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Photoperiod-induced differences in uterine growth in *Phodopus sungorus* are evident at an early age when serum estradiol and uterine estrogen receptor levels are not different

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ABSTRACT

Sexual development is inhibited in Siberian hamsters (*Phodopus sungorus*) in short days (SD), and a small uterus is an obvious indicator of photo-inhibition. The small uterus in SD is presumably due to the delayed onset of estrous cycles. However, in an earlier study, the investigators reported that serum estradiol (E2) concentration was significantly higher in young females raised in SD than in long days (LD), with the highest concentrations measured in SD at 4 weeks of age. These seemingly contradictory findings were investigated in the present study. First, uterine mass and body mass were measured in SD- and LD-reared hamsters from 1 to 12 weeks of age. Uterine mass was significantly greater in LD than in SD by 3 weeks of age and onward. Thereafter, our investigation focused on 4-week-old hamsters. Serum E2 concentrations in LD and in SD were not significantly different and there were no significant LD–SD differences in uterine estrogen receptors (ER), as measured by immunohistochemistry and quantitative real-time RT-PCR. Therefore, alternative explanations for the photoperiodic difference in uterine size in young Siberian hamsters are considered.

1. Introduction

Age at puberty can vary significantly in short-lived, temperate zone rodents depending on their season of birth. Females born before or near the summer solstice, i.e., when day length is either increasing or near its annual maximum, mature rapidly and produce litters during the year of their birth. Conversely, females born well after the summer solstice are very likely to delay puberty and their first breeding until the following spring (Negus et al., 1986, Butler et al., 2007). One of the hallmarks of delayed puberty in females is the stunted growth of the uterus. In Siberian hamsters, *Phodopus sungorus*, Ebling (1994) measured uterine mass at 5 and 10 weeks of age in females held in either short days (SD) or long days (LD). Whereas uterine mass increased more than two-fold in long day (LD) females, no change in uterine mass was detected in SD females. At both 5 and 10 weeks of age, Ebling (1994) determined that uterine mass in SD hamsters was significantly less than in LD females. Subsequent studies also documented significant uterine size discrepancies in SD and LD hamsters at 10 and 13 weeks of age (Place et al., 2004, Timonin et al., 2006).

Smaller uterine size in SD hamsters has been assumed to result from the indirect effects of the SD melatonin (MEL) signal, with MEL being secreted into the circulation from the pineal gland at night at higher concentrations and for a longer duration than in hamsters held in LD (Darrow and Goldman, 1985, Hoffmann et al., 1986). Stunted growth of the uterus has been presumed to result from the suppression of gonadotropins in SD (Dodge and Badura, 2002) and the associated reduction in the concentrations of estrogens (e.g., estradiol). To our knowledge, this hypothesis has never been formally tested and this conjecture has been called into question by a study that reported significantly higher serum E2 concentrations in SD than in LD hamsters between 4 and 11.4 weeks of age (van den Hurk et al., 2002). In that study, E2 in SD females was as much as eight-fold higher than in LD hamsters (8 weeks of age), and significantly higher concentrations were noted in SD at each of the ages studied (4, 8, and 11.4 weeks). The highest absolute concentration (~2400 pmol/L or ~654 pg/mL) was measured in SD females at 4 weeks of age. Given the growth promoting effects of E2 on the uterus (Scotti et al., 2007), we found the report of higher E2 in SD hamsters (van den Hurk et al., 2002) to be perplexing. Unfortunately, van den Hurk et al. (2002) did not report uterine mass data, thus the present study is the first to investigate the apparent mismatch between serum E2 concentrations and uterine growth in *P. sungorus*.

To that end, we collected uterine and body mass data at regular intervals from 1 to 12 weeks of age, to more clearly elucidate the differences when females develop in LD or in SD. Earlier investigations that reported the effects of photoperiod on uterine mass in *P. sungorus* evaluated females at only one or two ages (Ebling, 1994, Place et al., 2004, Timonin et al., 2006), and the youngest animals studied were 5 weeks of age (Ebling, 1994). When we determined that the photoperiod-induced differences in uterine size were evident by 3 weeks of age, we then focused our investigation on LD and SD

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hamsters at 4 weeks of age for the following reasons: 1. The highest E2 concentration reported by van den Hurk et al. (2002) was in SD females at this age and the LD–SD difference in serum E2 concentration at 4 weeks was substantial (approximately 5-fold), and 2. Neither LD nor SD females have matured by this age, as indicated by the lack of vaginal patency (Place et al., 2004) and the absence of any signs that ovulations had occurred (van den Hurk et al., 2002). Our investigations of 4-week-old hamsters avoided potentially confounding variables, such as stage of estrous cycle in LD females or LD–SD differences in reproductive state.

Using an assay previously validated for the measurement of E2 in Siberian hamsters (Scotti et al., 2007), we did not replicate the findings of van den Hurk et al. (2002), i.e., that serum E2 concentration is significantly higher in SD than in LD hamsters at 4 weeks of age. However, because we found serum E2 concentrations to be nearly identical in 4-week-old SD and LD females, we looked to the uterus for possible mechanisms to explain the photoperiod-induced difference in uterine size. To test the hypothesis that estrogen receptor (ER) abundance differs in uteri from SD and LD hamsters, we assessed ERs by immunohistochemistry for ERα and quantified mRNA levels by real-time RT-PCR for ERα (*Esr1*) and ERβ (*Esr2*).

2. Materials and methods

2.1. Experimental animals

Siberian hamsters from our colony (14 h of light per day, 14L) were transferred to LD (16L) or SD (10L) as breeding pairs to generate experimental LD and SD females. The time of lights-off was synchronized for all animals to 1700 Eastern Standard Time (EST). Animals were originally derived from wild-bred stock obtained from Dr. K. Wynne-Edwards, Queen's University. Experimental females were weaned on postnatal day 18, placed in polypropylene cages (2 to 4 siblings/cage), and maintained in the photoperiod in which they were born. Food (Teklad 8626, Madison, WI, USA) and water were available *ad libitum*. Ambient temperature and relative humidity were held constant at 21 ± 5 °C and 50 ± 10%, respectively.

Different sets of animals were used in Experiments 1 and 2, as well as for different determinations within Experiment 2. All experiments were carried out in accordance with the European Commission legislation on the protection of animals used for experiments (EC Directive 86/609/EEC).

2.2. Experiment 1: Uterine mass and body mass to 12 weeks of age

Measurements of uterine and body mass were made on six to seven SD and LD females at each of the following ages: 1, 2, 3, 4, 6, 8, 10, and 12 weeks. Sibling females were divided across age cohorts, thus siblings were never in the same age group. Body mass was measured just prior to death for each of the predetermined ages. Animals at 1 week of age were killed by decapitation, whereas older animals were given an overdose of pentobarbital sodium. Uteri were removed, dissected free of fat and connective tissue, and weighed on an analytical balance.

2.3. Experiment 2: Determinations at 4 weeks of age

2.3.1. Serum E2 and SHBG

Because female hamsters weigh less than 20 g at 4 weeks of age, serum samples from the entire SD and LD litters had to be pooled (females only) to assure adequate sample volumes. Five SD and six LD litters (three to five female pups per litter) were given an intraperitoneal overdose of pentobarbital sodium and exsanguinated by retro-orbital bleed. Blood was clotted at room temperature for 1 h and centrifuged at 1000*g* for 20 min in 4 °C. Drawn off serum was pooled by litter, frozen and maintained at −80 °C until assayed for E2. Serum samples were analyzed in duplicate by a radioimmunoassay (RIA) previously validated in Siberian hamsters (Scotti et al., 2007). Briefly, the RIA used was a solid-phase ^{125}I kit (Diagnostic Products Corporation, [now Siemens], Los Angeles, CA, USA), modified by addition of a pre-assay ether extraction. Following the addition of ^{3}H-estradiol (50 μL, ~1900 cpm) to determine extraction efficiencies, 300 μL serum samples were extracted in diethyl ether, dried under N_2, and reconstituted in 335 μL of an assay buffer. A 100 μL aliquot was counted on a scintillation counter to calculate recoveries for ^{3}H-estradiol and separate 100 μL aliquots were added to Coat-A-Count® tubes in duplicate for RIA. The cross-reactivity of the highly specific antibody to other estrogens is less than 2%, save for estrone (10%). A standard curve (15.6–1000 pg/mL) was made by serially diluting an E2 stock solution in the assay buffer. Tubes were incubated at room temperature (23 °C) for 3 h following the addition of ^{125}I-estradiol tracer. Tubes were aspirated of their contents then counted in a gamma counter. Volume and percent extraction recovery specific to each sample were used to calculate concentrations interpolated from the standard curve. The E2 assays met all quality assurance criteria and internal controls were run at the beginning, middle, and end of each assay. The intra- and inter-assay coefficients of variation were <10%, and the minimum detectable limit (MDL) of the assay was 18 pg/mL. Samples with an estradiol concentration below the level of detection were assigned this value for statistics and graphing.

As a biological validation to our E2 assay, we also measured serum E2 concentration in older hamsters (16–20 weeks of age), when LD females ($n = 12$) are expected to be cycling and should have higher E2 concentrations than immature SD females ($n = 5$). As expected, serum E2 concentration was highly variable in LD females (18.0–125.2 pg/mL), but uniformly low in photo-inhibited SD females (all below the MDL, 18.0 pg/mL). We have found that vaginal cytology does not reliably track the estrous cycle in *P. sungorus*, and no attempt was made to monitor the estrous cycle of LD females. Thus, the sampling likely represents LD females at different stages of the estrous cycle and SD females that were uniformly anestrus.

For analysis of SHBG, blood samples were collected from a separate cohort of LD and SD female hamsters at 4 weeks of age ($n = 12$ and 8, respectively). Competition assays were used to determine the affinities of hamster SHBG for E2 and testosterone relative to 5α-dihydrotestosterone (DHT). The serum concentration of SHBG was determined by the steroid binding capacity assay, employing ^{3}H-DHT as the labeled ligand and dextran-coated charcoal as the separation agent (Hammond and Lähteenmäki, 1983).

2.3.2. Histology and immunohistochemistry

A mid-portion of the uterine horn and an ovary from six SD and six LD females at 4 weeks of age were embedded separately in paraffin, sectioned at 6 μm, and mounted on glass slides for standard histology (uterus and ovary) or on Superfrost® Excell slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) for immunohistochemistry (uterus). Sections for standard histology were stained with hematoxylin and eosin (H&E).

For immunohistochemistry, sections from LD and SD uteri were alternately placed on each slide to control for potential staining variability between slides. Adjacent sections were mounted on separate slides for negative controls. After dewaxing and rehydration in a series of ethanols, slides were submerged in Antigen Unmasking Solution (1:100 v/v in H_2O; H-3300; Vector, Burlingame, CA, USA) and microwaved on hi-power for two 15-min bouts. Endogenous peroxides were quenched in hydrogen peroxide (0.5% in methanol) for 20 min. Sections were then incubated overnight at 4 °C in CleanVision™ Blocking solution (ImmunoVision Technologies, Norwell, MA, USA) plus 10% goat serum to block nonspecific binding sites. Monoclonal mouse anti-human ERα antibody (M7047, Dako, Carpinteria, CA, USA) was diluted 1:40 in dilution buffer and incubated with sections for 48 h at room temperature. Sections were incubated with the secondary antibody, biotinylated goat anti-mouse IgG (1:200 in dilution buffer; BA-9200; Vector, Burlingame, CA, USA), for 30 min. Negative controls excluded