Gonadal hormone effects on entrained and free-running circadian activity rhythms in the developing diurnal rodent *Octodon degus*

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Hummer, Daniel L., Tammy J. Jechura, Megan M. Mahoney, and Theresa M. Lee. Gonadal hormone effects on entrained and free-running circadian activity rhythms in the developing diurnal rodent *Octodon degus*. Am J Physiol Regul Integr Comp Physiol 292: R586–R597, 2007. First published August 17, 2006; doi:10.1152/ajpregu.00043.2006.—The slowly maturing, long-lived rodent *Octodon degus* (degus) provides a unique opportunity to examine the development of the circadian system during adolescence. These studies characterize entrained and free-running activity rhythms in gonadally intact and prepubertally gonadectomized male and female degus across the first year of life to clarify the impact of sex and gonadal hormones on the circadian system during adolescence. Gonadally intact degus exhibited a delay in the phase angle of activity onset ($\Psi_{on}$) during puberty, which reversed as animals became reproductively competent. Gonadectomy before puberty prevented this phase delay. However, the effect of gonadal hormones during puberty on $\Psi_{on}$ does not result from changes in the period of the underlying circadian pacemaker. A sex difference in $\Psi_{on}$ and free-running period ($\tau$) emerged several months after puberty; these developmental changes are not likely to be related, since the sex difference in $\Psi_{on}$ emerged before the sex difference in $\tau$. Changes in the levels of circulating hormones cannot explain the emergence of these sex differences, since there is a rather lengthy delay between the age at which degus reach sexual maturity and the age at which $\Psi_{on}$ and $\tau$ become sexually dimorphic. However, postnatal exposure to gonadal hormones is required for sexual differentiation of the circadian pacemaker. These data suggest that gonadal hormones modulate the circadian system during adolescent development and provide a new model for postpubertal sexual differentiation of a central nervous system structure.

adolescent development; puberty; phase angle; tau; sexual differentiation

ALTHOUGH DEVELOPMENTAL CHANGES within the circadian system have been well described, the existing research has focused primarily on changes during prenatal and early postnatal development or changes that occur with advanced age (23, 33). Adolescent development has received comparatively little attention, despite the well-known phase delays in circadian rhythms exhibited by human adolescents (2, 3, 5, 10). Adolescents develop has received comparatively little attention, despite the well-known phase delays in circadian rhythms exhibited by human adolescents (2, 3, 5, 10). There is little doubt that social factors play an important part in these developmental changes (e.g., increased academic responsibilities lead to later bedtimes); however, the phase delay in circadian rhythms is positively correlated with sexual maturity (5), which would suggest that gonadal hormones modulate the circadian system during adolescent development (6, 29, 32).

It is possible that the phase delay in the circadian rhythms in human adolescents is the result of gonadal hormones acting to lengthen the endogenous period of the central circadian pacemaker (2). This hypothesis has not received much empirical support; although free-running period ($\tau$) appears to be longer in adolescent humans than in adults (2, 7), these age groups have not been directly compared in the same experiment. The rodent models commonly used to study circadian rhythms are not well suited for studies of adolescent development, because rodents mature rather quickly. As a result, few data that model the influence of gonadal hormones on the central circadian pacemaker of adolescents are available.

However, it is well known that the suprachiasmatic nucleus of the hypothalamus (SCN) and the central circadian pacemaker contained within the SCN are sensitive to gonadal hormone exposure. For example, the size of the SCN in adult gerbils and the number of synapses in the SCN of adult rats are each sexually dimorphic, the result of exposure to testicular hormones at birth (15, 19). Estrogen exposure during adulthood decreases $\tau$ in female, but not male, hamsters and rats (1, 24); the sex-specific sensitivity of the circadian pacemaker to estrogen is a consequence of an organizational effect of perinatal testosterone exposure (1, 36). These data demonstrate that, at least in altricial species, exposure to gonadal hormones early in development leads to sexual differentiation of the SCN and the central circadian pacemaker contained within its boundaries.

The data presented here clarify the immediate and long-term impact of gonadal hormone exposure during adolescence on the central circadian pacemaker of a slowly maturing, long-lived, precocial rodent *Octodon degus* (degus). There is a $>30$-min difference in $\tau$ between adult male and female degus (23.1 and 23.7 h, respectively) (18, 21). In $>1$-yr-old degus, $\tau$ does not vary as a function of ovariectomy or castration, demonstrating that this sexual dimorphism is not a consequence of activation effects of circulating gonadal hormones during adulthood (17, 18). Instead, it is likely that the circadian time-keeping mechanism is sexually differentiated earlier in development. Since $\tau$ does not differ between males and females before puberty (16), we hypothesized that sexual differentiation of the underlying circadian pacemaker occurs during adolescence in response to gonadal hormone exposure at puberty.

The aim of these experiments was to address the following questions: 1) When does $\tau$ in *O. degus* become sexually dimorphic in relation to puberty? 2) Is postnatal exposure to gonadal hormones necessary for sexual differentiation of period? 3) Do changes in the endogenous period of the circadian...

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pacemaker underlie developmental changes in phase angle of entrainment? We hypothesized that 1) the sex difference in $\tau$ would emerge between 4 and 6 mo of age, when degus reach sexual maturity, and 2) the sex difference would fail to emerge in animals that were gonadectomized before puberty. We expected that phase angle of entrainment would become sexually dimorphic at the same age, supporting the notion that developmental changes in phase are associated with changes in the underlying period of the circadian pacemaker. These data would provide evidence that sexual differentiation of the central circadian time-keeping mechanism in degus depends on exposure to gonadal hormones during puberty and could provide a framework for understanding sexual differentiation of the central circadian pacemaker in other species.

**MATERIALS AND METHODS**

**Experiment 1a**

Animals. Twenty male and 20 female *O. degus* were selected from 10 litters born into an outbred colony at the University of Michigan. All procedures involving animals were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Housing. PRETESTING ENVIRONMENT. At 0–3 mo of age, degus were housed with parents and littermates in large (42.5 $\times$ 46 $\times$ 19.5 cm) plastic cages in a 12:12-h light-dark cycle (light intensity 250 lx) at 20 ± 1°C until they were weaned. The animals had free access to food (Prolab Laboratory Animal Diet Product 5P06) and acidified water (2.5 $\times$ 10^{-5%} HCl). Degus were weaned at 5–6 wk of age and housed in large cages with two or three same-sex siblings.

TESTING ENVIRONMENT. During testing (odd months, 3–11 mo of age), degus were individually housed in opaque plastic cages (42.5 $\times$ 22 $\times$ 19 cm) equipped with Nalgene running wheels (9 $\times$ 34.5 cm). Running-wheel activity data were collected in a 12:12-h light-dark cycle (lights on at 0600, 250 lx average) and in constant darkness (DD). Ambient room temperature was maintained at 20 ± 1°C, and animals had free access to food (5001 Rodent Diet, PMI Nutrition) and tap water. Cages were cleaned once a week during the light phase and under dim red light every other week during DD.

NONTESTING ENVIRONMENT. Between testing periods (even months, 4–12 mo of age), degus were housed in same-sex sibling groups as described above (see PRETESTING ENVIRONMENT), with the addition of a single running wheel to minimize potential ill effects of long-term isolation. Rooms were maintained on a 12:12-h light-dark cycle at 20 ± 1°C. Animals had ad libitum access to food (5001 Rodent Diet, PMI Nutrition) and tap water, and cages were cleaned once a week during the light phase.

Gonadectomy and group assignment. Males and females were evenly split across two conditions: gonadectomy (GDX) or sham GDX (intact). One to three siblings were randomly assigned to each group. Animals were gonadectomized under isoflurane gas (5% in O_2) anesthesia (Minrad) at 5–6 wk of age. This resulted in four groups ($n = 10$/group): intact males, intact females, GDX males, and GDX females. One GDX female died unexpectedly during the first test session and was excluded from all analyses.

Physical and pubertal development. Body weights were recorded every other month at 3–11 mo of age. Sexual development was assessed twice weekly at 2–6 mo of age, except when animals were in DD. During nontesting periods, females were examined for vaginal opening, which occurs only during estrus (34). When females were in running wheels, activity records were monitored for sharp increases in activity levels characteristic of estrus (18). Males were examined for the appearance of spikes on the glans of the penis. Penile spikes are a common trait among hystricomorphs (34); they are more accessible for inspection than the testes, which are held in the abdomen of degus.

The size and number of these spikes are positively correlated with circulating levels of testosterone (unpublished observations).

Testing procedure. Degus were transferred with cage mates into large cages equipped with a single running wheel ~10 days before they were 3 mo of age and allowed to habituate to the wheel (see NONTESTING ENVIRONMENT). At 3 mo of age, degus were housed individually with running wheels for 10 days in a 12:12-h light-dark cycle. Lights were disabled during DD and remained off for 3–4 wk, until animals reached 4 mo of age. Degus were then removed from isolation and housed with siblings in a 12:12-h light-dark environment. Animals had access to a shared wheel for 1 mo.

Animals were transferred again to individual wheels at 5 mo of age and spent 10 days in a 12:12-h light-dark cycle before they were released into DD. At 6 mo of age, they were again housed in a group with a shared running wheel in a 12:12-h light-dark cycle. In this way, animals alternated from month to month between the testing environment, in which activity data were collected from individually housed animals running in wheels, and the nontesting environment, in which animals were housed with same-sex siblings in a 12:12-h light-dark cycle. This procedure was repeated every other month until animals reached 12 mo of age. The design allowed entrained and free-running activity rhythms to be assessed in the same group of animals across development, with minimal isolation from conspecifics. Long-term social isolation is not typical of degus’ natural environment, and social isolation early in life leads to neurobiological (13, 14, 25–28) and behavioral anomalies in degus (unpublished observations).

Activity data collection and analysis. Activity data were recorded and stored in 10-min bins by the VitalView system (Mini Mitter). Actview software (Mini Mitter) was used to plot activity records. Average phase angles of entrainment ($\Psi_{on}$ and $\Psi_{off}$), daily duration of activity ($\alpha$), and activity amplitude were determined at 3, 5, 7, 9, and 11 mo while animals were in a 12:12-h light-dark cycle. The day of estrus and the day immediately before and the day after estrus were not included in these analyses, since estrus affects phase angle and activity levels in degus (18).

Phase angle of activity onset ($\Psi_{on}$) was calculated by subtraction of the time of daily onset in activity in the morning from the time of lights off. Activity onset was defined as the first 40-min window to contain at least three of four 10-min bins with ≥40 wheel revolutions each. This activity bout was preceded by ≥3 h of inactivity. In the same way, the phase angle of activity offset ($\Psi_{off}$) was calculated by subtraction of the time that daily activity ended in the evening from the time of lights off. Activity offset was defined with criteria similar to those used to define activity onset: the final 40-min window to contain at least three of four 10-min bins, each containing ≥40 wheel revolutions. This activity bout was followed by ≥3 h of inactivity. For calculation of $\alpha$, the time at which activity began in the morning was subtracted from the time at which it ended in the evening. Activity amplitude was calculated by subtraction of daily average wheel turns from daily maximum wheel turns. All measures were determined from 5 consecutive days of data before release into DD.

For calculation of $\tau$, 24-h frequency histograms were examined after an animal had established a steady free-running rhythm in DD. A regression line was drawn through the daily activity onsets from 7 consecutive days beginning 10–14 days after release into DD. Activity onset was defined as 40 wheel revolutions in each 10-min bin for ≥20 min following ≥4 h of inactivity. Period was calculated at 4, 6, 8, 10, and 12 mo of age by an observer blind to group assignment and confirmed by a second unbiased observer.

Statistical analyses. PREPLANNED ANALYSES. The main effects of, and interactions between, group and age were tested using mixed-effects repeated-measures ANOVA (Proc Mixed, SAS version 8); litter was included as a random effect to control for litter variance. Post hoc age comparisons were performed using Tukey’s honestly significant difference (HSD) test. Values are means ± SE. Group differences are considered significant if $P < 0.05$, except where otherwise indicated.
Four comparisons were planned in advance to test hypotheses based on data collected previously in our laboratory (16–18, 21). Pairwise contrasts were used to test the following planned comparisons at each age: 1) Is there a sex difference in entrained or free-running activity rhythms (intact males vs. intact females)? 2) Is the sex difference dependent on the gonads (GDX males vs. GDX females)? 3) Is the development of entrained or free-running rhythms dependent on the testes (intact vs. GDX males)? 4) Is the development of entrained or free-running rhythms dependent on the ovaries (intact vs. GDX females)? In this case, group differences were considered significant if $P < 0.0125$. This Bonferroni-adjusted criterion for statistical significance was established to correct for multiple comparisons ($\alpha' = 0.05 \div 4 = 0.0125$), thereby providing reasonable protection to the null hypothesis without losing so much power that type II error rates radically increase (22).

POST HOC ANALYSES. Many juvenile degus maintained a period close to 24 h for $\sim$10–14 days after release into DD before ultimately free running with a period 15–30 min shorter than these initial days in DD (see Fig. 11C). We hypothesized that this delayed free run may be an age-dependent process that disappears as degus mature, since $\tau$ rarely changes so long after adult degus are released into DD (unpublished observation). The number of individual cases in which $\tau$ suddenly shortened by $\geq 0.15$ h after $\geq 7$ days in DD was tallied. We used $\chi^2$ tests for independence to determine whether this behavior was related to age or group.

Experiment 1b

To further characterize pubertal development in male O. degus raised in captivity, paired testes weight, spermatogenesis rating, and circulating testosterone were measured in 1-mo to 3-yr-old male degus. These animals were unrelated to, and reared in different conditions (see below) from, animals tested in experiment 1a.

Animals. Degus were born into an outbred colony at the University of Michigan and weaned with two or three same-sex siblings into opaque plastic cages ($42.5 \times 22 \times 19$ cm) at 5–6 wk of age. They had free access to high-protein food (ProLab Laboratory Animal Diet Product 5P06) and acidified water ($2.5 \times 10^{-5}$ M HCl) until $\sim 3$ mo of age; after 3 mo, degus were fed an ad libitum diet of standard rodent chow (5001 Rodent Diet, PMI Nutrition) and tap water. Colony rooms were maintained on a 12:12-h light-dark cycle (light on at 0600, 250 lx average) at $20 \pm 1^\circ$C.

Paired testes weight and spermatogenesis. Male degus were perfused at 2–12 mo of age ($n = 5–10$/age). Animals received an overdose intraperitoneal injection of B-euthanasia (a drug cocktail containing pentobarbital sodium). Heparin (0.1 ml, 100 U/ml) and 0.1% sodium nitrite (0.1 ml) were injected directly into the left ventricle followed by 0.9% saline (250 ml). Animals were perfused with 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Testes were removed, weighed, and postfixed in 4% paraformaldehyde at 4°C until they were sliced.

Testes were dehydrated in increasing concentrations of ethanol followed by xylene and embedded in paraffin wax. Sections (5 mm) were collected on a sliding microtome and stained with Gill’s hematoxylin and eosin Y (11). An observer blind to age rated slices on a five-point spermatogenic index of seminiferous epithelium activity (adapted from Ref. 12).

Testosterone in circulation. Blood samples were collected from 1-mo to 3-yr-old gonadally intact male and female degus ($n = 5–8$/age). Blood was also sampled from a group of 1.5- to 2.5-yr-old GDX males ($n = 8$).

Blood (1 ml) was collected by cardiac puncture while males were anesthetized with 5% isoflurane. Serum samples were isolated by centrifugation (20 min, 3,000 rpm) and stored at $-20^\circ$C. Circulating levels of total unconjugated testosterone were determined in a single RIA (Double-Antibody Testosterone 125I RIA kit, MP Biomedicals; 6% reported intra-assay variation). The assay does not distinguish between protein-bound and free testosterone and has a reported 3.4% cross-reactivity with 5α-dihydrotestosterone. Undetectable values were replaced with a value equal to the lower limit of sensitivity for the RIA kit (0.03 ng/ml).

Statistical analyses. A one-way mixed-effects ANOVA (Proc Mixed, SAS version 8) was used to test the effect of age on testes weight and spermatogenesis. The spermatogenesis data were found to be normally distributed and, thus, were treated as a continuous variable. The testosterone data were converted to ranks to normalize the distribution before analysis by one-way ANOVA. Post hoc comparisons between ages were made using Tukey’s HSD test. Values are means ± SE, and group differences are considered significant when $P < 0.05$.

Experiment 2

In experiment 1a, gonadally intact degus exhibited delayed activity onsets relative to gonadectomized degus at 3 mo of age. This group difference occurred at the age at which degus showed initial signs of pubertal development, suggesting that gonadal hormone exposure during puberty may modulate phase angle of entrainment. Since data were not collected before 3 mo of age, it was unclear whether gonadally intact degus phase delay their activity onsets during the transition from prepuberty to puberty. Experiment 2 was conducted to answer this question.

Animals

Degus born into an outbred colony at the University of Michigan were initially housed with parents and littermates in large ($42.5 \times 46 \times 19.5$ cm) plastic cages in a 12:12-h light-dark cycle (lights on at 0600, 250 lx average). Ten males and 10 females from 3 litters were each weaned into an opaque plastic cage ($42.5 \times 22 \times 19$ cm) equipped with a Nalgene running wheel ($9 \times 34.5$ cm) at 5–6 wk of age. Animals had free access to food (ProLab Laboratory Animal Diet Product 5P06) and acidified water ($2.5 \times 10^{-5}$ M HCl). Room temperatures were maintained at 20 ± 1°C. Cages were cleaned once a week during the light phase.

Data collection and analysis. Activity data were collected, recorded, and stored in 10-min bins by the VitalView system (Mini Mitter) while animals were in a 12:12-h light-dark cycle (250 lx average). Phase angle of entrainment ($\Psi_{on}$) was assessed as described in experiment 1a at 8 and 12 wk of age. $\Psi_{on}$ was calculated from 3 consecutive days of data at each age.

A mixed-effects repeated-measures ANOVA (Proc Mixed, SAS version 8) was used to test the effects of sex and age on $\Psi_{on}$. Because litter was not a significant source of variance, it was not included in the model as a random effect. Values are means ± SE, and group differences are considered significant when $P < 0.05$.

RESULTS

Experiment 1a

Body weight. Body weight increased with age (main effect age: $F = 391.23, P < 0.0001$). There was an overall effect of group on body weight (main effect group: $F = 5.41, P = 0.0014$) but no significant difference in the rate of growth between groups (group $\times$ age interaction: $F = 1.75, P = 0.0614$).

The growth of intact males and females was similar during the first year of life (Fig. 1A). Females were lighter than males at 3 mo of age (pairwise contrast, $P = 0.0051$) but caught up to males at 5 mo and remained the same throughout the remainder of the first year. The sex difference in body weight was exaggerated in GDX animals. GDX males were heavier than intact males from $\sim$2 mo of age and this difference increased with age.
than GDX females throughout development, except at 5 mo of age (Fig. 1B; pairwise contrasts, \( P < 0.0125 \)).

GDX females were lighter than intact females at 3 mo of age (pairwise contrast, \( P = 0.0122 \)). There was also a tendency for GDX to be lighter than intact males at 3 mo; this group difference did not reach statistical significance (pairwise contrast, \( P = 0.0260 \)). Body weight of GDX males and females was the same as that of intact males and females by 5 mo of age, and they were similarly sized throughout the remainder of the first year.

**Pubertal maturation.** Intact females exhibited vaginal opening as early as 2 mo of age, with the majority showing vaginal opening for the first time at 3–3.5 mo of age (Fig. 2). GDX females never exhibited vaginal opening.

Intact males developed their full complement of penile spikes at 2.5–3.5 mo of age (Fig. 3, A and B). Castration at 5 wk prevented these spikes from appearing at any age. Spike size steadily increased between 3 and 5 mo of age (Fig. 3C).

\( \Psi_{on} \). There was a significant change in \( \Psi_{on} \) across the first year of development in *O. degus* (main effect age: \( F = 6.50, P < 0.0001 \); Figs. 4 and 5). The experimental groups exhibited different age-dependent changes in \( \Psi_{on} \) (group \( \times \) age interaction: \( F = 3.25, P = 0.0004 \)). \( \Psi_{on} \) changed significantly with age in intact males (1-way effect of age: \( F = 9.01, P = 0.0047 \)), intact females (1-way effect of age: \( F = 10.18, P = 0.0021 \)), and GDX females (1-way effect of age: \( F = 5.19, P = 0.0233 \)), but not GDX males (1-way effect of age: \( F = 2.52, P = 0.1352 \)).

Intact males phase advanced between 3 and 7 mo of age (Fig. 5, A and C; Tukey-corrected pairwise comparison of months 3 and 7, \( P = 0.0292 \)) and intact females between 3 and 5 mo (Fig. 5, A and D; Tukey-corrected pairwise comparison of months 3 and 5, \( P = 0.0281 \)). Intact females then phase...
delayed significantly between 7 and 9 mo of age (Tukey-corrected pairwise comparison of months 7 and 9, $P = 0.0110$), whereas $\Psi_{on}$ did not change in intact males after 7 mo of age. This resulted in a sex difference in $\Psi_{on}$ at 9 mo of age. Males were phase advanced relative to females at 9 and 11 mo of age (Fig. 5A; planned contrasts, $P < 0.0125$). This sex difference was absent in males and females gonadectomized before puberty (Fig. 5B).

Intact males tended to be phase delayed relative to GDX males at 3 mo of age, but the difference did not reach statistical significance (planned contrast, $P = 0.0166$). After 3 mo of age, intact males exhibited a significant phase advance in $\Psi_{on}$ with age, whereas $\Psi_{on}$ remained unchanged in GDX males (Fig. 5C). By 11 mo of age, intact males were phase advanced relative to GDX males, but the difference did not reach statistical significance (planned contrast, $P = 0.0352$).

At 3 mo of age, intact females tended to be phase delayed relative to GDX females, but the difference did not reach statistical significance (Fig. 5D; planned contrast, $P = 0.0216$). After 3 mo of age, intact and GDX females exhibited a similar $\Psi_{on}$ throughout the remainder of the first year.

$\Psi_{off}$. There was an age-dependent phase advance in $\Psi_{off}$ during the first year of life (main effect age: $F = 20.69$, $P = 0.0001$; Figs. 4 and 6). There was no overall effect of group or
significant interaction between group and age on \( \Psi_{\text{off}} \) (main effect group: \( F = 2.49, P = 0.0624 \); group \( \times \) age interaction: \( F = 1.49, P = 0.1351 \)).

Intact males and females exhibited similar developmental changes in \( \Psi_{\text{off}} \) (Fig. 6). Castration before puberty had no effect on the development of \( \Psi_{\text{off}} \) (Fig. 6A). In contrast, GDX females exhibited little change in \( \Psi_{\text{off}} \) across the first year (Fig. 6B). As a consequence, GDX females were phase delayed relative to intact females at 9 and 11 mo of age (planned contrast, \( P < 0.0100 \)). There was also a tendency for GDX females to be phase delayed relative to GDX males at 9 and 11 mo of age, but these differences were not statistically significant (planned contrast at 11 mo, \( P = 0.0195 \)).

\( \alpha \). As shown in Figs. 4 and 7, \( \alpha \) decreased with age (main effect age: \( F = 10.81, P < 0.001 \)). There was no effect of group on \( \alpha \) (main effect group: \( F = 1.71, P = 0.1667 \)), and group did not influence the age-dependent change in \( \alpha \) (group \( \times \) age interaction: \( F = 0.80, P = 0.6461 \)). Figure 7 illustrates the consistency in the development of \( \alpha \) across groups. At 9 mo of age, \( \alpha \) appeared to be longer in GDX than intact females; this group difference was not statistically significant (planned contrast, \( P = 0.0139 \)).

**Amplitude in wheel-running activity.** Activity amplitude changed significantly in *O. degus* during the first year of life, decreasing at 5–9 mo of age (main effect age: \( F = 32.93, P < 0.0001 \)). Amplitude varied as a function of group (main effect group: \( F = 6.62, P = 0.0003 \)), and group influenced the change in amplitude with age (group \( \times \) age interaction: \( F = 2.69, P = 0.0027 \)).

Each group exhibited a significant change in amplitude with age (1-way effect of age within each group: \( P < 0.0500 \)), but the age-dependent trajectory varied by group (Fig. 8). In general, the age-dependent decrease in amplitude was delayed in intact females relative to intact males and delayed in GDX animals relative to intact animals. This resulted in a sex difference in activity amplitude between intact males and females at 7 mo of age (Fig. 8A; planned contrast, \( P = 0.0031 \)). Similarly, activity amplitude was larger in GDX than in intact males (Fig. 8, B and C; planned contrast, \( P = 0.0003 \)) and in GDX than in intact females at 7 mo of age (Fig. 8D; planned contrast, \( P = 0.0013 \)).

\( \tau \). There was a significant decrease in \( \tau \) with age (main effect age: \( F = 33.06, P < 0.0001 \)). However, the development of \( \tau \) with age varied as a function of group (age \( \times \) group interaction: \( F = 5.25, P < 0.0001 \)). As shown in Figs. 9 and 10, tests of age within groups revealed a small, but significant, decrease in \( \tau \) with age in each group: intact males (\( F = 68.51, P < 0.0001 \)), intact females (\( F = 22.99, P < 0.0001 \)), GDX males (\( F = 5.78, P = 0.0011 \)), and GDX females (\( F = 13.00, P < 0.0001 \)).

In intact males and females, \( \tau \) was similar from 4 to 10 mo of age (Figs. 9 and 10A). At 12 mo of age, however, \( \tau \) was significantly shorter for intact males than females (planned comparison, \( P = 0.0010 \)). Similarly, period was the same in intact and GDX males until 12 mo of age (Figs. 9 and 10C). At 12 mo of age, \( \tau \) was significantly shorter in intact than in GDX males (planned comparison, \( P = 0.0021 \)). In GDX males and females, \( \tau \) did not differ at any age (Fig. 10B), nor was there a difference between intact and GDX females at any point in development (Figs. 9 and 10D).

The percentage of animals that exhibited an abrupt decrease in \( \tau \) after \( >7 \) days in DD was twice as high for 4- to 6-mo-old
degus as for 8- to 12-mo-old degus (Fig. 11A). Yet there was no overall effect of age on the tendency to exhibit this change in DD \( \chi^2 = 8.30, \text{df} = 4, P > 0.05 \). However, when months 4 and 6 were combined and compared with months 8-12, the peripubertal animals were significantly more likely to exhibit a delayed free run than postpubertal animals \( \chi^2 = 8.21, \text{df} = 1, P < 0.05 \).

At 4 mo of age, there was a significant effect of group on the propensity of \( \tau \) to abruptly change in DD \( \chi^2 = 9.16, \text{df} = 3, P < 0.05 \); Fig. 11B). No intact males exhibited an abrupt change in \( \tau \), whereas 20–60% of the animals in all other groups changed \( \tau \) suddenly at 4 mo of age. There was also a significant effect of group at 8 mo of age, inasmuch as four of the five animals that exhibited an abrupt change in \( \tau \) at this age were GDX females \( \chi^2 = 10.68, \text{df} = 3, P < 0.05 \). Overall, there was a tendency for GDX degus to exhibit this unusual pattern of behavior more than intact degus (Fig. 11B) until 10 mo of age, when an abrupt change of \( \tau \) became a rare occurrence.

Fig. 9. Double-plotted activity data collected at 5 ages from representatives from each group: animals 2364 (intact male), 2323 (intact female), 2315 (GDX female), and 2277 (GDX male). Free-running circadian period (\( \tau \), in h) is indicated beneath the corresponding activity record. Days of intense activity reflecting day of estrus are shown in examples for animal 2323 in months 6, 8, and 10.
Experiment 1b

Paired testes weight and spermatogenesis rating each significantly increased with age [main effect age: $F = 147.17, P < 0.0001$ (for testes weight); main effect age: $F = 58.87, P < 0.0001$ (for spermatogenesis)]. Testes grew rapidly from 2 to 6 mo and did not change after 6 mo of age (Fig. 12A). Similarly, from 2 to 4 mo of age, males exhibited dramatic changes in spermatogenesis, which reached a plateau at 4–6 mo of age (Fig. 12B).

Testosterone was undetectable in all females and many males. The exceptions were 2- and ≥18-mo-old males, in which six of six and five of seven samples, respectively, contained detectable levels of testosterone. There was an overall effect of group on circulating levels of testosterone in males ($F = 19.78, P < 0.0001$). Testosterone levels were significantly higher at 2 mo of age than in GDX males and all other ages except ≥18-mo-old males (Fig. 13; Tukey-corrected $P < 0.05$). Testosterone levels did not differ between any other pair of groups.

Fig. 10. Changes in $\tau$ for *O. degus* during the 1st yr of development. At 12 mo of age, $\tau$ was significantly shorter in intact males than intact females and GDX males (A and C). *$P < 0.0125$. GDX females did not differ from GDX males (B) or intact females (D).

Fig. 11. In some degus, $\tau$ becomes noticeably shorter after >1 wk of exposure to DD. A: percentage of all animals that exhibited a >0.15-h change in $\tau$ after 7 days in DD at various ages across the 1st yr of development. B: percentage of each group that exhibited a change in $\tau$ at various ages. C: double-plotted activity record of a 4-mo-old gonadally intact female showing a change in $\tau$, from 23.93 to 23.6 h, 11 days after release into DD.
Experiment 2

Sex and age each had a significant main effect on $\Psi_{on}$, with no significant interaction between factors. $\Psi_{on}$ was significantly phase delayed in male compared with female degus, regardless of age of testing (main effect sex: $F = 7.78, P = 0.0090$). Intact degus significantly phase delayed their activity onsets between 8 and 12 wk of age, independent of sex (Fig. 14; main effect age: $F = 15.22, P = 0.0005$).

DISCUSSION

The circadian rhythms of humans phase delay during adolescence (2, 3, 5, 10). It is likely that gonadal hormones modulate the circadian system during adolescent development, because these changes are related to sexual maturity (2, 5). The data presented here, collected from a slowly maturing, long-lived rodent, are consistent with the human data. O. degus exhibit a delay in $\Psi_{on}$ at approximately the age at which they begin to show signs of pubertal development. $\Psi_{on}$ in gonadally intact degus is phase delayed at this age compared with those that have been gonadectomized, demonstrating that the phase delay in $\Psi_{on}$ only occurs in the presence of gonadal hormones. The delay in $\Psi_{on}$ occurs in males and females, which indicates that males and females are equally sensitive to the same hormone (e.g., estradiol) or that different hormones have a convergent effect (e.g., testosterone in males and estradiol in females).

The largest delays in $\Psi_{off}$ were also observed in degus at 3 mo of age. In contrast to $\Psi_{on}$, the phase delay in $\Psi_{off}$ may have nothing to do with gonadal hormones; $\Psi_{off}$ was equally phase delayed in gonadally intact and gonadectomized degus. Thus, if the central circadian pacemaker is the target during puberty through which gonadal hormones modulate phase, it would appear that gonadal hormones modulate the putative morning and evening oscillators independently at this stage of development.

Two results indicate that the central circadian pacemaker of degus changes during adolescent development. 1) Peripubertal degus were more likely than postpubertal degus to exhibit an aftereffect of prior 24-h entrainment; in some cases, the underlying circadian pacemaker would not break free from the 24-h period of the previous light cycle until 10 –14 days in DD (Fig. 11C). The ~24-h period of the underlying circadian pacemaker in juveniles could serve an adaptive function by helping them maintain entrainment to their natural environment early in life. For example, this aftereffect of prior entrainment would make it easier for juveniles to maintain entrainment in situations where they are not consistently exposed to adequate light at dawn and dusk. This hypothesis has not been tested. Gonadal hormones appear to play some role in this developmental process, as the number of gonadally intact animals exhibiting an aftereffect of prior entrainment decreased at 4 – 6 mo of age, approximately the age at which they

Fig. 12. A: testes growth during the 1st yr of development in O. degus. Testes growth was accelerated between 2 and 6 mo of age and reached a plateau at 6 mo of age. B: spermatogenesis rating at various ages during the 1st yr of development. A rating of 5 indicates the most advanced level of spermatogenesis; a rating of 3 indicates some mature sperm. Spermatogenesis reached asymptote by 4 mo of age. Average Rating (A–D) indicate significant group differences (Tukey-corrected $P < 0.05$).

Fig. 13. Average serum testosterone concentrations in male degus. G, blood collected from >12-mo-old GDX males. Superscripted letters (a and b) indicate significant group differences (Tukey-corrected $P < 0.05$). Testosterone levels were significantly higher in 2-mo-old males than in all other age groups, except >18-mo-old males.

Fig. 14. Comparison of $\Psi_{on}$ in intact degus at 8 and 12 wk of age. In male and female degus, $\Psi_{on}$ was significantly phase delayed at 12 wk of age compared with 8 wk of age. *$P = 0.0005$ vs. 8 wk. Females were significantly phase advanced relative to males at 8 and 12 wk (main effect sex: $P = 0.0090$).
reach sexual maturity, and fewer gonadally intact degus exhibit an aftereffect of prior entrainment than gonadectomized animals at 6 and 8 mo of age. 2) Circadian period became progressively shorter in degus across the first year of life. Although degus exhibited the longest $\tau$ during puberty, at approximately the age at which they showed the most delayed onsets and offsets in their daily activity rhythm, the underlying circadian pacemaker does not appear to be the mechanism through which gonadal hormones modulate phase during puberty. There were no differences in $\tau$ between gonadally intact and gonadectomized animals until 12 mo of age, long after these animals had reached sexual maturity.

Across the first year of development, $\tau$ became progressively shorter and $\Psi_{\text{off}}$ increasingly phase advanced. This relation is reminiscent of the supposed connection in hamsters between the age-dependent advance in $\Psi_{\text{on}}$ and age-dependent decrease in circadian period (30, 35). The relation between age-dependent changes in $\Psi_{\text{on}}$ and circadian period is no longer widely accepted because recent well-controlled developmental studies have found no change in $\tau$ with age (7–9). However, in the present longitudinal study, using the same group of degus, we found a significant shortening of $\tau$ during the first year of development. The present study is the only one, to our knowledge, to describe a substantial change in circadian period so early in development; the change in $\tau$ occurred several months after degus reached sexual maturity, but long before degus would be considered aged [degus live 5–7 yr in the laboratory (20)]. The relation between $\tau$ and $\Psi_{\text{off}}$ is not perfect; for example, females ovariectomized before puberty exhibit an age-dependent decrease in $\tau$ with little corresponding change in $\Psi_{\text{off}}$. Similarly, postnatal hormone exposure has little effect on $\Psi_{\text{off}}$ in males but plays a significant role in changing circadian period after puberty in intact males (see below).

In gonadally intact males, $\tau$ became significantly shorter at 10–12 mo of age, resulting in a sex difference in $\tau$ at 12 mo of age. Period did not shorten at this age in prepubertally gonadectomized males, suggesting that exposure to testicular hormones is required in order for $\tau$ to shorten in males and become significantly different from females at 12 mo of age. Yet the sex difference in $\tau$ is not simply a function of a sex difference in circulating levels of testicular hormones, because gonadectomy of adult degus does not change $\tau$ (17, 18). Together, these data suggest that a postnatal organizational effect of testicular hormones sometime after 5 wk of age sexually differentiates $\tau$ in degus. Whether postnatal exposure to testicular hormones is sufficient for sexual differentiation of $\tau$ has not been tested; it is possible that prenatal and/or perinatal hormone exposure also plays a role in sexual differentiation of the period of the underlying circadian pacemaker.

There is a 6- to 8-mo delay between sexual maturity and the emergence of a sex difference in $\tau$. It is possible that the central circadian pacemaker is sensitive to testicular hormones earlier in development (i.e., during puberty), but sexual differentiation of the circadian time-keeping mechanism takes time. This seems unlikely given the many months that elapse between sexual maturity and the appearance of the sex difference. Instead, we hypothesize that the sex difference in $\tau$ in $O$. degus may truly represent a postpubertal process of SCN sexual differentiation. Humans become progressively phase delayed until 20 yr of age, years after they reach sexual maturity (29), and although social variables cannot be ruled out as important factors in this postpubertal change in phase preference, these data are consistent with the idea that the circadian system continues to change between puberty and adulthood. Organizational effects of gonadal hormones during puberty have been documented (31). To our knowledge, this is the only animal model of postpubertal sexual differentiation of the central nervous system. Such a model may lead to a better understanding of behavioral sex differences that emerge well after puberty.

A sex difference in $\Psi_{\text{on}}$ also emerges in gonadally intact animals several months after puberty. $\Psi_{\text{on}}$ does not become sexually dimorphic in animals gonadectomized before puberty. The data are somewhat ambiguous as to which gonadal hormone is involved, although there is a tendency for prepubertally gonadectomized males to exhibit a more delayed (i.e., more femalelike) $\Psi_{\text{on}}$ at 12 mo of age than gonadally intact males, which would indicate that testicular hormones underlie the sex difference in $\Psi_{\text{on}}$. However, because of the lengthy delay between puberty and sexual differentiation of $\Psi_{\text{on}}$, it is unlikely that the sex difference in $\Psi_{\text{on}}$ results from a developmental change in circulating hormone levels per se. Instead, postpubertal sexual differentiation of $\Psi_{\text{on}}$ is more likely a consequence of a change in the sensitivity of the circadian system to already elevated levels of testicular hormones (e.g., postpubertal change in the number or affinity of androgen receptors within the SCN). It does not appear that sexual differentiation of $\Psi_{\text{on}}$ in degus is related to sexual differentiation of $\tau$, since the sex difference in $\Psi_{\text{on}}$ appears at 9 mo of age, 2–3 mo before $\tau$ becomes sexually dimorphic. However, the sex difference in $\Psi_{\text{on}}$ may have adaptive consequences for $O$. degus. Since female degus exhibit large phase advances during estrus (18), early activity onsets in males may guarantee mating opportunities with females that are ovulating.

The emergence of sex differences in $\Psi_{\text{on}}$ and $\tau$ after puberty in degus is not the first report of a sexual dimorphism appearing postpubertally or even the first report of postpubertal sexual differentiation of the circadian system. Human sexual differentiation appears to continue well after puberty [e.g., phase angles of circadian entrainment (29)], and there is a great need for model systems to examine how such changes occur. $O$. degus provides a tremendous opportunity to examine the developmental variables that lead to sexual differentiation of the brain and behavior during and after adolescence.

In addition to the sex differences in period and phase, the amplitude of the wheel-running rhythm also developed differences at 5–7 mo of age between intact males and females and between intact and GDX animals of the same sex. It was surprising to find intact animals with a lower amplitude than same-sex GDX animals and then for the difference to disappear, because gonadectomy of adult males decreases amplitude significantly (17), whereas gonadectomy of adult females has no significant effect on amplitude (18). In addition, adult intact male degus produce a higher-amplitude activity rhythm than adult females, which is not true in this study at any age (unpublished data). These effects may be explained by the very early and repeated exposure of animals to running wheels. Interestingly, the data on adult phase and period match previous data very closely. This suggests that activity amplitude is controlled by other mechanisms in addition to the circadian timing mechanism, whereas period and phase are controlled only by the circadian system.
In conclusion, human adolescents exhibit delays in their circadian rhythms, including the daily pattern of sleep onset, spontaneous waking, and alertness, around the time of puberty (3–5). The data presented here, in the slowly maturing, long-lived rodent *Octodon degus*, are consistent with the human data. Gonadally intact degus phase delay their activity onsets at the age at which they begin to show signs of pubertal development; degus that have been gonadectomized before puberty begins do not exhibit this phase delay. Although gonadal hormones appear to be necessary for the phase delay to occur in degus during puberty, they do not appear to do so by changing the endogenous period of the underlying circadian pacemaker, because although degus exhibit the longest τ during puberty, gonadally intact degus and those gonadectomized before puberty do not exhibit differences in τ at this age. Perhaps gonadal hormones influence phase during puberty by modulating the sensitivity of the circadian system to light (2).

A sex difference in \( \Psi_{on} \) emerges in degus several months after puberty. This may have adaptive consequences for *O. degus*, since we predict that early activity onsets in males increase the likelihood of mating opportunities with females, in which phase advance occurs during estrus. Similarly, \( \tau \) becomes sexually dimorphic in degus after puberty. However, sexual differentiation of \( \tau \) does not appear to underlie sexual differentiation of \( \Psi_{on} \), since the sex difference in \( \Psi_{on} \) appears before the sex difference in \( \tau \). In both cases, the sex difference was absent in prepubertally gonadectomized degus, indicating that postnatal exposure to gonadal hormones is probably required for sexual differentiation of \( \Psi_{on} \) and \( \tau \). Apparently, sexual differentiation of \( \Psi_{on} \) and \( \tau \) is not simply a function of a developmental change in circulating hormone levels, since these sex differences do not emerge until several months after degus reach sexual maturity.

It appears that a sex difference in phase preference emerges in humans during late adolescence, well after they reach sexual maturity (29). Model systems are needed to examine how such changes occur and to understand the impact of gonadal hormone exposure during adolescence on the circadian system. The slowly maturing, precocial, long-lived rodent *O. degus* provides a unique opportunity to understand how gonadal hormones impact the circadian system during adolescence and beyond. Additional developmental studies in degus may clarify the underlying mechanism responsible for postpubertal sexual differentiation of the circadian system in degus and humans. These developmental changes in circadian rhythms may have lasting consequences for optimal work and school schedules.

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REFERENCES