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Effects of fluoxetine on the reproductive axis of female goldfish (*Carassius auratus*)

Jan A. Mennigen,[1,4] Christopher J. Martyniuk,[1,2] Kate Crump,[1] Huiling Xiong,[1] E. Zhao,[1] Jason Popesku,[1] Hymie Anisman,[3] Andrew R. Cossins,[4] Xuhua Xia,[1] and Vance L. Trudeau[1]

[1]*Centre for Advanced Research in Environmental Genomics, Department of Biology, University of Ottawa, Ottawa, Ontario, Canada;* [2]*Department of Physiological Sciences and Center for Environmental and Human Toxicology, University of Florida, Gainesville, Florida;* [3]*Institute of Neuroscience, Carleton University, Ottawa, Ontario, Canada; and* [4]*School of Biological Sciences, University of Liverpool, Liverpool, United Kingdom*

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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. First published September 2, 2008; doi:10.1152/physiolgenomics.90263.2008.—We investigated the effects of fluoxetine, a selective serotonin reuptake inhibitor, on neuroendocrine function and the reproductive axis in female goldfish. Fish were given intraperitoneal injections of fluoxetine twice a week for 14 days, resulting in five injections of 5 μg fluoxetine/g body wt. We measured the monoamine neurotransmitters serotonin, dopamine, and norepinephrine in addition to their metabolites with HPLC. Homovanillic acid, a metabolite in the dopaminergic pathway, increased significantly in the hypothalamus. Plasma estradiol levels were measured by radioimmunoassay and were significantly reduced approximately threefold after fluoxetine treatment. We found that fluoxetine also significantly reduced the expression of estrogen receptor (ER)β1 mRNA by 4-fold in both the hypothalamus and the telencephalon and ERα mRNA by 1.7-fold in the telencephalon. Fluoxetine had no effect on the expression of ERβ2 mRNA in the hypothalamus or telencephalon. Microarray analysis identified isotocin, a neuropeptide that stimulates reproductive behavior in fish, as a candidate gene affected by fluoxetine treatment. Real-time RT-PCR verified that isotocin mRNA was downregulated approximately sixfold in the hypothalamus and fivefold in the telencephalon. Intraperitoneal injection of isotocin (1 μg/g) increased plasma estradiol, providing a potential link between changes in isotocin gene expression and decreased circulating estrogen in fluoxetine-injected fish. Our results reveal targets of serotonergic modulation in the neuroendocrine brain and indicate that fluoxetine has the potential to affect sex hormones and modulate genes involved in reproductive function and behavior in the brain of female goldfish. We discuss these findings in the context of endocrine disruption because fluoxetine has been detected in the environment.

brain; estrogen receptors; isotocin; microarray; Prozac

FLUOXETINE (PROZAC), a selective serotonin reuptake inhibitor (SSRI) (46), has been used in several studies in fish to investigate the serotonergic modulation of the endocrine system (55). A racemic mixture of two lipophilic enantiomers, fluoxetine is metabolized by cytochrome *P*-450 isoenzymes to the active metabolite norfluoxetine (46). In humans, fluoxetine is primarily excreted in urine as ∼20%–30% unchanged parent compound, while metabolites are largely excreted as pharmacologically active norfluoxetine and inactive fluoxetine glucuronide (62).

Fluoxetine also has effects on reproduction in several vertebrates. For example, in the goldfish (*Carassius auratus*), combined intraperitoneal administration of 10 μg/g fluoxetine and 10 μg/g serotonin (5-HT) elevated luteinizing hormone (LH) levels relative to 5-HT administration alone 2 h after treatment (55). Japanese medaka (*Oryzias latipes*) exposed to waterborne 0.1 and 0.5 μg/l fluoxetine for 4 wk showed a significant elevation of plasma estradiol (E_2) levels (19). Conversely, 1 mo of chronic subcutaneous daily fluoxetine treatment (0.5–5 mg/kg body wt) suppressed circulating E_2 levels in estrogen-treated ovariectomized rats (*Rattus norvegicus*), with the highest estrogen reductions occurring in the group receiving the lowest dosage of fluoxetine (56). As well, administration of fluoxetine (10 mg/kg) disrupts estrous behavior, including lordosis, in intact female rats after a 3-wk treatment period (35). Similarly, in male rats, fluoxetine decreases sexual motivation and preference for females (63). Despite these studies, the molecular mechanisms underlying modulatory effects on reproductive parameters by fluoxetine are not well understood.

In this study, we investigated the transcriptomic response in the hypothalamus of female goldfish, using microarray analysis to identify candidate neuroendocrine genes modulated by fluoxetine. The brain regulation of reproduction in the goldfish has been well studied and is influenced by numerous neurotransmitters and neuropeptides (58). The monoamines 5-HT and norepinephrine (NE) have been shown to stimulate gonadotropin-releasing hormone (GnRH) release from the brain preoptic-anterior hypothalamic region in female goldfish in vitro, while dopamine (DA) exhibits potent inhibition of GnRH and LH release (69). In rainbow trout (*Oncorhynchus mykiss*), an increase in 5-HT turnover and a concurrent decrease in DA have been observed during the periovulatory period (51). In the Atlantic croaker (*Micropogus undulatus*), 5-HT and GnRH neurons have been shown to be in close proximity in the preoptic area of the hypothalamus (28). In the same species, Khan and Thomas (29) provided evidence for an involvement of a 5-HT_2-like receptor subtype, which mediates 5-HT stimulation of basal and GnRH-induced LH release in postvitellogenic, but not regressed, fish.

Address for reprint requests and other correspondence: J. A. Mennigen, Centre for Advanced Research in Environmental Genomics, Dept. of Biology, Univ. of Ottawa, Ottawa, ON, K1N 6N5, Canada (e-mail: jmenn090@uottawa.ca).

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Therefore, in the present investigation, concentrations of the neurotransmitters 5-HT, DA, and NE together with their primary metabolites were measured with high-performance liquid chromatography (HPLC) in the hypothalamus and telencephalon, the two major brain regions involved in neuroendocrine function. We hypothesized that fluoxetine would disrupt normal estrogen signaling and therefore measured plasma E_2 levels with radioimmunoassay (RIA) and estrogen receptor (ER)α, β1, and β2 mRNA expression levels in the neuroendocrine brain. Gene expression analysis by cDNA microarray and real-time PCR determined that isotocin mRNA was reduced significantly in the hypothalamus and telencephalon by fluoxetine. Isotocin is the fish homolog of the mammalian nonapeptide oxytocin (64), which has important roles in mammalian reproductive behavior (6) and physiology, such as modulation of GnRH-stimulated LH release (42). While studies in fish have revealed a role of isotocin in reproductive behavior (21), the impact of isotocin on the reproductive physiology of reproduction has not been investigated. Given the detected changes in isotocin mRNA in hypothalamus and telencephalon and the concurrent decrease in E_2 levels in the fluoxetine experiment, we studied the effect of isotocin on circulating LH and sex steroid levels in a second experiment.

MATERIALS AND METHODS

Experimental animals and design. Common female goldfish were purchased from a commercial supplier (Aleong's International, Mississauga, ON, Canada) and maintained at 18°C under a natural simulated photoperiod. The experiment was conducted in December, with lights (~50 lx) synchronized to Ottawa, ON, Canada day length (0700–1700 h). Fish were fed and maintained on standard flaked goldfish food. Goldfish were anesthetized with 3-aminobenzoic acid ethylester for all handling and dissection procedures. Care was taken to standardize all handling, injections, and sample protocols. All procedures used were approved by the University of Ottawa Protocol Review Committee and followed standard Canadian Council on Animal Care guidelines on the use of animals in research. Sexually recrudescent female goldfish with a body weight ranging from 15 to 51 g were injected intraperitoneally with fluoxetine obtained from Sigma-Aldrich (Oakville, ON, Canada). Fifty fish were treated twice a week for 17 days, for a total of five injections of 5 μg fluoxetine/g body wt with an injection volume of 1 μl/g. The dose represents a low pharmacological dose in the mammalian literature, because typically doses of 10 mg/kg are used in rat studies (10). The dose was chosen because it was the lowest injected dose at which mammalian studies revealed an impact on reproductive behavior (6). Furthermore, a study that investigated arginine vasotocin (AVT) gene expression in the preoptic area of male bluehead wrasses (*Thalassoma bifasciatum*) used a similar fluoxetine dose of 6 μg/g body wt (54). The length of the treatment was chosen to evaluate long-term effects that are known to be important in SSRI action (10) and that are a likely scenario in the environment because of pseudopersistency (1). We injected twice a week to account for the 2- to 6-day half-life of fluoxetine (62).

Fluoxetine was dissolved in physiological saline (0.6% NaCl). The control group ($n = 50$) received an equivalent volume of saline. One day after the final injection, goldfish were killed by spinal transection and brain tissues were rapidly dissected, immediately frozen on dry ice, and stored at −80°C until analysis.

We also performed experiments to determine the effects of isotocin on circulating sex steroid levels. Sexually regressed female goldfish with body weights ranging from 17 to 48 g were injected with isotocin (Chem Impex, Intl., Wood Dale, IL) dissolved in DMSO at a concentration of 1 μg/μl. The injection was carried out with 1 μl/g body wt, and fish were killed 12 h after the injection. Control groups received an equivalent volume of DMSO. The dose of the neuropeptide was chosen on the basis of other studies investigating effects of neuropeptides on LH release in goldfish (70). The time between injection and sampling was chosen to maximize the time window because oxytocin effects on LH in mammals are known to be relatively slow (16).

High-performance liquid chromatography. The HPLC protocol described by DiBattista et al. (13) was used. Individual telencephali and hypothalami ($n = 7$) were put in 1 ml of solution consisting of 0.1 mM Na_2EDTA, 0.3 mM monochloracetic acid, 50 ml of methanol, and distilled water. Until used, samples were kept on dry ice. After sonication, protein levels were determined with the BCA protein assay reagent kit (Pierce, Rockford, IL) and an ICK Multiscan/MCC 348 (Titertek, Huntsville, AL). Samples were centrifuged at 12,000 rpm for 3 min in a 4°C microcentrifuge. The supernatant was transferred into HPLC microvials. For HPLC analysis, a 1100 series Zorbac XDB/C8 4.62 column with a length of 150 mm was used (Agilent Technologies, Mississauga, ON, Canada). Chemicals used in standards were bought from Sigma-Aldrich. Data were log transformed to fit normal distribution and analyzed by Student's *t*-test with SYSTAT v10 (Systat Software, San Jose, CA) run on Windows XP.

Radioimmunoassay for luteinizing hormone. Blood (100–300 μl) was collected from anesthetized goldfish by caudal puncture from fluoxetine-injected ($n = 10$) and control ($n = 10$) animals at the end of the experiment. Blood was collected from isotocin-injected ($n = 15$) and control ($n = 15$) animals 12 h after isotocin injection. Plasma was harvested by centrifugation (10 min at 3,000 rpm) and stored frozen (−80°C) until analysis. The double-antibody RIA protocol of Zhao et al. (70) was used to analyze circulating LH levels.

Data from both experiments were tested for normality with SYSTAT v10 and were not normally distributed. A Mann-Whitney *U*-test was used to test for significant differences.

Radioimmunoassay for testosterone and estradiol. Sex steroids were extracted and testosterone (T) and E_2 levels were measured by RIA according to the protocol of McMaster et al. (36). Two hundred milliliters of the original plasma samples used in the LH assay was used to extract sex steroids ($n = 10$). Data were tested for normality with SYSTAT v10 and were not normally distributed. The Mann-Whitney *U*-test was used to test for significant differences in plasma E_2 concentration of blood samples taken from female goldfish at the end of the fluoxetine injection experiment. The detection limit (0.031 ng/ml) was defined as the first E_2 concentration significantly different from the zero point on the standard curve (ANOVA followed by Tukey's post hoc test). This value was assigned to samples that were below the detection limit.

In the second experiment, remaining plasma samples from those used for the LH measurements were pooled for a total of five, each representing three individual females. Two hundred milliliters was obtained from these samples to extract and measure the effects of isotocin on T and E_2. All samples were above detection limits. Data were not normally distributed and were therefore analyzed as described above.

RNA extraction and cDNA synthesis. RNA was isolated with the TRIzol method (Invitrogen, Burlington, ON, Canada) per the manufacturer's protocol. Total RNA concentration was evaluated with the GeneQuant spectrophotometer (Amersham Pharmacia Biotech, Oakville, ON, Canada). To remove genomic DNA, samples were treated with DNase and an RNAeasy Micro kit (Qiagen, Mississauga, ON, Canada). cDNA was prepared from 1 μg of total RNA and 200 ng of random hexamer primers (Invitrogen) with Superscript II RNase H^- reverse transcriptase as described by the manufacturer (Invitrogen). Each 20-μl reaction was diluted fivefold in nuclease-free water and used as the template for the real-time RT-PCR assays.

Multiplex real-time RT-PCR and SYBR Green assays. The Mx4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA) was used to amplify and detect the transcripts of interest. Primers were synthesized by Invitrogen, and gene-specific dual-labeled fluorescent

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probes were purchased from IDT (Toronto, ON, Canada). RNA was extracted from eight pools consisting of four or five individual hypothalami or telencephali from control and fluoxetine-injected female goldfish. The multiplex reaction for the endogenous reference gene β-actin in combination with ERα, ERβ1, and ERβ2 has been validated by our lab (33). Data were normalized to β-actin because the levels did not change significantly with fluoxetine treatment ($P = 0.56$ hypothalamus; $P = 0.99$ telencephalon).

A SYBR Green assay was developed to validate candidate differentially regulated genes identified by the microarray in the neuroendocrine brain. We used pooled samples that were used for microarray analysis ($n = 3$) and additional independent samples ($n = 7$) for the SYBR Green assay. Each PCR reaction was run with parameters described by Martyniuk and colleagues (34), with primer concentrations of 150 nM for all genes validated. Primers (Table 1) were designed with Primer3 and ordered from Invitrogen. Data were analyzed with the MX4000 software package. Standard curves relating initial template copy number to fluorescence and amplification cycle were generated by using the amplified PCR product as a template and were used to calculate mRNA copy number in each sample. β-Actin mRNA was also used as a normalizing gene in the SYBR Green assays because its expression did not significantly change with treatment.

Data were tested for normality with SYSTAT v10 and were not normally distributed. Significant changes in gene expression were therefore evaluated with the Mann-Whitney *U*-test.

Microarray hybridizations. We previously described and validated the production and use of our goldfish-carp cDNA microarray (33, 34). For a detailed description of the microarray the reader is directed to Williams et al. (68). A total of four microarray hybridizations were performed for hypothalamic tissue as the initial screen for the effects of fluoxetine on the neuroendocrine brain. We compared three independent treatment samples (containing 4 or 5 pooled hypothalami) to a collective pool of control samples (representing ~30 control fish). This experimental design allows for less technical variation because only one reference is handled while maintaining biological variation of the treatment samples (7). We also preformed a replicate of one treatment pool using the opposite dye (dye-swap design). Hybridizations and scanning protocols were used as described previously (33, 34). Briefly, hybridizations were performed according to the protocol supplied by Genisphere (Hatfield, PA). Microarrays were scanned at full-speed 10-μm resolution with the ScanArray 5000 XL system (Packard Biosciences/PerkinElmer, Woodbridge, ON, Canada) using both red and blue lasers. Images were obtained with ScanArray Express software using automatic calibration sensitivity varying photomultiplicator (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 analyzed with QuantArray (Packard Biosciences/Perkin Elmer), and raw signal intensity values were obtained for duplicate spots of genes. Raw intensity values for all microarray data and microarray platform information have been deposited in the NCBI Gene Expression Omnibus database (platform accession no. GPL3735; series accession no. GSE5420). We used Alien Spot Report Genes 1-10 (Stratagene) to normalize microarray data and the significance analysis of microarrays (SAM) method (59) for microarray analysis.

Candidate genes identified as being differentially regulated were further analyzed with the GOSSIP package in BLAST2GO (8) for Gene Ontology (GO) categorizations.

RESULTS

Fluoxetine increases homovanillic acid concentration and norepinephrine turnover in neuroendocrine brain. Levels of 5-HT and DA in the hypothalamus showed a trend for an increase of 35% and 50%, respectively, in fluoxetine-treated animals (Table 2). The DA metabolite homovanillic acid (HVA) increased significantly in fluoxetine-treated animals ($P = 0.04$). There were no significant changes in other neurotransmitters or metabolites measured. The ratio [metabolite]/[parent monoamine] was used as an index of brain monoaminergic activity. Calculation of this index showed no significant changes in monoamine turnover in the hypothalamus, but there was a significant increase in NE turnover in the telencephalon ($P = 0.01$), reflecting the observed trend of 3-methoxy-4-hydroxyphenylglycol (MHPG) increase and concurrent trend for a decrease of NE in the telencephalon (Table 2).

Effects of fluoxetine on circulating LH and E_2. In the female fish used for neurotransmitter and microarray analysis, fluoxetine treatment did not affect plasma LH levels (Fig. 1*A*). In contrast, E_2 levels were significantly decreased ($P = 0.028$) by approximately threefold in the blood plasma of fluoxetine-treated fish (Fig. 1*B*).

Fluoxetine decreases ERα and ERβ1 relative mRNA expression in the neuroendocrine brain. Since fluoxetine decreased circulating E_2 levels, we wanted to determine whether estrogen receptors in the brain were also affected. In both the hypothalamus and the telencephalon, ERβ1 mRNA was decreased significantly in fluoxetine-treated fish (Figs. 2*B* and 3*B*). ERβ1 mRNA was decreased ~4-fold in the telencephalon ($P = 0.001$) and 3.5-fold in the hypothalamus ($P = 0.001$). ERα (Fig. 3*A*) also decreased significantly in the telencephalon by ~1.7-fold ($P = 0.003$). Fluoxetine did not significantly change ERβ2 mRNA abundance in either tissue (Figs. 2*C* and 3*C*).

Microarray and real-time RT-PCR identified the neuropeptide isotocin as being downregulated by fluoxetine. To investigate potential mechanisms for the observed effects of fluoxetine on circulating E_2, we utilized microarray and real-time RT-PCR to identify fluoxetine-regulated target genes in the hypothalamus. We identified several candidate genes regulated

Table 1. *Genes found to be regulated by fluoxetine in microarray experiment and validation by real-time RT-PCR*

GO Term Categories	Gene	Accession No.	Microarray Fold Change	QRT-PCR Fold Change	Primer Sequence
Signal transduction	Glutathione peroxidase 4b	NP_002076	−2.0	−1.7	F: CCAAAGGCTACAAGGCAGAG R: TTGATCCGAAAGGCTACAGG
Signal transduction	Isotocin	NP_000481	−1.4	−6.4	F: ATCTTGGCTACTGGCAGCTT R: GTATCTGCTGTGGTGAAGGT
Regulation of cellular process, cellular component organization and biogenesis, anatomic structure morphogenesis, organ development	ATP inhibitory factor 1	NP_057395	+1.2	+1.8	F: GTGGCCCATATCAGGAGAAA R: ACTGTCCAGGGTGAATGGAA

GO, Gene Ontology; F, forward; R, reverse; QRT-PCR, quantitative RT-PCR.

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Table 2. *Amine and major metabolite concentrations in hypothalamus and telencephalon of control and fluoxetine-injected female goldfish*

	5-HT	HIA	DA	DO	HVA	NE	MHPG	HIA/5-HT	HVA/DO	MHPG/NE
Hypothalamus										
Control	10±1.2	3.4±0.40	8.7±0.6	3.4±0.4	0.6±0.5	13.0±1.35	2.4±0.4	0.34±0.09	2±0.45	0.18±0.15
Fluoxetine	13.6±1.4	3.8±0.20	13.1±2	4.5±0.6	9.9±1.4	15.6±1.5	1.8±0.4	0.32±0.04	2.2±0.45	0.12±0.02
P value	0.07	0.34	0.06	0.14	0.05	0.23	0.35	0.37	0.42	0.13
Telencephalon										
Control	7.6±1.7	4.3±0.5	8.5±2.3	6.0±1.2	11.9±3.6	22±6.9	2.4±0.7	0.68±0.1	1.99±0.41	0.13±0.03
Fluoxetine	9.6±1.5	6.5±1.7	6.1±1.2	4.2±0.6	7.7±1	14±1.8	5.2±1.4	0.68±0.34	2.05±0.36	0.75±0.08
P value	0.4	0.25	0.46	0.20	0.28	0.28	0.09	0.99	0.92	0.01

Data (in ng/mg protein) are means ± SE; $n = 7$. MHPG, 3-methoxy-4-hydroxyphenylglycol; NE, norepinephrine; DA, dopamine; DO, L-DOPA; HIA, 5-hydroxyindoleacetic acid; HVA, homovanillic acid; 5-HT, 5-hydroxytryptamine.

by fluoxetine treatment. Whereas a total of 17 mRNAs were found to be induced, a total of 70 mRNAs were downregulated. Of the induced mRNAs, 10 were identifiable, while 46 of the downregulated mRNAs were identified (listed in Supplemental Table S1).[1] An enrichment analysis for both sets of genes using the GOSSIP program of BLAST2GO (8) was computed, but no significant overrepresentation of any GO group was found. Therefore, results are represented as multilevel GO terms at a cutoff level of two sequences per GO term class. The results of a GO term-based classification of induced and downregulated genes are shown in Fig. 4, *A* and *B*, respectively. Genes in numerous processes were affected and include signal transduction, various metabolic pathways, organogenesis, and reproduction. A full list of up- and downregulated genes can be found in Supplemental Table S1.

Because of our interest in reproduction, we chose isotocin to further verify with real-time RT-PCR in the hypothalamus and telencephalon. Isotocin mRNA was significantly reduced with fluoxetine treatment in the hypothalamus (Fig. 5*A*) and the telencephalon (Fig. 5*B*). In the hypothalamus, isotocin was found to be downregulated 6.4-fold ($P = 0.001$). In the telencephalon, a 4.8-fold downregulation of isotocin mRNA ($P = 0.005$) was observed.

Isotocin increases circulating estradiol levels. We were interested in establishing a probable causal link between our gene expression analysis results and the observed decrease in E_2 levels following fluoxetine injections. Therefore, to investigate whether isotocin has an effect on circulating E_2, we measured LH, T, and E_2 levels in isotocin-injected animals. There was no change in circulating LH levels 12 h after injection (Fig. 6*A*). There was a threefold decrease ($P < 0.05$) in T (Fig. 6*B*) with a concurrent twofold increase ($P < 0.05$) of E_2 (Fig. 6*C*) in isotocin-injected females. The calculated E_2-to-T ratio increased fivefold ($P = 0.002$) in isotocin-injected fish (Fig. 6*D*).

DISCUSSION

Fluoxetine reduces plasma E_2 and changes expression of ER isoforms in the neuroendocrine brain. Fluoxetine injections in female goldfish significantly decreased circulating E_2, just as diminished circulating E_2 was also observed in female rats chronically treated with the SSRI fluvoxamine (48). Accompanying the decrease in blood E_2 was a decrease in ERα mRNA levels in the goldfish hypothalamus and telencephalon. The promoter of the teleost ERα contains one full estrogen response element (ERE) and one half-ERE, which have been shown to contribute to E_2-stimulated ERα expression in vitro (37). Therefore, the decrease in circulating E_2 may have resulted in the decreased transcription of ERα observed in our study. Although we did not specifically test the importance of the decrease of ERα in our study, it is well known that severe endocrine disruptions occur in both male and female ERα-knockout mice, among them alteration of sexual behavior, spermatogenesis, and infertility (14, 67). ERα plays a key role in mediating both positive and negative estrogen feedback over LH levels in female mice. The positive feedback appears to be mediated via classical ERE interaction, while positive feedback appears to be mediated via ERE-independent activity of ERα (20). ERβ1 mRNA expression was shown to decrease in

[1] The online version of this article contains supplemental material.

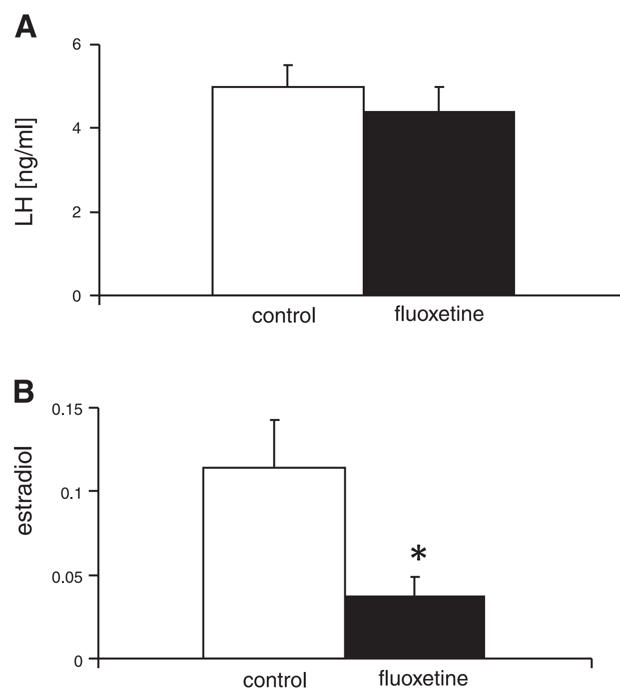

Fig. 1. Effects of fluoxetine on circulating reproductive hormone levels in female goldfish. *A*: luteinizing hormone (LH) concentrations in control (open bars) and fluoxetine-treated (filled bars) goldfish. *B*: estradiol (E_2) concentrations in control (open bars) and fluoxetine-treated (filled bars) female goldfish. Data are presented as means + SE ($n = 10$). *Significant difference at $P < 0.05$.

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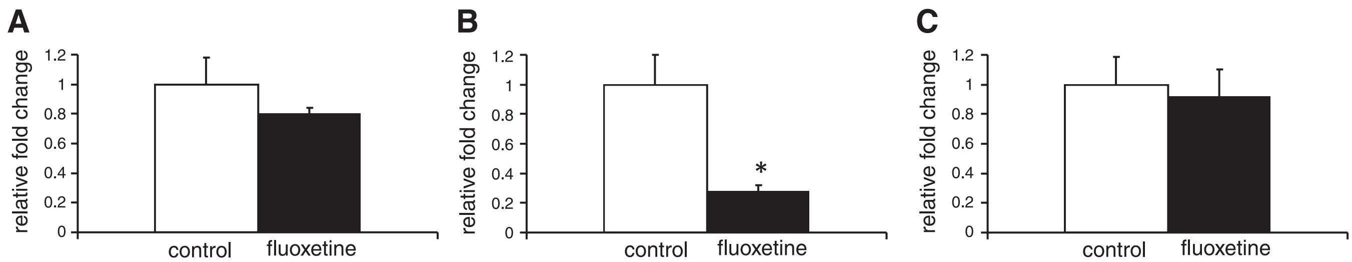

Fig. 2. Effects of fluoxetine on estrogen receptor (ER) expression in the hypothalamus of female goldfish. Relative mRNA levels for ERα (*A*), ERβ1 (*B*), and ERβ2 (*C*) are shown for control (open bars) and fluoxetine-injected (filled bars) goldfish. Data are means + SE (n = 7 or 8). *Significant difference at $P < 0.05$.

both hypothalamus and telencephalon in our experiment, but it is unclear whether this is directly related to the decreased circulating E_2 levels. The effect of E_2 on ERβ subtypes is less well defined, but there are both receptor subtype- and tissue-specific effects in goldfish (33). In whole female zebrafish (*Danio rerio*) brain, the serotonin agonist mianserin increased the expression of a putative ERβ2 subtype after 2 days (61). It is clear that ERs are affected in both species, but the magnitude and direction of expression changes differ. This could be related to differences in species, dose, exposure route, and chemical used. Nevertheless, mice deficient in ERβ show disruptions of the reproductive system (3). For example, homozygous ERβ-knockout mice exhibit subnormal fertility (31) and female ERβ-knockout mice have infrequent pregnancies, reduced litter size, and reduced ovulation (65), possibly due to morphological abnormalities observed in the brain of ERβ-knockout mice (66). ERβ2 did not change with fluoxetine treatment in either tissue in the goldfish. The significant reduction of ERα and ERβ1 in the neuroendocrine brain of fluoxetine-treated fish in our study is likely to have an important impact on the ability of the female brain to respond to either locally produced neuroestrogen or circulating E_2, contributing to the antireproductive actions of fluoxetine.

Modulation of the dopaminergic and noradrenergic pathway. To investigate possible mechanisms for the observed decrease in circulating E_2, monoamine neurotransmitter levels and levels of their metabolites known to be involved in modulating LH release in the neuroendocrine brain of goldfish (58, 69) were measured by HPLC. While moderate changes should be interpreted with caution, the increase in HVA reflects changes in DA activity, which is concurrent with the observed trend of a 50% increase of DA levels in the hypothalamus of fluoxetine-treated fish. A 1.3-fold downregulation of the DA transporter was observed with the microarray (see Supplemental Table S1), providing a putative mechanism for increased DA levels. Fluoxetine has been shown to increase both extracellular NE and DA levels in addition to 5-HT levels in the hypothalamus of the rat (30). Repeated SSRI exposure leads to a decrease in SSRI binding in fathead minnow (*Pimephales promelas*) (22) brain. However, the serotonin transporter (SERT) was not represented on the version of the array utilized, but it is possible that not only antagonism to SERT but also downregulation of SERT may contribute to the observed trend of a 36% increase in 5-HT. It is not known, however, whether the decrease in binding is mediated at the gene expression level.

The seasonal control of reproduction in some teleosts is linked to increased 5-HT levels (51) and decreased DA levels (51), and potential interactions between both systems in regulating neuroendocrine control of reproduction in some teleosts has been suggested (15). DA is the principal inhibitor of LH release (58, 69), while 5-HT and NE stimulate LH release (58, 69) in some teleost fish. The trends in the monoaminergic systems measured, however, were not associated with changes in plasma LH levels in fluoxetine-injected female goldfish. Microarray analysis did not identify GnRH mRNA as being affected. These data suggest that the GnRH-LH pathway is not a major contributor to the observed decrease in E_2 levels.

Fluoxetine modulation of gene expression in the hypothalamus. To investigate other potential mechanisms for the observed decrease in E_2, hypothalamic tissue was chosen for microarray analysis because the major hypophysiotrophic nuclei have been identified there. Interestingly, the enrichment analysis did not show a significant overrepresentation of any GO term group in either induced or downregulated genes. This likely reflects the widespread actions of 5-HT in the central nervous system, where it is known to modulate various physiological systems, including feeding, thermoregulation, sleep/arousal, nociception, and modulation of motor systems (27). It may also reflect the observation that different neurotransmitters and metabolites changed in fluoxetine-treated fish. This effect was similarly observed in the hypothalamus of fluoxetine-

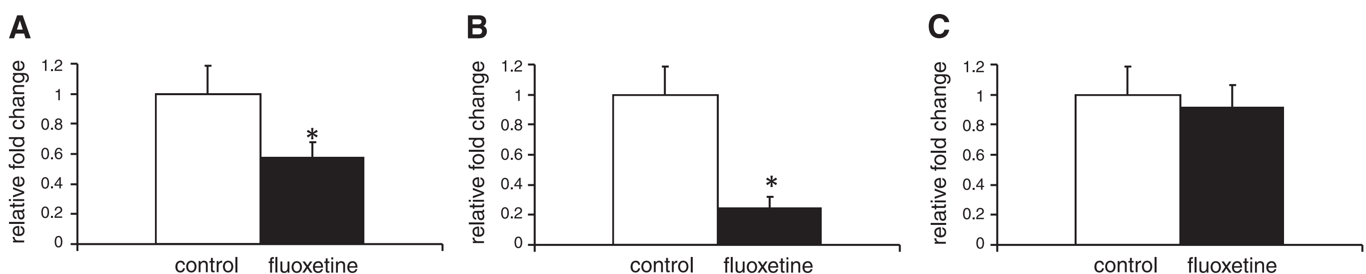

Fig. 3. Effects of fluoxetine on ER expression in the telencephalon of female goldfish. Relative mRNA levels for ERα (*A*), ERβ1 (*B*), and ERβ2 (*C*) are shown for control (open bars) and fluoxetine-injected (filled bars) goldfish. Data are means + SE (n = 7 or 8). *Significant difference at $P < 0.05$.

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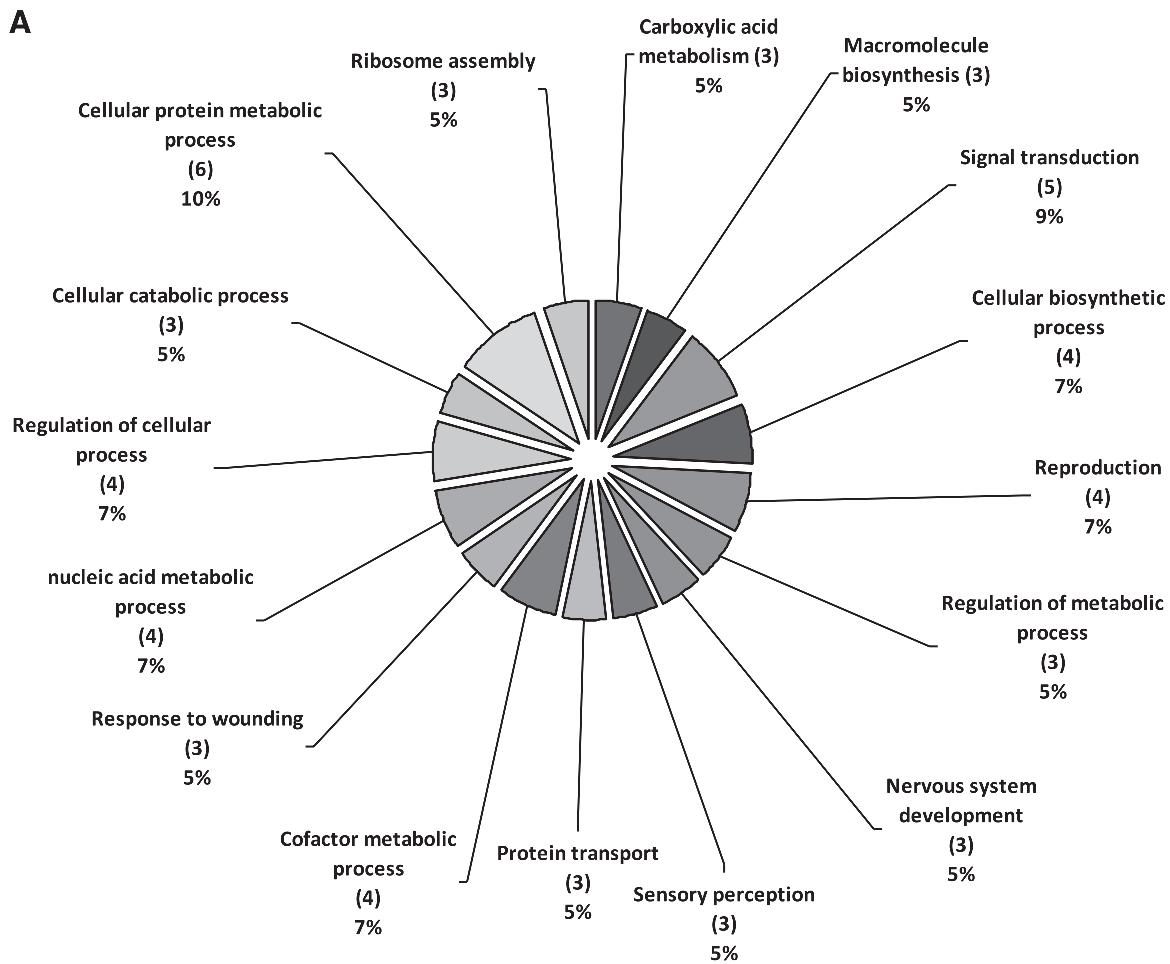

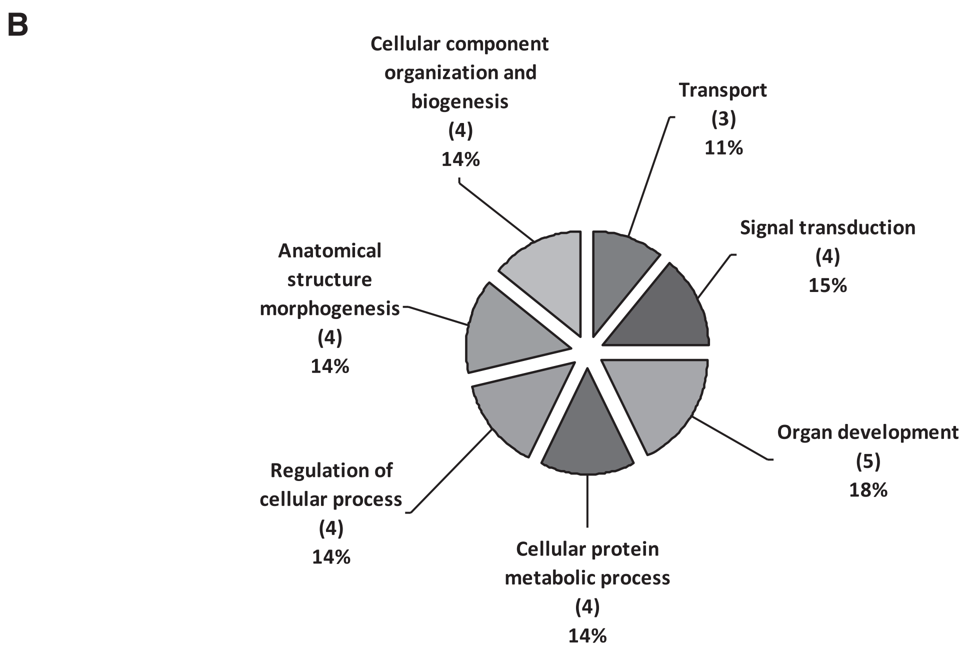

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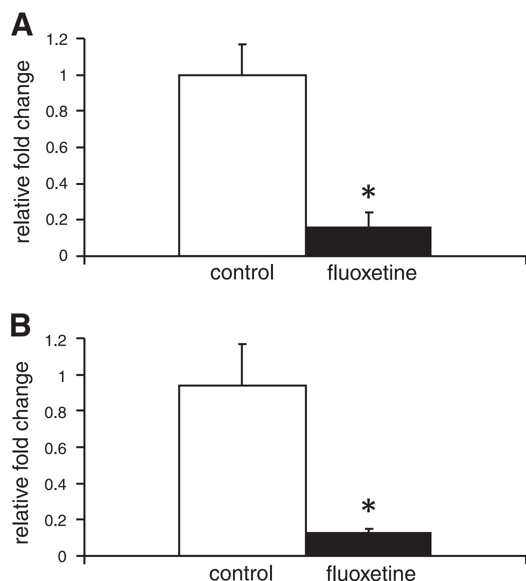

Fig. 5. Effects of fluoxetine on isotocin expression in the brain of female goldfish. Relative mRNA levels for isotocin in the hypothalamus (*A*) and telencephalon (*B*) are shown for control (open bars) and fluoxetine-injected (filled bars) goldfish. Data are means + SE (n = 10). *Significant difference at $P < 0.01$.

treated rats (30), and it was speculated that these changes explain fluoxetine-modulated changes in expression of numerous transcripts in hypothalamic tissue in rats (10). Several groups of fluoxetine-induced genes identified by multilevel GO analysis are related to development and morphogenesis, such as organ development, anatomic structure development, and regulation of cellular processes (Table 2). In a recent study in zebrafish, it was found that waterborne mianserin, an antidepressant and serotonin receptor agonist, affected numerous genes as determined with cDNA microarrays of whole brain (61). While fluoxetine and mianserin work though different therapeutic targets, there was some similarity in the genes affected. For example, in both goldfish hypothalamus and whole zebrafish brain, these drugs altered genes regulating cellular metabolism and transcription. Moreover, our work and the mianserin study in zebrafish (61) provide evidence for a neuroplastic action of fluoxetine in the brain that has also been shown in rat hypothalamic tissue and is believed to be part of the mechanism of this antidepressant drug (10).

ATP inhibitory factor 1 falls into GO term categories of organ development, anatomic structure development, and regulation of cellular processes and was confirmed by real-time RT PCR to be increased (Table 1). Primarily known to negatively regulate ATP catalysis in the mitochondrion (25), it has recently been detected in the surface of epithelial cells (11) with a potential role in epithelial cell signaling involved in angiogenesis (5). Interestingly, an endothelial tyrosine kinase receptor similar to angiopoietin receptor 1 was also found to be increased in the microarray experiment, suggesting effects on the neurovascular system. The enzyme glutathione peroxidase 4b was confirmed by PCR to be decreased in fluoxetine-treated goldfish. This isoform is expressed in neuronal cells (52) and was found to be induced in astroglial cells in proximity to damaged neurons, possibly to counteract further cell damage (53). The validated transcripts therefore provide evidence for the growing assertion that neuronal plasticity is a target of antidepressant drug action.

One notable difference between the studies for mianserin in zebrafish and fluoxetine in our goldfish model is the fact that brain aromatase did not change in our study, while it changed after 2 days but not 14 days in female zebrafish exposed to mianserin (61). Brain aromatase was represented on our microarray and was not affected by fluoxetine, a result we confirmed with real-time RT-PCR (not shown). This difference may partially reflect differences in length of treatment, tissue-specific expression changes, or drug-specific modes of action.

Among the downregulated genes, 4% fall into the GO category of reproduction. The disruption of normal reproductive function is a commonly observed side effect of SSRIs in mammalian models (17). We focused on this group to look for a potential mechanism to explain the observed decrease in circulating E_2.

Fluoxetine decreases the expression of isotocin, an important reproductive neuropeptide. We observed a significant reduction in isotocin mRNA levels after fluoxetine injection. Decreased transcription of the related AVT gene has also been observed in the brains of fluoxetine-treated male bluehead wrasses (54). Isotocin is abundant in the brain of teleosts and is the homolog to mammalian oxytocin (64). In rainbow trout, isotocin neurons are mainly located in the preoptic area, from where fibers project to pituitary and extrahypothalamic regions (60). In fish, there is evidence that isotocin has a central role to regulate sexual behavior (21, 57). Isotocin induces smooth muscle contraction in the ovary and oviducts, particularly in live-bearing teleosts (9). Facilitatory actions of GnRHs on isotocin neurons have also been observed in rainbow trout, suggesting that GnRH neurons modulate these classical neurosecretory neurons to control reproductive behavior (50). Seasonal changes in whole brain isotocin peptide levels were observed in the female three-spined stickleback (23), with the lowest levels occurring in recrudescent fish (December) and the highest in prespawning (July), suggesting a distinct role in the reproductive cycle of female fish. An increase in isotocin mRNA abundance in the reproductive phase was also detected in the preoptic nucleus in female masu salmon (*Oncorhynchus masou*) (41). In female medaka (*Oryzias latipes*), a significant decrease in vasotocin- and isotocin-immunoreactive neurons was observed after spawning (40). Thus in teleosts there is evidence that isotocin is an important regulator of reproductive processes. Mammalian studies provide evidence for serotonergic involvement in oxytocin release. In rats, a 14-day fluoxetine treatment attenuated oxytocin responses to 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist, possibly by reducing hypothalamic protein levels of G_{i1} and G_{i3} involved in signaling of this receptor (32). In male rats, fluoxetine treatment attenuated 5-HT_{2A} receptor-mediated stimulation of oxytocin (12).

Fig. 4. Gene Ontology (GO) analysis of differentially regulated genes in the brains of goldfish injected with fluoxetine. Multilevel GO term categories of genes increased (*A*) or decreased (*B*) by fluoxetine treatment as determined by BLAST2GO analysis of microarray results are shown. Number and % of sequences falling into each GO category are indicated.

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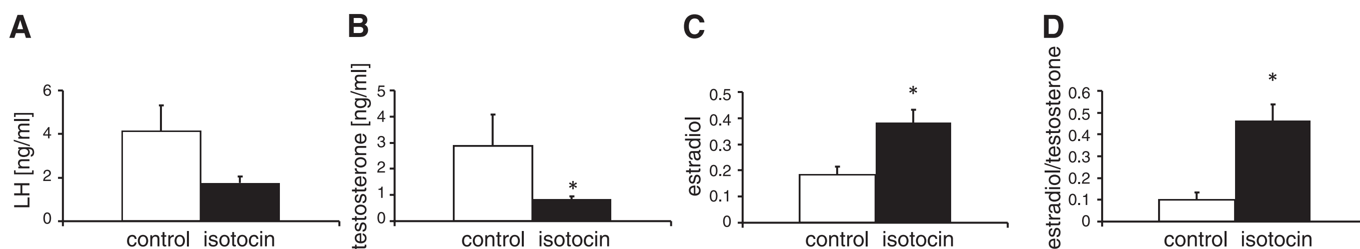

Fig. 6. Effects of isotocin on circulating reproductive hormones in female goldfish. Plasma levels of LH (*A*), testosterone (T; *B*), and E_2 (*C*) and the E_2-to-T ratio (*D*) 12 h after intraperitoneal injection are shown for control (open bars) and fluoxetine-treated (filled bars) goldfish. Data are means + SE (n = 15 for LH; n = 5 for T and E_2 steroids). *Significant difference at $P < 0.05$.

Recent mammalian studies provide evidence for a stimulatory effect of S-fluoxetine on allopregnalone at doses 10–50 times lower than the doses needed to inhibit serotonin reuptake (43). This neurosteroid in turn allosterically modulates the γ-aminobutyric acid A ($GABA_A$) receptor, with potential effects on reproductive function (26). $GABA_A$ receptor subunit mRNA and proteins are known to be expressed on oxytocin neurons (18). Therefore a possible influence of GABA on isotocin may potentially contribute to the observed changes in isotocin, as was also discussed with respect to the fluoxetine-induced decrease on AVP expression in male wrasses (54). However, in a recent meta-analysis using the same microarray platform as in this study, the goldfish hypothalamic transcriptome was compared between different pharmacological treatments (45). The microarray data set for fluoxetine from this study was also compared with a muscimol ($GABA_A$ agonist) study in goldfish. This comparison showed that isotocin was commonly regulated and muscimol resulted in an increase of isotocin mRNA in the hypothalamus, contrary to the observed decrease in our study. If $GABA_A$ modulation played a major role in the fluoxetine action, more similarities would be expected.

In our experiment, the decrease in isotocin mRNA may lead to decreased circulating isotocin. Isotocin could potentially act at several points of the reproductive axis. Oxytocin has been shown to stimulate GnRH-mediated LH release in rats (42) but also to increase ovarian E_2 production from porcine cells (44). The isotocin receptor is also expressed in both brain and ovaries in teleost fish (24), allowing for possible modulation of the reproductive axis at both levels.

Isotocin injection does not change LH but increases circulating E_2. In our first experiment we injected fluoxetine five times over 2 wk, and we observed no significant changes in circulating LH, yet E_2 levels were decreased. To investigate the role isotocin might play in controlling LH, we injected goldfish with isotocin and observed no significant effect on LH release after 12 h. It is therefore unlikely that decreased E_2 following fluoxetine treatment is related to effects on circulating LH. To study the potential functional significance of the observed decrease in transcript levels of the neurohypophysial hormone isotocin and the observed decrease in circulating E_2 levels, we also investigated the action of isotocin on circulating sex steroid levels. After 12 h a significant increase in E_2 and a concurrent decrease in circulating T levels were observed, resulting in a significant fivefold increase in the circulating E_2-to-T ratio in female goldfish injected with isotocin. This suggests that isotocin may act directly on the fish ovary to regulate estrogen production, as observed in porcine granulosa cells in vitro (44). While this is plausible, the hypothesis that isotocin acts on the goldfish ovary to regulate estrogen production requires further testing.

Conclusions. We show that fluoxetine injections impact the neuroendocrine axis of the female goldfish. We observed moderate changes in catecholamine dynamics in the telencephalon and hypothalamus similar to observations of fluoxetine action in mammalian models. Suppression of E_2 levels was accompanied by decreased expression of ERs in the neuroendocrine brain. Microarray analyses indicated that multiple genes in the brain were affected, consistent with the widespread importance of serotonergic systems in the vertebrate brain (27). In particular, we show that isotocin gene expression was decreased and this is likely linked to the observed decline in E_2 levels in the blood. Disruption of isotocin may also result in impairment of reproductive and social behaviors in fish (21, 57).

Significantly, there is now concern that fluoxetine and other SSRIs are environmental contaminants originating from sewage effluents (38). Detection of SSRIs in the brains of wild-caught fish (4) and bioconcentration in tissue under laboratory conditions (39) have also been reported. It is difficult to make predictions about pharmacokinetics in fish because of potential differences in metabolism of the drug, for example, by liver cytochromes. Multiple doses of 20 mg/day administered orally in humans lead to peak plasma concentrations of ~100 ng/ml (2). If an average human weight of 70 kg is assumed, the administered concentration would be around 0.3 mg/kg or 0.3 μg/g and therefore below the injected dose of 5 μg/g. Assuming similar metabolism in fish, blood levels similar to those found in therapeutic doses in mammals would be reached in fish. If one uses the brain-to-plasma ratio of 2.6 estimated for fluoxetine in mammals (49), therapeutic doses would lead to brain concentrations of 260 ng/ml compared with total detected SSRI and metabolite concentrations of 30 ng/g brain tissue in wild-caught fish (4). A low pharmacological dose similar to doses used in mammals elicited at least partially similar responses, especially with respect to isotocin. Effects of fluoxetine on oxytocin release are well documented in the mammalian literature at doses of 10 mg/kg per day (32). This is in line with a partially conserved mechanism of action for fluoxetine, which is further supported by the finding that binding kinetics of SSRIs to SERT in fish are similar to mammalian kinetics (22). Our studies raise the possibility that SSRIs are potential endocrine disruptors. We provide the mechanistic foundation needed to test the hypothesis that environmentally relevant levels of fluoxetine disrupt reproduction in fish.

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