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Gonadotropin-Releasing Hormone and Receptor Distributions in the Visual Processing Regions of Four Coral Reef Fishes

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Abstract
Gonadotropin-releasing hormone (GnRH) is widely distributed in the brain of fishes where it may function as a neuromodulator of sensory processing and behavior. Immunocytochemical and neuronal label experiments were conducted on species from four families of coral reef fishes (Chaetodontidae, butterflyfish; Pomacentridae, damselfish; Gobiidae, goby; and Labridae, wrasse) to assess conservation of GnRH targets in the visual processing retina and brain. In all species, GnRH-immunoreactive (-ir) axons from the terminal nerve project principally to the boundary between the inner plexiform (IPL) and inner nuclear (INL) layers of the retina, and are less prominent in the optic nerve, ganglion cell, IPL and INL. However, the density of GnRH innervation within the retina differed among fish species with highest concentrations in the damselfish and butterflyfish and lowest in the goby and wrasse. Experiments also show that GnRH receptors are associated with GnRH-ir axons within the fish retina primarily at the IPL-INL boundary, the region of light-dark adaptation and image processing of contrast, motion or color. GnRH-ir axons overlapped central projections of retinal ganglion cell axons primarily within the stratum album centrale and stratum griseum centrale of the tectum in all species, and were concentrated in several diencephalic visual processing centers. GnRH receptors are also localized to diencephalic visual centers and the stratum griseum periventriculare of the tectum, where motion perception and coordination of motor behavioral responses in three-dimensional space occur. This work demonstrates that the basic neural substrates for peptide-sensory convergence are conserved at multiple processing levels in the visual system of several reef fishes. Species differences in GnRH innervation to the retina and GnRH receptor distributions may be related to phylogeny, their use of vision in natural behaviors, or possibly binding properties of the antibodies. Future studies are needed to characterize the exact GnRH variants and receptor types found in these species so that possible functional consequences of GnRH influence on vision can be defined.

Introduction
Gonadotropin-releasing hormone (GnRH) is best known as an important regulator of reproductive physiology and behavior via the brain-pituitary-gonad axis. However, extensive projections of non-preoptic area
GnRH somata throughout the vertebrate brain indicate this decapeptide may also function as a neuromodulator or modulatory neurotransmitter that influences a variety of neural circuits [see Oka, 1997 and references therein]. The prevalence of GnRH in peripheral and central sensory regions [Munz et al., 1981; Rosen et al., 1997; Forlano et al., 2000; Wirsig-Wiechmann, 2001; Wirsig-Wiechmann and Oka, 2002; Wirsig-Wiechmann and Wiechmann, 2002] and its physiological effects on sensory cells [Stell et al., 1984, 1987; Walker and Stell, 1986; Eisthen et al., 2000; Park and Eisthen, 2003] indicate a function related to sensory processing and integration. Thus neuroanatomical evidence of GnRH peptide and receptors within specific sensory processing regions is required to test predictions regarding neuromodulatory function.

There are several lines of evidence that indicate GnRH influences visual information processing in fishes. First, although GnRH-ir axons occur in the optic nerve or retina of amphibians [Wirsig-Wiechmann, 1993], reptiles [Medina et al., 2005], birds [Fukuda et al., 1982], and mammals [Wirsig-Wiechmann and Wiechmann, 2002], the highest densities of GnRH-ir fibers are found in the fish retina [Munz et al., 1981, 1982; Stell et al., 1984, 1987; Grober et al., 1987; Oka and Ichikawa, 1990]. The origins of the retinal GnRH projections in fishes are GnRH somata associated with the terminal nerve (TN) [Munz et al., 1981, 1982; Springer, 1983; Stell et al., 1984, 1987; Behrens and Wagner, 2004] at the junction between the olfactory bulb and telencephalon (nucleus olfactoretinalis) and most frequently contain salmon GnRH (sGnRH or GnRH3) [Amano et al., 1997; Fernald and White, 1999].

Second, GnRH was also shown to modulate visual processing within the fish retina [Stell et al., 1984, 1987; Umino and Dowling, 1991; Behrens et al., 1993]. Although the functional consequence of this modulation upon sensory processing and behavior remains unclear, recent studies show that the effects are mediated through the TN dopamine-interplexiform cell pathway [Maaswinkel and Li, 2003; Behrens and Wagner, 2004; Grens et al., 2005]. Third, Oka and colleagues demonstrated that TN GnRH neurons in the gourami are rhythmically active and function as a general neuromodulator system influenced by physiological and environmental factors [Oka, 1992, 1997; Oka and Matsushima, 1993; Abe and Oka, 2002; Haneda and Oka, 2004]. Thus GnRH is an excellent candidate for modulation of visual processing such as light-dark adaptation, and the perception of contrast, motion or color that might be influenced by olfactory, visual or pineal inputs to the TN GnRH system.

Previous studies on reef fishes focused on GnRH1 in the preoptic area and its proximate control of sex reversal and mating behavior, with no focus on potential modulation of visual sensory cues [Grober and Bass, 1991; Elofsson et al., 1997, 1999; Grober, 1998; Bass and Grober, 2001]. Visual cues provide important social signals in fishes [reviewed by Myrberg and Fuiman, 2002] for conspecific recognition [Zumpe, 1965], location of mates [Reese, 1975], courtship and spawning [Rowland et al., 2002], social status [Ross, 1982], and territory defense and aggression level [Myrberg and Thresher, 1974; Muske and Fernald, 1987]. On Hawaiian reefs, terminal phase males of the protogynous saddleback wrasse, Thalassoma duperrey, possess a bright white vertical bar that is used in visual displays for spawning territory defense and to attract female mates for spawning [Ross, 1982; Barry and Hawryshyn, 1999]. The high-contrast black vertical bars on the territorial multiband butterflyfish, Chaetodon multicinctus, and planktivorous benthic spawning Hawaiian sergeant damselfish, Abudedefduf abdominalis, are used for mate and conspecific recognition [Helfrich, 1958; Hourigan, 1987; Tricas, 1989]. Male and female polygamous halfspotted gobies, Asterropteryx semipunctata, undergo changes in color patterns associated with courtship and aggressive behaviors, and males use elaborate fin and body motions coupled with pigment pattern changes to court and attract females for spawning [Privitera, 2002]. Thus, these and other species can be expected to possess neural adaptations to enhance their perception of visual signal contrast, color or motion during natural behaviors.

Fish GnRH receptors are part of the G-protein coupled receptor family with as many as five different subtypes found within a single species [Lethimonier et al., 2004; Moncaut et al., 2005]. Ligand specificity, regulation, function, and temporal expression patterns of different GnRH receptor subtypes within a single species are still complicated and unclear. GnRH receptors were detected with molecular techniques in retinal tissue of several different fish species [Madigou et al., 2000; Okubo et al., 2000; Robison et al., 2001; Moncaut et al., 2005], but were only recently localized to specific retinal cell types in a freshwater cichlid [Grens et al., 2005]. Descriptions of GnRH receptor localization (via in situ hybridization and immuno-cytochemistry) in the fish brain are only available for a few species [Madigou et al., 2000; Peter et al., 2003; Soga et al., 2005; Chen and Fernald, 2006], but examination within specific sensory processing areas such as the visual system are not available. Thus studies that examine target cells for GnRH action in the retina and visual brain...
and the modulatory potential of GnRH on visual processing at both retinal and central (tectum; diencephalon) levels within a single species are critical for understanding the functional significance of GnRH in visually-mediated behaviors.

In order for GnRH to function as a modulator of sensory function, regions of GnRH action should show neuroanatomical evidence of GnRH-ir axons and target cells that express GnRH receptors. The primary purpose of this study was to test for peptide and receptors in the visual system of fishes by examination of GnRH and GnRH receptor immunoreactivity in both the retina and visual brain of the same species. The majority of previous studies of GnRH influence on the visual system were performed on freshwater monochromatic fish such as the goldfish and perch [Stell et al., 1984, 1987; Walker and Stell, 1986; Umino and Dowling, 1991]. The present study addresses the question of whether co-occurrence of GnRH-ir axons and GnRH receptors in the retina and visual brain is a common trait among derived perciform species that live in clear reef waters, show species-specific color patterns, and exhibit complex social behaviors mediated by the visual system. Our results demonstrate that the co-occurrence of GnRH-ir axons and GnRH receptors are conserved in the retina and visual brain of several perciform reef fishes, and are consistent with the hypothesis that GnRH can influence processing of visual stimuli at both retinal and central levels. However, the density of GnRH innervation to the retina and the distribution of receptors in the retina and brain differed among species, which might be related to phylogeny, their use of vision in natural behaviors, or possibly due to binding properties of the antibodies. Nevertheless, the commonality of GnRH in the retina and visual brain among derived reef fishes indicates that GnRH neuropeptide might have conserved roles in visual processing, sensory integration, and behavior.

Materials and Methods

Animals

Reef fish species that represent four different perciform families were used for this study: multiband butterflyfish, Chaetodon multicinctus (Chaetodontidae); saddleback wrasse, Thalassoma duperrey (Labridae); Hawaiian sergeant damselfish, Abudefduf abdominalis (Pomacentridae); and halfspotted goby, Asteropteryx semipunctata (Gobiidae). These species were chosen because their reproductive and social behaviors are well-characterized and diverse, and visual cues are important for feeding, predator avoidance, courtship, spawning, and agonistic interactions. They are also part of an ongoing larger comparative neuroanatomical study. Adult fish (butterflyfish = 75–90 mm standard length (SL); wrasse = 110–135 mm SL; damselfish = 115–145 mm SL; goby = 25–35 mm SL) of each species were caught via hook and line or hand net from near shore waters on Oahu, transported back to the lab, and either used immediately (goby, wrasse, and damselfish) or maintained in aquaria (butterflyfish) for less than 24 h with flow-through aerated seawater on a 12 h light-dark cycle prior to use in experiments. Collection, maintenance, surgical, and perfusion procedures for fishes used in this study were approved by the University of Hawaii IACUC.

GnRH and GnRH Receptor Immunocytochemistry

Fish used for immunocytochemistry were deeply anesthetized with tricaine methanesulfonate (MS222 or FINQUEL; Argent Chemical Laboratories, Inc., Redmond, Wash., USA), then transcardially perfused with 0.9% sodium chloride followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). All fish were sacrificed during daylight hours to eliminate possible dark-adapted/light-adapted variations in GnRH immunostaining [Ball et al., 1989]. Brains and left eyes were removed from each fish, postfixed in 4% paraformaldehyde in 0.1 M PB for 12–24 h, rinsed in 0.1 M PB, cryoprotected in 30% sucrose in 0.1 M PB overnight, and sectioned in the sagittal or transverse plane at 24 μm with a cryostat. Sections were collected onto alternate gelatin-coated slides, dried flat overnight at room temperature, and stored at 4°C prior to immunocytochemistry.

Slides with brain or retinal tissue were first rinsed with 0.05 M phosphate-buffered saline (PBS), non-specific binding blocked with 0.3% Triton-X 100 in PBS with 2% normal goat serum (NGS; Vector Laboratories, Burlingame, Calif., USA) for 0.5–1 h, and incubated with primary GnRH or GnRH receptor antibody (1: 5000 final concentration) overnight (14–16 h) at room temperature in a sealed humidified chamber. The primary GnRH antibody was 7 CR-10 (donated by Nancy Sherwood, University of Victoria, British Columbia), which is a polyclonal antibody that labels multiple forms of GnRH including salmon (GnRH3) and chicken II GnRH (GnRH2) [see Forlano et al., 2000 for 7CR-10 cross-reactivity data]. The primary GnRH receptor antibody was GnRH-RIII (donated by Ishwar Parhar, Nippon Medical School, Tokyo, Japan) that was made against the extracellular loop 3 region of the type III GnRH receptor (anti-amberjack, anti-striped bass, anti-medaka GnRH-R type III; CLEGKVSIEL; ISPR3) [see Parhar et al., 2002 and Soga et al., 2005 for details on ISPR3 antibody specificity] and shows wide distribution in the cichlid brain [Soga et al., 2005]. Primary antibody incubation was followed by a PBS wash, incubation with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, Calif., USA) with 2% NGS for 1 h, PBS wash, quenching with 0.5–1% hydrogen peroxide in PBS, washed in PBS again, incubation with avidin–biotin complex (Elite ABC kit; Vector Laboratories, Burlingame, Calif., USA) for 2 h, washed in PBS, and reacted with a diaminobenzidine (DAB) substrate kit with nickel chloride intensification (Vector Laboratories, Burlingame, Calif., USA) for 5 min. Slides were then soaked in distilled water for 10 min, counterstained with 0.1% methyl green or 0.5% cresyl violet, dehydrated in an ethanol series (50–100%), cleared in toluene, and coverslipped with Cytoseal 60 (Richard Allen Scientific). In several cases, tissue was incubated with primary antibody at a final concentration of 1:1000 followed by a Fluorescein (FITC) or Texas Red goat anti-rabbit secondary antibody (Vector Laboratories,
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Burlingame, Calif., USA) and mounted with vectashield medium (Vector Laboratories, Burlingame, Calif., USA) for visualization with a fluorescent microscope. All tissue sections were observed on a Zeiss Axioskop 2 microscope and images captured with an Optronics Macrofire digital camera (Optronics®, Goleta, Calif., USA).

Immunocytochemistry controls for GnRH peptide localization included: (1) omission of primary antiserum, secondary antiserum, ABC solution or DAB all resulted in no staining (negative controls); (2) preabsorption of 7CR-10 primary antiserum with 8 μM salmon GnRH peptide (Bachem California Inc., Torrance, Calif., USA) eliminated staining in terminal nerve somata and retina, and reduced staining in diencephalon and midbrain regions; (3) incubation with a seabream specific GnRH antibody (ISPI, donated by Ishwar Parhar, Nippon Medical School, Tokyo, Japan) labeled somata and fibers only in the preoptic area, with an absence of staining in the retina or other visual regions of the brain (with the exception of goby tissue, which showed no label with this antibody). Controls for GnRH receptor immunocytochemistry included: (1) negative controls as described above for GnRH peptide; (2) pituitary tissue was used as a positive control, and ISPR3 labeled a subgroup of cells in the pituitary consistent with that described by Parhar et al. [2002] for this same antibody; (3) preabsorption of ISPR3 with 1–2 μg of corresponding antigen (ISPI-antigen, donated by Ishwar Parhar, Nippon Medical School, Tokyo, Japan) per ml of working primary antiserum 24 h prior to use eliminated all positive staining.

Quantification of GnRH in the Retina

In order to compare GnRH immunoreactivity within the retina among fish species, the number of axon varicosities was enumerated. Axon varicosities, or swellings, are thought to be non-synaptic release sites for GnRH peptide and therefore should be a proxy indicator of the amount of peptide available for release [see Oka and Ichikawa, 1992]. Quantification was performed only on mature males within the spawning season for the butterflyfish, damselfish, wrasse, and goby to eliminate any sex or temporal variations. The total number of GnRH-ir varicosities was counted along a 250 μm transect line at the INL-IPL boundary in six sections per fish (n = 3–8 individuals per species) at 400–1000X. Immunoreactive fibers not located at the INL-IPL boundary were not included, and counts were performed at approximately the same location in the eye (adjacent to a point where the optic nerve exits the eye) among individuals and species. Comparisons among species were made with one-way analysis of variance (ANOVA) and subsequent Tukey’s test for pairwise comparisons (SigmaStat 3.1).

Optic Nerve Tracing and Double Label Experiments

Optic nerves were labeled on several individuals of the wrasse (n = 3), goby (n = 4), and damselfish (n = 3), but not the butter- flyfish, to determine the central retinal projections and to test the prediction that GnRH-ir axons overlap regions of visual input. Fish were anesthetized with MS222, lightly clamped to a fish holder positioned in a shallow tank, and ventilated through the mouth with aerated MS222 seawater. The left eye was surgically removed by transecting the optic nerve and ocular muscles, the orbit dried, and a piece of paraffin placed beneath the cut optic nerve. The exposed optic nerve was then re-cut and several crystals of neurobiotin tracer (Vector Laboratories, Burlingame, Calif., USA) applied directly to the surface of the proximal stump and allowed to be absorbed by the axons. A second piece of paraffin was then glued on top to cover the exposed nerve, the orbit filled with gelfoam and sealed with parafilm and vetbond (3M). Fish were then revived in fresh seawater and returned to holding aquaria for recovery.

Following survival times of 2–3 days, fish were deeply anesthetized with MS222 and transcardially perfused as described above for immunocytochemistry except that 4% paraformaldehyde in 0.1 M PB with 1% glutaraldehyde was used as the fixative and post-fix solution. Brains were sectioned serially in the sagittal or transverse plane at 40 μm with a cryostat and collected in Corors™ spot plate wells that contained 0.4% Triton-X 100 in 0.05 M PBS and soaked for 0.5–1 h. Floating sections were then transferred with a paintbrush to wells containing avidin-biotin complex solution (Elite ABC kit, Vector laboratories, Burlingame, Calif., USA) for 3 h at room temperature in a sealed humidified chamber on a shaker table (70–90 R.P.M.), rinsed in 0.1 M PB, reacted with DAB for 5–8 min, transferred to 0.1 M PB to stop the reaction, soaked in distilled water for 5–10 min, and then mounted on gelatin-coated slides. Slides were then dried overnight at room temperature and counterstained with 0.1% methyl green or 0.5% cresyl violet as above. For double label experiments, the neurobiotin reaction was completed up to the PB step to stop the DAB reaction and then sections were transferred to the immunocytochemistry blocking solution described above. All subsequent immunocytochemistry steps were then performed as above, except Novared substrate (Vector Laboratories, Burlingame, Calif., USA) was used to visualize GnRH-ir neurons so that in double labeled tissue the optic nerve filled neurobiotin-containing axons were black (DAB), and GnRH-ir neurons were red (Novared). In some cases, neurobiotin label was visualized with Texas Red Avidin D (Vector Laboratories, Burlingame, Calif., USA) followed by immunocytochemistry with FITC goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, Calif., USA) for GnRH, mounted with vectashield (Vector Laboratories, Burlingame, Calif., USA) and viewed on a Zeiss Axioskop 2 fluorescent microscope. Line drawings were made with a drawing tube attached to an Olympus BH2 microscope.

Results

GnRH and GnRH Receptors in the Retina

Varicose GnRH-ir axons are found in the optic nerve and retina of all four fish species examined (fig. 1A–D). GnRH-ir fibers within the optic nerve (fig. 1C) consist of only a few axons that travel from the brain through the optic chiasm, and along the edge of the optic nerve into the retina where they branch extensively (fig. 1A, B, D). Immunoreactive fibers in the retina are most abundant at the interface between the inner plexiform and inner nuclear layers (fig. 1A, B) where they form a plexus that is evident in oblique sections (fig. ID). GnRH-ir fibers are also observed to form ‘basket-like’ projections around individual cells within the inner nuclear layer.
Labeled GnRH fibers are also found within the inner plexiform, inner nuclear, ganglion cell, and optic fiber layers of all species examined, but were sparse compared to the plexus at the INL-IPL boundary. In contrast, GnRH-ir axons are absent from the outer nuclear or photoreceptor layer in all species. GnRH-ir somata are not found within the retina of any species examined.

The mean total number of GnRH-ir varicosities along a 250 μm transect at the INL-IPL boundary of the retina differed among species. Mean numbers of GnRH-ir varicosities ranged from 74.8 to 99.7 for the sergeant damselfish, from 67.8 to 87.2 for the butterflyfish, from 55.5 to 74.0 for the wrasse, and from 44.5 to 77.5 for the goby (fig. 2). The Hawaiian sergeant damselfish had the greatest mean number of GnRH-ir varicosities (89.9 ± 3.8 SE, n = 6) along the INL-IPL border, whereas the halfspotted goby had the lowest (58.1 ± 4.4 SE, n = 8). The sergeant damselfish had more GnRH-ir varicosities than both the wrasse (1-way ANOVA, F = 14.32, d.f. = 3, 20, p < 0.001; Tukey’s test, p = 0.005) and goby (1-way ANOVA, F = 14.32, d.f. = 3, 20, p < 0.001; Tukey’s test, p < 0.001), but not the butterflyfish (1-way ANOVA, F = 14.32, d.f. = 3, 20, p < 0.001; Tukey’s test, p = 0.41) (fig. 2). The butterflyfish also had more GnRH-ir varicosities compared to the goby (1-way ANOVA, F = 14.32, d.f. = 3, 20, p < 0.001; Tukey’s test, p = 0.001), but not the wrasse (1-way ANOVA, F = 14.32, d.f. = 3, 20, p < 0.001; Tukey’s test, p = 0.06) (fig. 2).

GnRH receptor immunoreactivity was also observed within specific regions of the retina, but the staining pattern differed slightly among species (fig. 3). First, GnRH receptor immunoreactivity was absent in the retina of

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**Fig. 1.** Gonadotropin-releasing hormone-immunoreactive fibers in the retina and optic nerve of reef fishes. Transverse sections through the retina of the Hawaiian sergeant fish, *Abudesdus abdominalis* (A), and saddleback wrasse, *Thalassoma duperrey* (B), show varicose GnRH-immunoreactive (-ir) axons (arrowheads) at the interface of the inner plexiform (IPL) and inner nuclear (INL) layers. GnRH-ir axons also form ‘basket-like’ structures around individual cells within the INL (inset A). (C) Single varicose GnRH-ir axon (arrows) in the optic nerve of the goby, *Asteropteryx semipunctata*. (D) Oblique section through the butterflyfish retina shows the plexus of GnRH-ir axons (arrows) at the IPL-INL boundary. GCL, ganglion cell layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PL, pigmented layer. Scale bars = 50 μm (A, B), 5 μm (inset in A), 10 μm (C), 20 μm (D).
Thus the goby likely has a GnRH receptor with a sequence that is not bound by the ISPR3 antibody. In the other three species, GnRH receptor immunoreactivity was observed within both cell bodies and fibers within the retina (fig. 3). GnRH receptor label in the damselfish and butterflyfish was found in cells within the inner nuclear layer at the interface between the inner nuclear and inner plexiform layers (fig. 3A, B). Occasional fibers in these two species were also observed at the interface between the inner plexiform and inner nuclear layers, and within the inner plexiform layer (fig. 3A, B). Immunoreactive cells in the butterflyfish along this same interface often had long processes that extended in both directions parallel to this boundary (fig. 3B inset). In contrast, GnRH receptor immunoreactivity in the wrasse was found not only within cells at the INL-IPL boundary, but also within cell bodies of the inner nuclear layer near the outer plexiform layer, and within the ganglion cell layer (fig. 3C, inset). This pattern differed from the butterflyfish and sergeant damselfish not only in location, but also in the fact that many cell bodies showed positive label in the wrasse compared to only scattered-ir cells observed in the other two species. The specific cell type that exhibits GnRH receptor immunoreactivity was not determined in this study, but might be within amacrine, interplexiform, horizontal, or bipolar cells within the inner nuclear layer.

Central Projections of the Optic Nerve

Central projections of the optic nerve were determined by anterograde bulk labeling with neurobiotin in the wrasse, goby and damselfish (fig. 4). The purpose of this study was not to describe the central retinal projections of these species in detail, but to identify potential substrates for visual-GnRH convergence in the brain. Overall labeling was similar among species and is briefly described below. Labeled axons coursed through the optic chiasm to the contralateral tectum, with sparse fibers noted within the ipsilateral diencephalon. Projections from the optic nerve/retina are located primarily within the superficial layers of the tectum where terminals were observed in the stratum opticum (SO) and distinct layers within the stratum fibrosum et griseum superficiale (SFGS) (fig. 4). In some individuals, small vertically oriented cells (diameter = 6.25 ± 0.42 (SE) µm) were also filled within the stratum griseum centrale (SGC) (fig. 4C, D). These cells were evenly spaced (70.5 ± 6.9 µm apart) and had long processes that spanned the height of the SGC (116.5 ± 2.9 µm long) (fig. 4D). Trans-synaptic label is common for the low molecular weight neurobiotin tracer and it is possible that these cell bodies were labeled via this mechanism. However, this tectal cell type was also observed following optic nerve label with different tracer molecules in other species [e.g., Ekstrom, 1984]. All species also showed some label...
within 1–2 deeper tectal layers (SAC and SGC) close to the stratum griseum periventriculare (SGP) (fig. 4A–C). The damselfish and goby showed neurobiotin label in only a single layer within the stratum album centrale (SAC) (arrowheads in fig. 4A, B), whereas the wrasse showed an additional intermediate visual layer in the SGC between the SFGS and SAC (arrows in fig. 4C). These deeper tectal layers also contain abundant GnRH–ir fibers (see fig. 5).

Optic nerve fills also labeled several groups of small cells within the contralateral diencephalon (fig. 6). A large group of small cells (diameter = 8.4 ± 0.9 μm) was located in a distinct nucleus near the preoptic area, which corresponds to the location of the accessory optic nucleus in other species [Braford and Northcutt, 1983; Presson et al., 1985]. Labeled cell bodies and terminals were also localized to the nucleus of the posterior commissure, area pretectalis pars dorsalis, area pretectalis pars ventralis (pretecto-retinal pathway), nucleus supranchiasmaticus, nucleus dorsolateralis thalami, and nucleus ventrolateralis thalami (thalamo-retinal pathway). Although the locations and identity of these retinopetal nuclei are based primarily on neuroanatomical studies in other species, the anterograde fills performed here indicate they are part of the visual processing system in the species examined here. Optic nerve fills also labeled several somata within the terminal nerve ganglia at the junction of the olfactory bulb and telencephalon (nucleus olfactoretinalis), indicative of retinal efferents from the TN GnRH system (olfacto-retinal pathway).

**Fig. 3.** Distribution of gonadotropin-releasing hormone receptors in the retina of reef fishes. **A** GnRH receptor immunoreactivity was observed in cell bodies (arrowheads) within the inner nuclear layer of *A. abdominalis* at the interface between the inner plexiform (IPL) and inner nuclear (INL) layers. Scattered immunoreactive fibers were also observed within the inner plexiform layer (arrow) and near the ganglion cell layer (GCL). **B** GnRH receptor immunoreactivity in the butterflyfish also showed labeled cell bodies (arrowheads) and fibers within the inner nuclear layer along the IPL-INL boundary. In addition, cells with long processes that run parallel to the IPL-INL boundary (inset) were also labeled in the butterflyfish. **C** GnRH receptor immunoreactivity in the wrasse was localized to cell bodies (arrows) in multiple layers of the retina; at the IPL-INL border, in the distal INL near the outer plexiform layer (OPL), and in the ganglion cell layer (arrowheads). Inset shows higher magnification of cells labeled within the INL of the wrasse. ON, optic nerve; ONL, outer nuclear layer; PL, pigmented layer. Scale bars = 50 μm (A), 100 μm (B), 5 μm (inset in B); 25 μm (C), 10 μm (inset in C).
Fig. 4. Central projections of retinal axons to the midbrain in reef fishes. Transverse sections through the tectum of the damselfish (A), goby (B), and wrasse (C) show that retinal fibers project primarily to the contralateral stratum opticum (SO) and stratum fibrosum et griseum superficiale (SFGS) layers (dark black neurobiotin label in A–C; dorsal is towards the top of each micrograph). However, all species also show retinal projections to a deeper layer within the stratum album centrale (SAC) (arrowheads in A–C). In addition, there is an intermediate projection to the stratum griseum centrale (SGC) between the SFGS and the deep SAC layer, which is only observed in the wrasse (arrows in C). Cell bodies with long apical and ventral processes were also filled within the SGC of all species (arrows in D, and asterisk in C). E Neurobiotin-filled terminals (swellings on fibers) from the retina within the SFGS of the caudal tectum in the damselfish. SGP, stratum griseum periventriculare; SM, stratum marginale. Scale bars = 100 μm (A), 50 μm (B, C), 20 μm (D), 10 μm (E).
Retinal Inputs Overlap GnRH-ir Axons in the Brain

Anterograde fills of the optic nerve show that ganglion cell afferents project through the optic chiasm to the contralateral tectum. GnRH-ir axons are also abundant within the tectum of all four species. Although visual information is primarily concentrated within the superficial tectal layers (SO and SFGS), there are also retinal projections to 1–2 deep tectal layers in the SGC and SAC (see fig. 4A–C, 5A–C, 6). There are only scattered GnRH-ir fibers within the superficial visual layers (SFGS) of the tectum in all species, but there was extensive overlap within the SAC between visual and varicose GnRH-ir fibers (fig. 5A–C, 6). Axonal swellings, or terminals on retinal afferents were often observed in close (putative synaptic) contact with varicosities of GnRH-ir axons (fig. 5C, inset) in the tectum. Further, GnRH-ir axons were also observed in proximity to the vertically oriented filled perikarya within the SGC, and the intermediate visual input layer in the SGC of the wrasse. There was also considerable overlap between retinal projections and GnRH-ir axons within all diencephalic visual regions (fig. 6), most notably the accessory optic nucleus (fig. 5D), nucleus suprachiasmaticus, preoptic area, and pretectal and thalamic nuclei.

GnRH Receptors in the Visual Brain

Staining patterns for GnRH-RIII in the brain varied among the different reef fishes examined. Immunoreactive label in visual brain regions was weak-to-absent in both the goby and damselfish. Thus, only immunoreac-

Fig. 5. Co-occurrence of gonadotropin-releasing hormone-immunoreactive axons and retinal projections in the tectum and diencephalon of reef fishes. A Transverse section through the tectum of the damselfish illustrates the region of overlap (large arrowheads) between retinal projections and GnRH-immunoreactive (–ir) axons in the stratum album centrale (SAC) above the stratum griseum periventriculare (SGP). B Higher magnification of the SAC of the damselfish shows the overlap of the retinal projections (in red; large arrowheads) and the GnRH-ir varicose axons (in green; small arrows). C Terminals of retinal ganglion cell fibers (in red; large arrowheads) are seen in close proximity to GnRH-ir varicosities (in green; small arrows) within the stratum album centrale of the damselfish and all other species examined. Inset shows a higher magnification of GnRH-ir varicose fiber (in green; asterisk) in close proximity (small arrows) to a retinal fiber (in red; large arrowhead). D GnRH-ir axons (in green; small arrows) near labeled somata (in red; large arrowheads) within the retinopetal accessory optic nucleus of the damselfish. SFGS, stratum fibrosum et griseum superficiale; SGC, stratum griseum centrale. Scale bars = 100 μm (A), 50 μm (B), 10 μm (C and D), 5 μm (inset in C). See online version for color figure.
GnRH and Receptors in Visual Regions

GnRH and GnRH Receptors in the Retina

GnRH immunoreactivity in the optic nerve and retina of the reef fishes examined in this study was similar to that described for perciform [Munz et al., 1982; Grens et
al., 2005] and non-perciform fishes [Stell et al., 1984, 1987; Grober et al., 1987]. The number of GnRH-ir axons within the optic nerve is much lower than that observed within the retina, which indicates extensive branching of fibers within the retinal layers as suggested for other fishes [Munz et al., 1982; Stell et al., 1984, 1987; Behrens and Wagner, 2004]. The GnRH-ir axons form a plexus at the IPL-INL boundary and often form ‘basket-like’ processes around individual cell bodies within the inner nuclear layer. The identity of the cell types surrounded by these processes was not determined in this study, but these ‘basket-like’ projections were described associated with GABAergic amacrine and dopaminergic interplexiform cells by others [Stell et al., 1984, 1987; Behrens and Wagner, 2004]. Thus the similarities in the localization of GnRH-ir axons within the retina of fishes examined in this and previous studies indicate it is likely a common trait among fishes. GnRH-ir axons are also found in the retina or optic nerve of less-recently derived fishes such as lampreys, elasmobranchs, bichirs, sturgeons and gars [reviewed by Reperant et al., 2006], as well as amphibians [Wirsig-Wiechmann, 1993], reptiles [Medina et al., 2005], birds [Fukuda et al., 1982], and mammals [Wirsig-Wiechmann and Wiechmann, 2002], and therefore could be a conserved trait among all vertebrates. However, the functional consequence of GnRH release on processing of visual information within the retina requires further investigation across taxa.

There is now ample evidence that GnRH functions as a neuromodulator in central and peripheral tissues, without action at conventional synapses [Oka and Ichikawa, 1992; Oka, 1997; Eisthen et al., 2000]. Thus, GnRH

**Fig. 7.** Gonadotropin-releasing hormone receptor immunoreactivity in the brain of coral reef fishes. **A** Sagittal section through the tectum of the wrasse. Small GnRH-RIII-ir somata (arrows) are abundant in the SGP, and scattered within two main layers of the SGC (asterisks). Double asterisk indicates the layer that also receives retinal projections in this species. Inset shows GnRH-RIII-ir fiber staining within the SAC region of the tectum. **B** Transverse section through the butterflyfish tectum shows many small GnRH-RIII-ir somata within the SGP (arrows) and a higher magnification of a single cell shows label localized to the cytoplasm (inset). **C** GnRH-RIII-ir label within somata and processes in the visual thalamus of the butterflyfish. Inset shows a higher magnification of two labeled cells to illustrate staining within the cell membrane/cytoplasm and processes, but absence of immunoreactivity in the nucleus (arrows). Scale bars = 50 μm (A–C), 5 μm (inset in A and B), 10 μm (inset in C).
of visible spectrum cues, many reef fishes have the abil-
ty to use reflected patterns of ultraviolet (UV) radiation
during social interactions [Losey et al., 1999], and UV
patterns are used for mate assessment in several taxa
[Bennett et al., 1997; LeBas and Marshall, 2000; Kodric-
Brown and Johnson, 2002]. The species with the most
abundant retinal GnRH-ir innervation in the present
study were the damselfish and butterflyfish, which have
UV and violet color vision, respectively, whereas the
wrasse and goby lack UV-sensitivity [Losey et al., 2003].
Thus we speculate that one possible role of GnRH in the
retina of these species is in processing of short wave-
length visual communicative information, but this re-
quires further examination. The number of varicosities
quantified in the present study is a proxy measure for
the amount of GnRH available for release [Oka and Ichi-
kawa, 1992], but it is also possible that varicosity differ-
ences reflect neuronal structural differences among
species. Alternatively, the species differences in GnRH-
ir varicosities observed in the retina might also be at-
tributed to phylogenetic differences or related to other
ecological or behavioral factors that differ among these
perciform species (i.e., social system, feeding behavior,
aggression and territoriality, courtship and mating be-
havior). Future studies should compare GnRH and
GnRH receptor content in the retina among closely re-
lated species (i.e., congeners) that represent different
habitats, ecological niches, and behaviors in order to
separate the effects of phylogeny and behavioral ecology
on neuropeptide phenotype.

The presence of multiple GnRH variants within a sin-
gle fish species indicates there could also be multiple cog-
nate GnRH receptors, but ligand specificity and function
remain unclear. Studies in fishes indicate that up to five
different types of GnRH receptor genes are expressed in
the brain of a single species [Jodo et al., 2003; Lethimo-
nier et al., 2004]. Further, variation in spatiotemporal
patterns of multiple GnRH receptors indicates different
roles in GnRH action [Illing et al., 1999; Parhar et al.,
2002; Peter et al., 2003; Jodo et al., 2003; Soga et al.,
2005; Chen and Fernald, 2006]. In the butterflyfish and dam-
selfish, GnRH receptors were found on cell bodies and
fibers at the IPL-INL boundary of the retina, which coin-
cides with the distribution of GnRH-ir axons. In contrast,
we observed no receptor staining in the goby retina, and
staining in the wrasse was within the distal INL and gan-
glion cell layer in addition to the IPL-INL boundary.
These variations might be due to different binding prop-
erties of the antibody, or different receptor types that are
not yet characterized in these species. Future studies that
employ molecular techniques should help resolve these
immunocytochemistry staining differences among species and test for cognate GnRH receptors in the retina and brain of perciform and other fishes. For example, Grens et al. [2005] recently demonstrated type I GnRH receptor expression in a small percentage of cells in the amacrine cell layer, and abundant type 2 GnRH receptor expression in ganglion cells of the cichlid, Astatotilapia burtoni. However, the fact that cell processes are also labeled by GnRH receptor antibodies [this study; Soga et al., 2005] highlights the need to use techniques that both identify the cells that produce GnRH receptors (e.g., in situ hybridization) and those that detect the receptors at their final location in the neuronal membrane (e.g., receptor immunocytochemistry or ligand binding), that could be many microns away from the cell body [Jennes et al., 1997]. Nevertheless, GnRH might directly or indirectly influence multiple processing pathways in the retina via actions on different receptor types within a single species.

GnRH and GnRH Receptors in the Visual Brain

Projections from the retina terminate primarily in the superficial layers of the contralateral tectum, a region with only sparse GnRH innervation. In contrast, some retinal input to the fish tectum terminates within layers of the stratum album centrale and stratum griseum centrale where there is extensive GnRH-ir innervation. GnRH receptor immunoreactivity in the present study was found primarily within the SGP, with some staining within the SGC in the wrasse. Such regional mismatches between GnRH peptide and GnRH receptor immunoreactivity are reported in many species from fish to mammals [Wirsig-Wiechmann, 1993; Jennes et al., 1997; Soga et al., 2005], and GnRH might reach target neurons some distance away via diffusion, the cerebrospinal fluid or circulatory system. Teleosts contain several different cell types within the SGP, but the majority possess long apical dendrites that extend dorsally to ramify in secondary and tertiary branches within the retinal termination layers of the SFGS [Northcutt, 1983]. Thus GnRH released from the distant SAC or SGC likely diffuses into the SGP and influences GnRH-RII-containing processing cells that receive visual information from the retina via the long apical dendrites. GnRH receptors were also localized to the periventricular gray zone of the optic tectum in the tilapia (Orechromis niloticus) [same antibody used in the present study; Soga et al., 2005], goldfish [Peter et al., 2003], cichlid (Astatotilapia burtoni) [Chen and Fernald, 2006], trout [Madjigou et al., 2000], and are found in the visual processing superior colliculus of mammals [Jennes and Conn, 1993; Jennes et al., 1997]. Deep tectal layers are also known to receive input from other senses such as auditory, lateral line, and somatosensory information, are important for motion perception, and help coordinate motor behavioral responses in three-dimensional space [see Butler and Hodos, 1996]. Thus, GnRH in the tectum might function to modulate sensorimotor integration of complex social behaviors such as agonistic interactions, territoriality, and courtship and spawning in fishes, but this requires further study.

GnRH-ir axons and receptors were also present within retinopetal nuclei in the diencephalon of the butterflyfish and wrasse, including the olfacto-retinal, thalamo-retinal, and pretecto-retinal pathways. GnRH-ir axons are frequently found in these regions across fish taxa [Oka and Ichikawa, 1990; Foran et al., 1997; Reparant et al., 2006], and GnRH receptors were also localized to these regions in two different cichlid species [Soga et al., 2005; Chen and Fernald, 2006] and the goldfish [Peter et al., 2003], using both immunohistochemical and in situ hybridization techniques. Foran et al. [1997] also demonstrated that GnRH mRNA expression in the retino-recipient thalamus of the midshipman fish (Porichthys notatus) is influenced by retinal activity and could modulate visually-mediated seasonal reproductive behaviors. Although the interconnections and function of the centrifugal visual system is not fully understood in fishes [Reperant et al., 2006], GnRH might function as a neuromodulator in multiple processing pathways from the retina to the brain that regulate different aspects of visual information in fishes. Future neurophysiological and behavioral studies are needed to test the effects of GnRH on visual processing at the level of tectal and diencephalic processing centers.

All vertebrates examined to date show at least two different GnRH receptor subtypes expressed within the same species [Guilgur et al., 2006]. The GnRH-RII examined in this study is only identified and cloned in perciform fishes [Jodo et al., 2003; Lethimonier et al., 2004; Soga et al., 2005], but is related to the type I class of GnRH receptors in other vertebrates. Even though GnRH receptor staining within visual regions of the reef species examined here is similar to other fish studies that used multiple labeling techniques [Peter et al., 2003; Soga et al., 2005; Chen and Fernald, 2006], our current examination of a single GnRH receptor likely does not reveal the entire GnRH receptor distribution in these perciform species and we cannot rule out differential binding properties of the antibody as a cause for the
variations observed among species. However, with the exception of anti-GnRH-RIA and -RIB designed against the extracellular loop 3 of the two goldfish receptor subtypes [Peter et al., 2003], other general GnRH receptor antibodies are currently not available for fish tissue. In the tilapia, anti-GnRH-RIA showed no labeling in the brain, and GnRH-RIB immunoreactivity was restricted to the periventricular hypothalamic nuclei [Soga et al., 2005]. In contrast, anti-GnRH-RIII showed widespread label throughout the tilapia brain [Soga et al., 2005]. This widespread label was also observed in our experiments on the butterflyfish and wrasse, and therefore the staining observed with GnRH-RIII probably does reveal regions of the fish retina and brain involved in neuromodulation of visual processing. The significance of the apparent cytoplasmic/cell membrane distribution of GnRH-RIII observed in the retina and visual brain of fishes in the present study is unknown, but is consistent with the distribution seen in other immunocytochemical GnRH receptor studies in fishes [Peter et al., 2003; Soga et al., 2005]. It is also important to note that GnRH receptors produced in the soma might be transported within the neuron to different regions of the brain for insertion into dendritic or axonal membranes [see Jennes et al., 1997]. Our observation of GnRH-RIII staining within neuronal fibers throughout the retina and brain supports this idea, but it is also possible that other dendritic GnRH transmembrane receptors exist in low concentrations and were not detected by immunocytochemical techniques. Nevertheless, future studies with more specific GnRH receptor antibodies, ligand binding, or in situ hybridization experiments are needed before functional correlations between the visual system and GnRH can be defined in these perciform reef fishes.

Several previous researchers suggested that GnRH influences sensory processing of sexual stimuli and integrates sensory input with motor activities [see Munz et al., 1981, 1982; Jennes et al., 1988; Wirsig-Wiechmann and Lepri, 1991; Muske, 1993; Meredith and Fernandez-Fewell, 1994; Amano et al., 1997; Rosen et al., 1997; Sakuma and Suga, 1997; Chartrel et al., 1998; Forlano et al., 2000 for discussions]. More specifically, terminal nerve GnRH neurons were modeled as a ‘general neuromodulator system in vertebrates’ which can influence a wide variety of neuronal circuits [Oka, 1992, 1997]. Moore and Rose [2002; Rose and Moore, 2002] then proposed the ‘sensormotor processing hypothesis’ based on neurophysiological and neurochemical evidence in the roughskimnewt that another neuropeptide (arginine vasotocin) acts at multiple sites in the brain and multiple levels in a behavioral sequence from social recognition to motor output. This model can also be applied to other neuropeptides, including GnRH. However, as more evidence accumulates for the modulatory role of neuropeptides on animal behavior, it is appropriate to further refine these original ideas by independent examination of specific effects at the levels of sensory input, central processing, and motor processing. It is also significant that the neurophysiological effects of peptide hormones on sensory processing neurons should differ from those of motor circuits. Therefore, we propose here the ‘sensory neuromodulation hypothesis,’ to indicate that GnRH (or other neuropeptides) targets and has specific actions to modify processing of sensory information at peripheral or central levels. It is distinguished from the general ‘sensormotor processing hypothesis’ by the fact that the modulatory peptide affects sensory processing directly, prior to integration with motor output circuits and resultant behavioral responses. Although the criteria for a modulator substance changes as more is learned about neuropeptide actions, this ‘sensory neuromodulation hypothesis’ generates several testable predictions of sensory processing centers based on some of the traditional criteria for a neuromodulator [Barchas et al., 1978]: (1) GnRH should have access to and act upon GnRH receptor-containing target neurons within sensory processing regions; (2) exogenous application of GnRH should mimic natural release of peptide and produce consistent, repeatable physiological effects on target neurons; and (3) manipulations of GnRH concentrations (e.g., peptide synthesis or release, receptor availability) within sensory regions should cause observable changes in sensory-mediated behaviors of the animal. These neuroanatomical, physiological and behavioral effects of peptide-sensory convergence should have important neurosensory functions during social interactions, reproductive behavior, feeding, or predator avoidance that can ultimately influence survival and reproductive success. Future studies should test these predictions among different sensory systems to address hypotheses on the evolution of GnRH neuromodulatory function across taxa.

In summary, we demonstrate GnRH-ir axons and GnRH receptor immunoreactivity concentrated at the IPL-INL boundary of the retina, overlap of retinal fibers and GnRH-ir axons in deep layers of the tectum and diencephalic visual centers, and GnRH-RIII-ir in the tectum and diencephalon in several different families of corral reef fishes. These data provide neuroanatomical sup-
port for the hypothesis that GnRH targets and influences the processing of visual information at both retinal and central levels within a variety of recently derived teleost fishes. However, any functional significance of the differential distribution and concentration of GnRH fibers and receptors in the visual system remain to be demonstrated.

References


Maruska/Tricas

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GnRH and Receptors in Visual Regions


