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Auto-regulation of estrogen receptor subtypes and gene expression profiling of 17β-estradiol action in the neuroendocrine axis of male goldfish

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Abstract

Auto-regulation of the three goldfish estrogen receptor (ER) subtypes was examined simultaneously in multiple tissues, in relation to mRNA levels of liver vitellogenin (VTG) and brain transcripts. Male goldfish were implanted with a silastic implant containing either no steroid or 17β-estradiol (E2) (100 μg/g body mass) for one and seven days. Liver transcript levels of ERα were the most highly up-regulated of the ERs, and a parallel induction of liver VTG was observed. In the testes (7 d) and telencephalon (7 d), E2 induced ERα. In the liver (1 d) and hypothalamus (7 d) ERβ1 was down-regulated, while ERβ2 remained unchanged under all conditions. Although aromatase B levels increased in the brain, the majority of candidate genes identified by microarray in the hypothalamus (1 d) decreased. These results demonstrate that ER subtypes are differentially regulated by E2, and several brain transcripts decrease upon short-term elevation of circulating E2 levels.

Keywords: Estrogen receptors; Fish; Aromatase; Gene expression

1. Introduction

Estrogens in vertebrates influence a wide range of physiological processes including growth, reproduction, and general homeostasis by exerting effects on both reproductive and non-reproductive target tissues (Klinge, 2000; Nilsson et al., 2001; Martyniuk et al., 2006a). Many of the actions of the main estrogen, 17β-estradiol (E2), are mediated by the nuclear estrogen receptors (ERs), that act as ligand-activated transcription factors (Katzenellenbogen and Katzenellenbogen, 1996). The E2-activated ER dimerizes and binds to estrogen response elements in the promoters of estrogen-responsive genes, and modulates the transcription of these genes through interactions with other transcription factors and the components of the transcription initiation complex (Tsai and O'Malley, 1994; Klinge, 2000).

In fish, three nuclear ER genes have been identified and characterized as ERα, ERβ1 and ERβ2 or ERγ (Hawkins et al., 2000; Ma et al., 2000; Legler et al., 2002; Menuet et al., 2002), but their exact functions are not well understood. Previous studies in several species established that ERα is inducible by E2 and E2 mimics (Pakdel et al., 1997; Yadetie et al., 1999; Bowman et al., 2002), suggesting that the ERs are auto-regulated. However, possible ER auto-regulation including the ERβ1 and ERβ2 subtypes in most fish species has not yet been studied in detail.

The induction of hepatic vitellogenin (VTG), a precursor yolk protein, in response to estrogens by an ER-mediated pathway is well documented in several oviparous fish species (Ryffel, 1978; Pakdel et al., 1991), and VTG in males is widely accepted as a biomarker of exposure to environmental estrogens (Tyler et al., 1996; Flouriot et al., 1997; Arcand-Hoy and Benson, 1998; Denslow et al., 1999; Hutchinson and Pickford, 2002; Filby et al., 2006). Nonetheless, whether each ER subtype contributes equally to the transcriptional regulation of the VTG gene has not been determined in many species. In largemouth bass (*Micropterus salmoides*) and Atlantic salmon (*Salmo salar*), the ERα subtype is highly correlated with VTG mRNA levels in the liver (Sabo-Attwood et al., 2004; Meucci and Arukwe, 2006). Goldfish (*Carassius auratus*), a model aquatic species, exhibits overlapping tissue distributions of ERα, ERβ1 and ERβ2, however, the role of each ER subtype in the regulation of genes such as VTG *in vivo* is unknown.

Many studies have examined the receptor-mediated effects of E2 and environmental estrogens/anti-estrogens (xenoestrogens) in fish using *in vitro* and *in vivo* assays that examine hepatic, and

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to a lesser extent, gonadal ERα levels. In marked contrast, few studies have employed transcriptomic analysis to investigate the effects of E2 on the fish brain. This is surprising given that ERs are widely expressed in the brain and because neuroestrogen production is an evolutionarily conserved vertebrate trait (Callard et al., 2001). Brain regions of particular importance to neuroendocrine control are the hypothalamus and telencephalon. The hypothalamus is a major integrating center that receives neural inputs from both internal and external sources and converts them to hormonal signals via the pituitary, which ultimately regulates development, growth, and reproduction (Trudeau, 1997; McEwen, 2001). The telencephalon contains the preoptic area, which plays an important role in the central nervous system control of growth, reproduction and behaviour in fish (Blazquez et al., 1998). Sex steroids, including E2, exert both positive and negative feedback at the level of the preoptic area, hypothalamus and pituitary to regulate luteinizing hormone release (Trudeau, 1997; Blazquez et al., 1998). Obtaining gene expression profiles in the neuroendocrine axis of model fish species following exposure to E2 or xenoestrogens will help to establish links between genomic and physiological data, and will elucidate the impact of endocrine disrupting chemicals on aquatic vertebrates.

This study describes *in vivo* E2 exposure experiments using adult male goldfish as a model to test the following hypotheses: (1) ERα, ERβ1 and ERβ2 subtypes are differentially autoregulated by E2 within and between tissues; (2) liver VTG induction by E2 parallels the up-regulation of one or more ER subtypes by E2; and (3) E2 regulates novel transcripts and pathways in the hypothalamus.

2. Materials and methods

2.1. Experimental animals and experimental design

Common goldfish (*C. auratus*) were purchased in August 2005 (Aleong's International Inc., Mississauga, ON, Canada), and were acclimatized to 18 °C under a natural photoperiod and fed with standard floating trout pellets. The experiment was conducted in the October 2005. After anaesthesia with tricaine methanesulphonate (MS222; 0.1 g/L) in the water, 60 gonad-intact, sexually regressed male goldfish (mean body weight 16.6 ± 0.7 g) were implanted in the intraperitoneal cavity with a control (no steroid) or solid silastic implant containing 17β-estradiol (E2; 100 μg/g body mass) for one and seven days (15 fish/70 L tank). This implantation protocol produces physiological blood E2 levels in the range of levels found in females at ovulation (Trudeau et al., 1991). After the exposure periods, fish were anaesthetized in MS222 and blood was collected via the caudal vein using heparinized syringes and centrifuged (5000 g for 15 min) to collect plasma which was stored at −80 °C. Fish were sacrificed by cervical transection and gonadal tissue was removed and weighed to determine the gonadosomatic index (gonad weight/body weight × 100). Other tissues were immediately removed, frozen on dry ice and stored at −80 °C until RNA isolation. Hypothalami and telencephali were pooled (2–3 samples/tube) to increase RNA yield prior to isolation of total RNA.

2.2. E2 radioimmunoassay

Steroids were extracted from 200 μL of plasma and measured according to (McMaster et al., 1992). A non-parametric test (Mann–Whitney *U*) was used to test for significant differences between groups ($p < 0.05$).

2.3. RNA isolation

Total RNA was isolated from 20 to 30 mg of frozen liver and 40–50 mg of frozen testes using the TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol; tissues were disrupted and homogenized using a MM301 Mixer Mill (Retsch, Newton, PA, USA). A sample of the crude RNA was then treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) for 30 min at 37 °C to degrade any remaining genomic DNA, followed by a 10-min incubation at 65 °C with RQ1 DNase Stop Solution (Promega) to inhibit the reaction. For brain tissues, total RNA was isolated using Qiagen's RNeasy Plus Mini Kit (Mississauga, ON, Canada). Total RNA (2 μg) was reverse transcribed into cDNA in a volume of 20 μL using Superscript II RNase H$^-$ reverse transcriptase (SSII) according to the manufacturer's protocol for use with random primers (Invitrogen).

2.4. ER multiplex real-time RT-PCR

A set of specific primers and probes were designed for *C. auratus* ERα, ERβ1, ERβ2 and β-actin based on known sequences using Premier Biosoft International's Beacon Designer 2.1 (Table 1). Amplicons for each primer and probe set were cloned and sequenced to confirm specificity. Primers were synthesized (Invitrogen) and gene-specific dual-labelled fluorescent probes were purchased from IDT DNA (Coralville, IA, USA). Each 20 μL reaction was diluted 5-fold in nuclease-free water and used as the template for the real-time RT-PCR assay. The Mx4000® Multiplex Quantitative PCR System (Stratagene, Madison, WI, USA) was used to amplify and detect the endogenous reference gene β-actin

Table 1
Dual-labelled fluorescent probes and oligonucleotide primers used for real-time RT-PCR multiplex assays and Basic Local Alignment Search Tool matches from nucleotide sequence of amplicons

Gene target, Genbank accession no.	Element	Sequence 5′–3′	Amplicon length (bp)
ERα, AY055725	Sense probe	Cy5-AGTCACTGCACACCGCACAGAAGC-BHQ2	77
	Sense primer	GCAGGAGGGTTTGATTCTGAGA	
	Anti-sense primer	CCATAATGATAGCCGGACGCA	
ERβ1, AF061269	Sense probe	FAM-CACCATCGACAAGAACCGCCGCAA-BHQ1	96
	Sense primer	TGACTACATCTGCCCTGCCA	
	Anti-sense primer	CCAACTTCGTAACATTTTCGGAGA	
ERβ2, AF177465	Sense probe	ROX NHS-TCCCCACCCCACACACTCTACGCC-BHQ2	96
	Sense primer	TGGTCCCTTTAAATTCAGCAATCT	
	Anti-sense primer	GTGTTTCCCTGTAGGCCAGTG	
β-actin, AB039726	Sense probe	HEX-TCACGACCAGCCAGATCCAGACGC-BHQ1	142
	Sense primer	ACTACTGGTATTGTGATGGACTCC	
	Anti-sense primer	CGGTCAGGATCTTCATCAGGTAG	

bp, base pairs.

in multiplex with ERα, ERβ1 and ERβ2. The multiplex assay efficiency was optimized by evaluating different concentrations of primers (50–900 nM) and probes (50–250 nM). The optimal concentrations of forward and reverse primers used in this study were 50 nM for ERα, 225 nM for ERβ1 and 150 nM for both ERβ2 and β-actin. The optimized probe concentrations were 75 nM for ERα, 200 nM for ERβ1, 100 nM for ERβ2 and 150 nM for β-actin. Reactions were performed in a 25 μL reaction mixture containing primers, probes, 1× Qiagen PCR buffer, 5 mM $MgCl_2$, 800 μM dNTPs, 1.25 U HotStarTaq, and 5 μL of diluted cDNA template. The thermocycle program included an enzyme activation step at 95 °C (15 min) and 40 cycles of 95 °C (15 s) and 62 °C (1 min). On each plate, six serial 5-fold dilutions (12 wells) of pooled sample cDNA were run to assess the PCR efficiency and for data analysis. All samples were run in triplicate along with a no template control and a no reverse transcriptase control.

Data are expressed as mRNA levels relative to β-actin; β-actin mRNA in the brain was previously reported to be unaffected by E2 treatment in goldfish (Bosma et al., 2001). If β-actin levels changed between control and E2 treated tissues, β2 microglobulin was tested in real-time RT-PCR simplex reactions (see below for methods) and if appropriate was used as the endogenous reference gene for the ER real-time RT-PCR multiplex reactions. This gene was previously identified as a candidate endogenous reference gene in our lab (Martyniuk et al., 2006b). For statistical analysis, significant changes in gene expression in normally distributed data (log transformed) were evaluated using an Independent Samples *T*-Test ($p < 0.05$), and non-normally distributed data were evaluated using the Mann–Whitney *U*-test ($p < 0.05$; SPSS 13.0).

The relative amounts of ERα, ERβ1 and ERβ2 within each tissue in the real-time RT-PCR multiplex reactions were compared using the method described by Qiagen (Critical Factors for Successful Real-Time RT-PCR http://www1.qiagen.com/literature/brochures/pcr/QT/1037490_AG_PCR_0206_Int_lr.pdf) for targets with comparable amplification efficiencies. Samples of each tissue from the control fish at the one day time point were analyzed. Real-time amplification of serial dilutions of a cDNA sample were used to obtain standard curves of cycle threshold (C_T) values versus template amount for each target (i.e. ERα, ERβ1, ERβ2) and for the reference gene (β-actin). To compare the amplification efficiencies of the targets, the C_T values of each target were subtracted from the reference gene C_T values, and this difference was plotted against the logarithm of the template (cDNA) amount. Microsoft Excel (5.1)-fitted lines and equations were then obtained using standard regression analyses for each target. The slopes of the resulting straight lines were <0.1, and therefore the amplification efficiencies were comparable and the relative amounts of ERα, ERβ1 and ERβ2 within the control samples were evaluated (data not presented). For normally distributed data, significant differences between the mean, normalized, log transformed levels of ERα, ERβ1 and ERβ2 within each tissue for the one day control fish were assessed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test ($p < 0.05$), while non-normally distributed data were evaluated using the Kruskal–Wallis test ($p < 0.05$; SPSS 13.0).

2.5. Microarray hybridization and analysis

The complete details for the goldfish brain enriched microarray production, hybridization and scanning are found in Martyniuk et al. (2006b). Goldfish cDNAs were spotted along with common carp (*Cyprinus carpio*) cDNAs at the University of Liverpool Microarray Facility, UK (Gracey et al., 2004). Briefly, total RNA was extracted using TRIZOL Reagent (Invitrogen) as per the manufacturer's protocol. Total RNA was pooled from hypothalami (15 fish) into a single reference control RNA pool for one day. Independent pools ($n = 3$) of hypothalamic RNA from treated fish were hybridized to the goldfish–carp microarrays, and a fourth hybridization was a replicate dye-reversal of one of the three pooled RNA samples. This was done with hypothalamic RNA from the one day time point only (four arrays in total). Microarray hybridizations were completed using 2 μg total RNA and the Genisphere Array 900MPX™ cDNA microarray labeling kit (Genisphere, Hatfield, PA, USA).

As previously reported and validated by Martyniuk et al. (2006b), the normalization strategy used for the microarray was intensity-dependent Lowess normalization (Yang et al., 2002). The Significance Analysis of Microarray (SAM) method (Tusher et al., 2001) was used to assess the significance of the differential expression of the genes using a false discovery rate of 5%. The Gene Ontology (GO) analysis for the differentially expressed genes was the Blast2Go (http://www.blast2go.de/) and DAVID programs (http://david.abcc.ncifcrf.gov/home.jsp).

2.6. Real-time RT-PCR

Aromatase B, VTG and genes identified from the microarray analysis were evaluated using real-time RT-PCR in simplex reactions. All primers were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Invitrogen. Amplicons for each primer set were cloned and sequenced to confirm specificity. The primer sets used in this study are reported in Table 2. Each real-time RT-PCR reaction contained the following final concentrations: 25 ng first strand cDNA template, 1× QPCR buffer, 3.5 mM $MgCl_2$, 100 nM gene-specific primer, 0.25× SYBR Green (Invitrogen), 200 μM dNTPs, 1.25 U HotStarTaq (Invitrogen), and 100 nM ROX reference dye, in a 25 μL reaction volume.

The accumulation of PCR product was measured in real time as the increase in SYBR Green fluorescence using the Mx4000® Multiplex Quantitative PCR system (Stratagene). 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, 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 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 from 55 °C (+1 °C/30 s) to 95 °C. Data were analyzed using the Mx4000® Software Package. Standard curves relating initial template

Table 2
Oligonucleotide primers for SYBR Green real-time RT-PCR assays and Basic Local Alignment Search Tool matches from nucleotide sequence of amplicons

Gene target, Genbank accession no.	Element	Sequence 5′–3′	Amplicon length (bp)
Aromatase B, AB009335.1	Forward	TGCTGACATAAGGGCAATGA	153
	Reverse	GGAAGTAAAATGGGTTGT GGAT	
Vitellogenin, DQ641252	Forward	AGCTGTTGTGCTTGCCTTG	167
	Reverse	GGTGAACCTTGCTGTTGACTT	
Adenylate kinase 1, Id24l12[a]	Forward	TGGCTACACTCACCTGTCCTC	219
	Reverse	AACTCCTCTCCCTGTTTGACC	
Ubiquitin-conjugating enzyme E2D 1, Id09a14[a]	Forward	GGCCTTCTTTCTCACAATTCAC	287
	Reverse	TCTGGGTCCATTCTCTTGCT	
B2 microglobulin, L05536	Forward	GCCCTGTTCTGTGTGCTGTA	244
	Reverse	AAGGTGACGCTCTTGGTGAG	
Elongation factor-1, AB056104.1	Forward	GATTGTTGCTGGTGGTGTTG	227
	Reverse	GCAGGGTTGTAGCCGATTT	

[a] The Carp gene IDs are listed and can be retrieved in carpBASE2.1 (http://legr.liv.ac.uk/).

copy number to fluorescence and amplification cycle were created and used to calculate relative mRNA copy number in each sample.

For verification of microarray expression data using real-time RT-PCR simplex reactions elongation factor-1, was used as the endogenous reference gene. This gene did not to change in the hypothalamus and telencephalon in response to 17α-ethinylestradiol exposures in goldfish studies conducted in our lab (Martyniuk et al., 2006b). Real-time RT-PCR data are expressed as mRNA levels relative to elongation factor-1. For statistical analysis, significant changes in gene expression in normally distributed data (log transformed) were evaluated using an Independent Samples *T*-Test ($p < 0.05$), while data that were not normally distributed data were evaluated using Mann–Whitney *U*-test ($p < 0.05$; SPSS 13.0).

3. Results

3.1. Gonadosomatic index (GSI) and radioimmunoassay for plasma E2

There was no significant difference in GSI between E2 and control treated male goldfish after one and seven days of exposure ($p > 0.05$; data not shown). Male goldfish treated with E2 silastic implants had significantly higher levels of circulating E2 compared to fish with control silastic implants ($p < 0.05$; Fig. 1). Plasma E2 levels of control male fish were below the detection limits of the radioimmunoassay, and were assigned the lowest detectable concentration of the assay (0.063 ng/mL).

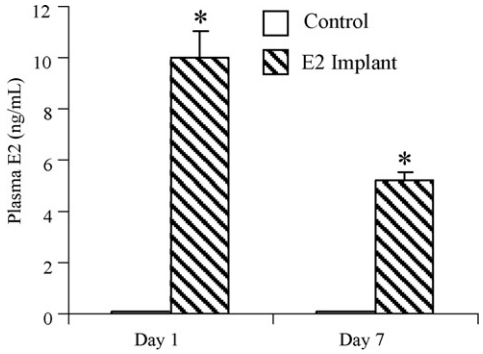

Fig. 1. Plasma 17β-estradiol (E2) levels measured by radioimmunoassay in male goldfish with control (no steroid) or E2 silastic implants (100 μg/g body mass) for one and seven days. Means (+S.E.) are presented and asterisks indicate significant differences between control and E2 treated fish for each time point ($n = 6$; Mann–Whitney *U*-test, $p < 0.05$).

3.2. ER expression in various tissues by multiplex real-time RT-PCR

Tissue mRNA levels of ERα, ERβ1, and ERβ2 in males with E2 implants were compared to control males to investigate the auto-regulation of the three ER subtypes by E2. The expression levels of the ERs in the liver, testes, hypothalamus and telencephalon are plotted as fold change above the control values (see Figs. 2 and 3). In all ER multiplex reactions the endogenous reference gene, β-actin, was not significantly different between the control and E2 implanted fish, with one exception. In the liver, β-actin mRNA levels were significantly higher (~2-fold, $p < 0.05$) in the one day E2 silastic implanted fish compared to control fish (data not shown). However, β2 microglobulin mRNA levels were examined using SYBR Green real-time RT-PCR and did not change (data not shown; $p = 0.064$) under these conditions, and therefore these levels served as the endogenous reference

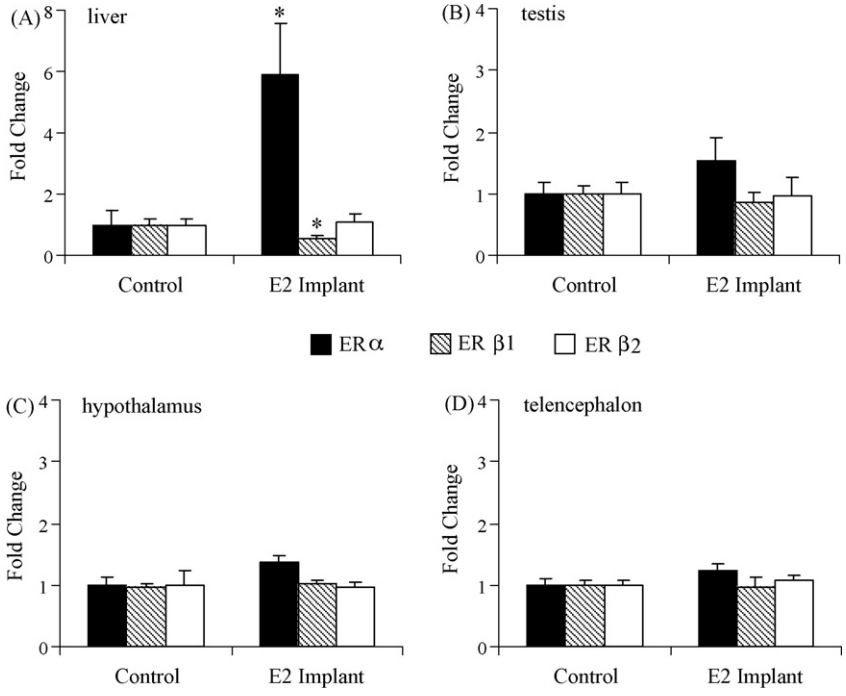

Fig. 2. Effects of control (no steroid) and E2 silastic implants (100 μg/g body mass) for one day on mRNA levels of estrogen receptor subtypes (α, β1, β2) in male goldfish. Relative fold change (means + S.E.) above controls determined by real-time RT-PCR in (A) liver ($n = 12$), (B) testes ($n = 12$), (C) hypothalamus ($n = 6$), and (D) telencephalon ($n = 6$). The expression level of each gene was normalized with respect to β-actin, except for liver in which case β2 microglobulin was used as the endogenous reference gene. Asterisks (*) indicate significant differences between control and E2 implanted fish for individual genes (Independent Samples *T*-Test, $p < 0.05$).

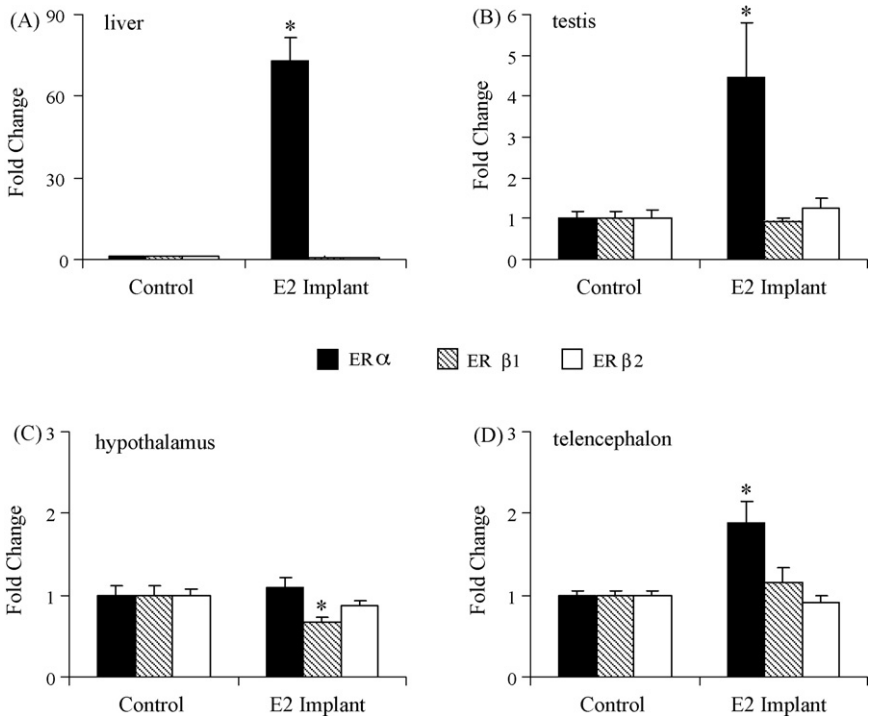

Fig. 3. Effects of control (no steroid) and E2 silastic implants (100 μg/g body mass) for seven days on mRNA levels of estrogen receptor subtypes (α, β1, β2) in male goldfish. Relative fold change (mean + S.E.) above controls determined by real-time RT-PCR in (A) liver ($n = 12$), (B) testes ($n = 12$), (C) hypothalamus ($n = 6$), and (D) telencephalon ($n = 6$). The expression level of each gene was normalized with respect to β-actin. Asterisks (*) indicate significant differences between control and E2 implanted fish for individual genes (Independent Samples *T*-Test, $p < 0.05$).

gene for normalization of liver samples for the one day time point.

The expression levels of the three ER subtypes varied in response to E2 exposure (Figs. 2 and 3). In the liver, a significant induction of ERα occurred after one (5.9-fold; $p < 0.05$) and seven (73-fold; $p < 0.05$) days of E2 implantation. However, ERβ1 in the liver showed a 2-fold decrease ($p < 0.05$) after one day, but was unchanged after seven days of E2 implantation compared to the control. Elevated E2 did not elicit any changes in liver ERβ2 mRNA levels. In the testes, E2 induced ERα levels 4.5-fold ($p < 0.05$) only after seven days of E2 implantation, while neither ERβ1 nor ERβ2 expression levels changed at either time point. Of the three ER subtypes in the hypothalamus, only ERβ1 mRNA levels after seven days of E2 treatment showed a significant decrease (∼1.5-fold, $p < 0.05$), while ERα and ERβ2 levels remained unchanged in this brain region. In contrast, in the telencephalon ERα significantly increased ∼1.9-fold ($p < 0.05$) after seven days of E2 treatment, while no changes in ERβ1 or ERβ2 levels were observed.

The relative proportions of the three ER subtypes within each tissue were analyzed in samples from the control fish at the one day time point. Liver (Kruskal–Wallis test, $p > 0.05$), hypothalamus and testes (one-way ANOVA, Tukey's HSD, $p > 0.05$) demonstrated no significant differences between the relative levels of ERα, ERβ1 and ERβ2 mRNA levels (data not shown). However, significant differences between the ER subtypes did exist within the telencephalon (one-way ANOVA ($p < 0.05$), Tukey's HSD; data not shown). The mean, normalized expression (±S.E.) of ERβ1 was significantly higher than ERα (1.6 ± 0.17-fold; $p < 0.05$), but not significantly higher than ERβ2 (1.2 ± 0.1-fold; $p > 0.05$).

To examine another E2-mediated response, VTG mRNA levels were measured in males treated with E2 compared to controls (Fig. 4). Significant increased expression of VTG after both one (∼50-fold, $p < 0.05$) and seven (∼170-fold, $p < 0.05$) days of exposure was observed.

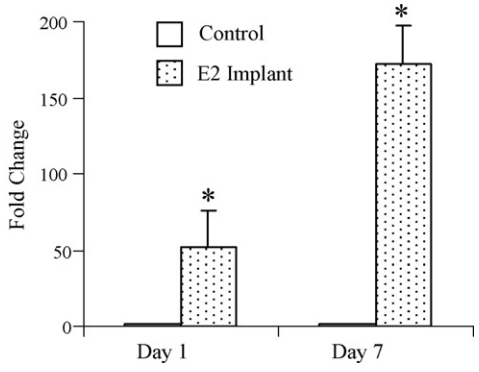

Fig. 4. Effects of control (no steroid) and E2 silastic implants (100 μg/g body mass) for one and seven days on liver vitellogenin mRNA ($n = 7$–8) in male goldfish. Relative fold change (mean + S.E.) above controls was determined by real-time RT-PCR. The expression level of each gene was normalized with respect to elongation factor-1. Asterisks (*) indicate significant differences between control and E2 implanted fish for individual genes at each time point (Mann–Whitney *U*-test for 1 d and Independent Samples *T*-Test for 7 d, $p < 0.05$).

Table 3
List of candidate genes identified by microarray analysis in male goldfish after one day of E2 silastic implantation (q value <5%, fold change $\geq \pm 1.35$)

Gene	Fold change	Carp database ID	Homologous gene with NCBI ID
3-Hydroxyanthranilate 3,4-dioxygenase	+1.83	05j07	NM_001007390
Aromatase B	+1.35	NA[a]	U18974
AMBP protein precursor	−2.51	08o13	BC055598
Hypothetical protein LOC445082	−2.34	18g19	NM_001003476
Spectrin alpha chain, brain	−2.07	22k20	XM_683356
Alpha-1,6-mannosyl-glycoprotein	−2.06	09l11	XM_690279
Adenylate kinase	−2.03	24l12	NM_001003993
Apolipoprotein A-I-2 precursor	−1.87	11b06	XM_696149
Matrix metalloproteinase 9	−1.8	16n24	BC053292
Ubiquitin-conjugating enzyme	−1.8	09a14	NM_199664
Similar to ataxin 2-binding protein 1 isoform 2	−1.74	18p10	XM_213535
Gelsolin precursor	−1.7	06i05	NM_178131
Proproteinase E precursor	−1.69	09i08	NM_001024408
39S ribosomal protein L12, mitochondrial precursor	−1.66	18d02	NM_001017696
Ribonuclease P protein subunit p40	−1.66	10b15	NM_001002148
Transitional endoplasmic reticulum ATPase	−1.6	24o22	XM_687038
Apolipoprotein A-I precursor	−1.54	6e10	NM_131128
Poly U binding splicing factor half pint	−1.52	22m20	NM_001002121
Proactivator polypeptide precursor	−1.51	5e19	NM_131883
RAB2, member RAS oncogene family	−1.51	19m19	BC044459
zgc:91894	−1.5	22o13	NM_001004116
Protein C20orf108 homolog	−1.49	06n20	BC096895
Coagulation factor V precursor	−1.48	06m14	NM_001007208
Lipin-1 (Fatty liver dystrophy protein)	−1.45	06o08	XM_679712
Proteasome subunit alpha type	−1.44	11i10	BC076206
Tubulin-specific chaperone A	−1.44	11a13	BC046032
Alpha-1-antitrypsin homolog precursor	−1.44	07d21	NM_001013259
Transcriptional repressor NF-X1	−1.43	17e21	XM_685467
Similar to heparan sulfate proteoglycan 2 (perlecan)	−1.43	22b07	CAG03827
Hypothetical protein XP_678073 isoform 1	−1.42	10i09	XM_678073
Dystrobrevin binding protein 1	−1.41	20g08	NM_201134
Transcription regulator protein BACH1	−1.41	10h22	NM_001020663
Hypothetical protein LOC406484	−1.41	06c14	BC065450
Vacuolar sorting protein 4a	−1.4	20j02	NM_013245
abl-interactor 1	−1.4	18o07	NM_200738
Fructose-bisphosphate aldolase B	−1.4	06g20	NM_194367
Similar to 14 kDa apolipoprotein	−1.39	07k09	AY773183
Sulfotransferase family cytosolic 2B member 1	−1.39	6e13	NM_198914
Troponin T, fast skeletal muscle isoforms	−1.36	19g13	NM_181653
Similar to putative nuclear protein	−1.36	23i02	XM_682749

The carp gene IDs are listed and can be retrieved in carpBASE2.1 http://legr.liv.ac.uk/. The homologous genes in NCBI database are also given. Not listed are sequences that showed no nucleotide or amino acid similarity to proteins in the NCBI/carp database.
[a] NA, not applicable.

3.3. Microarray analysis of hypothalamus

Putative E2-regulated transcripts identified by microarray analysis after one day of E2 implantation are listed in Table 3. The transcripts listed showed a significant change ($q < 5$) after one day of E2 implantation compared to controls, and the estimated microarray fold change values are presented. The majority of transcripts decreased in relative abundance following E2 implantation. GO biological function assignment was checked for the candidate genes. Six GO themes were identified based on biological process category, and included cell protein metabolism, response to stimuli, transport, nucleobase or nucleotide metabolism, cell organization, and regulation of cellular processes. In particular, cellular metabolism was significantly over-represented in 13 genes ($p < 0.05$; Fig. 5).

Real-time RT-PCR was used to verify a subset of genes in the hypothalamus and telencephalon that were identified as being modulated by E2 in the microarray analysis. In all real-time RT-PCR in simplex reactions, the endogenous reference gene, elongation factor-1, was not significantly different between the control and E2 implanted fish. The genes selected were chosen based on their relevance to the neuroendocrine control of reproduction (i.e. aromatase B) or to the cellular metabolism theme indicated in the GO analysis (Fig. 5, i.e. ubiquitin-conjugating enzyme E2D 1 (ube2d1) and adenylate kinase 1). After both one (~2-fold, $p < 0.05$) and seven (~2.5-fold, $p < 0.05$) days of E2 treatment, aromatase B was elevated in the hypothalami of treated males (Fig. 6A). Similarly, in the telencephalon males with the E2 silastic implants showed significantly increased levels of aromatase B after both one (~1.5-fold, $p < 0.05$) and seven (~2.5-fold, $p < 0.05$) days of exposure (Fig. 6B). Adenylate

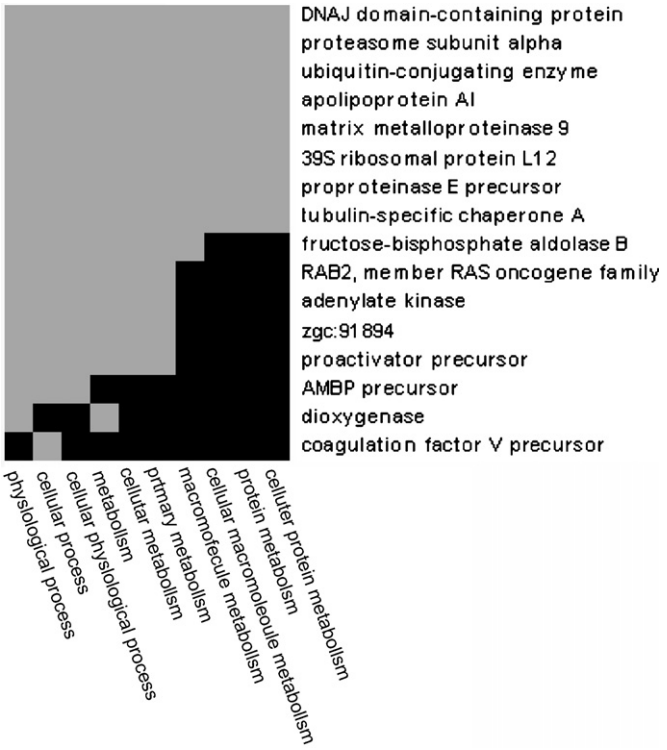

Fig. 5. Enrichment analysis of Gene Ontology terms of differentially expressed genes in the hypothalami of one day E2 implanted (100 μg/g body mass) male goldfish relative to control fish (silastic implants without E2). Differentially expressed genes were identified by cDNA microarray (genes listed in Table 3). GO analysis was performed using the Blast2Go (http://www.blast2go.de/) and DAVID programs (http://david.abcc.ncifcrf.gov/home.jsp). This 2-D view showed the relationships among highly related genes and their annotations. Gray corresponds to the GO term association positively reported, while black corresponds to the GO term association not reported yet. Cellular metabolism was significantly over-represented in 13 genes from DNAJ domain containing protein to proactivator precursor.

kinase 1 was significantly lower in the hypothalamus and telencephalon after both one and seven days of E2 treatment (ranging from ~1.7–2.4-fold, $p < 0.05$). However, real-time RT-PCR verified significant decreases in ube2d1 levels at both time points in the hypothalamus (ranging from 1.5- to 1.8-fold, $p < 0.05$; Fig. 6C–F), but no changes were observed in the telencephalon.

Two additional genes that have been shown to be seasonally responsive to E2 in the goldfish brain (Lariviere et al., 2005), glutamic acid decarboxylase (GAD) 65 and GAD67, were not identified by microarray analysis as being differentially expressed. This lack of effect was also verified by real-time RT-PCR to further increase our confidence in the microarray analysis.

4. Discussion

This is the first study to examine the auto-regulation of all three goldfish ER subtypes simultaneously within the neuroendocrine regions of the brain and within the testis and liver. We also quantified in the same animals the mRNA levels of two known E2-responsive genes, namely brain aromatase and liver VTG. The results of this study show that goldfish ER subtype levels in males are differentially regulated by E2, and that this regulation pattern varies between tissues. Microarray analysis also identified candidate genes not previously reported to be modulated by E2 in the hypothalamus of fish, and GO analysis revealed that the cellular metabolism theme was significantly over-represented. Collectively, these results provide a better understanding of the effects that estrogens have on genes regulated by ER pathways, while revealing novel E2-responsive genes in the brain of a model aquatic species.

4.1. Hepatic ER modulation by E2

Although several studies have shown hepatic ERα induction upon E2 exposure in several teleosts, this is the first to report a decrease in hepatic ERβ1 and no change in ERβ2 in goldfish. Soverchia et al. (2005) measured hepatic ERβ1 mRNA levels in juvenile goldfish (sex not specified) and reported an increase (~1.5–2.5-fold) following a three week waterborne exposure to E2 (10^{-7} M) and 4-nonylphenol (10^{-7} and 10^{-6} M). This discrepancy with the present study may be due to dose, route/length of exposure or age-specific differences. Another study also conducted in adult sexually regressed goldfish demonstrated an increased hepatic expression of ERα and ERβ1 in females injected with 2 μg of E2, and an increase of ERβ2 when injected with a lower dose of E2 (0.02 μg) after 36 h (Nelson et al., 2007). The males in this study exhibited a more sensitive response to E2 with an up-regulation of ERα and ERβ1 with both 0.2 and 2 μg E2 injections, but no doses affected ERβ2 expression levels (Nelson et al., 2007). Although the up-regulation of ERα in male goldfish was confirmed in the present study, the reported up-regulation of ERβ1 by Nelson et al. (2007) was not. However, Nelson et al. (2007) did not report the circulating plasma E2 levels nor the variation in weights of the fish which would have affected the dose each fish received, hence direct comparisons of the E2 doses used between these two studies are difficult. In addition, the method of E2 exposure (single injection versus silastic implant) may affect the spatial and temporal expression of ER subtypes. Additional experiments using the goldfish model are necessary to resolve the nature of the potential discrepancies in ERβ subtype auto-regulation by E2 in goldfish. A study examining all three ER subtypes in male largemouth bass (*M. salmoides*) reported that liver ERα was induced in a dose-dependent manner after 24 h with a single E2 injection (dose ranged from 0.5 to 2.5 mg/kg (Sabo-Attwood et al., 2004). In that study, liver ERβ1 levels did not change and ERβ2 levels were moderately up-regulated (Sabo-Attwood et al., 2004). In juvenile Atlantic salmon (*S. salar*; sex not specified) hepatic ERα was induced after three and seven days of exposure to the xenoestrogen 4-nonylphenol in a dose and time-dependent manner (Meucci and Arukwe, 2006). However, juvenile Atlantic salmon ERβ levels showed a slight decrease after three days of 4-nonylphenol exposure and a slight increase after seven days of exposure; ERβ2 has not been identified in this species (Meucci and Arukwe, 2006). Zebrafish (*Danio rerio*) ER subtype auto-regulation studies show the greatest similarity to the present findings in goldfish. A 48 h exposure of zebrafish (sex not specified) to waterborne 10^{-8} M E2 resulted in a strong stimulation

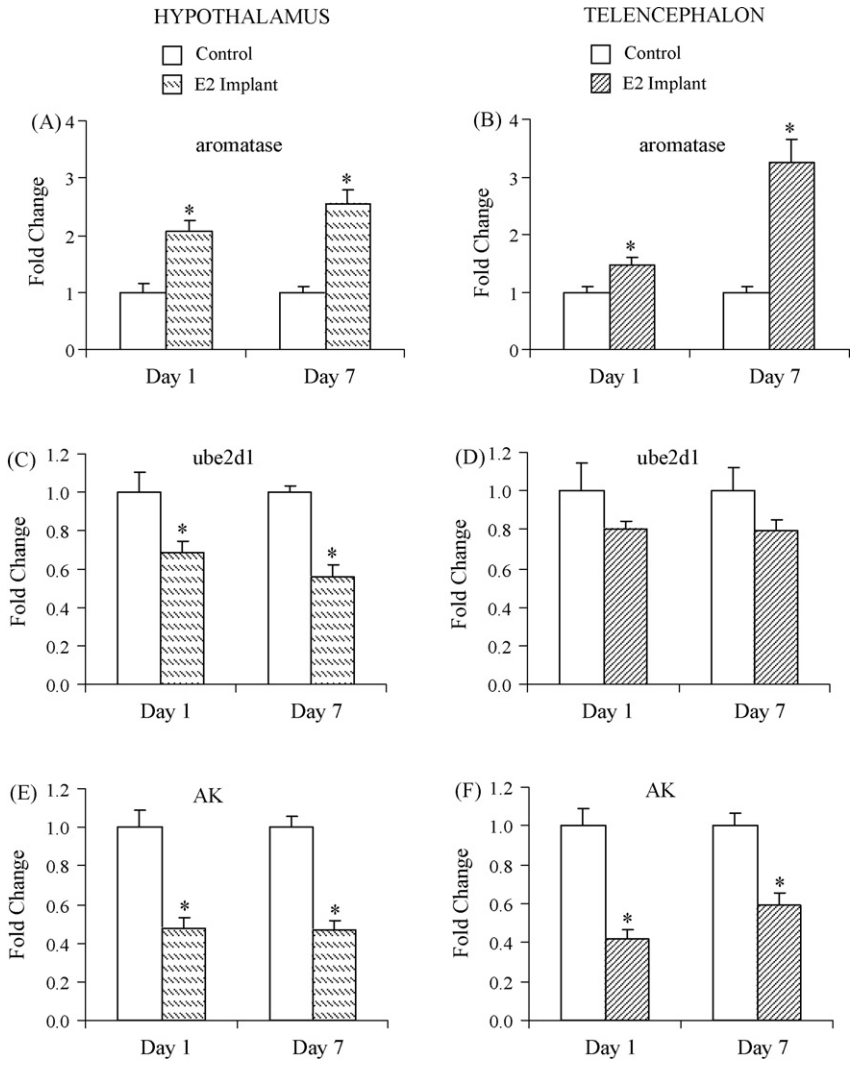

Fig. 6. Effects of control (no steroid) and E2 silastic implants (100 μg/g body mass) for one and seven days on mRNA levels of (A) hypothalamus aromatase B ($n = 6–7$), (B) telencephalon aromatase B ($n = 6–7$), (C) hypothalamus ubiquitin-conjugating enzyme E2D 1 (ube2d1; $n = 5–8$), (D) telencephalon ube2d1 ($n = 6–8$), (E) hypothalamus adenylate kinase 1 (AK; $n = 5–8$), and (F) telencephalon AK ($n = 6–8$) in male goldfish. Relative fold change (mean + S.E.) above controls was determined by real-time RT-PCR. The expression level of each gene was normalized with respect to elongation factor-1. Asterisks (*) indicate significant differences between control and E2 implanted fish for individual genes at each time point (Mann–Whitney *U*-test, $p < 0.05$).

of liver ERα, while ERβ1 was markedly reduced and ERβ2 remained unchanged (Menuet et al., 2004). To date, hepatic ERα induction appears to be a common response regardless of dose and exposure route, and strongly implicates ERα as the main ER subtype responsible for E2-induced VTG synthesis in the liver of oviparous teleosts. Our study also demonstrated that the relative abundance of the ER subtypes was not significantly different within the liver, indicating that ERα is more responsive to E2 auto-regulation than ERβ1 or ERβ2. The significance of the decrease of ERβ1 levels in goldfish liver, and in the few other teleosts studied to date is unknown.

4.2. Gonadal ER modulation by E2

The present study demonstrated that after seven days of E2 implantation ERα was up-regulated in the testes, while ERβ1 and ERβ2 mRNA levels remained unchanged after both one and seven days of E2 exposure. This auto-regulation of ERα in the testes parallels the high up-regulation of this subtype in the liver. However, the absence of altered ERβ1 levels in the testes compared to the down-regulation found in the liver reflects an organ specific auto-regulation of the ERβ1 by E2. The absence of changes in ERβ2 levels in the testis as well as in liver and brain in response to E2, suggests a less sensitive auto-regulatory loop for ERβ2 compared to ERα and ERβ1. There were no significant differences in the relative proportions of ERα, β1 and β2 within the testes in this study, and therefore ERα up-regulation upon E2 treatment results in a disproportionate increase in this subtype. In contrast to our findings, Nelson et al. (2007) reported an increase in all three goldfish ER subtypes in both male and female goldfish gonads after 36 h when injected with 2 μg E2. Again, ERα induction appears to be a common response regardless of the dose/route/length of exposure, while the response of the ERβ1 subtype appears to be more sensitive to such experi-

mental conditions. A study in male and female fathead minnow reported similar gonadal ERα induction and no change in ERβ2, but showed decreases in gonadal ERβ1 following a 14-d waterborne E2 exposure (35 ng/L; Filby et al., 2006). Although the consequences and specific molecular events resulting from elevated ERα levels in the testis are not well understood, several studies have documented the adverse effects of E2 and endocrine disrupting chemicals on testis development, testosterone production and spermiation (Billard et al., 1981; Trudeau et al., 1993; Panter et al., 1998).

4.3. ER modulation by E2 in the neuroendocrine brain

This is the first study to report differential regulation of ER subtypes in the brain of goldfish. After seven days of E2 treatment, only ERβ1 was down-regulated in the hypothalamus, while ERα was up-regulated in the telencephalon. The mRNA levels of ERβ2 were not affected by E2 treatment in either the hypothalamus or telencephalon. Previous autoradiography studies reported high levels of E2 target cells in the hypothalamus and telencephalon of the goldfish brain (Kim et al., 1978), and studies of whole brain ER mRNA levels in other teleosts have reported some similar trends of ER auto-regulation. Meucci and Arukwe (2006) reported a similar induction of ERα in whole brain of juvenile Atlantic salmon (*S. salar*) in a dose- (5–50 μg/L) and time-dependent manner; ERβ mRNA levels (ERβ2 has not been identified in this species) were inhibited after three days of exposure but were induced after seven days of exposure (Meucci and Arukwe, 2006). Whether this biphasic response of ERβ observed by Meucci and Arukwe (2006) is specific to nonylphenol exposure, due to the use of whole brain samples versus specific regions of the brain, and/or age- and species-specific effects has yet to be determined. In contrast to our findings and those reported by Meucci and Arukwe (2006), brain ERα isoforms in female rainbow trout fry did not respond to nonylphenol (2.2 μg/L to 2.2 mg/L) or E2 (2.7 μg/L) treatments (Vetillard and Bailhache, 2006). Martyniuk et al. (2006b) also did not observe any changes in male goldfish ER subtype levels in the hypothalamus and telencephalon after 15-d 17α-ethinylestradiol (0.1 and 1 nM) waterborne exposures. Nonetheless, based on our findings and the published literature, there is evidence for ERα induction and ERβ1 down-regulation in select brain regions of teleosts in response to some estrogenic compounds. However, chemical/species-specific, seasonal, temporal and dose-dependent responses are likely to effect the regulation of brain ER subtypes, and studies in our lab are currently underway to address some of these issues. In addition, further studies examining the significance of the higher proportion of ERβ1 observed in the telencephalon and lack of difference in subtype proportions in the hypothalamus, in relation to ER auto-regulation are warranted.

4.4. Liver VTG modulation by E2

A large increase in VTG mRNA levels in the liver was observed after both one and seven days of E2 implantation, and the only ER subtype that simultaneously increased was ERα. This suggests a more important role for ERα in liver vitellogenesis than the ERβ1 and ERβ2 subtypes. Recently, zebrafish ER transfection experiments in several cell lines demonstrated distinct transcriptional activation properties of zebrafish ERs *in vitro*. For example, both ERα and ERβ2 were capable of inducing a zebrafish ERα promoter construct, but ERβ1 showed minimal transcriptional activity (Menuet et al., 2004). It is well established that the efficiency of transcription is promoter-dependent (Madigou et al., 2001), therefore the examination of the promoter regions of the goldfish ER subtypes will be needed to further elucidate the differential regulation of these ERs by E2. Furthermore, to conclusively determine the contribution of each ER subtype to the VTG response, receptor binding experiments with each ER subtype to the promoter regions of the VTG gene are necessary.

4.5. Microarray analysis of the hypothalamus

Changes in mRNA levels following various treatments have previously been reported to be relatively modest in brain compared to other tissues, typically in the 1.5–2.5-fold range (Marnova et al., 2004; Martyniuk et al., 2006b), which was largely confirmed in the present experiment. Most candidate transcripts identified as being significantly modulated by E2 were categorized by GO analysis into cellular metabolism, demonstrating that one distinguishing feature for E2 treatment on goldfish brain was a change in metabolic processes. The aromatase B, GAD65 and GAD67 gene expression data also concurs with a similar study conducted in our lab that examined the gene expression responses of male goldfish exposed to waterborne 17α-ethynylestradiol for 15 days (Martyniuk et al., 2006b). However, Martyniuk et al. (2006b) did not observe changes in ube2d1 or in adenylate kinase 1, but whether this is due to the length, dose, route of exposure or synthetic versus endogenous estrogen requires further investigation. Similar to Martyniuk et al. (2006b), our microarray analysis also identified genes in the brain previously reported to be estrogen-responsive in other tissues. For example, apolipoproteins, which are involved in lipid mobilization and transport, were also up-regulated by E2 in the testis of sea bream (*Sparus auratus*; Pinto et al., 2006). The apolipoproteins in our study decreased in the hypothalamus in response to E2, consistent with what we have observed following waterborne 17α-ethinylestradiol exposures in males (Martyniuk et al., 2006b). Although the direction of change appears to depend on the tissue, dose and/or species, the identification of similar candidate genes within these studies, in addition to the identification of some unique candidate genes, exemplifies the utility of cDNA microarrays for gene profiling estrogen action in the vertebrate brain.

The present study showed that aromatase B was elevated in the hypothalamus and telencephalon after both one and seven days of exposure to E2. These results concur with several studies examining aromatase B induction upon E2 treatment in various teleost species, including goldfish (Gelinas et al., 1998). It has also been established that an auto-regulatory loop for aromatase B exists in adult goldfish (Pasmanik and Callard, 1988; Callard et al., 2001) and in zebrafish embryos (Kishida and Callard, 2001;

Kishida et al., 2001). In addition, an estrogen response element exists in the promoter region of the goldfish aromatase B gene (Callard et al., 2001). In the present study aromatase B induction by E2 does not appear to be tightly correlated to ER subtype induction in either brain region. Given that there were no obvious changes in the ERs after one day of treatment and higher ratio of ERβ1 relative to ERα in the telencephalon, it is possible that existing pools of certain ER subtypes and/or the putative membrane ER may be important for the rapid regulation of aromatase B. Two recently developed mouse hypothalamic neuronal cell lines were reported to express characteristic ratios of ERα and ERβ, and this ratio was proposed to account for the unique transcriptional responses of the cell lines to E2 (Titolo et al., 2006). These findings in addition to the distinct transcriptional activity of the zebrafish ER subtypes reported *in vitro* (Menuet et al., 2004) further supports the hypothesis that ER subtypes possess differential functions.

Ubiquitin-conjugating enzymes are important components of the ubiquitin-proteosome pathway, and act as enzymatic coactivators that modulate nuclear hormone receptor-dependent gene transactivation by degrading and/or modifying factors that influence gene transcription (Verma et al., 2004). Thus, the observed decrease in ube2d1, an indirect coactivator of steroid hormone-induced transactivation, could be interpreted as a negative feedback pathway of elevated E2 levels on the ubiquitin-proteosome pathway in the hypothalamus, but further studies detailing this process are needed to support this supposition. Similarly, the decrease in adenylate kinase 1, which is known to amplify metabolic signals and promote intracellular phosphoryl transfer, may also alter E2-responsive genes such as ERs. The phosphorylation of vertebrate ERs is known to be a major post-translational modification that results from the activation of various cellular kinases and tends to enhance ER action (Lannigan, 2003), hence a decrease in intracellular phosphoryl transfer processes could ultimately impede ER-mediated effects.

4.6. Conclusions

Our results indicate that differential auto-regulation of goldfish ER subtypes, α, β1 and β2, exists in various tissues within the neuroendocrine axis of adult males with elevated circulating E2 levels. Up-regulation of the ERα subtype was apparent in the liver, gonad and telencephalon, and a moderate down-regulation of ERβ1 was evident in the hypothalamus and liver, while no apparent auto-regulation was observed for ERβ2. Thus, different patterns of ER subtype expression during the reproductive cycle when E2 levels naturally fluctuate, may ultimately alter the ER-mediated processes of the neuroendocrine tissues examined. In the liver, VTG induction paralleled ERα induction by E2, which is further evidence to support the hypothesis that ERα is the main ER subtype involved in vitellogenesis. However, the induction of aromatase B by E2 in specific brain regions did not appear to closely parallel ER subtype induction. This integrative whole-organism approach outlined here will further aid in defining the effects that estrogens and xenoestrogens have on genes in the vertebrate neuroendocrine axis.

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