobindin 2a	1.34	BC046077.1
Downregulated genes		
similar to interferon-related developmental regulator I	−2.26	XM_701614.1
apolipoprotein A-I binding protein*	−2.26	BC075969.1
ferredoxin 2*	−1.49	BX005286.6
bone morphogenetic protein-15	−1.41	AY860977.1

Accession numbers for basic local alignment search tool (BLAST) hits are given for goldfish or carp (if available) and/or the closest similar sequence after a BLAST search. Not listed are sequences that showed no nucleotide or amino acid similarity to proteins in the National Center for Biotechnology Information (NCBI) database. EE2, 17α-ethinylestradiol. *Genes that were not similar to known nucleotide sequence after BLAST search but showed similarity to known protein after translation of nucleotide sequence. †A goldfish sequence on the goldfish-carp microarray.

change ($q < 5\%$) are listed for the 1.0 nM group, in addition to the estimated fold change. Real-time RT-PCR was used to verify a subset of genes that were identified as being regulated by EE2 exposure. We selected genes with different functions that *1*) showed high fold-changes and/or *2*) were of interest to the neuroendocrine control of reproduction. These included aromatase, secretogranin-III (Sg-III), ependymin, and interferon-related developmental regulator 1 (IFDR-1). Using gene expression data from both the hypothalamus and telencephalon, we determined that β-actin and β-2-microglobulin transcription are modulated by EE2 exposure and thus were not appropriate to normalize our data. It has been reported that β-actin is estrogen responsive in the liver of sheepshead minnows (*Cyprinodon variegatus*) (24). We determined that EF-1 (accession #AB056104) did not significantly change with the treatment and used this gene to normalize our real-time RT-PCR data. Brain aromatase transcription was significantly induced (approximately sixfold in the 1.0 nM EE2 exposure in the hypothalamus and approximately threefold in the telencephalon; Fig. 4, *A* and *B*). Our data also show that Sg-III is significantly upregulated in the hypothalamus at 1.0 nM EE2 but significantly reduced at both doses of EE2 in the telencephalon (Fig. 4, *C* and *D*). IFDR-1 mRNA was significantly induced in the telencephalon in the high dose of EE2 only (Fig. 4, *E* and *F*). There were no significant changes in the mRNA abundance of ependymin, GAD 65, GAD 67, and isotocin in the hypothalamus or telencephalon (not shown).

DISCUSSION

Testis size is reduced in high EE2 exposure group. We found that male goldfish exposed to 1.0 nM had smaller testis (~33% reduction) than both control fish and fish exposed to 0.1 nM EE2. This is comparable to what has been reported in other teleost fish exposed to waterborne EE2. Sexually maturing rainbow trout exposed to EE2 (100 ng/l or ~0.34 nM) for 62

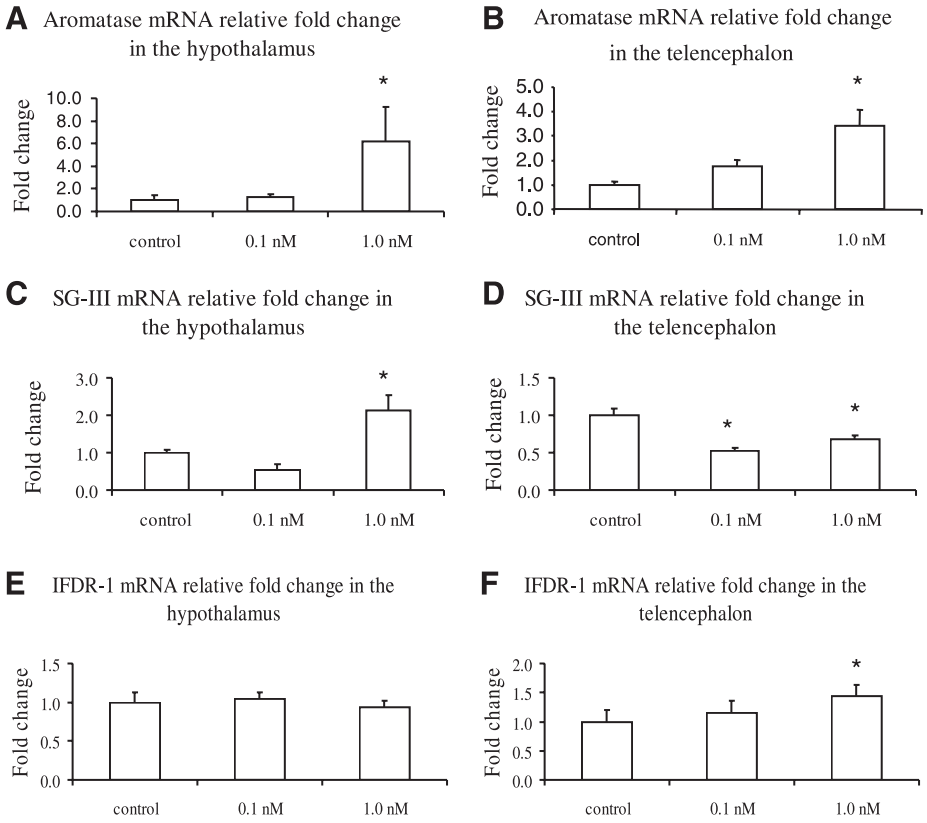

Fig. 4. Relative fold changes (±SE) determined by real-time RT-PCR SYBR green assay of aromatase mRNA (*top row*), secretogranin III (Sg-III) mRNA (*middle*), and interferon-related developmental regulator 1 (IFDR-1) mRNA (*bottom*) in the hypothalamus (*left*) and in the telecephalon (*right*). *Significant difference ($P < 0.05$) compared with controls ($n = 5–6$).

days showed a reduction in testis mass (41). Sexually maturing trout exposed to 10 ng/l (~0.034 nM) did not show a reduction in testis size. Goldfish exposed to a nominal concentration of 1–10 μg/l E2 in water had approximately a 50% reduction in testis size after 24–28 days of treatment (3). In the fathead minnow (*Pimephales promelas*), low doses of EE2 (0.034 nM) did significantly reduce testis size after a 3-wk treatment period (34).

Sex steroids are depressed after EE2 treatment. EE2 decreased serum T levels by 60–70%, similar to other studies in fish. Male goldfish implanted with E2 silastic implants (100 μg/g body wt) showed approximately a threefold reduction in both T and 11-ketotestosterone (11-KT) (50). In studies investigating effects of waterborne exposures to EE2, male mummichogs showed depression of T and 11-KT at 7 and 15 days static exposure to >250 ng/l EE2 (27). Tilton et al. (46) showed that T production was significantly depressed at an exposure to 500 ng/l EE2 in Japanese medaka (*Oryzias latipes*). These studies demonstrate that E2 negatively regulates androgen production.

Endogenous E2 in male teleost fish plays a critical role in spermatogenesis and gametogenesis (31). Our study detected an average of ~210 pg E2/ml in control fish, similar to what has been reported for other male teleost fish (36, 42). We found that levels of E2 were significantly reduced in both EE2 exposure groups. In contrast, male channel catfish (*Ictalurus punctatus*) injected with 1 mg/kg EE2 had a significant increase in serum E2 concentrations after 7 days postinjection (45). When exposing to estrogenic compounds, low doses may induce whereas higher doses of estrogens inhibit normal reproductive function. For example, in female mummichogs (*Fundulus heteroclitus*) high EE2 concentrations depressed ovarian production and circulating E2 levels but EE2 concentrations <100 ng/l caused increased E2 production (27). Current evidence suggests that there is a threshold effect of EE2 in which EE2 no longer stimulates, but inhibits, gonadal steroid production. This threshold concentration of exposure to estrogens will depend upon multiple factors such as age, sex, time of season, and species sensitivity to estrogens.

EE2 and gene expression in the brain. Previous studies show changes in relative abundance of mRNA transcripts in the brain are less in magnitude compared with other tissues, typically less than twofold (4, 29). In the present study, gene expression analysis detected similar fold changes in the hypothalamus, which typically ranged between 1.2- and 2-fold. Trudeau et al. (53) demonstrated using in vivo somatic gene transfer of an estrogen response element (ERE)-thymidine kinase-luciferase (ERE-TK-LUC) construct into the brain that male goldfish exposed to 10 nM EE2 had elevated brain luciferase activity (twofold). This indicates that waterborne estrogenic chemicals found in the environment can modulate gene transcription in the brain. Transcripts we identified as being significantly regulated by EE2 can be categorized into cell signaling and transduction, cellular metabolism (protein and nucleic acid metabolism, fatty acid), cell structure and growth, transcription/ translation, and others.

In general, our microarray data tended to underestimate the magnitude of the fold change compared with real-time RT-PCR data, an observation that has been documented previously after comparing the two techniques (57). Relative changes in brain aromatase, Sg-III, and IFDR-1 mRNA were confirmed by real-time RT-PCR. GAD65, GAD67, and isotocin mRNA,

genes that have an important role in reproduction in fish, were not identified by microarray analysis as being differentially regulated, and this was confirmed with real-time RT-PCR. This increased our confidence in the ability of our miroarrays to identify estrogen-responsive targets. We also identified genes in the hypothalamus previously reported to be estrogen responsive in other tissues in both the low and high EE2 treatment groups. For example, Pinto et al. (35) report that in the testis of the sea bream (*Sparus auratus*), apolipoproteins and fibrinogen beta and gamma are upregulated after E2 injections. We identified these genes as being upregulated in the brain after exposure to 1.0 nM EE2.

Neural aromatase, the enzyme that converts T into E2, is pronounced in the teleost brain and may be up to 100–1,000 times greater than in the mammalian brain (33). The 5′-flanking region in the promoter of the brain aromatase gene in goldfish contains two EREs and a half-ERE site (9). Neural aromatase can therefore be regulated both by effects of locally produced E2 and also E2 from gonadal sources (reviewed in Ref. 28). We detected a high amount of variation in brain aromatase mRNA in both control and treated fish. This is similar to what has been reported in the brain and gonad of male fathead minnows (*Pimephales promelas*) (16) and in the brain of male zebrafish (*Danio rerio*) (49). Goldfish exposed to 1.0 nM EE2 had a significant increase in brain aromatase steady-state mRNA in both the hypothalamus and telencephalon; however, we did not detect changes in brain aromatase mRNA in the lower EE2 dose. Lyssimachou and colleagues (26) report that aromatase mRNA in the brain of juvenile Atlantic salmon is induced by lower EE2 (<50 ng/l) exposure after a 3-day treatment. This contrasting data may reflect the age of the animals used or the length of the exposure. There is other evidence to suggest that endogenous E2 and exposure to environmental estrogens induce aromatase mRNA transcription in the teleost brain. In vivo steroid treatment in the goldfish with E2 and aromatizable androgens increase levels of brain aromatase mRNA eight- and fourfold in the forebrain and hindbrain, respectively (13). Similar findings of an induction in aromatase transcription in zebrafish 4–10 days posthatch (49), and activity in Japanese medaka 14 days (10) after EE2 exposure have been reported. In the present study, aromatase was induced in the brain despite the significant reduction in circulating serum T and E2. Forlano and Bass (12) report recently that in female plainfin midshipman (*Porichthys notatus*), higher doses of EE2 were correlated with lowered E2 levels and an increase in brain aromatase expression. Interestingly, we detected no change in estrogen receptor alpha, beta, or gamma mRNA abundance (data not shown) in either tissue, suggesting that the regulation of aromatase transcription may not be dependent upon increased ER transcription. The induction in aromatase mRNA may be a response of the male brain to maintain local production of neuroestrogen when circulating sex steroids are rendered low following estrogenic exposures. Alterations in brain aromatase expression during exposure to estrogenic compounds in the environment could also result in impaired sexual behavior in male fish.

Sg-III mRNA was significantly increased in the hypothalamus at 1.0 nM EE2 but decreased at both doses of EE2 in the telencephalon, suggesting tissue-specific regulation of Sg-III mRNA that is dependent upon the dose of EE2. Sg-III is a member of the granin family, which also includes chromogranin A, B and secretogranin II (Sg-II). Chromogranin members are often concentrated in secretory granules in CNS neurons. These proproteins are actively processed into small neuroactive peptides that can influence neurotransmitter release. Hosaka and colleagues (18) demonstrated that Sg-III has a role in prohormone-processing and is able to bind proopiomelanocortin-derived peptides, in addition to cholesterol-rich secretory granules in endocrine cells. The authors suggest that the targeting role of Sg-III and other proteins facilitates the efficient release of peptides at exocytosis. To date, little is known about the regulation of Sg-III, and this is the first report of Sg-III being sensitive to estrogens. Previous work with other members of the granin family in mammals suggests that, in general, transcription of this family is negatively responsive to estrogen feedback (2, 25). In the telencephalon, we show that EE2 decreases Sg-III, consistent to what has been shown in the mammalian pituitary for Sg-II. However, we detected a twofold increase in the hypothalamus of the 1.0 nM EE2 treatment group. In the goldfish, Samia et al. (39) found that goldfish pituitary Sg-II expression levels vary with the seasonal reproductive cycle, independently of sex steroids. The authors suggest that Sg-II modulation is most likely through other neuropeptides important for reproduction, for example, GnRH. In the mammalian pituitary, Sg-II is colocalized with LH and appears to be involved in secretory vesicle packaging in gonadotrophs (11). This raises the question of whether exposure to environmental estrogens disrupts synaptic transmission in the brain, specifically neurotransmitters and neuropeptides with a prominent role in reproduction.

Our microarray identified IFDR-1 mRNA as being reduced in the hypothalamus after 1.0 nM ng/l EE2 exposure, although we were unable to verify this with real-time RT-PCR in this tissue. However, there was a significant upregulation in the telencephalon of IFDR-1 mRNA after real-time RT-PCR. In the developing rat, this gene shows homology with interferon-γ (IFN-γ) and is expressed in differentiated tissues such as nervous tissue, kidney, and lung (8). IFNs belong to the cytokine family and will act as mitogens and growth factors. There is evidence that a large number of IFNs are regulated by estrogens and that they mediate reproductive physiology and development and remodeling of reproductive tissues (17, 20). Moreover, human interferon regulatory factor-1 (IRF-1), in addition to a number of cytokines, plays a role in signaling networks in breast cancer (59). Although speculative, disruption in the expression of these proteins by EE2 during critical phases of seasonal reproduction could have downstream consequences for reproductive neuroendocrine function.

Conclusions. We have produced a brain-enriched cDNA goldfish microarray and demonstrate its use in evaluating risks associated with endocrine disruption in aquatic vertebrates. Our goldfish-carp microarray is now being used to study neuroendocrine function in response to neurotransmitter receptor agonists and the effects of pharmaceuticals in the vertebrate brain. In the present study, we identified a number of candidate genes in the brain that are estrogen responsive. Gene profiling, coupled to more traditional estrogenic endpoints such as Vtg induction, will provide more sensitive and robust data for evaluating risks associated with endocrine disruption.

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