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# Hormones and Behavior

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## Gonadotropin-releasing hormone (GnRH) modulates auditory processing in the fish brain

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### ARTICLE INFO

#### Article history:

Received 3 October 2010

Revised 6 January 2011

Accepted 6 January 2011

Available online 14 January 2011

#### Keywords:

*Abudefduf abdominalis*

Damsel fish

Hearing

Midbrain

Neuromodulator

Teleost

Torus semicircularis

### ABSTRACT

Gonadotropin-releasing hormone 1 (GnRH1) neurons control reproductive activity, but GnRH2 and GnRH3 neurons have widespread projections and function as neuromodulators in the vertebrate brain. While these extra-hypothalamic GnRH forms function as olfactory and visual neuromodulators, their potential effect on processing of auditory information is unknown. To test the hypothesis that GnRH modulates the processing of auditory information in the brain, we used immunohistochemistry to determine seasonal variations in these neuropeptide systems, and *in vivo* single-neuron recordings to identify neuromodulation in the midbrain torus semicircularis of the soniferous damselfish *Abudefduf abdominalis*. Our results show abundant GnRH-immunoreactive (-ir) axons in auditory processing regions of the midbrain and hindbrain. The number of extra-hypothalamic GnRH somata and the density of GnRH-ir axons within the auditory torus semicircularis also varied across the year, suggesting seasonal changes in GnRH influence of auditory processing. Exogenous application of GnRH (sGnRH and cGnRHII) caused a primarily inhibitory effect on auditory-evoked single neuron responses in the torus semicircularis. In the majority of neurons, GnRH caused a long-lasting decrease in spike rate in response to both tone bursts and playbacks of complex natural sounds. GnRH also decreased response latency and increased auditory thresholds in a frequency and stimulus type-dependent manner. To our knowledge, these results show for the first time in any vertebrate that GnRH can influence context-specific auditory processing *in vivo* in the brain, and may function to modulate seasonal auditory-mediated social behaviors.

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### Introduction

Gonadotropin-releasing hormone 1 (GnRH1) is a decapeptide best known for its role in the regulation of reproduction via the hypothalamic-pituitary-gonad axis, but studies now show that other GnRH forms are widely distributed throughout the brain and may function as neuromodulators in both vertebrates and invertebrates (Kawai et al., 2010, 2009; Minakata et al., 2009; Oka, 1997; Wright and Demski, 1993). In derived perciform fishes, three different forms of GnRH are expressed in three different brain regions: GnRH3 (or salmon GnRH; sGnRH) associated with the terminal nerve ganglia, GnRH1 (seabream GnRH; sbGnRH, or other species-specific releasing form) in the hypothalamus and preoptic area, and GnRH2 (or chicken-II GnRH; cGnRHII) in the midbrain tegmentum (Amano et al., 1997; Fernald and White, 1999; Yamamoto, 2003). The function of GnRH1 as a releasing factor for gonadotropin release from the pituitary is well established (Clarke, 2002; Senthilkumaran et al., 1999; Yamamoto et al., 1998), but the wide distributions and high concentrations of GnRH3 and GnRH2 in fish brains indicate important yet undiscovered functions for

these neuropeptides (Kawai et al., 2009; Maruska and Tricas, 2007; Oka, 1997).

GnRH3 and GnRH2 immunoreactive (-ir) fibers and GnRH receptors are found in sensory and motivational centers of the vertebrate brain (Chen and Fernald, 2006; Forlano et al., 2000; Jennes and Conn, 1994; Kawai et al., 2009; Maruska and Fernald, 2010; Millar, 2003; Rosen et al., 1997; Soga et al., 2005), which provides neuroanatomical support for a modulatory function. Evidence across vertebrate taxa also shows that GnRH can influence the function of Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> ion channels and may regulate the excitability of, or neurotransmitter release from, target neurons (Oka, 1997). The GnRH2 variant is the most evolutionarily conserved form and is located in somata within the midbrain of most species studied to date. In addition to a role in motor reproductive pathways (Chartrel et al., 1998; Liu and Demski, 1993; Maney et al., 1997), coordination of reproduction and metabolic state (Kauffman and Rissman, 2004; Matsuda et al., 2008), and modulation of pineal functions (Servili et al., 2010), midbrain GnRH2 neurons are also hypothesized to modulate sensory processing of sexual or communicative stimuli (Maruska and Tricas, 2007; Muske, 1993; Oka, 1997). Likewise, GnRH3 neurons in fishes project widely throughout the brain (Oka and Matsushima, 1992), are involved in regulation of reproductive behavior (Abraham et al., 2010; Onuma et al., 2005;

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Uchida et al., 2005), and their spontaneous activity can be influenced by biologically-relevant sensory signals (Ramakrishnan and Wayne, 2009). In fishes and other vertebrates, the terminal nerve GnRH system also modulates processing of visual and olfactory information at the periphery (i.e., retina and olfactory epithelium) (Eisthen et al., 2000; Kawai et al., 2009; Park and Eisthen, 2003; Stell et al., 1987; Zhang and Delay, 2007). However, the hypothesis that these two extra-hypothalamic GnRH systems can directly modulate sensory processing in the brain has received only limited experimental testing (Kawai et al., 2010; Kinoshita et al., 2007), and little is known about how GnRH might influence other reproductively important senses such as audition.

In birds and frogs that rely on auditory signals for reproduction, there is evidence for an auditory-endocrine circuit that involves the GnRH1 system (Burmeister and Wilczynski, 2000; Cheng et al., 1998; Maney et al., 2007). For example, auditory stimuli from a mating chorus increase the number of hypophysiotrophic GnRH-ir cells and elevate circulating androgen levels in the green treefrog *Hyla cinerea* (Burmeister and Wilczynski, 2000), and acoustic stimulation with conspecific sounds triggers luteinizing hormone release in some birds (Cheng et al., 1998; Maney et al., 2007). While these studies are consistent with the hypothesis that perception of context-specific auditory cues can influence the GnRH1 system and reproductive physiology, it remains unclear whether similar actions exist for modulation of auditory function by the GnRH2 or GnRH3 systems.

Sound production is critically important during reproductive and agonistic behaviors in many fishes (Amorim, 2006), but the relationships between the auditory and GnRH systems are not known in this vertebrate group that contains over 25,000 species. The Hawaiian sergeant fish, *Abudefduf abdominalis*, is a colonial benthic-spawning damselfish that uses low-frequency, low-intensity pulsed sounds during reproductive and territorial behaviors (Maruska et al., 2007a), and the central projection patterns and response dynamics of auditory neurons in the brain were previously described (Maruska and Tricas, 2009a, 2009b). We therefore used this soniferous fish to test the hypothesis that GnRH2 and GnRH3 modulates processing of auditory information in the midbrain. Our results provide neuroanatomical and neurophysiological evidence that GnRH can influence context-specific auditory processing *in vivo* in the vertebrate midbrain. We also show seasonal changes in the modulatory GnRH systems, and that actions on the auditory system are consistent with the already described modulatory effects of GnRH on olfactory and visual senses.

## Methods

### Experimental animals

Adult sexually mature Hawaiian sergeant fish, *Abudefduf abdominalis* (SL = 136.7 ± 8.4 mm SD;  $\bar{x}$  BM = 110.3 ± 24.0 g SD), were caught with hook and line from Kaneohe Bay, Oahu, and used immediately in either neurophysiology or neuroanatomical experiments (average time from capture to anesthetization was 10 min). All fish were measured for standard length (SL), total length (TL), and body mass (BM), and sex was determined by examination of sexually dimorphic genital papillae under a dissection microscope and mature gonads were verified by dissection. All experimental procedures followed guidelines for the care and use of animals and were approved by the University of Hawai'i Institutional Animal Care and Use Committee.

### GnRH immunohistochemistry

Immunohistochemistry techniques were used to determine the distribution of GnRH-immunoreactive (-ir) neurons in the brain of *A. abdominalis*, and to test for sex and seasonal changes in GnRH-ir neurons. Sexually mature adult males (M) and females (F) were collected from four separate time periods: January (pre-spawn, minimal

gonadal indices; N = 6 M, 6 F), April (early-spawn; rising gonadal indices; N = 6 M; 7 F), June (peak-spawn; maximum gonadal indices; N = 6 M, 6 F), and October (post-spawn; minimal gonadal indices; N = 6 M, 6 F) (Helfrich, 1958; Maruska, 2009). Fish were deeply anesthetized (MS-222) and perfused transcardially with 0.9% heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed, postfixed in 4% paraformaldehyde in 0.1 M PB at 4 °C for 12 hrs, rinsed in 0.1 M PB, and cryoprotected overnight in 30% sucrose prior to sectioning.

The immunohistochemical reaction protocol was similar to that described previously (Dewan et al., 2008; Maruska, 2009; Maruska et al., 2007b; Maruska and Tricas, 2007). Briefly, cryoprotected brains were sectioned in the sagittal or transverse plane at 24  $\mu$ m with a cryostat, alternately collected onto chrom-alum-coated slides, surrounded with a hydrophobic barrier (Immedge pen; Vector Laboratories), rinsed with 0.05 M phosphate buffered saline (PBS), blocked with 0.3% Triton-X 100 (Sigma) in PBS with 2% normal goat serum (NGS; Vector Laboratories), and incubated with primary antibody (1:5000 final concentration) at room temperature overnight in a sealed humidified chamber. The GnRH primary antibody 7CR-10 (donated by Dr. Nancy Sherwood, University of Victoria, British Columbia), is a broad-based polyclonal antibody that labels multiple forms of GnRH (see Forlano et al., 2000 for cross-reactivity data). In *A. abdominalis*, this antibody does not label the GnRH1 cells in the preoptic area, but does label both the GnRH3 and GnRH2 cell groups, indicating that all of the GnRH-ir fibers in auditory regions originated from one of these two extra-hypothalamic groups (see controls below). Primary antibody incubation was followed by a PBS wash, incubation with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) with 2% NGS, PBS wash, quenching of endogenous peroxidases with 1.5% hydrogen peroxide in PBS, PBS wash, incubation with avidin-biotin-horseradish peroxidase complex (ABC Elite kit; Vector Laboratories), PBS wash, and reacted with a 3,3'-diaminobenzidine (DAB) chromogen substrate kit with nickel chloride intensification (Vector Laboratories). Slides were then soaked in distilled water, counterstained with 0.1% methyl green, dehydrated in an ethanol series (50–100%), cleared in toluene, and coverslipped (Cytoseal 60, Richard Allen Scientific).

Immunohistochemistry controls included: 1) omission of primary antisera, secondary antisera, ABC solution or DAB all resulted in no staining (negative controls), 2) preabsorption of antisera 7CR-10 with 8  $\mu$ M salmon (Bachem) or chicken II (Peninsula labs) GnRH peptide reduced, but did not eliminate all reaction product, 3) incubation with a seabream specific GnRH antibody (ISPI, donated by Dr. Ishwar Parhar, Nippon Medical School, Tokyo, Japan) labeled only GnRH1 somata in the preoptic area (POA) (but not GnRH2 or GnRH3 cells), and labeled fibers were restricted to the POA, hypothalamus and pituitary, 4) incubation with a salmon GnRH antibody (1668, donated by J. King, University of Cape Town, South Africa) labeled GnRH1 and GnRH3 somata and fibers (but not GnRH2 cells), and 5) incubation with a GnRH2 specific antibody (Adams-100, donated by T. Adams, University of California - Davis) preabsorbed with 8  $\mu$ M sGnRH peptide labeled midbrain GnRH2 somata and fibers only. Brain sections were visualized on a Zeiss Axioskop 2 microscope and images captured with an Optronics® Macrofire digital camera.

### Quantification of GnRH-immunoreactive somata

Unbiased estimates of the number and size of GnRH cells were acquired by quantification in alternate sagittal sections without knowledge of SL, BM, sex, or month collected. Our immunohistochemical experiments with multiple antibodies and preabsorptions showed that GnRH1 cells projected primarily to the pituitary and therefore were not a source of the fiber projections to auditory regions of the brain. Therefore, only GnRH2 and GnRH3 neurons were quantified. To assess whether somata could be counted more than once in adjacent alternate sagittal

sections, ten randomly chosen cell diameters from each group for two fish were measured along the medial-lateral brain axis in transverse sections. The largest cells were GnRH3 neurons and their diameters were smaller than section thickness ( $\bar{x}$  diam. =  $16.3 \pm 4.7 \mu\text{m}$  SD). Thus, there was no duplication of cell counts made on alternate  $24 \mu\text{m}$  sections.

Cell size was determined from digital images of somata photographed at  $400\times$  and cell profile area was calculated with Sigma Scan Pro 5.0 (SPSS, Inc.). For each fish, 5–10 GnRH2 and GnRH3 cells were measured. Fewer than 10 cells were measured only when  $<10$  cells were present, which only occurred in the GnRH2 cell group for a few individuals. To ensure that cell sizes were quantified equally across individuals, cell profile areas were only measured for cells with a visible nucleus and at least one neurite present.

#### Quantification of GnRH-immunoreactive axons within auditory regions

To test for sex and seasonal variations in innervation to the midbrain torus semicircularis, we also quantified the density of GnRH-ir fibers in sagittal sections. Axon varicosities, or swellings, are thought to be release sites for GnRH peptide and therefore quantification of varicose axons should be a proxy indicator of the amount of peptide available for release (Oka and Ichikawa, 1992). Gray-level thresholding with Scion Image software (NIH) was performed in the torus semicircularis of each animal from photomicrographs taken of a  $360 \mu\text{m}^2$  area as described previously (Maruska, 2009). The torus semicircularis is the primary midbrain processing region for auditory and mechanosensory lateral line information and fiber densities were measured in the lateral torus that included both auditory and lateral line regions. Measurements were taken from the same location among individuals based on neuroanatomical landmarks without knowledge of fish sex, size, or season. Photographs were all taken at the same time with identical camera and software settings. The number of pixels covered by immunoreactive fibers was then determined from each of 3 randomly chosen adjacent sections in each animal and expressed as a mean percentage of the  $360 \mu\text{m}^2$  area covered by GnRH-ir fibers. GnRH-ir fibers within the primary hindbrain octaval nuclei were also identified based on a previous study that used neural tracing techniques to delineate these nuclei (Maruska and Tricas, 2009a). However, GnRH-ir fibers were scattered and difficult to quantify in these hindbrain regions, so are only qualitatively described.

#### Neurophysiology experiments

To test how exogenous GnRH application might influence auditory-evoked responses, single neuron extracellular neurophysiology recordings were made *in vivo* from the midbrain TS in 17 fish ( $N=3\text{F}$ ,  $14\text{M}$ ; 1–3 neurons recorded per fish). In cases where multiple neurons were tested in the same fish, recorded neurons were either from opposite sides of the brain, or in different locations within the TS. Recording methods were similar to those described previously (Maruska and Tricas, 2009b), and are only briefly summarized below. Fish were anesthetized in a solution of 0.2% benzocaine, immobilized with pancuronium bromide ( $\sim 0.001 \text{ mg g}^{-1} \text{ BM}$ ), and positioned in an acrylic head holder within the experimental tank. The fish was suspended so that the inner ear was beneath the water surface and 10 cm above an underwater loudspeaker partially buried in gravel on the bottom of a 30 cm diameter Nalgene experimental tank [design similar to (Fay, 1990; McKibben and Bass, 1999; Sisneros and Bass, 2003)]. The tank rested on a vibration isolation table (Technical Manufacturing Corporation) inside a sound isolation chamber (Industrial Acoustics), and all recording and stimulus generation equipment were located outside of the acoustic booth. Fish were continuously ventilated with seawater (water temperature  $23\text{--}25^\circ\text{C}$ ; salinity 34–36 ppt; without anesthetic) that was pumped from the experimental tank through the mouth and over the gills during all experiments. The brain was exposed by dorsal craniotomy and the

cranial cavity filled with Fluorinert fluid (FC-75, 3 M) to enhance clarity, reduce bleeding, and prevent drying.

Extracellular single unit discharges were recorded with carbon fiber carbostar electrodes (400–800 k $\Omega$ ; Kation Scientific, Inc.) that were advanced manually through the overlying tectum and down into the TS. An auditory search stimulus (100 or 200 Hz tone bursts at 124–126 dB<sub>rms</sub> re:  $1 \mu\text{Pa}$ ; 10 ms rise and fall; 20 ms plateau; 8.3 Hz repetition rate) was presented to the animal while the electrode was advanced through the brain, and action potentials were monitored visually (oscilloscope) and acoustically (loudspeaker). Only those units that showed a clear response modulation to the underwater speaker stimulus were recorded and used for neuropeptide testing. Neural action potentials were amplified (500–10,000 $\times$ ) and filtered (100–5000 Hz) (Neurolog system; Digitimer Inc.), and then converted to digital files via a Cambridge Electronics Design (CED) power 1401 system and recorded onto a computer with associated Spike 2 software.

#### Stimulus generation

Acoustic stimuli were generated by the CED digital to analog converter controlled by Spike 2 software, attenuated (CED 3505 programmable attenuator), amplified (Peavey stereo amplifier UMA 352), and played through an underwater loudspeaker (UW-30, Lubbell Labs, frequency response 100 Hz–10 kHz) positioned beneath the fish. Prior to experiments, a calibrated mini-hydrophone (Bruel and Kjaer 8103) was positioned at the location normally occupied by the fish head, and a stimulus routine was run to generate a frequency-stimulus amplitude lookup table. This table was then used by the stimulus generation script to produce fixed stimulus sound pressure levels across test frequencies (80–800 Hz). Stimuli consisted of 100 repetitions of 40 ms ramped tone bursts (10 ms rise and fall; 20 ms plateau; 8.3 Hz repetition rate) at frequencies of 80–400 Hz (80, 100, 200, 300, 400 Hz), which corresponds to the region of best hearing sensitivity in this species (Maruska et al., 2007a; Maruska and Tricas, 2009b). The frequency response of the loudspeaker was also verified with the mini-hydrophone positioned in the tank at the spot normally occupied by the fish inner ear. Relative sound pressure levels (SPL) were calculated for each frequency and intensity by measuring the root mean square (rms) voltage at the position normally occupied by the fish head and converted to SPL in dB<sub>rms</sub> re:  $1 \mu\text{Pa}$ . Sound pressure levels used during these recordings ranged from 80–146 dB<sub>rms</sub> re:  $1 \mu\text{Pa}$ .

To test for GnRH influence on the response of TS neurons to biologically-relevant auditory signals, we also used stimulus playbacks of natural *A. abdominalis* sounds recorded from behaving fish in the wild. These sounds were recorded on a DAT recorder with a hydrophone placed near male nest sites. Two different natural sounds were tested: an aggressive pulse train ( $\sim 800$  ms 3-pulse train), and a courtship-female visit sound ( $\sim 2000$  ms train) (for details on field recordings and sound characteristics see (Maruska et al., 2007a)). These sounds were generated and presented through the underwater speaker as described above for the tone bursts except that 50 repetitions were used for aggressive pulse trains, and 10 repetitions for the courtship sound. Sound pressure levels in dB<sub>rms</sub> re:  $1 \mu\text{Pa}$  were determined with a calibrated hydrophone and calculated as described above. Thresholds for these natural sounds were determined as the lowest intensity to show a peri-stimulus time histogram with increased spike rates that were 2 standard deviations above background correlated in time with the stimulus waveform.

#### Neurophysiology data analysis

Data analyses were performed both on- and off-line with Spike 2 software (CED) as described in Maruska and Tricas (2009b). Following resting (unstimulated) rate recordings, the first stimulus frequency was tested. Thresholds were determined for each test frequency by starting with a supra-threshold intensity followed by decreasing



intensities in 5 dB steps until the neuron no longer responded to the stimulus. Threshold was defined as the lowest intensity to evoke a significant Rayleigh statistic  $Z$  value ( $\geq 4.5$ ;  $Z = R^2 * N$ , where  $R$  is the synchronization coefficient and  $N$  is the total number of spikes sampled), and best frequency (BF) was defined as the frequency with the lowest threshold (see Maruska and Tricas, 2009b for details).

### Neuropeptide application

To test for effects of exogenous GnRH on auditory-evoked response properties of TS neurons, we performed *in vivo* recordings from single neurons in adult male and female damselfish during the protracted spawning season (Feb–June). This period was chosen because acoustic communication is more prevalent during the colonial nesting and courtship season and any GnRH modulation of auditory processing was originally hypothesized to occur at this time. Time constraints precluded neurophysiology recordings during the non-spawning period for comparison. Multi-barrel electrodes (carbostar-4; Kation Scientific, Inc.) were filled with three different test solutions at the start of each experiment: 1) 0.9% saline (control), 2) neuropeptide (sGnRH or cGnRHIII), and 3) neural tracer [fluorescein isothiocyanate-biotinylated dextran amine (FITC-BDA), Vector Laboratories]. Each barrel was backfilled, attached to a picospritzer (Picospritzer II, General Valve Corp.) by individual tubes, and then verified under a dissection scope to release its contents by application of 30 ms pulses at 30–40 psi prior to use. Concentration of neuropeptides were based on those previously used in the literature, were within a physiologically-relevant range (Kinoshita et al., 2007; Saito et al., 2003), and were dissolved in 0.9% saline: sGnRH (50, 100 or 200 nM), and cGnRHIII (50 nM). Prior to neurochemical application, baseline resting discharge data and frequency response properties were recorded as described above. Once baseline measurements were taken, the saline control solution was applied (10–15 × 30 ms pulses at 25–35 psi), followed by presentation of the same test frequencies and conspecific sounds. If the saline application caused any decrease in amplitude of the action potential, the pressure and number of pulses was modified, and the neuron stabilized before re-acquiring pre-ejection values and continuing. Following saline application, GnRH was applied in the same manner and the neuron retested with the same paradigm. Attempts were made to hold the single cell recording as long as possible to determine recovery to baseline activity. Response properties obtained after application of GnRH were compared to those of the saline control and expressed as a percentage of the saline response. Neurons were classified with respect to the change in average spike rate between GnRH and saline application at 5–10 dB above threshold at 100 Hz as: decreased response ( $\geq 20\%$  reduction in spike rate), no change (0–19.9% increase or decrease in spike rate), or increased response ( $> 20\%$  increase in spike rate). To test whether the observed GnRH effects could be blocked by a GnRH receptor antagonist, we also recorded from several neurons that were pre-treated with 2  $\mu$ M antide (Sigma-Aldrich Co.) followed by the 100 nM sGnRH treatment. Multi-barrel electrodes filled with FITC-BDA neuronal tracer (Vector Laboratories) were also used to label recording sites (pressure eject ~5–20 × 30 ms pulses at 20–30 psi) as described in Maruska and Tricas (2009b).

### Statistical analyses

Linear regressions were used to determine the effect of body size on GnRH-ir cell number and size because our data did not meet the assumption of parallel slopes required for analysis of covariance. When separated by sex and season, there were no significant relationships between body size and cell number or cell size in either of the two GnRH-ir cell groups, nor was there a relationship between body size and GnRH-ir fiber density in the TS (linear regressions, all  $p > 0.05$ ). Our data are therefore presented without adjustment for body size. There was also no difference in body size (SL or BM) among sexes or among

sampling periods (2-way ANOVA,  $F_{\text{season}} = 0.55$ ,  $p = 0.65$ ;  $F_{\text{sex}} = 0.41$ ,  $p = 0.53$ ), so any sex or seasonal variations in GnRH-ir cells or fibers are not due to a size sample bias within a sex or sampling period. Differences in the number and size of GnRH-ir somata and fiber densities were determined with a two-way analysis of variance (ANOVA) with sex and season as factors, and subsequent post-hoc Tukey's tests for pairwise comparisons. In some cases, data were normalized by a log or square root transformation. Neurophysiological data were compared with Student's  $t$ -tests, one-way ANOVA, paired  $t$ -tests, or repeated measures ANOVA (RM ANOVA). All statistical analyses were performed with Sigma Plot 11.0 (Systat, Inc.).

## Results

### Distribution of GnRH-ir neurons and GnRH variants

GnRH-ir somata occur in three discrete neuron populations in the Hawaiian sergeant fish brain: GnRH3 cells associated with the terminal nerve ganglia at the junction between the olfactory bulb and rostral telencephalon, GnRH1 cells in the anterior preoptic area, and GnRH2 cells in the midbrain tegmentum. Immunohistochemistry experiments with different primary antibodies and preabsorption controls indicate that *A. abdominalis* likely contains salmon GnRH ([Trp<sup>7</sup>Leu<sup>8</sup>]-GnRH) in the terminal nerve (GnRH3), chicken II GnRH ([His<sup>5</sup>Trp<sup>7</sup>Tyr<sup>8</sup>]-GnRH) in the midbrain tegmentum (GnRH2), and seabream GnRH ([Ser<sup>8</sup>]-GnRH) in the preoptic area (GnRH1). However, we cannot discount the possibility that some neurons in the preoptic area also contain the salmon GnRH form, and future *in situ* hybridization studies are needed to confirm this antibody staining.

GnRH1 somata are located in the anterior parvocellular POA, and show prominent ventral projections in the preoptico-hypophyseal tract that courses along the rostral edge of the hypothalamus towards the pituitary (data not shown). Incubation of brain tissue with anti-sbGnRH (ISPI), which labels only GnRH1 cells, confirmed that the fiber projections from these cells are restricted to the POA, rostral hypothalamus and pituitary. Therefore, GnRH1 likely does not innervate midbrain and hindbrain auditory regions.

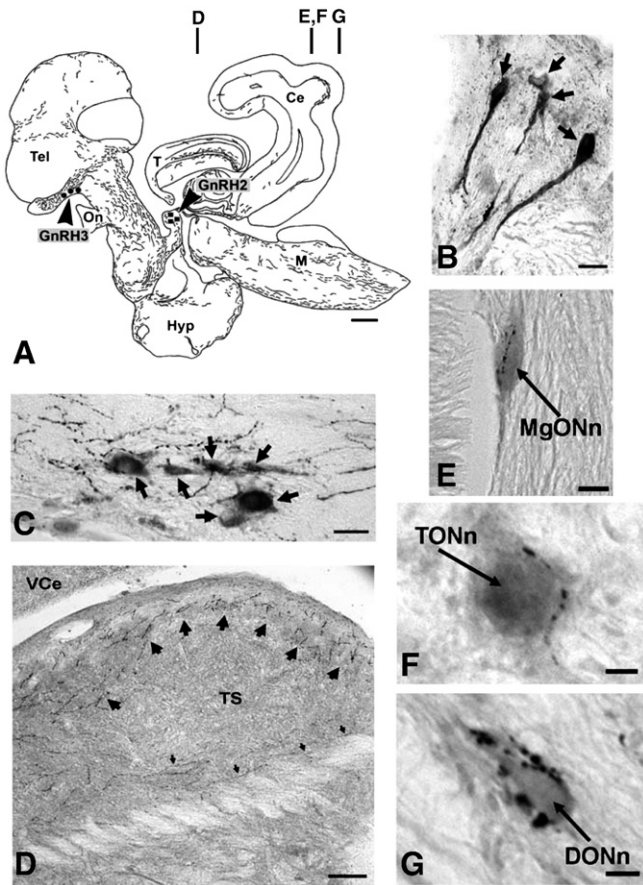
GnRH3 neurons form a discrete cluster of cells located ventrally at the junction of the olfactory bulb and rostral telencephalon (Fig. 1A, C), have their maximum diameter along the rostro-caudal body axis, and show prominent projections to forebrain regions such as the olfactory bulbs, telencephalon, preoptic area, thalamus, and hypothalamus. However, there are also significant projections of GnRH-ir fibers from GnRH3 cells into the tectum, cerebellum, midbrain including the torus semicircularis and medulla.

GnRH2 somata are located along the midline of the midbrain tegmentum below the fourth ventricle, are multi- or monopolar, and show numerous projections primarily to caudal brain regions such as the tegmentum, tectum, torus semicircularis, torus longitudinalis, cerebellum, medulla, and spinal cord (Fig. 1A, B).

### GnRH-ir axons in auditory processing regions

GnRH-ir axons were localized in several auditory processing regions of the midbrain and hindbrain (Fig. 1). In the midbrain, GnRH-ir fibers were found throughout the rostro-caudal and medial-lateral extent of the torus semicircularis in both the auditory (central nucleus of TS; TSc) and mechanosensory (ventrolateral nucleus of TS; TSvl) processing regions. In the auditory TSc, GnRH-ir fibers were prominent within the periventricular layer and along the ventrolateral laminae of the TS (Fig. 1D).

In the hindbrain, there were scattered GnRH-ir axons within all octavolateralis nuclei: medial (MON) and caudal (CON) octavolateralis; descending (DON), anterior (AON), posterior (PON), tangential (TON) and magnocellular (MgON) octaval nuclei (Fig. 1) (for detailed descriptions of these nuclei in *A. abdominalis*, see Maruska and Tricas, 2009a). GnRH-ir fibers in the primary mechanosensory processing MON



**Fig. 1.** Extra-hypothalamic GnRH2 and GnRH3 neurons project to auditory processing regions in the damselfish brain. A) Schematic sagittal section through the Hawaiian sergeant fish brain shows the location of immunoreactive (-ir) GnRH3 (dots) and GnRH2 (squares) somata and their combined fiber projections (black lines). Note that GnRH1 somata and fibers are not shown. B) Photomicrograph of GnRH2-ir somata (arrows) in the midbrain tegmentum. C) Photomicrograph of GnRH3-ir somata (arrows) in the terminal nerve ganglion at the junction between the olfactory bulb and rostral telencephalon. D) Cross section through the midbrain auditory torus semicircularis (TS) shows abundant GnRH-ir fiber innervation along the periventricular layer of the central nucleus of the TS (large arrows), and above the ventrolateral laminae of the TS (small arrows). Medial is to the left. E–G) Putative contacts between varicose GnRH-ir fibers and hindbrain octaval processing neurons in the magnocellular octaval nucleus (MgONn), tangential octaval nucleus (TONn), and descending octaval nucleus (DONn). Approximate locations of cross sections shown in D–G are indicated on the sagittal brain in A. Ce, cerebellum; Hyp, hypothalamus; M, medulla; On, optic nerve; T, tectum; Tel, telencephalon; VCe, valvula cerebelli. Scale bars: 1 mm (A); 20  $\mu$ m (B); 15  $\mu$ m (C, E); 50  $\mu$ m (D); 5  $\mu$ m (F); 10  $\mu$ m (G).

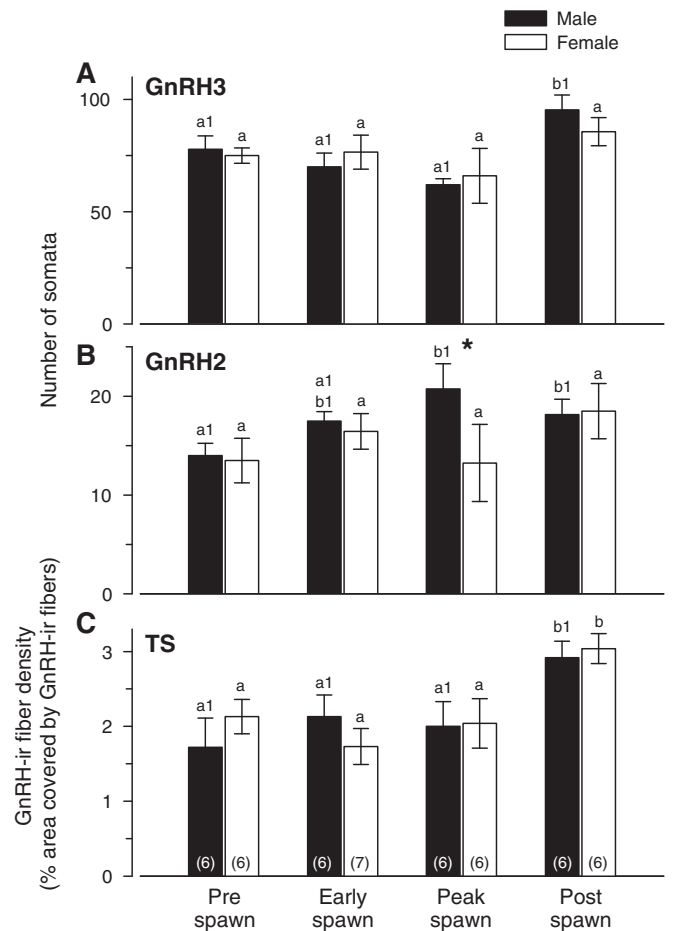
were diffuse and widely distributed but also concentrated in the principal cell region below the cerebellar crest. However, GnRH-ir axons were not observed to surround individual conspicuous somata of the mechanosensory MON. In contrast, varicose GnRH-ir fibers were often in close proximity to large octaval and auditory neuron somata within the AON, DON, TON, and dorsal MgON (Fig. 1E–G). The GnRH-ir axons in these octaval nuclei often surrounded individual cells with putative synaptic contacts between GnRH-containing varicosities and the cell body of the octaval neuron. Varicose GnRH-ir fibers were also found within the octavolateralis efferent nucleus.

*Sex and seasonal variations in GnRH-immunoreactivity*

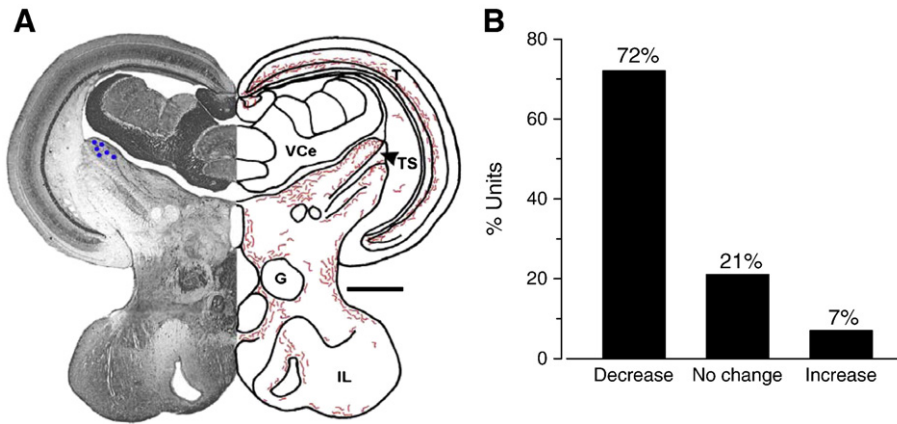
To test whether there were sex or seasonal changes in the two extra-hypothalamic GnRH cell populations (GnRH2 and 3) that innervate auditory processing regions, we used immunohistochemistry to

quantify cell number, cell size, and fiber density within the TS (Fig. 2). There were no sex differences in GnRH3 cell number, but in males there were more GnRH3 cells during the post-spawning period in October compared to all other times (2-way ANOVA,  $F_{\text{season}} = 6.434$ ,  $p = 0.001$ ;  $F_{\text{sex}} = 3.352$ ,  $p = 0.904$ ) (Fig. 2). A single sex difference was found in the number of GnRH2 cells, where males had more cells compared to females during the peak spawning period in June. In males, there were also more GnRH2 cells during peak and post spawn times compared to the pre spawn period, but there were no seasonal differences in females (2-way ANOVA,  $F_{\text{season}} = 3.711$ ,  $p = 0.029$ ;  $F_{\text{sex}} = 3.14$ ,  $p = 0.050$ ) (Fig. 2). There were no sex or seasonal differences in cell size for GnRH2 (2-way ANOVA,  $F_{\text{season}} = 1.22$ ,  $p = 0.313$ ;  $F_{\text{sex}} = 2.13$ ,  $p = 0.152$ ) or GnRH3 ( $F_{\text{season}} = 0.75$ ,  $p = 0.529$ ;  $F_{\text{sex}} = 0.0002$ ,  $p = 0.989$ ) neurons.

In both males and females, GnRH-ir fiber densities in the TS were higher during the post spawn period compared to all other times, but there were no sex differences (2-way ANOVA,  $F_{\text{season}} = 5.07$ ,  $p = 0.004$ ;  $F_{\text{sex}} = 0.04$ ,  $p = 0.852$ ) (Fig. 2). There was no relationship, however, between TS GnRH-ir fiber density and cell number or cell size for either GnRH2 or GnRH3 cell groups (all  $r < 0.36$ ,  $p > 0.05$ ).



**Fig. 2.** The density of GnRH-ir fibers in the torus semicircularis and the number of GnRH2 and GnRH3 somata varied across the seasonal reproductive cycle. A) The number of GnRH3 cells in males was higher during the post spawning period compared to all preceding times, but there were no seasonal changes in females. B) In males, the number of GnRH2 cells was higher during peak and post spawn times compared to pre-spawn, but there were no seasonal changes in females. Males also had more GnRH2 cells compared to females during the peak spawn period only (\*). C) The density of GnRH-ir fibers in the TS was higher during the post spawn period for both males and females, but there were no sex differences. Data are plotted as mean  $\pm$  SE, sample sizes are indicated within each bar on the bottom graph, and different letters indicate differences among time periods within each sex at  $p < 0.05$  (males: a1 or b1; females: a or b).



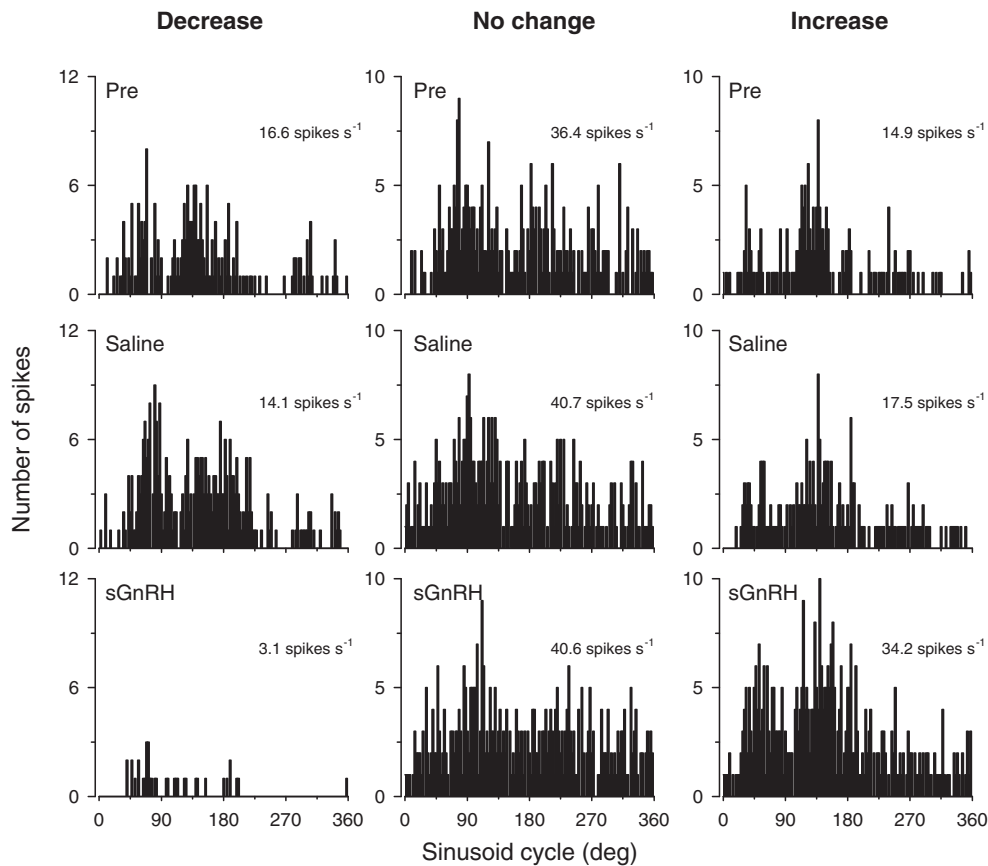
**Fig. 3.** GnRH application decreased auditory-evoked spike rates in the majority of recorded neurons in the torus semicircularis. A) Labeled recording sites and distribution of GnRH-ir fibers in the TS. Left side shows a cross section through the midbrain stained with cresyl violet to illustrate the location of several representative recording sites (blue dots) within the auditory central nucleus of TS. Right side shows the distribution of GnRH-ir axons (red lines) in the same cross section. Scale bar, 1 mm. G, nucleus glomerulosus; IL, inferior lobe of hypothalamus; T, tectum; TS, torus semicircularis; VCe, valvula cerebelli. B) Percentage of TS auditory neurons that showed a decrease (21 neurons), increase (2 neurons), or no change (6 neurons) in auditory-evoked spike rate after 100 nM sGnRH application.

#### Effects of GnRH application on midbrain auditory processing

We tested for effects of exogenous GnRH application on the auditory response properties of individual neurons in the torus semicircularis of the damselfish. Labeled recording sites confirmed that all of the neurons were located within the auditory central nucleus of TS, a region that also shows prominent GnRH-ir fiber innervation in this species (Fig. 3A). The mean resting discharge rate of TS auditory neurons analyzed in this

study was  $8.3 \pm 2.2$  spikes  $s^{-1}$  (range: silent–24 spikes  $s^{-1}$ ) and a high percentage (72%) of those neurons that received the full saline control and GnRH treatments were silent in the absence of auditory stimuli. Of the neurons that were active without acoustic stimulation, the resting discharge rates between pre-injection, saline, and GnRH application did not differ (RM ANOVA,  $F = 3.318$ ,  $p = 0.067$ ).

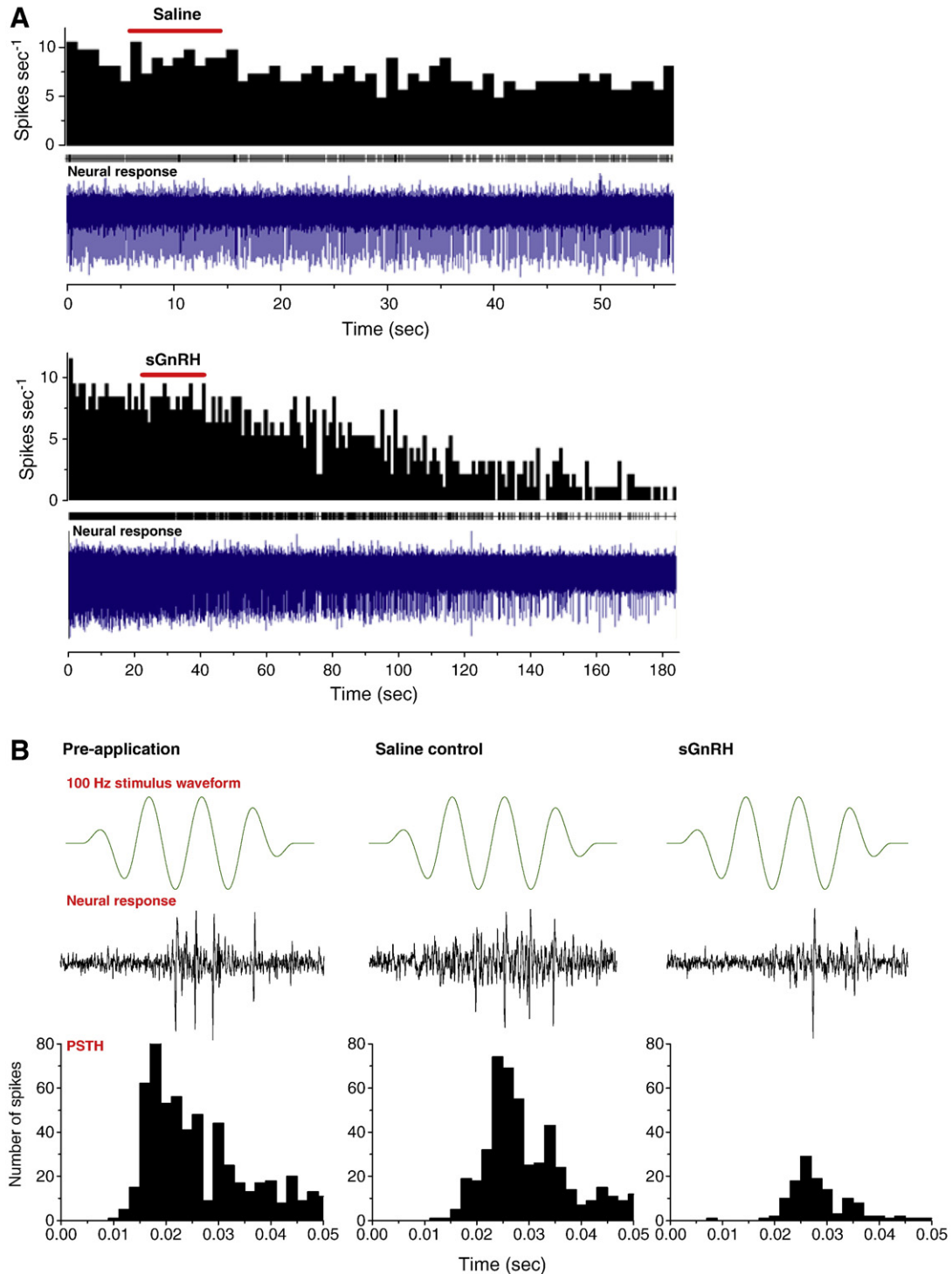
The majority of recorded neurons (21/29) showed a decrease in tone burst auditory-evoked spike rate after sGnRH application, while



**Fig. 4.** Representative examples of auditory-evoked responses by neurons in the torus semicircularis of the damselfish brain. Period histograms show the response of three individual neurons that responded with a decrease, no change or increase in spike rate to a 100 Hz tone burst stimulus (100 repetitions at 119 dB). Period histogram is shown for each neuron before treatment (pre; top row), after saline (Saline; middle row) and after 100 nM sGnRH application (sGnRH; bottom row). Auditory-evoked spike rates (spikes  $s^{-1}$ ) for each neuron in response to each treatment are shown. Resting rates for each neuron were: silent (decrease neuron), 5.5 spikes  $s^{-1}$  (no change neuron), and 0.73 spikes  $s^{-1}$  (increase neuron).

~21% (6/29) showed no change, and only two neurons increased their spike rates (2/29) (Figs. 3B, 4). Application of control saline solution did not alter auditory-evoked spike rates in TS neurons, while sGnRH application caused a distinct decrease in spike rate in the majority of neurons without changing the amplitude of the action potentials (Figs. 4, 5). The GnRH-induced change in spike rate was observed

at ~20 s–5 min after peptide application, and persisted for longer than 20 min in most cases without returning to pre-injection levels. Our longest recording was a neuron that was held stable for ~40 min after GnRH application and testing, a time also not sufficient for recovery. After sGnRH application and testing were complete, attempts were also made to recover several neurons by re-application



**Fig. 5.** Decrease in evoked spike rate after application of sGnRH in representative auditory neurons in the torus semicircularis. A) Example of saline and sGnRH application to a single auditory neuron in the TS. Bottom graph in each panel shows the neural response to the continuous 40 ms 100 Hz tone burst stimulus, middle shows the event spikes, and top shows the average neuron spike rate. Note the decrease in spike rate over time after application of 100 nM sGnRH. B) Representative examples of a single auditory neuron response to an individual tone burst. Top trace shows the 40 ms 100 Hz tone burst used as a stimulus. Middle trace shows a representative resultant neural response from a single neuron before treatment (pre-application), after application of saline control solution, and after application of 100 nM sGnRH. Peri-stimulus time histograms (PSTH) show the neural response binned over 100 successive stimulus presentations. Note the overall decrease in neural discharge rate after sGnRH application compared to both the pre-application and saline control.



of saline solution, but there was no change in evoked spike rate after this treatment compared to the preceding sGnRH application (paired *t*-test,  $t = 0.480$ ,  $p = 0.678$ ,  $N = 3$ ).

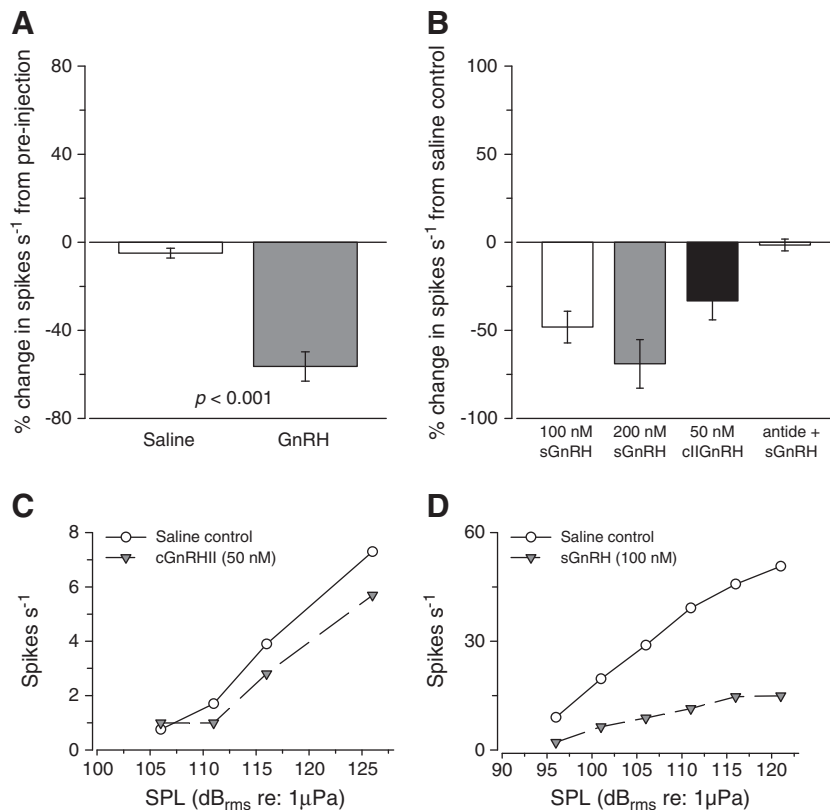
There was no difference in evoked spike rate of neurons between pre-injection and application of control saline solution (determined for 100 Hz tone bursts; paired *t*-test,  $t = 1.534$ ,  $p = 0.136$ ). Further, the decreased auditory-evoked spike rate observed after sGnRH application ( $56.4 \pm 0.11\%$ ; range, 20–99%) was much greater than that observed after control saline application ( $4.9 \pm 0.46\%$ ; range, 1.5–12%) (paired *t*-test,  $t = 7.29$ ,  $p < 0.001$ ) (Fig. 6A). To eliminate the possibility that the observed decrease in spike rate after sGnRH application was due to the sequential application of saline followed by sGnRH, we tested some auditory neurons ( $N = 4$ ) without saline application (e.g., only pre-injection and post-sGnRH application were tested). These neurons showed a similar percent decrease in spike rate ( $46.8 \pm 0.23\%$ ) compared to those that were sequentially treated with saline followed by sGnRH (*t*-test,  $p = 0.502$ ), which indicates the GnRH-induced decrease was likely not due to sequential testing.

We also tested whether both sGnRH and cGnRHIII variants affected auditory-evoked responses because our immunohistochemistry experiments indicated that both peptides likely innervate the TS. Application of both sGnRH and cGnRHIII variants caused a decrease in discharge rate compared to saline controls (Fig. 6B). The greatest decrease in spike rate was observed from the 200 nM sGnRH concentration, but it did not differ statistically from that of 100 nM sGnRH or 50 nM cGnRHIII (ANOVA,  $F = 1.89$ ,  $p = 0.182$ ). We also recorded responses of 3 neurons to application of 50 nM sGnRH, one of which showed no change, and the remaining 2 showed a 40–45% decrease in spike rate, but the low sample size precludes inclusion of this concentration in the statistical analysis. Auditory neurons pre-treated with the GnRH receptor antagonist antide

showed no change in spike rate after subsequent sGnRH application, which differed from neurons that received sGnRH application without antide (*t*-test,  $t = -3.16$ ,  $p = 0.008$ ). Both sGnRH and cGnRHIII peptides also showed a decrease in neural gain (i.e., sensitivity; spikes  $s^{-1}$  per SPL) (Fig. 6C, D). Response rate-intensity curves naturally vary widely in dynamic range and slope among individual neurons in the TS (Maruska and Tricas, 2009b), thus the low sample size for neurons treated with cGnRHIII precludes any comment on whether neural gain curves differ between GnRH forms, or are due to other factors such as neuron type or circuitry differences.

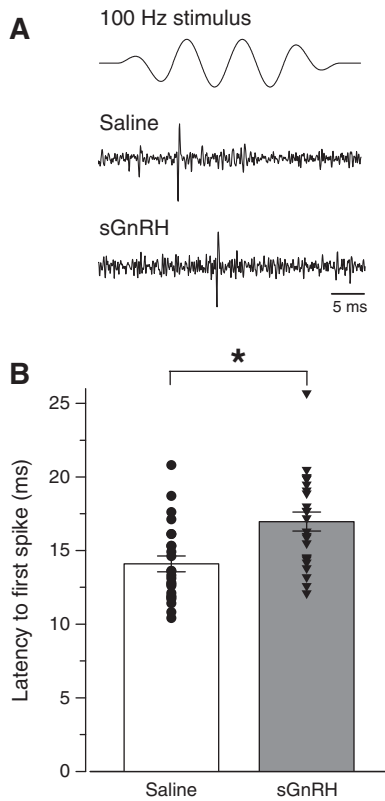
To examine whether sGnRH affected response latency in neurons that showed a decrease in spike rate, we also measured the latency to the first spike (time between stimulus onset and the first action potential of the response) in response to a 100 Hz tone burst stimulus (20 successive presentations at 10 dB above threshold) (Fig. 7). Application of 100 nM sGnRH caused an increase ( $\sim 2$ –5 ms) in overall auditory-evoked spike latency compared to saline controls (paired *t*-test,  $t = -9.37$ ,  $p < 0.001$ ). This change in latency was due to either a shift in the latency of the spike in neurons that responded with a single spike per tone burst, as shown for the neuron in Fig. 7A, or an absence of earlier spikes when a neuron responded to each tone burst with multiple spikes (e.g., neuron in Fig. 5B). There was no change in spike latency between pre-injection and saline controls (paired *t*-test,  $t = -1.57$ ,  $p = 0.131$ ).

To test whether sGnRH also influenced auditory thresholds, we compared auditory tuning curves for the same individual neurons after saline control treatment and after sGnRH application (Fig. 8). These results showed that sGnRH also caused a 5–10 dB increase in auditory thresholds (decrease in sensitivity) compared to saline controls, but only in the region of best hearing sensitivity from 80t



**Fig. 6.** GnRH inhibited auditory-evoked responses to tone bursts compared to saline controls in single auditory neurons in the torus semicircularis. A) GnRH application produced a greater mean percent decrease in spike rate from pre-injection values than did the saline application ( $p < 0.001$ ). Data are plotted as mean  $\pm$  SD ( $N = 34$ ). B) Effects of sGnRH concentration (100 nM and 200 nM), GnRH peptide variant (sGnRH and cGnRHIII), and antide (GnRH receptor antagonist) treatment on spike rates. Data are plotted as mean  $\pm$  SD ( $N = 21$ , 100 nM sGnRH; 5, 200 nM sGnRH; 4, 50 nM cGnRHIII; 4, 2  $\mu$ M antide + 100 nM sGnRH). C) Change in neural gain of an example auditory neuron in response to a 100 Hz tone burst after control saline application (open circles; solid line) and after 50 nM cGnRHIII application (inverted triangles; dashed line). D) Change in neural gain of an example auditory neuron in response to a 100 Hz tone burst after control saline application (open circles; solid line) and after 100 nM sGnRH application (inverted triangles; dashed line).





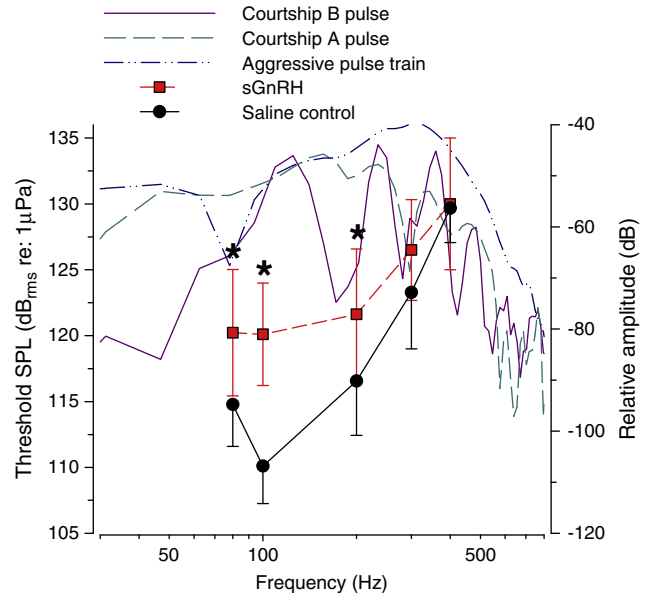
**Fig. 7.** GnRH increased the latency to first auditory-evoked spike in neurons of the torus semicircularis. A) Representative example of the response of a single neuron to a 100 Hz tone burst stimulus after saline and after 100 nM sGnRH application. The latency to the action potential is shifted by ~5 ms after sGnRH application. B) The average latency to first spike was greater after sGnRH application compared to the paired saline controls for the population of neurons that showed a decreased spike rate response. Bars show mean  $\pm$  SD, and symbols show latencies for individual TS neurons after saline (circles) and sGnRH (inverted triangles) treatment ( $N=24$  neurons). Asterisk indicates statistical difference at  $p < 0.001$ .

200 Hz (paired  $t$ -tests, 80 Hz,  $t = -2.27$ ,  $p = 0.053$ ; 100 Hz,  $t = -3.46$ ,  $p = 0.009$ ; 200 Hz,  $t = -3.06$ ,  $p = 0.018$ ). This is the same spectral region predominant in the natural sounds produced by this species (Fig. 8). In contrast, there was no difference in threshold at 300–400 Hz ( $p > 0.05$ ), but some neurons (14–30%) also no longer responded to these higher frequencies after sGnRH application (i.e., had a decreased bandwidth), even at the highest sound intensity tested.

GnRH also caused a decrease in spike rate in response to playbacks of complex conspecific sounds (Fig. 9). Both sGnRH and cGnRHII caused a decrease in spike rates in neurons responding to playbacks of aggressive ( $63.9 \pm 11.3\%$ ) and courtship sounds ( $70.1 \pm 8.6\%$ ) (RM ANOVA,  $p < 0.05$ ). While the overall spike rate was reduced after GnRH treatment, the temporal pulsed information of the natural sounds was still encoded by the neurons (Fig. 9A). There was also a behavioral context-dependent increase in auditory thresholds in response to sGnRH application (Fig. 9B). Auditory thresholds were higher for both the aggressive and courtship sounds after sGnRH application compared to saline controls, but this increase was greater for the courtship sound compared to both the aggressive pulse train and the 100 Hz tone burst (RM ANOVA,  $p = 0.009$ ).

## Discussion

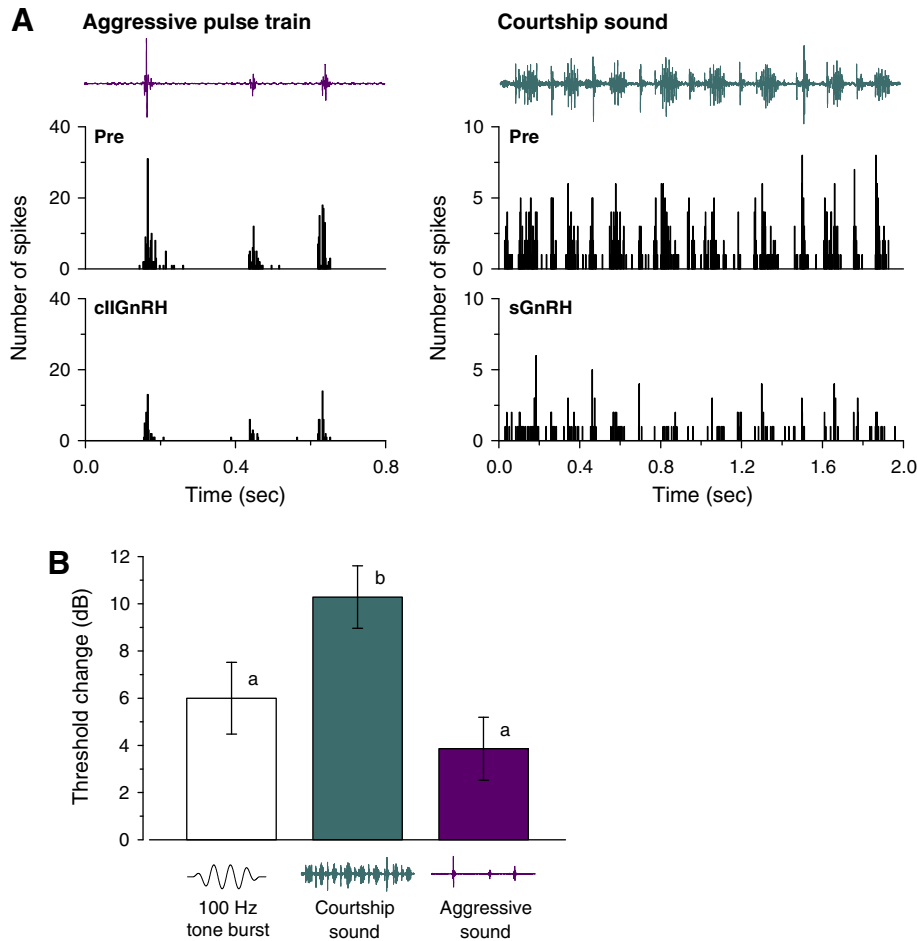
This study provides both neuroanatomical and neurophysiological evidence that GnRH can modulate auditory processing in the fish brain. To our knowledge, we show for the first time in any vertebrate that GnRH primarily causes an inhibitory action on auditory neurons



**Fig. 8.** GnRH caused an increase in auditory-evoked thresholds in TS neurons in the region of best hearing sensitivity. Auditory tuning curves for TS neurons in response to tone burst stimuli from 80 to 400 Hz after application of saline control solution (black circles, solid line) and 100 nM sGnRH (red squares, dashed line). Tuning curve data are plotted as mean  $\pm$  SD and referenced to the left axis. For clarity, error bars for the saline plot are shown in the negative direction only. Asterisks indicate a difference in mean threshold level at 80–200 Hz between saline and sGnRH treatments (paired  $t$ -test,  $p < 0.05$ ) ( $N = 10$ ). Power spectra (128 point FFT, Hanning window) of courtship (A and B pulse types; see Maruska et al., 2007b for details) and aggressive pulse train sounds used as playbacks are overlaid and plotted as relative amplitude in dB (right axis).

in the midbrain. This inhibitory effect was also dependent on frequency and the type of sound stimulus, and has important implications for auditory sensory processing, communication, and reproductive behavior of this species that are discussed below.

Our results show that both GnRH2 and GnRH3 can influence auditory processing in the TS of the damselfish. Immunohistochemistry experiments with multiple antibodies and preabsorption controls showed that both sGnRH-ir (GnRH3) and cGnRHII-ir (GnRH2) fibers are located within the TS, which is similar to other fishes (Yamamoto et al., 1995) and consistent with their hypothesized roles as neuromodulators. Our *in vivo* neurophysiology recordings also showed that exogenous application of both sGnRH and cGnRHII influenced the auditory-evoked responses of TS neurons in a similar manner, although our recordings with cGnRHII application were limited. Comparable physiological effects of different GnRH forms were also observed in the tectum (Kinoshita et al., 2007) and preoptic area (Saito et al., 2003) of the rainbow trout *Oncorhynchus mykiss*, however, cGnRHII often has greater effects on physiological and behavioral measures in fishes compared to GnRH3 (Kinoshita et al., 2007; Matsuda et al., 2008; Saito et al., 2003). While the distribution of GnRH receptors within auditory regions of the damselfish brain are not known, GnRH receptors are found within the TS of other perciform fishes in the same regions that receive GnRH-ir axons (Chen and Fernald, 2006; Soga et al., 2005). Multiple GnRH receptor subtypes within a single species are common in vertebrates, but they often show different distribution patterns in the brain that suggest functional specialization (Klausen et al., 2002). However, each receptor subtype has similar affinities for all GnRH ligands within a species (Flanagan et al., 2007; Lethimonier et al., 2004; Robison et al., 2001), which indicates complex regulatory mechanisms for ligand–receptor interactions within specific cell types. It is therefore possible that the differences in auditory-evoked response types found in the damselfish (e.g., decrease, no change, or increase in spike rate) are



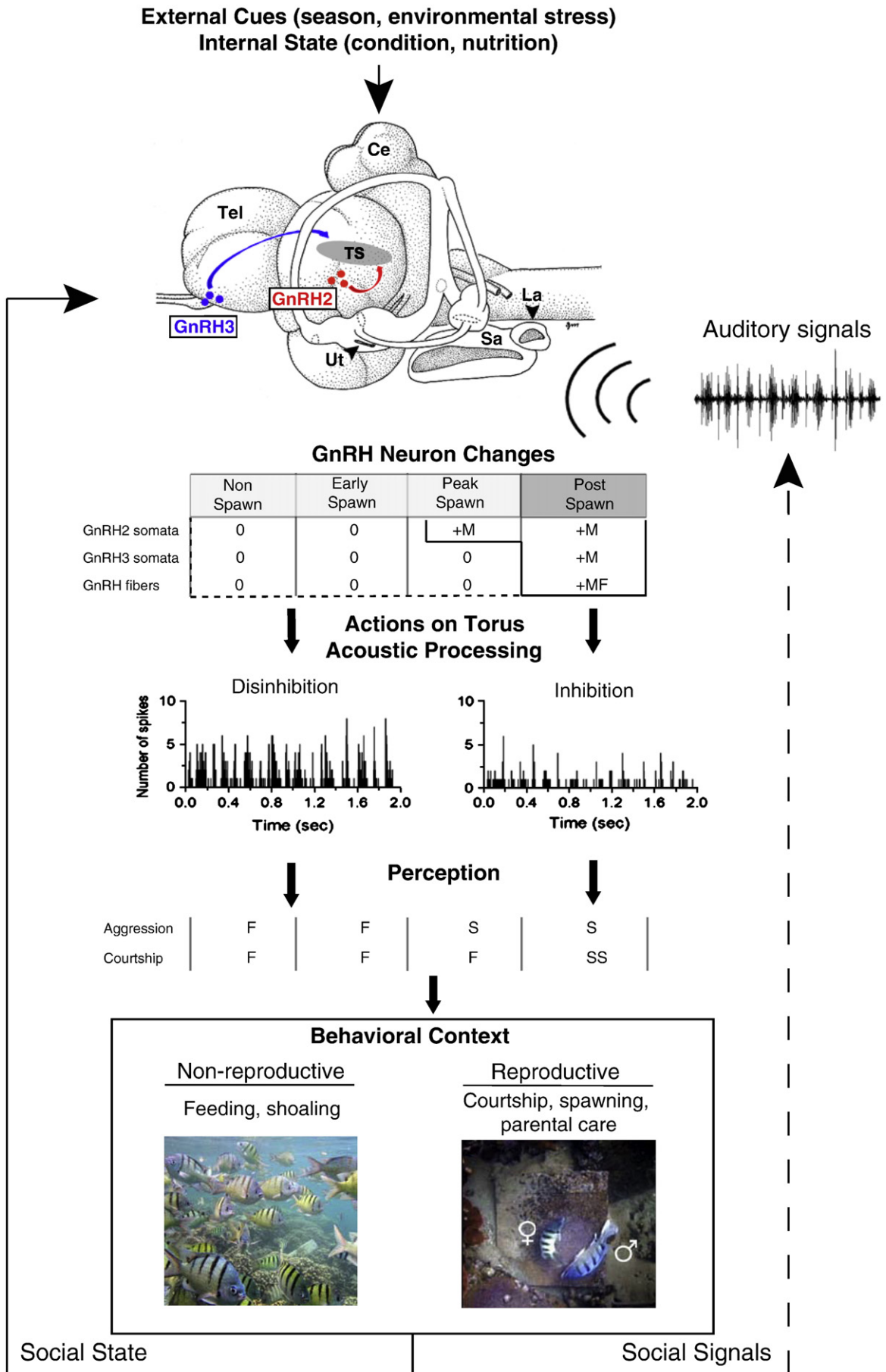
**Fig. 9.** GnRH caused a context-specific decrease in spike rate and an increase in auditory thresholds in torus semicircularis neurons. A) Representative examples of the response of TS neurons to playbacks of aggressive pulse train and courtship sounds to illustrate the decrease in spike rate after GnRH application in the absence of preceding saline application. Peri-stimulus time histograms before (pre) and after GnRH application (aggressive: 50 nM cGnRHII, 50 stimulus repetitions; courtship: 100 nM sGnRH, 10 stimulus repetitions) are shown for two different TS neurons. Top trace in each panel shows the stimulus waveform. B) GnRH application had the greatest effect on thresholds of the courtship-female visit sound, which was higher than both the 100 Hz tone burst and aggressive pulse train sound. Data are plotted as the mean  $\pm$  SE dB change in threshold level between control saline and 100 nM sGnRH values ( $N=7$ ). Insets show the stimulus waveform of each sound. Different letters indicate statistical differences at  $p<0.05$ .

mediated by neurons that express different receptor subtypes, have different transduction pathways, differ in neural connections (e.g., ascending vs. descending circuits, unimodal vs bimodal neurons, etc.), or that the GnRH effects occur pre- or post-synaptic to the recorded target neuron.

GnRH innervation to the TS in the Hawaiian sergeant fish was elevated during the minimum spawning period in both males and females, but was comparatively lower during the protracted spring-summer spawning season. In light of these seasonal variations and the primarily inhibitory effects of GnRH in the TS, several interpretations are possible. First, the reduced GnRH innervation during the protracted spawning season may function to improve detection of reproductively-relevant acoustic cues during this period (i.e., via disinhibition, or removal of the inhibitory effects of GnRH). Male Hawaiian sergeant fish spawn with females and defend egg nests in colonies during the spring-summer and auditory signals for both

courtship and aggression by both sexes are important for colony synchronization. Inhibitory effects of GnRH are also observed in isolated rat uterine muscles (Medeiros et al., 1988), midbrain central gray neurons in rats (Chan et al., 1985), bullfrog sympathetic neurons (Bley and Tsien, 1990; Boland and Bean, 1993; Elmslie et al., 1990), rat cervical ganglion neurons, (Lewis and Ikeda, 1997), immortalized GnRH GT1 neurons (Van Goor et al., 1999), and olfactory neurons in the mudpuppy and axolotl (Eisthen et al., 2000; Park and Eisthen, 2003; Zhang and Delay, 2007). Many of these inhibitory effects of GnRH are mediated by regulation of specific ion channels (e.g., calcium, sodium, or potassium channels), which can then have effects on membrane potential, frequency and duration of spikes, action potential shape, and neurotransmitter release. Additional techniques such as whole cell voltage clamp recordings, coupled with pharmacological blockers, are needed to examine the effects of GnRH on the function of different channel types in fish midbrain auditory neurons.

**Fig. 10.** A proposed model for GnRH modulation of auditory processing and acoustic-mediated behaviors in the Hawaiian sergeant damselfish. External cues from the environment (e.g. seasonal changes in photoperiod, temperature or environmental stressors), social cues from conspecifics (e.g. social state), and internal physiological cues (e.g. body condition, hormonal or nutritional state) (thin solid arrow at top and left) influence the synthesis and release of GnRH2 (red dots, arrow) and GnRH3 (blue dots, arrow) in the brain. GnRH neuron somata change is most evident in males (+M) during the post spawn period, and higher densities of GnRH fibers occur in the auditory TS of both males and females (+MF) during this time that affects the response properties of midbrain auditory neurons. When a fish hears an auditory signal such as a conspecific courtship sound (waveform at right; thin dashed arrow at right), release of GnRH in the TS can inhibit (right PSTH) or disinhibit/not change (left PSTH) midbrain auditory neuron responses. This modulatory action on midbrain sensory processing is sent to higher forebrain processing regions that facilitate or suppress perception of courtship and aggressive social signals, and ultimately influence context-dependent reproductive and non-reproductive behaviors. *Abbreviations:* Ce, cerebellum; F, facilitate; GnRH2, gonadotropin-releasing hormone 2—midbrain group; GnRH3, gonadotropin-releasing hormone 3—terminal nerve group; La, lagena; S, suppress; Sa, saccule; SS, super-suppress; Tel, telencephalon; TS, torus semicircularis; Ut, utricle.



An alternative explanation for the seasonal variation in GnRH-immunoreactivity is that the decrease in GnRH-ir fiber density in the TS during the protracted spawning period is due to an increase in the release of peptide from varicosities during this time, while the increased fiber density in the post spawn period results from reduced release and storage of peptide. This mechanism was suggested to explain the socially-mediated context-dependent decrease in GnRH-ir fiber densities (GnRH1 and 2) in the brain during the non-breeding season in female house sparrows *Passer domesticus* (Stevenson et al., 2008). In that study, GnRH synthesis was interpreted to be relatively stable because the number of GnRH-ir cells did not change, thus the decreased fiber density was explained as an increase in release. In our study, however, the elevated fiber densities in the TS during the post spawn period were coincident with greater numbers of GnRH cells, suggesting increased synthesis. Coupled with the physiological data that shows primarily inhibitory effects of GnRH on auditory-evoked responses, it seems more likely that GnRH release to the TS is higher during the post spawning period. This may serve to reduce the relative importance of reproductive auditory cues (which includes both courtship and aggressive sounds used during nest defense) to help prevent fish from engaging in costly reproductive activities during less optimal times (see also *Functional consequences* below). A related situation exists in the European starling *Sturnus vulgaris* where higher brain dopamine synthesis during the non-breeding season is associated with an inhibition of female behavioral responses towards male courtship songs (Riters et al., 2007). Future studies are needed, however, to test how seasonal changes in the release of GnRH within auditory regions of the fish brain might influence behaviorally-relevant neural circuits.

The inhibitory effects of GnRH in the damselfish TS were most prominent within the region of best hearing sensitivity, and varied with the type of sound stimulus. The greatest increase in GnRH-induced auditory threshold was observed when the complex courtship sound was used as a stimulus. This pulsed low-intensity sound is produced by the male as he leads a receptive female into the nest for spawning, and is likely intended for close-range communication (Maruska et al., 2007a). The 5- to 10-dB increase in threshold after GnRH treatment may also translate into decreased auditory-detection distance for acoustic communication. It is therefore possible that GnRH changes the TS neuron population response to species-specific vocalizations and acts as a gain control mechanism to alter detectability of certain acoustic signals, as shown for the neuromodulator serotonin in the auditory inferior colliculus (homolog of TS) of mammals (Hurley and Pollak, 2005a; Hurley et al., 2002). GnRH may also alter the integration of auditory and other sensory signals by differentially gating inputs from different sources, an idea supported by the fact that some TS neurons respond to bimodal and multimodal sensory cues in other fishes (e.g., visual, mechanosensory, auditory) (Schellart, 1983).

Another important finding in our study was that the GnRH-induced depression of auditory-evoked spike rates was long-lasting (>20–40 min). Similar persistent (30–60 min) inhibitory effects were observed for GnRH on oxytocin- and acetylcholine-induced contractibility of rat uterine muscles (Medeiros et al., 1988), and GnRH effects persisted for >20 min even after washout in periventricular neurons of the tectum in the rainbow trout *O. mykiss* (Kinoshita et al., 2007). Authors of the latter study suggested that the long-lasting facilitation caused by GnRH might enhance tectal sensitivity for retinal inputs, thus improving the detectability of visual signals used during the trout's homing migration from open water into natal streams. While the mechanisms are unknown, the long-lasting GnRH inhibition in the Hawaiian sergeant fish may also serve to alter acoustic detection of biologically-relevant sounds on a seasonal, diurnal, or lunar schedule to coordinate behaviors in this colonial species.

Application of exogenous GnRH also increased the response latency to auditory-evoked stimuli in the damselfish TS. In the auditory system, response latency encodes many features of the stimulus including frequency, intensity, temporal characteristics, and identity (Hurley and

Pollak, 2005b; Kossel and Vater, 1989; Rullen et al., 2005). Neuromodulators such as serotonin and noradrenaline were also shown to influence first spike latency in the auditory inferior colliculus of bats, and these latency changes may function to alter parallel stimulus-encoding strategies and gate the integration of different inputs that carry information about related sounds (Hurley and Pollak, 2005b; Kossel and Vater, 1989). In other studies that examined the effects of neuromodulators on neural response properties, increases in first spike latency often occur in conjunction with decreases in spike rate and vice versa (Hurley and Pollak, 2005b). This was also evident in the effects of GnRH on auditory neurons in the damselfish TS, and may be a conserved feature among peptide neuromodulators. The effects of neuromodulators on sensory systems, however, are complex and depend on many factors including receptor type and the role of the target neuron within the neural circuitry (Hurley et al., 2004), which requires further study.

#### *Functional consequences*

Our results show that the processing of midbrain auditory information can be influenced by GnRH, but how might information transfer in the auditory system be modified in the Hawaiian sergeant damselfish? Hawaiian sergeant fish are colonial, and during the protracted spawning season, males prepare a benthic spawning substrate, aggressively defend this territory, and perform visual and acoustic courtship displays to attract a female for spawning. After spawning, males remain to guard and care for the developing embryos until they hatch, but continue to court and spawn with other females so that they often guard multiple clutches simultaneously. Fig. 10 presents one proposed model for how GnRH might influence auditory processing and ultimately influence behaviors in this species. The abundance and release of GnRH2 and GnRH3 in the TS is likely influenced by external environmental cues (e.g., photoperiod, temperature, stressors) and social cues (e.g., social interactions, dominance status), as well as by the animals' internal physiological state (e.g., body condition, hormonal and nutritional state) (Abe and Oka, 2007; Kanda et al., 2010; Ramakrishnan and Wayne, 2009). The release and binding of GnRH to receptors in auditory regions can therefore be regulated on several temporal time scales (e.g., long-term seasonal, diurnal, or lunar, and short-term social interactions such as dominance, territory defense, courtship, or parental care). Mediated by these external and internal cues, changes in local GnRH release in the TS can then disinhibit, not change, or inhibit the response of auditory neurons depending on the type and context of the sound stimulus. This modulation can then affect the processing of auditory signals in the midbrain, influence input to higher integration and perception centers in the forebrain, and ultimately influence both non-reproductive (e.g., shoaling, feeding, predator avoidance) and reproductive (e.g., courtship, spawning, territory defense, parental care) behaviors. The fact that the inhibitory GnRH effects were long-lasting suggests that changes in GnRH release may alter the relative contribution of auditory inputs according to long-term temporal cues such as seasonal, diurnal, or lunar cycles. GnRH is also likely to alter many aspects of auditory stimulus coding because of its widespread distribution in hindbrain, midbrain, and forebrain auditory processing regions, which requires future study. In addition to the auditory-GnRH1 endocrine circuit involved in reproduction in other vocal vertebrates (Burmeister and Wilczynski, 2005; Cheng et al., 1998; Maney et al., 2007), our results indicate that a reciprocal relationship may exist between the GnRH2 and 3 cell groups and the auditory system. Future studies are needed to confirm whether there are sex or seasonal changes in GnRH-induced auditory effects and to test how GnRH-mediated auditory modulation influences natural behaviors. Our results provide insight into the functions of extra-hypothalamic GnRH systems and establish a framework to examine GnRH modulation of auditory function in other vertebrate taxa.



## Acknowledgments

We thank Drs. Nancy Sherwood, Ishwar Parhar, Tom Adams and Judy King for their generous gifts of GnRH antisera, Geoff Horseman of CED for his help with Spike 2 scripts, the reviewers for insightful comments to improve the manuscript, and Sigma-Xi Grants-in-Aid of Research and an NSF Doctoral Dissertation Improvement Grant (DDIG IBN 04-08197) for funding. This is contribution number 1428 from Hawai'i Institute of Marine Biology.

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