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Gonadotropin releasing hormone (GnRH) and gonadotropin inhibitory hormone (GnIH) in the songbird hippocampus: Regional and sex differences in adult zebra finches

Nicolette McGuire[b], Jennifer K. Ferris[a], Lutgarde Arckens[c], George E. Bentley[b], Kiran K. Soma[a,d,e,f,*]

[a] Department of Psychology, University of British Columbia, Vancouver, BC, Canada
[b] Department of Integrative Biology, Helen Wills Neuroscience Institute, University of California-Berkeley, Berkeley, CA, USA
[c] Laboratory of Neuroplasticity and Neuroproteomics, University of Leuven, Leuven, Belgium
[d] Department of Zoology, University of British Columbia, Vancouver, BC, Canada
[e] Graduate Program in Neuroscience, University of British Columbia, Vancouver, BC, Canada
[f] Brain Research Centre, University of British Columbia, Vancouver, BC, Canada

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ABSTRACT

Hypothalamic gonadotropin releasing hormone (GnRH) and gonadotropin inhibitory hormone (GnIH) are vital to reproduction in all vertebrates. These neuropeptides are also present outside of the hypothalamus, but the roles of extra-hypothalamic GnRH and GnIH remain enigmatic and widely underappreciated. We used immunohistochemistry and PCR to examine whether multiple forms of GnRH (chicken GnRH-I (GnRH1), chicken GnRH-II (GnRH2) and lamprey GnRH-III (GnRH4)) and GnIH are present in the hippocampus (Hp) of adult zebra finches (*Taeniopygia guttata*). Using immunohistochemistry, we provide evidence that GnRH1, GnRH2 and GnRH4 are present in hippocampal cell bodies and/or fibers and that GnIH is present in hippocampal fibers only. There are regional differences in hippocampal GnRH immunoreactivity, and these vary across the different forms of GnRH. There are also sex differences in hippocampal GnRH immunoreactivity, with generally more GnRH1 and GnRH2 in the female Hp. In addition, we used PCR to examine the presence of GnRH1 mRNA and GnIH mRNA in micropunches of Hp. PCR and subsequent product sequencing demonstrated the presence of GnRH1 mRNA and the absence of GnIH mRNA in the Hp, consistent with the pattern of immunohistochemical results. To our knowledge, this is the first study in any species to systematically examine multiple forms of GnRH in the Hp or to quantify sex or regional differences in hippocampal GnRH. Moreover, this is the first demonstration of GnIH in the avian Hp. These data shed light on an important issue: the sites of action and possible functions of GnRH and GnIH outside of the HPG axis.

1. Introduction

The decapeptide gonadotropin releasing hormone (GnRH) is essential to reproduction in all vertebrates [53]. Traditionally, GnRH is best known as a neuropeptide that is produced in the hypothalamus and secreted into the hypothalamo-hypophyseal portal system [18,19,29,30,51,52], particularly mammalian GnRH-I or chicken GnRH-I (GnRH1) [53]. GnRH1 binds to GnRH receptors in the anterior pituitary and stimulates secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) [24]. LH and FSH, in turn, bind to receptors in the gonads to stimulate sex steroid production and gamete maturation.

Many, if not all, vertebrates express multiple forms of GnRH in the brain, including brain regions that are not directly related to pituitary function [12,34,37,39]. In fact, chicken GnRH-II (GnRH2) is the most widely expressed form of GnRH among vertebrates [53]. GnRH1 and other forms of GnRH might regulate reproductive behavior and learning and memory [16] by acting as neurotransmitters or neuromodulators outside of the HPG axis [25,35]. In mammals, strong evidence exists for GnRH1 and GnRH2 in the brain [36]. Birds appear to express GnRH1, GnRH2, and lamprey GnRH-III (GnRH4) in the brain [5,57].

Gonadotropin inhibitory hormone (GnIH) is another neuropeptide that is produced in the hypothalamus and regulates vertebrate reproduction [28,40]. GnIH is secreted by hypothalamic neurons into the hypothalamo-hypophyseal portal system, binds to GnIH receptors on pituitary gonadotropes, and directly inhibits the synthesis and release of LH and FSH [10,32,64]. Like GnRH, GnIH also acts as a neuromodulator within the brain [13,72]. GnIH+

* Corresponding author at: Department of Psychology, University of British Columbia, 2136 West Mall, Vancouver, BC V6T 1Z4, Canada. Tel.: +1 6048275820.
E-mail address: ksoma@psych.ubc.ca (K.K. Soma).

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fibers project to the diencephalon and midbrain of quail [65]; to the septum, preoptic area, amygdala, bed nucleus of the stria terminalis, midbrain, and hindbrain of hamsters [28]; and to the arcuate nucleus, preoptic area, and midbrain of rhesus macaques [62].

The hippocampus (Hp), a brain region that is well-known for its role in learning and memory, has a high density of GnRH receptors in rodents [1,21]. Interestingly, GnRH treatment *in vitro* increases *de novo* estradiol production by rat hippocampal cells [43]. However, there is little evidence for GnRH1+ cell bodies or fibers in the mammalian Hp [33,69,71], and in rats, GnRH1+ neurons in the preoptic area do not project to the Hp [14]. Thus, it is unclear how GnRH reaches GnRH receptors in the Hp. GnRH1 might reach hippocampal GnRH receptors *via* the cerebrospinal fluid. Alternatively, other forms of GnRH (*e.g.*, GnRH2, GnRH4) might be present in the Hp, but most previous studies focused on GnRH1. In one study, GnRH2+ cells are present in the musk shrew Hp [45]. In birds, there are some data to suggest that the Hp expresses both GnRH2 and GnRH4 [5,56], but this issue has not been systematically examined. Further, the presence of GnIH in the Hp has not been widely examined. One study in rats used an antibody raised against a portion of the GnIH (RFRP) precursor peptide and detected fibers in the CA2/CA3 pyramidal layer [46], and another study detected GnIH mRNA in pig Hp [31].

The avian Hp lacks the distinct anatomical subdivisions of the mammalian Hp (*i.e.*, dentate gyrus, Ammon's horn, hilar region). However, avian and mammalian hippocampi display similarities in the distributions of neuropeptides and neurotransmitters, which suggests that there may be functionally similar subdivisions [15,27]. The similarities with the mammalian Hp include: connectivity, topological relationship to lateral ventricles, morphological and neuronal cell types, and neuropeptide distribution (substance P, neuropeptide Y, somatostatin, vasoactive intestinal peptide) [11,15,27]. Further, avian and mammalian hippocampi both express aromatase, the enzyme critical for estrogen synthesis.

There have been a few attempts to identify GnRH in the Hp. However, no study has systematically examined multiple forms of GnRH in the Hp, and only two previous studies have examined GnIH in the Hp. Furthermore, no study has quantified regional or sex differences in hippocampal GnRH or GnIH expression, which may yield important clues about the regulation and functions these neuropeptides in the Hp. Given the ability of GnRH to stimulate *de novo* estradiol synthesis in the rat Hp, we examined GnRH immunoreactivity (-ir) in hippocampal regions that express high levels of neuronal aromatase [42,49,50] and that are affected by estradiol or aromatase inhibitor treatments [49].

Here, we examined the distributions of GnRH1, GnRH2, GnRH4 and GnIH in the avian Hp using immunohistochemistry. We used the zebra finch (*Taeniopygia guttata*), the best-studied songbird model, and compared males and females. We also examined GnRH1 and GnIH mRNA in micropunches from the Hp using PCR.

2. Materials and methods

2.1. Subjects

Samples were collected from adult male and female zebra finches (*T. guttata*) that were group-housed in same-sex cages in a colony at the University of British Columbia. Birds were held under a 16L:8D light:dark cycle (lights on at 8:00 am), with *ad libitum* food and water. Protocols were approved by the UBC Animal Care Committee and conformed to the regulations established by the Canadian Council on Animal Care.

One set of subjects was used for immunohistochemistry. Adult males ($n = 8$) and adult females ($n = 8$) were captured (within 30 s of disturbance) and euthanized by rapid decapitation (within 2 min of disturbance). Collection occurred from 9:45 to 10:45 am on February 28, 2011 and April 24, 2011 (4 males and 4 females on each day). Brains were removed and immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) overnight at room temperature. Brains were washed in PBS for 1 h, then cryoprotected in 30% sucrose until they sank. Brains were then rapidly frozen on dry ice and stored at −80 °C until use.

A second set of subjects was used for PCR. Adult males ($n = 4$) and adult females ($n = 4$) were captured and euthanized by rapid decapitation (as above) between 9:45 and 10:45 am on August 23, 2011. Brains were fresh frozen immediately on dry ice and stored at −80 °C until use.

Gonadal measurements were taken from both sets of subjects to confirm reproductive condition. Ovary length (6.04 ± 0.49 mm) and largest follicle diameter (2.03 ± 0.44 mm) were measured in females ($n = 12$). Testes length and width were measured in males ($n = 12$), and then testes volume was calculated as an ovoid sphere [41] (left testis: 72.29 ± 9.16 mm^3, right testis: 56.71 ± 12.62 mm^3). These data verified that subjects were in reproductive condition [41].

Table 1
Primary antibodies used and the peptides they label in the zebra finch brain.

Antibody	GnRH1	GnRH2	GnRH4	GnIH	Reference
HU60	+	+	–	–	[8,67]
HU11B	–	–	–	–	[66]
Anti-cLHRH-II	–	+	–	–	[57,68]
Anti-lGnRH-III	+	+	+	–	[5,58]
PAC 123/124	–	–	–	+	[59,70]

Note: HU11B was raised against mammalian GnRH-I but did not produce any specific staining in the zebra finch brain under the present conditions.

2.2. Immunohistochemistry for GnRH forms and GnIH

Brain coronal sections were cut in 6 series of alternate sections at 40 μm on a cryostat. Sections were placed in antifreeze and stored at −20 °C prior to use. Immunohistochemistry was performed in two rounds, with four males and four females in each round. Brain sections were carefully transferred from antifreeze to 0.1 M Tris-buffered saline (TBS) in mesh well plates and washed in TBS (4×5 min). This wash step followed each step below, except where noted. Tissue was incubated for 15 min in 0.5% hydrogen peroxide in TBS to quench endogenous peroxidase activity, and then incubated in 10% normal goat serum (NGS; Vector Labs, Burlington, ON, S-1000) in TBS with 0.2% Triton X-100 (TBS-T) for 2 h. Immediately after blocking, sections were incubated in primary antibody (see below) or preadsorbed primary antibody (see below) in 2% NGS in TBS-T overnight on an orbital shaker at room temperature.

The primary antibodies used were: HU60 (1:2000), HU11B (1:2000), anti-chicken GnRH-II (1:2000), anti-lamprey GnRH-III (1:6400), and PAC 123/124 (1:5000). See below and Table 1 for details about each primary antibody. Optimal primary antibody concentrations and incubation time were empirically determined.

HU60 is a rabbit polyclonal antibody raised against mammalian GnRH-I that recognizes chicken GnRH-I, chicken GnRH-II, and salmon GnRH, with little to no cross-reactivity with GnRH fragments, atrial naturetic factor, growth hormone-releasing hormone, oxytocin, somatostatin, thyrotrophin-releasing hormone, and vasoactive intestinal peptide [67]. HU60 has been successfully validated for identifying GnRH1 and GnRH2 in birds [8,41], where cell bodies expressing these two forms of GnRH can be distinguished based on anatomical location (*i.e.*, GnRH1 in the preoptic area and GnRH2 in the midbrain). HU60 was a generous gift from Dr. Henryk Urbanski (Oregon Health Sciences University).

HU11B is a mouse monoclonal antibody with a high specificity for mammalian GnRH-I [66]. HU11B was raised against synthetic GnRH1 bound to bovine thyroglobulin. It binds GnRH1 and GnRH1 fragments, but not atrial naturetic factor, growth hormone-releasing hormone, oxytocin, somatostatin, thyrotrophin-releasing hormone, or vasoactive intestinal peptide [66]. HU11B was also a generous gift from Dr. Henryk Urbanski.

Anti-chicken LHRH-II is rabbit polyclonal antibody that is highly specific for GnRH2. Specificity was originally determined by solid and liquid phase adsorption with GnRH2 and a specific peptide fragment of GnRH2 (Lys-His-Gly-Trp-Tyr-Pro-Gly-NH2). Antigen spot tests showed that anti-cLHRH-II did not cross-react with GnRH1, vasopressin, vasotocin, oxytocin, isotocin, mesotocin, neurophysin I and II, somatostatin-releasing inhibiting factor, thyrotropin releasing hormone, corticotropin releasing factor, growth hormone releasing factor, adrenocorticotropic hormone, prolactin, ovine and bovine growth hormone, galanin, gastrin, cholecystokinin, substance P, neurotensin, vasoactive intestinal polypeptide, neuropeptide Y, secretin, neurokinin, angiotensin, glucagon, or β-endorphin [68]. Anti-cLHRH-II was further validated by dot blot (specific reaction with GnRH2, but not with GnRH1 or GnRH4) [57].

Anti-lamprey GnRH-III is a rabbit polyclonal antibody raised against GnRH4 [58]. Preadsorption with GnRH4, but not GnRH1, abolishes immunoreactivity [39]. This antibody may cross-react with somatostatin [55], so we preadsorbed this antibody with GnRH4 and somatostatin peptides as controls. Anti-lGnRH-III has been used in songbird studies [5] and was a generous gift from Dr. Stacia Sower (University of New Hampshire).

PAC 123/124 was raised against synthetic white-crowned sparrow GnIH peptide [59]. It is highly specific for GnIH (confirmed by preadsorption studies) and also binds GnIH-related peptides-1 and -2 [59]. It has been validated using preadsorption controls in birds, hamsters, rats, rhesus macaques and humans [26,28,62,63,70].

For preadsorption controls, primary antibodies were incubated with peptides on an orbital shaker at room temperature for 2 h, prior to incubation with sections. Preadsorbing peptides used were: LH-RH human (Anaspec, Fremont, CA, catalog #20781), LHRH II (Bachem, Torrance, CA, catalog #H-4278), LHRH lamprey III (Bachem, Torrance, CA, catalog #H-4258), and somatostatin 14 (Anaspec, Fremont, CA, catalog #24277).

After primary antibody incubation, tissue was washed in TBS (9 × 5 min) and then incubated for 2 h in secondary antibody (1:250 in TBS-T). The secondary antibody for HU60, anti-cLHRH-II, anti-lGnRH-III and PAC 123/124 was biotinylated goat anti-rabbit IgG (Vector Labs, Burlington, ON, catalog #BA-1000). The secondary antibody for HU11B was biotinylated goat anti-mouse IgG (Vector Labs, Burlington, ON, catalog #BA-9200). Excess secondary antibody was washed out (6 × 5 min in TBS; this wash step follows all subsequent steps) before incubation with the Vectastain Elite ABC kit (Vector Labs, Burlington, ON, catalog #PK-6100) according to manufacturer's instructions. After 1 h, the sections were washed and then incubated for 2 min with DAB (Vector Labs, Burlington, ON, catalog #SK-4100). Tissue was float-mounted onto gelatin-coated slides and coverslipped with Permount (Fisher Scientific).

2.3. Immunohistochemistry quantification

Immunoreactivity was quantified in two regions of the Hp: the dorsomedial cluster (DmC) and the ventrolateral band (VlB) [48,50]. The DmC and VlB of the zebra finch Hp are sites of high aromatase expression in neurons [42,49,50] and are affected by estradiol or aromatase inhibitor treatments [49]. These two hippocampal regions were sampled at three rostro-caudal levels: (1) rostral Hp, at the level of the tractus septopalliomesencephalicus (TrSM); (2) central Hp, at the level of the commisura anterior (CoA); and (3) caudal Hp, at the level of the cerebellum (Cb) [38].

The slides were coded, and the researcher was blind to the sex of the subjects. Images were taken bilaterally from each brain section on a Zeiss microscope using the 10× objective at the maximum resolution (2048 × 1536). Images were digitized in Image J. Background gray level measurements were taken from each image from four locations in the Hp not containing immunoreactive elements. A quantification threshold was set such that only immunoreactivity that was at least 1.5× the average background gray level for that image was counted.

Quantification of immunoreactivity was restricted to regions of interest (ROI) superimposed onto the acquired image in Image J. ROI shapes and sizes were designed to encompass 2/3 of the DmC (100 μm diameter circle) and VlB (300 × 150 μm rectangle) [50]. ROIs were always positioned within the boundaries of the Hp. After applying the quantification threshold to each image, the pixel area was calculated using Image J. In addition, when immunoreactive cell bodies were present in the Hp (for HU60 and anti-lGnRH-III only), the number of immunoreactive cells was counted manually. Cells on the edge of the ROI or cells without a visible unlabeled nucleus were excluded. Mean pixel area and cell counts were used for statistical analyses.

2.4. PCR for GnRH1 and GnIH mRNA

A thick (300 μm) section was taken at the level of the TrSM from each fresh-frozen brain using a cryostat. Using a 15 gauge sample corer (Fine Science Tools, Foster City, CA, cat #18035-01), 1 mm diameter punches were taken bilaterally from each thick section. Each punch therefore included both the DmC and VlB regions, from the rostral to the central Hp. Subsequently, 1 mm diameter punches were taken bilaterally from the preoptic area-hypothalamus of the same thick section. To avoid contamination, the sample corer was cleaned with 100% ethanol, nuclease-free water, and Eliminase between each punch. Tissue punches were stored in DNase/RNase-free microcentrifuge tubes at −80 °C until use.

Detection of cDNA encoding zebra finch GnRH1 followed the method of [60]. Briefly, total RNA was isolated using TRIZOL (Invitrogen, Carlsbad, CA, USA) and mRNA was isolated with an mRNA isolation kit (Roche Diagnostics, Mannheim, Germany). The mRNA was reverse-transcribed using oligo(dT)15 primer (Promega, cat #C1101) and M-MLV reverse transcriptase (Invitrogen, cat #28025). A negative control was prepared by substituting nuclease-free water for mRNA. cDNA for GnRH1 was amplified using the forward primer (5′-TCTCTCAGGCAGCAGGATGGA-3′) and the reverse primer (5′-CTTTCTTCTGCCTTGTTCCTCC-3′), which targets nucleotides 39–324 of FJ407188 [61]. Zebra finch β-actin was amplified as a positive control. PCR amplifications were performed using ExTaq polymerase (Takara Bio Inc., Shiga, Japan, cat #RR001A). Another negative control was prepared by substituting nuclease-free water for cDNA.

Detection of GnIH precursor mRNA was also attempted using this method, using the primers 5′-GGAAGAAAAGCAGAGGAGTCTC-3′ and 5′-TGGAGATCTCCCAAGCCTGT-3′ which are based on Gambel's white crowned sparrow (*Zonotrichia leucophrys gambelii*) GnIH precursor cDNA (GenBank accession #AB128164).

A sample of GnRH1 PCR products with bands of the appropriate size were ligated into pGEM-T Easy vectors (Promega, Madison, WI, cat #A1360). Ligated vectors then transformed JM109 competent cells (Promega, Madison, WI, cat #L2001). The inserts of positive clones were amplified using universal M13 primers. DNA was sequenced at the UC Berkeley DNA sequencing facility (Berkeley, CA) and compared to FJ407188 in BLAST. Bands of the appropriate size for GnIH precursor were detected in the hypothalamus, but not

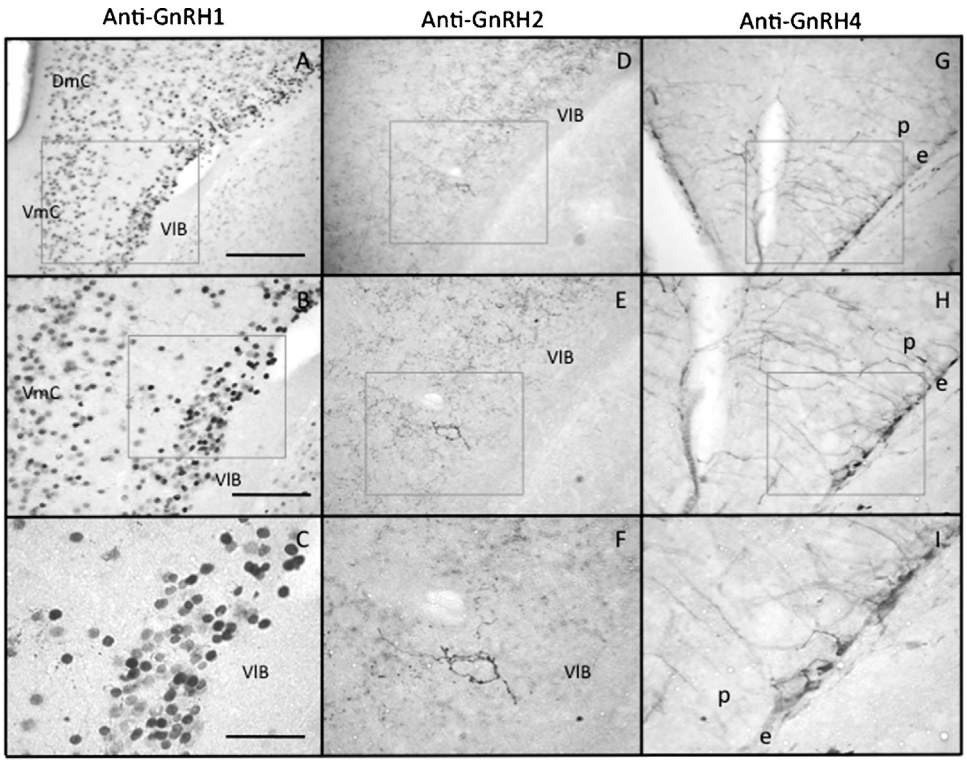

Fig. 1. GnRH forms in the zebra finch hippocampus (Hp). (A–C) GnRH1/2-ir cells and fibers are present in the ventrolateral band (VlB), ventromedial cluster (VmC), and dorsomedial cluster (DmC). (D–F) GnRH2-ir fibers are present in the VlB. (G–I) GnRH4-ir cells are present in the ependyma (e), with fibers projecting into the parenchyma (p). (B, E, H and C, F, I) are higher magnifications of respective boxed areas in (A, D, G and B, E, H). A, D, G scale bar = 100 μm. B, E, H scale bar = 50 μm. C, F, I scale bar = 25 μm.

in the Hp. Therefore no hippocampal PCR products for GnIH were cloned.

2.5. Statistics

Data were analyzed using GraphPad Prism software. In both the DmC and VlB, the effects of Sex and Region (rostro-caudal level) on immunoreactivity (mean pixel area) were analyzed using 2-way ANOVAs with Bonferroni *post hoc* tests. In addition, the effects of Sex and Region on the number of immunoreactive cells (for the HU60 and anti-lGnRH-III antibodies) were analyzed using 2-way ANOVAs with Bonferroni *post hoc* tests.

3. Results

3.1. HU60 immunoreactivity

HU60-ir was clearly visible in the zebra finch Hp (Figs. 1A–C and 2A, and Supplementary Fig. 1A–C). This immunoreactivity was observed from the rostral Hp to the caudal Hp. As seen in previous studies, HU60-ir cell bodies and fibers were observed in the preoptic area (POA, Fig. 2B) and midbrain (Fig. 2C), consistent with HU60 recognizing GnRH1 and GnRH2. Within the Hp, prominent immunoreactivity was observed in cells and fibers in the ventrolateral band (VlB) along the lateral ventricle. Immunoreactivity was also observed in the dorsomedial cluster (DmC) adjacent to the midline, in the ventromedial cluster (VmC), and in the dorsolateral Hp adjacent to the hyperpallium apicale (HA) (Figs. 1A–C and 2A and Supplementary Fig. 1A–C). Preadsorption with GnRH1 and GnRH2 peptides together abolished immunoreactivity in the Hp (Fig. 2D). In contrast, preadsorption with GnRH4 did not reduce labeling in the Hp.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2013.05.007.

The HU60 antibody labels both cell bodies and fibers, so we examined the effects of Sex and Region (*i.e.*, rostro-caudal level) on mean pixel area in the DmC and VlB. In the DmC, there was a significant effect of Sex on mean pixel area (female > male; $F = 14.75$, df = 1, $p = 0.0004$) (Fig. 3A). There was no significant effect of Region and no significant Sex × Region interaction (Region: $F = 2.48$, df = 2, $p = 0.09$; Interaction: $F = 2.63$, df = 2, $p = 0.08$). With regard to the number of immunoreactive cells in the DmC, there was a significant Sex × Region interaction ($F = 3.91$, df = 2, $p = 0.027$) (Fig. 3B). *Post hoc* tests revealed that males had more immunoreactive cells than females in the rostral Hp ($p < 0.05$).

In the VlB, there was a significant effect of Region on mean pixel area (central Hp < rostral Hp = caudal Hp; $F = 3.05$, df = 2, $p = 0.05$) (Fig. 3C). There was no significant effect of Sex and no significant Sex × Region interaction (Sex: $F = 0.27$, df = 1, $p = 0.61$; Interaction: $F = 1.89$, df = 2, $p = 0.15$). With regard to the number of immunoreactive cells in the VlB, there was a significant effect of Sex (female > male; $F = 26.74$, df = 1, $p < 0.0001$) and a significant effect of Region (central Hp > rostral Hp = caudal Hp; $F = 7.00$, df = 2, $p = 0.001$) (Fig. 3D). The Sex × Region interaction was not significant ($F = 2.29$, df = 2, $p = 0.10$).

3.2. HU11B immunoreactivity

The HU11B antibody did not produce any specific staining in the zebra finch brain, at least under the present conditions. No HU11B-ir was observed in the Hp, and labeled cell bodies were not observed in the POA, median eminence, or midbrain (data not shown).

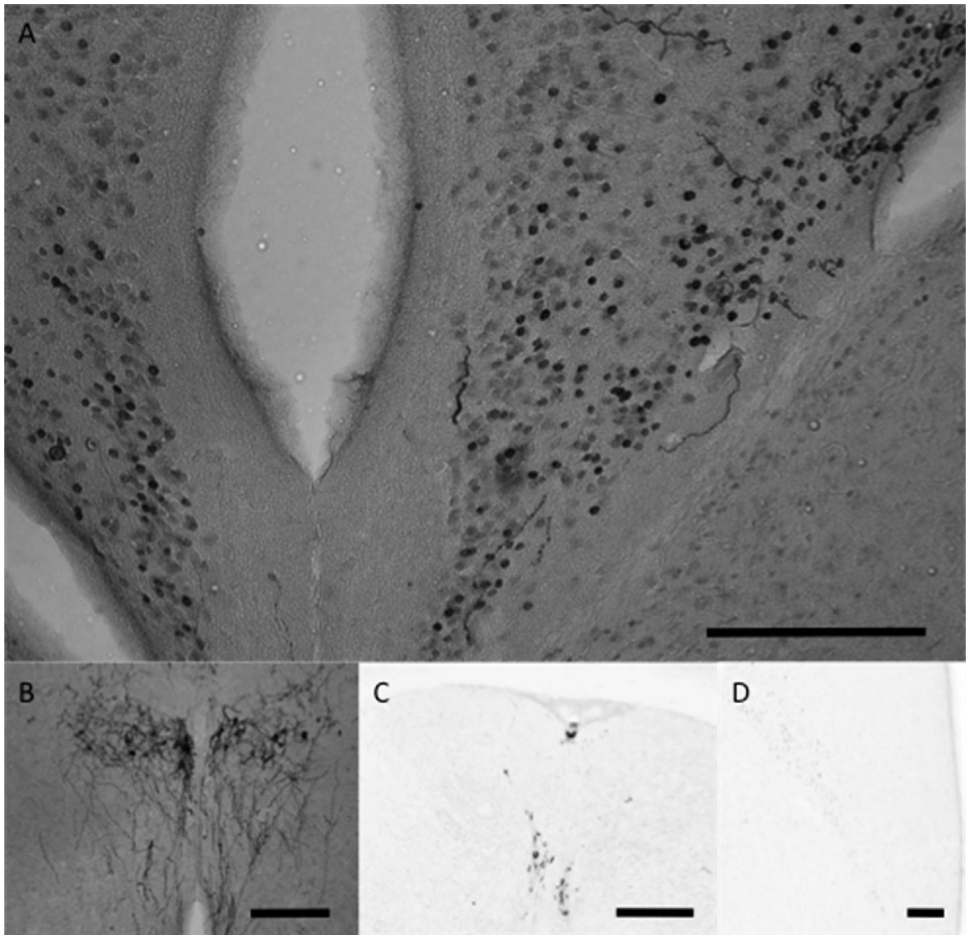

Fig. 2. HU60 immunoreactivity was present in the (A) Hp, (B) POA, and (C) midbrain. (D) Preadsorption of the primary antibody with GnRH1 and GnRH2 peptides abolished immunoreactivity in the Hp. Scale bar = 100 μm.

3.3. Anti-cLHRH-II immunoreactivity

Anti-cLHRH-II recognizes GnRH2 in quail and house sparrow brains [56,68]. GnRH2-ir was clearly present in fibers in the zebra finch Hp (Figs. 1D–F and 4A), from the rostral Hp to the caudal Hp. GnRH2+ fibers were also present in the POA and midbrain (Fig. 4B–C), while GnRH2+ cell bodies were visible only in the midbrain (Fig. 4C), confirming that the anti-cLHRH-II antibody is specific to GnRH2. Within the Hp, GnRH2-ir was most visible in the VlB and in the dorsolateral Hp adjacent to the HA (Figs. 1D and 4A). Preadsorption with GnRH2 peptide completely abolished immunoreactivity in the Hp (Fig. 4D). Preadsorption with GnRH1 or GnRH4 peptides did not affect immunoreactivity in the Hp (data not shown).

We examined the effects of Sex and Region on mean pixel area of GnRH2-ir. In the DmC, there was a significant effect of Sex (female > male; $F = 8.23$, df = 1, $p = 0.006$) and a significant effect of Region (caudal Hp > rostral Hp = central Hp; $F = 3.65$, df = 2, $p = 0.034$) (Fig. 5A). The Sex × Region interaction was not significant ($F = 0.329$, df = 2, $p = 0.72$).

In the VlB, there was a significant effect of Sex but not Region (Sex: $F = 10.68$, df = 1, $p = 0.0013$; Region: $F = 2.63$, df = 2, $p = 0.07$) (Fig. 5B). There was a significant Sex × Region interaction ($F = 3.33$, df = 2, $p = 0.038$). *Post hoc* tests revealed that females had more immunoreactivity than males in the rostral Hp and central Hp ($p < 0.05$).

3.4. Anti-lGnRH-III immunoreactivity

Anti-lGnRH-III was raised against GnRH4. In the Hp, immunoreactivity was observed from the rostral Hp to the caudal Hp (Figs. 1G–I and 6A). Consistent with prior observations in white-crowned sparrows (*Zonotrichia leucophrys*), immunoreactive cell bodies and fibers were observed in the caudomedial nidopallium (NCM) and parolfactory lobe (LPO) [5] (data not shown). Immunoreactive cell bodies were also observed in the POA and midbrain (Fig. 6B–C). Within the Hp, immunoreactive cell bodies were interspersed within or near the ventricular zone (VZ) along the lateral ventricles (Figs. 1I and 6A). These cells projected fibers into the parenchyma of the Hp (Fig. 1G–I). At the intersection of the Hp, HA and lamina pallio-subpallialis (LPS), cell bodies emanated dorsally into the parenchyma and fibers projected dorsally. A few long fibers were observed in the medial Hp. Preadsorption with GnRH4 greatly reduced immunoreactivity in the Hp (Fig. 6D) but not in the POA or midbrain. Preadsorption with somatostatin did not reduce immunoreactivity in the Hp (data not shown).

The anti-lGnRH-III antibody labels both cell bodies and fibers containing GnRH4 in both the DmC and VIB. We examined the effects of Sex and Region on mean pixel area and the number of immunoreactive cells. In the DmC, there were no significant effects of Sex or Region on mean pixel area (Sex: $F = 2.21$, df = 1, $p = 0.15$; Region: $F = 0.026$, df = 2, $p = 0.52$) (Fig. 7A) or on the number of immunoreactive cells (Sex: $F = 0.29$, df = 1, $p = 0.63$; Region: $F = 0.24$,

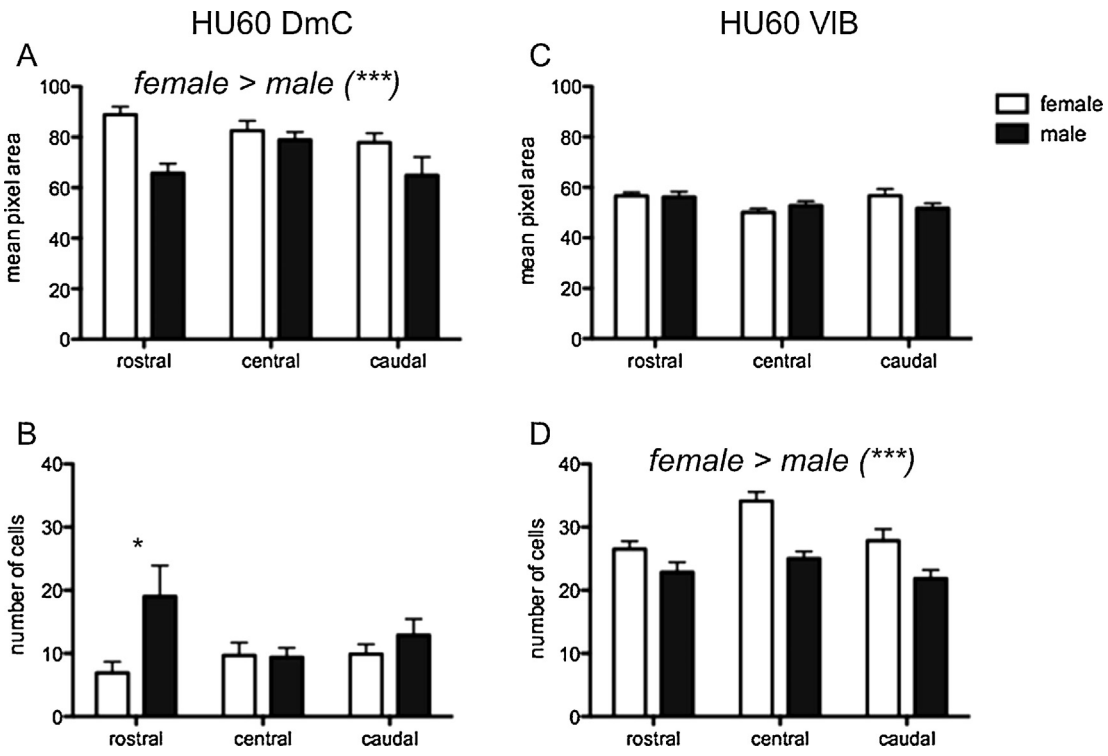

Fig. 3. Sex and regional differences in HU60-ir in the zebra finch Hp. (A) For total HU60-ir in the DmC, there was a significant main effect of Sex (female > male). (B) For HU60-ir cell number in the DmC, there was a significant sex difference in the rostral DmC only (male > female). (C) For total HU60-ir in the VlB, there was a significant main effect of Region (rostro-caudal level). (D) For HU60-ir cell number in the VlB, there was a significant main effect of Sex (female > male) and a significant main effect of Region (central Hp > rostral Hp = caudal Hp). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

df = 2, $p = 0.75$) (Fig. 7B). There were no significant Sex × Region interactions ($p > 0.05$ in both cases).

In the VlB, there were no significant effects of Sex or Region on mean pixel area (Sex: $F < 0.01$, df = 1, $p = 0.99$; Region: $F = 0.24$, df = 2, $p = 0.79$) and no Sex × Region interaction (Fig. 7C). With regard to the number of immunoreactive cells in the VlB, there was a significant effect of Region (caudal Hp < central Hp = rostral Hp; $F = 3.26$, df = 2, $p = 0.041$) but no effect of Sex ($F = 1.05$, df = 1, $p = 0.31$) and no Sex × Region interaction ($F = 0.12$, df = 2, $p = 0.89$) (Fig. 7D).

3.5. PAC 123/124 (GnIH) immunoreactivity

PAC 123/124 recognizes GnIH in songbirds [59]. In the Hp, GnIH+ fibers were present from the rostral Hp to the caudal Hp (Fig. 8A–D). As expected, GnIH+ cell bodies were only present in the hypothalamic paraventricular nucleus (PVN) (Fig. 8E), while GnIH+ fibers were observed in the POA (Fig. 8F), midbrain (Fig. 8G), and ME (Fig. 8H). Within the Hp, short beaded GnIH+ fibers were present in the VlB (Fig. 8A and B). Long beaded GnIH+ fibers in the DmC and VmC were parallel to the midline (Fig. 8A and C). GnIH+ fibers were also observed in the septum, just ventral to the Hp (Fig. 8D).

We examined the effects of Sex and Region on mean pixel area of GnIH-ir. In the DmC, there were no effects of Sex or Region (Sex: $F = 0.002$, df = 1, $p = 0.97$; Region: $F = 0.53$, df = 2, $p = 0.47$) and no Sex × Region interaction (Fig. 9A). In the VlB, there were also no effects of Sex or Region (Sex: $F = 1.37$, df = 1, $p = 0.25$; Region: $F = 0.03$, df = 2, $p = 0.86$) and no Sex × Region interaction (Fig. 9B).

3.6. GnRH1 and GnIH mRNA

GnRH1 mRNA is present in the zebra finch Hp (Fig. 10). GnRH1 transcripts were detected in male and female POA and Hp by PCR gel electrophoresis. Cloned and sequenced PCR products were homologous with *T. guttata* gonadotropin-releasing hormone 1 (luteinizing-releasing hormone) (GnRH1) mRNA (NM_001142320.1).

As expected, a band of the appropriate size for the GnIH precursor was detected in the hypothalamus. However, GnIH mRNA was not detected in the Hp, which matches the pattern of results seen for GnIH immunohistochemistry (*i.e.*, GnIH+ fibers but not cell bodies).

4. Discussion

This report presents evidence that multiple forms of GnRH and also GnIH are present in the Hp of a songbird. We employed several well-characterized primary antibodies and preadsorption controls, as well as PCR using brain micropunches. To our knowledge, this is the first study in any species to (1) systematically examine multiple forms of GnRH in the Hp or (2) quantify sex or regional differences in hippocampal GnRH. This is also the first study to demonstrate the presence of GnIH in the avian Hp. These data are useful because the roles of GnRH and GnIH outside of the HPG axis are largely overlooked and not well understood.

4.1. Distribution of GnRH and GnIH immunoreactivity in the songbird Hp

The HU60 antibody recognizes both GnRH1 and GnRH2 [8]. HU60-ir cells and fibers were visible throughout the rostro-caudal extent of the Hp. Immunoreactivity was most prominent in cells in the DmC (adjacent to the midline) and VlB (adjacent to the lateral ventricles). Short immunoreactive fibers were also observed in the DmC and VlB. Interestingly, aromatase is also highly expressed in neurons in the zebra finch DmC and VlB [42,48,50], and future studies will examine the co-localization of GnRH and aromatase in the Hp. Preadsorption controls indicate that labeled cells and fibers in the Hp contain GnRH1, GnRH2, or both peptides. Interestingly,

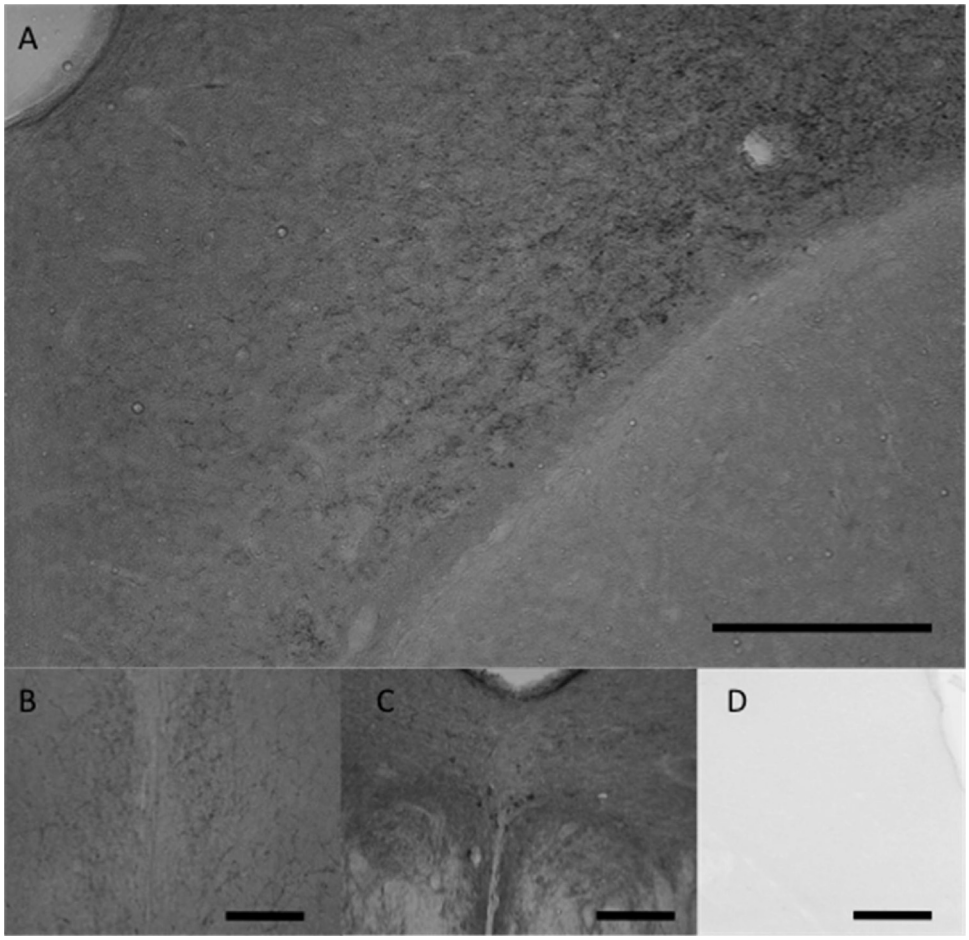

Fig. 4. GnRH2-immunoreactive fibers were present in the (A) Hp and (B) POA. (C) GnRH2+ cell bodies and fibers were present in the midbrain. (D) Preadsorption of the primary antibody with GnRH2 peptide abolished immunoreactivity in the Hp. Scale bar = 100 μm.

labeled cells in the Hp (but not in other regions) are round and do not have detectable fibers (Figs. 1A–C and 2A and Supplementary Fig. 1A–C). The immunostained elements appear to be cell bodies, and not cell nuclei, because their diameter is larger than the diameter of cell nuclei in the Hp (unpub. results). In a previous study of GnRH2-ir in the house sparrow brain, a similar pattern of staining was observed in the Hp [56]. Prior studies in birds and mammals have identified GnRH+ mast cells in the brain that are also round and lack processes [54], and thus the hippocampal HU60-ir cells seen here could be non-neuronal. This will be investigated in future double labeling studies. Also, future *in situ* hybridization studies will be useful to visualize GnRH1 mRNA in the Hp.

GnRH2+ fibers, recognized by the anti-cLHRH-II antibody, were visible throughout the rostro-caudal extent of the Hp. Immunoreactivity was most prominent in beaded fibers in the VlB and in the dorsolateral Hp adjacent to the HA. Preadsorption and positive controls confirm the high specificity of this antibody for GnRH2. Note that we did not observe GnRH2+ cell bodies in the zebra finch Hp, in contrast to results from the house sparrow Hp [56]; the reasons for this difference remain unclear. Taken together, the present data suggest that the hippocampal cell bodies labeled by HU60 (see above) are GnRH1+ rather than GnRH2+.

The anti-lGnRH-III antibody was raised against GnRH4. Immunoreactive cell bodies were interspersed within or near the

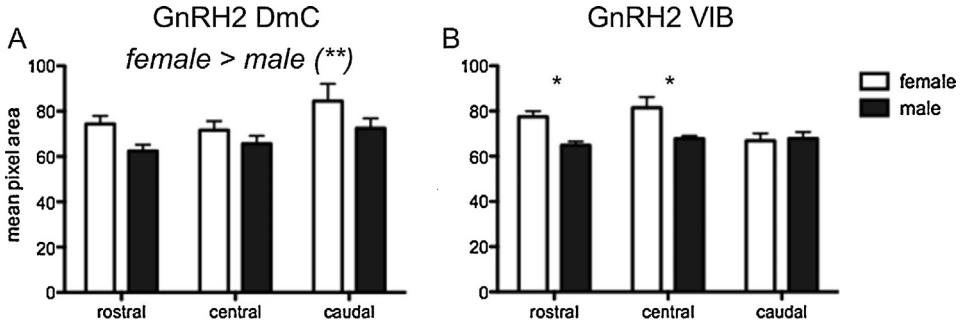

Fig. 5. Sex and regional differences in GnRH2-ir in the zebra finch Hp. (A) For total GnRH2-ir in the DmC, there was a significant main effect of Sex (female > male) and a significant main effect of Region (caudal Hp > rostral Hp = central Hp). (B) For total GnRH2-ir in the VlB, there were significant sex differences in the rostral VlB and central VlB only (female > male). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

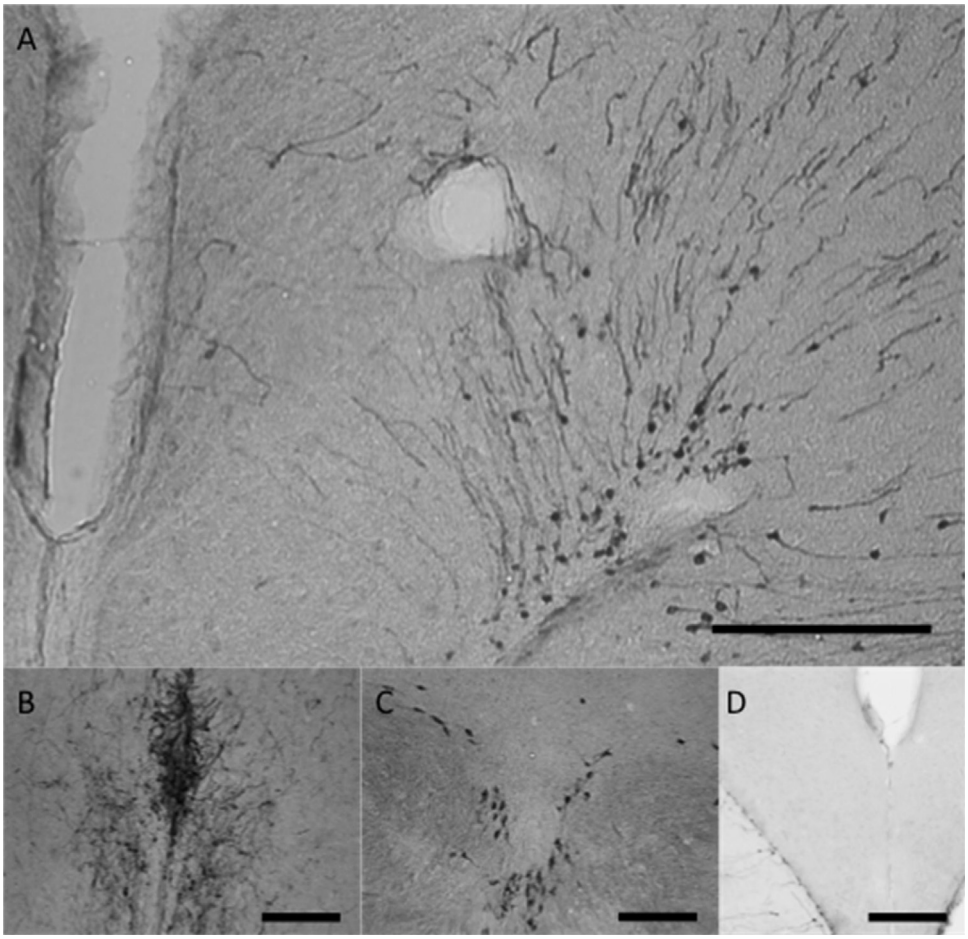

Fig. 6. GnRH4-immunoreactivity was present in the (A) Hp, (B) POA, and (C) midbrain. (D) Preadsorption of the primary antibody with GnRH4 peptide greatly reduced immunoreactivity in the Hp. Scale bar = 100 μm.

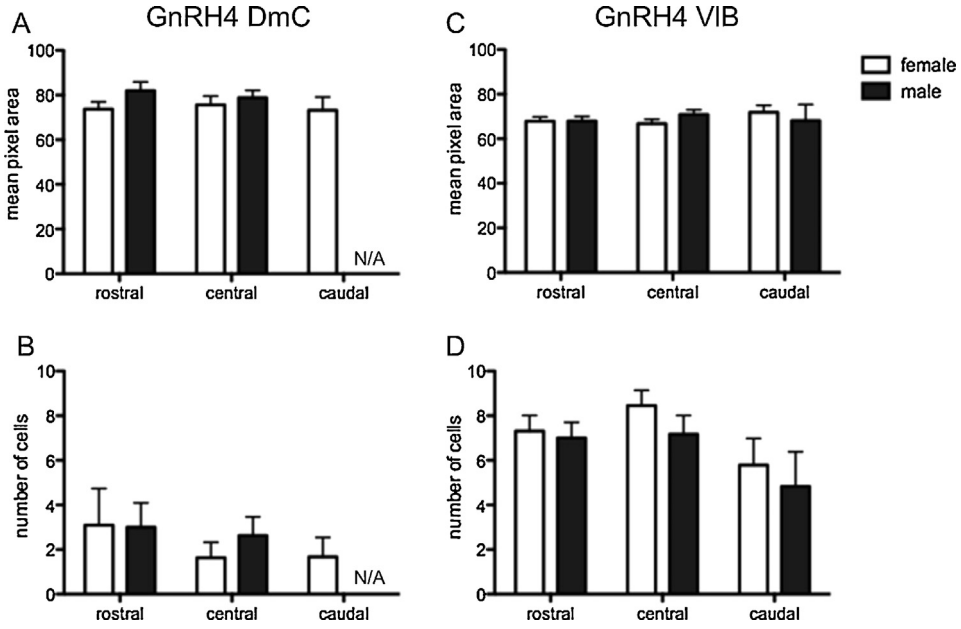

Fig. 7. Sex and regional differences in GnRH4-ir in the zebra finch Hp. There were no significant effects of Sex or Region on (A) total GnRH4-ir in the DmC, (B) GnRH4-ir cell number in the DmC, or (C) total GnRH4-ir in the VlB. (D) There was a significant main effect of Region on GnRH4-ir cell number in the VlB (central Hp > caudal Hp). N/A = not available (insufficient data from the caudal DmC of males for analysis).

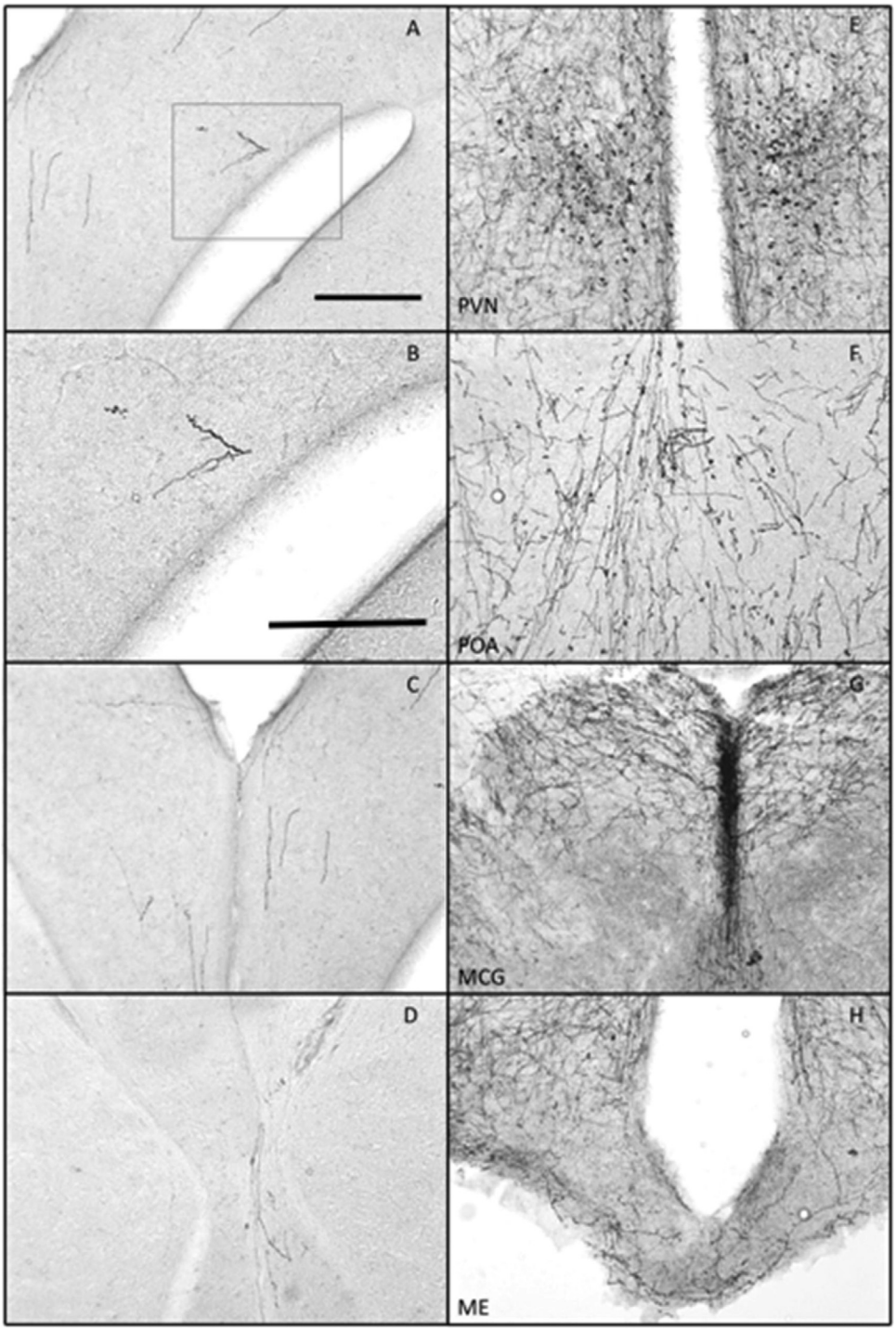

Fig. 8. GnIH-immunoreactive fibers were present in the Hp. (A) Short beaded GnIH+ fibers were present in the VlB. (B) Higher magnification view of boxed area in (A). (C) Long beaded GnIH+ fibers in the medial Hp were parallel to the midline and (D) present in the septum just ventral to the Hp. (E) GnIH+ cell bodies were present only in the PVN. GnIH+ fibers were present in the (F) POA, (G) midbrain central gray (MCG), and (H) median eminence (ME). Scale bar = 100 μm.

ventricular zone along the lateral ventricles. Cells were unipolar, with fibers projecting in parallel into the parenchyma of the Hp. Immunoreactive cells were also present in the POA and midbrain, and thus it is unclear whether this antibody also cross-reacts with GnRH1 and GnRH2. Preadsorption with the GnRH4 peptide greatly reduced immunoreactivity in the Hp and LPO, but not in the POA and midbrain, suggesting that the immunoreactivity in the Hp is indeed GnRH4-ir. As this antibody also recognizes somatostatin in lamprey [55], we performed a preadsorption with somatostatin. Preadsorption with somatostatin did not reduce immunoreactivity in the Hp or LPO but did reduce immunoreactivity in the NCM. Given the location and morphology of the immunoreactive cells in the Hp (see Figs. 1G–I and 6A), future double labeling studies will characterize the identity of these cells (*i.e.*, neurons vs. radial glia). Interestingly, following injury, radial glia and astrocytes express aromatase in the zebra finch Hp [42]. Future studies will also localize the GnRH4 transcript using *in situ* hybridization.

GnIH, a peptide that opposes the actions of GnRH1 in the HPG axis [3,4], is also present in the songbird Hp. GnIH+ fibers were visible throughout the rostro-caudal extent of the Hp. We observed

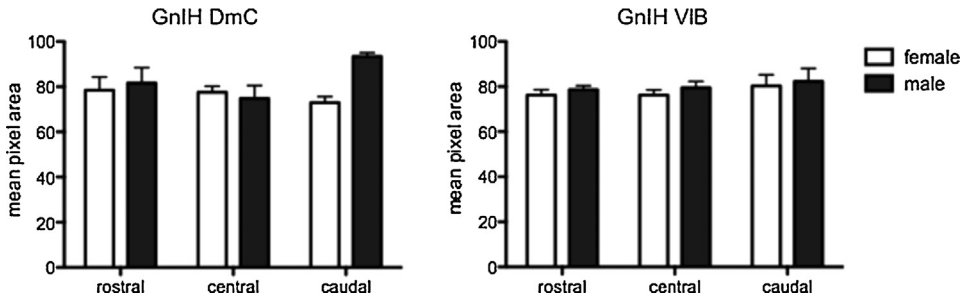

Fig. 9. Sex and regional differences in GnIH-ir in the zebra finch Hp. There were no significant effects of Sex or Region on total GnIH-ir in the DmC (A) or VlB (B).

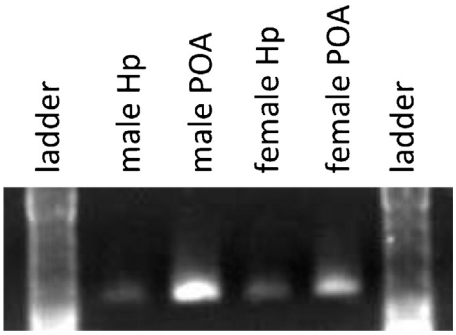

Fig. 10. GnRH1 mRNA in micropunches from the Hp and POA. The mRNA extracted from male and female Hp and hypothalamus was reverse transcribed to cDNA and used as a template for PCR. GnRH1 transcripts were detected in the Hp and POA of males and females. PCR products were cloned, sequenced and compared to *Taeniopygia guttata* gonadotropin-releasing hormone 1 (luteinizing-releasing hormone) (GnRH1) mRNA (NM_001142320.1) to confirm identities. Note that GnIH mRNA was not detected in the Hp but was detected in the hypothalamus (data not shown), consistent with the GnIH immunohistochemical data.

short beaded fibers in the VlB and longer beaded fibers along the midline. GnIH+ cells were observed only in the PVN of the hypothalamus, and GnIH transcripts were not detected in the Hp (see below). Thus it appears that GnIH+ fibers reach the Hp from the PVN *via* the septum. This is the first report of GnIH in the avian Hp and lays the foundation for future studies on the role of this neuropeptide in hippocampal function [31,46,73].

4.2. Expression of GnRH1 in the songbird Hp

The mRNA for GnRH1 was detected *via* PCR in micropunches from the Hp and hypothalamus. These PCR data are consistent with the immunohistochemical data, which showed the presence of HU60-ir cell bodies in the Hp. In contrast, mRNA for GnIH was detected in micropunches from the hypothalamus but not from the Hp. Again, these PCR data are consistent with the immunohistochemical data, which showed the presence of GnIH+ fibers but not cell bodies in the Hp. Taken together, the data indicate that GnRH1 is synthesized in the zebra finch Hp, but GnIH is synthesized in the PVN and then transported to the Hp.

The GnRH2 and GnRH4 genes have not yet been cloned in songbirds, and thus we did not examine their transcripts in the present study. This is an aim for future studies.

4.3. Comparison with hippocampal GnRH and GnIH in mammals

In contrast to the present data in zebra finches, there is little evidence for GnRH+ cell bodies or fibers in the Hp of rats and mice [21,33,45,71]. However, the expression of GnRH receptor in the rat Hp is very high [2,22,23,44]. In rats, GnRH may reach hippocampal GnRH receptors *via* the cerebrospinal fluid (*i.e.*, "volume transmission") [7]. Interestingly, GnRH receptors are co-localized with ERβ on CA1-4 pyramidal neurons in mouse and sheep hippocampi [1], and *in vitro* GnRH1 treatment increases local estradiol production by rat hippocampal cells [43]. These data suggest that GnRH stimulates local estradiol synthesis and action within the Hp, an idea that future studies will examine more closely.

In contrast to GnRH, recent evidence suggests that RFRP-3 (the mammalian ortholog of GnIH) is present in fibers in the rat Hp [46], specifically in the CA2/CA3 pyramidal layer. Retrograde tract tracing confirmed that the fibers originated from RFRP-3 cell bodies in the dorsomedial hypothalamus. These data are similar to the present results in zebra finches. Furthermore, RFRP-3 receptors have been detected in the pyramidal layer in the rodent Hp [20].

4.4. Sex and regional differences in GnRH immunoreactivity in the songbird Hp

Sex and region (*i.e.*, rostral-caudal level) affected hippocampal GnRH-ir but not GnIH-ir. Specifically, HU60-ir was greater in females than males in the DmC (Fig. 3A). In addition, the number of HU60-ir cells was greater in females than males in the VlB. Females also had more GnRH2-ir than males in the DmC, rostral VlB, and central VlB. Thus, sex differences were observed for GnRH1 and GnRH2 specifically.

These sex differences in GnRH-ir might be the result of sex differences in steroid synthesis or steroid receptors. Circulating or locally synthesized estradiol might regulate hippocampal GnRH levels. In the zebra finch Hp, males show higher levels of presynaptic aromatase in neurons than females [42], suggesting that high local levels of estradiol might downregulate GnRH-ir in the Hp. This hypothesis can be tested by locally inhibiting aromatase activity in the Hp of zebra finches. Alternatively, sex differences in hippocampal GnRH might be the result of sex differences in steroid receptors. For example, in rats, females have higher levels of hippocampal ERα than males [47]. In addition, in rats, hippocampal GnRH receptor expression differs between males and females [17]. Taken together, these data indicate that there are sex differences in hippocampal GnRH as well as hippocampal GnRH receptors, which may contribute to sex differences in learning and memory, stress responsiveness, or affect.

5. Conclusions

Using a combination of techniques, we present evidence for GnRH1, GnRH2, GnRH4 and GnIH in the songbird Hp. Previous studies in birds or mammals report that the Hp also contains GnRH receptors, GnIH receptors, LH, FSH, LH receptors, FSH receptors, and all the steroidogenic enzymes to synthesize estradiol from cholesterol [6,9]. The presence of all these signaling molecules within the Hp raises the interesting possibility of local regulation of hippocampal steroid synthesis. More generally, the present data are useful because the roles of GnRH and GnIH outside of the HPG axis are largely unknown, but these neuropeptides are likely to play key

roles in the Hp as well as other brain regions. Songbird models will continue to be valuable for understanding this important issue in neuroendocrinology.

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