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Photoperiod and Testosterone Interact to Drive Seasonal Changes in Kisspeptin Expression in Siberian Hamsters (*Phodopus sungorus*)

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Abstract

Kisspeptin, a neuropeptide product of the *KiSS-1* gene, has recently been implicated in the regulation of seasonal breeding in a number of species, including Siberian hamsters. In this species, kisspeptin expression is reduced in the anteroventral periventricular nucleus (AVPV) following exposure to inhibitory day lengths, and exogenous kisspeptin activates the reproductive neuroendocrine axis of reproductively quiescent animals. Because sex steroids can impact kisspeptin expression, it is unclear whether changes in kisspeptin occur in direct response to photoperiodic cues or secondarily in response to changes in sex steroid concentrations resulting from the transition to reproductive quiescence. The present study aimed to assess the relative contributions of photoperiod and testosterone in regulating kisspeptin expression in Siberian hamsters. Animals housed in long or short day lengths for 8 weeks were either castrated or received sham surgeries. Half of the hamsters in each photoperiod were given testosterone to mimic long-day sex steroid concentrations. The results obtained indicate that kisspeptin neurones in the AVPV and arcuate nuclei were influenced by both photoperiod and testosterone. In the AVPV, removal of testosterone or exposure to inhibitory day lengths led to a marked reduction in kisspeptin-immunoreactive cells, and testosterone treatment increased cell numbers across conditions. Importantly, long-day castrates exhibited significantly more kisspeptin cells than short-day castrates or intact short-day animals with empty capsules, suggesting the influences of photoperiod, independent of gonadal steroids. In general, the opposite pattern emerged for the arcuate nuclei. Collectively, these data suggest a role for both gonadal-dependent and independent (i.e. photoperiodic) mechanisms regulating seasonal changes in kisspeptin expression in Siberian hamsters.

Keywords

KiSS-1; reproduction; RF amide; seasonal breeding; gonadal steroids

The neuropeptide kisspeptin plays a prominent role in reproductive axis regulation, contributing to the timing of reproductive maturity and adult reproductive neuroendocrine function (1). Both mice and humans lacking functional *KiSS-1* and *GPR-54* genes, encoding the neuropeptide kisspeptin and its G-protein coupled receptor, GPR-54, respectively, fail to

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proceed through puberty and remain reproductively immature throughout adulthood (2–5). Exogenous administration of kisspeptin stimulates the gonadotrophin-releasing hormone (GnRH) system (6–18) and accelerates progression into puberty in rats and nonhuman primates (16,19).

Most animals alter their reproductive status to coordinate off-spring birth and rearing with favourable environmental conditions (20). In temperate zone breeding rodents, changes in photoperiod (i.e. day length) signal time of year and this cue is transduced into changes in the activity of the hypothalamo-pituitary-gonadal (HPG) axis (21,22). Recent studies suggest a key role for kisspeptin in regulating seasonal changes in reproductive function (11,23–25). Both Siberian (*Phodopus sungorus*) and Syrian (*Mesocricetus auratus*) hamsters held in simulated 'summer-like' or 'winter-like' photoperiods display marked differences in hypothalamic kisspeptin gene and protein expression (11,23,24). In our previous work in Siberian hamsters, we found that animals maintained on long days (16 : 8 h light / dark cycle) have high numbers of kisspeptin-immunoreactive (-ir) neurones in the anteroventral periventricular (AVPV) nucleus that are markedly reduced by exposure to short day lengths (11,23). Curiously, the arcuate nucleus (Arc) exhibits the opposite pattern of staining; few kisspeptin-ir neurones are found in long-day-housed animals, whereas a large number of kisspeptin-ir cells are present in short-day-housed animals (11,23). The specific mechanisms mediating these changes remain to be established.

In mice, sex steroids differentially modulate the expression of *KiSS-1* in the AVPV and Arc. In the AVPV, castration or ovariectomy reduces *KiSS-1* gene expression, whereas sex steroid replacement (testosterone or oestradiol respectively) restores the pattern of *KiSS-1* to pre-gonadectomy values; the opposite pattern is observed in the Arc (26,27). In photoperiodic rodents, inhibitory day lengths lead to gonadal involution and pronounced reductions in circulating gonadal steroids (28). As a result, the specific contribution of photoperiod and / or sex steroids to observed changes in the kisspeptin system need to be assessed independently. Photoperiod-driven changes in kisspeptin could precede the down-regulation of the HPG axis and subsequent decreases in testosterone, indicating a mechanism independent of gonadal steroids. Alternatively, short-day induced decreases in testosterone may drive observed alterations in hypothalamic kisspeptin via feedback mechanisms. Finally, these two processes may interact to precisely regulate kisspeptin. To assess the relative roles of testosterone and photoperiod to changes in kisspeptin expression, adult male Siberian hamsters were housed in long- and short-day photoperiods and circulating levels of testosterone were manipulated by castrations and testosterone replacement.

Materials and methods

Animals and housing

Adult (> 60 days of age) male Siberian hamsters (*Phodopus sungorus*) (n = 49) were obtained from our breeding colony maintained at Indiana University. The progenitors of these animals were generously provided by Dr Randy Nelson (Ohio State University) and Dr Timothy Bartness (Georgia State University). All animals were group-housed at weaning with same-sex siblings in a long-day photoperiod (16 : 8 h light / dark cycle). Prior to the start of the study, animals were housed individually in polypropylene cages (27.8 × 17.5 × 13.0 cm). Temperature was kept constant at 20 ± 2 °C and relative humidity was maintained at 50 ± 5%. Food (Purina Rat Chow; Ralston Purina Co., St Louis, MO, USA) and tap water were available *ad libitum* throughout the experiments. All experimental procedures follow NIH guidelines for the Care and Use of Experimental Animals and were approved by the Bloomington Institutional Animal Care and Use Committee.

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Experimental procedures

To investigate the relative contribution of photoperiod and gonadal steroids to changes in kisspeptin expression, hamsters were randomly assigned to either long- (16 : 8 h light / dark cycle) (n = 18) or short- (8 : 16 h light / dark cycle) (n = 31) photoperiods (long-day room: lights on 04.00 h; short-day room: lights on 08.00 h). After 8 weeks in photoperiod, hamsters were weighed to the nearest 0.1 g and pelage colouration was noted. Hamsters that did not lose ≥ 10% of their original body mass and did not display the typical progression of pelage colouration from summer grey to winter white were considered to be unresponsive to the short-day photoperiods (called ‘nonresponders’) (29,30). This assignment was visually confirmed via examination of testis size during surgery. Twelve hamsters were deemed nonresponsive to short-day photoperiods and were removed from the remainder of the study. Animals then received castrations or sham surgeries, followed by Silastic capsules either filled with testosterone or left empty, resulting in one of six treatment groups: (i) long day, sham operated, empty capsule (n = 6), (ii) long day, castrated, empty capsule (n = 6), (iii) long day, castrated, testosterone-filled capsule (n = 6), (iv) short day, sham operated, empty capsule (n = 7), (v) short day, castrated, empty capsule (n = 6) (vi) short day, sham operated, testosterone-filled capsule (n = 6). Two weeks after surgery and capsule implantation, a blood sample was collected from all hamsters and animals were perfused; brain tissue and serum were collected as described below. All sampling was conducted between 09.00 and 12.30 h EST.

Surgical procedures

Surgical procedures have been described previously (31). Briefly, hamsters were anaesthetised with 0.05 ml of a ketamine (20 mg / ml) / xylazine (4 mg / ml) cocktail in 0.9% saline. Lateral abdominal incisions were made and testes were excised, the abdominal cavity was sutured and skin was closed with 9-mm surgical clips (Clay Adams, Parsipany, NJ, USA). All above procedures were followed for the sham operation, except the testes were left in tact. Immediately after surgery, hamsters received either a 10-mm long Silastic capsule implant (inner diameter 1.47 mm, outer diameter 1.95 mm; American Scientific Product, McGraw Park, IL, USA) filled either with testosterone (Sigma, St Louis, MO, USA) or left empty as described previously (31). Implants were applied subcutaneously via a 5-mm incision on the intrascapular surface made perpendicular to the midline. The incision was closed with a 9-mm surgical clip. Both castration and implant sites received an application of nitrofurazone antibacterial ointment (Squire laboratories, Revere, MA, USA) to prevent infection and animals received meloxicam (Boehringer Ingelheim Vetmedica, Inc., St Joseph, MO, USA) orally for analgesia. Animals were returned to their respective photoperiods for an additional 2 weeks.

Blood sampling and perfusion

At the conclusion of the experiment (i.e. 2 weeks after surgery and implantation) a blood sample was collected via the retro-orbital sinus. Clots were removed and the blood spun for 30 min at 5000 g, serum was collected and stored at −80 °C until assayed for testosterone. Hamsters were weighed to the nearest 0.1 g and then deeply anaesthetised with 0.3 ml of ketamine cocktail and perfused transcardially with 50 ml of 0.9% saline, followed by 100–150 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.3). Brains were post-fixed 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 brainstem.

Immunohistochemistry, microscopy, cell counts, and optical density

Kisspeptin-immunoreactive cells were labelled using a rabbit anti-human kisspeptin serum (T-4771; Peninsula Laboratories Inc, Bachem, San Carlos, CA, USA) 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. Preliminary trial runs with this commercially available antiserum revealed immunoreactivity in the dordomedial hypothalamus (23), an area where the homologue of the avian RFamide gonadotrophin inhibitory hormone (GnIH) has been detected in rodents (32,33), and an area where KiSS-1 mRNA is not expressed in other rodents. To eliminate potential cross-reactivity of the kisspetpin antibody with this RFamide peptide, kisspeptin antiserum was first preadsorbed with GnIH peptide (diluted 1 : 5000) overnight. This antiserum is polyclonal, and thus contains a host of antibodies targeting specific epitopes of the peptide of interest. Preadsorption with GnIH allows the blocking of antibodies that are promiscuous for GnIH while maintaining those specific to kisspeptin. This procedure has been previously described and shown to specifically block GnIH labelling and maintain kisspeptin expression in this species (23). Sections were then mounted onto gelatin-coated slides, dehydrated in a graded series of ethanol solutions (70, 95 and 100%), and cleared in xylenes (Fisher Scientific Co. Pittsburgh, PA, USA) before the application of cover slips.

Slides were examined under bright field illumination on a Zeiss Z1 microscope (Carl Zeiss, Oberkochen, Germany) 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 the Arc. All cells were confirmed at a minimum magnification of × 400. Counted cells were photographed with a Zeiss Axiocam Cooled CCD camera at × 400 magnification for cell size and density analyses. All cells in every fourth section were counted through the rostro-caudal extent of the AVPV and 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 prior to data analysis.

Soma size and optical density (OD) measurements were performed on images captured at × 400 magnification for all cells on those two sections in the AVPV and Arc containing the highest number of cells. For each animal and brain region, a single mean optical density and cell size was calculated by taking the average value from the two sections examined. All cells examined had optical densities at least two standard deviations above the mean background OD measures for an individual brain. Cell bodies were outlined and the two-dimensional area was calculated using ImageJ, version 1.32 (National Institutes of Health, Bethesda, MD, USA). Each pixel in the greyscale 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 normalised to minimise differences between replications of immunohistochemistry. First, a background measurement was taken by placing a square outline, four times, on non-overlapping, 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 ImageJ, and subtracting the background OD from the OD of each cell.

Testosterone measurement

To confirm the effectiveness of castrations and testosterone implants, serum testosterone levels were measured via a commercial EIA kit (Correlate-EIA Kit #900–065; Assay Designs, Ann Arbor, MI, USA). Serum samples were run on two separate plates. Samples were diluted 1 : 20 and run in duplicate for each sample. The sensitivity of the assay was 3.82 pg / ml and the intraassay coefficient of variation was less than 5% on both plates and the inter-assay coefficient of variation was 5.5%. This assay has been previously validated for use in Siberian hamsters (34).

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Statistical analysis

The effect of experimental treatment on circulating levels of testosterone, body mass, and the number of kisspeptin immuno-reactive cells, cell size and optical density in the AVPV and Arc were analysed using a one-way analysis of variance (ANOVA). When an ANOVA revealed a significant effect of treatment, differences between treatment groups were probed using a Tukey HSD test. In addition, we probed for a relationship between circulating levels of testosterone and the number of kisspeptin cells, cell size and optical density in both the AVPV and Arc, regardless of treatment, using a Pearson's correlation. Prior to statistical examination, variables not meeting assumptions of parametric statistics were transformed accordingly. All statistics were performed using Minitab 15 (Minitab Inc., State College, PA, USA) for Windows, with $\alpha < 0.05$.

Results

Treatment effects on circulating testosterone titres

Treatment had a significant effect on serum testosterone titres ($F_{5,28} = 6.73$, $P < 0.001$; Fig. 1). Post-hoc analysis revealed that hamsters receiving testosterone implants, regardless of photoperiod, had the highest titres, and did not differ from each other ($P > 0.05$). Long-day castrates given testosterone treatment and short-day intact animals receiving testosterone did not differ from long-day sham controls receiving empty implants ($P > 0.05$). Short-day hamsters that did not receive testosterone, regardless of surgical condition, did not differ from each other, and had significantly lower concentrations of testosterone than both long- and short-day animals receiving testosterone ($P < 0.05$); neither group differed from long-day castrates or long-day sham groups that received empty implants ($P > 0.05$).

Treatment effects on body mass

Treatment had a significant effect on the change in body mass across the 10 weeks of the study ($F_{5,31} = 16.12$, $P < 0.001$) (Table 1). Hamsters in short days lost significantly more body mass than long-day-housed hamsters, regardless of treatment ($P < 0.05$). Within each photoperiod, treatment did not influence body mass ($P > 0.05$ in all cases).

Treatment effects on kisspeptin-ir cells in the AVPV

There was a significant effect of treatment on the number of kisspeptin-ir neurones in the AVPV ($F_{5,31} = 27.99$, $P < 0.001$; Fig 2 and Fig 3). Short-day-housed animals receiving sham surgeries or castrations combined with empty implants displayed the fewest kisspeptin-ir neurones in the AVPV; all other groups had significantly more kisspeptin-ir neurones ($P < 0.05$). Castrated, long-day-housed hamsters with empty implants displayed significantly fewer kisspeptin-ir neurones than long-day hamsters receiving testosterone implants; both castrated long-day and sham-operated short-day-housed hamsters given testosterone had significantly more kisspeptin-ir neurones than long-day-castrated hamsters with empty implants ($P < 0.05$). Long-day-housed castrated hamsters with empty implants displayed fewer, but not a statistically different numbers of kisspeptin-ir neurones, compared to long-day-housed sham operated hamster-with empty implants ($P > 0.05$).

Optical density and cell size in short-days castrated hamsters receiving empty implants was not calculated due to a lack of kisspeptin-ir neurones. When comparing the remaining groups, no effect of treatment was found on cellular optical density ($F_{4,22} = 0.15$, $P > 0.05$). Treatment did have a significant effect on cell size in the remaining groups ($F_{4,22} = 6.00$, $P = 0.002$). Castrated long-day animals with empty implants had significantly smaller cells than short-day sham-operated hamsters receiving testosterone ($T = 4.85$, $P < 0.001$), and tended to have

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smaller cells compared to short-day sham-operated hamsters with empty implants (T = 2.74, P = 0.079). All other treatment groups had kisspeptin-ir cells of similar size (P > 0.05).

Treatment effects on kisspeptin-ir cells in the Arc

There was a significant effect of treatment on the number of kisspeptin-ir neurones in the Arc ($F_{5,22} = 2.80$, $P < 0.05$; Fig 2 and Fig 3). Short-day, sham-operated hamsters with blank implants tended to have more Arc kisspeptin-ir neurones compared to long-day castrated hamsters provided with exogenous testosterone (P < 0.1); however, post-hoc analyses revealed no significant pair-wise differences between groups (P > 0.05 in all cases). Treatment had no effect on either cellular optical density (P > 0.05) or cell size (P > 0.05) (Table 2).

Relationship between circulating testosterone levels and kisspeptin

The number of kisspeptin-ir cells in the AVPV was positively correlated with circulating testosterone concentrations ($r^2 = 0.22$, $P < 0.05$; Fig. 4). Additionally, in detectable cells in the AVPV, the size of kisspeptin-ir cells was positively correlated with serum testosterone concentrations ($r^2 = 0.16$, $P < 0.05$; Fig. 4). Serum testosterone and optical density did not covary in AVPV kisspeptin-ir cells (P > 0.05). Additionally, a significant negative relationship between testosterone and the number of Arc kisspeptin-ir neurones was observed ($r^2 = 0.31$, $P < 0.01$; Fig. 5). Circulating concentrations of testosterone did not correlate with either optical density (P > 0.05) or cell size (P > 0.05; Fig. 5) in the Arc.

Discussion

The present study examined the relative contributions of photoperiod and gonadal steroids to kisspeptin regulation in seasonally breeding animals. Specifically, studies were designed to determine whether photoperiodic signals drive the down-regulation of the HPG axis and decreases in testosterone via kiss-peptin, if short-day induced decreases in testosterone drive observed alterations in kisspeptin, or whether both variables contribute to kisspeptin control. The present findings indicate that both photoperiod and circulating testosterone concentrations affect expression of kisspeptin in the AVPV and Arc, with low circulating sex steroids and short day lengths leading to low AVPV and high Arc expression of kisspeptin. Whereas the effects of short days on kisspeptin-ir were reversed with testosterone treatment, removal of testosterone via castration was either unable to induce short-day-like kisspeptin-ir expression in long-day animals (AVPV), or had no effect (Arc). Taken together, these findings indicate that both photoperiod and testosterone impact the kisspeptin system in Siberian hamsters.

The finding that testosterone influences kisspeptin expression in Siberian hamsters is consistent with observations in mice in which gonadectomised males and females display fewer *KiSS-1* neurones in the AVPV and an increase in neurones in the Arc compared to intact animals and individuals provided exogenous sex steroids (26,27). Although the present study investigated peptide content, future studies will be needed to investigate treatment effects on *KiSS-1* gene expression, as changes at the transcriptional level likely contribute to seasonal changes in kisspeptin peptide content.

When combined across all treatment groups, a significant positive relationship between circulating concentrations of testosterone and the number of AVPV kisspeptin-ir neurones and a negative relationship in the Arc was observed. This finding suggests potential positive and negative feedback effects of testosterone on AVPV and Arc kisspeptin expression, respectively, in this species. The Arc plays an important role in negative feedback of sex steroids in mammals (35,36), whereas, in females, the AVPV is an important locus for positive feedback effects of oestradiol necessary to stimulate the pre-ovulatory luteinising hormone (LH) surge (37,38). Recently, Arc kisspeptin has been proposed to mediate the negative

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feedback effects of gonadal hormones (26,27), possibly via alterations of the GnRH pulse-generator (39). Consistent with the roles of these brain regions, sex differences in expression patterns of kisspeptin have been noted, with females having higher expression in the AVPV compared to males (40,41), and AVPV kisspeptin neurones playing a prominent role in the pre-ovulatory LH surge (42). The role of steroid positive feedback on AVPV kisspeptin neurones in male rodents remains unclear, but has been suggested to potentially play a role in sexual behaviours (43). The noted differential effects of sex steroids on the AVPV and Arc in hamsters and mice, combined with noted sex differences in kisspeptin expression in these regions, suggest differential regulation between the sexes. It is noteworthy, however, that our present results suggest that testosterone may positively drive kisspeptin expression in the AVPV of males, an exciting opportunity for further exploration.

In addition to the effects of gonadal steroids on kisspeptin labelling, the present studies provide evidence for photoperiodic influences on kisspeptin that are independent of gonadal steroids. Short-day hamsters that display naturally low levels of circulating testosterone had similar testosterone titres to long-day-castrated hamsters. Importantly, however, even though these hamsters had comparable testosterone titres, short-day hamsters (both intact and castrated) displayed significantly fewer kisspeptin-ir neurones in the AVPV, and greater numbers in the Arc, compared to castrated long-day-housed animals; post-hoc analyses, however, did not reveal these differences to be significant in the Arc. When considering this comparison in isolation, this finding suggests that AVPV kisspeptin may be more sensitive to the negative feedback effects of testosterone in short days, rather than representing a gonadal steroid-independent effect of photoperiod. If true, then exogenous testosterone replacement should lead to suppression of kisspeptin in short-day animals. By contrast, testosterone treatment instead leads to a robust increase in kisspeptin in short-day animals. Taken together with all other group differences, these observations suggest that both photoperiod and circulating testosterone concentrations affect expression of kisspeptin.

It is possible that the alterations in kisspeptin staining seen in short days compared with long-day-castrated hamsters can be attributed to short-day animals experiencing a prolonged period of reduced gonadal steroids (i.e. approximately 6 weeks) relative to castrated long-day animals (i.e. 2 weeks). In common with all studies using castration and steroid replacement, these manipulations do not fully mimic natural changes in testosterone titres experienced over short (e.g. circadian changes in testosterone) or long time scales (e.g. gradual declines in testosterone in animals transitioning through gonadal regression). Although these possibilities cannot be ruled out, it is unlikely to fully explain these findings, as testosterone has significant effects on androgen receptor levels in as little as 11 days in this species (44), and castration has noticeable effects on HPG axis activity in as little as 1 week in another seasonal breeder, the prairie vole (*Microtus ochrogaster*) (45).

Photoperiod, independent of sex steroid hormones, is known to alter a variety of physiological and behavioural traits in seasonal mammals (46–57). For example, photoperiod alters circulating levels of gonadotrophins independent of gonadal steroids in Siberian hamsters (48,51). This observation, combined with the results of the current study, indicates that these steroid-independent changes in HPG axis activity may be, at least in part, attributable to steroid-independent changes in kisspeptin levels. The mechanisms by which photoperiod impacts the kisspeptin system independent of gonadal steroids represents an exciting area for further empirical investigation, and may help to clarify the observed variation in kisspeptin expression that is not explained by sex steroid concentrations. Future studies aiming to examine the interactive effects of photoperiod and energy status on the kisspeptin system, for example, represent an important area for further inquiry. Siberian hamsters provide an ideal model for such investigations; food intake and body mass is reduced in Siberian hamsters held on short days (58). Similarly, pronounced effects of energetics on kisspeptin have been noted; kisspeptin

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expression is reduced in laboratory animals held on a restricted diet (59–61) and *ob* / ob mice exhibit increased kisspeptin following leptin administration (62).

A significant interaction between photoperiod and circulating testosterone titres on androgen receptor levels in the Arc has been observed in Siberian hamsters (44). In the present study, although treatment had a significant effect on the number of kisspeptin-ir neurones in the Arc, no significant relationship was observed between any groups. If Arc kisspeptin-ir neurones are responsive to both photoperiod and sex steroids, then the interaction between photoperiod and testosterone concentrations may modulate Arc kisspeptin expression such that sex steroid feedback has a differential effect on kisspeptin immunoreactivity in long versus short days. The majority of Arc kisspeptin neurones in mice express mRNA for both androgen receptor and oestrogen receptor-α (26), supporting the possibility that Arc kisspeptin neurones are modulated by the interactive effects of photoperiod and testosterone on sex steroid receptors.

In one previous study, 4 weeks of testosterone treatment provided to reproductively quiescent Syrian hamsters did not alter Arc *KiSS-1* expression (24). In Syrian hamsters, Arc *KiSS-1* mRNA levels are opposite to the pattern observed in Siberian hamsters (23), with higher mRNA levels observed in reproductive than nonreproductive individuals (24). It is important to note that, in the present study, testosterone levels in manipulated hamsters were within the physiological range of values reported for long-day sham controls (i.e., individuals with highest testosterone levels in both long-day sham control and long-day castrate plus testosterone had approximately 17 ng / ml testosterone). Testosterone titres were negatively correlated with the number of kisspeptin-ir cells in the Arc, and, although not significant, testosterone treatment in short-day hamsters reduced the number of kisspeptin-ir cells to long-day like levels, suggesting additional species differences. In female sheep, which also breed seasonally, the majority of kisspeptin neurones are found in the Arc, and, similar to observations in laboratory mice, ovariectomy increases *KiSS-1* mRNA, whereas sex steroids (oestradiol, progesterone) return *KiSS-1* mRNA levels to values observed in intact animals (25). In Medaka fish, *KiSS-1* in the nucleus posterioris periventricularis is not responsive to changes in ovarian steroids, whereas *KiSS-1* neurones in the nucleus ventral tuberis are positively regulated by oestrogens (63). Future studies, in a greater variety of animals, will be needed to determine the relative contribution of sex steroids to kisspeptin regulation in discrete nuclei at the transcriptional and post-transcriptional levels of control.

The present observations provide further support for the role of kisspeptin as a key regulator of seasonal changes in reproductive function and as a mechanism for transducing and relaying environmental / internal signals to the GnRH system. Through manipulations of testosterone and photoperiod, the present study provides strong evidence indicating that both photoperiodic signals and gonadal steroid feedback work together to precisely regulate kisspeptin expression in Siberian hamsters. Studies aimed at uncovering the specific mechanisms by which these steroid independent effects are generated (e.g. melatonin regulation versus direct neural input), as well as the relative contributions of other relevant stimuli that also vary seasonally (e.g. temperature or food availability) to the regulation of this system, are required.

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Fig. 1.

Circulating testosterone titres to experimental treatment: circulating levels of testosterone were significantly altered in response to experimental manipulations ($P < 0.05$). Animals were held in either long- or short-day lengths, and received either surgical castration or a sham operation, combined with a silastic capsule either filled with testosterone or left blank. Different numbers denote that groups significantly differed from each other ($P < 0.05$).

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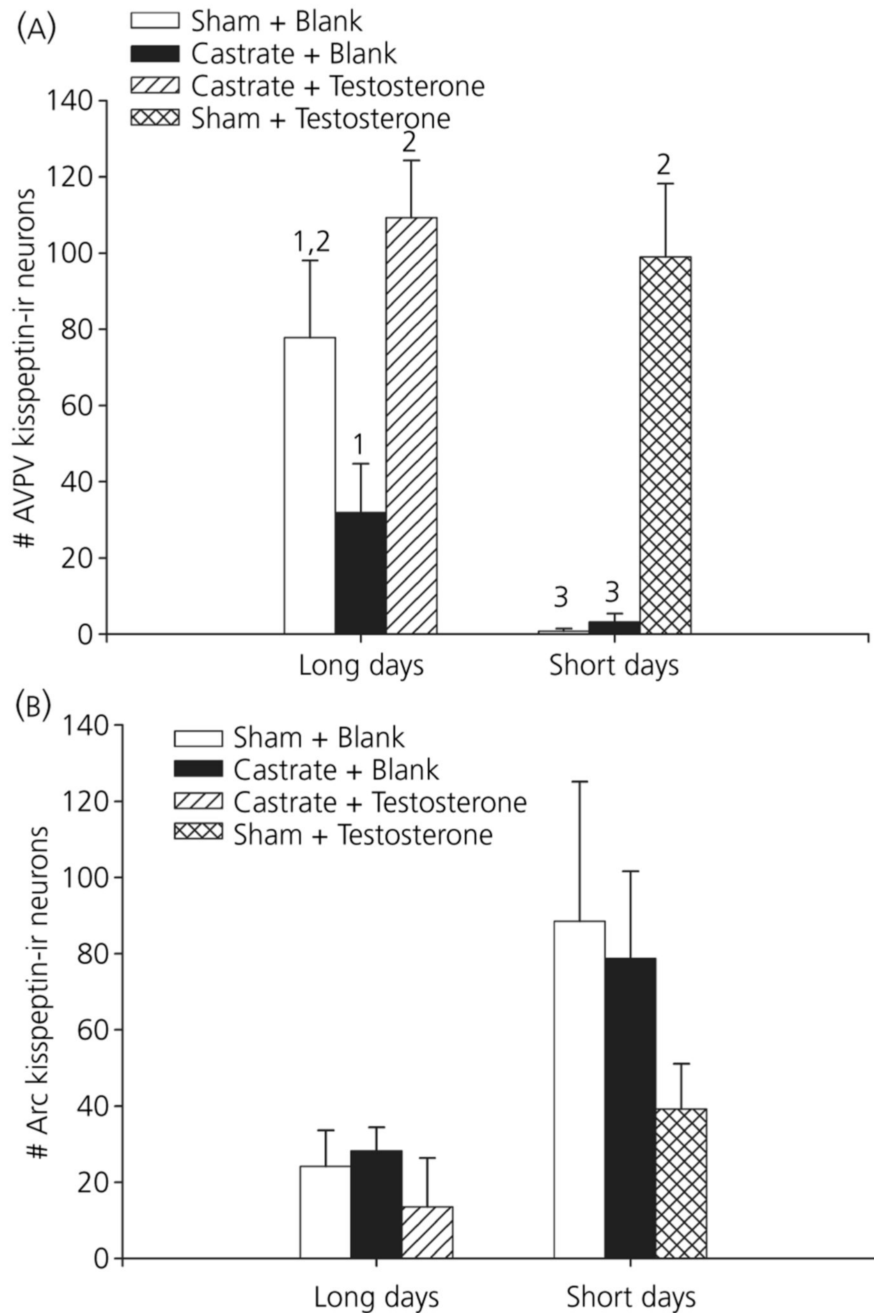

Fig. 2.

Effect of treatment on the number of kisspeptin-immunoreactive (-ir) neurones: experimental treatment had a significant effect on the number of kisspeptin-ir neurones in both the anteroventral periventricular nucleus (AVPV) (A) and arcuate nucleus (Arc) (B) of the hypothalamus ($P < 0.05$). Animals were held in either long or short day lengths, received either surgical castration or a sham operation, combined with a silastic capsule either filled with testosterone or left blank. Different numbers denote that groups significantly differed from each other ($P < 0.05$).

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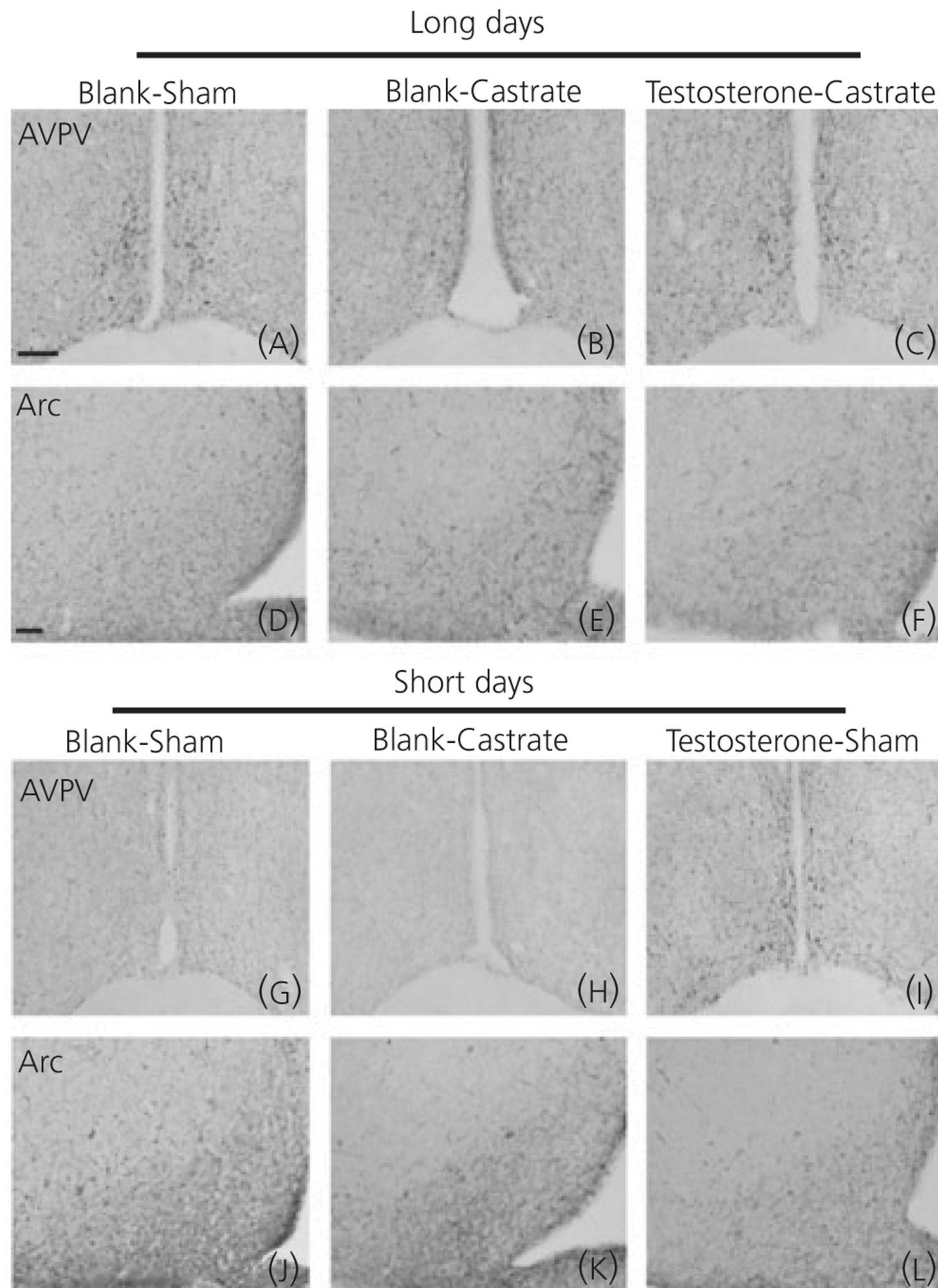

Fig. 3.

Response of kisspeptin-immunoreactive (-ir) neurones to experimental treatment: low-power photomicrographs demonstrating the effect of photoperiod and testosterone on kisspeptin expression in Siberian hamsters. Animals were held in either long- (A–F) or short- (G–L) day lengths and either castrated or left intact. Castrated long-day animals were either treated with testosterone or vehicle. Because intact short-day hamsters exhibit testosterone values equal to those of long-day castrates, one group of intact short-day hamsters (I,L) was treated with testosterone. Scale bars = 50 µm for anteroventral periventricular nucleus and 100 µm for arcuate nucleus. AVPV, anteroventral periventricular nucleus; Arc, arcuate nucleus.

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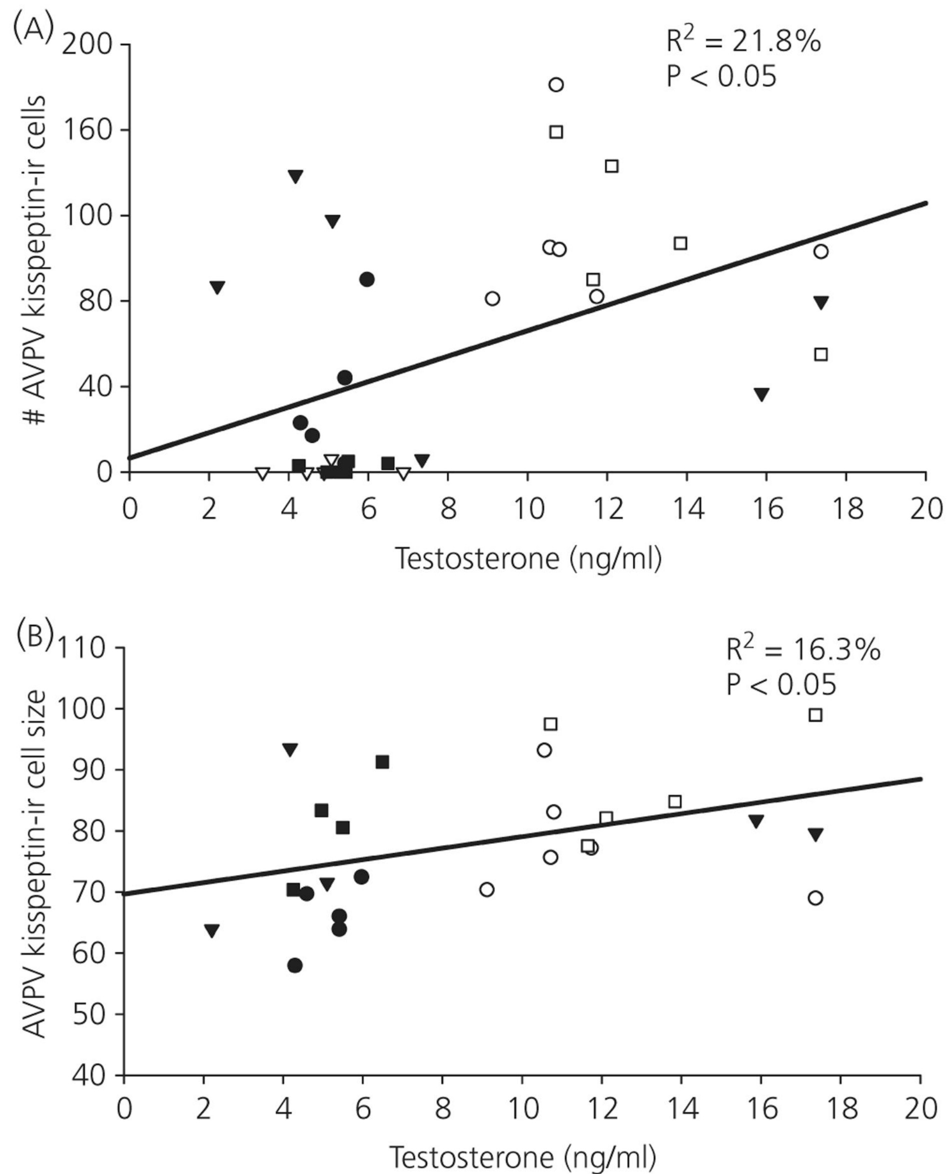

Fig. 4.
Relationship between testosterone and kisspeptin in the anteroventral periventricular nucleus (AVPV): across all individuals, testosterone levels were significantly positively related with both the number (A) and size (B) of kisspeptin-immunoreactive (-ir) neurones in the AVPV. ▼, long-day controls (sham + blank capsule); ●, long-day castrated + blank capsule; ○, long-day castrated + testosterone; ■, short-day controls (sham + blank capsule); ▽, short-day castrates + blank capsules; □, short-day sham + testosterone.

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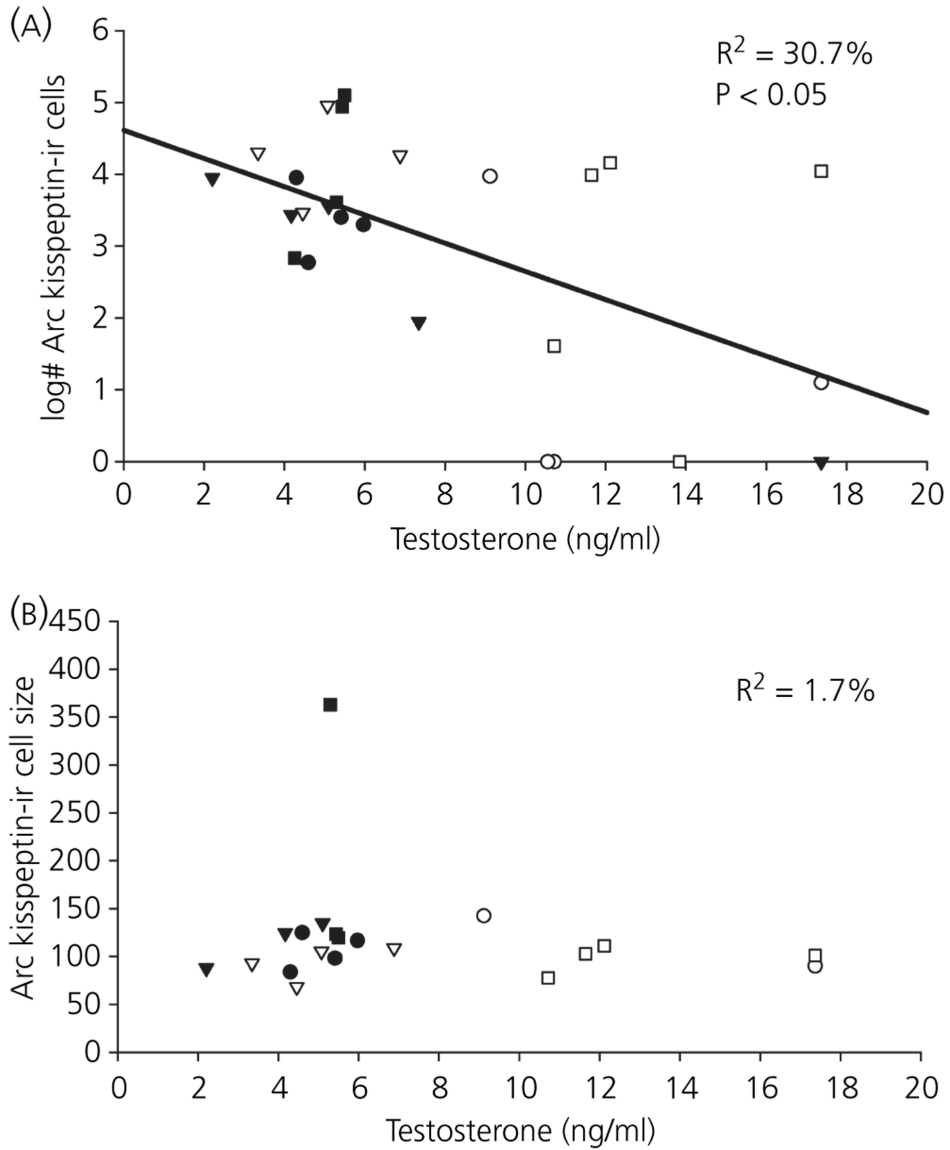

Fig. 5.

Relationship between between testosterone and kisspeptin in the arcuate nucleus (Arc): across all individuals, testosterone levels were significantly negatively related with the number of kisspeptin-immunoreactive (-ir) neurones in the Arc (A). There was no relationship between testosterone and the size of Arc kisspeptin-immunoreactive neurones (B). ▼, long-day controls (sham + blank capsule); ●, long-day castrated + blank capsule; ○, long-day castrated + testosterone; ■ short-day controls (sham + blank capsule); ∇, short-day castrates + blank capsules; □, short-day sham + testosterone.

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Table 1

Mean ± SEM Body Mass Values for Each Experimental Group.

Treatment	Initial mass	Week 8 mass	Week 10 mass
LD-sham-empty	41.73 (2.45)	42.11 (2.78)	41.12 (2.61)[1]
LD-cast-empty	38.47 (1.74)	41.63 (2.00)	37.66 (2.51)[1]
LD-cast-T	38.47 (1.79)	38.05 (2.71)	35.90 (2.23)[1]
SD-sham-empty	41.51 (1.18)	32.93 (1.59)	31.62 (1.50)[2]
SD-cast-empty	36.70 (2.69)	28.76 (1.11)	28.66 (1.02)[2]
SD-sham-T	37.57 (1.53)	29.56 (1.72)	31.46 (1.20)[2]

Superscript numbers denotes groups differed significantly ($P < 0.05$) in the change in body mass across the 10-week period of the study. LD, long-day photoperiod; sham, sham operation; empty, empty capsule; cast, castrated; T, testosterone-filled capsule.

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Table 2

Mean ± SEM of Optical Density (OD) and Cell Size.

Treatment	AVPV OD	Arc OD	AVPV cell size	Arc cell size
LD-sham-empty	29.75 (3.94)	25.75 (4.24)	78.07 (5.00)	115.85 (14.25)
LD-cast-empty	28.82 (4.59)	23.39 (2.76)	65.18 (2.19)*	104.60 (7.28)
LD-cast-T	31.05 (4.40)	24.98 (5.82)	78.07 (3.66)	116.36 (26.08)
SD-sham-empty	26.03 (6.19)	24.28 (1.96)	81.38 (4.32)	176.65 (62.31)
SD-cast-empty	—	22.54 (2.73)	—	93.83 (9.16)
SD-sham-T	28.91 (3.43)	21.15 (2.02)	90.81 (4.37)*	98.16 (5.55)

AVPV, anteroventral periventricular nucleus; Arc, arcuate nucleus; LD, long-day photoperiod; SD, short-day photoperiod; sham, sham operation; cast, castrated; empty, empty capsule; T, testosterone-filled capsule.

*Signifcantly differ from each other ($P < 0.05$).