

Perinatal Influences of Melatonin on Testicular Development and Photoperiodic Memory in Siberian Hamsters

C. R. Tuthill,* D. A. Freeman,‡ M. P. Butler,† Tori Chinn,* J. H. Park* and I. Zucker*†

Departments of *Psychology and †Integrative Biology, University of California, Berkeley, CA, USA.

‡Department of Biology, University of Memphis, Memphis, TN, USA.

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Abstract

We assessed the influence of perinatal melatonin on reproductive development and adult responsiveness to melatonin. Testicular growth in an intermediate day length (14 : 10 h light/dark cycle) was substantially reduced in Siberian hamsters gestated by pinealectomised compared to pineal-intact females; gonadal development was normalised in offspring of pinealectomised dams that were pinealectomised at 3–4 days of age. Hamsters deprived of melatonin only during gestation, or both pre- and postnatally, underwent testicular involution during treatment with melatonin in adulthood. Photoperiodic histories acquired prenatally did not endure as long as those acquired by adult hamsters. Hamsters first exposed to melatonin in adulthood were not more proficient in acquiring photoperiodic histories than were normal males. These findings indicate that pre- versus postnatal differences in melatonin signal duration determine rates of testicular development. Exposure to melatonin perinatally does not appear to organise the neuroendocrine substrate that mediates effects of day length and melatonin on the gonads of adult hamsters.

Melatonin of pineal origin, which is secreted almost exclusively at night, transduces effects of day length (DL) on the neuroendocrine axis. The duration of nocturnal melatonin secretion is directly proportional to the duration of the dark phase (1, 2), thereby providing an accurate endocrine representation of DL. The longer DLs of spring and summer are associated with shorter intervals of melatonin secretion, typically 5–6 h per night; in winter DLs, melatonin secretion typically endures for 8–12 h per night. The summer and winter phenotypes of body mass, pelage, and gonadal status can be induced in pinealectomised (pinx) Siberian hamsters by daily melatonin infusions 5–6 h and 10–12 h long, respectively (3–6).

Long DLs and their associated melatonin signals exert enduring effects on Siberian hamsters. A long-day photoperiodic history, acquired over the course of 2 weeks in adulthood, affects testicular responses to intermediate DLs 6.5 weeks later (7, 8). Fetal hamsters also acquire photoperiodic histories, despite lacking a functional visual system, or the ability to secrete melatonin; cross-placental maternal transmission of melatonin during the latter part of gestation influences pups' postnatal responses to intermediate DLs (9–11).

Hormones and environmental perturbations restricted to sensitive periods during development permanently change mammalian structure and function (12). Steroids naturally

present during gestation or early postnatal life permanently alter mammalian reproductive physiology and behaviour (13), as well as adult responsiveness to hormones (14). Elimination of hormone secretion, as achieved by endocrine organ ablation during 'critical' periods, was instrumental in establishing the profound influence of thyroid and gonadal hormones on brain development and sex differentiation (13, 15). We are unaware of comparable studies of reproductive function in photoperiodic mammals deprived of circulating melatonin both pre- and postnatally.

Melatonin binding on postnatal day 3 is increased by approximately 29% in the suprachiasmatic nucleus (SCN) of rats born to pinealectomised mothers compared to intact mothers (16). Pinealectomy of male rats on postnatal day 23 significantly decreases melatonin binding capacity in the SCN and pars tuberalis (17). Animals deprived of melatonin both pre- and postnatally may therefore sustain substantial changes in melatonin binding in SCN and pars tuberalis at different life stages; these tissues are implicated in photoperiodic time measurement (18, 19).

In the present study, we determined whether melatonin availability during intervals before and after birth influences responsiveness to melatonin in adulthood. Reproductive development and subsequent responsiveness to melatonin were assessed in juvenile and adult hamsters that had not been exposed to melatonin during gestation and/or postnatally.

Materials and methods

Animals

Siberian hamsters (*Phodopus sungorus*) were obtained from a local colony maintained at an ambient temperature of 21 ± 2 °C. Rooms were illuminated with fluorescent light under a 14 : 10 h light/dark cycle (14 L), unless specified otherwise. Food (mouse chow no. 5015, Purina Mills, St Louis, MO, USA) and tap water were available *ad lib*. Pups were weaned at 18 days of age and individually housed for the remainder of the experiment.

Somatic and reproductive measures

Hamsters were weighed (± 0.1 g) at predetermined intervals. Under light anaesthesia induced by isoflurane vapors, the length and width of the hamster's left testis were measured externally (± 0.1 mm). The product of testis width squared times testis length is a measure of estimated testis volume (ETV) that is highly correlated with testis mass (20). ETV and body mass (BM) were recorded at several predetermined times and paired testis mass (PTM) at autopsy.

Pinelectomy

Pineal glands of adult hamsters were removed under ketamine cocktail anaesthesia according to the procedure of Bartness and Goldman (21). Briefly, hamsters were secured in a stereotaxic apparatus. A circular opening (approximately 2 mm in diameter) was drilled in the skull and the pineal gland was removed with a pair of microdissecting forceps. For sham-pinx hamsters, the circular opening was drilled but the skull flap was not removed. Hamsters were administered the analgesics acetaminophen and codeine (1% solution in drinking water) for 3 days in some experiments and injected *s.c.* with buprenorphin postoperatively in the remaining studies.

Pinelectomies were performed on neonates under cryogenic anaesthesia without the use of a restraining device. A bone flap was retracted and the pineal gland exposed and removed; after the bone flap was returned to its original position and the wound was closed with a tissue adhesive (Nexaband Liquid, Veterinary Products Laboratories, Phoenix, AZ, USA), pups were rewarmed for several minutes under a lamp and then returned to their mothers. All members of a litter were either pinx or sham-pinx, except for several litters that served as unoperated controls.

Catheterisation/infusion

A *s.c.* polyethylene catheter was implanted as described elsewhere (22). Each catheter was attached to a swivel mounted inside the cage lid, which permitted unrestricted locomotion and burrowing. Infusions were delivered by a syringe pump (flow rate = 0.017 ml/h; Razel Scientific Instruments, Stamford, CT, USA) loaded with 1-ml syringes and controlled by a digital timer (Intermatic, Spring Grove, IL, USA). Hamsters received daily melatonin infusions (100 ng/infusion in a saline vehicle) or the vehicle over the course of 5, 7 or 10 h per day, as appropriate.

Determination of responsiveness to melatonin

Melatonin infusions, 10 h per day, over the course of 6 weeks induce gonadal regression in photoresponsive hamsters (4), defined as a decrease of 48% in ETV, as validated previously (20, 23, 24).

Statistical analysis

The Statview statistics package (Abacus Concepts Inc., Berkeley, CA, USA) was used for all statistical analyses. Differences in ETV and BM were analysed separately by repeated measures ANOVA. Where significant F-ratios were obtained, pair-wise comparisons between treatment groups on a given week were conducted using the Tukey–Kramer test. Within-group changes in BM and ETV over successive weeks were analysed by paired *t*-tests. Terminal PTM and BM values were analysed separately by between-subjects ANOVA, and pair-wise differences were assessed with the Tukey–Kramer test. Differences were considered significant if $P < 0.05$ and are reported as such regardless of the actual *P*-value. Comparisons involving frequencies were made using chi-square or Fisher's exact test.

Procedures

All procedures were approved by the Animal Care and Use Committee of the University of California at Berkeley.

Experiment 1: influence of pre- and/or postnatal melatonin deprivation on gonadal and somatic development

This experiment assessed the extent to which male reproductive development is affected by the absence of circulating melatonin during pre- and postnatal life.

Adult females maintained from birth under a 14 : 10 h light/dark cycle (lights on 05.00 h) were pinx or sham-pinx. Several weeks later, females were paired with intact 14 L males. All pups in a given litter were either pinx or sham-pinx at 3–4 days of age. ETVs and BM were recorded at 34–37 days of age. In the designations pinx-pinx, pinx-sham and sham-sham, the left and right terms denote the dam's pineal status at the time of insemination and the pineal status of the pup beginning on postnatal day 3–4, respectively.

Experiment 2: influence of perinatal melatonin on responsiveness to long-duration melatonin signals in adulthood

This experiment assessed the impact of melatonin deprivation during prenatal or prenatal and postnatal life on adult responsiveness to long-duration melatonin signals; such signals induce gonadal regression in adult pinx Siberian hamsters (4). Hamsters from experiment 1, approximately 6–7 months of age, and with ETVs > 400 were tested. The pinx-pinx hamsters had never been exposed to circulating melatonin, whereas the pinx-sham hamsters only received melatonin signals postnatally. The pineal glands of the pinx-sham hamsters were removed 2 weeks before hamsters were infused.

Subcutaneous catheters were implanted in all hamsters; ETV and BM were recorded on the day infusions began (week 0) and again after 6 weeks of daily infusions with melatonin (100 ng/infusion) or the vehicle. Infusions were 10 h long, and began each day at the onset of darkness.

Experiment 3: effects of melatonin deprivation on acquisition of a photoperiodic history

Adult hamsters require 8–14 days to acquire a photoperiodic history, assessed by their subsequent responses to intermediate DLs (7; 8) (i.e. the antecedent day length affects subsequent responses to intermediate day lengths) (25). Fetuses acquire a similar photoperiodic history in 2–3 days (26).

Fetuses, unlike adults, have no prior photoperiodic histories when first exposed to maternal melatonin signals *in utero*. Normal adults, without exception, have a history of prior melatonin exposure that may interfere with encoding of new melatonin durations and contribute to the longer latency for acquisition of photoperiodic histories. If this reasoning is valid, then adult hamsters with no prior melatonin histories might acquire photoperiodic histories more rapidly than normal adults, and perhaps as readily as fetuses. The present experiment tested this hypothesis.

Sexually mature pinx-pinx hamsters were infused for 7 consecutive days with melatonin; the infusion duration simulated melatonin durations produced endogenously by hamsters under a 16 L photoperiod (phase 1). This was followed by 6 weeks of daily melatonin infusions that mimicked durations secreted by intact animals under an intermediate 13–13.5 h DL (phase 2). If the phase 1 melatonin infusions establish a long-day photoperiodic history, then the phase 2 melatonin signals should induce gonadal regression.

Pinx-pinx pups, prepared as described in Experiment 1, were maintained from the time of conception under a 14 L photoperiod; at approximately 6 weeks of age, they were fitted with *s.c.* catheters. Only hamsters with baseline ETVs > 400 were included in the data analysis. During phase 1, hamsters were infused at a constant rate for 5 h per day with the saline vehicle ($n = 11$) or melatonin (100 ng; $n = 21$) for 7 consecutive days. At the end of phase 1, all hamsters were infused for 6 consecutive weeks for 7 h per day with melatonin (100 ng/infusion), followed by measurement of testes and body masses. In every case, the onset of infusions was coincident with dark onset.

Experiment 4: duration of photoperiodic histories acquired prenatally

Photoperiodic histories acquired by adult hamsters persist for > 6.5 weeks. The longevity of photoperiodic histories acquired prenatally is unknown. Siberian hamsters begin secreting melatonin rhythmically at approximately 15 days of age (27); the effects of the prenatal DL on gonadal responses to intermediate DLs are readily detectable in pineal-intact males by postnatal day 28 (9–11); the duration of the prenatal photoperiodic history remains

unknown. To address this question, we determined whether a prenatally acquired photoperiodic history influences the gonads of males first challenged with exogenous melatonin signals at 6 weeks of age.

Adult females maintained under a 15 L photoperiod from birth were paired with males. Male offspring from these pairings were pinx at 3–4 days of age and weaned 2 weeks later. When they were approximately 6 weeks old, each animal received an s.c. catheter and, for the next 6 weeks, was infused daily with melatonin (100 ng/infusion; $n = 22$) or saline ($n = 9$) for 7 h per day after which PTM was determined. The 7-h melatonin infusion simulates the duration of endogenous nocturnal melatonin secretion of hamsters housed under a 13 h DL. In adult pinx hamsters previously maintained under 15 L, this melatonin treatment induces gonadal regression when infusions are initiated either 0 or 6.5 weeks after pinealectomy (7).

Results

Experiment 1

Pinx-pinx ($n = 11$), pinx-sham ($n = 11$) and sham-sham ($n = 7$) groups were 35.4, 35.4 and 35.0 days old, respectively, at the time measurements were taken.

ETV

There was a significant treatment effect (ANOVA; $P < 0.05$). Sham-sham and pinx-pinx groups underwent substantial testicular growth by 5 weeks of age; their ETV values did not differ from each other ($P > 0.05$) and were almost equivalent to those of fully mature adult males (Fig. 1A). By contrast, ETVs of the pinx-sham males were much lower and

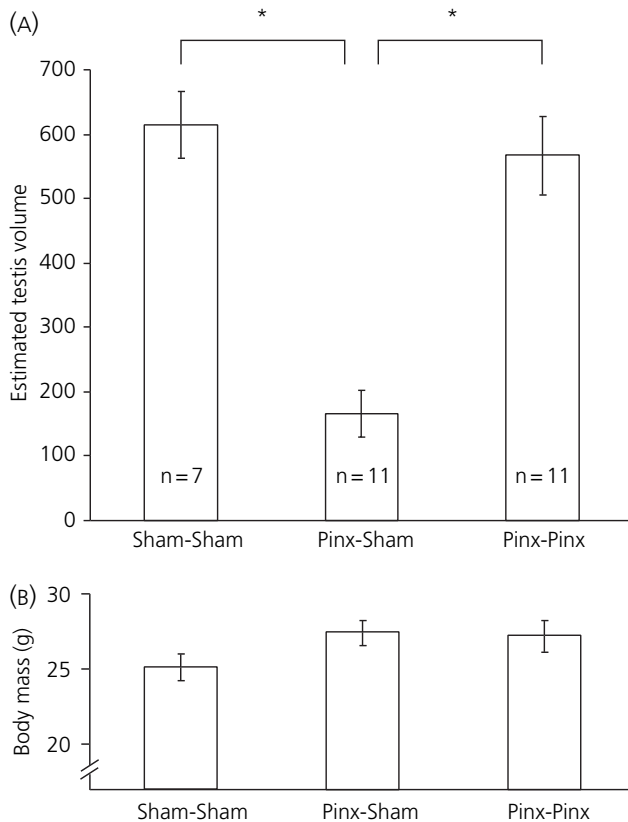


Fig. 1. Mean \pm SEM testis volume (A) and body mass (B) at 35 days of age. Hamsters were maintained under a 14 L photoperiod. Dams were either pinealectomised (pinx) or sham-pinx prior to mating; pups were either pinx or sham-pinx at 3–4 days of age (see text for details) (* $P < 0.05$).

differed significantly from those of the other two groups (Fig. 1).

An ETV value of 400 is the threshold above which testes are considered functional: 9/11 (82%), 6/7 (86%) and 1/11 (9%) of pinx-pinx, sham-sham and pinx-sham males had ETVs > 400 at 35 days of age ($P < 0.05$). Pineal-intact pups born to pinx dams evidently undergo much slower gonadal growth compared to normal pups, or the pinx offspring of pinx dams.

Body mass

There was no significant treatment effect ($F = 1.47$; $P > 0.05$). Pinx-sham hamsters were marginally heavier than sham-sham hamsters (Fig. 1B) ($P = 0.082$).

Gonadal and body mass status in adulthood

To assess whether the retarded testicular growth of pinx-sham hamsters relative to hamsters deprived of melatonin both pre- and postnatally (pinx-pinx) persisted into adulthood, we determined testis volume for two groups of hamsters at 6–7 months of age; ETVs of pinx-pinx hamsters were greater than those of pinx-sham hamsters (Fig. 2) ($P < 0.05$). Eight of 16 (50%) pinx-sham and 1/24 (4%) of pinx-pinx males had ETVs < 400 ($P < 0.05$) (i.e. well into adulthood half the pinx-sham hamsters failed to undergo normal gonadal development). The BM of the pinx-sham (45.4 ± 2.0 g) and pinx-pinx groups (48.2 ± 1.2 g) did not differ significantly ($P > 0.05$). BM of pinx-sham hamsters with ETVs < 400 (45.4 ± 3.6 g) and > 400 (45.5 ± 2.0 g) also did not differ significantly.

Experiment 2

Groups designated for melatonin and saline infusion within each treatment condition were well equated for ETV and BM

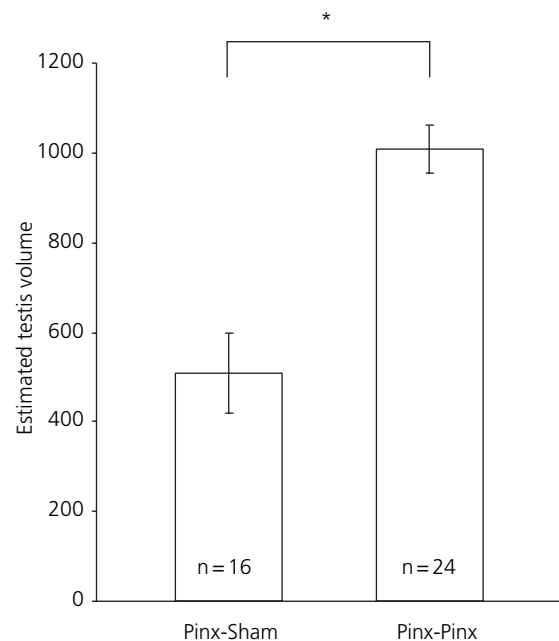


Fig. 2. Testis volume at 6–7 months of age of hamsters born to pinealectomised (pinx) dams. Other conventions are as described in Fig. 1.

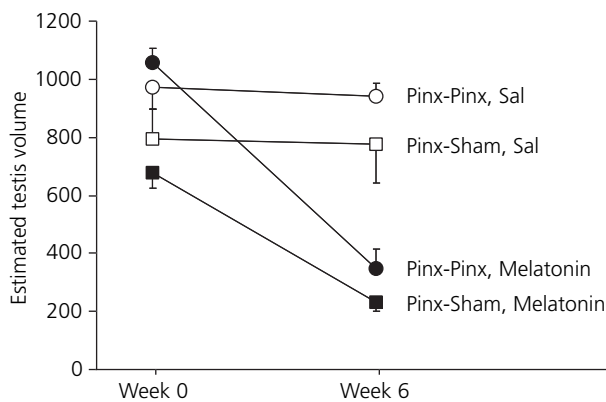


FIG. 3. Testicular responsiveness to melatonin or saline (Sal) infusions for 10 h per day for 6 weeks beginning when hamsters were approximately 6.5 months old. After 6 weeks of infusions, hamsters treated with melatonin had significantly lower ETVs than corresponding controls treated with Sal ($P < 0.05$).

at the time infusions began (not illustrated). Only hamsters with well developed gonads were tested. ETVs of pinx-pinx ($n = 8$) and pinx-sham ($n = 7$) hamsters did not change significantly over the course of 6 weeks of vehicle infusions (Fig. 3) ($P > 0.05$) but hamsters in both groups treated with melatonin underwent substantial and comparable gonadal regression of $> 60\%$ (Fig. 3) ($n = 17$ and 3 for pinx-pinx and pinx-sham groups, respectively). For both treatment conditions, decreases in ETV were significantly different for melatonin- versus vehicle-treated groups ($P < 0.05$). Males deprived of melatonin prenatally or both prenatally and postnatally did not differ in their responsiveness to melatonin in adulthood and apparently were as responsive to melatonin as normal males pinx and treated in adulthood (4).

Experiment 3

At the onset of phase 1 infusions ETVs did not differ between the groups (777 ± 33 and 733 ± 25 for melatonin and saline groups, respectively; $P > 0.05$). Paired testis mass at the end of phase 2 also did not differ significantly between the groups that had received melatonin or saline during phase 1 (Fig. 4) ($P > 0.05$). Two of 21 hamsters that received the 5-h melatonin treatment during phase 1 underwent testicular regression (PTM < 300 mg) when infused with melatonin for 7 h per day during phase 2, compared to 0/11 for the group treated with saline during phase 1 (chi squared, $P = 0.29$).

Experiment 4

After 6 weeks of infusions, the paired testis mass of hamsters treated with saline did not differ from that of males infused with melatonin (693 ± 39 versus 626 ± 57 , respectively; $P > 0.05$) (Fig. 5); 0/9 saline-treated and 4/22 melatonin-infused hamsters had ETVs < 400 ($P > 0.05$). Thus, for the majority of hamsters, exposure to short duration melatonin signals *in utero* did not influence responsiveness to intermediate duration melatonin signals at 6 weeks of age.

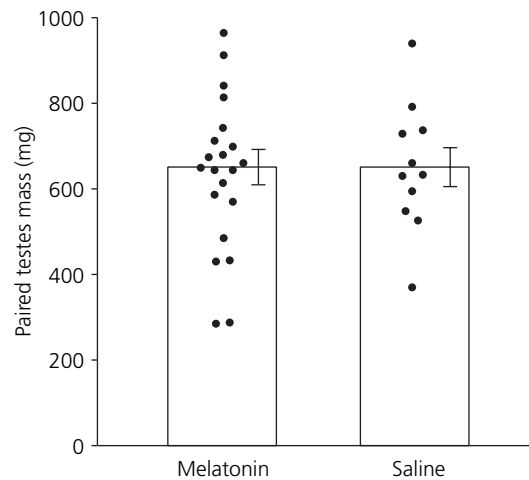


FIG. 4. Mean \pm SEM testis mass of pinx-pinx hamsters after daily infusion for 1 week with melatonin (5 h per day) followed by 6 weeks of daily infusions for 7 h per day with melatonin or Sal. Dots indicate values for individual hamsters. Testis mass did not differ between groups ($P > 0.05$).

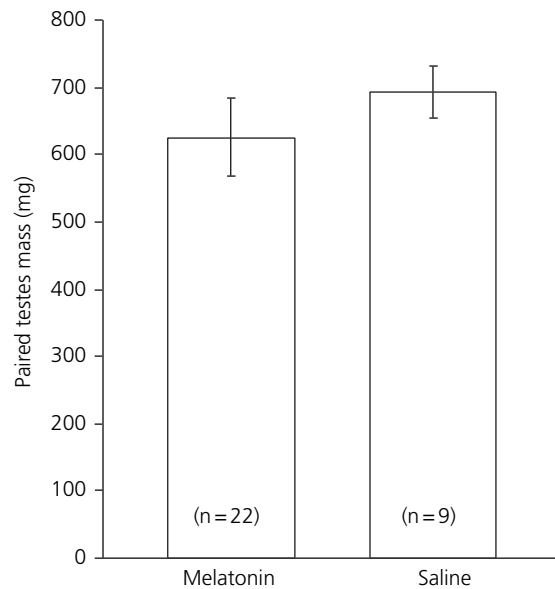


FIG. 5. Mean \pm SEM testis mass of pinx offspring of pineal-intact dams maintained under a 15 L photoperiod. Males were pinx at 3–4 days of age; beginning at 6 weeks of age they were infused for 7 h per day with melatonin for 6 weeks. Testis mass did not differ between treatment groups ($P > 0.05$).

Discussion

Siberian hamsters deprived of circulating melatonin both pre- and postnatally, or only postnatally, underwent gonadal regression at a normal rate when first challenged with long-duration melatonin signals at 6–7 months of age. The absence of melatonin during development does not appear to compromise adult responsiveness of the hypothalamic-pituitary-gonadal (HPG) axis to melatonin under the test conditions of this study. The neural substrates that decode long-duration melatonin signals indicative of short day lengths are not subject to organisational actions by melatonin during critical

periods in development. This is in contrast to organisational effects of steroids on the HPG axis; perinatal androgens profoundly and permanently alter adult responsiveness to steroid hormones (13).

The response of Siberian hamsters to intermediate day lengths (14 : 10 h light/dark cycle) depends on the animals' photoperiodic history (10, 11, 25). A 14 : 10 h light/dark cycle induces the short-day phenotype in hamsters previously maintained on longer day lengths, and the long-day phenotype in those formerly kept in shorter day lengths. Adult hamsters acquire long-day photoperiodic histories in 2 weeks; the photoperiodic 'memory' is retained for at least 6.5 weeks, and undergoes a decline after 13 weeks; it is completely absent after 20 weeks (7). Long-day photoperiodic histories acquired by hamsters prenatally also fade with time and are not retained in tests initiated at 6.5 weeks of age (Experiment 4). The prenatally acquired photoperiodic histories appear to be more short-lived than those acquired in adulthood. The difference may be related to the immaturity of the prenatal neuroendocrine system or to the smaller number of melatonin signals hamsters receive prenatally versus postnatally. On the basis of four of 22 hamsters (18%) retaining the prenatal melatonin history for at least 6 weeks, we speculate that the prenatally acquired photoperiodic history endures in most hamsters for approximately 4–5 weeks.

Seven consecutive days of melatonin infusion to sexually mature hamsters previously deprived of all circulating melatonin did not impart a long-day photoperiodic history. As few as 2 days of similar infusions instate a photoperiodic history in late-term fetuses (26). Thus, the absence of melatonin during gestation and postnatally (Experiment 3) does not result in adult hamsters that compare with fetuses in respect of the ease by which they acquire photoperiodic histories, although different methods were used in testing juveniles and adults. Unknown factors other than circulating melatonin must account for the diminished responsiveness of postnatal Siberian hamsters to melatonin signals.

Juvenile hamsters gestated by pinealectomised dams and thereby deprived of maternal melatonin signals, failed to undergo normal testicular development through 35 days of age when maintained under the 14 L intermediate day length (Experiment 1). This corroborates earlier reports that charted testicular growth of 14 L offspring of intact and pinealectomised dams at 28 and 34 days of age (10, 26, 28). The present study suggests that this is an enduring effect: testicular growth remained depressed through 6 months of age in a subset of hamsters deprived of maternal melatonin during fetal life. This extends preliminary observations (10) in which testis weights at 50 days of age of normal hamsters housed under 14 L were approximately 460 mg compared to 300 mg for intact males born to pinealectomised mothers.

Reproductive development in juvenile Siberian hamsters is determined in large part by the difference between prenatal and postnatal melatonin signal durations. When the postnatal melatonin signal duration is longer than the prenatal signal, testicular growth is inhibited (Fig. 1) (6). However, testicular growth in males deprived of melatonin prenatally was completely normalised by 35 days of age in those that were pinealectomised at 3–4 days of age. Because pineal secretory activity begins after postnatal day 4 (27, 29), these offspring

were deprived of circulating melatonin both pre- and postnatally; their pre- and postnatal melatonin signals were of equal duration (0 h). This circumstance was compatible with testicular growth being indistinguishable from that of pineal-intact males born to pineal-intact females. Within limits, the absence of prenatal melatonin signals appears to be consequential only when the hamster begins to secrete melatonin in a rhythmic fashion at the end of the second week of postnatal life. Because all endogenous melatonin signals are performed longer than the 0-h signals transmitted to fetuses by pinealectomised dams, postnatal gonadal development is impaired in pineal-intact pups. The reversal of this effect by early postnatal pinealectomy of pups is compatible with a mechanism that compares prenatal to early postnatal melatonin signals and that interprets the absence of melatonin signals prenatally as the equivalent of the ultimate long day (26).

Factors other than circulating melatonin have been implicated in the transfer of photoperiodic information from the dam to fetuses. This conclusion was based on studies in which pregnant Siberian hamsters were treated with s.c. melatonin-filled capsules that swamped endogenous melatonin signals; these dams and their progeny were subsequently housed in constant light (LL) (30). LL suppresses melatonin secretion in this species (31, 32), but the present results suggest that, in the context of the photoperiodic history phenomenon, LL treatment and pinealectomy are not equivalent. Offspring of intact and pinealectomised dams housed in LL during gestation and postnatally had testes weights of approximately 95 mg, compared to values of 280 mg for intact males born to pineal-intact dams housed under 14 L (28). In the present study (i.e. Experiment 1), testis dimensions of pinealectomised offspring born to pinealectomised dams were equal in size compared to those of normal males housed under 14 L. Because neither LL treatment nor pinealectomy mimics physiological conditions, any conclusions made regarding normal function on the basis of these manipulations must remain tentative.

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