Identification, localization, and regulation of passerine GnRH-I messenger RNA

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Abstract

The neuropeptide GnRH-I is critical for the regulation of reproduction in all vertebrates. Study of the regulation of GnRH-I in passerine songbirds has been the focus of studies on subjects as diverse as photoperiodism, puberty, stress, nutrition, processing of auditory information, migration, global climate change, and evolutionary biology. Until now, analysis of GnRH-I in songbirds has been limited to measurement of immunoreactive peptide. Measurement of mRNA regulation has been impossible because of lack of knowledge of the GnRH gene sequence, despite many attempts in the last 20 years to identify it. Thus, the relative roles of environmental, social, physiological, and evolutionary influences upon passerine GnRH regulation have remained enigmatic. Here, we report the first cloning of GnRH-I cDNA from a songbird, *Taeniopygia guttata*, its localization and regulation. Although the homology of its translated precursor

polypeptide between chicken GnRH-I precursor polypeptide was only 54%, zebra finch GnRH-I precursor contained an amino acid sequence that can be processed into chicken GnRH-I peptide (pEHWSYGLQPG-amide). *In situ* hybridization combined with immunocytochemistry showed co-localization of GnRH-I mRNA and immunoreactive peptide in the preoptic area of sexually mature birds. GnRH-I mRNA signal was greatly reduced in sexually immature birds. Ovary mass of female birds was positively correlated with GnRH-I mRNA level in the brain. These data will now permit molecular analysis of the regulation of songbird reproduction by physical, social, and physiological cues, along with fine scale analysis of selection pressures acting upon the reproductive system of songbirds. (244/250).

Journal of Endocrinology (2009) **201**, 81–87

Introduction

Vertebrate reproduction is primarily regulated by GnRH, of which there are at least three distinct forms, namely GnRH-I, -II, and -III. One or more of these forms has been found in all vertebrates studied to date, regulating gonadotropin release and reproductive activity. Some forms of GnRH have also been isolated from invertebrates such as tunicates (Powell *et al.* 1996) and a cephalopod (Iwakoshi *et al.* 2002).

In several orders of birds, namely Galliformes, Anseriformes, Columbiformes, and Passeriformes, chicken-GnRH-I and -II (cGnRH-I and -II) are involved in regulation of the reproductive axis (Millar & King 1984, Miyamoto *et al.* 1984, Millar *et al.* 1986, Millam *et al.* 1989, Sharp *et al.* 1990, Wingfield & Farner 1993). The main population of cGnRH-I-ir cell bodies in the avian brain is a bilateral cluster in the pre-optic area (POA) that extends from the anterior commissure to the supracommissural division of the organum vasculosum of the lamina terminalis. Cell bodies immunoreactive for cGnRH-II are situated in the midbrain (Juss *et al.* 1992, Millam *et al.* 1993). GnRH-I immunoreactive neurons typically project to the median eminence,

allowing regulation of pituitary gonadotropin release. GnRH-II is generally considered not to influence pituitary gonadotropin release directly, although there is some evidence for the presence of GnRH-II in the median eminence in quail (D'Hondt *et al.* 2001).

Songbird reproduction has long been of interest to biologists who study behavior, physiology, and evolution, to name a few key areas of research. As with all vertebrates, GnRH-I is central to songbird reproduction. The study of this neuropeptide is critical if we are to understand selection pressures acting upon the avian reproductive system, and thus the evolution of the vast diversity of mating systems in songbirds, photoperiodism, migration, puberty, and reproductive response to rapid climate change. Understanding the mechanisms responsible for early termination of seasonal breeding in warmer temperatures (Dawson 2005, Silverin *et al.* 2008), for example, is key to conservation biology and environmental policy. Research into the mechanistic control of songbird reproduction has been limited because of the inability to identify the passerine GnRH-I cDNA sequence. Despite the cDNA cloning of GnRH-I in Galliformes (chicken, quail, turkey), Anseriformes (goose, duck), and

Columbiformes (dove), the Passeriform (songbird) GnRH-I gene sequence has remained unknown for many years, although it is thought to encode chicken GnRH-I (Sherwood *et al.* 1988). Here, we report the first cloning of GnRH-I cDNA from a songbird, *Taeniopygia guttata*, and its localization. We also demonstrate a correlation between reproductive status and GnRH-I mRNA level in female birds, along with differences in GnRH-I mRNA between juvenile and adult zebra finches.

Materials and methods

Brain material

Mature (90 days and older) male and female, and immature (30–50 days old) female zebra finches were used in this study. Animals were raised in the University of California at Berkeley (Berkeley, CA, USA). Brains were collected immediately after terminal anesthesia by isoflurane. Ovaries were also collected from mature female birds and weighed. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under an approved protocol from the University of California.

Identification of a cDNA encoding zebra finch GnRH-I precursor

Manually dissected preoptic area of the sexually mature birds (three males and four females) was used for the identification of a cDNA encoding zebra finch GnRH-I precursor. Total RNA (including rRNA and mRNA) was isolated by using TRIZOL (Invitrogen). Total RNA was reverse-transcribed using oligo(dT)-anchor primer (5'/3' RACE Kit, 2nd Generation; Roche Diagnostics) and reverse transcriptase (M-MLV reverse transcriptase; Invitrogen). Various forward primers targeting the GnRH-I coding region and a PCR anchor primer (Roche Diagnostics) were used to amplify the 3' end of the zebra finch GnRH-I precursor cDNA. Out of 24 forward primers tested, only two forward primers (5'-CAACACTGGTCCTACGG-3' and 5'-CAGCACTGGTCCTACGG-3') produced a clear single band of PCR product, revealed by electrophoresis. All PCR amplifications were performed in a reaction mixture containing Taq polymerase (TaKaRa Ex TaqTM; Takara Bio Inc., Shiga, Japan). PCR products were sub-cloned into a pGEM-T Easy vector (Promega) and the DNA inserts of the positive clones were amplified by PCR with universal M13 primers. Amplified DNA was sequenced at the UC Berkeley DNA sequencing facility (Berkeley, CA, USA) using 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and the 3' end (314 bases) of zebra finch GnRH-I precursor cDNA was determined.

To identify the 5' end of the zebra finch GnRH-I precursor cDNA, the template cDNA was reverse transcribed using a gene-specific reverse primer based on the identified 3' end of zebra finch GnRH-I precursor cDNA sequence

(5'-TCTCCATGGCTTCCCTCAG-3'), followed by poly(A) tailing of the cDNA with dATP and terminal transferase (Roche Diagnostics). The tailed cDNA was amplified with the oligo(dT)-anchor primer and a nested gene-specific reverse primer (5'-GGAATTCTGGTGC-GAGCCTG-3' or 5'-GCTCCTCCTCTAATTTCTCCA-3'). A second PCR was performed using PCR anchor primer and a further nested gene-specific reverse primer (5'-CAATCTCCTGGAATGGTC-3'). The second PCR products were sub-cloned and sequenced as described above.

In situ hybridization of zebra finch GnRH-I precursor mRNA

Coronal sections of four immature and six mature female zebra finch brains at 20 µm thickness were collected on a cryostat at -20 °C for histological studies. *In situ* hybridization was carried out with slight modifications of our previous method (Ubuka *et al.* 2005) using a digoxigenin (DIG)-labeled antisense RNA probe. The DIG-labeled antisense RNA probe was produced using a standard RNA labeling kit (Roche Diagnostics) by using partial zebra finch GnRH-I precursor cDNA (nt 8–295 in Fig. 1) as a template. Defrosted sections were first fixed in 4% paraformaldehyde (PFA) for 30 min. After washing the sections three times in (PBS; 10 mM phosphate buffer, 0.14 M NaCl, pH 7.4), they were incubated in 1 µg/ml proteinase K (Sigma-Aldrich) in PBS at 37 °C for 30 min. Sections were fixed again in 4% PFA for 10 min, and then treated with 0.2 N HCl for 10 min after rinsing the sections in DEPC treated water. The sections were again rinsed in DEPC treated water twice and pre-incubated in 50% deionized formamide in 5X SSC (Roche Diagnostics) before the hybridization. Hybridization was carried out overnight at 50 °C in 50% deionized formamide, 50% hybridization solution (2X concentrate, buffered with SSC, Sigma-Aldrich) at the probe concentrations of 200 ng/ml. After hybridization, the sections were washed twice in 2X SSC in 50% formamide, and twice in 1X SSC in 50% formamide for 15 min each. After rinsing the sections in PBS, they were incubated with alkaline phosphatase-labeled sheep anti-DIG antibody (Roche Diagnostics) in 1.5% DIG blocking reagent (Roche Diagnostics) in PBS. After rinsing the sections three times in PBS and once in alkaline phosphate buffer (pH 9.5), the immunoreactive product was visualized by immersing the sections in a substrate solution (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate stock solution; Roche Diagnostics) in alkaline phosphate buffer. Control for specificity of *in situ* hybridization was performed by using a DIG-labeled sense RNA probe, the sequence of which was complementary to the antisense probe.

Immunocytochemistry of GnRH-I and -II peptide

Immunocytochemical analysis of GnRH was conducted with slight modifications of our previous method (Ubuka *et al.* 2008). Sections were first fixed in 4% PFA for 30 min, and incubated in 0.3% H₂O₂ in absolute methanol for 20 min to

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      ATTTTCCAGAGGAGAAGAGCTGCAGTTACTCCACAACCTCTCTCAGGCAGCAGG   54
ATGGAGAAGCCCCGGAGGATGGTGGCCACTGTCTGCTGTGTGTGCTGGCCGTGGGGCTG 114
  M   E   K   P   R   R   M   V   A   T   V   L   L   C   V   L   A   V   G   L   20

GGGCTGGCTCAGCACTGGTCCCTACGGCCTCCAGCCAGGGGGCAAGAGGAGCACCCAGCTC 174
  G   L   A   Q   H   W   S   Y   G   L   Q   P   G   G   K   R   S   T   Q   L   40

CTGCTGGGACCATTCCAGGAGATTGCAAATGAAATGGAGAAATTAGAGGAGGAGCAGCAG 234
  L   L   G   P   F   Q   E   I   A   N   E   M   E   K   L   E   E   E   Q   Q   60

AGCGCGTGCCAGGCTCGCACCAGAATTCCAGGAGTGGGGCTCTGAGGGAAGCCATGGAG 294
  S   A   C   P   G   S   H   Q   N   S   R   S   G   A   L   R   E   A   M   E   80

AGGCTGCTGGAGGAACAAGGCAGAAGAAAGAACGTTTAAAAATTTCCAAAGTGAAATGGA 354
  R   L   L   E   E   Q   G   R   R   K   N   V   *   92

GCCACAGAGCCAGAGTGAGGTTTCAAACTGTGTTAAAATAAATTCCTCCTCAAATGGTC 414
TTTGCTTGGATCAATATGTGAATAAATACTCTGAAATGGC-poly (A) 454

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Figure 1 Nucleotide sequence and deduced amino acid sequence of zebra finch GnRH-I precursor cDNA. The open reading frame begins with the putative start codon ATG at the position 55–57 according to the Kozak's rule (Kozak 1987), and terminates with a stop codon TAA at the position 331–333. The stop codon is indicated with an asterisk. The poly(A) adenylation signal AATAAA is underlined with a broken line at position 435–440. The decapeptide hormone sequence (QHWSYGLQPG) with Gly residue as an amidation signal and endoproteolytic residues Lys and Arg (amino acid 24–36) is underlined. GenBank FJ407188.

suppress endogenous peroxidase activity after washing the sections three times in PBS-T (0.2% Triton X-100 in PBS). Sections were then washed three times in PBS-T and incubated overnight at 4 °C in the primary antibody at a concentration of 1:5 000 in PBS-T. The primary antibody used to label GnRH neurons was rabbit anti-GnRH (HU60H; kindly donated by Dr H Urbanski). Although the antibody we used does not distinguish between GnRH-I and GnRH-II neurons, we can identify them based on their separate locations in the brain (GnRH-I neurons are located in the preoptic area, whereas GnRH-II neurons are located in the midbrain) and from their distinctive appearance (GnRH-II neurons are smaller in size and stubby with thinner neurites; Ubuka *et al.* 2008). The next day, three subsequent washes in PBS-T were followed by incubation in biotinylated goat anti-rabbit IgG (1:250 in PBS-T) for 1 h. After washing the sections in PBS-T three times, they were then incubated for 1 h in avidin-biotin complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) in PBS-T. The resulting complex was visualized using 0.03% 3, 3'-diaminobenzidine after washing the sections three times in PBS-T. The specificity of the primary antibody was assessed by preadsorption tests of the antibody with 1×10^{-5} M synthetic chicken GnRH-I (pEHWSYGLQPG-NH₂).

Double-labeling of zebra finch GnRH-I precursor mRNA and GnRH-I peptide

After hybridizing the sections with DIG-labeled antisense RNA probe, sections were washed and incubated with

GnRH-I antibody (HU60H) overnight. After washing the sections in PBS three times, the sections were incubated with rhodamine-labeled goat anti-rabbit IgG together with alkaline phosphatase-labeled sheep anti-DIG antibody in PBS containing 1.5% DIG blocking reagent. After washing the sections three times in PBS and rinsing once in alkaline phosphate buffer, the sections were incubated in a substrate solution of alkaline phosphatase.

Image processing and statistics

Microscopic images were acquired digitally on an AxioImager A1 microscope (Carl Zeiss AG, Gottingen, Germany) with an AxioCam MRc5 digital camera (Carl Zeiss AG) using AxioVision Rel. 4.5 software package (Carl Zeiss AG). The density of GnRH-I mRNA *in situ* hybridization signal was measured using Image J (National Institutes of Health, Bethesda, MD, USA) as a gray scale value from 0 (white) to 256 (black) and expressed as the mean density per cell in arbitrary units, which was obtained by subtracting background gray values. The GnRH-I mRNA density value of each bird was obtained by calculating the mean density of ten randomly chosen cells in the brain section which had the largest numbers of GnRH-I neurons. Quantification of non-radioactive *in situ* hybridization signal was validated as in previous studies (Ubuka *et al.* 2005, Kogami *et al.* 2006). GnRH-I mRNA density levels were compared between mature and immature female birds using Student's *t*-test. The correlation of ovary mass with GnRH-I mRNA signal density in mature female birds was analyzed using linear regression analysis.

Results

Nucleotide sequence and deduced amino acid sequence of zebra finch GnRH-I precursor cDNA

We cloned the entire zebra finch GnRH-I precursor cDNA from the zebra finch hypothalamus by a combination of 3' and 5' RACE. Figure 1 shows that zebra finch GnRH-I precursor cDNA (GenBank FJ407188) is composed of 454 nucleotides, containing a short 5' untranslated sequence of 54 nt, a single open reading frame of 276 nt, and a 3' untranslated sequence of 124 nt with the addition of various lengths of poly(A) tail. The open reading frame begins with the putative start codon ATG at the position 55–57 according to the Kozak's rule (Kozak 1987), and terminates with a stop codon TAA at the position 331–333. A single polyadenylation signal (AATAAA) was found in the 3' untranslated region at position 435–440. The open reading frame region encoded a 92-residue polypeptide. Zebra finch genome resources became available only very recently. According to the genome database, zebra finch GnRH-I precursor mRNA is transcribed from zebra finch DNA in chromosome 22 (NW_002197932).

The translated zebra finch GnRH-I precursor polypeptide comprised a signal peptide sequence (amino acid 1–23 in Figs 1 and 2), decapeptide hormone sequence (QHWSYGLQPG) with Gly residue as an amidation signal and endoproteolytic residues Lys and Arg (amino acid 24–36 in Figs 1 and 2) followed by GnRH-associated peptide (GAP) sequence (amino acid 37–92 in Figs 1 and 2; Seeburg & Adelman 1984, Dunn *et al.* 1993). The homology of zebra finch GnRH-I precursor polypeptide sequence (FJ407188) with chicken GnRH-I precursor polypeptide (NP_001074346)

was 54%, 52% with ring dove (ACD80081.1), 39% with rat (NP_036899.1), 37% with human (NP_000816), and 35% with cattle (NP_001071605.1) (Fig. 2).

Co-localization of zebra finch GnRH-I mRNA and GnRH-I peptide in the zebra finch brain

We investigated the histological localization of GnRH-I precursor mRNA using *in situ* hybridization in the zebra finch brain. An intense bilateral expression of zebra finch GnRH-I precursor mRNA was observed in the preoptic area of the hypothalamus (Fig. 3A). GnRH-I precursor mRNA-containing neurons were distributed along the third ventricle from the preoptic area to the region around the anterior commissure and the medial septum (Fig. 4). Immunocytochemistry for GnRH-I peptide was conducted on the same sections. As shown in Fig. 3B, intense immunoreactivity with GnRH-I peptide was also found in the preoptic area. Clear cellular co-localization of GnRH-I precursor mRNA and GnRH-I peptide was identified in all immunoreactive cells by merging the images of GnRH-I precursor mRNA *in situ* hybridization and GnRH-I immunocytochemistry (Fig. 3C), and by comparing the location of the signals (Fig. 3D and E). Co-localization of GnRH-I precursor mRNA and GnRH-I peptide was observed from the preoptic area to the region around the anterior commissure and the medial septum (Fig. 4). No co-localization of GnRH-I precursor mRNA and GnRH-II peptide was observed. *In situ* hybridization using sense RNA probe served as control (Fig. 3F). Preadsorption of the antibody with 1×10⁻⁵ M synthetic chicken GnRH-I produced no immunoreactivity (data not shown).

Cattle	MKPTPKLLAGLILLILCVGCSG QHWSYGLRPGGKR NAENVIDSFQEIAK	50
Human	MKPIQKLLAGLILLTWCEGCSS QHWSYGLRPGGKR DAENLIDSFQEIVK	50
Rat	METIPKLMAAVLLTVCLEGCSS QHWSYGLRPGGKR NTEHLVDSFQEMGK	50
Ring dove	MEKSRKILVGVLILLVLSMETCSA QHWSYGLQPGGKR NAENLLSLQEIAK	50
Chicken	MEKSRKILVGVLFTASVAICLA QHWSYGLQPGGKR NAENLVESFQEIAN	50
Zebra finch	MEKPRRMVATVLLCVLAVGLGLA QHWSYGLQPGGKR STQLLLGPFEQIAN	50
	Signal peptide (1–23) GnRH-I (24–36) GAP (37–)	
Cattle	EVDQPV EPKCCG CIVHQSHSPLRDLKAALES LI EEETGQRKI	92
Human	EVGQLA ETQRFECT THQPR SPLRDL KGALES LI EEETGQKKI	92
Rat	EEDQMA EPQNFECT VHWPR SPLRDL RGALER LI EEEEAGQKKM	92
Ring dove	EM EKL GEVQK SECPG STQHSRLGELKEAVGSL LI EEETR----	88
Chicken	EMESLGEGQKA EC PGSYQHPRLSDLK ETMAS LI EG EARKEI	92
Zebra finch	EM EKL EEEQSACPGSHQNSRSGALREAMERLL EE QGRRKNV	92
	GAP (–92)	

Figure 2 Alignment of cattle, human, rat, ring dove, chicken, and zebra finch GnRH-I precursor polypeptide. The translated zebra finch GnRH-I precursor polypeptide is comprised of a signal peptide sequence (amino acid 1–23), a decapeptide hormone sequence (QHWSYGLQPG) with Gly residue as an amidation signal and endoproteolytic residues Lys and Arg, which is shown in bold (amino acids 24–36; GnRH-I), followed by GnRH-associated peptide sequence (amino acids 37–92; GAP). The homology of the zebra finch GnRH-I precursor polypeptide sequence (FJ407188) with chicken GnRH-I precursor polypeptide (NP_001074346) is 54%, 52% with ring dove (ACD80081.1), 39% with rat (NP_036899.1), 37% with human (NP_000816), and 35% with cattle (NP_001071605.1). Amino acids identical to those in the zebra finch GnRH-I precursor polypeptide are shaded.

Figure 3 Expression of zebra finch GnRH-I precursor mRNA and GnRH-I peptide in the preoptic area of the hypothalamus. *In situ* hybridization using an antisense RNA probe for zebra finch GnRH-I mRNA (A) and immunocytochemistry for GnRH-I (B) were performed on the same section. The merged image (C) of pictures (A) and (B) shows the colocalization of GnRH-I precursor mRNA and GnRH-I peptide in identical neurons. (D) and (E) show the highlighted area in (A) and (B) respectively. Arrows in (D) and (E) indicate identical cells. (A)–(C) and (F) are shown at the same magnification (Bars, 50 µm). Similar results were obtained in ten different birds. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-08-0508>.

Correlation of reproductive status and the expression level of GnRH-I precursor mRNA in female zebra finches

Despite differences in gonadal status, no study has yet identified changes in GnRH peptide in zebra finches. Thus, we wished to investigate potential changes in GnRH-I mRNA expression according to gonadal status. We quantified GnRH-I mRNA expression level in immature versus mature female birds. All birds in this study, whether mature or immature, expressed GnRH-I mRNA, but the mean density of GnRH-I mRNA signal in immature birds was significantly lower than that of mature birds ($P < 0.01$, Fig. 5A). Furthermore, the ovary mass of mature female birds was positively correlated with GnRH-I mRNA expression in the brain as assessed by linear regression analysis ($df = 1$, $F = 7.89$, $R^2 = 0.66$, $P < 0.05$, Fig. 5B).

Discussion

Here, we report the first cloning of GnRH-I cDNA from a songbird, *T. guttata*, its localization and its regulation

Figure 4 Schematic representation of zebra finch brain sections including GnRH-I neurons. Clusters of GnRH-I neuronal cell bodies as shown by dots were distributed along the third ventricle from the preoptic area (POA) to the region around anterior commissure (CoA) and the medial septum (SM). TrSM, tractus septomesencephalicus; TeO, tectum opticum; ME, median eminence.

according to sexual maturity and adult gonadal status. We understand that many genes are cloned and sequenced on a daily basis. What is special about this case is that songbird research has been restricted to relatively gross analysis of GnRH regulation and has thus been severely hampered in terms of advance. For example, studies on GnRH-I immunoreactive peptide in zebra finches have been unable to detect changes in the peptide according to gonadal status because of the limitations of immunocytochemistry (Perfito *et al.* 2006, 2007). Our data on the correlation between gonadal status and GnRH-I mRNA indicate that measurement of GnRH-I mRNA allows us to determine small-scale differences in hypothalamic reproductive status of adult birds. This allows us to determine the effects of subtle environmental inputs on the GnRH system.

Based on our information about the identity of zebra finch GnRH, we have also cloned European starling (*Sturnus vulgaris*) GnRH-I cDNA (GenBank FJ514493). Thus, we, and others in the field, will be able to investigate regulation of GnRH-I synthesis in highly photoperiodic songbirds (as opposed to the opportunistic zebra finches). A key

Figure 5 Correlation of ovarian mass and the expression level of GnRH-I precursor mRNA in the female bird. (A) The mean density of GnRH-I precursor mRNA signal in the cells of mature and immature female birds. The columns and the vertical lines represent the mean $\pm$ S.E.M. of mature ($n=6$) and immature ($n=4$) birds. ***, $P < 0.001$ by Student's t -test. (B) Correlation of ovary mass (mg) with the mean density of GnRH-I precursor mRNA signal in mature female birds ($n=6$, $df=1$, $F=7.89$). $R^2=0.66$, $P < 0.05$ by linear regression analysis.

question that has remained unanswered for some time is when (and if) GnRH-I synthesis is terminated at the end of the breeding season. In other words, what is the mechanism of termination of breeding? For example, it is considered that in Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*), a decrease in GnRH-I secretion is the initial step for the onset of photorefractoriness and not a decrease in GnRH-I biosynthesis (Meddle *et al.* 2006). It is also considered that decreased synthesis of cGnRH-I is not the proximate cause of gonadal regression at the end of the breeding season in European starlings (Dawson 2005). Deviche *et al.* (2008) came to a different conclusion on the regulation of seasonal breeding in Cassin's sparrows (*Aimophila cassini*), in that gonadal regression in this species is a consequence of reduced GnRH synthesis. Measurement of GnRH-I mRNA in these, and other commonly studied passerine species will elucidate the mechanisms of termination of breeding.

It is notable that sexually immature female zebra finches express GnRH-I mRNA (albeit at lower levels than in sexually mature adults) despite having completely undeveloped gonads. Even juveniles (20 days of age) of seasonally

breeding photoperiodic species such as house sparrows (*Passer domesticus*) express high amounts of immunoreactive GnRH-I in the POA and in the external ME (Bentley, unpublished data). This is despite having fully regressed gonads, not yet having lost the yellow beak flange used for food-begging and still retaining a few juvenile down feathers on their heads. Taken together, these data indicate that GnRH-I is synthesized and transported to the ME prior to the onset of gonadal growth and puberty in some passerine species. Thus, puberty in these species is likely to be regulated via mechanisms that control release of GnRH-I, either at the level of GnRH-neurons or at the median eminence, or both.

In summary, identification of passerine GnRH-I mRNA allows study of the passerine reproductive system at a higher level and a finer scale than has been previously possible. We believe that the new information presented here not only provides a significant advance in the field of avian neuroendocrinology, but also for key areas such as passerine evolutionary biology, captive breeding programs, and stress physiology.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

Funding was provided by NSF IOS-0641188 (to G E B).

Acknowledgements

We thank Yuka Minton for her expert help with this study.

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Received in final form 30 December 2008

Accepted 9 January 2009

Made available online as an Accepted Preprint
9 January 2009