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Interactions of Gonadotropin-Releasing Hormone (GnRH) and Gonadotropin-Inhibitory Hormone (GnIH) in Birds and Mammals

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ABSTRACT Gonadotropin-releasing hormone (GnRH) regulates secretion of both of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone. Thus, it is a key hormone for vertebrate reproduction. GnRH was considered to be unusual among hypothalamic neuropeptides in that it appeared to have no direct antagonist, although some neurochemicals and peripheral hormones (opiates, GABA, gonadal steroids, inhibin) can modulate gonadotropin release to a degree. Five years ago, a vertebrate hypothalamic neuropeptide that inhibited pituitary gonadotropin release in a dose-dependent manner was discovered in quail by Tsutsui et al. (2000. Biochem Biophys Res Commun 275:661–667). We now know that this inhibitory peptide, named gonadotropin-inhibitory hormone, or GnIH, is a regulator of gonadotropin release in vitro and in vivo. Its discovery has opened the door to an entirely new line of research within the realm of reproductive biology. In our collaborative studies, we have begun to elucidate the manner in which GnIH interacts with GnRH to time release of gonadotropins and thus time reproductive activity in birds and mammals. This paper reviews the distribution of GnIH in songbirds relative to GnRHs, and our findings on its modes of action in vitro and in vivo, based on laboratory and field studies. These data are simultaneously compared with our findings in mammals, highlighting how the use of different model species within different vertebrate classes can be a useful approach to identify the conserved actions of this novel neuropeptide, along with its potential importance to vertebrate reproduction. *J. Exp. Zool. 305A:807–814, 2006.*

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Many of the neuropeptide pathways involved in transduction of environmental stimuli have been well-studied. A classic example of this is the gonadotropin-releasing hormone (GnRH) system. GnRH is a key hormone for vertebrate reproduction. Since the initial definitive demonstration in 1971 that one hypothalamic polypeptide (GnRH) regulates secretion of both of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (Schally et al., '71), several other GnRHs have been identified (15 to date and there is potential for more). It has also been generally accepted that GnRH alone regulates the release of pituitary gonadotropins and that no other neuropeptide has a direct influence on the reproductive

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axis. Some neurochemicals and peripheral hormones (e.g., GABA, opiates, gonadal steroids, inhibin) can modulate gonadotropin release to a degree, but GnRH was considered to be unusual among hypothalamic neuropeptides in that it appeared to have no hypothalamic antagonist (c.f. GHRH and somatostatin regulating growth hormone, for example). As a challenge this dogma, a vertebrate hypothalamic neuropeptide that inhibited pituitary gonadotropin release in a dose-dependent manner was discovered 6 years ago (Tsutsui et al., 2000).

In a search for novel peptides regulating the release of pituitary hormones, Tsutsui's laboratory isolated a native peptide from quail brain which was confirmed as a 12 amino acid sequence (SIKPSAYLPLRFamide) with RFamide at the C-terminus (Tsutsui et al., 2000; and see Tsutsui et al., 2006a, this issue). This neuropeptide had not been previously reported in vertebrates. After raising an antibody to this quail peptide, it was found not only to be located in the quail hypothalamus, but also to decrease gonadotropin release from the cultured anterior pituitary (Tsutsui et al., 2000). This neuropeptide was therefore dubbed gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2000; also see Tsutsui et al., 2006a this issue).

From the past 6 years of research, we now know that GnIH is a regulator of gonadotropin release in vitro and in vivo. The findings uncovering the functional role of GnIH in birds and mammals are discussed herein. Its discovery has opened the door to an entirely new line of research within the realm of reproductive biology, particularly in terms of negative effects of the external physical and social environment on reproduction (e.g., stress). Similar peptides are also present in amphibians, fish, and mammals (including humans, based on a gene database search). Furthermore, the GnIH precursor polypeptide is cleaved into three separate mature peptides in birds and possibly two in mammals (GnIH, GnIH-related peptide 1, or -RP-1, and GnIH-RP-2 in birds; RFamide-related peptides 1 and 3 in mammals), and GnIH inhibits gonadotropin common α-and β-subunit production, as well as release (Ciccone et al., 2004; Tsutsui et al., 2006a,b; Ubuka et al., 2006). This means that, including the GnRHs, there are now up to five neuropeptides that are potentially involved in the regulation of reproduction (GnRH-I, GnRH-II, GnIH, GnIH-RP-1, GnIH-RP-2), whereas it was generally accepted a few years ago that only one neuropeptide (GnRH-I) was directly involved. In the last 6 years, we have had to change our way of thinking about regulation of the reproductive axis drastically, and we are only at the beginning of an exciting new era of research on reproductive biology.

GNIH DISTRIBUTION IN SONGBIRDS AND MAMMALS

Our initial studies investigated the neural distribution of GnIH cell bodies and fibers in several highly photoperiodic songbird species using immunocytochemistry. We considered that GnIH might play a pivotal role in the termination of the breeding season in species such as the intensely studied Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*), song sparrow (*Melospiza melodia*), and house sparrow (*Passer domesticus*). Dense populations of GnIH-immunoreactive (GnIH-ir) neurons were found only in the paraventricular nucleus (PVN) of all birds, regardless of sex or species. These GnIH-ir neurons were bipolar or tripolar (Fig. 1). No GnIH-ir neurons were detected elsewhere in the brain. We have also found a similar distribution of GnIH-ir neurons in several species of cardueline finch and a tropical sparrow (unpublished data). Preadsorption control sections exhibited no immunoreactivity (i.e., use of primary antiserum that prior to use on brain sections had been preincubated in a saturating solution of the peptide it was raised against). Following cloning of the cDNA encoding GnIH, we performed in situ hybridization studies in white-crowne sparrows to extend our findings by determining the cellular source of synthesis of GnIH. As in quail, the PVN appears to be the sole source of GnIH production in this species (Ukena et al., 2003; Osugi et al., 2004).

In addition to the dense bilateral population of GnIH-ir neurons within the hypothalamus of white-crowned sparrows, song sparrows and house sparrows, there are extensive networks of branching beaded fibers emanating from those cells, presumably transporting GnIH. Thus, it appears that all of the immunoreactive fibers originate in the PVN. Some of the fibers extend to terminals in the ME, consistent with a role for GnIH in pituitary gonadotropin regulation. In all birds studied, other fibers extend through the brain caudally at least as far as the brainstem and possibly into the spinal cord, consistent with multiple regulatory roles for GnIH (data only

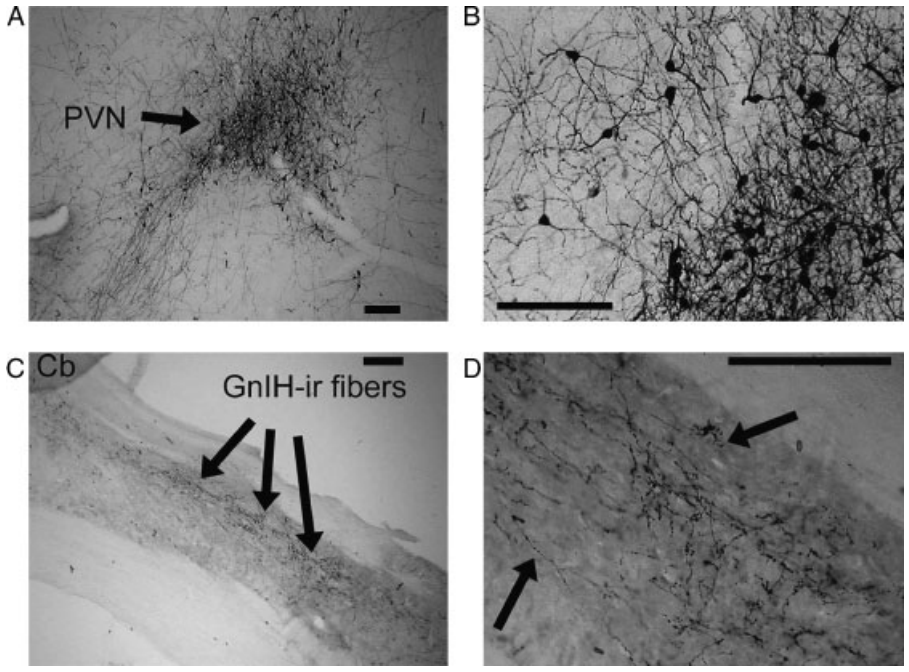

Fig. 1. (**A**) Sagittal section from female house sparrow brain with dense GnIH immunoreactivity in the PVN. (**B**) Higher magnification of the PVN of male house sparrow brain in sagittal section showing individual GnIH-ir neurons and fibers emanating from the cell bodies. (**C**) Sagittal section showing GnIH-ir fibers in the fasciculus longitudinalis medialis (FLM) of the brainstem of a female house sparrow. Arrows indicate GnIH-ir fibers. Cb = cerebellum. (**D**) Higher magnifications of (C). Scale bars, 100 μm. Modified from Bentley et al., 2003.

shown for house sparrows, Fig. 1C and D). In the brainstem, fibers appear to be restricted to the fasciculus longitudinalis medialis (FLM). In addition to hypothalamic and preoptic areas (POA), GnIH fibers and terminals are also present in septal areas, the ventral paleostriatum and the optic tectum.

Given the extensive projections of GnIH in avian species and the important role uncovered for GnIH in the regulation of the gonadotropins, it is critical to determine the generality of these findings. To date, GnIH-ir has been investigated in several rodent species, photoperiodic Syrian hamsters (*Mesocricetus auratus*), and less photoperiodic rats (*Rattus novegicus*) and mice (*Mus musculus*) (Ukena and Tsutsui, 2001; Kriegsfeld et al., 2006). As in birds, GnIH fibers form an extensive network extending along a midventral and dorsal continuum from the tenia tectum to the hindbrain. Cell bodies occupy a location slightly caudal to that of birds, being confined to the rostral-caudal extent of the dorsomedial hypothalamus (DMH). As with song birds, GnIH cell bodies are not seen outside the cluster of cells confined to the DMH. Fiber terminals were particularly concentrated in the tenia tecta, medial septum/diagonal band of Broca, lateral and ventrolateral septum, POA, amygdala, retrochiasmatic area, PVN, and arcuate. Diffuse fiber staining was seen as caudally as the brain stem. Unlike results seen in avian species (Bentley et al., 2003; Ukena et al., 2003), fibers were not detected in the external layer of the median eminence (ME) but were present in the internal layer. These findings suggest that GnIH might not reach fenestrated capillaries via a traditional pathway to regulate gonadotropins at the level of the pituitary as it likely does in birds. GnIH-induced LH release is rapidly pituitary (Anderson et al., 2005) inhibited by GnIH in vivo in rats, however presumably at the findings in mammals suggest an important role for GnIH in the regulation of reproduction across taxa and underscore the importance of studying how this novel system integrates with other well-established mechanisms of reproductive control.

SEASONALITY OF GNIH IN SONGBIRDS AND MAMMALS

Photoperiodic songbirds and mammals rely on the annual cycle of changing photoperiod to restrict their reproductive activity to the most

opportune time of year. In photoperiodic songbirds, long days cause not only reproductive maturation but also subsequent regression to a prepubertal state, in sequential order (see Nicholls et al., '88; Dawson et al., 2001 for reviews). Based upon research by Tsutsui and co-workers (Tsutsui et al., 2000; Satake et al., 2001), we predicted that GnIH may be important for the timing of reproduction in all photoperiodic bird species, as is the case for GnRH.

In song sparrows subjected to a simulated annual cycle of changing photoperiod, GnIH-ir neuron area was significantly greater at the termination of the breeding cycle (long day) when compared to non-reproductively active (short day) or reproductively active (long day) birds (Bentley et al., 2003). Without performing concurrent in situ hybridization analysis, the exact dynamics of GnIH synthesis and release in different photoperiodic conditions are unclear. It is clear that further study is needed to elucidate the seasonal dynamics of GnIH synthesis and release, but preliminary results from plasma radioimmunoassay (RIA) indicate that (a) GnIH can be released into the bloodstream and (b) the increase in neuron size at the onset of photorefractoriness represents an inhibition of release into the plasma (G.E. Bentley et al., unpublished data). These results are not consistent with our original hypothesis that GnIH may regulate termination of breeding. Even if it is the case that GnIH release into the bloodstream is regulated over the annual cycle, it might be that the other two peptides (GnIH-related peptides 1 and 2) that are also cleaved from the common precursor polypeptide continue to be produced at all times of year. Indeed, the presence of dense immunoreactive fibers throughout the brain in birds collected at all times of year provides evidence for this possibility, as the antibody we employed does not distinguish between GnIH, GnIH-RP-1, and GnIH-RP-2, which are all encoded by a single gene producing a large precursor polypeptide (Osugi et al., 2004).

The distribution of GnIH in multiple brain areas is consistent with it having multiple roles within the CNS. Thus it is entirely possible that the change in GnIH-ir neuron size and apparent change in plasma GnIH at the termination of reproduction is related to factors other than regulation of the HPG axis. For example, copulation solicitation, a reproductive behavior is enhanced in response to treatment with exogenous chicken GnRH-II (c-GnRH-II) (Maney et al., '97). The fact that GnIH-ir fibers appear to be in contact with c-GnRH-II neurons implies that GnIH or its related peptides might have a direct effect upon reproductive behavior.

Seasonality of GnIH has not yet been fully investigated in mammals. However, melatonin strongly influences GnIH production in quail (Ubuka et al., 2005), and similar effects of melatonin have been found in the mammalian homolog of GnIH (Inoue et al., unpublished observation). Thus it is likely that the GnIH system is an integral part of the ''photoperiodic machinery'' that drives seasonal reproduction in photoperiodic mammals.

GNIH RELATIVE TO GNRH-I AND -II IN SONGBIRDS AND MAMMALS

In birds and mammals, the widespread distribution of GnIH fibers and terminals to brain regions known to contain GnRH neurons suggested that GnIH might directly influence GnRH secretion. Likewise, in birds, projections to the outer layer of the ME suggested potential direct regulation of gonadotropin secretion (Tsutsui et al., 2000). To investigate these possibilities, we assessed the relative distributions of GnIH and GnRHs using double-label immunocytochemistry and a combination of brightfield, fluorescence, and confocal microscopy (Bentley et al., 2003; Kriegsfeld et al., 2006).

In songbirds, there is close proximity of GnIH-ir fibers to the cGnRH-I neurons and fibers in the POA (Fig. 2A). The PVN also contains cGnRH-I fibers which pass directly through and in close proximity to the population of GnIH-ir neurons and fibers as they project to the ME. It is clear that GnIH-ir fibers are also in close proximity to cGnRH-II neurons in the midbrain (Fig. 2B). (Data taken from house sparrows—see Bentley et al., 2003). Close appositions from GnIH-ir fibers can be seen in hamsters, using light microscopy (Fig. 2C and D—Kriegsfeld et al., 2006). Distribution of GnIH relative to GnRH is similar in quail (Ukena et al., 2003). Thus, the spatial association of GnIH and GnRH appears to be conserved, possibly with a similar mechanistic basis.

Given the resolution limitations of light microscopy, it is impossible to determine whether or not GnIH-ir fibers are in contact with GnRH-I or -II neurons. To this end, we turned to confocal microscopy. The following data are presented with the caveat that only electron microscopy

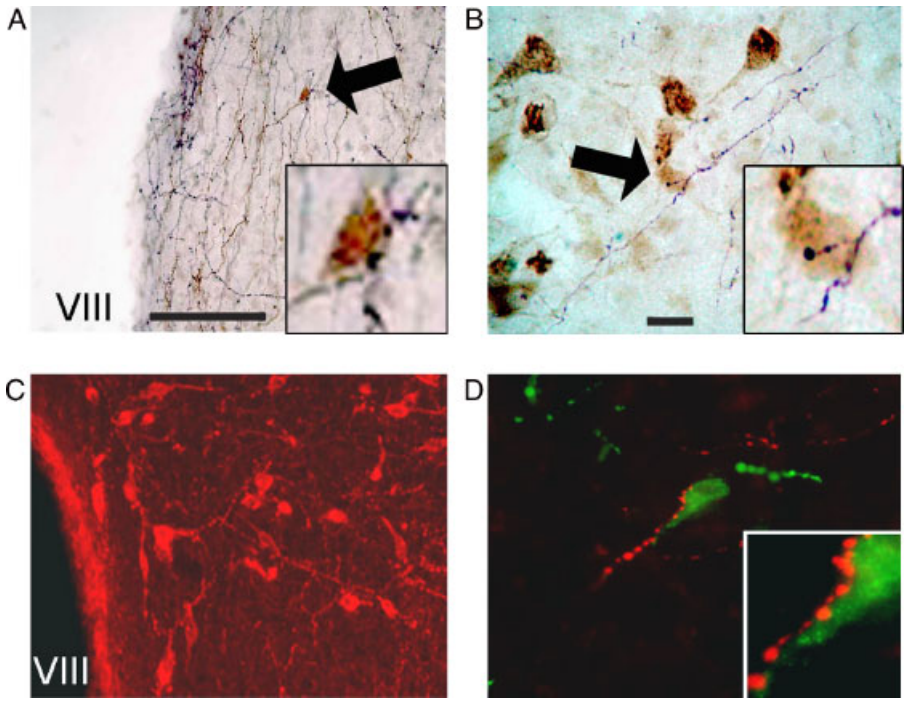

Fig. 2. (**A**) Coronal section through the main population of cGnRH-I neurons (brown, marked by black arrow) in the POA of a male house sparrow. Note the purple GnIH fibers. Inset shows higher magnification of the area indicated by the black arrow in the main photomicrograph. VIII = third ventricle; scale bar = 100 μm. (**B**) Coronal section through midbrain showing the main population of cGnRH-II neurons (brown), with GnIH-ir fibers (purple) in close proximity. Inset shows higher magnification of the area indicated by the black arrow in the main photomicrograph. Scale bar = 10 μm. (**C**) High-power coronal section depicting cell bodies for GnIH clustered in the DMH of a female Syrian hamster. (**D**) A GnIH (red) fiber projecting upon a GnRH (green) cell body and fiber. High-power inset allows one to see presumptive GnIH-ir boutons along the length of the GnRH fiber and cell body. Adapted from Bentley et al., 2003 and Kriegsfeld et al., 2006.

can provide unequivocal data on synaptic contact between GnIH-ir fibers and GnRH neurons and fibers.

In songbirds and hamsters, confocal microscopy indicates that the cGnRH-I and GnIH proteins are on the same scanned optical plane suggesting important functional interactions. A single cGnRH-I-ir neuron from the POA of a house sparrow is shown in Figure 3A (Bentley et al., 2003). A GnIH-ir fiber appears to be in contact with a cGnRH-I-ir neuron in this 0.2 μm optical plane. The same is true for GnIH-ir fibers and GnRH neurons in hamsters, rats, and mice (Fig. 3B; Kriegsfeld et al., 2006). The functional significance of these interactions is unclear at present, but provides potential for GnIH regulation of GnRH production and/or release. Additionally, apparent contact of GnIH-ir and GnRH-I fibers at the level of the ME in songbirds gives rise to potential for direct effects of GnIH on GnRH-I release to the portal blood system (Bentley et al., 2003).

GNIH EFFECTS ON LH RELEASE IN SONGBIRDS AND MAMMALS

Data collected in vitro indicated a direct effect of GnIH of pituitary release of LH in quail (Tsutsui et al., 2000). We built upon these findings in a series of laboratory and field experiments in different songbird species, as well as in hamsters (*Mesocricetus auratus*).

GnIH inhibits GnRH-induced LH release in a laboratory setting

Photorefractory (non breeding) male song sparrows ($n = 7$ per group) were used for this experiment (See Osugi et al., 2004 for details). Photorefractory birds were used so that we could control the amount of GnRH given to each bird (eliminating any confounding effects of endogenous GnRH). The pituitary gland of photorefractory birds remains responsive to exogenous GnRH even though little or no endogenous GnRH is released in this reproductive condition (Wingfield et al.,

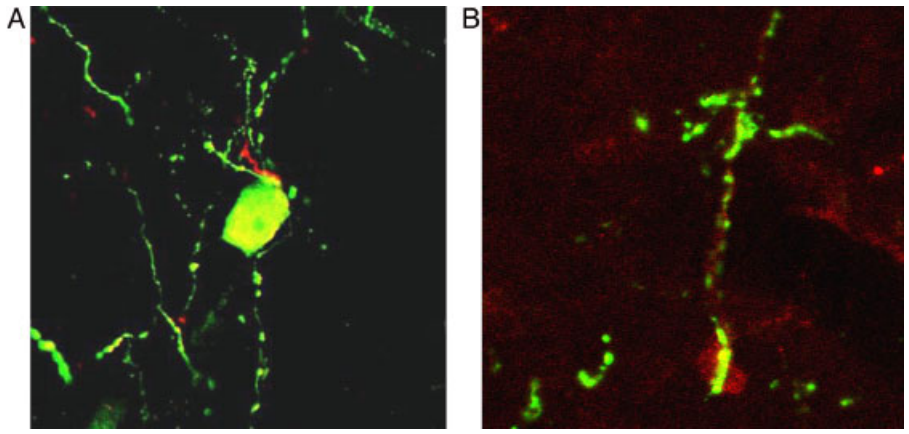

Fig. 3. (**A**) Confocal image of a cGnRH-I neuron and GnIH fibers in male house sparrow brain. Green depicts cGnRH-I-ir neuron and fibers, and red depicts GnIH-ir fibers. Note the GnIH-ir fiber apparently in contact with the center cGnRH-I-ir neuron. Optical plane of section = 0.2 μm. (**B**) Confocal image of a GnRH neuron and GnIH fibers in female Syrian hamster brain; GnIH fibers are seen in green while the GnRH cell is shown in red. Note the GnIH-ir fiber apparently in contact with the center GnRH-ir neuron and the fiber appears to repeatedly contact the cell along its process. Optical plane of section = 1.0 μm. Modified from Bentley et al., 2003 and Kriegsfeld et al., 2006.

'79; Nicholls et al., '88; Dawson et al., 2001). Group I (control) was given an intravenous (i.v.) injection (into the right jugular vein) of 10 ng GnRH in 20 μl physiological saline. Group II (experimental) was given an i.v. injection of a mixture of 10 ng GnRH plus 1,000 ng GnIH in 20 μl physiological (0.9%) saline. Blood samples were taken from the alar vein at 2, 5, and 10 min after injection. This protocol has been used previously to demonstrate rapid gonadotropin-releasing activity of GnRH in songbirds (e.g., Wingfield et al., '79; Wingfield and Farner, '93). The LH data from this experiment are shown in Figure 4A (taken from Osugi et al., 2004). At 2 min post-injection, birds injected with GnRH alone had higher plasma LH at 2 min than at 10 min. Birds injected with the GnRH/GnIH cocktail also had elevated plasma LH at 2 min post-injection, but this increase in LH was much attenuated compared with the control group. The difference in plasma LH concentration between the two groups was no longer present at 5 and 10 min post-injection, by which time plasma LH had returned to baseline values in both groups.

We saw a similar rapid effect of quail GnIH on LH release in castrated, photostimulated white-crowned sparrows (Osugi et al., 2004). Further, administration of GnIH over a 30 sec period via intracerebroventricular (i.c.v.) infusion into the third ventricle caused a rapid decrease (within 5 min) of plasma LH in photostimulated female white-crowned sparrows. This decrease was no longer apparent 20 min after infusion of GnIH (Bentley et al., 2006).

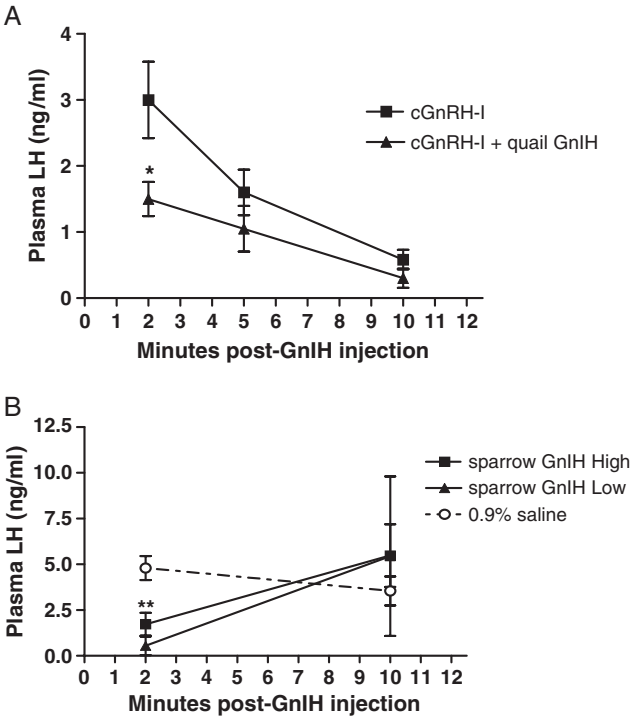

Fig. 4. (**A**) The effects of intra-jugular injection of quail GnIH on GnRH-elicited LH release in photorefractory male song sparrows. Note that plasma LH was lower in the GnRH+GnIH group than in the control (GnRH alone) group at 2 min post-injection. $^{*}P<0.05$ GnRH alone vs. GnRH+GnIH by ANOVA, followed by Fisher's PLSD for post-hoc analysis. (**B**) The effects of sparrow GnIH on plasma LH in field-injected, breeding Gambel's white-crowned sparrows. Plasma LH was lower in the GnIH-injected group at 2 min but at no other time. $^{**}P<0.01$ GnIH high vs. vehicle by ANOVA, followed by Fisher's PLSD for post-hoc analysis. See Osugi et al. (2004) for details.

GnIH inhibits plasma LH release in a field setting

White-crowned sparrow GnIH with the deduced amino acid sequence SIKPFSNLPLRF-NH_2 (see Osugi et al., 2004; and Tsutsui et al., 2006a,b) was synthesized and used for i.v. injections in field-caught Gambel's white-crowned sparrows on their breeding grounds in Alaska in June. Plasma LH is typically high during this period of the breeding life-history stage. Birds were captured in mist nets and saline or GnIH were injected into the jugular vein within 3 min of capture. Blood (approx 100 μl) was subsequently drawn from the alar vein at 2 and 10 min post-injection. Birds that were injected with 1,000 ng GnIH had significantly lower plasma LH at 2 min than the saline-injected group (Fig. 4B from Osugi et al., 2004). At 2 min post-injection, the same pattern was seen in birds injected with 500 ng GnIH, but the sample size in this group was too small for statistical analysis. Thus, GnIH can rapidly affect LH release in birds in vivo in laboratory and field settings, as well as in vitro.

GnIH inhibits plasma LH release in hamsters

Effects of GnIH upon plasma LH were found to be similar in Syrian hamsters providing evidence for conserved properties of the physiological actions of GnIH (Kriegsfeld et al., 2006). Administration of 600 ng GnIH intraperitoneally (i.p.) caused a significant suppression of plasma LH at 15, 30, and 60 min post-administration, but not at 5 min (Fig. 5). These findings are similar to those found in quail (Ubuka et al., unpublished data and see Tsutsui et al., 2006a, this volume). i.c.v. infusion of GnIH into hamsters also caused a rapid reduction in plasma LH, as in birds (Fig. 5). Strikingly, the reduction in LH after i.c.v. infusion lasted for up to 30 min (Kriegsfeld et al., 2006) compared with 5 min or so in white-crowned sparrows. However, the peptide was infused over a longer period (15 min) in the hamster study.

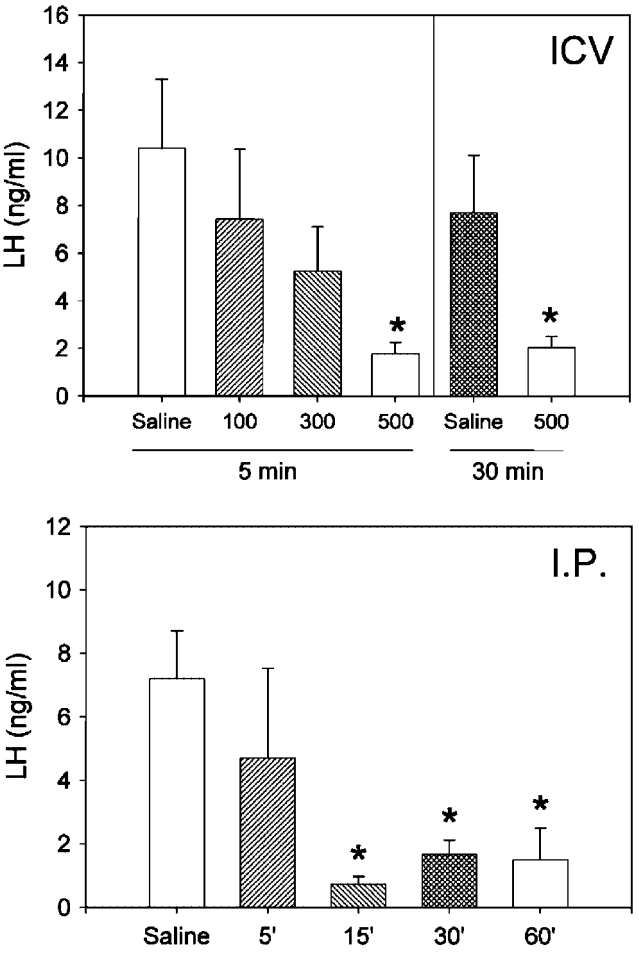

Fig. 5. GnIH delivery rapidly and dose-dependently inhibits LH. Ovariectomized Syrian hamsters were injected with GnIH either ICV (5 μl into the lateral ventricle of 0, 100, 300, or 500 ng in saline) or peripherally (0.2 cc, i.p. with 0 or 600 ng). When injected ICV, GnIH reduced LH concentrations in a dose-dependent manner with suppression sustained 30 min following the most effective dose. Similar results were obtained with peripheral injections, although the time course of suppression was slightly slower. Modified from Kriegsfeld et al., 2006.

CONCLUSIONS

Although it remains to be confirmed whether GnIH is present in all vertebrates, we have been struck by the similarities in our findings on GnIH in birds and mammals. The overall distribution of GnIH, its distribution relative to GnRH, and its action on LH release appear to be conserved between birds and mammals. The fact that melatonin can regulate GnIH production in birds and mammals indicates that the mechanisms regulating its expression are also conserved. The as yet unknown actions of the other two peptides that are cleaved from the GnIH precursor polypeptide, GnIH-RP-1 and -RP-2, are therefore also possibly conserved across vertebrate classes. In sum, we have only just begun to uncover the actions of GnIH; it is likely that GnIH and its related peptides will prove to be involved in an increasing number of fundamental aspects of physiology and behavior during coming years.

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