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Rapid inhibition of female sexual behavior by gonadotropin-inhibitory hormone (GnIH)

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Abstract

Gonadotropin-releasing hormone (GnRH) is largely responsible for the initiation of sexual behaviors; one form of GnRH activates a physiological cascade causing gonadal growth and gonadal steroid feedback to the brain, and another form is thought to act as a neurotransmitter to enhance sexual receptivity. In contrast to GnRH, gonadotropin-inhibitory hormone (GnIH) inhibits gonadotropin release. The distribution of GnIH in the avian brain suggests that it has not only hypophysiotropic actions but also unknown behavioral actions. GnIH fibers are present in the median eminence (ME) and are in apparent contact with chicken GnRH (cGnRH)-I and -II neurons and fibers. In birds, cGnRH-I regulates pituitary gonadotropin release, whereas cGnRH-II enhances copulation solicitation in estradiol-primed females exposed to male song. In the present study, we determined the effects of GnIH administered centrally to female white-crowned sparrows. A physiological dose of GnIH reduced circulating LH and inhibited copulation solicitation, without affecting locomotor activity. Using rhodaminated GnIH, putative GnIH binding sites were seen in the ME close to GnRH-I fiber terminals and in the midbrain on or close to GnRH-II neurons. These data demonstrate direct effects of GnIH upon reproductive physiology and behavior, possibly via separate actions on two forms of GnRH.
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Keywords: GnRH; GnIH; Lordosis; Copulation; Sexual behavior; Proceptivity; Receptivity; LH

Introduction

Female sexual behaviors (proceptivity and receptivity) are likely to induce copulatory behavior and, presumably, successful fertilization by males of the same species (Beach et al., 1976). To understand sexual selection, we also need to understand the physiological mechanisms regulating female mate choice and copulation solicitation. Songbirds possess two forms of gonadotropin-releasing hormone (GnRH), a neuropeptide that regulates reproductive status. One form of GnRH is produced by a population of hypothalamic neurons in the pre-optic area (POA) and projects fibers to the median eminence, where chicken GnRH-I (cGnRH-I) is released to the hypothalamo-hypophysial portal system, causing gonadotropin release (Sharp et al., 1990). The second form of GnRH, cGnRH-II, is produced in the midbrain (region of the oculomotor complex) and possibly acts as a neurotransmitter (Temple et al., 2003). Administration of GnRH-I and GnRH-II in vitro and in vivo causes pituitary gonadotropin release (Millar et al., 1986; Wingfield and Farner, 1993). Despite its potential role as a gonadoliberin when administered exogenously, GnRH-II neurons in songbirds are not hypophysiotropic, thus precluding a direct role for GnRH-II in pituitary hormone release. Although the function(s) of cGnRH-II has remained enigmatic, recent reports indicate a critical role for GnRH-II in the regulation of female sexual behavior (Maney et al., 1997; Kauffman and Rissman, 2004).

A recently discovered neuropeptide, gonadotropin-inhibitory hormone (GnIH), acts in opposition to GnRH-I (Tsutsui et al., 2000). GnIH is a novel hypothalamic dodecapeptide having a

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C-terminal RF-amide motif (first identified in quail as SIKPSAYLPLRF-NH2; Tsutsui et al., 2000). GnIH administration decreases plasma LH in vitro and in vivo in birds and in mammals (Tsutsui et al., 2000, 2005; Osugi et al., 2004; Bentley et al., in press). Furthermore, in vivo GnIH administration to quail for 2 weeks via osmotic pumps decreases the expressions of gonadotropin common α and LH β-subunit mRNAs in a dose-dependent manner, with a knock-on effect of reducing plasma testosterone (Ubuka et al., in press). It appears that GnIH binds to the pituitary gland via specific G-protein-coupled receptor for GnIH to exert rapid action on pituitary gonadotropin release (Yin et al., 2004). In addition to rapid action on the pituitary gland, the distribution of GnIH in the avian brain is suggestive of potential for direct action upon cGnRH-I and -II (Bentley et al., 2003; Ukena et al., 2003). Fibers that are immunoreactive for GnIH (GnIH-ir) are in close proximity to, and possibly in contact with, cGnRH-I neurons in the POA and fiber terminals in the median eminence. Furthermore, GnIH-ir fiber terminals are in close proximity to (or in contact with) cGnRH-II neurons in the midbrain.

Sexual receptivity in female rodents is indicated by lordosis (Beach et al., 1976). Central administration of GnRH (via intraventricular infusion) facilitates lordosis in estrogen-primed rats (Moss and McCann, 1973, 1975; Pfaff, 1973; Dudley et al., 1983). Further, central infusions of an antibody to GnRH reduce lordosis (Sakuma and Pfaff, 1983). As in rats, estrogen-primed songbirds exhibit a lordosis-like behavior in response to central administration of GnRH—in this case, GnRH-II (Maney et al., 1997). Because of the antagonistic actions of GnRH and GnIH upon gonadotropin release, it is likely that they act antagonistically upon reproductive behavior as well—especially considering the distribution of GnIH-ir fibers. If this turns out to be the case, then rapid activation or deactivation of the GnIH system could provide a mechanism by which female songbirds respond correctly at a specific time of year to social cues and copulate with the appropriate partner (i.e., of the correct age and species). Captive female songbirds, once they are estradiol-primed, will solicit copulation in response to an audio recording of male song. When a female solicits copulation, she raises her tail and head while at the same time fluttering her wings and giving a characteristic vocalization. This display can be considered a combination of sexual proceptivity and receptivity. A solicitation display assay (SDA) is commonly used in passerine research to quantify the responsiveness of females to song (King and West, 1977; Brenowitz, 1991; Searcy, 1992). Given the potential for interactions of GnIH and the two avian GnRHs, we sought to determine what effects centrally administered GnIH might have on the known functional outputs of cGnRH-I (gonadotropin release) and cGnRH-II (copulation solicitation).

Materials and methods

Birds and treatments

All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the approval of the University of Washington Animal Care and Use Committee.

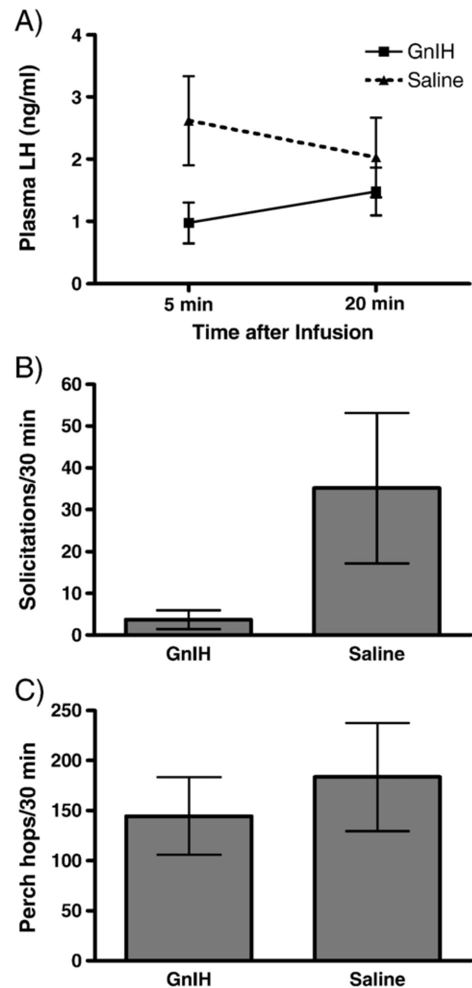

Fig. 1. (A) The effect of intra-cerebroventricular infusion of white-crowned sparrow GnIH (ICV GnIH) on plasma LH in photostimulated female Gambel's white-crowned sparrows. Plasma LH was lower in the GnIH-infused birds at 5 min post-infusion but not at 20 min. (B) The effect of ICV GnIH on copulation solicitations made by estradiol-implanted female Gambel's white-crowned sparrows in response to male song playback. GnIH-treated birds solicited exhibited fewer solicitation displays than saline-treated birds during the 30-min period following infusion. (C) The effect of ICV GnIH on locomotor activity in female Gambel's white-crowned sparrows. GnIH-treated birds did not become less active than controls, as measured by the total number of perch-hops during the 30-min period following infusion. In all cases, $n = 6$ and bars depict SEM.

Adult female white-crowned sparrows, *Zonotrichia leucophrys gambelii*, were captured in Central Washington during their autumn migration. Thus, they were in non-breeding condition (photorefractory) with low circulating gonadotropins. The birds were housed in outdoor aviaries under naturally changing day lengths until the end of January (natural photoperiod approximately 9-h light and 15-h dark per 24-h period; 9L:15D). At this point, the reproductive system is termed photosensitive—i.e., it is able to be stimulated by increasing spring day lengths. Using a stereotaxic frame customized for passerine birds (MyNeuroLab.com), each bird was chronically implanted with a 10-mm, 26-gauge steel cannula guide (Plastics One; C315G) aimed at the third ventricle. Anesthesia was achieved using 1%–4% isoflurane/oxygen gaseous mix. The cannula guide tip was placed 2.3 mm anterior to the intersection of the midsagittal and transverse sinuses and 6.0 mm ventral to the surface of the brain. The guide was affixed with dental acrylic and kept clear of obstruction using a 33-gauge internal dummy cannula (Plastics One, C315DC). Birds were immediately transferred to single-occupation cages within an

environmental chamber and allowed to recover for 5 days prior to testing of cannula patency. Photoperiod within the chamber was set to 16L:8D to mimic long, spring-like photoperiods and to stimulate gonadotropin production. Patency of the cannulation was tested using an infusion of 1 μg angiotensin-II (Ang-II, human, Bachem Inc., Torrance, CA). This peptide stimulates drinking behavior within 2 min of infusion in white-crowned sparrows (Richardson and Boswell, 1993; Maney et al., 1997). Paired saline control infusions were performed at the same time, and treatments were reversed 24 h later. Any birds that did not exhibit drinking behavior within 2 min of angiotensin-II infusion were excluded from the experiment. Birds infused with saline did not drink within 5 min of the infusion. Infusions for all experiments were performed as follows: each bird was captured from its cage and held in the experimenter's hand during infusion. It was conscious at all times. The internal dummy cannula was removed and replaced with an infusion cannula connected to plastic tubing pre-loaded with the infusate and a Hamilton syringe. The plunger of the syringe was depressed slowly over a 30-s period, and the infusion cannula was held in place for a further 60 s. The dummy cannula was then replaced, and the bird was released into its cage. The fact that the birds solicited copulation very soon after this procedure indicates that it entailed minimal stress.

Experiment 1: effect of ICV GnIH upon plasma luteinizing hormone (LH)

To determine the effects of central administration of GnIH upon circulating LH, pairs of birds (total, $n = 6$) were infused i.c.v. with either GnIH (200 ng in 4 μl sterile avian saline) or 4 μl sterile avian saline. Dosage of GnIH was calculated based upon amounts of GnIH detected in quail brain (Tsutsui et al., 2000), and infusions took place 7 days after initial cannulation (2 days after testing of cannula patency). Blood samples were collected from the brachial vein at 5 and 20 min post-infusion. Treatments were reversed 5 days later. In this way, birds acted as their own controls. Plasma was then separated via centrifugation and stored at −20°C until assayed for LH.

Experiment 2: effect of ICV GnIH upon copulation solicitation behavior

Four days after the final infusions and bleeds in Experiment 1, all birds were implanted subcutaneously with one 12-mm estradiol (E2) silastic implant (see Moore, 1983; Maney et al., 1997). After a further 17 days, all birds received a second estradiol implant, as solicitation in response to audio playback of male white-crowned sparrow song was intermittent. After a further 2 days, all birds avidly solicited copulation in response to male song playback. Infusions and behavioral trials began the following day.

Infusions and behavioral trials

For each trial, a pair of birds was transferred (in their respective cages) from the home chamber to an empty environmental chamber. Thus, each pair had visual and auditory contact with its neighbor from the home chamber. The pair of birds was allowed to habituate to the test chamber for 1 h, at which point one of the pair was infused i.c.v. with GnIH (200 ng in 4 μl sterile avian saline) and the other with 4 μl sterile avian saline. Birds were infused one after the other; total infusion time for the pair of birds was under 5 min (time taken to remove birds from their cages, infuse them and return them to their cages). Video recording began immediately, as did audio playback of male white-crowned sparrow song. Audio playbacks consisted of a selection of field recordings of male white-crowned sparrow song ($n = 6$ individuals) interspersed with periods of silence. Playbacks and videotape continued for 30 min, at which point the trial was terminated and birds returned to their home chamber. Treatments were reversed the following day or over the subsequent 2 days. All trials were carried out at the equivalent time of day. The same audio playback tape (song from $n = 6$ individuals) was used for each trial. Thus, each bird acted as its own control (saline vs. GnIH).

Once all trials had been completed, videotape was scored by two individuals who were unaware as to the treatment status of each bird. The number of total copulation solicitations per individual was tallied, as was the number of perch-hops (a measure of locomotor activity). Blood samples were also collected for measurement of plasma estradiol by radioimmunoassay.

Experiment 3: localization of putative GnIH binding sites within the brain: in vivo receptor autoradiography

Following behavioral trials, birds were infused with either rhodaminated GnIH (rhod-GnIH; 4 μg in 4 μl saline; $n = 4$) or free rhodamine (28 μg in 4 μl saline; $n = 2$). They were then heavily anesthetized with 4% isoflurane/oxygen and decapitated 2 min post-infusion. Brains were rapidly extracted on to dry ice for processing at a later date. Rhodaminated GnIH was custom-made (Pacific Immunology, CA) and consisted of (rhodamine B)SIKPFSNLPLRF-NH2; code: Bentley 3N05-2-PAC(GnIH-12); MW: 1844.5 (theoretical), 1843.7 (detected); >90% purity.

Frozen brains were sectioned coronally at 40 μm, thaw mounted, and coverslipped using an aqueous mounting medium. Putative rhod-GnIH binding sites were visualized using fluorescence microscopy.

Hormone assays

Plasma was assayed for LH using the homologous chicken LH radioimmunoassay (RIA) (Follett et al., 1972). All samples were run in duplicate in a single assay. Intra-assay coefficient of variation was 4.2%, and the detection limit was 0.039 ng/ml. Following the second phase of the experiment involving estradiol implants, plasma estradiol was also measured via radioimmunoassay (Wingfield and Farner, 1975). All samples were run in duplicate in a single assay. Intra-assay coefficient of variation was 3.45%, and the detection limit was 0.019 ng/ml.

Results

Experiment 1: effect of ICV GnIH upon plasma luteinizing hormone (LH)

Five min post-infusion, plasma LH was lower in GnIH-treated birds than in saline-treated birds (repeated measures ANOVA; $F = 4.201$ (1,11) $P < 0.04$. This difference was not apparent 20 min post-infusion. Results are summarized in Fig. 1A.

Experiment 2: effect of ICV GnIH upon copulation solicitation behavior

In the 30 min following central infusions, GnIH-treated birds solicited copulation significantly fewer times than those treated with saline (repeated measures ANOVA; $F = 6.311$ (1,11) $P < 0.04$. These data are shown in Fig. 1B. All birds solicited copulation when treated with saline, but at different frequencies. Not all birds solicited copulation when treated with GnIH, yet overall locomotor activity was the same in both groups (repeated measures ANOVA; $F = 0.345$ (1,11) $P = 0.56$. These data are shown in Fig. 1C.

Following implantation with estradiol, mean plasma estradiol concentration was 6.79 ± 0.44 ng/ml.

Experiment 3: localization of putative GnIH binding sites within the brain: in vivo receptor autoradiography

When infused directly into the brain, rhodamine-conjugated GnIH appeared to bind specifically to areas in the diencephalon and the midbrain. Major areas of binding were the internal layer of the median eminence (ME), the nST (nucleus stria terminalis) and the oculomotor complex, the midbrain area that contains

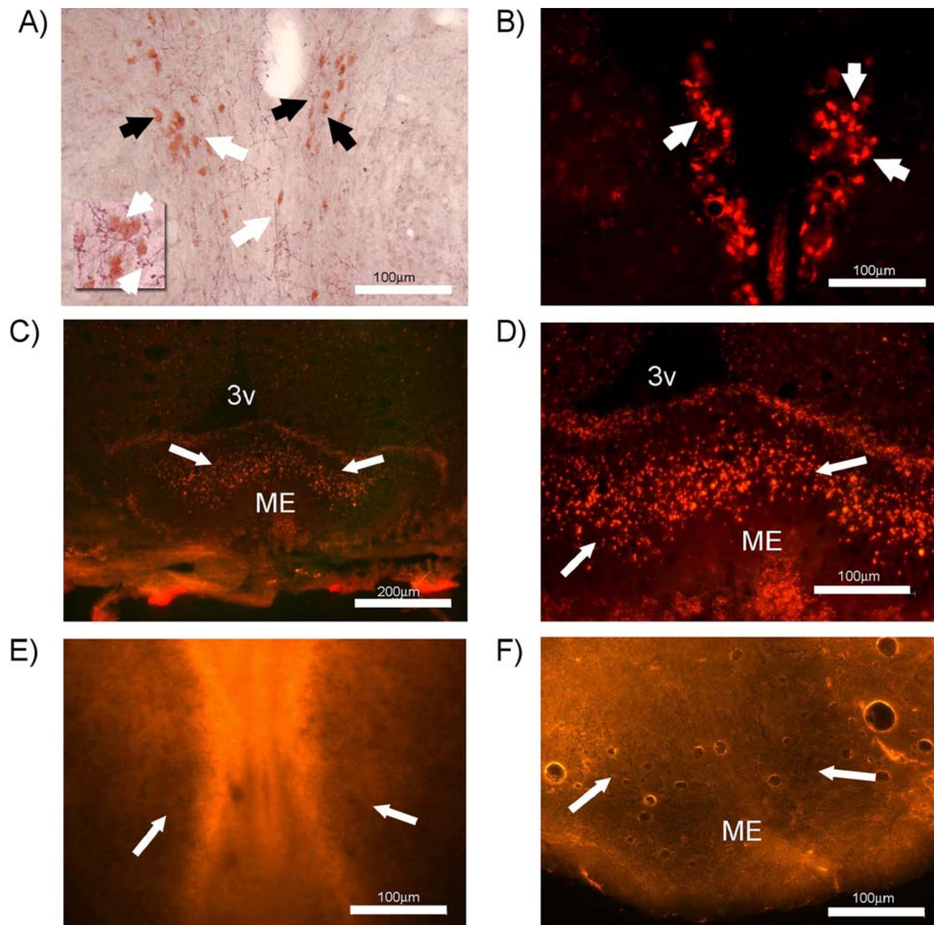

Fig. 2. GnRH-II/GnIH double-label immunocytochemistry and in vivo receptor autoradiography using rhodaminated GnIH (rhod-GnIH). (A) Coronal section through midbrain (oculomotor complex) showing the main population of cGnRH-II neurons (brown, marked by black arrows) with GnIH-ir fibers in close proximity (purple, marked by white arrows). Inset: higher magnification of section from the same animal. Immunocytochemically labeled section prepared as in Moss and McCann (1973). (B) Coronal section through midbrain (oculomotor complex) showing putative rhod-GnIH binding sites (marked by white arrows) on GnRH-II neuron-like entities in the vicinity of the oculomotor complex. (C) Coronal section through the median eminence, showing putative Rhod-GnIH binding sites on the internal layer of the median eminence (marked by white arrows). 3V—third ventricle; ME—median eminence. (D) Higher magnification of panel C. (E) Coronal section through midbrain (oculomotor complex, region marked by white arrows) showing no visible binding of free rhodamine after ICV infusion. (F) Coronal section through median eminence (region marked by white arrows) showing no visible binding of free rhodamine after ICV infusion.

cGnRH-II immunoreactive neurons. These data are shown in Figs. 2A–D. Unconjugated rhodamine showed no binding in any area of the brain, including the ME and oculomotor complex, as shown in Figs. 2E–F.

Discussion

Since its discovery, the inhibitory action of GnIH on pituitary release of gonadotropins has been firmly established in birds and mammals (Tsutsui et al., 2000, 2005; Osugi et al., 2004; Bentley et al., in press; Ciccone et al., 2004). Despite the focus of attention upon regulation of the release of gonadotropins, the distribution of GnIH-ir fibers in the central nervous system of birds and mammals has been suggestive of more than one function of this RF-amide peptide (Tsutsui et al., 2000; Ukena and Tsutsui, 2001; Bentley et al., 2003, in press; Ukena et al., 2002, 2003). Clear projections, with possible contact, of GnIH-ir fibers to cGnRH-II neurons in the songbird brain led us to hypothesize that GnIH might be involved in regulation of cGnRH-II-mediated behavior, as well as in regulation of gonadotropin release. Given the inhibitory nature of the effect of GnIH upon gonadotropin release, we predicted that GnIH would also inhibit cGnRH-II-mediated behavior, in this case, copulation solicitation.

Taken together, the data from the present study strongly indicate central effects of GnIH upon reproductive physiology and behavior. We observed a decrease in circulating LH in response to GnIH infusion, as well as a reduction in the frequency of copulation solicitation. We saw no apparent effect upon locomotor activity after GnIH infusion, suggesting that the observed effects of GnIH were not an effect of general debilitation or somnolence. Birds remained alert and active after infusions. In a separate, pilot experiment, a cocktail of GnIH and angiotensin-II was infused in the manner described in the present study, and drinking behavior was not different from that observed in birds infused solely with angiotensin-II. All birds drank within 2 min of infusion. Again, this is suggestive that the effect of GnIH at this dose (200 ng) is specific.

A further line of evidence that is consistent with a direct and specific effect of GnIH upon the central components of the reproductive system comes from the use of rhodaminated GnIH. Binding of rhod-GnIH was observed in the median eminence, the nST and the vicinity of cGnRH-II neurons in the oculomotor complex. Very little binding was observed elsewhere in the brain, and no binding of "free" rhodamine was observed anywhere in the brain. Given the previously published histological data indicating the location of GnIH fiber terminals (Bentley et al., 2003), it might be expected that GnIH would bind in the ME and oculomotor complex. It is therefore compelling that binding of rhod-GnIH was restricted to those areas of the brain, and that the physiological and behavioral data in the present study are in line with these findings. Of course, GnIH might be acting on reproductive physiology and behavior via some other, as yet unknown, hormonal or neural mechanism. This could certainly be true if the putative GnIH binding sites turn out not to be true locations of GnIH receptor. In addition, i.c.v. GnIH effects upon reproductive physiology and behavior have yet to be demonstrated in free-living breeding birds that are not artificially steroid-primed.

It is perhaps noteworthy that the effect of GnIH upon LH release was consistent with an effect on cGnRH-I terminals, but that the majority of the binding of rhod-GnIH was in the internal layer of the ME, and not in the external layer, where the cGnRH-I terminal fields are located. The internal layer of the ME corresponds to where some GnIH fiber terminals are located; it also corresponds to where many glia are (Yamamura et al., 2004). Thus, it is possible that GnIH (a) does not affect cGnRH-I release, (b) influences cGnRH-I release to the ME indirectly via action upon glial cells (Yamamura et al., 2004), or (c) acts directly on cGnRH-I fibers—just not at the terminal. Finally, the fact that an effect of GnIH was seen at 5 min does not preclude a more rapid effect via some other mechanism.

In summary, the experiments described in the present study describe central effects of GnIH upon reproductive physiology and behavior. Centrally administered GnIH reduces plasma LH. It also inhibits copulation solicitation behavior in female white-crowned sparrows in response to male song. Rhodaminated GnIH appears to bind to areas involved both in the regulation of gonadotropin release and copulation solicitation. Therefore, we conclude that GnIH is likely to be a major component in the regulation of reproductive behavior of birds as well as a known component in the regulation of reproductive physiology. Given that GnIH-ir fibers are located in other brain areas (e.g., visual system, nST, brainstem), we expect that other actions of GnIH and its related peptides are likely to exist.

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