Pubertal Development of Sex Differences in Circadian Function

An Animal Model

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ABSTRACT: The development of adult circadian function, particularly sexual dimorphism of function, has been well studied only in rapidly developed rodents. In such species development is complete by weaning. Data from adolescent humans suggest that significant development occurs during the pubertal period. We hypothesized that a more slowly developing rodent might better mimic the changes in circadian function around puberty in humans and allow us to determine the underlying neural changes. Entrained and free-running circadian rhythms were analyzed and correlated with pubertal development in male and female *Octodon degus* (degu) that remained gonadally intact or were gonadectomized at weaning. Brains were collected during development to measure androgen and estrogen receptors in the suprachiasmatic nuclei (SCN) Adult circadian period does not develop until 10–12 months of age in degus, long after the onset of gonadal maturation (3–5 months). The timing of circadian period maturation correlates with the appearance of steroid receptors in the SCN. Changes in free-running rhythms only occurred in gonadally intact degus. Adult phase angles of activity onset develop between 2 and 3 months of age (comparing results of two experiments), soon after the onset of pubertal changes. Conclusion: The development of sexually dimorphic adult circadian period occurs after gonadal puberty is complete and requires the presence of gonadal steroids. The delay in development until after gonadal puberty is likely due to the delayed appearance of steroid receptors in the SCN. Phase is not sexually dimorphic and changes in the absence of steroid hormones.

KEYWORDS: *Octodon degus*; degu; activity; phase angle; period; tau; development

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INTRODUCTION

The study of development of circadian rhythmicity in humans has a long history, beginning with the observations by Kleitman and Engelmann[1] that infants have essentially ultradian sleep/wake rhythms at birth that take approximately 8 weeks to begin producing night-time consolidation of sleep (see Ref. 2 for example). During the period of time that rhythmicity is developing, the parents also demonstrate altered sleep/activity patterns,[3] which frequently leads to sleep deprivation and the associated problems. However, many infants begin to demonstrate weak circadian rhythmicity within a few weeks of birth and parents quickly recover normal sleep/wake cycles. Work with altricial rodents suggests that this neonatal circadian pattern may be the result of maternal entrainment of the infant until the infant's own circadian system takes over completely, around the time of weaning.[4–7] Once the individual is independently producing robust circadian rhythms, it was long assumed that the system was stable until we became aged. Studies in both animal models and humans demonstrated that relatively old individuals have marked changes in expression of circadian rhythms (see Ref. 8 for review). Interestingly, very little work has been done to examine changes that may occur in the expression of circadian rhythms between weaning (and early independence) and the development of adult circadian rhythms. This is surprising, given the number of papers that have demonstrated that sex differences exist in the expression of numerous circadian rhythms for multiple species, including humans.[9–13] It is clear that at least some of these differences develop at puberty, and therefore something about circadian expression, if not the timing mechanism itself, must be changing during the transition from the juvenile to the adult state. The exception, of course, is Carskadon's data on sleep/wake regulation in adolescent humans, which does not appear to be sexually dimorphic, but clearly does change around the time of puberty (see Ref. 14 for review).

BRIEF REVIEW OF CIRCADIAN RHYTHMS AND THEIR IMPORTANCE

A circadian rhythm is defined as "an observed biological activity that oscillates under constant environmental conditions with a period length close to but not exactly equal to 24 h" (p. 15).[15] The circadian mechanism is the result of biochemical oscillation driven by feedback loops, providing an internal clock (or sense of time) for the individual. This clock may act to time events only within the tissue where it resides (e.g., the clock in the testes of the fly[16]) or it may provide information as a pacemaker to numerous other tissues. In mammals, the suprachiasmatic nucleus (SCN) is where the primary light-sensitive circadian pacemaker cells reside and co-ordinate their output signals. The endogenous period of pacemaker can be determined by housing an organism in constant conditions and measuring daily changes in one or more of the outputs, such as the rest/activity, body temperature, cortisol, or melatonin daily patterns. The free-running rhythm of these different behavioral and physiological measures will be the same and reflects the period (τ) of the SCN clock.

The non–24 h period of the endogenous circadian clock requires that outside information be obtained daily to synchronize the internal clock with the environment. The most important time cue (i.e., zeitgeber) is the light:dark cycle (LD), although many other non-photic cues can also act as weak cues on their own or can enhance

the effect of the LD cycle (e.g., hot:cold cycles, daily periods of exercise, daily periods of social contact).[17–19] The process of synchronizing the endogenous circadian system to the environment is known as entrainment. Entrainment results from light (or other cues) altering the period of the endogenous clock. This occurs in a phase dependent manner, such that light exposure late in subjective day and early in subjective night causes a delay in the circadian oscillation and results in a lengthened period. Light signals during late subjective night and very early subjective day cause a phase advance in the oscillation and a shortening of the period. The relationship between the direction and size of phase shifts in response to zeitgeber signals at various times across the subjective day is known as the phase response curve (PRC). These phase shifts occur each day at times that shift the period by the difference between 24 h and the endogenous τ, resulting in synchrony between the 24-h period of the environment and the internal circadian mechanism.

As a result of entrainment, a specific relationship develops between the biological rhythm being measured and the entraining zeitgeber, described as the phase angle of entrainment. The phase angle of entrainment (ψ) is the difference between a specific phase of the biological rhythm being measured and a phase marker in the environmental zeitgeber. For example, activity onset for a diurnal organism may begin before, after, or at the same time that lights come on. The difference between activity onset and lights-on in hours or degrees of arc between the two reference points describes this relationship. When the zeitgeber controls the circadian rhythm very well (i.e., it is a "strong zeitgeber" because the individual is very sensitive to its effects), the ψ will remain constant unless the zeitgeber cycle is shifted, then the biological rhythm will also shift to regain proper alignment. This process of recovery is called reentrainment and the duration of the recovery period is positively correlated with the size of the shift. During the days of recovery the various behavioral and physiological rhythms recover at different rates resulting in the symptoms of circadian desynchronosis (or "jetlag"). If the zeitgeber is not very strong, then ψ will be unstable.

The phase angle of entrainment is influenced by two things: the endogenous period of the circadian pacemaker that needs to be adjusted to 24 h and the phase-response curve defining the effect of equal zeitgeber stimulation at various times. If two individuals have different free-running periods and the same PRC, then the phase angle of entrainment must differ to accommodate the difference in the circadian time when light must impinge on the system to entrain the endogenous mechanism. As we will see below, if ψ is the same in two individuals, it does not mean that the underlying period and PRC are the same, PRC differences may compensate for period differences. However, when ψ differs, then either τ or the PRC must also differ between individuals.

Why are circadian rhythms so important? Under the normal entrained state, circadian rhythmicity allows the organism to anticipate regular changes in the external environment and prepare to respond to them. If food or predators regularly appear soon after sunrise or sunset, anticipation of those events with appropriate physiological and behavioral responses can improve health and survival. Equally important is the internal coordination between different physiological events provided by the circadian system, such that appropriate combinations of physiological changes occur at the same time. For example, in preparation for meal time, the parasympathetic system controlling digestion, hunger drive, and intestinal motility are increased. At bedtime, heart rate, body temperature, and arousal all decline. During desynchrony of

the circadian system, such as occurs during jetlag or shift work, the suites of rhythms are not well-coordinated and we suffer discomfort. Interestingly, the value of this circadian organization is particularly well reflected during early development in appropriately timed birth[20,21] and better growth patterns in pups synchronized with their mothers.[22–26] These findings carry over to comparisons of human infant growth patterns in different hospital nurseries where circadian signals vary from nearly absent to moderate.[27,28]

DEVELOPMENT OF CIRCADIAN RHYTHMS

Descriptive analysis of the development of circadian rhythms during infancy has been conducted in several species, including hamsters, rats, mice, and humans (see Ref. 2 for review). Studies of neonatal animals demonstrate that the expression of circadian rhythms is not fully developed at birth, even though the circadian mechanism in the suprachiasmatic nucleus (SCN) appears to be fully functioning.[29] The lack of fully developed rhythmicity at birth is likely due to the incomplete innervation of the SCN in altricial species, and the still developing output pathways from the SCN. At the time when circadian rhythms appear, they are initially of low amplitude, developing their full adult pattern only after days or weeks.[30] Thus, it is clear that the expression of circadian rhythmicity develops for some time after birth. Davis and Reppert argued, however, that while there are "systematic changes in sensitivity to stimuli and in amplitude, there is no evidence for a systematic change in the free-running period of circadian oscillations" (p. 257).[2] This is not to say that the environment can not affect the circadian mechanism, since it is clear that previous photoperiodic history can influence the free-running period (τ) for a very long time.[31] Similarly, it is clear that sex differences exist in the expression of circadian rhythms of adults and in SCN anatomy and function.[10–12] We present data below that demonstrate that the free-running period can be permanently altered postnatally and the change is dependent upon steroid hormones.

Sex Differences in Circadian Rhythms

A variety of species exhibit sex differences in circadian rhythms, including rats, mice, degus, hamsters, and humans. Sex differences are apparent in τ,[9,12,32–34] the amount and distribution of daily activity,[35] time spent sleeping,[34] the time and variability of activity onsets,[12] phase responses to light pulses,[12,36] the upper limits of entrainment,[12] susceptibility to splitting,[37] rates of reentrainment,[38] sensitivity to non-photic social cues during reentrainment,[39] and the age at which circadian rhythms first emerge.[40]

Some of the sex differences persist in the absence of circulating hormones in adulthood (organizing effects that are independent of adult hormones). For example, Zucker and colleagues[41] demonstrated that the circadian system of hamsters is sexually differentiated as a result of perinatal androgen exposure, such that period length in adult males is rendered insensitive to estrogen (E) exposure. That is, while E shortens τ in adult females, it fails to alter τ in adult males or adult females exposed perinatally to testosterone (T). Albers and colleagues[9] reported that perinatal exposure to T, either endogenously in male rats or exogenously in females, leads to

an adult τ that is shorter than untreated females. In the absence of perinatal T exposure, exogenous E treatment shortens τ in adulthood. However, the same adult E treatment lengthens or shortens τ in males and perinatally androgenized females depending on the length of the animal's pretreatment period.[9]

Adult gonadal hormones also modulate the expression of circadian rhythms leading to some of the sex differences mentioned above (activational/reversible effects). Castration of males rapidly alters phase angles of activity entrainment (ψ) and rhythm amplitude (intensity of activity change across the day), but does not alter τ or PRC. Similarly, ovarian hormones modulate circadian rhythms in adult females. The amount and distribution of activity, amount and quality of sleep, ψ for activity onset, and τ fluctuate with the estrous cycle. Female hamsters phase advance and run significantly more during proestrous and estrus compared to other days of the cycle.[42–44] Rats exhibit earlier activity onsets, longer active phases (α), and shorter τ's on estrus.[45] Female degus increase activity and phase-advance activity onset during estrus, and then decrease activity and phase-delay activity onset the day after behavioral estrus.[33] Women sleep longer, have more REM, and experience fewer sleep disturbances during the luteal phase of the menstrual cycle than the follicular phase.[34] It seems that gonadal steroids are capable of rapidly influencing the circadian system, since many of these changes occur within hours of changes in peripheral hormone concentrations.[42]

These two sets of data suggest that sex differences in circadian rhythms may be due to organizational effects, that pubertal hormones may elicit sex differences, or an interaction between early organizational and later activational hormones may produce uniquely male and female circadian patterns. This latter possibility suggests that pre/perinatal exposure to steroid hormones may cause differences in expressed rhythms that are not apparent until pubertal hormones activate the differences. However, to date, no one has examined the timing of the development of sex differences in fundamental circadian properties of a precocial species or a more slowly developing species, with the exception of the data from humans.

OCTODON DEGUS: AN ANIMAL MODEL
FOR CIRCADIAN DEVELOPMENT

Octodon degus is a moderate-sized, precocious, but slowly maturing, hystricomorph rodent from central Chile. We have used them to study a variety of questions about circadian rhythms in a diurnal mammal that readily adapts to most laboratory settings. They are born fully furred, eyes open, and are mobile within hours of birth. Teeth begin erupting within a few days, but they don't begin to consume significant solid food until two weeks of age and continue to nurse for 4–5 weeks. Although pubertal changes (measured by penile development and vaginal opening; Jechura, Hummer, and Lee, unpublished data) occur at 3 to 4 months of age (range is 10–14 weeks for males and 10–24 weeks for females) and reproductive activity is successful after 4.5 months for most animals, adult body size is not reached until 6–7 months of age (Young, Lee, and Rush, unpublished data). When compared with altricial myomorph rodents (e.g., hamsters, rats, mice) they have a slower pace of development marked by a relatively long juvenile period. In our laboratory we have routinely considered degus to be "adults" at 6 months and "elderly" at 4 years. The

latter cut-off in a lifespan of 5-7 years was chosen as a marker of aging because female pregnancy rate drops off rapidly after 4 years (Lee, unpublished data).

Several sex differences have been described in the circadian system of degus, including rates of reentrainment,[38] the effect of social cues on reentrainment,[38,39] sensitivity to light intensity (Stimpson & Lee, unpublished data), the phase-response curve (PRC),[36] and τ.[32,33,36]

Adult male degus exhibit shorter free-running period lengths $(23.2 \pm 0.1 \text{ h})$[32] than adult females $(23.75 \pm 0.1 \text{h})$[33] Period does not vary as a function of ovariectomy (OVX) with or without hormone replacement[33] or castration[32] in adulthood. Rather, we hypothesized that the sex difference in τ probably results from a sex-specific organization of the brain earlier in development. In altricial species this organizational effect occurs pre- or perinatally. However, no one has looked at when sex differences might occur in a precocious species, particularly a relatively slowly developing and long-lived one. We also hypothesized that slower development might reveal the type of pubertal phase change in the circadian mechanism that Carskadon describes for the sleep/wake cycle of adolescent humans.[14] The three experiments described below explore the age-dependent change in phase angle of entrainment, timing of permanent sexual differentiation of circadian period, and a potential mechanism to explain the latter phenomenon.

AGE-RELATED CHANGES IN PHASE ANGLE OF ENTRAINMENT

For the studies described here only wheel-running activity was used as an indicator of circadian function. We also use core temperature rhythms in our circadian studies, but have not found any significant difference in τ, PRC, or rates of reentrainment between activity and core temperature. In the first experiment we tested the hypothesis that juvenile degus would have a different phase angle of entrainment than adults and that they would require more time to recover from a phase advance in the light cycle than do adults. The male and female pups were placed into cages with running wheels with their same-sex siblings on the day of weaning for 1 week to adapt to the environment. They were then housed individually with a running wheel and we collected 7 days of baseline data while they were entrained to LD 12:12 at 300 lux. Adult males and females were treated similarly. The light cycle was then phase advanced 6 hours and the animals were allowed to recover. We determined the phase angle of entrainment at 8 weeks of age for the pups and for the adults (age 1– 3 years) and the rate of recovery from the phase shift.

The phase angle of entrainment did not differ between adult males and females, nor between male and female pups.[46] However, 8-week-old pups had a phase angle 50 min earlier than adults $(F = 7.365, P < 0.015;$ FIG. 1). We found that juvenile pups required the same length of time to recover from a phase shift as the adults. These data are consistent with data comparing pre- and post-pubertal adolescents.

In a second experiment, phase angle of entrainment was determined in 3-, 6-, and 12-month-old animals that remained intact or were gonadectomized (GDX) at weaning. The GDX animals demonstrated a significant reduction in the phase angle $(P < 0.001;$ FIG. 2) between 3 and 12 months, with data at 6 months intermediate between and not significantly different from the other two ages.[46] Interestingly, the intact animals do not show a significant change across these ages, which differs from the
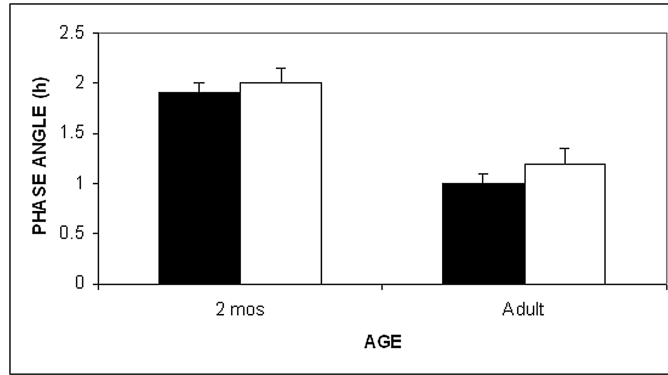
FIGURE 1. Entrained phase angles of activity onset (h) for 2-month-old pups and adults (1–3 years). Data are mean ± SEM time at which animals become active prior to lights on each day. Males are *dark bars* and females are *open bars*. Adults had significantly delayed phase angles compared with prepubertal juveniles.
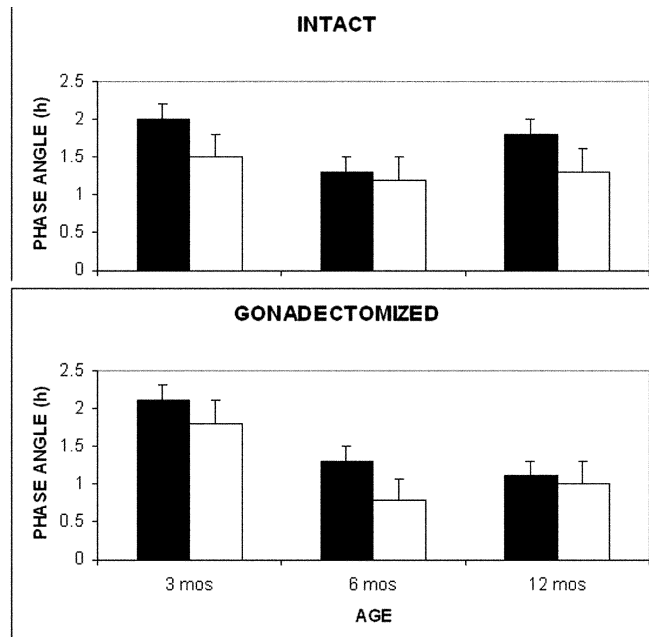

FIGURE 2. Mean ± SEM entrained phase angles of activity onset (h) at 3, 6, and 12 months of age for intact and gonadectomized male (*dark bars*) and females (*open bars*). Intact animals did not significantly change during the year, while both male and female gonadectomized animals significantly decreased the phase angle between 3 and 12 months.

comparison between 2-month-old and adult animals. This is most likely due to the effect of steroid hormones on phase, since we have found that gonadal steroids modify phase in both males and females.[32,33]

These two studies lead us to conclude that there is an age-dependent delay in the phase of activity onset that is steroid hormone independent. The change is equivalent in males and females. Intact adult animals are less delayed than they might otherwise be, because gonadal steroids slightly alter the phase. Interestingly, GDX of adult females results in no change in ψ (compared to females prior to estrus, when a large advance in ψ occurs). In contrast, GDX of adult males results in an advance of ψ for activity onset, which is not what one would expect from comparing 12-month-old animals that were intact or GDX since weaning. This would suggest that exposure to steroid hormones during the first six months also produces an organizational change in the underlying PRC that is steroid dependent.

GONADAL STEROID DETERMINED CHANGES IN PERIOD LENGTH

It is interesting that phase does not differ between adult males and females, because we have reported that period differs by an average of 36 min.[32,33] As noted above, GDX of adults does not alter τ, suggesting that organizational effects produce these differences. In the following study we tested the hypothesis that organization occurs during puberty. The free-running period of intact pups and pups GDX at 5 weeks of age was determined at 3, 6, and 12 months of age.[46] At 3 and 6 months of age, male and female degus, whether intact or GDX did not differ from each other and all had period lengths intermediate to those previously found in adult males and females. At 12 months of age, about 8 months after puberty onset, the sex difference in τ was evident for the intact animals ($P < 0.034$; FIG. 3 and 4). The decrease for intact males between 6 and 12 months was significant ($P < 0.03$), while τ did not change significantly for intact females from 3 to 12 months. In contrast, those GDX at 5 weeks demonstrated no difference in τ between males and females at 12 months and no change between 6 and 12 months of age. Intact and GDX male τ differed significantly at 12 months ($P < .048$). These data strongly suggested that gonadal hormones that increase during puberty were necessary for the development of the sex difference in τ, but that the difference actually appears long after the hormones began to increase and results from masculinization of the SCN. This poses an interesting dilemma. While there are other behaviors that first develop during puberty and take some time to become completely organized (e.g., male hamster sexual behavior),[47] we can find no report of another steroid-dependent behavior that is so delayed relative to the timing of puberty.

STEROID HORMONE RECEPTORS IN THE SCN

These data, of course, raised interesting questions about the precise timing of the post-pubertal change in τ and what might be happening in the SCN when the changes finally occur. The most obvious hypothesis was that steroid hormone receptors arrive in the SCN long after puberty, but at the time when τ changes. We repeated the earlier longitudinal study, but have examined the behavior of animals every 2 months

12-Month Male Representing Group Mean = 23.10±0.03
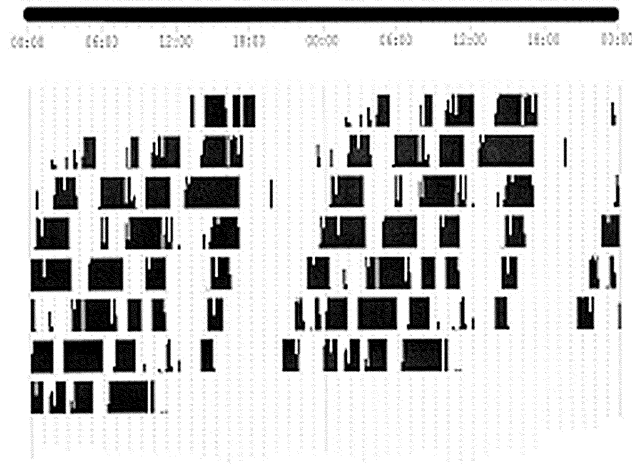
12-Month Female Representing Group Mean = 23.58±0.12

FIGURE 3. Example actograms of male and female 12-month-old degus housed in constant darkness. The free-running wheel-running activity of males is significantly faster than that of females. Prior to 12 months males and females alike had periods similar to the female in this example.

from 4–12 months of age. We found again that the sex difference does not emerge until 12 months of age. Brains have been collected from other animals between 3 and 12 months of age at monthly intervals. With the use of immunocytochemistry, we are determining whether and when androgen receptor-immunoreactivity (AR-ir) and estrogen receptor immunoreactivity (ER-ir) occurs in the SCN. Preliminary data from
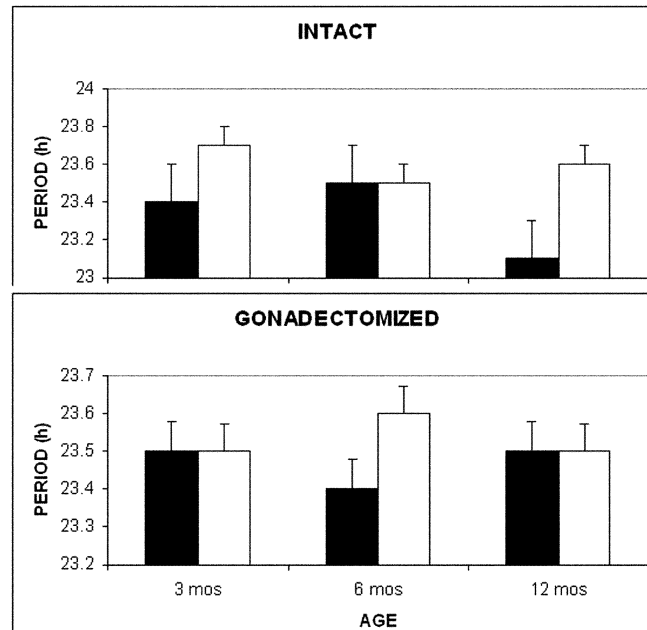
FIGURE 4. Mean free-running period (τ) $\pm$ SEM for intact and gonadectomized males and females at 3, 6, and 12 months of age. *Closed bars* represent males and *open bars* represent females. Gonadectomized animals do not change significantly and males do not differ from females. Intact females did not change, but males decreased the period significantly and differed from females at 12 months of age.

3, 7, and 12 month old males supports our hypothesis. There is essentially no AR-ir in the SCN of males at 3 months of age. At 7 months of age we find AR-ir in the SCN, primarily in the ventral area where retinohypothalamic tract neurons synapse on SCN neurons. This location for AR-ir would suggest a role for androgens in modulating the light input signal. We hypothesize that receptors in this area might modulate phase angle of entrainment, for example.

In the brains of 12-month-old males we found more AR-ir than at 7 months, and it was distributed into the dorsal SCN as well as the ventral area. In hamsters, the cells in the SCN that oscillate and also send axons to other areas of the brain are predominantly in the dorsal SCN. If the same were true for degus, the AR-ir we find in the dorsal SCN might be important for modulating output signals. More importantly, these late-appearing receptors may be those that permanently reduce τ in males.

We have also begun looking at ER-ir in females. A similar pattern appears to be emerging with virtually no ER-ir in the SCN at 3 months of age and stained cells evident in the ventral SCN at 7 months of age. There are far fewer ER-ir cells visible in the SCN of females than there are AR-ir cells in males. This may be because we are currently using an ERα antibody. Significant amounts of ERβ have been reported in the SCN of rats and humans,[48,49] but we have not yet identified an ERβ antibody

that recognizes ERβ receptors in degus. It is entirely possible that female degus have as much ER-ir as males have AR-ir, but we are not yet visualizing the right protein. On the other hand, the behavioral data suggest that estrogen can modulate ψ in the adult intact females, which would be consistent with the emergence of ER receptors in the ventral SCN. We find few if any ER-ir–labeled cells in the dorsal SCN of females, again consistent with the apparent lack of change in τ during development that we find in females.

At this time, it is still unclear which steroid hormones cause the change in τ. It may be that males change τ in response to exposure to testosterone. Alternatively, androgens may only be modulating adult circadian rhythms, while estrogen is critical for reorganization of SCN function. This would follow the common hypothalamic sexual differentiation pattern of androgens being aromatized to estrogen, which then provides the organizational effect. If the latter is true, then we will need to explain why males and females respond differently to the same hormone. For example, this sort of effect might occur if there is an early pre- or perinatal period of organization that determines whether τ will shorten following exposure to aromatized T or E in adulthood. Thus, we have several important follow-up studies using replacement hormones (estrogen and dihydrotestosterone, which cannot be converted to estrogen) to determine which hormone is critical for the organizational effects, and/or for the activational responses, and at what age they have their effects. In addition, we will determine whether males also have ER in the SCN to allow aromatized testosterone to organize the SCN.

CONCLUSION

Our data reveal a somewhat surprising developmental program in the emergence of circadian sex differences in degus. We can find no other case where steroid hormones are necessary for an organizational change in behavior or physiology so long after adult hormone secretory patterns are constantly available. We do not know what triggers the rise in steroid receptors in the SCN since it is apparently not the rise in gonadal steroids per se. This model offers an opportunity to examine what cellular and genetic changes are occurring just prior to the rise in receptors. We hypothesize that the rise in receptors that leads to the change in τ is an age-dependent phenomenon that might also be related to the non-steroid–dependent changes that are occurring with phase angle of entrainment.

These data also offer us an opportunity to examine our understanding of the relationship between period, phase responses, and phase angles of entrainment—and how these may change as a function of age and hormone exposure.

ACKNOWLEDGMENTS

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