Physiol Genomics 38: 351–361, 2009.
First published June 9, 2009; doi:10.1152/physiolgenomics.00051.2009.

Profiling neuroendocrine gene expression changes following fadrozole-induced estrogen decline in the female goldfish

Dapeng Zhang, Jason T. Popesku, Christopher J. Martyniuk, Huiling Xiong, Paula Duarte-Guterman, Linhui Yao, Xuhua Xia, and Vance L. Trudeau

Centre for Advanced Research in Environmental Genomics (CAREG), Department of Biology, University of Ottawa, Ottawa, Ontario, Canada

Submitted 13 March 2009; accepted in final form 5 June 2009

Zhang D, Popesku JT, Martyniuk CJ, Xiong H, Duarte-Guterman P, Yao L, Xia X, Trudeau VL. Profiling neuroendocrine gene expression changes following fadrozole-induced estrogen decline in the female goldfish. *Physiol Genomics* 38: 351–361, 2009. First published June 9, 2009; doi:10.1152/physiolgenomics.00051.2009.— Teleost fish represent unique models to study the role of neuroestrogens because of the extremely high activity of brain aromatase (AroB; the product of *cyp19a1b*). Aromatase respectively converts androstenedione and testosterone to estrone and 17β-estradiol (E2). Specific inhibition of aromatase activity by fadrozole has been shown to impair estrogen production and influence neuroendocrine and reproductive functions in fish, amphibians, and rodents. However, very few studies have identified the global transcriptomic response to fadrozole-induced decline of estrogens in a physiological context. In our study, sexually mature prespawning female goldfish were exposed to fadrozole (50 μg/l) in March and April when goldfish have the highest AroB activity and maximal gonadal size. Fadrozole treatment significantly decreased serum E2 levels (4.7 times lower; $P = 0.027$) and depressed AroB mRNA expression threefold in both the telencephalon ($P = 0.021$) and the hypothalamus ($P = 0.006$). Microarray expression profiling of the telencephalon identified 98 differentially expressed genes after fadrozole treatment (q value <0.05). Some of these genes have shown previously to be estrogen responsive in either fish or other species, including rat, mouse, and human. Gene ontology analysis together with functional annotations revealed several regulatory themes for physiological estrogen action in fish brain that include the regulation of calcium signaling pathway and autoregulation of estrogen receptor action. Real-time PCR verified microarray data for decreased (activin-βA) or increased (calmodulin, ornithine decarboxylase 1) mRNA expression. These data have implications for our understanding of estrogen actions in the adult vertebrate brain.

aromatase; microarray; fish; brain

MAIN ESTROGENS estradiol-17β (E2) and estrone (E1) play fundamental regulatory roles in neuroendocrine and reproductive systems. Through binding to its nuclear estrogen receptors (nER)-α and nER-β, E2 regulates transcriptional process of target genes whose promoters contain estrogen-responsive elements (ERE) (57). In addition to this classical nuclear action, E2 also has membrane actions in which a unique membrane receptor is utilized to rapidly activate a series of signaling pathways (52, 70). Since the signaling pathway activation will ultimately influence the transcriptional activities of downstream transcription factors including nERs, membrane actions of E2 provide another mode to regulate genomic gene expression (70). Fish are unique models in which to study E2 action because there is high activity of the B-subtype aromatase (AroB; the product of *cyp19a1b*), the enzyme responsible for synthesizing E2 from testosterone (T), in the neuroendocrine brain, telencephalon (Tel) and hypothalamus (Hyp) (42). Moreover, in contrast to mammals where aromatase and nER are commonly expressed in neurons, current studies in fish and birds strongly suggest that AroB is exclusively expressed in radial glial cells and nER is mostly expressed in neurons (16, 43). Thus, the local production of glial estrogen and signaling to neurons is an important interaction between these cell types that has fundamental implications for neurogenesis, neuronal regeneration, neuroprotection, and neuroendocrine function (16, 19, 37).

Profiling the physiological effects of fadrozole, a reversible competitive inhibitor of aromatase, has been used successfully in studies investigating estrogen action in fish (2, 4, 15, 38, 45, 69), frog (41), and rodent (23) models. By inhibiting aromatase activity, fadrozole decreases serum E2 levels (2, 4), impairs gonadal development (2), and potentially induces sex reversal in fish (4, 15, 69). However, the associated gene expression changes caused by fadrozole-induced E2 decline in neuroendocrine systems are still largely unknown. Several studies have utilized real-time PCR analysis of selected genes (61, 72) and low-density microarrays (60) to profile fadrozole-induced changes in gene expression. Recently, the zebrafish (*Danio rerio*) model was used to identify the relationship of the global transcriptomic changes between brain, testis, and ovary after fadrozole treatments (62). However, in this latter study, there were no attempts to relate the changes in the transcriptome to changes in physiology.

In the present study, we assessed the effects of fadrozole on neuroendocrine and reproductive systems in the goldfish model (46) using both physiological and transcriptomic information. In goldfish, serum E2 levels exhibit a distinct seasonal profile with an increase around April and May, corresponding to the prespawning period (55). Accordingly, a similar seasonal profile for aromatase activity (42) and AroB expression (71) has been observed in the neuroendocrine brain Tel and Hyp. All these data indicate that the prespawning period from April to May is a critical period for physiological E2 action in neuroendocrine systems. This allows us to target this particular period and block E2 action by treating goldfish with fadrozole between March and April. We then evaluated the effect of fadrozole by gonadal histology, serum E2 levels, global gene expression profiling of the neuroendocrine brain, and subsequent gene ontology analysis.

Address for reprint requests and other correspondence: V. L. Trudeau, Centre for Advanced Research in Environmental Genomics, Dept. of Biology, Univ. of Ottawa, Ottawa, ON, K1N 6N5, Canada (e-mail: trudeauv@uottawa.ca).

MATERIALS AND METHODS

Experimental animals and design. Female common goldfish *(Carassius auratus)* were purchased in January 2005 from Aleong's International (Mississauga, ON, Canada) and were maintained at 18°C under a natural simulated photoperiod typical of Ottawa, Canada. Fish were fed everyday with standard goldfish food. The experiment was conducted from March to April. Goldfish ($n = 64$) were housed in 70-liter round tanks ($n = 16$ per tank; four tanks in total) supplied with constant aeration. Fadrozole was kindly provided by Novartis Pharma and diluted in water at a final concentration of 50 μg/l. The water and fadrozole were renewed every 3 days. After the exposure period (40 days), goldfish were anesthetized with 3-aminobenzoic acid ethylester (MS222; Aquatic Eco-Systems, Apopka, FL) for all handling and dissection procedures according to the guidelines of the Animal Care Committee, University of Ottawa, and the Canadian Council on Animal Care. Care was taken to standardize all handling and sampling protocols. Samples were rapidly dissected and immediately frozen on dry ice. Two or three hypothalami and telencephali were pooled together for a total number of 12 pools in each group. At the time of sampling, body weights and gonadal weights were recorded and gonadosomatic index (GSI; gonad weight expressed as percentage of the body weight) was calculated. An independent identical treatment was conducted in March-April 2006 with a second set of fish (Aleong's International) to confirm microarray result using real-time PCR (see below).

E2 radioimmunoassay. E2 was measured by radioimmunoassay (RIA) according to an established protocol (34). Sex steroids were extracted from 100 μl of serum from female goldfish. Since data were not normally distributed, a nonparametric test (Mann-Whitney *U*-test; SigmaStat3.5) was used to test the significant difference ($P < 0.05$).

Gonadal histology. Ovaries (three females per treatment) were fixed in Cal-Ex II (Fisher Scientific) for 3 days. Gonads were stored in 70% ethanol until paraffin embedding. Embedded samples were sectioned at 5 μm, and cross sections were stained with hematoxylin and eosin following conventional histological procedure. Images of the sections were captured with a Micro Publisher 3.3 Digital microscope camera (Qimaging, Surrey, BC, Canada).

RNA isolation and cDNA synthesis. Homogenization and disruption of brain tissue samples were done using an MM301 Mixer Mill (Retsch, Newton, PA) at 20 Hz for 2 min. The RNeasy Plus Mini kit (Qiagen) was then used for RNA isolation as described in the manufacturer's protocol. Concentrations of RNA were determined using GeneQuant spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ). cDNA was prepared from 2 μg total RNA and 200 ng random hexamer primers (Invitrogen, Burlington, ON, Canada) using Superscript II RNase H-reverse transcriptase (SSII) as described by the manufacturer (Invitrogen). A no reverse transcriptase control (NRT) was made where RNase-free water was added to the cDNA synthesis reaction instead of the SSII enzyme.

Microarray hybridization and data analysis. The description, validation, and use of our goldfish-carp microarrays have been published previously (31, 32, 35, 46, 64). Version 1.1 of the cDNA array was used in the present study and contains 8,664 clones printed in duplicate. Detailed microarray platform information has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (platform accession no. GPL7056). For the microarray hybridizations, a common reference pool was made from all control Tel RNA samples. The reference pool and five separate pools of Tel RNA from fadrozole-treated fish were analyzed. 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 a 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 have been deposited in the GEO database with series accession GSE14558.

In preparation for data analysis, we first removed the outlier spots that were generated due to the scratches or dust on the surface or imperfections during array production or hybridization. The spots whose estimated fluorescence intensity was below or equal to the estimated background signal intensity in either channel were also removed. We further removed the genes whose intensities could not be detected in >20% replicate spots. Other missing values were imputed by using K-nearest neighbor averaging scheme (54). Lowess normalization (68) was used to decrease within-slide bias. For between slide normalization, either the Scale method (68) or a novel General Procrustes Analysis (GPA) method (65) were used. Significance analysis of microarray (58) was performed to assess the significance of differential expression of the genes. Genes with false discovery rate <0.05 are considered as differentially expressed ones. We finally compared the performance of GPA and Scale normalizations based on positive identification of known E2-responsive genes that were retrieved by PubMed searching. Compared with the Scale normalization, GPA normalized data generated fewer differentially expressed genes; however, it retrieved more known estrogen-regulated genes. For example, both somatostatin type 2 and somatostatin type 5, which are differentially regulated by E2 in rat pituitary (26), were identified in the GPA normalized data but not in the Scale normalized data. This result provides a practical support for reliable performance of GPA method in microarray data analysis.

Functional annotations. Gene Ontology (GO) analysis for differentially expressed genes was carried out using the Blast2Go program (http://www.blast2go.de/). The specific GO terms were selected with the confluence score higher than cutoff values (9 for biological process category; 5 for molecular function category). This Blast2Go score takes into account the number of sequences converging at one GO term and penalizes by the distance to the term where each sequence actually was annotated (20).

Gene function was also verified by retrieving and comparing homologous sequences from the GenBank database and profiling domain information in the Pfam database (http://pfam.sanger.ac.uk/). To illustrate the regulatory relationship between E2 and the identified gene candidates, we retrieved research papers by searching key words of "estrogen" and an individual gene name in PubMed database.

Real-time PCR assay. SYBR green real-time PCR assays were conducted to measure the relative gene expression of AroB, ornithine decarboxylase 1 (ODC1), activin-βA, and calmodulin, aldolase c, elongation factor (EF) 1α, 18s rRNA in both control and treated groups. Primers (Table 1) were designed using Primer3 (http://frodo.wi.mit.edu/) and purchased from Invitrogen. The Mx4000 Quantitative PCR System (Stratagene, La Jolla, CA) was used to amplify and detect the transcripts of interest. Each PCR reaction contained the following final concentrations: 25 ng first-strand cDNA template, 1× QPCR buffer, 3.5 mM MgCl2, optimized concentrations (150–300 nM) of gene-specific primers, 0.25× SYBRGreen (Invitrogen), 200 μM dNTPs, 1.25 U HotStarTaq (Invitrogen), and 100 nM ROX reference dye in a 25 μl reaction volume. The thermal cycling parameters were an initial 1 cycle Taq activation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, optimized

Table 1. *Oligonucleotide primers used for real-time PCR assays*

Gene Target	GenBank ID	Sequence 5′-3′	Amplicon Size, bp
EF1-α	AB056104	F: GATTGTTGCTGGTGGTGTTG R: GCAGGGTTGTAGCCGATTT	227
18 s rRNA	AF047349	F: AAACGGCTACCACATCCAAG R: CACCAGATTTGCCCTCCA	166
Activin-βA	AF169032	F: TTTAAGGACATCGGGTGGAG R: TGATTGATGACGGTGGAATG	237
Ornithine decarboxyase 1	AY640230	F: TTGACATTGGAGGAGGCTTT R: GATGACCTTTTTGGCGATG	194
Calmodulin	AY656699	F: CATTTCCATCAGCGTCCA R: GCACCATCACGACCAAAGA	108
Aromatase B	AB009335	F: TGCTGACATAAGGGCAATGA R: GGAAGTAAAATGGGTTGTGGA	153
Aldolase C	U36777	F: GGCACGTCCATAAGAGAAGG R: GGAGGTCAGAGTGAGGAGGA	100

F, forward; R, reverse.

annealing temperature (58–60°C) for 5 s, 72°C for 30 s, and a detection step at 80°C for 8 s. Dilutions of cDNA (1:10 to 1:31,250) from all samples were used to construct a relative standard curve for each primer set. After the reaction was complete, a dissociation curve was produced starting from 55°C (+1°C/30 s) to 95°C. For each PCR reaction, negative controls were also introduced including a no template control where RNase-free water was added to the reaction instead of the template (cDNA) and a NRT control (previously described). The SYBR green assay for every target gene was optimized for primer concentration and annealing temperature to obtain for the standard curve an $R^2 > 0.98$, amplification efficiency between 90 and 110% and a single sequence-specific peak in the denaturation curve. For each gene, real-time PCR samples were run in duplicate.

Data were analyzed using the MxPro software package. The relative standard curve method was used to calculate the relative mRNA level of target and reference genes between samples. The copy numbers of target genes were normalized to those of EF1α, an internal reference gene whose expression did not change significantly with the treatment. By contrast, 18s rRNA expression was significantly increased in both Tel (1.6-fold; $P < 0.001$ by *t*-test) and Hyp (1.8-fold; $P < 0.001$ by *t*-test) after fadrozole exposure, indicating that 18s rRNA is not a proper internal reference gene for our experiment. The final normalized data from biological replicates (12 RNA samples per group) are presented as means + SE of fold changes of the treated group relative to the control group, which was set at 1. For statistical analysis, significant changes in gene expression in normally distributed data were evaluated using an Independent Samples *t*-test ($P <$

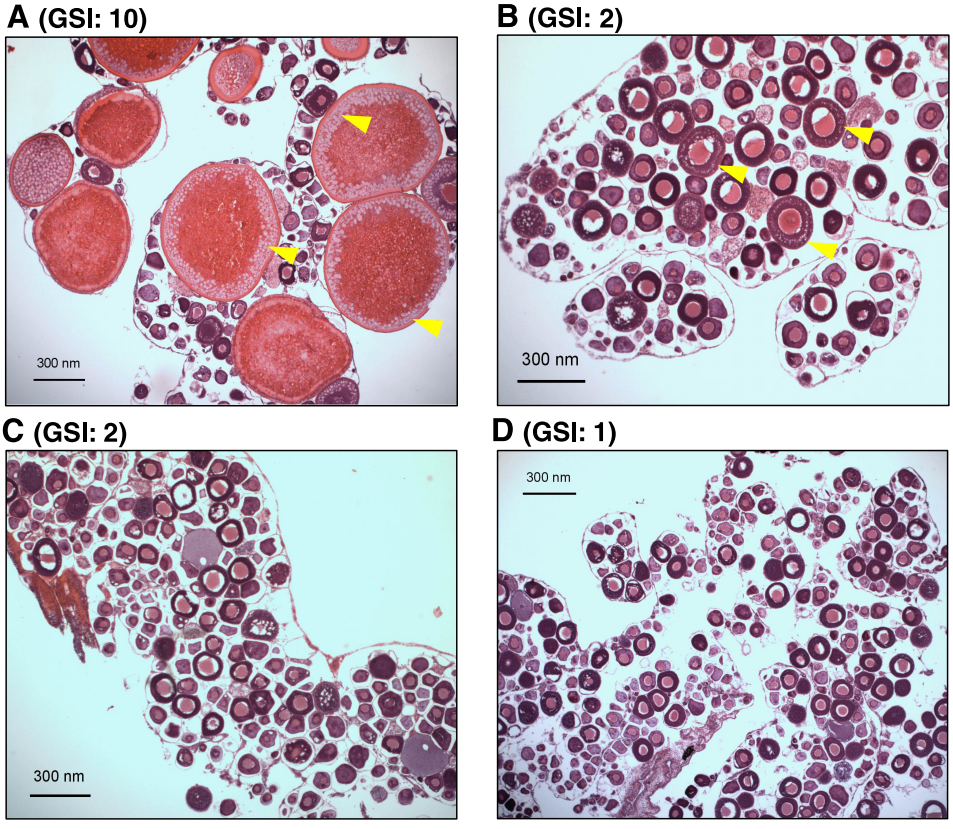

Fig. 1. Histological photos of ovary from control (*A, B*) and fadrozole treated (*C, D*) female goldfish. Gonadosomatic index (GSI) values of sampled goldfish are indicated next to the panel letters. Vesicles or cortical alveolus are indicated by yellow arrows.

0.05; SigmaStat3.5), and nonnormally distributed data were evaluated using the Mann-Whitney *U*-test ($P < 0.05$).

RESULTS

GSI and histological examinations. Ovarian GSI (2.2 ± 0.3; mean ± SE) in the fadrozole-treated female goldfish was 43% lower than controls (3.9 ± 0.9). However, this was not statistically significant ($P = 0.251$ by *U*-test). This is very likely due to large variation within samples.

Histology was used to determine the effect of fadrozole on female goldfish since effects on gonad development have been observed in other fish (2, 4, 15, 38, 45) and frog species (41). In the female control goldfish, the oocytes were evident in the cortical alveolar/vitellogenic stages (Fig. 1, *A* and *B*). However, in females from fadrozole-treated group, the oocytes were only at early stages of development and no vesicles (cortical alveolus) were observed (Fig. 1, *C* and *D*). The current results suggest that inhibition of aromatase delayed the expected seasonal maturation of the ovary over the duration of the experiment.

Fadrozole treatment decreases serum E2 level and AroB expression. Considering the histological differences in ovary between control and treated groups, we next measured serum E2 level in female goldfish. Compared with the control group, significantly lower (4.7 times) levels of circulating serum E2 were found in the fadrozole-treated fish ($P = 0.027$; Fig. 2). This indicates that fadrozole treatment effectively reduced E2 production in goldfish.

We also wanted to know whether E2 reduction by fadrozole affected expression of AroB, which is known to be directly regulated by E2 in fish (12). Real-time PCR indicated that fadrozole treatment was effective; in the treated group, AroB expression was three times lower in both the Tel ($P = 0.021$; Fig. 3*A*) and the Hyp ($P = 0.006$; Fig. 3*B*) than that in the control group. This indicates that the E2 reduction by fadrozole is achieved not only by inhibiting aromatase activity, but also by further decreasing AroB expression in the brain.

Microarray analysis of gene expression in Tel. Since AroB is a typical target of E2 transcriptional action (12, 31), its expression change suggests that E2 reduction may have effects on the expression of other E2-regulated genes. The goldfish-carp cDNA microarray was then used to identify these potential E2-regulated genes in Tel. Microarray data analysis identified 98 differentially expressed genes (*q* value <0.05) in which 68 were upregulated (Table 2) and 30 were downregulated (Table 3) following fadrozole treatment.

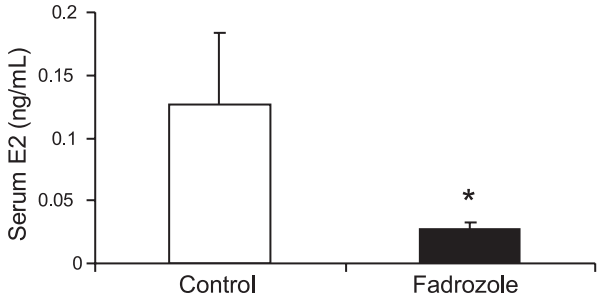

Fig. 2. Serum estradiol-17β (E2) level change in female goldfish after fadrozole treatment. Mean + SE values are shown for each group ($n = 12–13$). *Significant differences between control and fadrozole-treated groups ($P < 0.05$ by *t*-test).

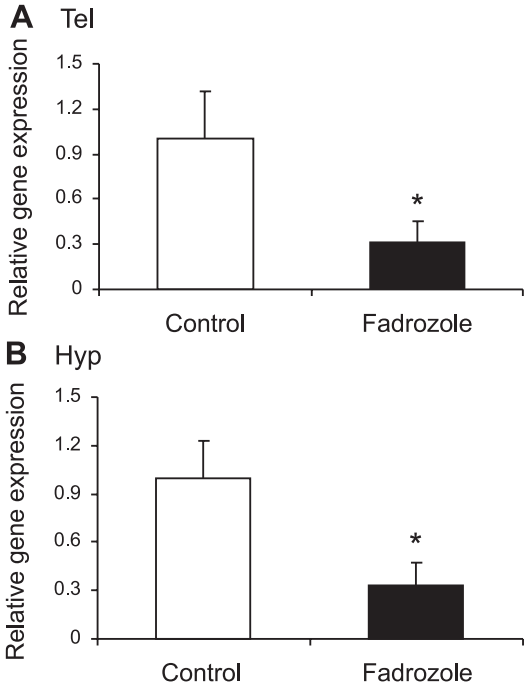

Fig. 3. Relative gene expression of B-subtype aromatase (AroB) in telencephalon (Tel) and hypothalamus (Hyp) after fadrozole treatment. The expression of AroB was normalized to elongation factor (EF) 1α. Mean + SE values are shown for each group ($n = 10–12$). *Significant differences between control and treatment groups ($P < 0.05$ by *t*-test).

Some of the identified genes have been previously shown to be estrogen responsive in fish or other species (Tables 2 and 3). For instance, in our previous microarray analysis of the Hyp (31), matrix metalloproteinase-9 was decreased in male goldfish after exposure to E2, while it is upregulated in the present study. There is also evidence showing that some identified candidate genes have regulatory relationships to estrogen in other tissues of other species. These include SNAP25, somatostatin-14, somatostatin receptor 2 and 5, neuropeptide Y, lactate dehydrogenase a, ubiquitin carboxy-terminal hydrolase L1, parvalbumin, matrix metalloproteinase-13. We list the PMID of the retrieved references for this information (Tables 2 and 3).

Functional significance of differentially expressed genes. We used GO analysis to explore the potential biological processes or molecular functions related to differentially expressed genes (Fig. 4). Grouping of genes into functional categories is set by a user-defined cutoff parameter. When the cutoff value of classification is 9, the GO biological processes include the terms transport, multicellular organismal process, signal transduction, response to stress, cellular metabolic process, response to chemical stimulus, negative regulation of cellular process and calcium ion transport (Fig. 4*A*). When cutoff value of classification is 5, the specific GO molecular functions identified include the terms catalytic activity, ATP binding, zinc ion binding, DNA binding, receptor binding, calcium ion binding, calcium channel activity, and calcium channel inhibitor activity (Fig. 4*B*). Interestingly, in both categories, GO terms related to calcium signaling are retrieved with very high confluence scores. This suggests that fadrozole treatment and the associated reduction in estrogen synthesis

Table 2. *List of upregulated genes identified by microarray analysis in female goldfish after fadrozole treatment (q value < 5%)*

Gene Name (up, 68)	GenBank ID[1]	Fold[2]	PMID[3]	Function[4]
Calmodulin	AAT73046	+1.58		C, E
SNAP25	P36978	+1.41	17293448 (rat) 9576603 (rat)	C
EPD I	P13506	+1.45		C
EPD II	P12958	+1.39		C
Neurogranin	ACJ64077	+1.38		C
Visinin-like 1	XP_689658	+1.32		C
Similar to testican 3	AAH96871	+1.37		C
EF hand domain containing 2	NP_077305	+1.42		C
Secretogranin II precursor	AAC94994	+1.36	8321414 (rat)	C
S100 calcium binding protein, beta (neural)	AAH76251	+1.32		C
Synuclein beta	ACA96673	+1.43		
Rho guanine nucleotide exchange factor 9	XP_689415	+1.36		
Receptor-type tyrosine phosphatase N polypeptide 2	AAI63485	+1.56		
Fatty acid desaturase 2	NP_571720	+1.31		
Ornithine decarboxyase 1	AAV34288	+1.48		
GABA A receptor beta 2	NP_001019558	+1.37		
Solute carrier family 6 (GABA transporter), Member 1	AAT58230	+1.28		
Glula (glutamine synthase a)	NP_853537	+1.29		
Glutamate-cysteine ligase, catalytic subunit	NP_954971	+1.31		
Delta-aminolevulinic acid dehydratase	NP_001017645	+1.46		
Gamma-glutamyl cyclotransferase	NP_998170	+1.47		
Hematological and neurological expressed 1	NP_991176	+1.47		
MAP-kinase activating death domain-containing	CAF99926	+1.47		
Hsp 90b	O57521	+1.43		E
Hsp binding protein 1	NP_957048	+1.33		
Mdkb (midkine-related growth factor b)	NP_571791	+1.42		
Na,K-ATPase alpha subunit 3	BAB60722	+1.37		
ATPase, H+ transporting, lysosomal, V0 subunit c	NP_001098606	+1.22		
O-GlcNAc transferase	XP_694454	+1.48		E
Ubiquitin thiolesterase	NP_958885	+1.48	14667807 (human)	U
Ubiquitin-conjugating enzyme E2 variant 1	AAI07636	+1.37		U, E
Ubiquitin c-terminal hydrolase l1	NP_958885	+1.30	16734731 (human)	U
Cdc23	NP_957227	+1.35		U
SMT3 suppressor of MIF two 3 homolog 3	AAH68341	+1.30		U
Beta actin	P83750	+1.45		
Alpha actin	BAA08755	+1.27		
F-actin capping protein beta	ACH70650	+1.28		
FKBP12-rapamycin associated protein	AAC94996	+1.37		
Apolipoprotein Eb	NP_571173	+1.33		
Aldolase C	NP_919365	+1.43		
Pyruvate kinase	AAH67143	+1.27		
Enolase alpha nonneuron	AAH59511	+1.30		
Ckb (creatine kinase, brain)	NP_775329	+1.40		
Puromycin sensitive aminopeptidase	XP_684042	+1.37		
Myelin basic protein	AAW52552	+1.35		
GAPDH	NP_998259	+1.35		
Isotocin neurophysin	NP_840076	+1.34		
Neuropeptide Y	AAA49186	+1.26	16675543 (mouse)	
PACAP	NP_999880	+1.32		E
Stathmin	NP_001017850	+1.29		
Gpia (glucose phosphate isomerase a)	NP_658909	+1.35		
Protein tyrosine phosphatase, receptor D isoform 3	XP_685643	+1.35		
Jumonji containing 3, histone lysine demethylase	NP_001025349	+1.28		
HMP19; neuron specific gene family member 2	AAI52653	+1.32		
Lactate dehydrogenase-a	AAD40736	+1.40	17010207 (rat)	
Interferon-related developmental regulator 1 (ifrd1)	NP_001070023	+1.68		
Hypothetical protein LOC447815	NP_001004554	+1.31		
Meiotic recombination 11 homolog A	NP_001001407	+1.48		
Integral membrane protein 2B	NP_955940	+1.31		
DAZ associated protein 1	AAI53535	+1.52		
High-mobility group box 2	AAH81415	+1.26		
High mobility group-T protein	NP_001092721	+1.24		
zgc:110141	NP_001017856	+1.32		
wu:fj40 g07	XP_695097	+1.28		
Similar to phospholysine phosphohistidine inorganic	NP_001092251	+1.64		
Matrix metalloproteinase 9	NP_998288	+1.40	11245078 (rat) 18083300 (goldfish)	

Continued

Table 2.—*Continued*

Gene Name (up, 68)	GenBank ID[1]	Fold[2]	PMID[3]	Function[4]
Somatostatin receptor type five, subtype B	AAN76495	+1.46	9528936(rat)	S
Glucagon receptor precursor	AAS93685	+1.20		

[1]GenBank ID: Protein ID for homologs sequences from fish except NP_077305, for which we could not find sequence entry for fish homolog. [2]Fold: Gene expression fold change from microarray data analysis. [3]PMID: PubMed ID for reference that shows regulation of E2 on the candidate gene in specific species. [4]Function: Functional themes that include the effect on E2 action via either regulating nuclear estrogen receptor (nER) expression or activity (E), calcium binding activity (C), ubiquitin-proteasome pathway-related (U), and somatostatin-related (S). Gene name in boldface indicates that transcript was further analyzed by real-time PCR.

affect calcium signaling in the brain by changing the expression of the calcium binding proteins. This is further supported by functional annotations with domain information for these proteins. From the current analysis, there are 12 proteins shown to have calcium binding activity: calmodulin, SNAP25, EPD I, EPD II, neurogranin, visinin-like 1, similar to testican 3, EF hand domain containing 2, secretogranin II precursor, S100 calcium binding protein-β, parvalbumin-α, and fish-egg lectin (functional theme C in Tables 2 and 3).

Literature searches also retrieved several genes whose homologs are somehow functionally related to E2 actions (functional theme E in Tables 2 and 3). These genes fall into two types of regulation of E2 actions: one is direct regulation of nER activity, which includes calmodulin (5), HSP90b (29), O-GlcNAc transferase (7), ubiquitin-conjugating enzyme E2 (UCE2) variant 1 (39), activin βA (11), and the dynein light chain (22, 48). The second type is regulation of nER mRNA levels by such factors as PACAP (9) and activin-βA (28). While speculative, observed changes in the expression of PACAP and activin-βA following fadrozole treatment could potentially affect ER expression further modulating E2 actions in the brain. Other gene groups identified include the ubiquitin-proteasome (UP) pathway-related genes, ubiquitin thiolesterase, ubiquitin-conjugating enzyme E2, ubiquitin c-terminal hydrolase L1, and cdc23 and SMT3 suppressor of MIF two 3 homolog 3 (functional theme U), and three somatostatin-

Table 3. *List of downregulated genes identified by microarray analysis in female goldfish after fadrozole treatment (q value < 5%)*

Gene Name (down, 30)	GenBank ID[1]	Fold[2]	PMID[3]	Function[4]
Parvalbumin alpha	NP_991137	−1.32	15276619 (rat)	C
Fish-egg lectin	AAK01373	−1.31		C
Preprosomatostatin-14	NP_898893	−1.87	12097812 (goldfish)	S
Somatostatin receptor type 2	XP_694885	−1.51	9528936 (rat) 9705075 (human)	S
Fatty acid binding protein 2, intestinal	AAH75970	−1.41		
zgc:55455	AAI64355	−1.45		
Activin beta A	AAD50448	−1.94		E
RD RNA binding protein	NP_001002375	−1.40		
Kinectin 1	AAH44389	−1.43		
Hypothetical protein LOC567341	NP_001020721	−1.33		
Solute carrier family 7 (cationic amino acid transporter) member 3	NP_001007330	−1.55		
Ribosomal protein S4, X-linked (rps4x)	NP_001005589	−1.34		
U6 snRNA-associated Sm-like protein LSm7	AAI33958	−1.30		
BolA-like 3	AAH78189	−1.29		
Death associated protein 1a (dap1a)	ACI70034	−1.31		
Farnesyltransferase, CAAX box, beta	NP_001002128	−1.34		
Matrix metallopeptidase 13 (Collagenase 3)	XP_685843	−1.31	12893436 (rat) 16919424 (rabbit)	
Cadherin 1, epithelial (cdh1)	NP_571895	−1.28		
Pecanex-like protein 3	XP_690089	−1.30		
sc:d0375	XP_001923590	−1.27		
Small Hsp B15	ABQ57502	−1.30		
cL41a ribosomal protein L41 mRNA	AAM77969	−1.26		
PRP6 premRNA processing factor 6	NP_997820	−1.27		
Pyridoxine (pyridoxine, vitamin B6) kinase	NP_001119921	−1.28		
Muscle-specific beta 1 integrin binding protein	AAI52227	−1.31		
Titin-like (ttnl)	NP_997066	−1.34		
Cytokine receptor-like factor 3	CAJ80719	−1.28		
Dynein, light chain, LC8-type 2	NP_998189	−1.25		E
Heterogeneous nuclear ribonucleoprotein U	NP_001028767	−1.28		
wu:fc14a08	XP_696080	−1.28		

[1]GenBank ID: Protein ID for homologs sequences from fish. [2]Fold: Gene expression fold change from microarray data analysis. [3]PMID: PubMed ID for reference that shows regulation of E2 on the candidate gene in specific species. [4]Function: Functional themes that include the effect on E2 action via either regulating nER expression or activity (E), calcium binding activity (C) and somatostatin-related (S). Gene name in boldface indicates that transcript was further analyzed by real-time PCR.

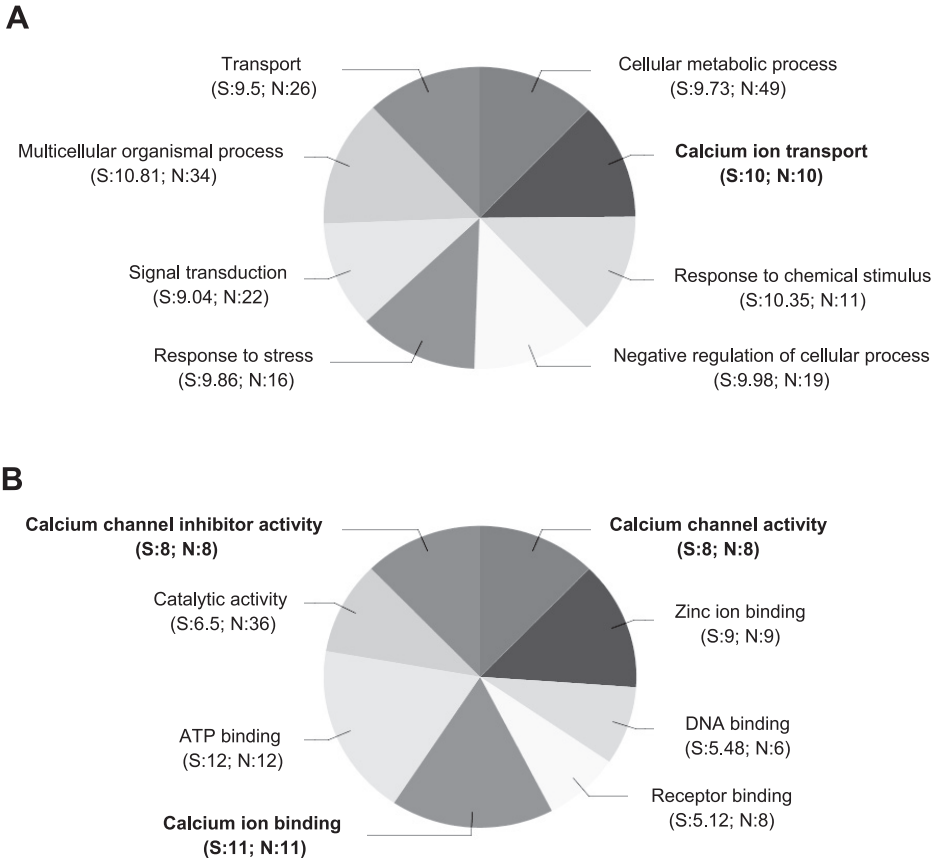

Fig. 4. Gene Ontology (GO) term analysis of differentially expressed genes after fadrozole treatment. *A*: sequence distribution based on GO biological process terms whose confluence scores are higher than cutoff value of 9. *B*: sequence distribution based on GO molecular function terms whose confluence scores are higher than cutoff value of 5. The specific score (S) and the number of genes (N) for the individual GO term are indicated.

related genes, preprosomatostatin-14, somatostatin receptor type 2 and somatostatin receptor type 5 subtype B (functional theme S), which are known E2 targets (26, 49, 66).

Real-time PCR verifications. Real-time PCR was used to validate the expression change of four genes in Tel as determined in the microarray analysis. Expression changes of these same genes in the Hyp, another major neuroendocrine region, were also examined. Except for aldolase c, three other genes including calmodulin, activin-βA, and ODC1 exhibited significant changes in both the Tel and the Hyp (Fig. 5). Calmodulin is a calcium binding protein as identified by GO and domain analysis. After fadrozole treatment, calmodulin mRNA level increased 1.3-fold ($P = 0.031$) in the Tel and 1.4-fold ($P = 0.004$) in the Hyp (Fig. 5*A*). Activin-βA is a member of the transforming growth factor-β (TGF-β) superfamily (25). It was chosen for its functional relevance to ER action (E2 function theme in Tables 2 and 3) (11). After fadrozole treatment, activin-βA expression is decreased ~30% in both the Tel ($P = 0.009$) and the Hyp ($P = 0.018$) (Fig. 5*B*).

ODC1 is the key enzyme responsible for catalyzing the reaction of ornithine to putrescine in the ODC/polyamine system (51). Putrescine has been shown to be an alternative precursor source of the neurotransmitter γ-aminobutyric acid (GABA) (53). We observed an increase in the ODC1 mRNA level in both Tel (2-fold; $P = 0.002$) and the Hyp (1.7-fold; $P = 0.008$) after fadrozole treatment (Fig. 5*C*). Aldolase c expression was not significantly affected ($P = 0.087$; Fig. 5*D*); however, the direction of the expression change (1.2-fold) was consistent with the microarray analysis. It should be noted that all significant expression fold changes ranged from 1.3 to 2, which is typical in central nervous tissues and highly similar to previous observations in fish brain (32).

DISCUSSION

In the present study, we used the goldfish model to evaluate the effects of fadrozole on neuroendocrine and reproductive functions. In contrast to other studies, we treated the female goldfish from March to April, the seasonal period when goldfish have maximal brain aromatase activity and high serum E2 levels (42, 55). We intended to produce an E2-deficient phenotype by fadrozole treatment. Thus, we hypothesized that, compared with control goldfish that have been exposed to the normal seasonal E2 profile and physiological expression changes of E2-regulated genes, fadrozole-treated fish will exhibit opposite and contrasting effects on physiology and gene expression.

We first confirmed and characterized the physiological effects of fadrozole by two approaches. There was a significant decrease in serum E2 level for the female goldfish treated with fadrozole. We also observed that fadrozole treatment affected ovarian development. The GSI of controls was ~3.9, while those receiving fadrozole was ~2.2, which is more typical of midrecrudescent fish in December or January (71). Although based on a small subsample, fadrozole-treated fish ovaries only contained oocytes at early stage of the development, which was in contrast to control fish ovaries that exhibited more advanced development. Our results are comparable to the histological observations in the fadrozole-treated adult female fathead minnow (*Pimephales promelas*) (2).

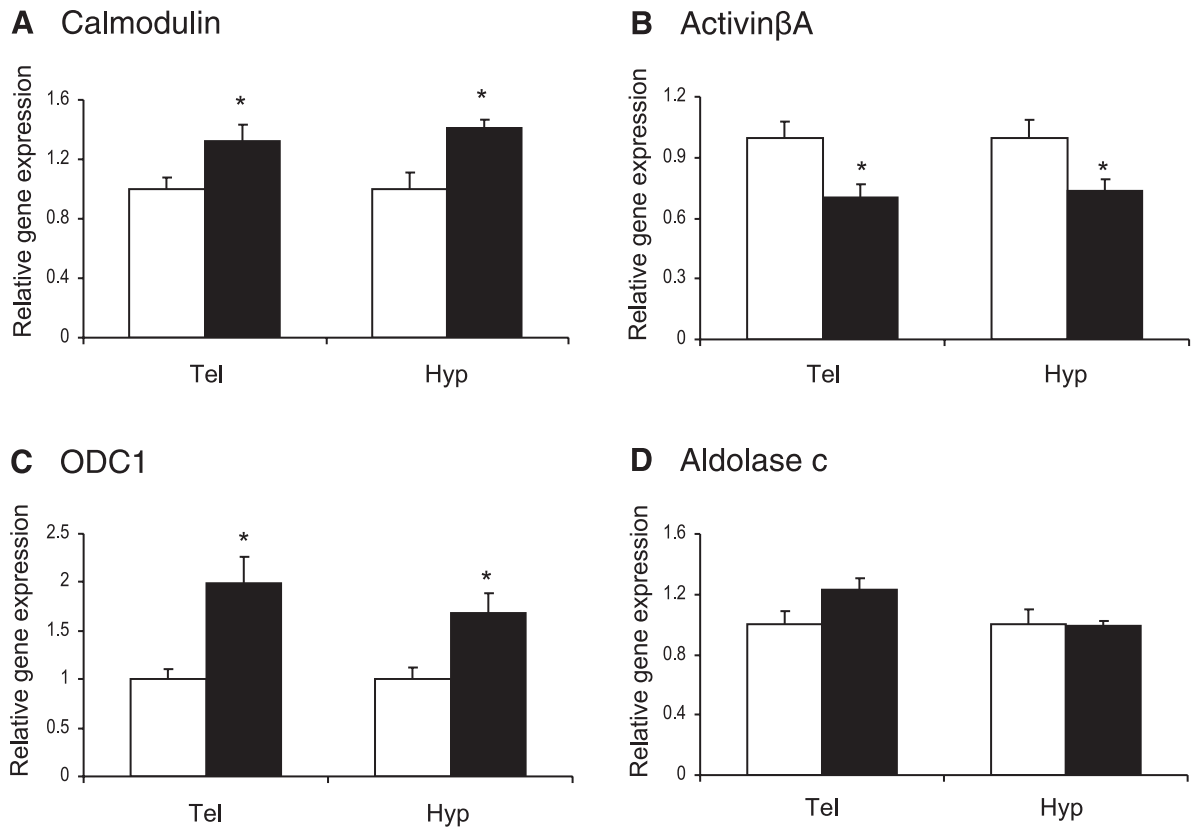

Fig. 5. Relative gene expression change in both Tel and Hyp after fadrozole treatment as determined with real-time PCR. Four genes were tested including calmodulin (*A*), activin-βA (*B*), ornithine decarboxylase 1 (ODC1) (*C*), and aldolase c (*D*). EF1α was used as the internal reference gene. Control samples are shown as white bars, whereas fadrozole-treated samples are shown as black bars. Mean + SE values are shown for each group ($n = 10–12$). *Significant differences between control and treatment groups ($P < 0.05$ by *t*-test).

As expected, we found that AroB mRNA is significantly lower in both the Tel and the Hyp after fadrozole treatments. AroB is a direct E2 target in the fish brain and the estrogen-response element in the goldfish promoter has been characterized (12). Decreased AroB expression in Tel and Hyp therefore suggests an effective inhibition of E2 production and further inhibition of nER-mediated action. We used cDNA microarrays to profile global gene expression changes in the Tel that may be associated with fadrozole-induced E2 reduction. We identified numerous candidate estrogen-responsive genes and confirmed the expression change of calmodulin, activin-βA, and ODC1 by real-time PCR. Some other genes, for example MMP9 (31) and preprosomatostatin-14 (13), have also been shown to be E2-regulated in the goldfish brain. We also identified additional genes whose homologs are subjected to E2 regulation in other species. These observations support the hypothesis that fadrozole treatment can reverse E2 action to regulate gene expression. Moreover, the conserved transcriptional regulation by estrogen of those genes in different species may indicate an ancestral regulatory mechanism that are most likely located at some evolutionarily conserved EREs in promoter regions. Further comparative genomic analyses and functional characterizations are needed to substantiate this hypothesis.

Gene expression changes in the brain associated with E2 reduction provide functional insights into physiological actions of estrogens. Firstly, GO analysis together with domain annotations identified 12 gene products, including calmodulin, that possess calcium binding activity. This indicates that E2 deprivation impacted calcium signaling via E2-dependent transcriptional pathways. It has been extensively documented that in neurons of mammals (rat, pig, and monkey), E2 can regulate cellular calcium concentration (24, 47) and oscillations (1, 40). This E2 effect has been shown to be mediated by a membrane-initiated signaling pathway of E2 involving the G protein-coupled receptor 30 (1, 40). Moreover, in rat pituitary cells, E2 can also regulate calcium signaling by affecting the expression of some calcium-binding proteins, such as SNAP25 (63) and parvalbumin (17). In teleost fish, E2 is known to induce an increase in circulating levels of vitellogenin (8, 36), a calcium binding protein from the liver, and a parallel increase in blood plasma calcium (21, 44). However, the molecular mechanisms involved in estrogen-regulated calcium signaling and associated physiological effects in fish are still largely unknown. Our findings suggest that E2 can regulate the calcium signaling pathway in fish brain by modulating the gene expression of calcium-binding proteins.

Numerous differentially regulated genes have been implicated in ER-mediated actions (functional theme E in Tables 2 and 3), which are typified by calmodulin (18, 30), hsp90b (10, 29), O-GlcNAc transferase (7), UCE2 variant 1 (39, 59), and the dynein light chain (22, 48). The expression change of calmodulin was verified by real-time PCR in the current study. Calmodulin, hsp90b, and the dynein light chain are required for

the transcriptional activity of ERα. By directly interacting with ERα (14), calmodulin modulates the ability of ERα to bind to a ligand (6) or to the ERE (5) and promotes receptor stability by preventing UP degradation (30). Hsp90b also contributes to protecting the ER against the UP pathway (29), whereas dynein light chain has been recently shown to link nER to other chromatin remodeling complexes (22). In contrast, O-GlcNAc transferase and UCE2 variant 1 and other proteins including ubiquitin thiolesterase, ubiquitin c-terminal hydrolase L1, cdc23, and SMT3 suppressor of MIF two 3 homolog 3 (functional theme U in Table 2) are all functionally related to the UP degradation pathway, which regulates nER stability (7, 39, 59). The differential expression of these modulatory factors after fadrozole treatments suggests an autoregulation mechanism of E2 action in the neuroendocrine brain. Moreover, in another study where male goldfish were treated with E2, a decrease in the expression of another UCE2 enzyme was observed in the Hyp (31), further indicating estrogenic regulation of these pathways.

The differential expression of activin-βA provides another regulatory mechanism for the cross talk between E2 and activin signaling in fish. The activin-βA subunit along with activin-βB, -αA, and -αB are members of the TGF-β superfamily. Activin is a dimeric growth factor formed by two subunits and binds the membrane serine/threonine kinase receptors (33), which then induce the activation of downstream signaling pathways and affect activities of transcriptional factors such as Smad (50). Very recently, activin signaling was shown to regulate ERα activity in the human breast cancer cells (11) and induce expression of ERβ in the mouse granulosa cells (28). Moreover, E2 has been shown to regulate the expression of the activin-β subunits in the mouse neonatal ovary (27) and type II activin receptor in rat Hyp (56). Therefore, it seems that there is a cross talk between E2 and activin signaling in mammals. In our study, fadrozole treatment resulted in a decrease in activin-βA expression in the Tel, which supports a regulatory cross talk between activin and E2 signaling in the fish brain.

ODC1 is another gene whose expression was confirmed as increased after fadrozole treatment. ODC1 catalyzes the formation of putrescine from ornithine, a rate-limiting step during the biosynthesis of the polyamines spermidine and spermine (51). This ODC/polyamine system has been shown to be important for neuronal cell development (51) and modulates the ion channel and calcium-dependent transmitter release (3). Moreover, there is emerging recognition that putrescine is an alternative precursor source of GABA (53, 67), a major neurotransmitter that has an important reproductive role because of its stimulatory actions on gonadotropin-releasing hormone and luteinizing hormone release in goldfish (46). The increased ODC1 expression after fadrozole treatment may indicate an enhancement of the ODC/polyamine system and/or suggests a new pathway for E2 to regulate GABA production in addition to the known effects of E2 on glutamic acid decarboxylase in goldfish (46).

In conclusion, we used fadrozole to impair estrogen production in the female goldfish at a crucial stage in the reproductive cycle and evaluated the associated physiological effects on neuroendocrine and reproductive systems. We profiled gene expression changes in the brain upon fadrozole-induced E2 reduction that was further verified by the real-time PCR. Functional annotations and GO analysis revealed several regulatory themes for physiological E2 actions in the fish brain, including the regulation of the calcium signaling pathway and autoregulation of nER action by influencing global gene expression. Our results may serve as a basis for understanding estrogen action and estrogenic endocrine disruption of neuroendocrine function in the vertebrate brain.

ACKNOWLEDGMENTS

The authors acknowledge Bill Fletcher for animal care and Mandy Woodhouse and Vicki Marlatt for help and discussions of RIA. We also thank Prof. Andrew Cossins and Dr. Margaret Hughes (Liverpool Microarray Facility) for microarray production, assistance, and advice.

GRANTS

This work was supported by a University of Ottawa International scholarship (D. Zhang, P. Duarte-Guterman), Ontario Graduate Scholarships (C. J. Martyniuk, J. T. Popesku), Ontario Graduate Scholarship in Science and Technology (H. Xiong), Natural Sciences and Engineering Research Council of Canada Discovery and Strategic programs (V. L. Trudeau, X. Xia).

REFERENCES

1. **Abe H, Keen KL, Terasawa E.** Rapid action of estrogens on intracellular calcium oscillations in primate luteinizing hormone-releasing hormone-1 neurons. *Endocrinology* 149: 1155–1162, 2008.
2. **Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, Leino RL.** Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). *Toxicol Sci* 67: 121–130, 2002.
3. **Bernstein HG, Muller M.** The cellular localization of the L-ornithine decarboxylase/polyamine system in normal and diseased central nervous systems. *Prog Neurobiol* 57: 485–505, 1999.
4. **Bhandari RK, Komuro H, Higa M, Nakamura M.** Sex inversion of sexually immature honeycomb grouper (*Epinephelus merra*) by aromatase inhibitor. *Zoolog Sci* 21: 305–310, 2004.
5. **Biswas DK, Reddy PV, Pickard M, Makkad B, Pettit N, Pardee AB.** Calmodulin is essential for estrogen receptor interaction with its motif and activation of responsive promoter. *J Biol Chem* 273: 33817–33824, 1998.
6. **Bouhoute A, Leclercq G.** Antagonistic effect of triphenylethylenic antiestrogens on the association of estrogen receptor to calmodulin. *Biochem Biophys Res Commun* 184: 1432–1440, 1992.
7. **Bowe DB, Sadlonova A, Toleman CA, Novak Z, Hu Y, Huang P, Mukherjee S, Whitsett T, Frost AR, Paterson AJ, Kudlow JE.** O-GlcNAc integrates the proteasome and transcriptome to regulate nuclear hormone receptors. *Mol Cell Biol* 26: 8539–8550, 2006.
8. **Bradley JT, Grizzle JM.** Vitellogenin induction by estradiol in channel catfish, Ictalurus punctatus. *Gen Comp Endocrinol* 73: 28–39, 1989.
9. **Bryant WM, Gibson MA, Shupnik MA.** Stimulation of the novel estrogen receptor-alpha intronic TERP-1 promoter by estrogens, androgen, pituitary adenylate cyclase-activating peptide, and forskolin, and autoregulation by TERP-1 protein. *Endocrinology* 147: 543–551, 2006.
10. **Buchner J.** Hsp90 & Co - a holding for folding. *Trends Biochem Sci* 24: 136–141, 1999.
11. **Burdette JE, Woodruff TK.** Activin and estrogen crosstalk regulates transcription in human breast cancer cells. *Endocr Relat Cancer* 14: 679–689, 2007.
12. **Callard GV, Tchoudakova AV, Kishida M, Wood E.** Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. *J Steroid Biochem Mol Biol* 79: 305–314, 2001.
13. **Canosa LF, Lin X, Peter RE.** Regulation of expression of somatostatin genes by sex steroid hormones in goldfish forebrain. *Neuroendocrinology* 76: 8–17, 2002.
14. **Castoria G, Migliaccio A, Nola E, Auricchio F.** In vitro interaction of estradiol receptor with Ca^{2+}-calmodulin. *Mol Endocrinol* 2: 167–174, 1988.
15. **Fenske M, Segner H.** Aromatase modulation alters gonadal differentiation in developing zebrafish (*Danio rerio*). *Aquat Toxicol* 67: 105–126, 2004.
16. **Forlano PM, Schlinger BA, Bass AH.** Brain aromatase: new lessons from non-mammalian model systems. *Front Neuroendocrinol* 27: 247–274, 2006.

17. **Fujimoto N, Igarashi K, Kanno J, Honda H, Inoue T.** Identification of estrogen-responsive genes in the GH3 cell line by cDNA microarray analysis. *J Steroid Biochem Mol Biol* 91: 121–129, 2004.
18. **Gallo D, Jacquot Y, Laurent G, Leclercq G.** Calmodulin, a regulatory partner of the estrogen receptor alpha in breast cancer cells. *Mol Cell Endocrinol* 291: 20–26, 2008.
19. **Garcia-Ovejero D, Azcoitia I, Doncarlos LL, Melcangi RC, and Garcia-Segura LM.** Glia-neuron crosstalk in the neuroprotective mechanisms of sex steroid hormones. *Brain Res Brain Res Rev* 48: 273–286, 2005.
20. **Gotz S, Garcia-Gomez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talon M, Dopazo J, Conesa A.** High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res* 36: 3420–3435, 2008.
21. **Guerreiro PM, Fuentes J, Canario AV, Power DM.** Calcium balance in sea bream (*Sparus aurata*): the effect of oestradiol-17beta. *J Endocrinol* 173: 377–385, 2002.
22. **Hu Q, Kwon YS, Nunez E, Cardamone MD, Hutt KR, Ohgi KA, Garcia-Bassets I, Rose DW, Glass CK, Rosenfeld MG, Fu XD.** Enhancing nuclear receptor-induced transcription requires nuclear motor and LSD1-dependent gene networking in interchromatin granules. *Proc Natl Acad Sci USA* 105: 19199–19204, 2008.
23. **Huddleston GG, Paisley JC, Clancy AN.** Effects of estrogen in the male rat medial amygdala: infusion of an aromatase inhibitor lowers mating and bovine serum albumin-conjugated estradiol implants do not promote mating. *Neuroendocrinology* 83: 106–116, 2006.
24. **Joels M, Karst H.** Effects of estradiol and progesterone on voltage-gated calcium and potassium conductances in rat CA1 hippocampal neurons. *J Neurosci* 15: 4289–4297, 1995.
25. **Keah HH, Hearn MT.** A molecular recognition paradigm: promiscuity associated with the ligand-receptor interactions of the activin members of the TGF-beta superfamily. *J Mol Recognit* 18: 385–403, 2005.
26. **Kimura N, Tomizawa S, Arai KN.** Chronic treatment with estrogen up-regulates expression of sst2 messenger ribonucleic acid (mRNA) but down-regulates expression of sst5 mRNA in rat pituitaries. *Endocrinology* 139: 1573–1580, 1998.
27. **Kipp JL, Kilen SM, Bristol-Gould S, Woodruff TK, Mayo KE.** Neonatal exposure to estrogens suppresses activin expression and signaling in the mouse ovary. *Endocrinology* 148: 1968–1976, 2007.
28. **Kipp JL, Kilen SM, Woodruff TK, Mayo KE.** Activin regulates estrogen receptor gene expression in the mouse ovary. *J Biol Chem* 282: 36755–36765, 2007.
29. **Lee MO, Kim EO, Kwon HJ, Kim YM, Kang HJ, Kang H, Lee JE.** Radicicol represses the transcriptional function of the estrogen receptor by suppressing the stabilization of the receptor by heat shock protein 90. *Mol Cell Endocrinol* 188: 47–54, 2002.
30. **Li L, Li Z, Howley PM, Sacks DB.** E6AP and calmodulin reciprocally regulate estrogen receptor stability. *J Biol Chem* 281: 1978–1985, 2006.
31. **Marlatt VL, Martyniuk CJ, Zhang D, Xiong H, Watt J, Xia X, Moon T, Trudeau VL.** Auto-regulation of estrogen receptor subtypes and gene expression profiling of 17beta-estradiol action in the neuroendocrine axis of male goldfish. *Mol Cell Endocrinol* 283: 38–48, 2008.
32. **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 17alpha-ethinylestradiol. *Physiol Genomics* 27: 328–336, 2006.
33. **Mathews LS.** Activin receptors and cellular signaling by the receptor serine kinase family. *Endocr Rev* 15: 310–325, 1994.
34. **McMaster ME, Munkittrick KR, Van Der Kraak GJ.** Protocol for measuring circulating levels of gonadal sex steroids in fish. *Canadian Technical Report of Fisheries and Aquatic Sciences* 1–19, 1992.
35. **Mennigen JA, Martyniuk CJ, Crump K, Xiong H, Zhao E, Popesku J, Anisman H, Cossins AR, Xia X, Trudeau VL.** Effects of fluoxetine on the reproductive axis of female goldfish (*Carassius auratus*). *Physiol Genomics* 35: 273–282, 2008.
36. **Mosconi G, Gallinelli A, Polzonetti-Magni AM, Facchinetti F.** Acetyl salmon endorphin-like and interrenal stress response in male gilthead sea bream, Sparus aurata. *Neuroendocrinology* 68: 129–134, 1998.
37. **Mouriec K, Pellegrini E, Anglade I, Menuet A, Adrio F, Thieulant ML, Pakdel F, Kah O.** Synthesis of estrogens in progenitor cells of adult fish brain: evolutive novelty or exaggeration of a more general mechanism implicating estrogens in neurogenesis? *Brain Res Bull* 75: 274–280, 2008.
38. **Navarro-Martin L, Blazquez M, Piferrer F.** Masculinization of the European sea bass (*Dicentrarchus labrax*) by treatment with an androgen or aromatase inhibitor involves different gene expression and has distinct lasting effects on maturation. *Gen Comp Endocrinol* 160: 3–11, 2009.
39. **Nawaz Z, Lonard DM, Dennis AP, Smith CL, O'Malley BW.** Proteasome-dependent degradation of the human estrogen receptor. *Proc Natl Acad Sci USA* 96: 1858–1862, 1999.
40. **Noel SD, Keen KL, Baumann DI, Filardo EJ, Terasawa E.** Involvement of G protein-coupled receptor 30 (GPR30) in rapid action of estrogen in primate LHRH neurons. *Mol Endocrinol* 23: 349–359, 2009.
41. **Olmstead AW, Kosian PA, Korte JJ, Holcombe GW, Woodis KK, Degitz SJ.** Sex reversal of the amphibian, *Xenopus tropicalis*, following larval exposure to an aromatase inhibitor. *Aquat Toxicol* 2008.
42. **Pasmanik M, Callard GV.** Changes in brain aromatase and 5 alpha-reductase activities correlate significantly with seasonal reproductive cycles in goldfish (*Carassius auratus*). *Endocrinology* 122: 1349–1356, 1988.
43. **Pellegrini E, Menuet A, Lethimonier C, Adrio F, Gueguen MM, Tascon C, Anglade I, Pakdel F, Kah O.** Relationships between aromatase and estrogen receptors in the brain of teleost fish. *Gen Comp Endocrinol* 142: 60–66, 2005.
44. **Persson P, Sundell K, Bjornsson BT.** Estradiol-19beta induced calcium uptake and resporption in juvenile rainbow trout *Oncorhynchus mykiss*. *Fish Physiol Biochem* 13: 379–386, 1994.
45. **Piferrer F, Zanuy S, Carrillo M, Solar II, Devlin RH, Donaldson EM.** Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males. *J Exp Zool* 270: 255–262, 1994.
46. **Popesku JT, Martyniuk CJ, Mennigen J, Xiong H, Zhang D, Xia X, Cossins AR, Trudeau VL.** The goldfish (*Carassius auratus*) as a model for neuroendocrine signaling. *Mol Cell Endocrinol* 293: 43–56, 2008.
47. **Qiu J, Bosch MA, Jamali K, Xue C, Kelly MJ, Ronnekleiv OK.** Estrogen upregulates T-type calcium channels in the hypothalamus and pituitary. *J Neurosci* 26: 11072–11082, 2006.
48. **Rayala SK, den Hollander P, Balasenthil S, Yang Z, Broaddus RR, Kumar R.** Functional regulation of oestrogen receptor pathway by the dynein light chain 1. *EMBO Rep* 6: 538–544, 2005.
49. **Scanlan N, Dufourny L, Skinner DC.** Somatostatin-14 neurons in the ovine hypothalamus: colocalization with estrogen receptor alpha and somatostatin-28(1-12) immunoreactivity, and activation in response to estradiol. *Biol Reprod* 69: 1318–1324, 2003.
50. **Shi Y, Massague J.** Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113: 685–700, 2003.
51. **Slotkin TA, Bartolome J.** Role of ornithine decarboxylase and the polyamines in nervous system development: a review. *Brain Res Bull* 17: 307–320, 1986.
52. **Thomas P, Pang Y, Filardo EJ, Dong J.** Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* 146: 624–632, 2005.
53. **Tillakaratne NJ, Medina-Kauwe L, Gibson KM.** gamma-Aminobutyric acid (GABA) metabolism in mammalian neural and nonneural tissues. *Comp Biochem Physiol A Physiol* 112: 247–263, 1995.
54. **Troyanskaya O, Cantor M, Sherlock G, Brown P, Hastie T, Tibshirani R, Botstein D, Altman RB.** Missing value estimation methods for DNA microarrays. *Bioinformatics* 17: 520–525, 2001.
55. **Trudeau VL, Peter RE, Sloley BD.** Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. *Biol Reprod* 44: 951–960, 1991.
56. **Trudeau VL, Pope L, de Winter JP, Hache RJ, Renaud LP.** Regulation of activin type-II receptor mRNA levels in rat hypothalamus by estradiol in vivo. *J Neuroendocrinol* 8: 395–401, 1996.
57. **Tsai MJ, O'Malley BW.** Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 63: 451–486, 1994.
58. **Tusher VG, Tibshirani R, Chu G.** Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 98: 5116–5121, 2001.
59. **Verma S, Ismail A, Gao X, Fu G, Li X, O'Malley BW, Nawaz Z.** The ubiquitin-conjugating enzyme UBCH7 acts as a coactivator for steroid hormone receptors. *Mol Cell Biol* 24: 8716–8726, 2004.
60. **Villeneuve DL, Knoebl I, Larkin P, Miracle AL, Carter BJ, Denslow ND, Ankley GT.** Altered gene expression in the brain and liver of female fathead minnows Pimephales promelas Rafinesque exposed to fadrozole. *J Fish Biol* 72: 2281–2340, 2008.
61. **Villeneuve DL, Larkin P, Knoebl I, Miracle AL, Kahl MD, Jensen KM, Makynen EA, Durhan EJ, Carter BJ, Denslow ND, Ankley GT.**

A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. *Environ Sci Technol* 41: 321–330, 2007.

62. **Wang RL, Bencic D, Biales A, Lattier D, Kostich M, Villeneuve D, Ankley GT, Lazorchak J, Toth G.** DNA microarray-based ecotoxicological biomarker discovery in a small fish model species. *Environ Toxicol Chem* 27: 664–675, 2008.
63. **Weiss JM, Huller H, Polack S, Friedrich M, Diedrich K, Treeck O, Pfeiler G, Ortmann O.** Estradiol differentially modulates the exocytotic proteins SNAP-25 and munc-18 in pituitary gonadotrophs. *J Mol Endocrinol* 38: 305–314, 2007.
64. **Williams DR, Li W, Hughes MA, Gonzalez SF, Vernon C, Vida MC, Jeney Z, Jeney G, Dixon P, McAndrew B, Bartfai R, Orban L, Trudeau VL, Rogers J, Matthews L, Fraser EJ, Gracey AY, Cossins AR.** Genomic resources and microarrays for the common carp *Cyprinus carpio L. J Fish Biol* 72: 2095–2117, 2008.
65. **Xiong H, Zhang D, Martyniuk CJ, Trudeau VL, Xia X.** Using generalized procrustes analysis (GPA) for normalization of cDNA microarray data. *BMC Bioinformatics* 9: 25, 2008.
66. **Xu Y, Berelowitz M, Bruno JF.** Characterization of the promoter region of the human somatostatin receptor subtype 2 gene and localization of sequences required for estrogen-responsiveness. *Mol Cell Endocrinol* 139: 71–77, 1998.
67. **Yamasaki EN, Barbosa VD, De Mello FG, Hokoc JN.** GABAergic system in the developing mammalian retina: dual sources of GABA at early stages of postnatal development. *Int J Dev Neurosci* 17: 201–213, 1999.
68. **Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP.** Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res* 30: e15, 2002.
69. **Zerulla M, Lange R, Steger-Hartmann T, Panter G, Hutchinson T, Dietrich DR.** Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (*Pimephales promelas*). *Toxicol Lett* 131: 51–63, 2002.
70. **Zhang D, Trudeau VL.** Integration of membrane and nuclear estrogen receptor signaling. *Comp Biochem Physiol A Mol Integr Physiol* 144: 306–315, 2006.
71. **Zhang D, Xiong H, Mennigen JA, Popesku JT, Marlatt VL, Martyniuk CJ, Crump K, Cossins AR, Xia X, Trudeau VL.** Defining neuroendocrine gene expression patterns associated with reproductive seasonality in fish. *PLoS ONE* 4: e5816, 2009.
72. **Zhang X, Hecker M, Park JW, Tompsett AR, Jones PD, Newsted JL, Au DW, Kong R, Wu RS, Giesy JP.** Time-dependent transcriptional profiles of genes of the hypothalamic-pituitary-gonadal axis in medaka (*Oryzias latipes*) exposed to fadrozole and 17 beta-trenbolone. *Environ Toxicol Chem* 27: 2504–2511, 2008.