Distributions of Two Gonadotropin-Releasing Hormone Receptor Types in a Cichlid Fish Suggest Functional Specialization

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ABSTRACT
Gonadotropin-releasing hormone 1 (GnRH1) from the brain controls reproduction in vertebrates via a GnRH-specific receptor in the pituitary; however, other forms of GnRH are found in all species, suggesting additional roles for this family of peptides. GnRH action depends critically on the location of its cognate receptors in the brain. To understand the potential roles of additional GnRH forms, we localized two known GnRH receptor types in a cichlid fish, Astatotilapia burtoni, in which GnRH1 is socially regulated. Using in situ hybridization, we describe the mRNA expression pattern of these GnRH receptor (GnRH-R) subtypes in the brain, specifically with respect to GnRH-producing neurons. Our data suggest that following a gene duplication, the two GnRH receptors have evolved to serve different functions. The type 1 receptor (GnRH-R1) is expressed less widely than the type 2 receptor (GnRH-R2). Specifically, GnRH-R1 is expressed in groups of neurons in the telencephalon, preoptic area, ventral hypothalamus, thalamus, and pituitary. In contrast, GnRH-R2 is expressed in many more brain areas, including the olfactory bulb, telencephalon, preoptic area, hypothalamus, thalamus, midbrain, optic tectum, cerebellum, hindbrain, and pituitary. The specific distribution of GnRH-R2 suggests that the GnRH ligands may act via this receptor to influence behavior in A. burtoni. Moreover, only GnRH-R2 mRNA is colocalized in the three known groups of GnRH-containing neurons, suggesting that any direct feedback regulation of GnRH by itself must act through this receptor type. Taken together, these data suggest that the two GnRH receptor types serve different functional roles in A. burtoni. J. Comp. Neurol. 495:314–323, 2006. © 2006 Wiley-Liss, Inc.

Indexing terms: GnRH receptor; GnRH autocrine regulation; teleost; A. burtoni

Gonadotropin-releasing hormone 1 (GnRH1) was originally thought to act only in controlling reproduction, but data have shown that all vertebrates actually express two or three GnRH forms, encoded by different genes (White and Fernald, 1998). These decapeptides fall into three distinct classes: GnRH1, GnRH2, and GnRH3 (White and Fernald, 1998; Somoza et al., 2002) and are distributed differently in the brain (Kasten et al., 1996; White and Fernald, 1998; Lethimonier et al., 2004). Understanding the yet unknown roles of these GnRH forms will depend on the locations and responses of their cognate receptors. To understand how the various GnRH systems might function, we used a cichlid fish (Astatotilapia burtoni) model system in which GnRH1 is socially regulated and the three different GnRH forms are localized in distinct sites in the brain. We mapped the locations of the two GnRH receptor types using in situ hybridization to map their general distribution and their specific relation to GnRH-containing neurons.

GnRH1 is delivered by the brain to the pituitary, where it stimulates the gonadotrophs via GnRH receptors to release gonadotropins. In some

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species, GnRH1 has also been associated with the control of growth hormone (Marchant et al., 1989; Klausen et al., 2002) and prolactin secretion (Weber et al., 1997). In contrast, the role(s) of GnRH2 are less clear. GnRH2 may be important in sexual behavior (Volkoff and Peter, 1999; Kauffman and Rissman, 2004a) and/or in controlling appetite (Kauffman and Rissman, 2004b), depending on species. GnRH3, which, to date, has been found primarily in teleost fish, projects widely throughout the telencephalon (Kim et al., 1995; Yamamoto et al., 1995; Amano et al., 2002; Gonzalez-Martinez et al., 2002) and in one fish species, dwarf gouramis, it influences nesting behavior (Yamamoto et al., 1997). Given the sparse data on GnRH2 and GnRH3, there is no consensus about their possible functions, in striking contrast to GnRH1, which is known to control reproductive capacity in all vertebrates.

Since so little is known about the release and function of GnRH2 and GnRH3, identifying their roles will depend in part on discovering where they act through localizing GnRH receptors in relation to the GnRH-producing neurons. In an African cichlid fish species, *A. burtoni*, all three forms of GnRH ligands and two forms of GnRH receptors (GnRH-Rs) have been identified (White and Fernald, 1998; Robison et al., 2001). This species provides an opportunity to identify putative roles for different GnRH forms through analyzing the distribution of their cognate receptors. Male *A. burtoni* exist in one of two distinct social phenotypes: dominant (territorial, T) males, which comprise ~10~30% of the population, and subordinate (nonterritorial, NT) males, which make up the remaining 70~90% of the population (Fernald and Hirata, 1977b). *Astatotilapia burtoni* live in a lek-like social system in shore pools of Lake Tanganyika, Africa. T males are brightly colored, large, reproductively capable, and defend territories containing a food resource used to entice females to spawn with them. NT males are camouflaged, smaller, have regressed gonads, and school with females. Importantly, males shift between these social states, depending on their success in aggressive encounters.

GnRH receptors belong to the superfamily of seven-transmembrane G-protein-coupled receptors. These receptors are composed of polypeptides with canonical hydrophobic transmembrane domains and hydrophilic extracellular and intracellular loops (Stojilkovic et al., 1994; Ruf et al., 2003; Millar et al., 2004). Multiple GnRH receptor types have previously been reported in mammals, birds, fish, and amphibians (Wang et al., 2001; Lethimonier et al., 2004; Millar et al., 2004) and, as with any duplicated gene, one key question is whether the functions of duplicated gene products may have diverged over evolutionary time. Phylogenetic analysis of fish GnRH receptors indicates the presence of at least two subtypes (Lethimonier et al., 2004; Millar et al., 2004), and we have cloned and identified representatives of these GnRH receptors: GnRH-R1 and GnRH-R2 (GenBank Access. Nos.: AY705931 and AY028476, respectively), named based on phylogenetic analysis by Lethimonier et al. (2004).

In experiments reported here, we describe the mRNA expression pattern of these GnRH receptor subtypes in the brain, specifically with respect to the locations of GnRH-producing neurons. In *A. burtoni*, the distribution of the two receptor types is distinctly different. GnRH-R1 is mainly found in brain areas related to reproductive function and is highly expressed in the pituitary. These

**Abbreviations**

- 3: layer three
- An: anterior nucleus
- AOn: Accessory optic nucleus
- aPPn: anterior part of parvocellular preoptic nucleus
- C: cerebellum
- CEPn: central pretectal nucleus
- Cn: central nucleus of the inferior lobe
- cPn: central posterior nucleus
- CTn: central thalamic nucleus
- Dc: central nuclei of dorsal telencephalon
- Dd: dorsal part of dorsal telencephalon
- DH: dorsal part of the hypothalamus
- Dll: dorsal nuclei of lateral part of dorsal telencephalon
- Dlv: ventral nuclei of lateral part of dorsal telencephalon
- Dm: median divisions of dorsal part of the telencephalon
- Dm3: median division 3 of dorsal part of the telencephalon
- Dm4: median division 4 of dorsal part of the telencephalon
- Dn: diffuse nucleus of inferior lobe
- Dp: posterior nucleus of dorsal telencephalon
- DT: dorsal thalamus
- dPn: dorsal posterior nucleus
- DW: deep white zone
- E: entopeduncular nucleus
- ECL: external cell layer
- G: granular cell layer
- gMPn: gigantocellular portion of the magnocellular preoptic nucleus
- Gn: glomerular nucleus
- H: habenula
- ICL: internal cell layer
- LH: lateral hypothalamus
- LT: longitudinal torus
- M: molecular cell layer
- maPn: magnocellular portion of the anterior preoptic nucleus
- MB: mammillary body
- MO: medulla oblongata
- MPn: magnocellular preoptic nucleus
- nMLF: nucleus of the medial longitudinal fasciculus
- OB: olfactory bulb
- ON: optic nerve
- OT: optic tectum
- P: Purkinje cell layer
- Pin: pineal gland
- Pit: pituitary gland
- PG: periventricular gray zone
- PCGn: preglomerular commissural nucleus
- PGN: preglomerular nucleus
- pMPn: parvocellular portion of the magnocellular preoptic nucleus
- Pu: posterior nucleus
- pPT: periventricular nucleus of the posterior tuberculum
- PPn: parvocellular preoptic nucleus
- S: superficial white and gray zone
- Sn: suprachiasmatic nucleus
- Tel: telencephalon
- Tn: tuberal nucleus of the hypothalamus
- TN: terminal nerve ganglion
- V: ventral telencephalon
- Vc: commissural nucleus of the ventral telencephalon
- Vd: dorsal nucleus of the ventral telencephalon
- VH: ventral part of the hypothalamus
- VI: lateral nucleus of the ventral telencephalon
- VLN: ventrolateral nucleus
- VM: ventromedial nucleus
- Vp: postcommissural nucleus of the ventral telencephalon
- vPPn: central portion of the periventricular preoptic nucleus
- Vs: supracommissural nucleus of the ventral telencephalon
results suggest that GnRH-R1 may be important for regulating reproduction. Conversely, GnRH-R2 was detected widely throughout the brain, from the olfactory bulb to the medulla, as well as in the pituitary. Thus, GnRH-R2 is in a position to allow GnRH to modulate sensory, metabolic, and motor systems. Interestingly, by colocalizing neurons producing the three forms of GnRH and the two GnRH receptors using double in situ hybridization, we found that only GnRH-R2 is expressed in the cell bodies of all three types of GnRH-containing neurons, suggesting it plays a critical role in autocrine regulation of these cells. Taken together, these data suggest that the two GnRH receptor types in A. burtoni may have specialized since their duplication, and now serve related but separate functions in the animal. Whether the three forms of GnRH all act in the service of reproduction remains unknown.

MATERIALS AND METHODS

Subjects

African cichlid fish, *Astatotilapia (Hoplochromis) burtoni*, bred from wild-caught stock, were maintained under conditions simulating those of their natural environment (pH 7.8–8.2, temperature 29°C, 12:12-hour light/dark cycle) with full spectrum lighting. Animals were kept in colonies with 2–5 males and 7–12 females in aquaria with terra cotta shelters. All work was performed in compliance with the animal care and use guidelines of the Stanford University Administrative Panel on Laboratory Animal Care.

Behavioral observations and analysis

Males were identified by attaching combinations of colored beads just below the dorsal fin to allow observation of known individuals. Each male was observed three times per week for 3 minutes at the same time (5 hours after light onset) for 4 weeks. Male A. burtoni exist in one of two distinct social phenotypes: dominant or territorial (T) males are brightly colored, large, reproductively capable, and defend territories containing a food resource used to entice females to spawn. Nonterritorial (NT) males are camouflaged, smaller, have regressed gonads, and school with females (Fernald and Hirata, 1977a,b; Fernald, 1982; Fraley and Fernald, 1982; Fernald and Shelton, 1985). Males shift between these social states depending on their success in aggressive encounters (White et al., 2002). From observations, males were classified as T or NT on the basis of their coloration patterns and behavior (Hirata and Fernald, 1975; Fernald and Hirata, 1977a,b). To quantify behavioral social status, we used a “dominance index” (DI) (White et al., 2002). DI is calculated as the sum of the number of aggressive acts minus the number of submissive acts that occurred during a given observation period. These daily mean values of DI were used to determine T or NT status. We also measured the relativeigon size (gonadosomatic index; GSI = [gonad mass/body mass] × 100). To be considered a T, a male must have had DIs greater than 3 during all the observation periods and GSI greater than 0.5 (White et al., 2002).

Tissue preparation for immunocytochemical staining

To localize the GnRH-producing neurons and their processes for comparison with previous work, reproducively active fish (T males; n = 3) were killed by rapid cervical transection, their brains removed and placed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) overnight, and then transferred into a 30% sucrose solution overnight. Fixed tissue was frozen in mounting medium (OCT, Tissue-Tek, Torrance, CA) on dry ice and stored at −80°C. Three series of sections were cut on a cryostat (Microm, Zeiss, Thornwood, NY) in coronal or sagittal planes at 14 μm.

Sections were rehydrated (PBS) and incubated in blocking solution (0.3% Triton X-100, 0.2% bovine serum albumin, 10% normal goat serum diluted in PBS). Slides were incubated in primary antisera (a rabbit polyclonal anti-GnRH antibody that labels GnRH neurons in A. burtoni, generously provided by Dr. H. Urbanski [Lot #HU60]) diluted 1:1,000 in blocking solution (Urbanski et al., 1990) at 4°C overnight. The other set of slides were incubated in blocking solution instead of primary antisera for the control group. All of the sections were washed in PBS and the signal was amplified using an ABC kit (Vector Laboratories, Burlingame, CA). Finally, the sections were incubated in 3′,3′-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) to visualize the bound antibody and counterstained with cresyl violet to visualize the cells. The slides were dehydrated in an ethanol/xylene series and coverslipped with Permunt medium for microscope viewing.

Tissue preparation for in situ hybridization

Preliminary data comparing T, NT males and females (both gravid and nongravid reproductive status) revealed no significant differences across animals in GnRH receptor distribution in the brain. Consequently, reproducively active fish (T males; n = 6) were used in this study. Animals were killed by rapid cervical transection, brains removed, and placed in sterile PBS. Tissue was frozen in OCT on dry ice and stored at −80°C. The brain tissue was sectioned coronally in three series as described above at 14 μm on a cryostat and mounted on glass slides that were stored at −80°C until use.

In situ hybridization probes

To localize expression of GnRH receptor mRNAs, probes for GnRH-R1 and GnRH-R2 were synthesized, based on GenBank sequences (GnRH-R1: GenBank Access. No.AY705931; GnRH-R2: GenBank Access. No.AY028476; note that the names of these sequences conform to the system of Lethimonier et al. [2004] and Grens et al. [2005]). Plasmids were linearized using BamHI and Xho I to generate both sense and antisense templates for each receptor. GnRH-R1 and GnRH-R2 probes were from 151–1035 basepairs (bp) and from 55–1129 bp, respectively, in the full sequences from GenBank, and were generated by transcribing with SP6 or T7 polymerase in the presence of [35S]-UTP (Maxiscript kit; Ambion, Austin, TX).

To localize the GnRH ligand, plasmids for GnRH1 (Ser5-GnRH), GnRH2 (His5, Trp7, Tyr8)-GnRH), and GnRH3 (Trp7, Leu8)-GnRH) were extracted from glycerol stocks (White et al., 1995) using a commercial kit (QiAspin MiniPrep Kit, Qiagen, Valencia, CA) and sequenced (BioCore, Palo Alto, CA) to confirm their integrity. Plasmids for each of the three GnRH types were amplified, purified, and linearized to generate sense and antisense templates for each type of GnRH. Three types of GnRH probes were designed from the cDNA sequences of
mature peptides (GenBank Access. Nos. were U31865 for GnRH1, L27435 for GnRH2, and S63657 for GnRH3) separately: GnRH1 probe was from 18–206 bp; GnRH2 probe was from 1–249 bp, GnRH3 probe was from 67–322 bp. Transcripts were labeled with digoxigenin (DIG) to produce sense and antisense in situ probes using T3 or T7 polymerase (Maxiscript, Ambion, Austin, TX).

In situ hybridization

In situ hybridization followed standard procedures as modified by our laboratory (Burmeister and Fernald, 2005; Grens et al., 2005). Briefly, slides were brought to room temperature, fixed for 10 minutes in 4% paraformaldehyde in PBS, rinsed twice for 3 minutes each in PBS, immersed in 0.1 M triethanolamide (TEA) buffer for 3 minutes, acetylated in 0.25% acetic anhydride in 0.1 M TEA for 10 minutes, rinsed twice for 3 minutes each in 2× sodium citrate sodium chloride (SSC) buffer, dehydrated in an ethanol series, and air dried. Radioactive probes were diluted to $\sim 5 \times 10^6$ cpm/ml and DIG probes were diluted to 1 ng/ml in hybridization solution (Sigma-Aldrich, St. Louis, MO) supplemented with 1 g/ml dithiothreitil (DTT). Preheated probe mix was added to each slide and then slides were coverslipped and immersed overnight in a 60°C mineral oil bath. After removing slides from the mineral oil, residual oil was removed by immersion in chloroform. Probe and coverslips were removed in two rinses in 4× SSC, then washed by 2× SSC with DTT. To detect the GnRH probe, slides were incubated in anti-DIG-peroxidase primary antibody (Roche, Indianapolis, IN) then amplified (Tyramide Signal Amplification kit, NEN Life Sciences, Boston, MA), and stained using DAB. Finally, slides were dehydrated in ethanol, air dried, and dipped in nuclear emulsion (NBT-2; Eastman Kodak, Rochester, NY) diluted 1:1 in water, air dried, and stored in a light-tight box at 4°C for 3–4 weeks. Following development, slides were stained with cresyl violet, dehydrated in an ethanol/xylene series, and coverslipped as described above.

We used sense versions of each probe to test the specificity of our in situ hybridization results, and in every case no signal above background was seen. Midbrain regions in A. burtoni sections were identified using the nomenclature of Fernald and Shelton (1985), and telencephalic nuclei were identified using zebrafish and tilapia brain atlases (Ando et al., 1999; Wullimann and Mueller, 2004), as used in A. burtoni by Burmeister and Fernald (2005). Photomicrographs were captured digitally (SPOT camera system; Diagnostic Instruments, Sterling Heights, MI) and adjusted using PhotoShop 7 software (Adobe, San Jose, CA).

RESULTS

Localization of GnRH neurons and fiber tracts in A. burtoni

Immunohistochemical staining confirmed the location of GnRH-containing neurons (Fig. 1) to be in separate groups. The types of GnRH were distinguished as previously reported (White et al., 1995): GnRH1 cell bodies in the preoptic area (Fig. 1C), GnRH2 cell bodies in the midbrain tegmentum (Fig. 1D), and GnRH3 cell bodies in
the terminal nerve ganglion (Fig. 1B). GnRH1 fibers from the preoptic area aggregate into larger tracts as they enter the pituitary stalk. Other GnRH-containing fibers appear in the olfactory bulbs, ventral and dorsal telencephalon, diencephalon, pretectum, optic tectum, cerebellum, and medulla. A schematic illustration of the location of GnRH containing fibers is shown in Figure 2A.

**Localization of GnRH-R1 mRNA in the brain and pituitary of *A. burtoni***

We found GnRH-R1 mRNA to be highly expressed in the pituitary, hypothalamus, and preoptic area (POA) and expressed at a much lower level in the telencephalon and thalamus. A schematic summary of the distribution of GnRH-R1 is shown in Figure 2B.

In the telencephalon, most cells expressing GnRH-R1 mRNA were found in the median division 3 of dorsal part of the telencephalon (Dm3; Fig. 3A) that receives a thalamic projection (Wullimann and Mueller, 2004). In the diencephalon, GnRH-R1 mRNA was expressed in the preoptic area (POA), especially in the magnocellular preoptic nucleus (MPn; Fig. 3B). GnRH-R1 mRNA expression was also found in the tuberal nuclei (Tn) and the ventral part of the hypothalamus (VH; Fig. 3D). In addition, the central posterior nucleus of dorsal thalamus nucleus (cPn) expressed GnRH-R1 mRNA and label was also found in the ventral portion of the periventricular pretectal nucleus (vPPn; Fig. 3E). The highest level of GnRH-R1 expression was in the pituitary gland (Pit; Fig. 3C).

**Localization of GnRH-R2 mRNA in the brain and pituitary of *A. burtoni***

Generally, GnRH-R2 gene expression was more widespread than GnRH-R1 expression. Expression of GnRH-R2 was seen in the olfactory bulb, telencephalon, synencephalon, diencephalon, pretectum, tectum, pituitary, pineal gland, cerebellum, and medulla (schematic summary in Fig. 2C). In the olfactory bulb (OB), GnRH-R2 mRNA was expressed in the inner cellular layer and in the rostral telencephalon it was expressed in the terminal nerve ganglion (TN; Fig. 4A). In the telencephalon, the cells expressing GnRH-R2 mRNA were found in the ventral nucleus of the ventral telencephalon (Vv; Fig. 4A), the medial division 4 of dorsal part of the telencephalon (Dm4), and the dorsal and lateral part of dorsal telencephalon (Dd and Dld). GnRH-R2 was found in brain regions associated with olfaction (e.g., olfactory bulb, dorsal and lateral part of dorsal telencephalon, ventral nuclei of the ventral telencephalon) (Wullimann, 1998; Wullimann and Mueller, 2004). In addition, GnRH-R2-expressing cells were found in the parvocellular preoptic nucleus (Ppn) and MPn of the preoptic area (Fig. 4B), and habenula (H; Fig. 4C). All nuclei in the thalamus have GnRH-R2 signal (Fig. 4C), including the anterior nucleus (An), the posterior thalamus nucleus (Pn), the ventromedial nucleus (VMn), the central posterior nucleus (CPn), preglomerular commissural nucleus (PGCn), and periventricular nucleus of the posterior tuberculum (pPT). In the hypothalamus, GnRH-R2 signals were found in several divisions, including the tuberal nuclei (Tn), ventral and dorsal hypothalamus (VH and DH; Fig. 4F), the mamillary body (MB), all periventricular regions, and the preglomerular nuclear complex (PGn). These nuclei of the thalamus and hypothalamus are related to several sensory functions, including the lateral line system (Wullimann, 1998; Wullimann et al., 2001).
and Mueller, 2004). GnRH-R2 was also expressed in the pineal gland (Pin; Fig. 4C), the pretectum and layer three of optic tectum (3; Fig. 4D), the pituitary gland (Fig. 4E), the Purkinje cell layer of cerebellum (P; Fig. 4G), and the medulla, which is involved in fish movement (Wullimann, 1998). The accessory optic nucleus of the pretectum (AOn) which projects to the cerebellum also had GnRH-R2 mRNA expression.

Only GnRH-R2 is colocalized with GnRH containing neurons

As noted above, previous data showed that three groups of neurons, each containing one form of GnRH, were located in distinct regions of the A. burtoni brain from anterior to posterior (White and Fernald, 1998): GnRH3 neurons in the terminal nerve ganglion (TN), GnRH1 neurons in the preoptic area, and GnRH2 neurons in the nuclei of medial longitudinal fascicules (nMLF). Using in situ hybridization labeling for both GnRH ligands and receptors, we found that only GnRH-R2 was coexpressed with all three types of GnRH-containing neurons (Fig. 5).

DISCUSSION

The two known A. burtoni GnRH receptor forms have distinctly different distributions in the brain, with GnRH-R2 more widespread than GnRH-R1. The widespread of the distribution of GnRH receptor expression suggests that the GnRH forms may act as neuromodulators. In the pituitary, GnRH1 may act on different types of cells via two distinct GnRH-Rs. Only GnRH-R2 appears to be involved in autocrine function of GnRH based on our colocalization experiment. These data, together with evidence that they arose from a gene duplication (Robison et al., 2001; Fernald and Illing, in prep.), suggest that subfunctionalization of these two receptors may be in process.

Localization of GnRH receptors (GnRH-Rs)

As previously reported for A. burtoni, GnRH fibers can be seen throughout the brain from the olfactory bulb to the cerebellum. Importantly, all three types of GnRH ligand can act on each of the two GnRH receptors, as demonstrated biochemically (Illing et al., 1999; Bogerd et al., 2002; Lethimonier et al., 2004; Fernald and Illing, in prep.), and as implied from evidence from the retina, where both receptors are expressed but only GnRH3 is present (Grens et al., 2005). Both GnRH-Rs are expressed in brain areas involved in sexual behavior and reproduction, including the magnocellular preoptic nucleus of the preoptic area, the tuberal nuclei, and the ventral part of the hypothalamus (Fernald and Shelton, 1985; Ando et al., 1999; Gilchriest et al., 2000). Further, both GnRH receptor types are found in the thalamic nuclei associated with reproductive functions, such as sex change and gamete release (Demske and Dulka, 1986). In particular, GnRH-R2-expressing cells are located in the parvocellular preoptic nucleus, a well-known site of control for fish reproduction (Sas et al., 1990; Anglade et al., 1994; Linard et al., 1995; Rodriguez-Gomez et al., 2000). The preoptic area and hypothalamus express both GnRH-R types and these areas are known to control appetite and feeding (Sas et al., 1990; Batten et al., 1993), as well as stress responses (Ando et al., 1999; Gilchriest et al., 2000) and reproduction. This suggests that GnRH might not only regulate reproduction but also metabolic functions and stress responses via GnRH-R types in A. burtoni.
In the visual system, we have recently shown that both GnRH-R1 and GnRH-R2 have distinct expression patterns in the *A. burtoni* retina with GnRH-R1 in a subset of amacrine cells and GnRH-R2 in ganglion cells (Grens et al., 2005). Interestingly, here we show that GnRH-R2 is also expressed in several nuclei known to receive retinal projections, including the ventral thalamus, the dorsal thalamus, the pretectal complex, and the optic tectum (Fernald, 1982). These results suggest that GnRH may be influencing visual processing in visual centers extending from the retina to the brain.

**Localization of GnRH receptor mRNAs in the pituitary of *A. burtoni***

GnRH1 plays a major role in controlling reproduction by stimulating gonadotrophs in the pituitary to release gonadotropins into the circulation. In *A. burtoni*, GnRH1 is the only form delivered to the pituitary (Bushnik and Fernald, 1995), where we found both GnRH-R1 and GnRH-R2 expressed. GnRH-R1 is most highly expressed in the ventral-anterior and posterior part of pituitary, which preliminary data suggest contains luteinizing hormone (LH) and follicle-stimulating hormone (FSH) cells. In contrast, GnRH-R2 is expressed in the dorsal-anterior and posterior parts of the pituitary. These results are similar to those described for tilapia, where two different types of GnRH-Rs are also expressed in different cell populations in the pituitary: GnRH-R1 subtypes (called GnRH-R IA and IB) in gonadotropic cells releasing FSH and LH, and the other type (called GnRH-RIII) in growth hormone cells (Parhar et al., 2002). Thus, GnRH1 may be regulating both growth and reproduction via distinct receptor subtypes. Previous experiments have shown that in *A. burtoni* there is an interaction between growth and

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**Fig. 4.** In situ hybridization showing the expression pattern of GnRH-R2 mRNA in selected nuclei of an *A. burtoni* brain. The bright-field views show the coronal sections of brain areas (A1–G1). The darkfield pictures show the GnRH-R2 mRNA expressing cells in the brain area (A2–G2). Silver grains labeling GnRH-R2 mRNA was seen in A: the terminal nerve ganglion (TN) and the ventral nucleus of the ventral telencephalon (Vv); B: the preoptic area (POA including aPPn, mMPn, and pMPn); C: the pineal gland (Pin), and the habenula (H), as well as the nuclei of the thalamus; D: the optic tectum and the pretectum; E: the pituitary gland; F: the hypothalamus; G: the cerebellum. Scale bars = 250 μm (applies also to A2–G2).
Fig. 5. GnRH-R2 is colocalized with three types of GnRH-expressing neurons in _A. burtoni_. GnRH is labeled with DAB (brown) and cell bodies are counterstained with cresyl violet (purple), and GnRH-Rs mRNA are labeled with silver grains (white). Left column shows GnRH-R1; right column shows GnRH-R2. The rows represent sections taken from the GnRH neuron locations from anterior to posterior. Scale bars = 20 μm.
reproduction consistent with these data (Hofmann et al., 1999; Hofmann and Fernald, 2000).

**Only GnRH-R2 is expressed in GnRH-containing neurons**

Electrophysiological recordings have shown that GnRH and its antagonists can influence the firing patterns of both hypothalamic and terminal nerve GnRH-containing neurons, raising the possibility that there is a feedback system (Oka, 1992; Dellovade et al., 1998; Abe and Oka, 2000, 2002; Kremsanovic et al., 2003). Using immunoreactive labeling, GnRH-RIII was weakly identified only in GnRH3 neurons (Soga et al., 2005), whereas in contrast using in situ hybridization we found that only GnRH-R2 is colocalized with GnRH in all three types of GnRH-containing neurons. This strongly suggests that the demonstrated GnRH autocrine feedback control acts via GnRH-R2.

**Functional diversity of GnRH receptors**

Taken together, these data suggest that GnRH receptors have assumed distinct but related functions in the A. burtoni brain. Their spatial distribution in the pituitary emphasizes the point that different functional pathways may be served by each receptor type. In other parts of the brain, the distribution of GnRH ligands in A. burtoni are segregated to specific areas (White et al., 1995), suggesting that each of the three distinct GnRH types could be detected with either receptor type, depending on the brain area, but that it is unlikely that they compete for receptors. Thus, the response to GnRH ligands in these areas depends on the binding properties of individual receptor types. For GnRH-R2, we know that the response to GnRH2 as measured using inositol phosphate response is ~3 orders of magnitude more sensitive than to the other two ligands (Robison et al., 2001), suggesting that lower levels of ligand would be needed to elicit a response. In other species, GnRH receptors are also expressed differentially (Madigou et al., 2000; Bogerd et al., 2002; Ikemoto et al., 2004). Gonzalez-Martinez et al. (2004) reported in the European sea bass that GnRH-R1 type receptor is also found in the telencephalon, preoptic area, thalamus, and hypothalamus. In tilapia, different types of GnRH-Rs protein display both widespread and restricted distribution in the brain, similar to our findings here (Soga et al., 2005). In the goldfish (Illing et al., 1999), for example, two receptor subtypes have distinctly different response properties, suggesting that these have also diverged in their function.

Gene duplication events are known to have three different outcomes: 1) the product of one gene duplicate becomes nonfunctional; 2) the two genes acquire different but related functions (e.g., subfunctionalization); 3) one duplicate gene assumes a new function (neofunctional). Both copies of the GnRH-R gene remain responsive to GnRH (Robison et al., 2001) and several GnRH-expressing fibers contact different types of GnRH receptors (Soga et al., 2005). Thus, it appears likely that the receptors have become subfunctionalized, although it is not yet clear exactly how their functions differ (Lynch, 2002).

Our data support the idea that different forms of GnRH alone and in combination acting via cognate receptors may play important roles in the physiology and behavior of vertebrates. The potential for new roles for GnRH needs to be tested with assays that identify how this ancient peptide functions in different brain regions.

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**LITERATURE CITED**


DIFFERENTIAL DISTRIBUTIONS OF GnRH RECEPTORS


