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Environmental Control of Kisspeptin: Implications for Seasonal Reproduction

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The *KiSS-1* gene encodes the peptide hormone kisspeptin, which acts as a principal positive regulator of the reproductive axis by directly stimulating GnRH neuron activity. To gain insight into a potential role for kisspeptin in integrating and relaying reproductively relevant stimuli to the GnRH system, we investigated changes in kisspeptin peptide expression associated with photoperiodic changes in reproductive state as well as pituitary and gonadal responses to peripheral kisspeptin injections. Seasonally breeding rodents undergo pronounced fluctuations in reproductive state in response to changing day lengths. In common with other rodent species, a majority of male Siberian hamsters (*Phodopus sungorus*) exhibit reproductive decline after exposure to short-day lengths. A subset of individuals fails to respond to day length information, however, and maintains their reproductive function. We exploited these individual differences to examine whether kisspeptin may act at the interface between external stimuli and the reproductive system. After extended exposure to short days, animals with a quiescent reproductive axis displayed a marked reduction in kisspeptin cell labeling in the anteroventral periventricular nucleus but robust kisspeptin-immunoreactive staining in the arcuate nucleus. In contrast, animals with functional reproductive systems displayed high numbers of kisspeptin-immunoreactive neurons in the anteroventral periventricular nucleus but a paucity of expression in the arcuate nucleus. Kisspeptin injections significantly elevated LH over preinjection levels regardless of photoperiod or reproductive state. Collectively, these findings suggest an important role for kisspeptin in coordinating and relaying environmentally relevant information to the reproductive axis as well as a role for this peptide in regulating seasonal changes in reproductive function. (*Endocrinology* 148: 1158–1166, 2007)

TO MAXIMIZE REPRODUCTIVE success and avoid breeding during inappropriate conditions, animals must integrate neural signals that convey both external and internal status and appropriately alter the activity of the hypothalamo-pituitary-gonadal (HPG) axis. The final common pathway in which these stimuli are integrated to influence reproductive function is the GnRH neuronal system (1–3). The GnRH system has traditionally been considered the pinnacle of hierarchical control regulating downstream pituitary and gonadal function. Upstream mechanisms responsible for interpreting internal or external status and relaying this information to the GnRH system, however, remain largely unspecified.

Examining changes in neuropeptide levels in response to environmental stimuli in specific neuronal populations that mediate reproductive function can provide important insight into potential systems that act at the interface between the environment and the GnRH system. One recently identified class of peptide hormones, kisspeptins, are the product of the antimetastatic *KiSS-1* gene, which encodes a large 145-amino acid chain that is subsequently enzymatically cleaved into shorter, biologically active peptides (*i.e.* kisspeptin-54, -14, -13, -10) (4). These peptides are the natural ligands of the previously orphaned G protein-coupled receptor GPR54 (4, 5) and exert a profound influence on the HPG axis (6, 7). Specifically, administration of exogenous kisspeptin leads to marked, dose-dependent increases in the gonadotropins LH and FSH across all mammalian species studied to date (7–13), including humans (14). This response appears to be mediated via the actions of kisspeptin on the GnRH system, rather than a direct action on the pituitary; kisspeptin depolarizes GnRH neurons (15), and gonadotropin release can be blocked by pretreatment with a GnRH antagonist (7, 8, 16, 17). Furthermore, treatment of pituitary tissues or cultured cells with kisspeptin *in vitro* fails to elicit gonadotropin release (10, 16; but see Ref. 12). Within the brain, kisspeptin cell bodies are concentrated in the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei of the hypothalamus, with scattered cells in the periventricular and anterodorsal preoptic nuclei (8, 18, 19). These regions likely play an important role in kisspeptin regulation of HPG activity and thus reproductive functions.

An ideal model system to investigate the mechanisms by which endogenous and exogenous stimuli impact the GnRH neuronal system is seasonally breeding rodents. Temperate zone rodents breed seasonally, restricting reproduction to the time of year when environmental conditions are optimal (*i.e.* spring/summer). Virtually all seasonally breeding rodents use photoperiodic signals, which provide a noise-free

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Abbreviations: ARC, Arcuate nucleus; AVPV, anteroventral periventricular; DMH, dorsomedial hypothalamus; GnIH, gonadotropin inhibitory hormone; HPG, hypothalamo-pituitary-gonadal; ir, immunoreactive.

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cue to precisely time reproduction. Changes in photoperiod alter HPG axis activity and reproductive function, allowing reproduction to be coordinated with favorable ambient conditions (20–23). Under controlled laboratory conditions, animals maintained on long summer-like photoperiods (>12 h light/d) remain reproductively active with fully functional gonads, whereas animals maintained in short winter-like photoperiods (<12 h light/d) exhibit down-regulation of HPG axis activity and pronounced regression of the gonads and internal reproductive ducts (20).

For the present studies, we hypothesized that kisspeptin acts as a relay point for integrating and interpreting reproductively relevant stimuli, including photoperiod. We used Siberian hamsters (*Phodopus sungorus*) to capitalize on several aspects of their reproductive physiology. As with other seasonally breeding rodents, Siberian hamsters display marked changes in reproductive physiology in response to changes in photoperiod (20). Furthermore, this species displays an interesting polymorphism in which a subset of individuals, called reproductive nonresponders, fails to respond to photoperiodic information and remain reproductively active despite exposure to short days (24–26); the remaining animals, in contrast, display the typical gonadal regression in response to short days. This differential response to short days provides a powerful tool to assess how the same environmental stimuli can be differentially interpreted by the central nervous system and relayed to the reproductive axis. The goal of the present study was to determine the role of kisspeptin in mediating the pronounced changes in reproductive state observed in seasonal breeders exposed to differing photoperiodic stimuli. We previously reported that kisspeptin staining in the AVPV is significantly reduced in short-day, compared with long-day hamsters (27). Based on these initial observations, we hypothesized that expression of the neuropeptide kisspeptin in the hypothalamus would change in response to photoperiod and that these changes would track the reproductive state of the animal. In addition, we hypothesized that exogenous kisspeptin would stimulate the HPG axis regardless of photoperiod treatment. Collectively, these data will elucidate a possible key role for kisspeptin in mediating reproductive responses to relevant environmental stimuli.

Materials and Methods

Animals and housing

Adult (>60 d of age) male Siberian hamsters (*Phodopus sungorus*) (n = 56) were obtained from our breeding colony maintained at Indiana University. All animals were group housed at weaning with same-sex siblings in a long-day photoperiod (light-dark 16:8). Before the start of the study, animals were housed individually in polypropylene cages (27.8 × 7.5 × 13.0 cm) for 1 wk and then placed in either a long- (16:8) or short-day (8:16) photoperiod. Temperature was kept constant at 20 ± 2 C and relative humidity was maintained at 50 ± 5%. Food (rat chow; Purina, St. Louis, MO) and tap water were available *ad libitum* throughout the experiments.

Experiment 1: effects of photoperiod and reproductive state on kisspeptin neurons

Hamsters were held for either 2 or 8 wk in long- (2 wk n = 5; 8 wk n = 5) or short-day (2 wk n = 5; 8 wk n = 10) photoperiods. These two time points were chosen because animals responsive to short days display fully regressed gonads and basal sex steroid levels by 8 wk in photoperiod. In addition, we chose to include an additional time point, 2 wk, because gonadal regression has not yet occurred and circulating testosterone remains elevated (Demas, G. E., A. Lutz, and D. A. Zysling, unpublished data). This allowed us to capture any dynamic changes in kisspeptin labeling that may occur before full reproductive regression.

Perfusions and tissue preparation

At the conclusion of the experiment, hamsters were weighed to the nearest 0.1 g and then deeply anesthetized with 0.3 ml of a ketamine (20 mg/ml)/xylazine (4 mg/ml) cocktail in 0.9% saline and perfused transcardially with 50 ml of 0.9% saline, followed by 100–150 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.3). Brains were postfixed for 3 h at room temperature in 4% paraformaldehyde and cryoprotected in 20% sucrose in 0.1 M PBS and stored at 4 C until processed. Coronal sections (40 μm) were cut on a cryostat and processed as free-floating sections beginning rostrally at the medial septum/diagonal band of Broca and extending caudally to the brain stem.

Necropsies were performed and paired testes were collected, cleaned of fat and connective tissue and weighed. Animals that, after 8 wk in short days, had paired testes weighing more than 0.15 g (n = 6, mean = 0.65 ± 0.12 g) were classified as short-day nonresponders; animals with paired testes weighing < 0.15 g (n = 4, mean = 0.07 ± 0.03 g) were classified as short-day responders.

Antibody characterization and immunohistochemistry

Kisspeptin-immunoreactive (ir) cells were labeled using a rabbit antihuman kisspeptin serum (T-4771; Peninsula Laboratories Inc., Bachem, San Carlos, CA) raised against the following amino acids Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2, corresponding to amino acids 4–13, diluted at 1:7500. In preliminary trial runs, nonspecific staining strikingly similar to the distribution of gonadotropin inhibitory hormone (GnIH) peptide and mRNA was noted in the dorsomedial hypothalamus (DMH) whereas labeling in the AVPV and ARC resembled that of kisspeptin mRNA across species (8, 18, 19, 28, 29). This nonspecificity likely resulted from the fact that kisspeptin and GnIH share common amino acids at their C terminus (see Ref. 30 for review). Double-label immunohistochemistry using anti-Syrian hamster GnIH (PAC1365) and kisspeptin antisera resulted in colabeling of all cells in the DMH, whereas cells in the AVPV and ARC remained single labeled for kisspeptin only (Fig. 1). To eliminate potential GnIH staining, we preadsorbed the kisspeptin antiserum with GnIH peptide (generous gift of Dr. George Bentley, University of California, Berkeley, Berkeley, CA) for 24 h at 4 C before application. This procedure eliminated the DMH population of cells, whereas maintaining the AVPV and ARC populations in all cases (Fig. 1). Preadsorption with both GnIH and kisspeptin eliminated all staining.

We further confirmed the specificity of Bachem T-4771 (preadsorbed with GnIH) by double-labeling tissue with T-4771 and a rabbit antihuman kisspeptin antibody raised against amino acids 43–52 (a generous gift of Dr. Alain Caraty and Dr. Isabelle Brailiou, UniversityTours/Haras Nationaux, Nouzilly, France). This second antibody has been previously validated to show high specificity for kisspeptin (29). Antisera were detected using two biotinylated goat antirabbit secondary antibodies (CY2-T4771 and CY3-Caraty antibody; Vector Laboratories Inc., Burlingame, CA). The dilution of T-4771 was 10-fold greater (1:7500) than that which is optimal for direct immunohistochemistry (*i.e.* 1:750). This dilution prevented the second, secondary antibody from nonspecifically binding to the first primary. Amplification of the T4771 signal was accomplished by using a modified biotinylated tyramide procedure previously described (31). The antibody provided by Dr. Caraty and colleagues was directly labeled using a CY3 goat antirabbit secondary (Vector Laboratories). All double-label experiments using this procedure resulted in 100% colabeling of cells in the AVPV and ARC (Fig. 2). To confirm that this procedure was effective at preventing nonspecific labeling with the second rabbit antibody, the second kisspeptin antibody (*i.e.* provided by Dr. Caraty and colleagues) was eliminated and all other procedures implemented. In these controls trials, CY3 did not label T4771-ir neurons. Sections were mounted onto gelatin-coated slides, dehydrated in a graded series of ethanol solutions (70, 95, and 100%), and cleared in xylenes (Fisher Scientific, Hanover Park, IL) before the

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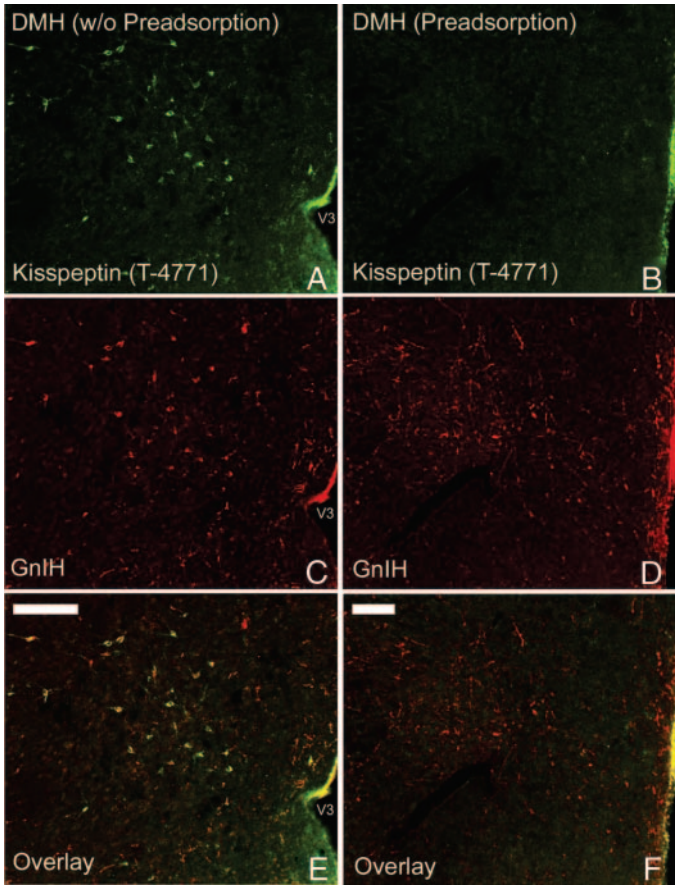

FIG. 1. Elimination of nonspecific GnIH staining with peptide preadsorption. Medium-power photomicrographs showing putative GnIH neurons in the DMH staining positively using the Bachem kisspeptin antiserum (A). GnIH staining confirms that the same neurons labeled using the kisspeptin antiserum are colabeled for GnIH in single- (C) and double-label (E) images. Putative GnIH labeling using the kisspeptin antiserum is abolished after GnIH peptide preadsorption (B). GnIH staining in the same tissue confirms that the kisspeptin antibody does not label GnIH cells after GnIH peptide preadsorption (D and F). *Scale bars*, 100 μM.

application of coverslips. Brains were processed immunohistochemically in three separate immunohistochemical runs (n = 10, 10, and 5 brains per assay). Variability between immunohistochemical replications was controlled by having an equal number of animals for each group in each run of immunohistochemistry. For each run, incubation times for every procedure were strictly controlled.

Microscopy, cell counts, and OD

Slides were examined under bright field illumination on a Zeiss Z1 microscope by independent observers naïve to the experimental conditions. Kisspeptin-ir cells were located by visually scanning the brains under ×200 magnification. Cell populations were restricted to the AVPV region of the preoptic area and ARC. All cells were confirmed at a minimum of ×400. Counted cells were photographed with a Axiocam Cooled CCD camera (Zeiss, New York, NY) at ×400 magnification for cell size and density analyses. All cells in every fourth section were counted through the rostrocaudal extent of the AVPV and ARC. For all animals this resulted in counting three sections through the AVPV and eight sections through the ARC. Both cells with a clearly discernable nucleus and cells showing clear soma and processes without a clear, unstained nucleus were counted. Because the inclusion of cells without a clearly defined nucleus may result in counting overestimates, an Abercrombie correction was applied before data analysis.

Soma size and OD measurements were performed on images captured at ×400. All cells examined had ODs at least 2 SD above the mean background OD measures for an individual brain. Cell bodies were outlined and the two-dimensional area was calculated using Image J v1.32. Each pixel in the gray-scale image capture has a measurable specific intensity, with values ranging from 0 (white) to 256 (black). The average value for all pixels in an outlined area is taken as the mean intensity of staining for a given region of the image. OD measures were normalized to minimize differences between replications of immunohistochemistry. First, a background measurement was taken by placing a square outline, four times, on nonoverlapping, unstained areas of each section. The mean of these four measures provided the background OD for each section. The OD for each cell body was assessed by outlining the cell body, obtaining a density measure using Image J, and subtracting the background OD from the OD of each cell.

Experiment 2: endocrine response to exogenous kisspeptin

Hamsters were held in long- (n = 11) or short-day (n = 19) photoperiods for 8 wk before kisspeptin injections. Hamsters were injected with kisspeptin-10 [KiSS-1 (112–121)/metastin (45–54) (human); Phoenix Pharmaceuticals, Inc., Belmont, CA], a commercially available product with known ability to stimulate the HPG axis (8), or a 0.1 M PBS vehicle injection based on a previously published protocol (32). Briefly, an initial blood sample was drawn from all hamsters via the retroorbital sinus to measure baseline hormone levels. Next, long- and short-day hamsters received ip injections of either 100 μl PBS (long day: n = 5; short day: n = 10) or 100 μl of a PBS solution containing 10 μM kisspeptin-10 (Phoenix Pharmaceuticals) (long day: n = 6; short day: n = 9) every 30 min for a total of four injections. Thirty minutes after the last injection, all hamsters were again bled. The injection protocol, described in more detail elsewhere (32), was chosen because it previously demonstrated the ability to elicit a significant increase in serum LH levels in mice, a similarly sized rodent to Siberian hamsters (32). Blood was centrifuged at 2500 rpm for 30 min, and serum was collected and stored at −80 C until assayed for hormones.

After the last blood sample was collected, necropsies were preformed and paired testes were removed and weighed. Animals were categorized *post hoc* as either short-day responders or nonresponders based on paired testes mass as described in experiment 1. One short-day animal that received kisspeptin injections displayed the nonresponsive phenotype (gonadal mass > 0.15 g), whereas five animals that received PBS injections displayed this phenotype (gonadal mass > 0.15 g), leaving five short-day responders injected with vehicle and eight short-day responders injected with kisspeptin.

Hormone measurements

Serum LH concentrations were measured in duplicate via a single RIA with reagents obtained from the National Institutes of Health based on a previous protocol (33). The antiserum was rLH-S-11 and the standard was rLH-RP3. The sensitivity was 0.01 ng/tube and the intraassay coefficient of variation was 2.9% for the low pool and 8.5% for the high pool. Serum testosterone was measured from samples with adequate serum after LH analysis (long day kisspeptin n = 6; long day vehicle n = 5; short day kisspeptin n = 4; short day vehicle n = 6) via a commercial enzyme immunoassay kit (Correlate-EIA kit no. 900–065; Assay Designs, Ann Arbor, MI). Serum samples were diluted 1:20 and run in duplicate for each sample. The sensitivity of the assay was 3.82 pg/ml, the intraassay coefficient of variation was 9.2%, and the interassay of variation was 2.14%. The antisera used in both assays were highly specific for the hormones measured, with low cross-reactivity with other hormones. Both the LH and testosterone assays have been previously validated for use in Siberian hamsters (34, 35).

Statistical analyses

Data in experiment 1 were grouped according to the photoperiod, duration in photoperiod, and reproductive state, yielding five groups: long days/2 wk, long days/8 wk, short days/2 wk, short days/8 wk (responders), and short days/8 wk (nonresponders). The effects of photoperiod and reproductive state on body and gonadal masses as well as

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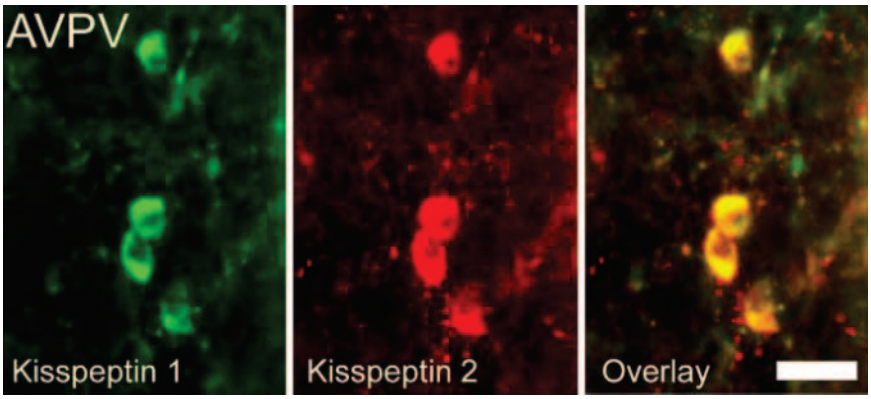

FIG. 2. Kisspeptin staining in AVPV with two kisspeptin antibodies. Representative photomicrographs of neurons staining for kisspeptin using the Bachem kisspeptin antibody (kisspeptin 1) preadsorbed with GnIH peptide, the kisspeptin antibody kindly provided by Dr. Alain Caraty and Dr. Isabelle Brailiou (kisspeptin 2). *Scale bar*, 25 μM. Both antibodies label 100% of the same cells in the AVPV (shown here) and the Arc (data not shown).

kisspeptin-ir neuron number, size, and OD were each analyzed in separate one-way ANOVAs. Pair-wise comparisons were probed with Tukey's *post hoc* tests when the overall ANOVA was significant.

In experiment 2, only one short-day, nonresponsive morph received kisspeptin injections (five received vehicle); thus, statistical comparisons between responsive and nonresponsive morphs were not possible. As such, all nonresponsive animals (n = 6) were removed from subsequent analysis. The effects of photoperiod on body and gonadal mass and baseline levels of the hormones LH and natural log (ln) testosterone were assessed using a one-way ANOVA; testosterone levels were natural log transformed to meet the parametric assumption of equal variance. The effects of peripheral kisspeptin injections on LH and testosterone were each analyzed using separate repeated-measures ANOVAs, with pre- and posthormone levels as the within-subjects factor and photoperiod and injection as the between-subject factors. In all cases, differences were considered statistically significant if $P < 0.05$. All analyses were performed using SPSS 14 for Windows (SPSS, Inc., Chicago, IL).

Results

Experiment 1: effects of photoperiod and reproductive state on kisspeptin neurons

Consistent with other rodent species, kisspeptin-ir neurons were concentrated in the anterioventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) (Fig. 3). Photoperiod and reproductive state significantly affected the number ($F_{4,20} = 3.989$, $P = 0.015$) and size ($F_{4,20} = 3.819$, $P = 0.018$) but not OD ($P > 0.1$) of kisspeptin-ir neurons in the AVPV (Fig. 4). Animals that regressed their gonads in response to 8 wk of short-day photoperiod displayed significantly fewer ($P < 0.05$) and smaller ($P < 0.05$) kisspeptin-ir neurons than all other groups; all other groups displayed a similar number and size of kisspeptin-ir neurons ($P > 0.05$) (Fig. 4).

The number of kisspeptin-ir neurons in the ARC was significantly affected by photoperiod and reproductive state ($F_{4,20} = 9.144$, $P < 0.001$) (Fig. 5), with animals reproductively responsive to 8 wk of short-day photoperiod (*i.e.* regressed gonads) displaying significantly more kisspeptin-ir neurons than all other groups ($P < 0.05$). Sixty percent of hamsters with regressed gonads had the most robust increase in ARC kisspeptin-ir cells labeling, whereas a modest increase was seen in the remaining 40% of hamsters. In sharp contrast to reproductively competent hamsters that had between 0 and 3 labeled cells in ARC, 100% of animals with regressed reproductive axes had cell counts between 20 and 65. Groups with functional gonads did not differ in the number of kisspeptin-ir neurons observed in the ARC ($P > 0.05$) with a mean of 2.3 ± 0.38 cells across conditions. Because of the extremely small number of ARC cells labeled for kisspeptin in reproductively competent animals, OD and cell size measures were not taken in this brain region because these results may be misleading due to measurement of one to two cells in most animals.

Photoperiod significantly affected gonadal ($F_{4,20} = 13.76$, $P < 0.001$) (Fig. 5B) and body mass ($F_{4,20} = 9.798$, $P < 0.001$) (Table 1), with responsive animals held on short days for 8 wk having significantly smaller testes ($P < 0.001$ in all cases) and lower mass ($P < 0.02$ in all cases); no difference was observed between the other groups ($P > 0.05$ in all cases).

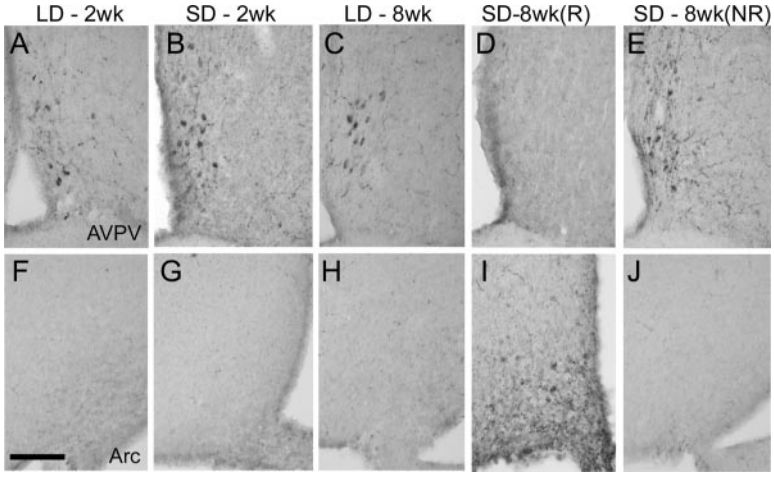

FIG. 3. Response of kisspeptin-ir neurons to photoperiodic treatment. Photomicrographs of kisspeptin-ir neurons in the AVPV (A–E) and the ARC (F–J) in the brains of animals held in long-day photoperiod either 2 (LD-2wk) or 8 wk (LD-8wk), or short-day photoperiod for 2 wk (SD-2wk), and short-day-responsive [SD-8wk (R)] and nonresponsive [SD-8 wk (NR)] animals held on short-day photoperiod for 8 wk. *Scale bar*, 100 μM. Animals responsive to short-day photoperiod [SD-8wk (R)] display a marked reduction in kisspeptin-ir neurons in the AVPV and a significant increase in the ARC, compared with all other groups.

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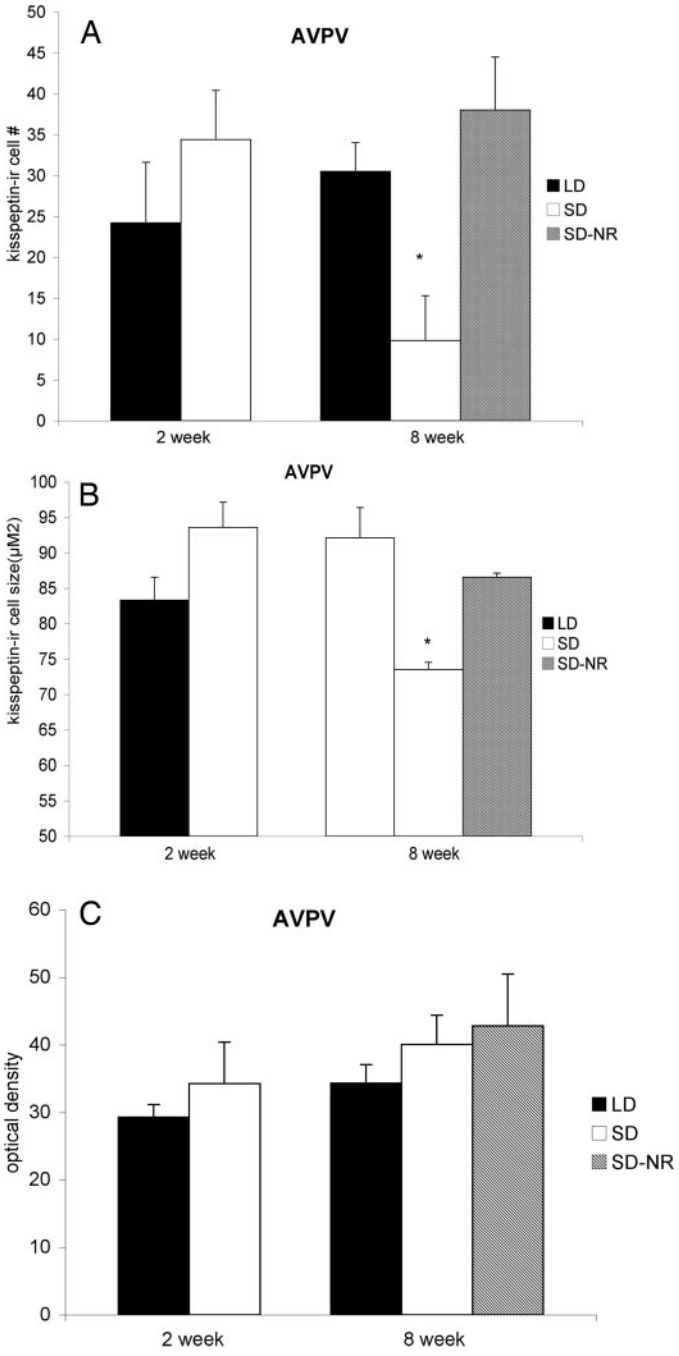

FIG. 4. Effects of photoperiod on AVPV kisspeptin-ir neurons. Short-day-responsive animals (SD-R) held 8 wk on short days display significantly fewer numbers of cells (A) and smaller kisspeptin-ir neurons (B) in the AVPV; no change in OD was observed (C). An *asterisk* denotes $P < 0.05$. LD, Long day; SD, short day; SD-NR, short-day nonresponsive.

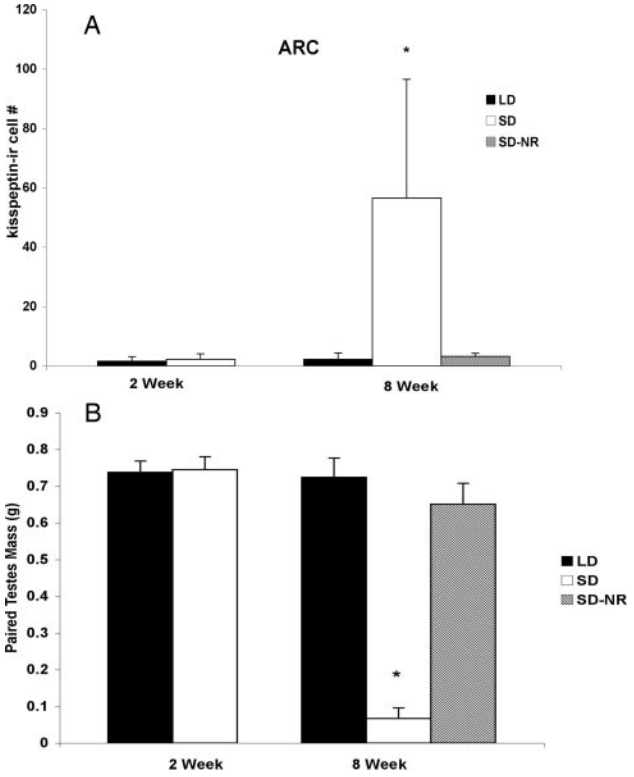

FIG. 5. Effects of photoperiod on ARC kisspeptin-ir neurons. After 8 wk on short days, responsive animals had significantly more kisspeptin-ir neurons in the ARC, compared with animals held on long days (LD) or short days (SD) for 2 wk or nonresponsive (SD-NR) animals held on short days for 8 wk (A). Short-day-responsive animals had significantly smaller paired testes than all other groups. An *asterisk* denotes $P < 0.05$.

Experiment 2: endocrine response to exogenous kisspeptin

Animals held in long days for 8 wk had significantly heavier paired testes ($F_{1,20} = 411.59$, $P < 0.001$), higher body mass (long day: 42.1 ± 1.8; short day: 32.7 ± 1.06) ($F_{1,20} = 18.98$, $P < 0.001$) and higher levels of testosterone (lnT) ($F_{1,16} = 4.67$, $P = 0.046$), compared with animals held on short days (Fig. 6). Baseline LH levels were not affected by photoperiod ($P > 0.05$).

Animals that received injections of kisspeptin displayed significantly elevated LH levels, compared with animals receiving PBS ($F_{1,23} = 19.04$, $P < 0.001$) (Fig. 6), regardless of photoperiod. Photoperiod treatment had no main effect on LH level ($P > 0.05$), and there was no interaction between photoperiod and kisspeptin treatment ($P > 0.05$).

Kisspeptin, compared with vehicle, significantly elevated levels of testosterone in long- but not short-day animals (photoperiod * injection; $F_{1,14} = 5.44$, $P = 0.035$) (Fig. 6). There was a main effect of photoperiod on testosterone ($F_{1,14} = 16.18$, $P = 0.001$), with long-day animals displaying higher

TABLE 1. Mean (± SEM) final body and paired testes masses (grams) in response to photoperiodic treatment for 2 or 8 wk in experiment 1

Experimental treatment	Body mass (g)	Paired testes mass (g)
Long day/2-wk	42.9 ± 1.7	0.74 ± 0.03
Short day/2-wk	41.25 ± 1.8	0.75 ± 0.03
Long day/8-wk	49.92 ± 3.6	0.72 ± 0.05
Short day/8-wk responder	29.72 ± 1.0[a]	0.07 ± 0.03[a]
Short day/8-wk nonresponder	43.46 ± 1.7	0.65 ± 0.12

[a] Significant differences between group means if $P < 0.05$.

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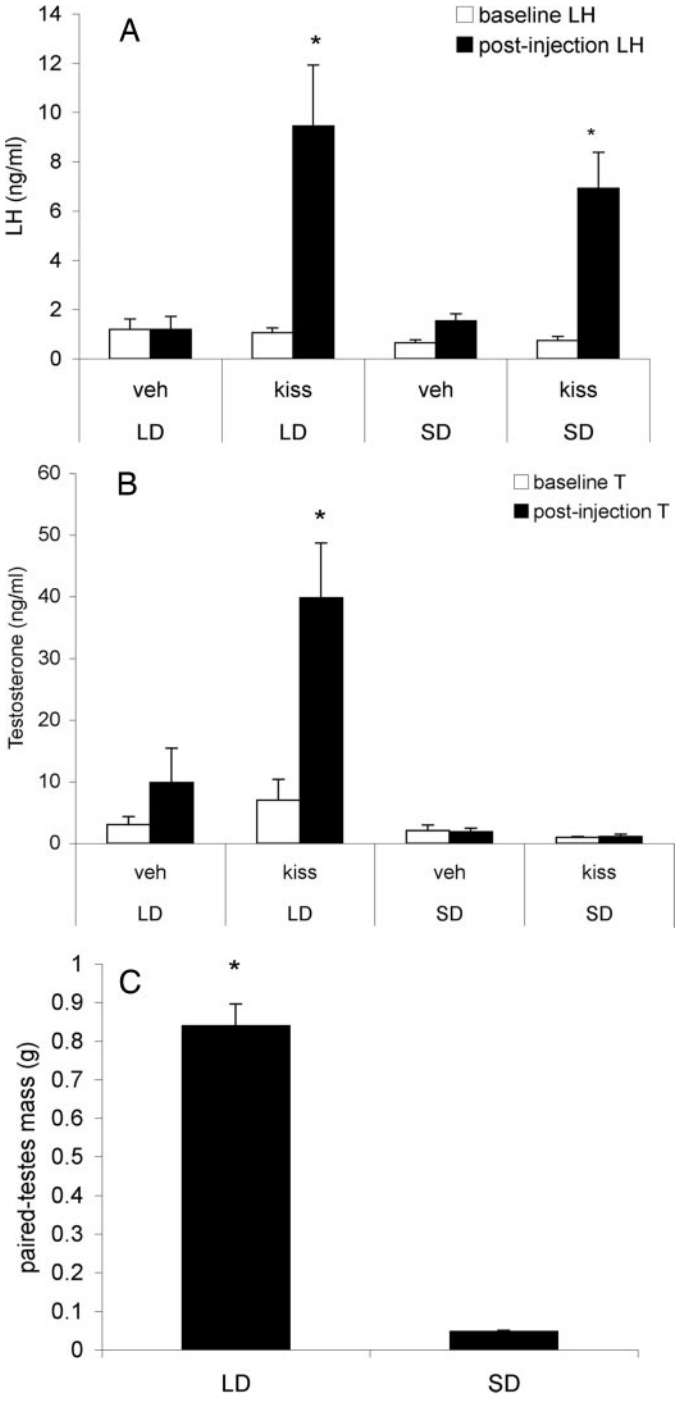

FIG. 6. Effect of kisspeptin on gonadotropin release. Peripheral injections (ip) of kisspeptin (kiss) significantly elevated pituitary LH, compared with either baseline levels or vehicle (PBS injected) animals (A). Hamsters held in both long- (LD) and short-day (SD) photoperiods for 8 wk displayed a similar increase in LH levels in response to kisspeptin. In addition, animals held in long days displayed significantly elevated testosterone (T) concentrations in response to kisspeptin (B). Short-day animals had significantly smaller gonads than animals housed in long-day photoperiod (C). An *asterisk* denotes $P < 0.05$. veh, Vehicle.

levels of testosterone than short-day animals. There was no effect of injection treatment on testosterone ($P = 0.06$) because short-day animals displayed no elevation of testosterone.

Discussion

The results of the present study demonstrate an important role for the peptide kisspeptin in the interpretation of environmental information and subsequent regulation of the neuroendocrine reproductive axis. Furthermore, the differences observed in the staining patterns between animals exhibiting a polymorphism in reproductive responsiveness to short-day lengths demonstrates a role for kisspeptin in driving the disparate response of the reproductive system to the same reproductively relevant stimulus, namely photoperiod. Specifically, in experiment 1, kisspeptin-ir neurons in the ARC and AVPV tracked reproductive state, with significantly more staining in the AVPV of animals reproductively active, compared with those with regressed gonads. Interestingly, the ARC kisspeptin cell population exhibited the opposite pattern of staining with regressed animals showing robust cell staining that was virtually absent in animals with active reproductive systems. In experiment 2, peripheral injections of exogenous kisspeptin resulted in robust increases in gonadotropin release in both long- and short-day hamsters, demonstrating that the GnRH system remains sensitive to kisspeptin regardless of reproductive condition. These results suggest that differential release of kisspeptin from the hypothalamus in response to differing day lengths mediates reproductive physiology in seasonally breeding animals.

As mentioned previously, kisspeptin staining in the hypothalamus (AVPV and ARC) was altered in response to photoperiodic treatment and reproductive state. This observed pattern may be regulated directly via photoperiodic signals (*i.e.* the duration of melatonin secretion) or may be the result of changes in circulating sex steroids due to photoperiod-induced changes in gonadal morphology (*e.g.* regressed gonads in short day responsive animals). Patterns of melatonin secretion are known to influence reproductive state in many seasonal rodents, including Siberian hamsters (20). The nonresponsive morph of Siberian hamsters produces the same melatonin signal as a long-day hamster, causing these hamsters to code for long days, even while in short-day photoperiods (24). The observation that short-day nonresponsive hamsters displayed the same pattern of brain kisspeptin expression as long-day hamsters supports a possible mechanistic role for melatonin in the regulation of kisspeptin expression. Similar staining patterns as those observed in the present studies have been reported previously for *KiSS-1* mRNA expression; gonadectomized mice have low *KiSS-1* expression in the AVPV but high expression in the ARC, whereas testosterone replacement results in the opposite expression pattern (18). Additionally, *KiSS-1* neurons express both androgen and estrogen receptors (18) suggesting that kisspeptin may be responding to photoperiodic changes in sex steroids. Future studies examining melatonin receptor expression in kisspeptin cells along with manipulations of melatonin, photoperiod, and gonadal steroids will address the relative contribution of these potential regulatory factors to hypothalamic kisspeptin expression.

The two hypothalamic nuclei staining positively for kisspeptin, the AVPV and ARC, contain neurons projecting to the medial preoptic area, a brain region containing GnRH cell bodies (36). Furthermore, more than half of GnRH neu-

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rons express mRNA for the kisspeptin receptor, *GPR54* (15, 32). The specific contribution of AVPV *vs.* ARC kisspeptin neurons in regulating GnRH cell function remains to be determined. It has been suggested that the opposing peptide expression patterns observed between the AVPV and ARC (19) and in the present study may participate in positive and negative feedback, respectively (19). It is noteworthy, however, that a peptide able to potently stimulate the HPG axis is expressed in high concentrations in the ARC in nonreproductive animals, a finding incompatible with a stimulatory action of ARC kisspeptin on the HPG axis. It remains possible that, whereas the kisspeptin neurons in the AVPV may act as a potent stimulator of the HPG axis, the kisspeptin neurons within the ARC may instead serve other, yet-unidentified neuromodulatory functions unrelated to reproduction. Alternatively, increased kisspeptin-ir labeling in the ARC may be the result of inhibited peptide release in this brain region, allowing greater immunodetection. Further research aimed at determining the precise neuroendocrine functions of kisspeptin within these two brain regions will help to select among these hypotheses.

As with kisspeptin staining in the ARC, mRNA expression for *Vgf* mRNA in the ARC is greater in short- compared with long-day animals (37). The function of this gene is still unknown, but it has been implicated in energy balance (38). Kisspeptin neurons in the ARC respond to signals of energy availability and balance (39–41), and Siberian hamsters are typically used as models for studies of energy balance because they exhibit marked seasonal changes in food intake and metabolism (42). Given the pronounced role of the ARC in feeding regulation, along with seasonal changes in energy balance in Siberian hamsters, it is possible that kisspeptin neurons in the ARC are altered in response to energy status in addition to modulation through negative feedback in response to sex steroids (18) and photoperiod. Interestingly, a previous report (43) has demonstrated kisspeptin staining in sheep in brain regions comparable with those seen in Siberian hamsters (*e.g.* the ARC and periventricular nuclei). Sheep, like hamsters, are a seasonally breeding species. In contrast to hamsters, however, sheep are short-day breeders, restricting reproduction to the short days of winter and inhibiting reproduction during long day lengths. Although the effects of photoperiod on kisspeptin staining were not assessed in this sheep study, it would be interesting to examine whether staining patterns in responses to photoperiodic manipulations were opposite those seen in long-day breeders (*e.g.* Siberian hamsters).

All individuals, regardless of photoperiod or reproductive state, displayed significant elevation in LH in response to peripheral injections of kisspeptin, demonstrating that animals are able to respond to the peptide regardless of photoperiodic signal. In addition, animals with functional gonads exhibited a robust increase in testosterone in response to exogenous kisspeptin, presumably stimulated by the observed elevated LH response to kisspeptin. In the current study, only one dose of kisspeptin (*i.e.* 10 μM/injection across four injections) was used in a manner known to elicit a significant LH surge in mice (32). Although both long-day and short-day-responsive animals displayed a comparably robust response to kisspeptin administration in the current study, the dose of kisspeptin used may have been sufficiently high to mask potential subtle differences in hypothalamic sensitivity to the peptide. Future investigations using a range of kisspeptin doses will allow a more direct examination of this possibility. Despite these potential subtle alterations in responsiveness to kisspeptin, the present results demonstrate that short-day animals are capable of activating the hypothamo-pituitary system in response to a kisspeptin signal.

Both long-day and short-day animals displayed elevated LH levels in response to kisspeptin. However, short-day-responsive animals did not alter serum testosterone concentrations. The fact kisspeptin administration does not increase testosterone in short-day responders is likely due to the regressed, nonfunctional state of the testes in these animals. Whether this is driven by a reduction in LH receptors or a lack of functional Leydig cells remains to be determined. Because kisspeptin has previously been shown to act at the level of the hypothalamus, and not the pituitary, to directly stimulate pituitary release of LH (10,17 but see Ref. 12), the peripheral injections used in the present study likely exerted their effects centrally. In seasonally breeding rodents, short-day lengths results in a marked down-regulation of the HPG axis and subsequent gonadal regression, whereas exposure to long days induces an up-regulation of the HPG axis followed by gonadal recrudescence. These changes result from actions upstream of the pituitary, as GnRH injections stimulate pituitary LH and FSH to a comparable degree in short-day, regressed and long-day, reproductively competent animals (44–47). Whereas the pituitary response to GnRH is not altered by photoperiod or reproductive state, GnRH release is markedly reduced in animals with regressed reproductive gonads (44, 47, 48). In the present study, alterations in kisspeptin staining, combined with our results demonstrating comparable LH responses to exogenous kisspeptin in long- and short-day animals, indicate that kisspeptin is likely driving seasonal changes in GnRH. Furthermore, these data uncover a novel upstream mechanism of GnRH regulation whereby environmental factors can be interpreted, integrated, and relayed to the GnRH system.

The results of the present study demonstrate that the investigation of photoperiodic polymorphisms can provide a powerful tool for understanding how kisspeptin affects the HPG axis independent of photoperiod. Kisspeptin-ir expression in short-day nonresponders did not differ from long-day hamsters in any of the measurements. Interestingly, although the subset of kisspeptin injected animals used in experiment 2 included only one short-day nonresponsive animal (making statistical comparisons impossible), LH and testosterone levels in response to kisspeptin injections in this animal displayed the same pattern of values observed in long-day animals (baseline LH = 0.68 ng/ml, postinjection LH = 11.60 ng/ml; baseline T = 5.00 ng/ml, postinjection testosterone = 33.92 ng/ml). Collectively, these results indicate that kisspeptin may provide a mechanism for differential interpretation and response to the same reproductively relevant stimuli and lends insight into the neural mechanisms mediating individual differences in reproductive regulation. For example, the nonresponsive phenotype may fail to inhibit hypothalamic kisspeptin synthesis in the AVPV, leav-

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ing high levels of the peptide available for continued stimulation of the GnRH neuronal system. Although our data provide both morphological (kisspeptin staining in the AVPV) and functional (HPG response to kisspeptin) support for this hypothesis, future studies are necessary to directly examine this possibility.

The combined results of this investigation provide the first evidence for an important regulatory role of kisspeptin in mediating reproductive consequences resulting from exposure to reproductively relevant stimuli. In addition, these findings indicate an important role for kisspeptin in mediating seasonal changes in reproductive function and individual differences in responsiveness to seasonal information. Seasonally breeding species serve as an important tool with which to explore the role of kisspeptin in mediating reproductive consequences in response to a wide range of environmental factors including the social environment, disease states, and energy availability.

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