

Female pheromones stimulate release of luteinizing hormone and testosterone without altering GnRH mRNA in adult male Syrian hamsters (*Mesocricetus auratus*)

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Abstract

In many species chemosensory stimuli function as important signals that influence reproductive status. Neurons synthesizing the peptide gonadotropin-releasing hormone (GnRH) are critical mediators of reproductive function via their regulation of the hypothalamic–pituitary–gonadal (HPG) axis, and they are thought to be responsive to chemosensory information. In the present study, we sought to elucidate the effects of female chemosensory stimuli on the HPG axis in sexually naive adult male Syrian hamsters. In Experiment 1, serial blood samples were collected from catheterized male hamsters following exposure to female pheromones in order to characterize the luteinizing hormone (LH) response to this chemosensory stimulus. In Experiment 2, brains and terminal blood samples were collected from animals 0, 60, and 120 min following pheromone exposure. GnRH mRNA was measured in brain tissue sections using in situ hybridization, and plasma concentrations of LH and testosterone were measured using radioimmunoassay. Data from Experiment 1 indicated that female pheromones elicited a rapid rise in plasma LH that peaked at 15 min and returned to baseline 45 min after exposure. In Experiment 2, testosterone was elevated in terminal blood samples obtained 60 min, but not 120 min, after exposure to pheromones. LH levels were unaffected at both of these time points. The chemosensory-induced increases in LH and testosterone release were not accompanied by subsequent changes in GnRH mRNA over the time course studied. These data suggest that while activation of the male HPG axis by female pheromones involves release of GnRH, it does not involve increases in GnRH mRNA 1–2 h after pheromonal stimulation as a mechanism for replenishment of released peptide.

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1. Introduction

In many species, including humans, chemosensory stimuli function as social cues that impact reproductive hormones and behavior (Doty, 2001; Jacob et al., 2001; McCoy and Pitino, 2002; Meredith, 1998; Wirsig-Wiechmann, 2001). In rodents, female odors or pheromones activate neurons in limbic circuits mediating male reproductive behavior and elicit gonadotropin and gonadal steroid release in sexually naive males (Meredith, 1998). Furthermore, female chemosensory stimuli can be used to establish a classically conditioned

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endocrine response to a neutral stimulus, confirming their roles as unconditioned stimuli for evoking reproductive responses in males (Graham and Desjardins, 1980).

The hypothalamic peptide gonadotropin-releasing hormone (GnRH) is a critical mediator of the male's endocrine and neural responses to female chemosensory stimuli (Fernandez-Fewell and Meredith, 1995; Meredith, 1998). First, via its endocrine role in the hypothalamic pituitary gonadal (HPG) axis, GnRH governs gonadotropin and gonadal steroid hormone secretion (Cattanach et al., 1977). Second, GnRH functions centrally as a neuromodulator of behavioral circuits. For example, mating behavior of male hamsters is abolished by removal of the vomeronasal organ, but this behavioral deficit can be reversed by *icv* administration of GnRH (Fernandez-Fewell and Meredith, 1995; Meredith and Howard, 1992; Pfeiffer and Johnston, 1994). Thus, female chemosensory stimuli presumably activate GnRH neurons, either directly or indirectly.

While the expression of reproductive behavior in sexually naïve male Syrian hamsters is absolutely dependent on female pheromones present in vaginal secretions and their transduction by the vomeronasal system (Meredith, 1986; Meredith and Howard, 1992), details underlying this process are not well-understood. In males, exposure to female pheromones elicits a rise in testosterone within 60 min (Romeo et al., 1998), but the LH response that presumably precedes the gonadal steroid response has not been documented in this species. Similarly, it is not known which subpopulation(s) of GnRH cells are involved in neuroendocrine and behavioral response to pheromones. As GnRH cellular function is thought to differ depending on location within the brain and surrounding neurochemical environment (Petersen et al., 1995; Porkka-Heiskanen et al., 1994; Richardson et al., 2002; Selmanoff et al., 1991; Spratt and Herbison, 1997; Tang et al., 1997), it is important to establish which GnRH cell populations respond to chemosensory input. C-fos expression has thus far not proved to be a useful marker of pheromone-induced activation of GnRH neurons in males (Meredith, 1998). On the other hand, under some conditions changes in GnRH mRNA have been associated with changes in GnRH release, providing an alternative approach to identifying activated GnRH cells (Gore and Roberts, 1994, 1995; Gruenewald and Matsumoto, 1993; Parfitt et al., 1999; Richardson et al., 2002; Spratt and Herbison, 1997). Whether female pheromones elicit changes in GnRH mRNA in male Syrian hamsters is not presently known. Thus, the purpose of this study was twofold: (1) to characterize the LH and testosterone responses to female pheromones in male hamsters, and (2) to determine whether increases in GnRH mRNA are associated with activation of GnRH neurons by female pheromones, and if so, whether pheromone-induced changes in

mRNA occur selectively in certain populations of GnRH neurons.

2. Methods

2.1. Animals

Sexually naïve adult male Syrian hamsters (Charles River, Kingston, NY) were used in the following two experiments. Animals were singly housed in clear polycarbonate cages (30.5 × 20.5 × 30.5 cm). All animals had ad libitum access to food (Teklad Rodent Diet No. 8640, Harlan, Madison, WI) and water throughout the study. The animal rooms were on a 14:10 light–dark cycle and ambient temperature was maintained at 21 ± 2°C. Experiment 1 was conducted between 09:00 and 13:00h, with lights off at 13:00h. Experiment 2 was conducted between 09:00 and 12:00h, with lights off at 20:00h. All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Michigan State University All-University Committee for Animal Use and Care.

2.2. Experimental design for Experiment 1: LH response to pheromones

Under sodium pentobarbital anesthesia (80 mg/kg, *ip*) six males were fitted with jugular catheters. Catheterization and blood sampling protocols were modifications of a previously published paradigm (Gillespie et al., 1999). Animals were allowed at least 48 h of recovery following surgery before blood sampling.

In order to fully characterize the LH response to pheromones across time we needed to obtain frequent (5 min intervals) blood samples before, during, and following pheromone exposure. Thus, throughout the sampling period animals were given back their own red blood cells (diluted 1:1 with heparinized saline), which were obtained following centrifugation and removal of plasma from samples obtained at earlier time points of the experiment. Pilot work demonstrated that blood replacement did not alter plasma LH levels. Sampling began with five baseline samples (0.2 cc each) taken at 5 min intervals. A cotton swab that was either dry (control) or contained vaginal secretions from estrous female hamsters (pheromone) was placed in the animal's cage immediately after the fifth baseline sample. Blood sampling continued at 5 min intervals for 30 more minutes. The final three samples were taken at 15 min intervals so that the last sample was withdrawn 75 min after the swab was first presented to the animal. Because of the technical complexity of this experiment, and to maximize data obtained from these subjects, four of the six hamsters were sampled twice (with at least 24 h between

sampling periods) and two were sampled once for a total of 10 sampling periods. Whether the animal was exposed to pheromone or control swabs was randomly determined on the day of sampling. Six of the sampling periods followed exposure to pheromone swabs and four followed exposure to control swabs.

The first two baseline samples were used to obtain red blood cells for replacement but did not produce enough plasma to measure LH. Therefore, averaging the third, fourth, and fifth baseline samples determined baseline for each sampling period. The percent change from baseline was then calculated for each subsequent sample after this point. Plasma LH response to pheromone and control swab exposure are plotted as percent change from baseline over time.

2.3. Experimental design for Experiment 2: effect of pheromones on GnRH mRNA

A separate group of animals was used for Experiment 2. Animals were left in their home cage and cotton swabs containing vaginal secretions from estrous females were placed in the cage. Animals were then killed by decapitation at 0 (no swab dropped), 60, and 120 min after exposure to female pheromones ($n = 5/\text{time point}$). These time points were chosen in order to assess whether GnRH mRNA levels were increased as a mechanism of replenishment of released GnRH peptide. Terminal blood samples were obtained for measurement of testosterone and LH by radioimmunoassay, and brains were collected, snap-frozen on dry ice, and stored at -80°C until sectioning on the cryostat. Coronal sections ($10\mu\text{m}$) were collected and thaw-mounted onto poly-L-lysine coated slides, which were stored with desiccant at -80°C until in situ hybridization histochemistry was performed.

2.4. Plasma hormone radioimmunoassays (Experiments 1 and 2)

For Experiment 1, plasma LH levels were determined in single samples in two separate assays. LH was measured by a double antibody RIA using reference preparation RP-3 and reagents in the rat LH kit were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases and Dr. A.F. Parlow. This assay has been validated for measurement of LH in the Syrian hamster (Richardson et al., 2002). The intra-assay CVs were 8.7 and 13.1% and the inter-assay CV was 11.5%. The minimum detectable levels of LH were 0.38 and 0.39 ng/ml in the two assays. For Experiment 2, plasma LH was measured in terminal blood samples using the same methods except that samples were run in duplicate in a single assay. The intra-assay CV was 12.4% and the lowest detectable level of LH was 0.73 ng/ml. Plasma testosterone levels were also measured in terminal samples from Experiment 2.

Samples were run in duplicate in a single assay using the Coat-A-Count Total Testosterone Kit (Diagnostic Products, Los Angeles, CA). This radioimmunoassay (RIA) has been validated in our laboratory for the measurement of plasma testosterone concentrations in Syrian hamsters (Parfitt et al., 1999). The intra-assay CV was 16.4% and minimum detectable was 0.1 ng/ml.

2.5. GnRH mRNA in situ hybridization histochemistry (Experiment 2)

Brain sections (at $80\mu\text{m}$ intervals) from each brain were processed for in situ hybridization using a ^{35}S -cRNA probe generated from Syrian hamster GnRH cDNA (generously donated by Dr. Heiko Jansen, Washington State University). The antisense probe was transcribed in a reaction mixture containing $1\mu\text{g}$ of linearized DNA (*Bam*HI linearized plasmid), $5\times$ transcription buffer (Epicentre Technologies, Madison, WI, USA), $80\mu\text{Ci}$ [^{35}S]UTP, $120\mu\text{Ci}$ [^{35}S]CTP, $150\mu\text{M}$ ATP, $150\mu\text{M}$ GTP, 12.5mM dithiothreitol, 20U RNase inhibitor, and 6U T7 RNA polymerase (Epicentre Technologies). Following incubation at 37°C for 2 h, unincorporated nucleotides were separated by Sephadex G50-50 chromatography and the antisense probe diluted in hybridization buffer (Amresco, Solon, OH, USA) to obtain $\sim 1.0 \times 10^6$ CPM/ $70\mu\text{l}$ of buffer.

Slides were removed from the -80°C freezer and placed immediately in 4% paraformaldehyde for 1 h. They were then washed several times in $2\times$ NaCl/Na citrate (SSC) before incubating for 10 min in 0.1 M triethanolamine (TEA) containing 0.25% acetic anhydride. Slides were washed in dH_2O and dehydrated through a series of alcohols. Diluted probe ($70\mu\text{l}$) was applied onto each slide and a glass coverslip was gently placed over the sections to prevent evaporation of the probe during hybridization. Slides were placed in plastic boxes lined with filter paper saturated with 50% formamide. The boxes were covered with plastic lids, wrapped with plastic wrap, and incubated at 55°C for 16 h. Following hybridization, the coverslips were removed by washes in $2\times$ SSC and the slides were then incubated in RNase A buffer ($200\mu\text{g}/\text{ml}$) for 1 h at 37°C . This incubation was followed by several washes in decreasing concentrations of SSC ($2\times$, $1\times$, $0.5\times$, and $0.1\times$) and an incubation in $0.1\times$ SSC for 1 h at 70°C . Afterwards, slides were washed in $0.1\times$ SSC and dH_2O rinses, dehydrated in graded alcohols, and air-dried.

Once completely dry, slides were exposed to XAR film (Eastman Kodak, Rochester, NY, USA). After removal from film, they were emulsion-dipped (NTB2 emulsion from Eastman Kodak diluted 1:1 in distilled water), stored in light-tight boxes at 4°C for 3 days, and developed. Sections were then lightly counterstained with thionin to visualize cell bodies, dehydrated in alcohols, cleared, and coverslipped. Incubation of tissue

sections with a sense probe does not result in labeling of cells (Parfitt et al., 1999).

2.6. Microscopic and statistical analysis of GnRH-mRNA labeled brains (Experiment 2)

Microscopic analysis was performed with a Leitz Laborlux S microscope equipped with a CCD video camera (Sony, XC-77) by an investigator blind to treatment conditions. Labeled cells were located in dark-field at 100× or 200× magnification and then were analyzed individually at a magnification of 400× in brightfield. A blue number 47 filter (Tiffen, Hauppauge, NY, USA) was used to subtract some of the Nissl staining. Images were captured and analyzed using NIH Scion Image 1.57 on a Power Macintosh 7100 computer.

2.6.1. Cell grain area

After each cell profile was traced and the Nissl area measured, a threshold was set so that only silver grains were visualized, and the area (μm^2) covered by silver grains was measured. For this initial analysis, only silver grain labeling over the Nissl stain was measured (Fig. 1A). The cell tracing was then moved to a nearby region without specific hybridization to determine background silver grain area. A GnRH-mRNA expressing cell was defined as a cell in which the area covered by silver grains was at least five times greater than that of background. Background grain area was subtracted from the Nissl cell grain area to obtain GnRH mRNA grain area for each individual cell (termed *cell grain area*). Approximately 100 cells were analyzed for each animal.

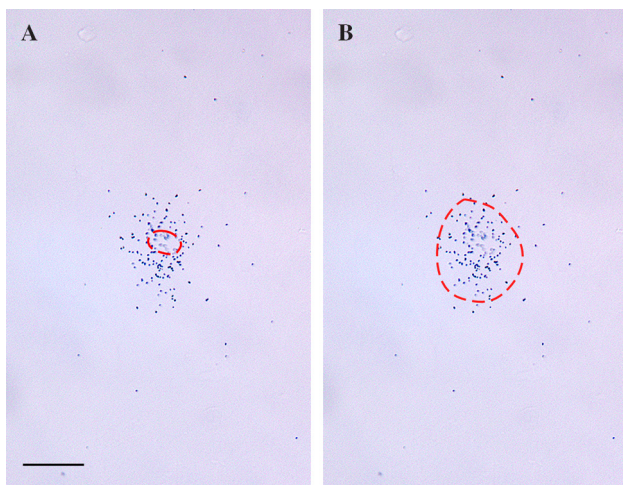


Fig. 1. Photomicrographs of a GnRH mRNA labeled cell demonstrating the two different methods by which ^{35}S labeling was quantified. The dotted line outlines the area in which the silver grain labeling was measured. For the cell grain area analysis, only the silver grain labeling over the Nissl stain was measured (A). For the cluster grain area analysis, the silver grain labeling within and surrounding the Nissl stain was measured (B). Bar, 25 μm .

2.6.2. Cluster grain area

Because the entire cluster of silver grains associated with a particular cell sometimes extended beyond the Nissl defined cell body, a second analysis was performed in which the area covered by the whole cluster of grains was measured (termed *cluster grain area*, Fig. 1B). Both analyses were performed to determine if the pattern of results was similar when a more (cell grain area) or less (cluster grain area) conservative estimate of labeling was used. Thus, three indices of GnRH mRNA were measured in each animal: (1) number of labeled cells, (2) cell grain area, and (3) cluster grain area.

The majority of GnRH cells reside within four main populations in the Syrian hamster: tenia tecta, medial septum (MS), diagonal band of Broca/organum vasculosum of the lamina terminalis (DBB/OVLT), and caudal preoptic area (cPOA) (Richardson et al., 2002). These regions were analyzed separately in the current study. Each of the GnRH mRNA variables (cell number, cell grain area, and cluster grain area) was averaged for each animal within a brain region. For each region, these measures were averaged across animals within each treatment group to obtain means for the tenia tecta, MS, DBB/OVLT, and cPOA.

2.7. Statistical analyses

Mean number of labeled cells, mean cell grain area, and spread grain cluster area were compared across the different time points using separate one-way ANOVAs for each brain region. Mean plasma testosterone levels from terminal samples were compared among the groups using one-way ANOVAs. Significant main effects were probed using Fisher's PLSD tests. Differences were considered significant when $p \leq 0.05$.

3. Results

3.1. Experiment 1: LH response to pheromones

Fig. 2 shows plasma LH data from sampling periods grouped according to swab exposure. Plasma LH is expressed as percent change from baseline. When a swab containing vaginal secretions was introduced, plasma LH began to rise within 10 min, peaked at 15–20 min, and returned to baseline 45 min after initial exposure to pheromone. In contrast, plasma LH levels remained relatively constant over the sampling period when a control swab was introduced.

3.2. Experiment 2: pheromonal regulation of GnRH mRNA

Plasma LH levels from animals killed 60 and 120 min after exposure to pheromone were not significantly

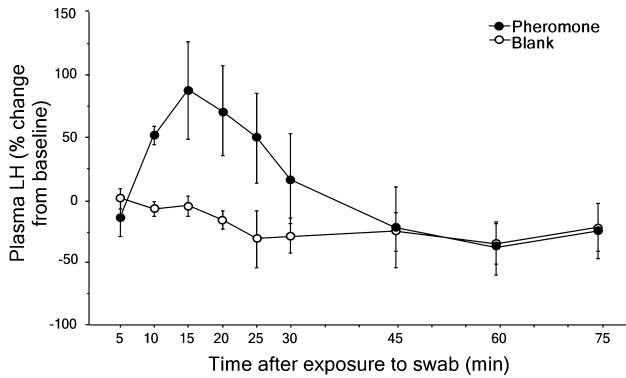


Fig. 2. Time course changes in mean plasma LH (\pm SEM) following exposure to pheromone or blank swabs. Data are expressed as percent change from baseline, averaged across all sampling periods in either the pheromone or control swab condition.

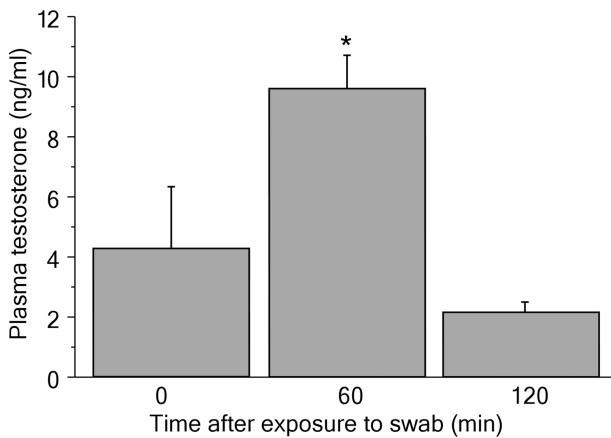


Fig. 3. Mean (\pm SEM) plasma testosterone levels (ng/ml) 0, 60, and 120min following exposure to swabs containing pheromone. Testosterone was significantly elevated 60min, as compared to 0min ($p = 0.010$) and 120min ($p = 0.002$).

different compared with LH levels from animals sampled 0min after exposure (0min = 3.37 ± 0.45 , 60min = 5.22 ± 1.072 , 120min = 5.26 ± 1.31 , $p > 0.05$).

In contrast, plasma testosterone was significantly higher 60min, as compared to 0min ($p = 0.010$) and 120min ($p = 0.002$), after exposure to pheromone (Fig. 3).

GnRH mRNA data are shown in Table 1. Pheromone exposure did not affect the number of GnRH-mRNA labeled cells in any of the four brain regions ($p > 0.05$, Table 1). There was no effect of exposure to pheromone on GnRH mRNA single cell labeling (mean cell grain area or mean grain cluster area) in any of the four brain regions ($p > 0.05$, Table 1).

4. Discussion

These experiments demonstrate that chemosensory information contained in vaginal secretions from conspecific females activates the HPG axis in sexually naive adult male Syrian hamsters. Female pheromones elicit a rapid rise in plasma LH and subsequent rise in testosterone. However, chemosensory-induced increases in LH and testosterone release do not appear to be accompanied by subsequent changes in GnRH mRNA. These data suggest that while activation of the male HPG axis by female pheromones involves release of GnRH, it does not involve increases in GnRH mRNA 1–2h after pheromonal exposure as a mechanism for replenishment of released peptide.

This is the first documentation of an LH response to female pheromones in male Syrian hamsters. We and others previously reported increased plasma androgen levels within 60min of exposure to a female or vaginal secretions (Macrides et al., 1974; Pfeiffer and Johnston, 1992; Romeo et al., 1998). Plasma LH levels rise 50% above baseline 10min after exposure to pheromone, which is consistent with rapid changes in LH in other species. Male Siberian hamsters elicit a rise in plasma LH within 15min of exposure to a female (Anand et al., 2002), and this response is thought to be elicited primarily by pheromonal cues produced by the female

Table 1

Mean (\pm SEM) number of GnRH mRNA expressing cells, GnRH mean cell grain area, and GnRH mean grain cluster area 0, 60, and 120min after exposure to a swab containing pheromone

Time after exposure to swab (min)	Brain region			
	Tenia tecta	MS	DBB/OVLT	cPOA
	Number of labeled cells			
0	18.67 \pm 3.84	31.80 \pm 4.57	42.00 \pm 11.00	9.00 \pm 2.30
60	9.00 \pm 2.27	38.80 \pm 4.01	46.00 \pm 8.67	9.60 \pm 5.77
120	13.60 \pm 2.52	29.20 \pm 2.08	38.60 \pm 5.80	6.00 \pm 5.52
	Mean cell grain area			
0	12.62 \pm 1.09	11.88 \pm 1.24	12.38 \pm 0.65	11.25 \pm 2.34
60	9.84 \pm 1.90	12.87 \pm 1.26	12.64 \pm 0.75	12.22 \pm 0.86
120	11.73 \pm 1.34	11.92 \pm 1.63	13.43 \pm 1.59	10.22 \pm 1.62
	Mean grain cluster area			
0	46.64 \pm 6.17	36.27 \pm 4.86	38.17 \pm 4.03	39.11 \pm 5.32
60	36.69 \pm 10.23	40.86 \pm 6.53	43.07 \pm 5.76	36.30 \pm 4.31
120	39.47 \pm 5.79	38.51 \pm 6.47	44.69 \pm 5.30	32.67 \pm 4.31

(Anand et al., 2004). We infer from the rise in LH that activation of the GnRH neurons is a neural response to the chemosensory stimulus. Pheromone-induced GnRH release could potentially play two roles. First, GnRH released into the median eminence would initiate the neuroendocrine response and second, GnRH released centrally may play a role in eliciting male sexual behavior (Fernandez-Fewell and Meredith, 1995; Meredith, 1998; Meredith and Fernandez-Fewell, 1994; Meredith and Howard, 1992; O'Connell et al., 1978; Wysocki and Lepri, 1991).

In contrast to the proposed behavioral role for pheromone-stimulated release of GnRH, the increase in testosterone that occurs 30–60 min after exposure to pheromones is unlikely to be directly involved in the *initial* activation of reproductive behavior, as males typically engage in the entire sequence of copulatory behaviors within minutes of being placed with a receptive female (Meek et al., 1997; Romeo et al., 1999, 2001). Additionally, male reproductive behavior is expressed in testosterone treated castrated males, which are not capable of showing a pheromone-induced rise in testosterone (Meek et al., 1997). Instead, the rise in testosterone 30–60 min after exposure to chemosensory information from the female more likely serves long-term functions, e.g., altering neural or behavioral responses to females in future encounters, or reinforcing the behavior (Wood et al., 2004).

Surprisingly, female pheromones did not result in significant changes in GnRH mRNA, as measured either by number of cells expressing GnRH mRNA or intensity of ^{35}S labeling per cell. The only notable changes were trends of reduced cell number and mean cell grain area within tenia tecta and medial septum 60 min following exposure to pheromones. As testosterone levels are high at this time and these areas have been implicated in testosterone negative feedback upon GnRH mRNA (Richardson et al., 2002), trends in this direction are to be expected. However, there were no measurable changes in GnRH mRNA that appear to be directly related to pheromone exposure. Because GnRH mRNA was assessed only at 60 and 120 min after exposure to vaginal secretions, we cannot rule out the possibility of more rapid or more protracted changes in GnRH mRNA. However, in a recent report, GnRH mRNA was not altered in male mice exposed to bedding containing female urine 45 min following exposure, even though plasma LH levels were significantly higher than control animals 45–90 min after exposure (Gore et al., 2000). In addition, the failure to detect changes in GnRH mRNA when plasma LH levels are altered has been shown under other conditions in the Syrian hamster. Plasma LH levels are increased in long days, as compared to short days, both under normal conditions (Sisk and Turek, 1983) and following glutamate stimulation (Ebling et al., 1994) in male Syrian hamsters. However, in agree-

ment with the current study, GnRH mRNA does not appear to be significantly altered by photoperiod, regardless of changes in plasma LH under similar photoperiodic conditions (Brown et al., 2001; Ronchi et al., 1992).

The absence of a demonstrable change in GnRH mRNA in response to the presumed increased release of GnRH suggests either that the magnitude of the GnRH response to female pheromones does not require significant increases in GnRH synthesis for replenishment of released peptide, or that GnRH mRNA and release are impacted by pheromones in a relatively small number of GnRH neurons. Another possible reason pheromone exposure did not significantly alter GnRH mRNA is that the synthesis-release relationship may differ across the various GnRH subpopulations. In our experience, GnRH neurons in the cPOA do not show measurable changes in GnRH mRNA under conditions in which GnRH release is high (Richardson et al., 2002). This fact is especially interesting when considered together with the recent demonstration of Fos expression in GnRH neurons *only* in the cPOA following electrical stimulation of the vomeronasal organ (Meredith and Fewell, 2001). Thus, it is possible that pheromones activate GnRH neurons in the cPOA to release GnRH, initiating both the behavioral and neuroendocrine responses, but that this activation does not result in measurable changes in GnRH mRNA in this group of cells.

Overall, the current study demonstrates that chemosensory information from females elicits a rise in LH and subsequent elevation in testosterone. Although this hormone response likely reflects increased GnRH release shortly after exposure to pheromones, GnRH secretion is not reflected in changes in GnRH mRNA. Thus, either the GnRH response is not large enough to stimulate synthesis and measurable changes in mRNA, the timing of such changes occurs earlier or later than 1–2 h after exposure to pheromones, or release of peptide from GnRH neurons specifically activated by pheromones does not result in alterations in GnRH mRNA in these cells.

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