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GP_{ER}/GPR30, a Membrane Estrogen Receptor, Is Expressed in the Brain and Retina of a Social Fish (*Carassius auratus*) and Colocalizes With Isotocin

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Abstract

Estradiol rapidly (within 30 minutes) influences a variety of sociosexual behaviors in both mammalian and nonmammalian vertebrates, including goldfish, in which it rapidly stimulates approach responses to the visual cues of females. Such rapid neuromodulatory effects are likely mediated via membrane-associated estrogen receptors; however, the localization and distribution of such receptors within the nervous system is not well described. To begin to address this gap, we identified GP_{ER}/GPR30, a G-protein-coupled estrogen receptor, in goldfish (*Carassius auratus*) neural tissue and used reverse-transcription polymerase chain reaction (RT-PCR) and *in situ* hybridization to test if GPR30 is expressed in the brain regions that might mediate visually guided social behaviors in males. We then used immunohistochemistry to determine whether GPR30 colocalizes with isotocin-producing cells in the preoptic area, a critical node in the highly conserved vertebrate social behavior network. We used quantitative (q)PCR to test whether GPR30 mRNA levels differ in males in breeding vs. nonbreeding condition and in males that were socially interacting with a female vs. a rival male. Our results show that GPR30 is expressed in the retina and in many brain regions that receive input from the retina and/or optic tectum, as well as in a few nodes in the social behavior network, including cell populations that produce isotocin.

Keywords

estrogen receptor; GPR30; GP_{ER}; isotocin; social behavior network; teleost fish

In many vertebrate species the expression of socio-sexual behaviors are rapidly (within 30 minutes) modulated by 17 β -estradiol (E₂) acting on limbic, sensory, and motor systems. For example, E₂ rapidly stimulates sexual receptivity in female rats via actions in the hypothalamus (Dewing et al., 2007) and increases the expression of male sexual behavior in

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Japanese quail, mice, and rats via actions in the preoptic area (Cross and Roselli, 1999; Cornil et al., 2006; Taziaux et al., 2007). E₂ also functions in the rapid modulation of social communication circuits by influencing auditory processing and song preferences in the zebra finch telencephalon (Ramage-Healey et al., 2010a, 2012; Tremere and Pinaud, 2011) and vocal patterning in the hindbrain of a teleost fish (Ramage-Healey and Bass, 2004, 2005). Thus, it is clear that estrogens are capable of dynamically regulating the activity of brain networks involved in the expression of a diversity of behaviors that guide social interactions, perhaps through actions in pathways that are evolutionarily conserved among vertebrates. However, the receptors that mediate these rapid estrogenic effects and their localization within the brain still remain largely unclear.

One possibility is that at least some rapid behavioral effects of E₂ are mediated by receptors that initiate nongenomic effects within neural pathways that influence social behavior or the processing of social cues (for review, see Ramage-Healey et al., 2010b; Maney and Pinaud, 2011; Cornil et al., 2012). Although some rapid actions of estrogens can be mediated via the classical estrogen receptors (ERs) ER α and ER β inserted into the neuronal membrane, G-protein-coupled receptors may also mediate some of E₂'s rapid behavioral and physiological effects (Lebesgue et al., 2009; Noel et al., 2009; Sun et al., 2010; Thomas, 2012; Liu et al., 2012). GPR30 (also known as GPER) is a membrane-bound G-protein-coupled ER that has been identified in brain tissue of rat, bird, and zebrafish (Brailoiu et al., 2007; Canonaco et al., 2008; Liu and Zhu, 2009; Archaya and Veney, 2012), but its distribution in networks that control sexual and social functions has not been well described to date. A common feature of GPR30 in all vertebrates studied thus far is its abundance in the hypothalamus and preoptic area (Sakamoto et al., 2007; Canonaco et al., 2008; Liu and Zhu, 2009), and one study in mammals has shown that, within these regions, GPR30 is primarily expressed in cells that produce the neuropeptide oxytocin (Sakamoto et al., 2007). Oxytocin and its homologs mesotocin (birds, reptiles, some cartilaginous fish) and isotocin (bony fish) influence a variety of social functions in vertebrates (for review, see Goodson, 2013; Anacker and Beery, 2013; Goodson and Thompson, 2010), indicating a great deal of conservation in these peptide circuits. However, the possibility that E₂ might rapidly modulate neuropeptide-producing cells through GPR30 receptor mechanisms has not been explored in any nonmammalian species.

To begin to address the issue of whether GPR30 could mediate the rapid effects of E₂ on the expression of sociosexual behaviors, we characterized GPR30 mRNA and protein in goldfish brain in regions with known functions related to social behavior or the perception of social cues, and we asked whether GPR30 colocalizes with isotocin. Goldfish are a good model in which to investigate this issue for two reasons. First, the teleost fish nervous system, and that of goldfish in particular, contains a large quantity of aromatase enzyme, and thus is capable of rapid, *de novo* synthesis of estrogens (Diotel et al., 2010). Second, goldfish are a highly social species (Kavaliers, 1989; Magurran and Pitcher, 1983) in which behavior has been well studied in the context of sex steroid modulation. During the breeding season, as testosterone levels rise, olfactory cues play a predominant role in eliciting courtship-related behaviors (reviewed in Stacey and Sorensen, 2009), although visual cues are used for sexual discrimination (Thompson et al., 2004) and may help males maintain proximity to ovulating females and thus better compete with other males during spawning

(Thompson and Mangiamele, 2013). In goldfish males, rapid increases in social approach in response to the visual stimuli of females (in the absence of other cues) can be induced within 30–45 minutes of peripheral testosterone (T) injection, an effect that is blocked by aromatase inhibition, and within 10–25 minutes of peripheral estradiol injection (Lord et al., 2009). Although olfactory cues are critical for initiating courtship in male goldfish, we have no evidence to suggest that E₂ rapidly modulates responses to olfactory signals in this species.

E₂ could rapidly influence pathways that mediate visually guided approach responses of males to females via actions in limbic structures associated with the social brain network (SBN), a collection of highly conserved, interconnected nuclei that respond to sex steroids and mediate multiple social behaviors, including sexual responses, across vertebrates (Newman, 1999; Goodson, 2005; O'Connell and Hofmann, 2011; O'Connell and Hofmann, 2012). In goldfish, proposed homologous regions include the supracommissural nucleus of the ventral telencephalon (Vs), the putative fish homolog of the medial extended amygdala, and/or the preoptic area (POA). Both the Vs and the POA are areas that express aromatase in goldfish (Gelinas and Callard, 1993; Gelinas and Callard, 1997) and are involved in the regulation of courtship behavior (Kyle and Peter, 1982). The preoptic area receives direct retinal projections (Springer and Gaffney, 1981), as well as olfactory input (Levine and Dethier, 1985; Lado et al., 2014), so it could play a critical role in integrating sexual stimuli processed in multiple sensory modalities into behavioral output. Interestingly, isotocin produced in the preoptic area can, like E₂, stimulate approach responses to the visual stimuli of conspecifics within 5–20 minutes in goldfish males (Thompson and Walton, 2004), raising the possibility that estrogenic effects on these peptidergic neurons, perhaps through GPR30 signaling mechanisms, could also play some role in E₂'s behavioral effects.

Alternatively, or in addition, rapid estrogenic effects on male goldfish social approach behavior could be mediated through actions in visual processing areas, including the retina, where increased visual sensitivity could help males detect potential mates, and/or its primary target, the optic tectum (OT), which plays a critical role in visual orienting (Springer et al., 1977). Notably, both the retina and one of its major primary targets, the OT, contain high levels of the enzyme aromatase in goldfish (Gelinas and Callard, 1997), thereby supporting the hypothesis that local synthesis of estrogens in this primary visual pathway could play a particularly important role in mediating visually guided behaviors. Estrogens could also stimulate approach responses to females through influences on the processing of visual information in ascending visual pathways that ultimately reach the dorsal telencephalon, including the lateral, medial, and central divisions (Dl, Dm, and Dc, respectively) via a relay in the preglomerular complex (Northcutt, 2006; Yamamoto and Ito, 2008), although the precise role these pathways play in visual processing is not yet known.

The main aim of this study was to test the hypothesis that GPR30 is expressed in brain regions that potentially mediate rapid, estrogen-modulated visually guided social behaviors in goldfish, particularly in those regions that synthesize estrogens. Thus, our focus is on localizing GPR30 mRNA and protein in primary retinal targets that express aromatase and that mediate rapid orienting responses (in particular, the OT), and in forebrain regions that express aromatase and likely generate approach responses to sexual stimuli. In particular, we focused on several nodes of the SBN, particularly the Vs and preoptic area, both of which

contain aromatase in goldfish and are critical for the expression of courtship-related behaviors in males (Kyle and Peter, 1982). In addition, we predicted that GPR30 in the preoptic area of goldfish would be localized on isotocin-producing cells, as it is in mammals. Because brain aromatase and circulating sex steroid levels in goldfish fluctuate seasonally (Pasmanik and Callard, 1988) and during social interactions (Kobayashi et al., 1986), we also predicted that GPR30 mRNA levels would differ in the brains of males in breeding vs. nonbreeding condition and in males that were socially interacting with a female vs. a rival male.

MATERIALS AND METHODS

Animals

Adult comet goldfish (12–15 cm length) were purchased from Hunting Creek (Thurmont, MD) and Blackwater Creek Fisheries (Eustis, FL) and housed in the lab in 200-gallon stock aquaria with 15–50 other same-sex individuals. We held fish in long daylight conditions (14L:10D) at 20°C during the spring and summer breeding season (late April to June) and in short daylight conditions (12L:12D) at 18°C during fall and winter (October–December). In all experiments, animals were sacrificed by immersion in 0.1% MS-222 (Sigma-Aldrich, St. Louis, MO) followed by decapitation. All animal procedures were approved by the Research Oversight Committee (IACUC) at Bowdoin College.

Identification of partial goldfish GPR30 mRNA sequence

We extracted total RNA from pooled male and female goldfish brain homogenates using Trizol reagent (Invitrogen, Grand Island, NY) and synthesized cDNA using Superscript III reverse transcriptase and oligo dT primers. To identify goldfish GPR30, we designed degenerate primers using Codehop (Table 1) and performed polymerase chain reaction (PCR) using the following parameters: 10 cycles of 94°C for 10 seconds, 65°C for 30 seconds, 72°C for 1 minute, and 25 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute. We sequenced the resulting 564 bp fragment. We then performed 5' rapid amplification of cDNA ends (5' RACE) using the SMARTer RACE system (ClonTech, Palo Alto, CA) with a universal primer mix and a reverse gene-specific primer (Table 1) to amplify a 1046 bp fragment that included a partial coding sequence. We sequenced subclones of all fragments and assembled a contig of all available sequence (1222 bp) of goldfish GPR30 (GenBank Accession No. KR941551).

To determine the degree of similarity between goldfish GPR30 and that of other vertebrate species, we compared goldfish GPR30 predicted protein with the known protein sequences of two other teleost fish (zebrafish [*Danio rerio*; GenBank Accession: NP_001122195.1] and Atlantic croaker [GenBank Accession: ABY51613]), zebra finch (GenBank Accession: XP_004175714.1), rat (GenBank Accession: NP_598257.1), and human (GenBank Accession: NP_001496.1). We aligned the sequences using Clustal Omega multiple sequence alignment and displayed the resulting multiple sequence alignment using Boxshade (http://www.ch.embnet.org/software/BOX_form.html) (Fig. 1).

Reverse transcription (RT)-PCR

To examine GPR30 expression in brain and body tissues using RT-PCR, we collected brain, retina, and liver tissue from male goldfish ($n = 4$) injected with Ovaprim (contains GnRH) and held overnight at 20°C to induce reproductive behavior. Using a scalpel, we first removed the OT. We then made a cut posterior to the optic nerve to divide the remaining brain tissue into two halves: one half containing the telencephalon plus the anterior diencephalon (“forebrain”), and the other half containing the posterior diencephalon, mesencephalon—excluding the OT—and rhombencephalon (“brainstem”) (Fig. 2A, dotted line). We extracted total RNA from pooled tissue homogenates using Trizol (Life Technologies, Grand Island, NY), treated it with DNase (DNA Free, Ambion, Grand Island, NY), and synthesized cDNA from 1 µg total RNA using Superscript III reverse transcriptase and oligo dT primers. We performed PCR using gene-specific primers (Table 1) designed to amplify a 166 bp segment of our original 564 bp fragment of GPR30. We used touchdown PCR cycling parameters as follows: 10 cycles of 94°C for 30 seconds, 60–55°C for 30 seconds (: 0.5°C every cycle), 72°C for 60 seconds, and 20 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 60 seconds. To check for the presence of genomic DNA contamination, we also performed RT-PCR on RNA samples that lacked reverse transcriptase (No RT). As a positive control, we amplified the housekeeping gene EF1α according to Marlatt et al. (2008). PCR products were analyzed on a 1% gel stained with ethidium bromide.

In situ hybridization

To examine the neuroanatomical distribution of GPR30 mRNA, we performed *in situ* hybridization on goldfish brain tissue. Male goldfish ($n = 11$) were individually housed overnight in 10-gallon tanks with a female that had been treated with prostaglandin F2a (PGF2α) and were sacrificed shortly after lights-on the following morning (07:00). We embedded brains in M-1 embedding medium (Thermo Scientific, Kalamazoo, MI), rapidly froze them on dry ice, sectioned them on a cryostat at 20 µm in four series, and mounted them on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). We generated radioactively (³⁵S) labeled sense and antisense riboprobes by reverse transcription from a cDNA template of *C. auratus* GPR30 using a MAXIscript kit (Ambion, Austin, TX). The sequence of the 539 bp antisense probe is as follows: GGTTCAGGCAGCTGTTG GAGAATGCGGCCAGGTTGACGATGTGTCCAGTCAACGGGT AGTCGTGTACAGTGTGGTGTGCTGCGTTTCGACGGGTCA GCTGTGCCTTGAGCAGCTGGATGCTAATGAAAACATTTTCA GGCAGCCAGCAAATGAAGAACACCAGCACCACCACCACAA TCATGCGCAGGGCCTTCTGCCGGCGCGGCCACAGTCCCCT GTGCTTCTGGGATCGCATGAGGGTGCGGACGATCAGGGAG TAGCACAGGCCGATGATGGAGAAGGGCACCAGAAATCCGA TCGTCACCTCGAGCCACTGGATCTCAAAGACATTGGCGAAG CAGAAGTGCACCTCGCCGGTGTGTTGTGTCTGCACAATGGT GAAAGGAAGCAAAGTAGCCAGGATAGAGGCCATCCAGATG AGGCTGCAGCTGAGTTTAGCGTGCTGCATGGTCCGGAGTGG ACTGCTGCTCAAAGAGTTAGCGAGAGCAATGTATCGGTTCGAA ACTCATCCATGTCAGGAAGAAGACGCTGCTGTAC. We performed *in situ*

hybridization according to the protocol described in Mangiamele et al. (2013), except that slides were hybridized overnight at 50°C. To visualize the bound riboprobe, we dipped slides in Kodak NTB autoradiography emulsion, allowed them to dry, and stored them in light-proof boxes at 4°C for 1 month. Slides were then developed and counterstained with cresyl violet. We confirmed the specificity of our riboprobe for GPR30 mRNA by noting the absence of binding (i.e., silver grains over tissue sections) on slides treated with sense probe (Fig. 3A1).

Immunohistochemistry

We used immunohistochemistry to identify specific cell populations that expressed GPR30. We collected brains of male and female goldfish in and out of breeding condition ($n = 1$ male with milt and 2 without, 1 female with eggs and 1 without) and fixed whole brains in 4% paraformaldehyde at 4°C overnight. We then washed them in 1× phosphate-buffered saline (PBS), cryoprotected in 30% sucrose solution overnight at 4°C, embedded in M-1 embedding medium, and stored them at -80°C until processing. We sectioned brains on a cryostat at 20 µm in four series and thaw-mounted them onto Superfrost Plus slides. We localized GPR30 on brain sections using a rabbit polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX; for additional details see Table 2), raised against the C-terminus of GPR30 of human origin. We postfixed sections on slides for 10 minutes in 4% paraformaldehyde, then washed two times in 1× PBS, and performed antigen retrieval by incubating the slides in boiling citrate buffer (10 mM citric acid; pH = 6.0) 3× for 5 minutes each. Slides were then rinsed 2× in PBS and incubated for 1 hour in blocking solution (2% bovine serum albumin [BSA] in 0.3% Triton X). We incubated slides in primary antibody (1:100 in blocking solution) overnight at 4°C in a humid chamber. After incubation, we rinsed them 2× in PBS and incubated in the dark for 2 hours with goat anti-rabbit secondary Alexa Fluor 488 antibody (1:200; Invitrogen, Cat# A11008, RRID:AB_143165). We rinsed the tissue 2× in PBS, 1× in distilled water, and cover-slipped using HardSet Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA).

Alternate series of four brains of both males and females were double-labeled with anti-GPR30 primary antibody (1:100) and a mouse monoclonal anti-oxytocin antibody (1:5,000; Millipore, Billerica, MA; for additional details see Table 2) to label cells in the goldfish preoptic area that produce isotocin, the nonmammalian homolog of oxytocin. GPR30 was visualized in brain tissue using the same method described above, while isotocin was visualized with horse anti-mouse Dylight 594 secondary antibody (1:500; Vector Laboratories, Cat# DI-2594-1.5)

Antibody characterization

Details of the primary antibodies used in this study are given in Table 2. A comparison of the human-directed immunizing sequence of our anti-GPR30 antibody (aa 290–340, GenBank Accession: Q99527.1) and the corresponding peptide sequence of GPR30 in zebrafish (aa 273–325, GenBank Accession: NP_001122195.1) suggests that our primary antibody is likely to be highly specific to fish GPR30: the two sequences are 74% identical and 84% of the residues show strongly similar properties. Although we were not able to obtain sequence for goldfish GPR30 over the entire 50 amino acid immunizing sequence,

there is an exact match between the available goldfish sequence and the zebrafish sequence in the first eight residues of the immunizing sequence, and it is reasonable to expect that goldfish and zebrafish sequences are highly similar over the remaining 42 residues, given that the available predicted protein sequences are 93% identical.

For each primary antibody, we conducted preliminary experiments to determine the optimal dilution. To test the specificity of the GPR30 antibody, we preadsorbed GPR30 primary antibody with a 5-fold excess of GPR30 peptide fragment (Santa Cruz Biotechnology, Cat# sc-48523-P) for 1 hour at room temperature. We then incubated alternate sections in the preadsorbed antibody overnight at 4°C in a humid chamber and visualized using the secondary antibody as described above. We observed no fluorescent staining in tissue sections treated with preadsorbed antibody (Fig. 3C2).

The anti-oxytocin primary antibody has been previously characterized by the manufacturer using a competitive enzyme-linked immunosorbent assay (ELISA) assay and showed no reactivity to arginine vasopressin or vasotocin. To further test the specificity of the antioxytocin primary antibody for isotocin, we conducted preadsorption assays on goldfish tissue. We preadsorbed the OT antibody with 50 μ M vasotocin or isotocin for 1.5 hours at room temperature. Both aliquots were then used in double-labeling experiments with GPR30 antibody. Alternate sections incubated with pre-adsorbed vasotocin appeared qualitatively similar to sections that were double-labeled with anti-GPR30 and anti-oxytocin antibody, while sections incubated with isotocin appeared qualitatively similar to series treated with only GPR30 antibody, indicating that the oxytocin antibody is specific for isotocin and does not recognize vasotocin. To rule out the possibility of nonspecific binding of our secondary antibody, we used alternate series as no primary controls in every immunohistochemistry experiment conducted and observed no fluorescent signal in any experiment.

Western blot characterization of GPR30 antibody

To confirm that our GPR30 antibody binds specifically to goldfish GPR30, we extracted total cellular proteins from whole goldfish brain using RIPA lysis and extraction buffer (Thermo Scientific, Tewksbury, MA) according to the manufacturer's instructions. We separated the proteins in the whole brain extract by size using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred them to a nitrocellulose membrane for 1.5 hours. The membrane was blocked in 0.5% dry nonfat milk in wash buffer (1 $\times$ Tris-buffered saline, 0.1% Tween 20), then incubated in our anti-GPR30 primary antibody (1:200) for 2 hours at room temperature, washed 3 times for 5 minutes each in wash buffer, and then incubated in goat antirabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:20,000; BioRad, Hercules, CA, Cat# 170-5046) for 1 hour at room temperature. After washing 3 times for 5 minutes each in wash buffer, the membrane was exposed to HRP substrate (TMB Membrane Peroxidase Substrate System, KPL, Gaithersburg, MD) for 15 minutes, per the manufacturer's instructions. We visualized two bands: one faint band at 55 kD and a darker band around 110 kD (Fig. 3B1). Previous reports show multiple forms of GPR30 that vary in size, with nonglycosylated forms at 38 and 55 kDa and mature glycosylated forms at 80 and 120 kDa in human tissue (Maiti et al., 2011), although the size/form of the protein may vary by tissue type, availability of estrogen,

etc. Preadsorption of the primary antibody with 5-fold excess of the blocking peptide for 1 hour at room temperature blocked visualization of all bands (Fig. 3B2). We were not able to characterize our anti-oxytocin antibody using Western blot; however, preadsorption of the anti-oxytocin primary antibody with 50 μ M isotocin for 1.5 hours at room temperature prior to immunohistochemistry blocked all signal.

Microscopy

For brain sections processed for *in situ* hybridization (ISH), we qualitatively assessed presence or absence of GPR30 mRNA expression relative to background silver grain levels while viewing the tissue under darkfield illumination at low (4 $\times$) and high (10 $\times$, 20 $\times$, 40 $\times$) magnification. Brightfield illumination at high magnification was used to visualize the ISH signal simultaneously with Nissl stain in order to delineate neuroanatomical boundaries. Photomicrographs of mRNA expression were taken at 10 $\times$ under darkfield illumination with a digital monochrome camera (Nikon OS-Fi2, Melville, NY) attached to a Nikon Ti Eclipse microscope equipped with a motorized scanning stage. We created image mosaics composed of multiple fields of view using Nikon Elements BR software. If necessary, the final images were brightness- and contrast-enhanced in Adobe Photoshop (San Jose, CA) for clarity. We defined neuroanatomical boundaries using the goldfish atlases of Bradford and Northcutt (1983) and Nieuwenhuys (1998), and qualitatively assessed the presence or absence of GPR30 mRNA expression relative to background silver grain levels under darkfield illumination.

We viewed fluorescently labeled sections on an Olympus BX51 microscope (Olympus, Center Valley, PA), which was outfitted with epifluorescence, and took photographs with a digital monochrome camera (Evolution VF, Media Cybernetics, Rockville, MD). For double-labeling experiments, the same field of view was photographed sequentially using FITC and Texas red filter cubes and the images were later merged using Adobe Photoshop. Some fluorescent sections were also examined and photographed using a confocal laser-scanning microscope (Leica TCS, Leica Microsystems, Buffalo Grove, IL) and Leica LSM software to adjust image brightness and contrast.

Quantitative real-time polymerase chain reaction (qPCR)

We used quantitative real-time PCR to measure mRNA expression levels of GPR30 in the brains of goldfish males sacrificed during the breeding season (May–June; $n = 4$) and compared it with those in nonbreeding condition (October and December; $n = 4$). Before arriving at our laboratory, males were kept outdoors in mixed-sex ponds. We kept them in single-sex 200-gallon tanks and sacrificed them within 10 days of arrival. We confirmed that all males sacrificed in the breeding season were in reproductive condition by noting the expression of milt (sperm and seminal fluid) upon application of light pressure to the abdomen. In the nonbreeding season, males lacked expressible milt. Additionally, testes were removed and weighed to calculate the gonadosomatic index [GSI 5 (gonad mass/body mass) $\times$ 100]. The average GSI of males in breeding condition was 3.5 ± 0.7 and in nonbreeding condition it was 2.6 ± 0.8 .

In a second experiment, we used qPCR to compare GPR30 mRNA expression levels in male goldfish brains in response to different social stimuli. The day before the experiment, all males were injected with Ovaprim and housed with either a female that was injected with (PGF2 α , dose: 0.1 μ g per g body weight) ($n = 5$) or another male fish ($n = 6$). Females injected with PGF2 α become sexually receptive within 30 minutes GPER/GPR30 in the goldfish brain (Stacey and Goetz, 1982). Males were in reproductive condition and expressing milt. The following day, we recorded male following behavior for 1 hour after lightson. Males exposed to females displayed stereotypical courtship behavior, including following, nudging, and in some cases spawning. No sexual behavior was observed in males exposed to other males.

Males in both experiments were sacrificed shortly after lights-on (07:00), their brains were removed, immediately frozen on dry ice, and stored at -80°C until RNA extraction. We extracted total RNA using Trizol reagent (Life Technologies, Carlsbad, CA) and synthesized cDNA by reverse transcription using a qScript cDNA Supermix kit (Quanta Biosciences, Gaithersburg, MD). qPCR was performed using a Step One Real-Time PCR system (Applied Biosystems, Foster City, CA). We amplified GPR30 in 20 μ l reactions in triplicate using Quanta PerfeCTa SYBR Green Fastmix with Rox reference dye, 0.25 μ M of each primer, and 5 ng/ μ l cDNA. PCR parameters were as follows: 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. We used PCR Miner to calculate reaction efficiencies (E) and threshold cycle number (CT) from the raw fluorescence readings (Zhao and Fernald, 2005). Reaction efficiencies were between 0.85–1.0. We normalized the expression of GPR30 relative to the expression of a reference gene (EF1 α) using the following equation: relative target mRNA levels $5 [1/(11E_{\text{target}})^{\text{CT}_{\text{target}}}] / [1/(11E_{\text{reference}})^{\text{CT}_{\text{reference}}}]$, where E is the reaction efficiency and CT is the average cycle threshold of triplicate reactions. Mean CT values for EF1 α were analyzed using a Student's t-test to verify that there was no significant difference between the test groups being compared ($P > 0.13$). Primer sets for both genes are shown in Table 1 and produced a single melting curve peak when cDNA template was used in the reaction, but no amplification when reverse transcriptase was omitted from the cDNA synthesis reactions (negative controls).

Statistical analysis

qPCR data were not normally distributed, so we compared mRNA levels between groups using the Mann-Whitney U-test. We used JMP 9.0 (SAS, Cary, NC) for statistical analyses.

RESULTS

Identification of partial sequence *C. auratus* GPR30

We identified a 1222 bp fragment of *C. auratus* GPR30 mRNA, which coded for a predicted 283 amino acid protein. Comparison of the partial sequence of goldfish GPR30 predicted protein with full-length zebrafish, Atlantic croaker, zebra finch, rat, and human proteins showed that GPR30 is highly conserved among vertebrates. Goldfish GPR30 had, on average, 91% identity and 96% positive similarity to the two other teleost fish species, 69% identity and 81% similarity to zebra finch, and 73% identity and 84% similarity to rat and

human (Fig. 1). As is the case for most G-protein-coupled receptors, the *C. auratus* GPR30 predicted protein was highly conserved at known transmembrane domains, where goldfish GPR30 is, on average, 96% identical to that of other fish species, 81% identical to bird, and 84% identical to mammals, suggesting that it likely has a similar subcellular localization to GPR30 in other vertebrate species.

RT-PCR

We demonstrated that GPR30 is expressed in the brain and retina of male goldfish using RT-PCR. Compared to the uniform abundance of a housekeeping gene in all tissues sampled, we observed GPR30 to be relatively more abundant in the OT and brainstem samples compared to forebrain samples, and completely absent from liver tissue (Fig. 2B). This distribution is consistent with that found in fish and mammals, although the liver cells of humans and one fish species (gilthead seabream) do appear to express GPR30 mRNA (Liu and Zhu, 2009; Olde and Leeb-Lundberg, 2009; Cabas et al., 2013). We did not amplify GPR30 in control samples that lacked reverse transcriptase (no RT; Fig. 2B) or in no template controls (not shown), indicating that our samples and reagents were free of DNA contamination.

In situ hybridization

More precise localization of GPR30 mRNA in individual nuclei of interest in the goldfish brain was assessed via in situ hybridization. Overall, in male goldfish that were housed overnight with a PGF2 α -injected female, we found GPR30 expression in at least some nuclei of the forebrain, midbrain, and hindbrain, a pattern that is consistent with our RT-PCR results. Here we report only the most robust and unequivocal GPR30 hybridization signal, which we define as silver grain density in the brain region of interest that is at least 23 background levels. As already noted, we were primarily interested in whether GPR30 was found in brain regions that could play a role in mediating the rapid effects of estradiol on visually guided social approach behavior in male goldfish, although we also note other regions that showed strong hybridization signal. Table 3 summarizes GPR30 expression in primary and secondary visual pathways, as well as expression in the SBN, which includes isotocin-producing cells in the preoptic area and many of their target nuclei. In addition, Table 3 summarizes sites within visual pathways and the social behavior network that express aromatase, based on previous studies in goldfish and/or other teleost fish (Gelinas and Callard, 1997; Forlano et al., 2001; Goto-Kazeto et al., 2004). Given that many rapid estrogen effects depend on neural aromatization of androgens into estrogens (Maney and Pinaud, 2011), it is important to understand the distribution of GPR30 in the context of brain regions in which estradiol can be locally and rapidly produced.

Primary and secondary visual pathways

GPR30 mRNA is abundant in several cell populations in the forebrain and midbrain that receive either direct input from the retina or secondary visual input from the OT, but not in those pallial regions that receive secondary or tertiary visual input. Of the retinorecipient cell populations, we found robust hybridization signal in the suprachiasmatic nucleus and in the parvocellular and magnocellular preoptic area (Fig. 4B,C), and consistently throughout most of the rostral-caudal extent of the OT (Fig. 4C,D,F), which is the primary visual center in fishes. Of the six cell layers that make up the OT, hybridization signal was most dense in the

stratum fibrosum et griseum superficiale (SFGS) and stratum album centrale (SAC) (Fig. 3A2). We also found GPR30 expression in the central pretectal nucleus and dorsal accessory optic nucleus, (Fig. 4D) which receive both retinal and tectal efferents (Grover and Sharma, 1981). Although visual information does reach the dorsal telencephalon via ascending relays through pregomerular complex (Northcutt, 2006; Yamamoto and Ito, 2008), GPR30 expression was undetectable or at very low levels in the pallium (not shown). GPR30 mRNA was also very abundant in several fiber pathways that carry visual information, including the optic tract, which carries information from the retina (Fig. 4C,D) and the horizontal commissure (Fig. 4C,D), which carries bundles of fibers from the OT to the magnocellular superficial pretectal nucleus. Although fibers themselves cannot produce GPR30 mRNA, it is possible that mRNA localized to fibers is being transported for local translation elsewhere in the cells.

Social behavior network

In the SBN, we found that the anterior part of the parvocellular preoptic area showed the strongest hybridization signal (Fig. 4B), with strong expression also noted in the anterior magnocellular preoptic area (which receives some direct retinal input). Less consistent and weaker signal was also seen in periventricular regions of the hypothalamus, including the ventral hypothalamus (Fig. 4D), and in the periaqueductal gray (Fig. 4E) of some animals. Notably, areas of the forebrain that receive olfactory inputs, with the exception of the preoptic area, were largely devoid of GPR30 signal. These include the supracommisural nucleus of the ventral telencephalon (Vs) and the ventral nucleus of the ventral telencephalon (Vv), which receive inputs from medial divisions of the olfactory tract that process pheromone stimuli, and the posterior division of the dorsal telencephalon (Dp), which primarily receives information from the lateral olfactory tract (Levine and Dethler, 1985). We did not thoroughly investigate the olfactory bulbs themselves, but there did not appear to be intense signal in the few that were examined.

Other sites of GPR30 mRNA expression

GPR30 was also present in brain areas not directly implicated in sociosexual regulation in goldfish. Although outside the scope of our analysis, we consistently observed strong GPR30 expression between the medial boundary of the torus semicircularis and valvula cerebelli and some diffuse labeling throughout the tegmentum (for an example, see Fig. 4F) that was at least twice background levels, although the pattern of expression in specific nuclei/cell populations was not consistent among animals. Therefore, we have not specified here individual regions of the midbrain tegmentum that express GPR30. In a few animals, we observed strong GPR30 hybridization signal throughout the rostral hindbrain, although most of it appeared to be outside of well-defined cell populations, possibly in fiber tracts (not shown). Because we only observed GPR30 expression in hindbrain regions in two animals, we have not conducted extensive analyses on the specific hindbrain areas in which GPR30 was found.

Immunohistochemical detection of GPR30 in isotocin-producing cells in the preoptic area GPR30 immunolabeling was clearly extranuclear, and primarily cytoplasmic, in all brain regions in which it was observed in goldfish (Fig. 5A1-2), which is consistent with previous

observations in rat using the same primary antibody (Sakamoto et al., 2007). The labeling of GPR30-immunoreactive (ir) cell bodies was most intense in the preoptic area (Fig. 5B,C), with lower levels or no signal in midbrain regions where we observed GPR30 mRNA, with the exception of some labeled cells and fibers in and around the periaqueductal gray (Fig. 5E). We observed strong GPR30 immunolabeling of fibers in the preoptic area projecting to/from the parvocellular cell population (PPa; Fig. 5B), along the preoptico-hypophyseal tract from the preoptic area to the ventral hypothalamus (Fig. 5D), and along the lateral aspect of the periaqueductal gray (Fig. 5E). Many GPR30-immunolabeled fibers had varicosities that appeared along their entire length or a portion of their length (Fig. 5A3). Sections treated with GPR30 primary antibody preadsorbed with GPR30 peptide showed no immunolabeling (Fig. 3C2).

To test whether GPR30 is present in isotocin (IT)-producing cells, we double-labeled for GPR30 protein and IT in goldfish brain tissue. We observed GPR30/IT colocalization along the entire rostrocaudal extent of the preoptic area; however, we observed the largest population of labeled cells in the parvocellular (PPa) and the magnocellular (PM) preoptic area (Fig. 6F–H) in a pattern that was consistent with that observed in our GPR30 *in situ* hybridization. Axonal projections from these cells were also colabeled, particularly in or around the large swellings/varicosities (Fig. 6H,I). Preadsorption with excess vasotocin (VT) did not block IT (Fig. 6A) or GPR30 (Fig. 6B) immunoreactivity, whereas preabsorption with excess IT blocked the anti-oxytocin signal (Fig. 6D) but not GPR30 signal (Fig. 6E). Although we did not quantify the percentage of GPR30/IT colocalizing cells, nor systematically examine GPR30/IT colocalization across sexes or seasons, in the sections we examined we were unable to identify GPR30 cells in the POA that lacked IT, or IT cells that lacked GPR30 (Fig. 6H) in male goldfish, even in sections in which the antibodies were preincubated with excess VT (Fig. 6C). However, we did not have access to a non-rabbit VT antibody that does not crossreact with IT and so were unable to perform GPR30/VT double labeling. We therefore cannot completely rule out the possibility that GPR30 could also be found in non-IT preoptic neurons, including VT neurons. We observed similar patterns of colocalization in males and females sacrificed during and outside of the breeding season.

Seasonal and social modulation of GPR30 gene expression

To test whether GPR30 varies in the brain with season or social experience, we quantified and compared GPR30 mRNA levels using qPCR. We found no difference in the level of GPR30 expression in whole brains of males in breeding vs. nonbreeding condition (Mann–Whitney $U = 8$, $P = 0.99$; Fig. 7A), and no difference between males that interacted socially with a female vs. a rival male (Mann–Whitney $U = 14$, $P = 0.90$; Fig. 7B). In addition, GPR30 levels did not correlate with GSI (Pearson's $r = 0.21$, R-squared = 0.05, $P = 0.61$; Fig. 7C).

DISCUSSION

Estradiol (E_2) has rapid influences on social behaviors in fish, influencing visually guided social approach behaviors in goldfish within 25 minutes (Lord et al., 2009) and the firing of neurons involved in vocal displays in midshipman fish within just 5 minutes (Remage-

Healey and Bass, 2004, 2006). The rapid onset of such behaviors is consistent with E_2 's action on a nonnuclear and/or membrane-associated estrogen receptor, yet such a receptor had not been demonstrated in the brain pathways that are involved in processing or generating social signals in these species. Here we identified GPR30, a G-protein-coupled estrogen receptor, within brain regions that could mediate rapid, estrogen-modulated visually guided sociosexual behaviors in goldfish, although it is not exclusively produced within these pathways. In the following, we compare the patterns of GPR30 expression that we observed to previously published patterns of expression of aromatase in goldfish and other teleost fish in order to understand whether GPR30 receptor mechanisms might mediate the rapid, estradiol-induced changes in social behavior in fish previously observed by our lab and others.

In support of the possibility that estradiol stimulates visual orienting behaviors in goldfish via GPR30 mechanisms, we show that GPR30 mRNA is expressed in goldfish retina, throughout the optic tract, and at high levels in some nuclei in primary and secondary visual pathways. These nuclei include the OT (specifically, the SFGS and SAC cell layers), the suprachiasmatic nucleus (SCN), and magnocellular preoptic area, all of which receive direct retinal projections in fish (Springer and Gaffney, 1981; Vanegas and Ito 1983; Northcutt and Wulliman, 1988) and/or are physiologically responsive to a variety of visual stimuli (Guthrie and Sharma, 1991). We also found GPR30 in the central pretectal nucleus, which receives direct projections from the optic tract (Bradford and Northcutt, 1983). Interestingly, expression of the enzyme aromatase is highest in many of the same visual pathways in which we observed GPR30 expression in goldfish (see Table 3). Aromatase is found within the retina, processes within the optic tract, cells in the suprachiasmatic nucleus and preoptic area, and cells/fibers of the SFGS and SAC layers of the OT (Gelinas and Callard, 1993; Gelinas and Callard, 1997). However, it should be noted that GPR30's expression is more extensive than what has thus far been described for aromatase in goldfish brains. On the other hand, GPR30 expression was not high in telencephalic or diencephalic areas that receive ascending visual input, most notably areas within DI, Dm, or Dc (Northcutt, 2006; Yamamoto and Ito, 2008). Although some of these areas do express high levels of aromatase in goldfish and other teleost fishes (Gelinas and Callard, 1997; Forlano et al., 2001), in particular Dc, we could find almost no GPR30-expressing cells in the dorsal telencephalon. Thus, our work suggests that if GPR30 does mediate rapid estrogen effects on visual responses related to courtship and social approach, it likely influences early stages of visual processing that might influence initial detection of and/or orienting responses to socially relevant visual cues, but not higher-level processing of those stimuli. Notably, GPR30 expression was also not found in the olfactory bulb, nor in telencephalic areas that receive direct olfactory input, which is consistent with our lab's observation that olfactory responses appear to be androgen-mediated, not estrogen-mediated (Thompson et al., unpublished).

We also found GPR30 in two important nodes of the SBN: the preoptic area and the periaqueductal gray. We found the strongest GPR30 mRNA and protein abundance in the parvocellular and magnocellular preoptic area. Like in other vertebrate species, ablation of the preoptic area impairs (but does not completely abolish) courting and spawning behavior in male goldfish (Kyle and Peter, 1982), suggesting that it has a central role in the regulation of behaviors related to reproduction. The preoptic area likely also plays an important role in

responding to social cues, as it receives visual as well as olfactory inputs in goldfish (Springer and Gaffney, 1981), and some POA cells are strongly activated by female preovulatory pheromones (Kawai et al., 2015), which are well known to stimulate large increases in testosterone in goldfish males (Stacey et al., 1989). Several studies note strong aromatase expression in the preoptic area of teleosts (see Table 3), especially in the parvocellular preoptic area, where we noted extensive GPR30 labeling, suggesting that the physiology of these cells could be rapidly modulated by locally synthesized estradiol. We also found GPR30 signal in cells of the periaqueductal gray (PAG) and in the fibers surrounding it. The PAG has been shown to play a central role in modulating motor circuits related to social communication in fish; for example, neuronal firing patterns in cells of the PAG directly predict the initiation and duration of vocal motor output in midshipman fish (Kittelberger et al., 2006). Although it has not yet been described in goldfish, the distribution of aromatase mRNA and protein in the PAG of other teleosts (*Porichthys notatus*) closely matches the GPR30 expression noted in our study (Forlano et al., 2001). Taken together, the apparent overlap in distribution of GPR30 and aromatase in brain pathways related to the modulation of social behaviors raises the possibility that brain-derived estrogen could act on GPR30 to play a role in rapidly modulating these processes in fish.

It is interesting to note that, unlike ER α and ER β , GPR30 is not expressed in all nuclei of the social behavior network in goldfish. We found that GPR30 mRNA was present in the preoptic area and less consistently in the PAG, but not in the supracommisural nucleus of the ventral telencephalon (Vs; medial amygdala homolog), anterior tuberal nucleus (aTn; ventromedial hypothalamus homolog), or ventral tuberal nucleus (vTn; anterior hypothalamus homolog). This pattern is similar to the divergent profiles of GPR30 and the other ERs in mice, where GPR30-ir in the SBN does not overlap to a high degree with ER α or ER β . For example, GPR30 labeling is very intense in the mouse POA, ventromedial hypothalamus (VMH), and PAG, while labeling for the ERs is high in the POA but low or absent in parts of the VMH and PAG (Hazell, 2009). Conversely, ER α and ER β are both highly expressed in the bed nucleus of the stria terminalis and amygdala in mice, whereas GPR30 is very low in both of these regions (Hazell, 2009). In short, the distribution of GPR30 in the SBN appears to be distinct from that of ER α and ER β in multiple vertebrate species, suggesting that the fast, nongenomic actions of E₂ could occur in different cell populations, and potentially different subnetworks, than the genomic effects of E₂ within this highly conserved system (although the distribution of membrane versions of ER α and ER β , which could also mediate rapid E₂ effects independent of, or in concert with, GPR30 have not been determined in these species). Given the proposed importance of patterns of activity across the nodes of the social behavior network in determining appropriate behavioral responses to social stimuli (Newman, 1999; Goodson, 2005), our lab is planning to further investigate coexpression of ERs and GPR30, and their functional roles, in the social behavior network of goldfish.

Importantly, we found that GPR30 protein expression in the goldfish POA is colocalized in isotocin-producing cells. Although we did not systematically quantify it, we found that virtually all IT-ir1 cells in the parvocellular and magnocellular preoptic area were also GPR30-ir1. This finding is consistent with a previous study in rats, which found that greater than 93% of cells in the paraventricular and supraoptic nuclei of the hypothalamus expressed

both GPR30 and oxytocin (Sakamoto et al., 2007) and one in mice that found between 60–80% of oxytocin neurons coexpressed GPR30 (Hazell, 2009). We also observed GPR30-positive and isotocin-positive fibers associated with a large proportion of labeled cells in the anterior preoptic area. In fish, isotocin is produced by neurons located in the parvocellular, magnocellular, and gigantocellular nuclei of the preoptic area, which project to the pituitary and numerous extra-hypothalamic structures, including the lateral division of the dorsal telencephalon, the medial part of the ventral forebrain, the OT, hindbrain, and spinal cord (Goodson et al., 2003; Saito et al., 2004). We observed that many of the GPR30- and isotocin-positive fibers emanating from the POA, as well as those present in the PHT, had swellings or varicosities with antibody colabeling, suggesting that GPR30 may be present at membrane sites associated with vesicle release, although this does not necessarily mean that GPR30 is located on the neurosecretory vesicles themselves. In all vertebrates, oxytocin/isotocin-containing vesicles are transported axonally from the POA to the pituitary for secretion of the neuropeptide into the cerebral vasculature, and for release at axon terminals in the anterior pituitary and in various forebrain structures, which in mammals include the amygdala and parts of the SBN (Buijs, 1978; Sofroniew, 1980; Knobloch et al., 2012). Thus, one possibility is that the rapid release of oxytocin/isotocin is mediated via nongenomic, GPR30-associated mechanisms. Although few physiological experiments have been done to test this hypothesis, one study found that activation of GPR30 rapidly (within 1 hour) increases prolactin release in rats, while RNA interference of GPR30 expression in the preoptic area, paraventricular nucleus, and ventromedial hypothalamus blocks local elevation of prolactin in response to E₂, potentially via stimulation of oxytocin release (Lebesgue et al., 2009). Further research is clearly necessary to parse out the influence of GPR30 activation on neuro-endocrine cell populations.

We also used qPCR to explore, for the first time, the effect of season or social context—two important aspects of the natural environment that regulate sensory processing in many species—on GPR30 transcript abundance. Given that goldfish show seasonal changes in their social behavior (Thompson et al., 2004), we expected to find GPR30 levels in the brain that were lower in winter and higher during the spring breeding season. Additionally, if GPR30 is involved in modulating rapid responses to the social and reproductive environment, we predicted that males exposed to an ovulating female might show higher levels of GPR30 than males housed with another male fish. However, we did not find that GPR30 mRNA levels were modulated by season or social experience in the brains of male goldfish, which could indicate that GPR30 is not associated with seasonal or context-dependent E₂ effects on courtship behavior. However, the lack of a seasonal effect may be a result of the experimental water temperature (18°C) not being cold enough to fully mimic wintertime conditions, although males in the nonreproductive condition did show regressed gonads, or it may reflect the fact that seasonal changes in behavior could be achieved by modulating substrate availability, and not sensitivity of the brain to estradiol. Both testosterone and brain aromatase levels are higher during the breeding season in goldfish (Pasmanik and Callard, 1988; Gelinas et al., 1998), so availability of brain-derived E₂ should be higher in the spring as well, and GPR30 levels may not need to change seasonally. Alternatively, changes in GPR30 levels with a season or social context may occur, but perhaps only in specific brain regions or cell populations, as seen for the ERs in other

teleosts (Forlano et al., 2005; Maruska and Fernald, 2010), which our qPCR assay of the entire brain was not sensitive enough to detect. It may also be the case that preovulatory stimuli that predict upcoming sexual interactions, such as the preovulatory pheromone, 17, 20 bP, drive up expression of GPR30 more strongly than do postovulatory stimuli, such as PGF2 α . To fully assess whether GPR30 participates in rapid social modulation in goldfish, and whether this varies with season or social context, future studies will test whether blocking the receptor pharmacologically can block T and E₂'s rapid effects on visually guided social behavior.

CONCLUSION

The neuroanatomical distribution of GPR30 in goldfish in brain regions associated with visually guided social approach is consistent with a possible role in rapidly modulating social signal processing and the production of social behaviors. We found the highest density of GPR30 mRNA in the retina, in some nuclei that receive primary visual input, and in some fiber tracts that transmit visual information, suggesting that E₂ may act via GPR30 to rapidly modulate sensory processes that mediate visually guided social approach behavior in this species. In contrast, we found little to no GPR30 in olfactory pathways, which are important in processing pheromonal signals. GPR30 colocalizes with isotocin in specific cell populations of the preoptic area that are well known to be associated with the modulation of social behavior, the expression of distinct social phenotypes, and/or other neuroendocrine processes. This latter pattern of GPR30 colocalization appears to be a conserved feature of vertebrate brains, and needs to be better studied to elucidate the role of E₂ in rapidly modulating the neurochemistry and functional activation of the brain's social behavior network.

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Abbreviations

aTn	Anterior tuberal nucleus
CAq	Cerebral aqueduct
CPN	Central pretectal nucleus
HC	Horizontal commissure
IH	Inferior lobe of hypothalamus
MLF	Medial longitudinal fasciculus
NA-OD	Dorsal accessory optic nucleus

OT	Optic tectum
OPT	Optic tract (inclusive of dorsomedial and ventrolateral subdivisions)
PAG	Periaqueductal gray
PM	Magnocellular nucleus of the preoptic area
PPa	Parvocellular nucleus of the preoptic area
SAC	Stratum album centrale (of the optic tectum)
SCN	Suprachiasmatic nucleus
SFGS	Stratum fibrosum et griseum superficiale (of the optic tectum)
SGC	Stratum griseum centrale (of the optic tectum)
SO	Stratum opticum (of the optic tectum)
SM	Stratum marginale (of the optic tectum)
SPV	Stratum periventriculare (of the optic tectum)
Teg	Tegmentum
TS	Torus semicircularis
VC	Valvula cerebelli
VH	Ventral hypothalamus
III_n	Oculomotor nerve/oculomotor nerve root

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Human	1	MDVTSQARGVGEI	MYPCQAQ	PAAP	TT	SPF	NI	SHPLLGTALANGT	GELS	SHQOYVIGLF
Rat	1	MAATPAQDVGV	EIYLG	W	PAPS	NS	PLAL	NLS	LALREDAPGNL	TCGLS
ZFinch	1	-----	MEIYSASV	SPVLC	NST	TFNL	NGSHLC	NESISSRLAD	KSS	SHQOYVIGLF
Croaker	1	-----	MEBOTSLV	WYVNST	E	OLNTSY	---	EYNT	TYLIES	SDKYQSYVIGLF
ZFish	1	-----	MEBOTTNV	IQIYVNG	E	DFN	N	ADVK	ESD	TDYEFYVIGLF
Goldfish	1	-----	MEBOTTNV	IQIYVND	E	KFN	N	ADVK	ESD	TDYEFYVIGLF
Human	61	L	SCLY	TIFL	PPIGFV	G	NILILV	VN	ISPREKMT	IPDLYF
Rat	61	L	SCLY	TIFL	PPIGFV	G	NILILV	VN	ISPREKMT	IPDLYF
ZFinch	49	L	SCLY	TIFL	PPIGFV	G	NILILV	VN	ISPREKMT	IPDLYF
Croaker	46	L	SCLY	TIFL	PPIGFV	G	NILILV	VN	NHREKMT	IPDLYF
ZFish	46	L	SCLY	TIFL	PPIGFV	G	NILILV	VN	NHREKMT	IPDLYF
Goldfish	46	L	SCLY	TIFL	PPIGFV	G	NILILV	VN	NHREKMT	IPDLYF
TMD1										
Human	121	E	KYID	L	AVLCTF	M	SFLQ	VNMYS	V	FFLTWMSPDRY
Rat	121	E	KYID	L	AVLCTF	M	SFLQ	VNMYS	V	FFLTWMSPDRY
ZFinch	109	E	KYID	L	AVLCTF	M	SFLQ	VNMYS	V	FFLTWMSPDRY
Croaker	106	E	KYID	L	AVLCTF	M	SFLQ	VNMYS	V	FFLTWMSPDRY
ZFish	106	E	KYID	L	AVLCTF	M	SFLQ	VNMYS	V	FFLTWMSPDRY
Goldfish	106	E	KYID	L	AVLCTF	M	SFLQ	VNMYS	V	FFLTWMSPDRY
TMD2										
Human	181	I	WMA	S	ATLV	P	FFTA	V	HLQHT	DEAC
Rat	181	I	WMA	S	ATLV	P	FFTA	V	HLQHT	DEAC
ZFinch	169	I	WMA	S	ATLV	P	FFTA	V	HLQHT	DEAC
Croaker	166	I	WMA	S	ATLV	P	FFTA	V	HLQHT	DEAC
ZFish	166	I	WMA	S	ATLV	P	FFTA	V	HLQHT	DEAC
Goldfish	166	I	WMA	S	ATLV	P	FFTA	V	HLQHT	DEAC
TMD3										
Human	241	L	VR	A	H	R	H	R	G	L
Rat	241	L	VR	A	H	R	H	R	G	L
ZFinch	229	L	VR	A	H	R	H	R	G	L
Croaker	226	L	VR	A	H	R	H	R	G	L
ZFish	226	L	VR	A	H	R	H	R	G	L
Goldfish	226	L	VR	A	H	R	H	R	G	L
TMD4										
Human	301	A	H	P	L	T	G	H	I	V
Rat	301	A	H	P	L	T	G	H	I	V
ZFinch	289	A	H	P	L	T	G	H	I	V
Croaker	286	A	H	P	L	T	G	H	I	V
ZFish	286	A	H	P	L	T	G	H	I	V
Goldfish	286	A	H	P	L	T	G	H	I	V
TMD5										
Human	361	P	D	S	E	Q	S	D	V	R
Rat	361	P	D	S	E	Q	S	D	V	R
ZFinch	349	P	D	S	E	Q	S	D	V	R
Croaker	346	P	D	S	E	Q	S	D	V	R
ZFish	346	P	D	S	E	Q	S	D	V	R
Goldfish	346	P	D	S	E	Q	S	D	V	R
TMD6										
Human	361	P	D	S	E	Q	S	D	V	R
Rat	361	P	D	S	E	Q	S	D	V	R
ZFinch	349	P	D	S	E	Q	S	D	V	R
Croaker	346	P	D	S	E	Q	S	D	V	R
ZFish	346	P	D	S	E	Q	S	D	V	R
Goldfish	346	P	D	S	E	Q	S	D	V	R
TMD7										
Human	361	P	D	S	E	Q	S	D	V	R
Rat	361	P	D	S	E	Q	S	D	V	R
ZFinch	349	P	D	S	E	Q	S	D	V	R
Croaker	346	P	D	S	E	Q	S	D	V	R
ZFish	346	P	D	S	E	Q	S	D	V	R
Goldfish	346	P	D	S	E	Q	S	D	V	R

Figure 1.

Alignment of GPR30 predicted proteins in selected fish (goldfish, zebrafish, and Atlantic croaker), bird (zebra finch), and mammal (rat and human) species. Conserved amino acid residues are shaded by their similarity to the consensus sequence (not shown). Black shading represents >50% of residues in a column are identical to the consensus sequence. Residues that are similar, but not identical, to the consensus sequence are shaded in gray. Seven transmembrane domains (TMD) of the GPR30 predicted protein are noted by lines underneath the corresponding sequences. Note that goldfish predicted protein is a partial sequence.

Figure 2.

RT-PCR localization of GPR30 in goldfish brain and retina. **A:** The goldfish brain was divided into three sections for RT-PCR. An initial cut (not shown) isolated the OT, and the remaining tissue was then divided in half by cutting posterior to the optic nerve (dotted line shows point of sectioning). **B:** RT-PCR amplification of GPR30 and the housekeeping gene EF1 α in goldfish tissues. BS, brainstem (including posterior diencephalon, mesencephalon—excluding OT—and rhombencephalon); For, forebrain (telencephalon, plus anterior diencephalon); Liv, liver; OT, optic tectum; Ret, retina.

Figure 3.

Confirmation of *in situ* probe and antibody specificity. **A1,A2:** Darkfield images of the OT in sections hybridized with sense and antisense GPR30 riboprobes. Boundaries of the SM, SO, SFGS, SGC, SAC, and SPV are shown for clarity. **B1:** Western blot of GPR30 primary antibody against goldfish whole brain protein extract showing bands representing glycosylated (gGPR30) and nonglycosylated (ngGPR30) forms. **B2:** Immunodetection of all bands is completely blocked when the antibody is preadsorbed with the antigen peptide prior to Western blotting. **C1:** Strong immunolabeling of cells and fibers in the preoptic area is blocked (**C2**) by preadsorption of the primary antibody with a GPR30 peptide fragment. Scale bars = 100 μ m in A, C.

Figure 4.

GPR30 mRNA expression in the forebrain and midbrain. Sections shown from rostral (A) to caudal (F). **A:** Low-magnification (4 $\times$) brightfield image of Nissl counterstained anterior preoptic area (PPa) section with box showing region depicted in high-magnification (20 $\times$) darkfield image (**B**). **C,D:** Stitched darkfield images taken at 10 $\times$ of sections of goldfish midbrain showing robust GPR30 expression in some fiber tracts and nuclei that receive retinal inputs. **E:** High-magnification (40 $\times$) darkfield image showing GPR30 mRNA in the periaqueductal gray (PAG) with inset brightfield image (5 $\times$) showing cytoarchitecture of the caudal PAG. **F:** Stitched darkfield image taken at 10 $\times$ showing GPR30 expression in cell populations and fiber tracts at the level of the oculomotor nerve (IIIIn). Scale bars = 50 μ m in B; 500 μ m in C,D,F; 100 μ m in E.

Figure 5. GPR30 immunofluorescence is extranuclear (green = GPR30; blue = DAPI counterstain) (A1-2). Fibers emanating to/from the preoptic area with punctate GPR30 labeling of varicosities (A3). All images in panel A were taken at 100 $\times$ on a confocal microscope. Immunolabeled cells and fibers in the parvocellular preoptic area (B), magnocellular preoptic area (C), the preoptico-hypophyseal tract (D), and the ventrolateral side of the periaqueductal gray (E). Scale bars = 2 μ m in A; 100 μ m in B–E.

Figure 6.

GPR30 and isotocin (IT) immunofluorescence colocalize in cells of the preoptic area in male goldfish. In all images, green = GPR30 primary antibody and magenta = anti-oxytocin primary antibody. **A–E:** Blocking experiments confirm the specificity of the anti-oxytocin (OXT) antibody for isotocin: anti-OXT primary and anti-GPR30 primary preadsorbed with 50 μm vasotocin does not block IT signal (A), GPR30 signal (B), or their colocalization (C), whereas anti-OXT primary and anti-GPR30 primary preadsorbed with 50 μm isotocin blocks IT signal (D) but not GPR30 signal (E). All images taken at 20× magnification. **F–I:** Confocal images taken at 40× magnification show GPR30 (F) and IT (G) immunofluorescence in the parvocellular (PPa) and magnocellular (PM) nuclei of the preoptic area. GPR30 and IT are colocalized in the same cells (H; 40 ×), and all immunolabeling is extranuclear (I; same section at 100 ×). Scale bars = 100 μm in A–E; 20 μm in F–H; 1 μm in I.

Figure 7.

Comparison of GPR30 mRNA levels in goldfish brain in breeding vs. nonbreeding condition (**A**), and in males housed with a male vs. female (**B**) using qPCR. Data are graphed as mean relative mRNA level $\pm$ SEM. $P > 0.05$ for all comparisons. **C**: GPR30 mRNA levels were not correlated with GSI in goldfish males sacrificed in spring or fall.

TABLE 1

Primer Sequences (5' to 3') Used to Clone and Amplify Goldfish GPR30

	Forward primer	Reverse primer	Amplicon size
Degenerate primers	ACATCGCCGTCCTGtgyacnttyatg	CGGCCAGGTTACGatrtgncnct	564
5' RACE primers	UPM Long: CTAATACGACTCACTATAGGGCAAG CAGTGGTATCAACGCAGAGT UPM Short: CTAATACGACTCACTATAGGGC	CGACGGGTCAGCTGTGCCTTGGAGC	1,046
RT-PCR primers	GGTGACAATCGGATTTCTGG	CAGCCAGCAAATGAAGAACA	166
qPCR primers			
GPR30	TTTATGTCGCTGTTCTCCA	GCTGCTCAAAGAGTTAGCCAGA	102
EF1 α	GATTGTTGCTGGTGGTGTG	GCAGGGTTGTAGCCGATTT	227

UPM, universal primer mix.

TABLE 2

Primary Antibodies Used in This Study

Target	Antigen	Source	Working dilution
GPR30	C terminal of GPR30; Amino acids 290-340 of human GPR30/GPER1 (GenBank Accession Q99527.1)	Santa Cruz Biotechnology; sc-48523-R; RRID:AB_2112500; rabbit polyclonal	1:100
Isotocin	Oxytocin conjugated to thyroglobin	Millipore; MAB5296; RRID: AB_2157626; mouse monoclonal	1:5,000

TABLE 3

Comparison of Sites of Reported Aromatase Expression (See References Below) and GPR30 Expression in the Brain Areas Examined in This Study

Anatomical location	Aromatase	GPR30
Primary visual pathways <i>From Springer and Gaffney, 1981; Yamamoto and Ito, 2008; Northcutt, 2006; Saidel et al, 2001</i>	Enzyme in goldfish (1); mRNA transcript and enzyme in midshipman fish (2); mRNA in zebrafish (3)	Present study: RT-PCR (4), In situ (5); (6)
Retina	+ (1)	+ (4)
Optic tract (OPT)	+ (1, 2, 3)	+ (5)
Optic tectum (OT)	+ (1), but not (2)	+ (4,5)
Dorsal accessory optic nucleus (NA-OD)	–	+ (5)
Central pretectal nucleus (CPN)	–	+ (5)
Parvocellular superficial pretectal nucleus	–	–
Dorsal accessory optic nucleus (NA-OD)	–	+ (5)
Suprachiasmatic nucleus (SCN)	+ (1,3)	+ (5)
Preoptic area (PPa, PM)	+ (1,2,3)	+ (5,6)
Ventrolateral nucleus of the torus semicircularis (retinal target)	+ (3)	–
Anterior tuberal nucleus (aTn; retinal target)	+ (1,3)	–
Dorsal thalamus	+ (1,2)	–
Secondary Visual Pathways (Tectal, Thalamic and Preglomerular Targets or Visually-responsive areas without direct retinal input)		
Dorsolateral tegmentum (tectal efferent target)	–	Diffuse (5)
Horizontal commissure (HC) (Tectal efferent pathway)	–	+ (5)
Medial / Dorsal / Central regions of the dorsal telencephalon (preglomerular/thalamic targets)	+ (1,3)	–
Reticular formation	+ (1, 3)	–
Nucleus isthmi	–	–
Magnocellular superficial pretectal nucleus	–	–
SBN Nuclei (olfactory targets are indicated)		
Lateral nucleus of the ventral telencephalon (Vl; medial olfactory tract target)	+ (2,3)	–
Supracommissural nucleus of the ventral telencephalon (Vs; medial olfactory tract target)	+ (1,3)	–
Ventral nucleus of the ventral telencephalon (Vv)	+ (1,3)	–
Preoptic area (PPa, PM, PG) (medial olfactory tract and retinal target)	+ (1,2,3)	+ (5,6)
Anterior tuberal nucleus (also a retinal target; see above)	+ (1,3)	–
Ventral tuberal nucleus	+ (1,3)	–
Periaqueductal gray (PAG)	+ (1,2,3)	+ (5,6)
Additional Sites of Expression		
Olfactory bulb	+ (3)	–

Anatomical location	<u>Aromatase</u>	GPR30
Posterior division of dorsal telencephalon (Dp, lateral olfactory tract target)	+ (3)	–
Inferior hypothalamus	+ (3)	–
Cerebellum	+ (3)	–
Valvula cerebelli (VC)	+ (1)	+ (5)
Reticulospinal neurons (MLF)	+ (1)	Diffuse (5)
Midbrain tegmentum	+ (2)	Diffuse (5)