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Response to exogenous kisspeptin varies according to sex and reproductive condition in Siberian hamsters (*Phodopus sungorus*)

Timothy J. Greives, Kimberly L. Long, Christine M. Bergeon Burns, Gregory E. Demas *

Department of Biology, Program in Neuroscience and Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington, IN 47405, USA

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ABSTRACT

Most animals experience marked changes in reproductive status across development that are regulated by changes in the hypothalamo–pituitary–gonadal (HPG) axis. The upstream mechanisms regulating this axis remain less well understood. The neuropeptide kisspeptin serves as a positive regulator of reproduction; the precise actions of kisspeptin on the HPG axis in animals of differing developmental and seasonal reproductive states, however, remain unresolved. Further, sex differences in response to kisspeptin have not been fully explored. In Experiment 1, we investigated whether sensitivity to a broad range of kisspeptin doses differed in adult male and female Siberian hamsters held on reproductively inhibitory or stimulatory photoperiods. In Experiment 2, we asked whether the response to kisspeptin differed across stages of reproductive development. Males and females displayed elevated luteinizing hormone (LH) in response to kisspeptin; however, the sexes differed in this response, with males showing greater LH responses to kisspeptin than females. Hamsters responded to kisspeptin across all stages of reproductive development, although the magnitude of this response differed between animals of differental ages and between the sexes. Males showed significant increases in LH at an earlier developmental age than females; females also showed blunted LH responses during early adulthood whereas males remained relatively constant in their response to kisspeptin. These findings suggest that reproductively active and inactive hamsters are responsive to kisspeptin, but that the sexes differ in their responsiveness. Collectively, these data provide further insight into the basic actions of kisspeptin in the regulation of reproduction and provide a potential mechanism for the regulation of differential reproductive responses between the sexes.

1. Introduction

All vertebrates experience marked changes in reproductive physiology during the developmental transitions from a sexually immature, pre-pubertal state to a post-pubertal, reproductively-active state. In addition to these relatively permanent developmental changes in reproductive status, seasonally breeding animals must also transition between reproductively active and inactive states on an annual basis. These seasonal changes in reproduction have previously been likened to a form of "seasonal puberty" (Ebling and Foster, 1990).

Changes in the activity of the hypothalamo–pituitary–gonadal (HPG) axis are responsible for regulating both pubertal and seasonal transitions in reproductive function. The fundamental role that the hypothalamic hormone gonadotropin-releasing hormone (GnRH) plays in regulating reproduction and fertility has been unequivocally established (Herbison, 2005; Levine, 2003). Most animals do not display continuous reproduction, however, and must encode and integrate salient internal and environmental signals (e.g., energy stores, photoperiod), and subsequently adjust GnRH release to regulate seasonal reproductive timing (Baker, 1938; Bronson, 1989; Ebling, 2005; Wingfield, 2008). Specifically, most rodents respond to short "winter-like" day lengths by down-regulating HPG activity and inhibiting reproduction; reproductive function is restored after prolonged exposure to long "summer-like" day lengths. The upstream mechanisms that integrate these signals to regulate HPG activity, however, are less well understood. Further, it remains unclear whether such regulatory mechanisms act in a similar manner during both developmental and seasonal reproductive transitions (Clarke and Pompolo, 2005; Ebling and Foster, 1990; Lehman et al., 1997). Seasonally breeding animals, therefore, serve as an excellent model system to address these questions (Ebling and Cronin, 2000; Ebling and Foster, 1990).

Recently, the neuropeptide kisspeptin has been identified as a potential regulator of both developmental and photoperiodic changes in reproduction (Caraty et al., 2007; de Roux et al., 2003; Funes et al., 2003; Greives et al., 2007; Mason et al., 2007; Revel et al., 2006; Seminara et al., 2003; Shahab et al., 2005; Smith et al., 2007). Kisspeptin acts as a potent positive regulator of GnRH

* Corresponding author. Address: Department of Biology, Indiana University, 1001 E. 3rd St., Bloomington, IN 47405, USA. Fax: +1 812 855 6705.
E-mail addresses: gdemas@indiana.edu, gdemas@bio.indiana.edu (G.E. Demas).

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release in all mammals studied to date and is the endogenous ligand for the kisspeptin receptor (Kiss1R)(previously called GPR54) (Gottsch et al., 2009). The importance of kisspeptin in normal reproductive maturation has been demonstrated by the observation that mutations in either the *Kiss1* gene or the gene for its cognate receptor *Kiss1R* render the animal unable to reach puberty (d'Anglemont de Tassigny et al., 2007; de Roux et al., 2003; Funes et al., 2003); animals expressing these mutations display pre-pubertal reproductive morphology and physiology for the remainder of their lives. Additionally, in non-human primates and rodents, *Kiss1* gene expression and kisspeptin protein are upregulated in the hypothalamus during reproductive pubertal development (Clarkson and Herbison, 2006; Han et al., 2005; Keen et al., 2008; Navarro et al., 2004; Shahab et al., 2005). Furthermore, kisspeptin content in the hypothalamus of rodents changes in response to manipulations of sex steroids as well as changes in photoperiod in seasonally breeding rodents (Greives et al., 2008a, 2007; Mason et al., 2007; Revel et al., 2006; Smith et al., 2007, 2008, 2005a,b). These findings highlight a potential role for kisspeptin to serve as an integrative signal to the HPG axis; kisspeptin responds to relevant internal and environmental signals and alters activity of the HPG axis.

Although kisspeptin has been shown to regulate GnRH release in mammals (Clarke et al., 2009; Crown et al., 2007; Fernandez-Fernandez et al., 2006; Greives et al., 2008b; Herbison, 2007; Kriegsfeld, 2006), its basic functions are still being determined. For example, it remains unclear whether kisspeptin activates the HPG axis similarly in developmentally non-reproductive (i.e., pre-pubertal) animals as it does in seasonally non-reproductive (i.e., short-day) animals (Caraty et al., 2007; Greives et al., 2007; Messager et al., 2005), and whether kisspeptin acts in a similar fashion in both sexes, where the costs of activating or maintaining reproductive physiology may differ substantially (Ball and Ketterson, 2008). Interestingly, *Kiss1* gene expression differs between male and female rats in one hypothalamic nucleus, the anteroventral periventricular nucleus (AVPV) (Kauffman et al., 2007), and female Siberian hamsters display reduced levels of the pituitary gonadotropin luteinizing hormone (LH) compared with males following repeated injections of a single dose of kisspeptin (Greives et al., 2007; Mason et al., 2007). These findings support the idea that kisspeptin and its downstream effects may differ between the sexes in certain contexts.

In the current study, we examined the ability of the HPG axis of male and female Siberian hamsters (*Phodopus sungorus*) to respond to an injection of exogenous kisspeptin in differing photoperiod-induced reproductive states (Experiment 1) and at different time points across reproductive development (Experiment 2). Additionally, in both experiments we investigated potential sex differences in sensitivity to kisspeptin. By comparing the actions of kisspeptin across reproductive conditions, this study will help elucidate the potential role of kisspeptin as a key mechanism regulating activity of the GnRH neuronal system.

2. Materials and methods

2.1. Animals and housing

All animals were obtained from a breeding colony maintained at Indiana University. All animals were group-housed at weaning with same-sex siblings in a long-day photoperiod (L:D 16:8). Breeders and pre-weaned offspring were housed together in large polypropylene cages (45 × 23 × 15 cm) until weaning at 18 days of age; weaned and adult individually housed animals were housed in smaller 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 (PMI LabDiet 5012, Rat Diet, St. Louis, Mo) 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 (BIACUC).

2.2. Experiment 1: effect of photoperiod on HPG axis sensitivity to exogenous kisspeptin

The aims of this experiment were to: (1) test the sensitivity of the HPG axis to a range of kisspeptin doses, (2) investigate potential sex differences in sensitivity to kisspeptin, and (3) determine whether photoperiod alters endocrine response to kisspeptin. To accomplish this, both males and females were housed individually in long days (L:D 16:8) and allowed to acclimate for 5–7 days. The hamsters were then weighed to the nearest 0.1 g and placed in either long-day (L:D 16:8) or short-day (8:16) photoperiods for >8 weeks. Due to space limitations, males and females were run separately and each sex was run in cohorts; all other procedures were identical between sexes and cohorts. Short-day hamsters that did not respond to photoperiod, so called "non-responders" (Lynch and Lynch, 1986), were identified by a lack of body mass loss and pelage coloration (males and females) and by non-regressed testes (males). These animals were excluded from further analysis.

2.2.1. Kisspeptin injections and blood sampling

A baseline blood sample was collected from the retro-orbital sinus prior to the animals receiving a single i.p. injection of 100 μl of a 0.1, 1, 5, or 10 μM kisspeptin-10 [KiSS-1 (112–121)/metastin (45–54) (human); Phoenix Pharmaceuticals, Inc., Belmont, CA], or PBS vehicle, creating 20 distinct treatments: long-day males receiving a 100 μl injection of vehicle ($n = 9$), 0.1 μM ($n = 9$), 1 μM ($n = 10$), 5 μM ($n = 10$), or 10 μM kisspeptin ($n = 10$); short-day males receiving vehicle ($n = 8$), 0.1 μM ($n = 8$), 1 μM ($n = 6$), 5 μM ($n = 7$), or 10 μM kisspeptin ($n = 6$); long-day females receiving vehicle ($n = 10$), 0.1 μM ($n = 10$), 1 μM ($n = 10$), 5 μM ($n = 9$), or 10 μM kisspeptin ($n = 10$); and short-day females receiving vehicle ($n = 8$), 0.1 μM ($n = 8$), 1 μM ($n = 10$), 5 μM ($n = 10$), or 10 μM kisspeptin ($n = 7$). A second blood sample was obtained 30 min after this injection for luteinizing hormone (LH) analysis. A subset of long-day males that had sufficient serum left after LH analysis were combined with additional long-day males following the same procedures and assayed only for testosterone (Final sample sizes: vehicle, $n = 8$; 0.1 μM, $n = 8$; 1 μM, $n = 11$; 5 μM, $n = 11$; 10 μM $n = 8$). Blood samples were left at room temperature to allow clots to form. Clots were then removed, samples were centrifuged at 2500 RPM for 30 min, and serum was collected and stored at −80 °C until assayed for reproductive hormones (see below for assay details). Following blood sampling, necropsies were performed and reproductive tissues were extracted, cleaned of fat and connective tissue and weighed to the nearest 0.1 g.

2.3. Experiment 2: effect of development on HPG axis sensitivity to exogenous kisspeptin

To capture any dynamic changes in the ability of the HPG axis to respond to exogenous kisspeptin over the course of reproductive development, pups from a given litter were assigned pseudo-randomly (controlling for initial body mass) on the day of their birth to be sampled either prior to entering puberty (on Day 15 [D15]), during pubertal development (D30), as sub-adults with developed gonads but undeveloped accessory organs (D45), during young adulthood when animals are fully reproductively capable (D60), and during adulthood (D75) (Adam et al., 2000; Hoffmann, 1978; Stetson et al., 1986). Pups were obtained from 7 breeding

pairs producing multiple litters, and pups from at least 4 different breeding pairs were represented at each of the designated sampling periods (e.g., D15, D30).

2.3.1. Kisspeptin injections and blood sampling

To assess HPG axis activation in response to kisspeptin, animals were injected with kisspeptin-10, or a 0.1 M PBS vehicle injection. Specifically, hamsters received a single i.p. injection of either 100 μl PBS or 100 μl of a PBS solution containing 10 μM kisspeptin-10 (Phoenix Pharmaceuticals, Inc.), yielding 20 treatment groups: males injected with vehicle on D15 ($n = 7$), D30 ($n = 6$), D45 ($n = 7$), D60 ($n = 5$), D75 ($n = 7$); males injected with kisspeptin on D15 ($n = 7$), D30 ($n = 12$), D45 ($n = 8$), D60 ($n = 6$), D75 ($n = 7$); females injected with vehicle on D15 ($n = 10$), D30 ($n = 5$), D45 ($n = 7$), D60 ($n = 10$), D75 ($n = 6$); and females injected with kisspeptin on D15 ($n = 7$), D30 ($n = 7$), D45 ($n = 8$), D60 ($n = 8$), D75 ($n = 8$). Thirty minutes after the injection, a blood sample was collected via the retro-orbital sinus. Blood was left at room temperature to allow clots to form, the clots were removed the sample was centrifuged at 2500 RPM for 30 min., and serum was collected and stored at −80 °C until assayed for LH (see below for assay details). Following blood sampling, the animals were killed and necropsies were performed. Gonads were removed to confirm sex and reproductive status, cleaned of fat and connective tissue, and weighed.

2.3.2. Hormone assays

Serum LH concentrations were measured in duplicate via a radioimmunoassay (RIA) with reagents obtained from the National Institutes of Health based on a previous protocol (Chappell et al., 1997). The antiserum was rLH-S-11 and the standard was rLH-RP3. The sensitivity was 0.01 ng/tube and the intra-assay coefficient of variation (CV) was 5.87% for the low pool and 5.25% for the high pool, the inter-assay CV was 6.13% for the low pool and 5.91% for the high pool; samples from both sexes were run in each assay. Serum testosterone was measured via a commercial EIA kit (Correlate-EIA Kit #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 and the intra-assay coefficient of variation for the assays was <8.1%; the inter-assay coefficient of variation was 5.24%. The antisera used in both assays were highly specific for the hormones measured, with low cross-reactivity with other hormones. Both the LH and T assays have been previously validated for use in Siberian hamsters (Demas et al., 2004; Wolfe et al., 1995).

2.4. Statistical analyses

In Experiment 1, the effects of differing doses of kisspeptin or vehicle injection on testosterone in long-day males and luteinizing hormone in all animals held in either long- or short-day photoperiods were analyzed using a General Linear Model (GLM). For testosterone analysis, injection dose was the independent variable, post-injection testosterone was the dependent variable, and baseline testosterone values were included as a covariate in the model. For LH analysis, injection dose, sex, and photoperiod and all interactions were set as the independent variables, post-injection LH as the dependent variable, and baseline LH levels were included as a covariate in the model. Significant effects were subsequently probed with separate ANOVAs combined with Tukey HSD post-hoc tests.

In Experiment 2, the effects of kisspeptin or vehicle injection on serum luteinizing hormone levels across development were analyzed using a GLM with injection type, sex, and age as main effects and including all interaction effects. To meet the assumptions of normality and equality of variances, LH values were log-transformed prior to analysis. As in Experiment 1, significant interactions were probed with further ANOVAs. Differences in body and gonad mass between the age groups within each sex were analyzed separately using a one-way ANOVA. To meet the assumption of normality of the residuals, female gonad masses were log-transformed prior to analysis. Tukey's HSD post-hoc tests were employed to probe pair-wise differences between the age groups.

3. Results

3.1. Experiment 1: effect of photoperiod on HPG axis sensitivity to exogenous kisspeptin

The GLM revealed significant main effects of sex ($F_{1,154} = 15.80$, $p \leqslant 0.001$), injection dose ($F_{4,154} = 40.95$, $p \leqslant 0.001$) and photoperiod ($F_{1,154} = 8.51$, $p \leqslant 0.01$). A significant interaction between injection dose and sex was also revealed ($F_{4,154} = 4.35$, $p \leqslant 0.01$); all other interactions were not significant ($p > 0.05$ in all cases) (Fig. 1). To probe the above effects, separate ANOVAs were performed with the sexes split to investigate the effect of differing doses and photoperiod and their interaction on post-injection LH levels in these groups. Males displayed a significant response to differing doses ($F_{4,73} = 24.95$, $p \leqslant 0.001$), but neither photoperiod ($p > 0.05$), nor the interaction of photoperiod and injection dose significantly affected post-injection LH levels, ($p > 0.05$). Post-hoc analysis revealed that males receiving an injection with 10 μM kisspeptin had significantly higher LH levels compared with all injection doses. Female post-injection LH levels were significantly affected by injection dose ($F_{4,\ 82} = 14.31$, $p \leqslant 0.001$), photoperiod ($F_{1,82} = 6.94$, $p \leqslant 0.01$) and the interaction between dose × photoperiod ($F_{4,82} = 2.47$, $p \leqslant 0.05$). To probe the dose × photoperiod interaction in the females, the data were further split by photoperiod to investigate how the responses to differing doses of kisspeptin varied in animals held in differing photoperiod treatment. In separate ANOVAs, the dose of kisspeptin significantly affected post-injection LH levels in both long-day ($F_{4,44} = 6.37$, $p \leqslant 0.001$) and short-day females ($F_{4,38} = 9.27$, $p \leqslant 0.001$). Tukey HSD post-hoc analysis revealed that long-day females receiving the 10 μM dose significantly elevated LH levels compared with all other long-day treatment groups ($p < 0.01$ in all cases). Post-hoc analysis of short-day females revealed that females receiving a 10 μM dose did not differ from females receiving a 5 μM dose ($p > 0.05$), while females receiving the 10 μM kisspeptin dose had significantly higher LH values compared with the 1.0 and 0.1 μM dose and higher values compared with vehicle treated animals ($p \leqslant 0.001$ in all cases). Short-day females receiving a 5 μM dose of kisspeptin did not differ from females receiving a 10 μM dose ($p > 0.05$), but had significantly higher post-injection LH levels when compared with short-day females receiving 1.0 and 0.1 μM doses ($p \leqslant 0.05$ in all cases). Short-day females receiving vehicle injection tended to have lower post-injection LH values compared with those receiving the 5 μM dose ($p < 0.1$).

A significant main effect of injection dose on serum testosterone levels was observed ($F_{4,40} = 10.63$, $p \leqslant 0.001$)(Fig. 2). Post-hoc analysis revealed that males injected with 10 or 5 μM of kisspeptin had significantly elevated post-injection LH levels compared with vehicle-injected animals and animals injected with a dose of 0.1 μM ($p \leqslant 0.05$ in all cases). No other significant pair-wise comparisons were revealed ($p > 0.05$ in all cases).

Photoperiod had a significant effect on both male ($F_{1,79} = 65.36$, $p < 0.001$) and female body masses ($F_{1,79} = 65.28$, $p < 0.001$) (Fig. 2). No differences in body mass were observed between injection treatment groups within each photoperiod ($p > 0.05$ in all cases). Photoperiod had a significant effect on paired-testes masses ($F_{1,78} = 963.23$, $p < 0.001$)(Fig. 2). No differences in paired-testes

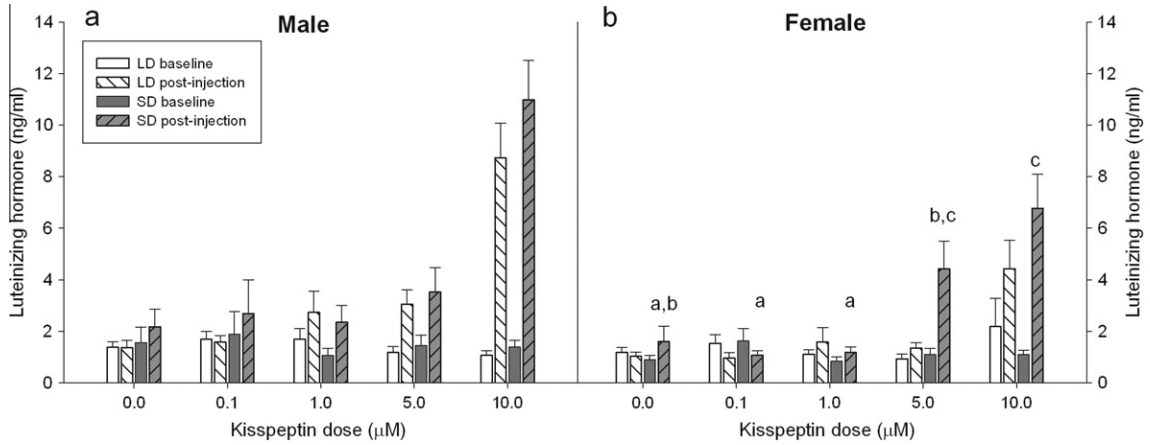

Fig. 1. Effect of differing doses of kisspeptin injections on serum levels of luteinizing hormone (LH). Male (a) and female (b) hamsters housed in either long-day (white bars) or short-day (dark-bars) photoperiods were injected with 100 μl of vehicle, 0.1 μM, 1 μM, 5 μM or 10 μM kisspeptin. Baseline (solid bars) and post-injection (striped bars) blood samples were assayed for LH. A significant main effect of injection dose was revealed in both sexes. Differing letters indicate significant differences in post-injection LH in the females revealed by post-hoc analysis probing the significant dose × photoperiod interaction in this sex. Figure legend: LD = long-day, SD = short-day. Significance is set at $p < 0.05$.

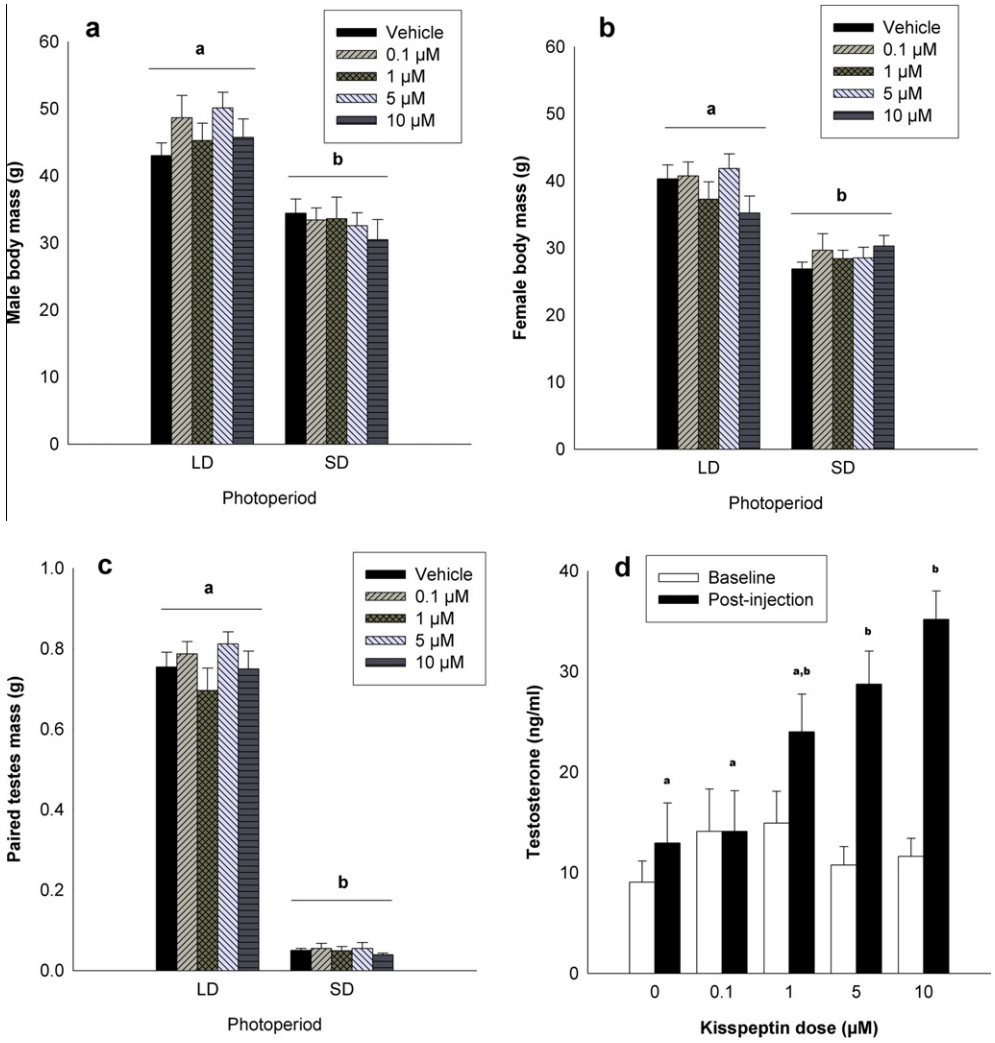

Fig. 2. Photoperiod treatment affects body mass and paired testes masses. Body masses of long-day male (a) and female (b) as well as paired testes masses of male (c) Siberian hamsters that received an injection of 100 μl of vehicle, 0.1 μM, 1 μM, 5 μM or 10 μM of kisspeptin were significantly affected by photoperiod treatment. Different letters indicate that groups differed significantly. Long-day hamsters injected with 100 μl of vehicle, 0.1 μM, 1 μM, 5 μM or 10 μM of kisspeptin displayed differences in post-injection testosterone titers compared with baseline titers (d). Post-injection serum testosterone concentrations differed significantly between injection dose, and is indicated by differing letters. Significance is set at $p < 0.05$.

mass were observed between injection treatment groups within each photoperiod ($p > 0.05$ in all cases).

3.2. Experiment 2: effect of development on HPG axis sensitivity to exogenous kisspeptin

The GLM revealed significant main effects of injection treatment ($F_{1,128} = 45.09$, $p \leqslant 0.001$), age ($F_{4,128} = 7.96$, $p \leqslant 0.001$), and sex ($F_{1,128} = 6.85$, $p \leqslant 0.01$) as well as a sex × age × injection interaction ($F_{4,128} = 3.50$, $p \leqslant 0.01$) on serum LH levels (Fig. 3); all two-way interactions were not significant ($p > 0.05$). To probe the 3-way interaction, the data was split by sex and then run in separate ANOVAs to investigate the effects of age and injection and the age × injection interaction to uncover the nature of the sex × age × injection interaction. These analyses revealed that in males, a main effect of injection type (kisspeptin vs. vehicle) was significant ($F_{1,62} = 31.92$, $p \leqslant 0.001$), while age and injection × age interactions were not significant ($p > 0.05$). In females, significant main effects of both injection type ($F_{1,66} = 15.48$, $p \leqslant 0.001$) and age ($F_{4,66} = 6.89$, $p \leqslant 0.001$) were revealed, as well as a significant interaction between age and injection ($F_{4,66} = 3.99$, $p \leqslant 0.01$).

The significant age × injection interaction was further probed by splitting the data by age and investigating the effect of the injection treatment (kisspeptin vs. vehicle) on females of different ages. These data revealed significant differences between kisspeptin and vehicle treated females at D30 ($F_{1,12} = 11.00$, $p \leqslant 0.01$), and D45 ($F_{1,13} = 20.20$, $p \leqslant 0.001$); 15 day old females tended to have higher LH levels in kisspeptin injected females compared with vehicle injected females ($p \leqslant 0.1$). No significant effect of kisspeptin injection on circulating LH was found in adult (D60 and D75) females ($p > 0.05$ in both cases).

In male hamsters, there was a significant effect of age on body mass ($F_{4,62} = 84.05$, $p < 0.001$) and paired-testes mass ($F_{4,65} = 65.03$, $p < 0.001$)(Fig. 4). Pre-pubertal (D15) males weighed less than all other groups ($p < 0.05$ in all cases) and had smaller paired-testes masses. Pubertal males (D30) weighed less and had smaller testes than sub-adult (D45), young adult (D60) and adult males (D75) ($p < 0.05$ in all cases). Sub-adult males (D45) weighed less than young adult and adult males ($p < 0.05$ in all cases); paired-testes masses did not differ between sub-adult males and young adult and adult males ($p > 0.05$ in all cases). The body mass and paired-testes masses of young adult (D60) and adult (D75) males did not differ ($p > 0.05$), and both groups were heavier than all other groups ($p < 0.05$ in all cases).

In female hamsters, there was a significant effect of age on body masses ($F_{4,65} = 52.03$, $p < 0.001$) and uterine horn and ovarian mass ($F_{4,68} = 37.38$, $p < 0.001$)(Fig. 4). Pre-pubertal (D15) females weighed significantly less and had smaller gonads than all other groups ($p < 0.05$ in all cases). Pubertal females (D30) were significantly lighter than sub-adult (D45), young adult (D60) and adult (D75) females, and had lighter gonads than young adult and adult females ($p < 0.05$ in all cases); pubertal and sub-adult gonad masses did not differ ($p > 0.05$). Sub-adult (D45) females were heavier than pre-pubertal and pubertal females, and weighed less than adult females ($p < 0.05$ in all cases). Young-adult (D60) weights were not significantly different than sub-adults (D45) or adults (D75) ($p > 0.05$). Gonadal masses did not differ between sub-adult (D45) and adult (D75) females, nor between young adult (D60) and adult (D75) females ($p > 0.05$ in all cases); young-adult females had heavier gonads than all other groups ($p < 0.05$ in all cases).

4. Discussion

Overall, significant activation of the reproductive neuroendocrine axis (as measured by serum LH) in response to kisspeptin injections was observed in both long- and short-day hamsters and across all stages of reproductive development; however, the magnitude of this response differed depending on the age of the animals. Additionally, sex differences were observed in response to kisspeptin; males and females displayed different patterns of LH responses to an intermediate dose of kisspeptin depending on reproductive status. Sex differences were also observed across pubertal development; kisspeptin injected females tended to have higher LH levels compared with males, and responded more robustly to kisspeptin at 30 days of age. Taken together, these data demonstrate a modest sex difference in the sensitivity to exogenous kisspeptin depending on the reproductive status of the animals. Differences in kisspeptin sensitivity during periods of reproductive transition may serve as one potential mechanism for the differential regulation of reproductive responses between male and female animals.

Potential differences in the sensitivity of the HPG axis of hamsters housed either in reproductively inhibitory short-day photoperiods or stimulatory long-day photoperiods were investigated both within and between the sexes in Experiment 1. The results of this experiment demonstrated that the sensitivities to kisspeptin differed between the sexes. Specifically, male hamsters demonstrated dose-dependent increases in LH in both photoperiods, with both

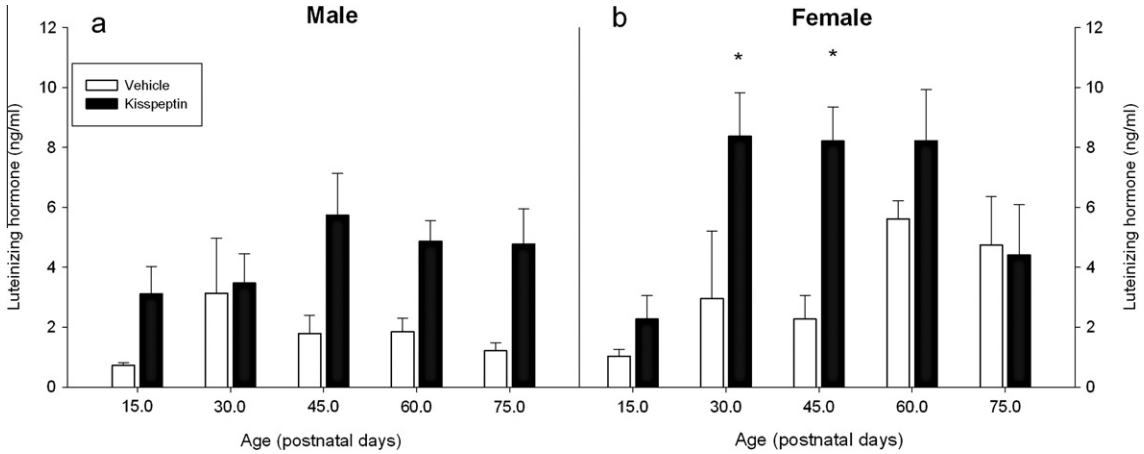

Fig. 3. Kisspeptin affects serum luteinizing hormone (LH) concentrations in Siberian hamsters at different stages of reproductive development. Male (a) and female (b) Siberian hamsters were injected with either 100 μl of vehicle or kisspeptin (10 μM) before puberty (D15), during puberty (D30), as sub-adults (d45), young adults (D60) or adults (D75). A significant main effect of kisspeptin in males was observed. In females a significant main effect of kisspeptin was also found, as well as an injection × age interaction. Post-hoc analysis in females revealed significant effects of kisspetpin compared with vehicle on circulating LH levels ($p < 0.05$); these effects are denoted by an asterisk (*).

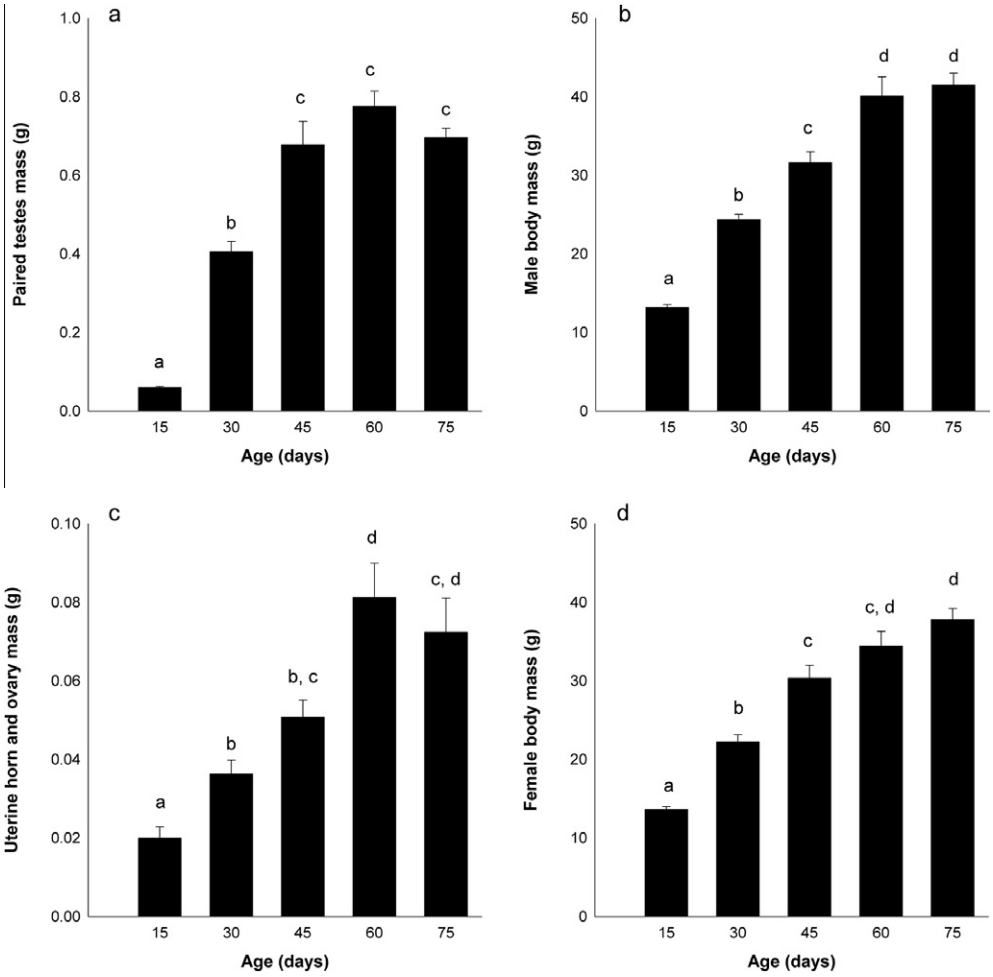

Fig. 4. Observed changes in body mass and reproductive organs across reproductive development. Male paired testes mass (a) and body mass (b), and female uterine and ovarian masses (c) and body masses (d) were recorded from individuals before puberty (D15), during puberty (D30), as sub-adults (d45), young adults (D60) or adults (D75). Different letters indicate that the groups significantly differed from each other ($p < 0.05$).

long- and short-day males injected with 10 μM kisspeptin elevating LH levels over baseline; males injected with 5 μM kisspeptin also elevated LH levels over baseline. These observations are consistent with a previous finding indicating a similar capability for kisspeptin to activate the HPG axis in both reproductive and non-reproductive male hamsters (Greives et al., 2007).

Both long-day reproductive and short-day non-reproductive female hamsters displayed a significant increase in serum LH in response to an injection of 10 μM kisspeptin. However, female hamsters differed in their response to an injection with an intermediate dose of 5 μM kisspeptin. Whereas long-day reproductive male hamsters displayed a robust increase in LH over baseline in response to kisspeptin, long-day reproductive females failed to elevate LH levels, while short-day non-reproductive females significantly increased serum LH levels. These observations suggest the possibility that males and females differ in sensitivity to differing concentrations of kisspeptin depending on their reproductive status.

The source of the observed sex differences in sensitivity to kisspeptin reported here remains unknown and most studies to date have focused on sex differences in *Kiss1* gene expression. In female mice, for example, treatment of neonatal mice with testosterone grossly reduces AVPV *Kiss1* expression (Kauffman et al., 2007). Likewise, neonatal castration of male mice leads to markedly enhanced *Kiss1* gene expression in the AVPV (Kauffman et al., 2007). Interestingly, this sex difference remains in gonadectomized animals, indicating the importance of early sex steroid exposure in the extent of AVPV *Kiss1* expression, although estrogen generally up-regulates *Kiss1* expression in adult females (Kauffman et al., 2007). Given these differences, even in the absence of gonadal steroids, it is likely that receptor numbers are similarly sexually differentiated. Additionally, photoperiod may differentially alter the number of receptors for kisspeptin, Kiss1R, between the sexes, although this idea remains to be tested. Sex differences in expression of the *Kiss1R* gene have been observed in rhesus monkeys of differing developmental reproductive states; Kiss1R mRNA increased during puberty in female but not male monkeys (Shahab et al., 2005). Whether or not differences in gene expression translate to actual differences in receptor number, and whether sex differences in seasonal changes in receptor number are observed in seasonally breeding animals requires further exploration. Photoperiod-induced differences in inhibitory neuropeptides, such as gonadotropin-inhibitory hormone (avian GnIH) (called RF-amide related peptide [RFRP] in mammals) (Bentley et al., 2003; Kriegsfeld et al., 2006; Tsutsui et al., 2000) may also act as a potential mechanism regulating sex differences in the ability of the HPG axis to respond to the stimulatory cue of kisspeptin (Greives et al., 2008b; Kriegsfeld, 2006).

In a previous study, female hamsters receiving repeated injections of 10 μM kisspeptin, a protocol that had been found to significantly elevate LH levels in adult wild-type male mice and adult male hamsters (Greives et al., 2007; Messager et al., 2005), displayed significant differences in LH response based on their photoperiodically-induced reproductive condition; long-day

reproductive females displayed significantly elevated serum LH over baseline in response to injections of kisspeptin, whereas serum LH levels in short-day females did not differ from baseline or from animals injected with vehicle (Mason et al., 2007). In the current study both long-day reproductive and short-day non-reproductive female hamsters receiving one injection of 10 μM kisspeptin displayed a significant increase in serum LH, demonstrating that, while short-day females are capable of responding to kisspeptin, other factors are capable of altering the ability of the axis to respond to subsequent presentations of kisspeptin. One likely candidate for the observed differences in LH responses to differing kisspeptin regimes is an increase in short-day induced steroidal negative feedback. Negative feedback to gonadal steroids is more pronounced in photo-inhibited seasonal breeders (Bittman et al., 1983; Ellis and Turek, 1979; Karsch et al., 1993; Moffatt et al., 1995). Thus, the up-regulation of this axis induced by a single injection of kisspeptin combined with short-day induced increases in negative feedback of the axis could facilitate the previously reported basal levels of LH measured in female hamsters after 4 total injections, 2 h after the initial injection (Mason et al., 2007). It remains unclear, however, whether the ovaries of photo-inhibited females are capable of elevating sex steroid levels in response to kisspeptin, and this question warrants further study. If photo-inhibited females do not alter circulating levels of sex steroids in response to kisspeptin, a down-regulation of this axis in response to multiple kisspeptin injections may be regulated via steroidal independent mechanisms acting on the kisspeptin–HPG system (Bittman and Goldman, 1979; Bittman et al., 1992; Greives et al., 2008a; Meikle and Fisher, 1996; Smith et al., 2008; Turek and Ellis, 1981; Zucker and Licht, 1983).

Experiment 2 documents for the first time the effects of a single injection of kisspeptin on serum LH levels in male and female seasonally breeding rodents in different developmental reproductive states. Specifically, male hamsters injected with kisspeptin displayed higher LH levels compared with vehicle-injected animals. This observed sensitivity to kisspeptin, regardless of developmental reproductive status, is similar to previous observations in adult male hamsters and female sheep in differing reproductive conditions; both reproductive and non-reproductive animals display significant elevations in LH in response to kisspeptin (Caraty et al., 2007; Greives et al., 2007). Female hamsters also demonstrated significantly elevated LH levels in kisspeptin-injected animals compared with those injected with vehicle. This elevation, however, was only significantly different in pubertal (D30) and sub-adult (D45) females; neither pre-pubertal (D15) nor adult (D60 and D75) females injected with kisspeptin displayed elevated LH levels compared with vehicle injected controls. The lack of a detectable kisspeptin-induced LH surge in adult females may be due to naturally occurring increased variation in LH levels in control females that are now cycling adults.

Because many of the physiological processes experienced during developmental and photoperiodic transition from a non-reproductive state to a reproductive state are similar, seasonally breeding animals have served as useful models for the study of the neuroendocrine mechanisms regulating reproductive status (Ebling and Cronin, 2000; Ebling and Foster, 1990). For example, GnRH secretion is reduced during the pre-pubertal and seasonal non-reproductive period, while the ability of the pituitary to respond to GnRH remains (Foster and Ryan, 1979; Goodman et al., 1982; Meredith et al., 1998; Pickard and Silverman, 1979; Sisk, 1987; Sisk and Foster, 2004). The reduction of GnRH release and subsequent inhibition of LH surges in both pre-pubertal and seasonal non-reproductive animals is regulated by increased steroidal negative feedback; ovariectomy or castration increases LH pulse frequency and circulating LH in both pre-pubertal and seasonally non-reproductive animals and sex steroid replacement suppresses circulating LH (Ebling et al., 1989; Glass and Dolan, 1988; Karsch et al., 1984, 1993; Olster and Foster, 1988; Richardson et al., 2004; Tilbrook et al., 1999). The current findings are consistent with this idea; significant similarities in the actions of kisspeptin on the HPG axis in hamsters of differing developmental and photoperiod-induced reproductive states were observed.

Additionally, significant sex differences in the responsiveness to kisspeptin were observed. The mechanisms regulating these differences, however, remain unresolved. Sex differences in the maintenance and timing of changes of reproductive function often differ between the sexes, and these differences likely reflect differing selective pressures between the sexes (Ball and Ketterson, 2008). The observed difference in sensitivity to kisspeptin may act as one such sexually differentiated mechanism that has been shaped by differing selective pressures. Collectively, these results provide further insight into the role of kisspeptin in the developmental and seasonal regulation of reproductive physiology.

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