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# Sexual Differentiation of Circadian Clock Function in the Adrenal Gland

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Sex differences in glucocorticoid production are associated with increased responsiveness of the adrenal gland in females. However, the adrenal-intrinsic mechanisms that establish sexual dimorphic function remain ill defined. Glucocorticoid production is gated at the molecular level by the circadian clock, which may contribute to sexual dimorphic adrenal function. Here we examine sex differences in the adrenal gland using an optical reporter of circadian clock function. Adrenal glands were cultured from male and female Period2::Luciferase (PER2::LUC) mice to assess clock function in vitro in real time. We confirm that there is a pronounced sex difference in the intrinsic capacity to sustain PER2::LUC rhythms in vitro, with higher amplitude rhythms in adrenal glands collected from males than from females. Changes in adrenal PER2::LUC rhythms over the reproductive life span implicate T as an important factor in driving sex differences in adrenal clock function. By directly manipulating hormone levels in adult mice in vivo, we demonstrate that T increases the amplitude of PER2::LUC rhythms in adrenal glands of both male and female mice. In contrast, we find little evidence that ovarian hormones modify adrenal clock function. Lastly, we find that T in vitro can increase the amplitude of PER2::LUC rhythms in male adrenals but not female adrenals, which suggests the existence of sex differences in the mechanisms of T action in vivo. Collectively these results reveal that activational effects of T alter circadian timekeeping in the adrenal gland, which may have implications for sex differences in stress reactivity and stress-related disorders. **(*Endocrinology* 157: 1895–1904, 2016)**

Glucocorticoid synthesis and release are regulated by the circadian system (1), which is a hierarchical collection of biological clocks located throughout the brain and body (2). At the cellular level, circadian rhythms are generated by a molecular oscillator involving a family of clock genes (3). At the core of this molecular oscillator is a 24-hour negative-feedback loop involving daily changes in transcription and translation. Briefly, the basic helix loop helix transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein (BMAL1) stimulate transcription of three *Period* genes (*Per1*, *Per2*, *Per3*) and two *Cryptochrome* genes (*Cry1*, *Cry2*), whose protein products feedback to inhibit their own transcription once every 24 hours. This core oscillator controls daily changes in cellular function by regulating the expression of numerous other clock-controlled genes in a tissue-specific manner (4). By regulating hormone synthesis and release, the molecular circadian clock plays an important role in maintaining optimal function in the face of daily environmental change.

Glucocorticoid production is regulated by the master clock in the brain and local clocks in downstream tissues (5). Importantly, the adrenal gland itself contains a circadian clock that is necessary for generating circadian rhythms in glucocorticoid release (6–10). Furthermore, the local circadian clock within the adrenal gland regulates the sensitivity to ACTH and the capacity to synthesize glucocorticoids. For example, circadian clock proteins regulate transcription of key mediators of glucocorticoid

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Abbreviations: GDX, gonadectomy; HPA, hypothalamo-pituitary-adrenal; PER2, Period2; PER2::LUC, PER2::Luciferase.

responses, including melanocortin 2 receptor and steroidogenic acute regulatory protein (6, 8, 10, 11). Additionally, ACTH can influence circadian clock proteins and the adrenal clock displays a daily rhythm in its sensitivity to ACTH (12–15). Changes in adrenal clock function may have widespread consequences within the circadian system because the daily glucocorticoid rhythm critically regulates other clock tissues (5). Thus, obtaining a deeper understanding of the intrinsic function of the adrenal clock is an important goal.

Although nearly all research on the circadian regulation of adrenal physiology has been conducted using male animals, glucocorticoid release patterns are known to be sexually diergic (16, 17). For instance, relative to male rodents, female rodents show greater corticosterone release under basal conditions (18) and in response to stress (19, 20). Although it is clear that sex differences exist in central components of the hypothalamo-pituitary-adrenal (HPA) axis (21, 22), there is also evidence that the adrenal gland itself is sexually differentiated. For example, female rodents display ACTH-induced glucocorticoid responses that are larger, faster, and persistently elevated relative to male rodents, even during dexamethasone-induced inhibition of the pituitary gland (23). Moreover, ACTH-stimulated glucocorticoid production in vitro is higher in adrenal cultures collected from female rodents relative to those collected from males (24). In contrast to extensive knowledge regarding sex differences in higher-order HPA structures, the cellular and molecular mechanisms underlying sex differences in the intrinsic function of the adrenal gland are not well understood.

An important question that remains unanswered is the extent to which the circadian clock in the adrenal gland contributes to sexually distinct patterns of glucocorticoid release. There is evidence that the function of the adrenal clock differs between male and female mice (25), but the basis of this effect has yet to be examined fully. Here we examine sex differences in adrenal clock function using a genetically encoded optical reporter of Period 2 (PER2) protein expression. Using this real-time bioluminescence assay to track intrinsic circadian clock function within adrenal glands of male and female mice, we verify that adrenal explants from male mice sustain higher-amplitude PER2 rhythms than those from female mice. Interestingly, we find that this sex difference in adrenal clock function is influenced by reproductive age, which implicates a role for gonadal steroids in the sexual differentiation of adrenal clock function. When examined directly, we find that the adrenal clock is affected by changes in circulating sex steroids during adulthood, with evidence for strong activational effects of T in both males and females in vivo. Lastly, in vitro application of T can directly influence PER2::Luciferase (PER2::LUC) rhythms in male adrenals but not female adrenals, suggesting that there are sex differences in the process by which T alters adrenal clock function in vivo. Collectively, these findings suggest the novel hypothesis that sexual differentiation of adrenal clock function may contribute to sexually dimorphic patterns of glucocorticoid release and stress reactivity.

## Materials and Methods

### Mice

Homozygous PER2::LUC knock-in mice (26), backcrossed onto a C57BL/6 background for at least 12 generations, were bred and raised under a 24-hour light-dark cycle with 12 hours of light and 12 hours of darkness (lights off at 6:00 PM CST). Throughout life, ambient temperature was maintained at 22°C ± 2°C, and mice had ad libitum access to water and food (Teklad Rodent Diet number 8604). At weaning, mice were group housed in cages without running wheels. Mice remained group housed until tissue collection unless they received a surgical treatment or were older than 20 weeks (to prevent fighting). All procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee at Marquette University.

### PER2::LUC tissue culture

PER2::LUC mice were killed with isoflurane anesthesia and cervical dislocation prior to tissue collection 4–6 hours before lights-off. Adrenal glands were excised and placed in chilled Hanks' balanced salt solution supplemented with HEPES, $NaHCO_3$, and penicillin-streptomycin. Adrenal glands were cleaned of adipose tissue and bisected manually with a scalpel before being cultured on a membrane with 1.2 mL of serum-free, air-buffered DMEM (Gibco 12100-046) supplemented with HEPES, $NaHCO_3$, penicillin-streptomycin, and beetle luciferin (Gold Biotechnologies). Bioluminescence rhythms were measured for at least 6 days with a luminometer (Actimetrics Inc) housed inside a light-tight incubator set to 36°C. To test effects of T in vitro, male and female adrenals were cultured for 9 days before medium exchange using DMEM with either 2 $\mu$M T (Sigma; catalog number T-1500) or vehicle (100% EtOH). The final concentration of EtOH in DMEM for both T and vehicle cultures was less than 0.001%. During dissection, two samples were extracted from each mouse to test the effects of T using a within-subjects design. Whether the left or right adrenal sample was stimulated with T was counterbalanced across mice.

### Gonadectomy

To test whether sex differences in adrenal clock function arise from differences in circulating T during adulthood, gonadectomy (GDX) or sham surgery was performed in male mice 8–11 weeks of age. GDX or sham surgery was performed under isoflurane anesthesia, with carprofen gel for pre- and postoperative analgesia. Testes were removed after laparotomy and clamping of the testicular artery. Mice with sham castrations underwent laparotomy, but the testes were left intact. Incisions were closed

with sterile suture, and nitrofurozone was applied to the incision site. Mice were singly housed after surgery, and adrenal glands were collected 4 weeks later for PER2::LUC recording.

### Steroid replacement

To further test the role of activational effects of T, male and female mice were implanted at 8–11 weeks of age with a sc SILASTIC brand capsule (Dow Corning Corp; outer diameter 2.16 mm, 15 mm in length) that was filled with 10 mm of T, 10 mm of $\beta$-estradiol (Sigma; catalog number E-8875) or left empty. Capsules were sealed with SILASTIC brand adhesive, washed in 70% ethanol, and primed in sterile saline at 36°C overnight to prevent a bolus of hormone upon implantation. This procedure has been shown to effectively restore T to physiological levels for more than 4 weeks (27, 28). Capsules were placed between the scapulae under isoflurane anesthesia, and the incision was closed with wound clips before being treated with nitrofurazone. Mice were singly housed after surgery, and adrenal glands were collected 4 weeks later for PER2::LUC recording.

### Estrous cycle determination

To determine whether the function of the adrenal clock varies with the estrous cycle, vaginal smears were obtained from 22- to 27-week-old female mice immediately prior to adrenal dissection. Vaginal smears were obtained by injecting and retrieving 100 $\mu$L of sterile saline into the vaginal lumen. Each sample was transferred to a glass microscope slide, dried, and stained using the Papanicolaou method (29) using hematoxylin, orange G, and eosin Y). Phase of estrous stage was determined by an observer blind to the results of PER2::LUC analyses using the following criteria. Proestrus was identified by the presence of a large proportion of nucleated epithelial cells (>70% of total cells), estrus by a large proportion of lightly stained enucleated and cornified cells (>50% of total cells), and diestrus by a large proportion of leukocytes (>80% of total cells).

### PER2::LUC rhythm analyses

PER2::LUC rhythms were analyzed with Lumicycle analysis software (Actimetrics Inc). First, the PER2::LUC time series was detrended by subtracting the 24-hour running average from the raw data. Next, PER2::LUC rhythms were analyzed by fitting a damped sine wave to the first five cycles in vitro, starting with the time of the first trough in vitro. Period, damping rate, and the goodness of fit of the sine wave were then recorded. Finally, the times of peak and trough PER2::LUC were recorded for each sample in vitro, and the corresponding values were extracted from the exported baseline subtracted time series using Excel. From these parameters, we quantified PER2::LUC amplitude (difference between peak and trough values) and period length (difference between two consecutive peak times) for each cycle in vitro. For each sample, we calculated the average period and the precision of period (inverse of SD) for the five cycles in vitro. Statistical analyses were performed with JMP software (SAS Institute). Data are represented in figures and tables as mean ± SEM.

## Results

Given the role of the circadian clock in gating glucocorticoid production and the influence of sex on stress reactivity, we examined sex differences in adrenal clock function using a genetically encoded optical reporter of PER2 clock protein expression (Figure 1A). Adrenals cultured from 12-week-old PER2::LUC mice displayed a marked sex difference in the amplitude of PER2 rhythms (Figure 1A, repeated measures ANOVA, $P < .0005$), consistent with an earlier report (25). Specifically, adrenal explants from male mice sustained higher-amplitude PER2 rhythms than those collected from female mice (Figure 1B). Consequently, male adrenals displayed a slower damping rate of PER2::LUC rhythms relative to female adrenals (Table 1). In contrast, period and phase did not differ by sex (Table 1). To test whether sex differences in rhythm amplitude were driven by the size of the tissue sample, we measured the weight of the adrenals after recording. Sample weight was not significantly correlated with damping of PER2::LUC rhythms in either females ($r^2 = 0.26$, $P > .1$) or males ($r^2 = 0.24$, $P > .1$), although overall adrenal weight was larger in females than males (Table 1, $t[14] = 5.67$, $P < .0001$), as reported previously (30). This pattern of results suggests that sexually dimorphic PER2::LUC rhythms are due to intrinsic differences in the function of the local circadian clock in the adrenal gland.

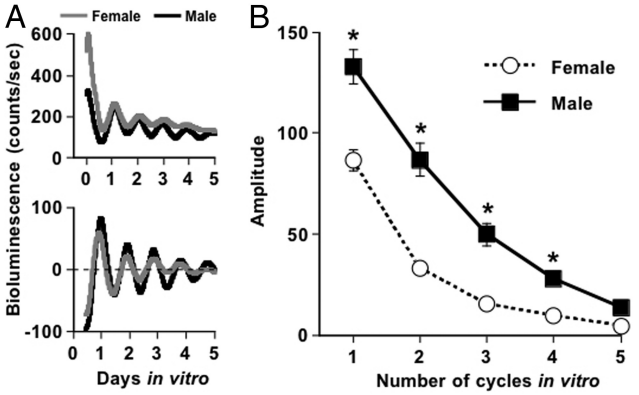


**Figure 1.** At 12 weeks of age, adrenal glands collected from male mice display higher amplitude PER2::LUC rhythms than adrenal glands collected from female mice. A, Representative PER2::LUC bioluminescence rhythms from adrenal glands collected from a male and female mouse before (top panel) and after (bottom panel) baseline subtraction. B, Adrenal glands collected from adult males displayed larger amplitude PER2::LUC rhythms on each cycle in culture (repeated measures ANOVA: sex, $F[1,14] = 85.80$, $P < .0001$; time in vitro, $F[4,11] = 106.11$, $P < .0001$; sex × time in vitro, $F[4,11] = 9.47$, $P < .0005$). Number of cultures per group is eight per sex (see Table 2). *, Male vs female, least squares means contrasts, $P < .01$.

To further examine the sex difference in adrenal clock function, we analyzed PER2::LUC rhythms from adrenal glands in male and female mice over the first year of life (Figure 2, A and B). In prepubescent mice, the sex difference in PER2::LUC amplitude was attenuated relative to adult mice but nevertheless detectable at each age examined. At 3 weeks of age, PER2::LUC amplitude was overall

**Table 1.** Parameters of Adrenal PER2::LUC Rhythms in Both Sexes Across the Reproductive Life Span

| | Weight, g | | Peak1 Time, h | | Period, h | | Damping, d | | Precision | | n | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age, Wk | Female | Male | Female | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| 3 | 0.6 ± 0.1 | 0.6 ± 0.1 | 41.9 ± 0.2 | 41.4 ± 0.3 | 22.5 ± 0.1 | 22.5 ± 0.2 | 1.2 ± 0.1 | 1.4 ± 0.1 | 1.0 ± 0.1 | 1.4 ± 0.2 | 8 | 8 |
| 4 | 0.6 ± 0.1 | 0.7 ± 0.1 | 41.9 ± 0.3 | 40.3 ± 0.3 | 22.5 ± 0.1 | 22.6 ± 0.2 | 1.4 ± 0.1 | 1.6 ± 0.1 | 1.8 ± 0.3 | 1.4 ± 0.3 | 10 | 10 |
| 5 | 1.4 ± 0.1 | 1.1 ± 0.1* | 40.5 ± 0.4 | 39.8 ± 0.2 | 22.7 ± 0.2 | 22.7 ± 0.1 | 1.3 ± 0.1 | 1.3 ± 0.1 | 0.5 ± 0.1 | 1.4 ± 0.2* | 8 | 8 |
| 8 | 1.9 ± 0.1 | 1.4 ± 0.1* | 42.7 ± 0.2 | 42.2 ± 0.5 | 23.0 ± 0.1 | 23.2 ± 0.2 | 1.4 ± 0.1 | 1.6 ± 0.1 | 1.2 ± 0.2 | 1.1 ± 0.2 | 12 | 12 |
| 12 | 1.6 ± 0.1 | 1.0 ± 0.1* | 42.2 ± 0.2 | 41.8 ± 0.2 | 22.8 ± 0.2 | 23.1 ± 0.5 | 1.3 ± 0.1 | 1.7 ± 0.1* | 1.0 ± 0.2 | 1.8 ± 0.4 | 8 | 8 |
| 26 | 2.4 ± 0.2 | 1.0 ± 0.1* | 44.3 ± 0.3 | 43.9 ± 0.1 | 21.9 ± 0.4 | 22.6 ± 0.2 | 1.2 ± 0.1 | 2.1 ± 0.2* | 0.5 ± 0.1 | 1.8 ± 0.3* | 10 | 8 |
| 52 | 1.5 ± 0.2 | 1.1 ± 0.2 | 41.6 ± 0.3 | 42.2 ± 0.4 | 23.4 ± 0.7 | 22.8 ± 0.1 | 0.9 ± 0.1 | 2.1 ± 0.2* | 1.9 ± 0.6 | 0.5 ± 0.1* | 14 | 6 |
| Overall | 1.4 ± 0.1 | 1.0 ± 0.1* | 42.1 ± 0.2 | 41.6 ± 0.2 | 22.6 ± 0.1 | 22.8 ± 0.1 | 1.2 ± 0.1 | 1.7 ± 0.1* | 1.2 ± 0.2 | 1.3 ± 0.1 | 70 | 60 |

* Student's *t* test ($P < .05$).

higher in males relative to females (Figure 2A, repeated measures ANOVA, $P < .05$), but the two sexes did not differ on any cycle in vitro (Figure 2, A and B). In 4-week-old mice, the sex difference in PER2::LUC amplitude was more pronounced than at 3 weeks of age (Figure 2A, repeated measures ANOVA, $P < .01$), with males displaying higher PER2::LUC amplitude on the second and third cycles in vitro (Figure 2, A and B). This pattern was maintained at 5 weeks of age, with a stronger overall statistical difference than evident at 3 or 4 weeks of age (Figure 2A, repeated measures ANOVA, $P < .005$). Similarly, males displayed higher amplitude rhythms relative to females at 8 weeks of age, which is near puberty in this strain of mouse (Figure 2A, repeated measures ANOVA, $P < .05$). The sex difference remained evident as mice grew older (Figure 2A), with higher PER2::LUC amplitude in males at both 26 weeks of age (repeated measures ANOVA, $P < .0001$) and 52 weeks of age (repeated measures ANOVA, $P < .0001$). Across the reproductive life span, no other rhythmic parameters systematically differed by sex (Table 1). Overall, the pattern of results indicates that the sex difference in adrenal clock function develops prior to puberty (Figure 2B). Given that T is measurable in male mice starting at 4 weeks old (31), these data suggest that circulating levels of T may regulate adrenal clock function.

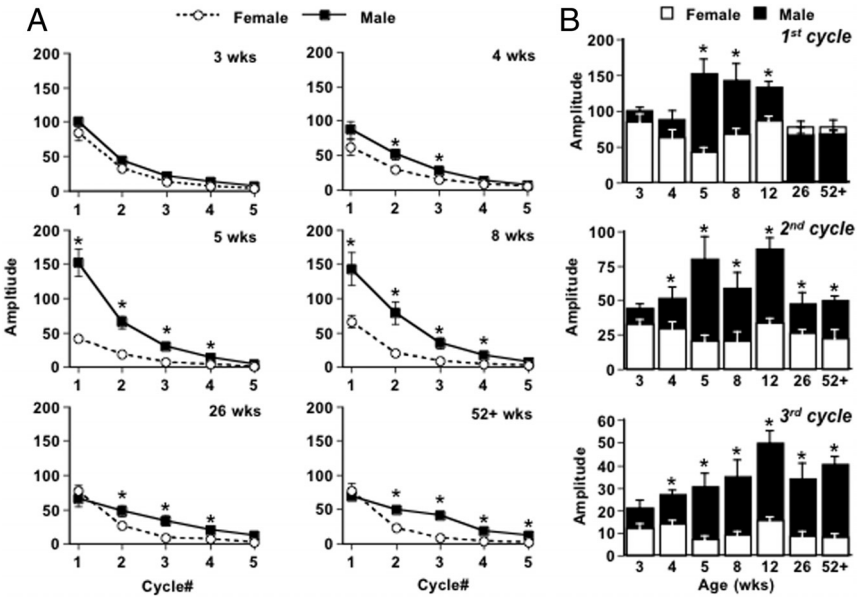


**Figure 2.** The magnitude of the sex difference in adrenal clock function changes systematically over the first year of life. A, Sex influenced the amplitude of adrenal PER2::LUC rhythms at each developmental age (repeated measures ANOVA: 3 wk, sex, $F[1,14] = 4.83$, $P < .05$; time in vitro, $F[4,14] = 59.51$, $P < .0001$; sex × time in vitro, $F[4,11] = 0.81$, $P = .54$; 4 wk, sex, $F[1,18] = 4.42$, $P < .05$; time in vitro, $F[4,15] = 20.62$, $P < .0001$; sex × time in vitro, $F[4,15] = 5.03$, $P = .009$; 5 wk, sex, $F[1,14] = 28.15$, $P = .0001$; time in vitro, $F[4,11] = 31.46$, $P < .0001$; sex × time in vitro, $F[4,11] = 8.43$, $P = .002$; 8 wk, sex, $F[1,22] = 11.68$, $P < .005$; time in vitro, $F[4,19] = 17.46$, $P < .0001$; sex × time in vitro, $F[4,19] = 3.61$, $P = .05$; 26 wk, sex, $F[1,16] = 3.28$, $P = .08$; time in vitro, $F[3,13] = 24.01$, $P < .0001$; sex × time in vitro, $F[4,13] = 9.03$, $P < .0001$; 52 wk, sex, $F[1,18] = 7.26$, $P < .02$; time in vitro, $F[4,15] = 21.59$, $P < .0001$; sex × time in vitro, $F[4,15] = 40.36$, $P < .0001$). B, Mean amplitude (±SEM) of PER2::LUC rhythms from male and female adrenal glands on the first three cycles in culture. Reproduction of data in Figures 1 and 2A is designed to highlight age-related changes. Number of cultures per group is 8–12 per sex (see Table 1). *, Male vs female, least squares means contrasts, $P < .01$.

Based on the results of our developmental time course, we hypothesized that T modulates the intrinsic capacity of the adrenal gland to sustain high amplitude PER2::LUC rhythms. To directly test this hypothesis, we performed castration or sham surgeries in adult male PER2::LUC mice. In addition, mice were implanted with T capsules to assess the effects of hormone replacement. Four weeks later, adrenal glands were collected from each group for PER2::LUC recording. Manipulating T levels in adult male mice influenced the amplitude of PER2::LUC rhythms displayed by the adrenal gland in vitro (Figure 3A, repeated measures ANOVA, $P < .0001$). Specifically, castration reduced the amplitude of PER2::LUC rhythms compared with sham surgical controls on the first three cycles in vitro (Figure 3A). Moreover, the amplitude of PER2::LUC

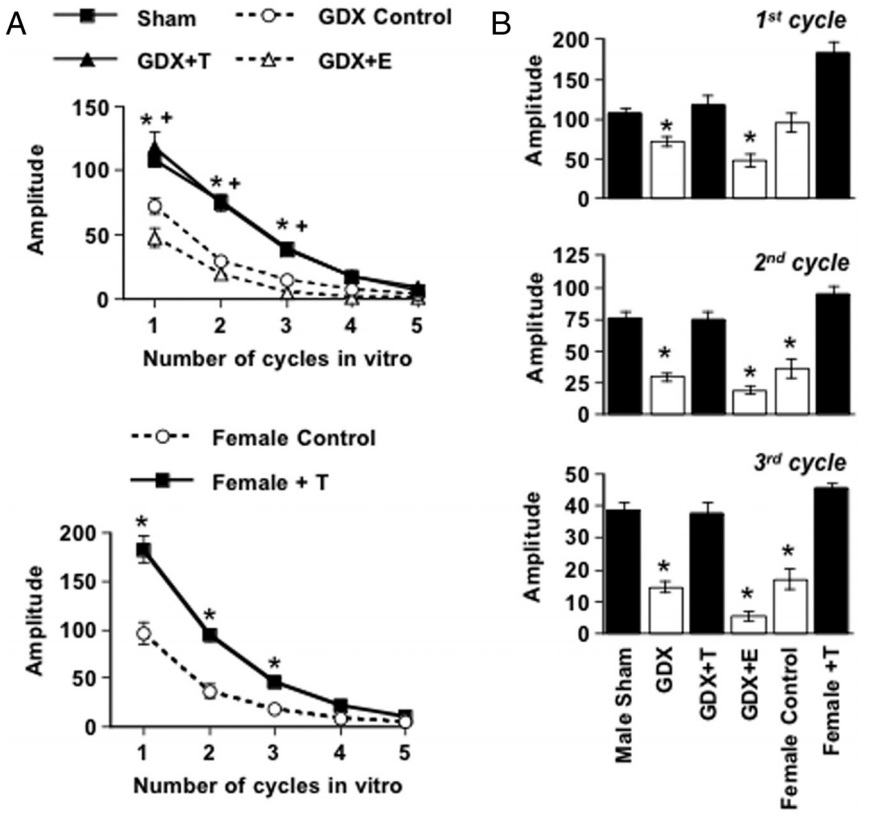


**Figure 3.** T increases amplitude of adrenal PER2::LUC rhythms in adrenal glands of both male and female mice. A, Manipulating T levels in adult male mice influenced PER2::LUC amplitude (repeated measures ANOVA: group, F[3,52] = 26.64, $P = .0001$; time in vitro, F[4,49] = 86.99, $P < .0001$; group × time in vitro, F[4,51] = 22.73, $P < .0001$). GDX+E, GDX + estrogen replacement; GDX+T, GDX + T replacement; Sham, sham GDX. *, Sham vs GDX (least squares means contrasts, $P < .001$; +, GDX vs GDX+T (least squares means contrasts, $P < .001$). B, In adult female mice, T increased PER2::LUC amplitude (repeated measures ANOVA: group, F[1,14] = 41.20, $P < .0001$; time in vitro, F[4,11] = 64.54, $P < .0001$; group × time in vitro, F[4,11] = 11.69, $P < .001$). Female control, sham hormone pellet; female+T, T pellet, female control vs female + T. *, Least squares means contrasts ($P < .01$). C, Mean amplitude (±SEM) of PER2::LUC rhythms on the first three cycles in culture. Reproduction of data in Figure 3, A and B, is designed to highlight T-dependent effects in both sexes. Number of cultures is 8–16 per group (see Table 2). *, Tukey's honestly significant difference, $P < .005$.

rhythms was restored by replacement of T but not estrogen (Figure 3A). Collectively, these data indicate that androgens during adulthood modulate the amplitude of PER2::LUC rhythms of adrenal glands in male mice.

We next tested whether adrenal clock function in adult female mice remains sensitive to T in adulthood. Adult female PER2::LUC mice were implanted with sham or T pellets, and adrenal glands were collected 4 weeks later for PER2::LUC recording. Provision of T to females in vivo elevated the amplitude of PER2::LUC rhythms displayed by the adrenal gland in vitro (Figure 3B, repeated measures ANOVA, $P < .001$). PER2::LUC amplitude was increased significantly on the first three cycles in vitro (Figure 3B). Interestingly, when effects of hormone manipulation are compared across sex (Figure 3B), groups with T displayed similar amplitude of PER2::LUC rhythms on both cycles 2 and 3, regardless of sex. In contrast, sample weight and other PER2::LUC rhythmic parameters were not systematically affected by hormonal manipulations (Table 2). These data indicate that the adrenal clock of females remains sensitive to activational effects of T during adulthood, which complement our results using hormonal manipulations in male mice.

Next we investigated whether changes in ovarian hormones over the estrus cycle influence adrenal clock function in female mice. To determine the estrous phase, we performed vaginal cytology on samples collected from females during adrenal dissection (Figure 4A). No estrus-related differences were detected in adrenal PER2::LUC rhythms (Figure 4B and Table 3; repeated measures ANOVA, $P > .2$). Together with the lack of change produced by estrogen replacement in castrated males, these results suggest that ovarian hormones do not alter adrenal clock function.

Because androgen receptors are expressed in mouse adrenal glands (32, 33), we tested whether T can influence PER2::LUC rhythms directly in vitro. Adrenals were cul-

**Table 2.** Parameters of Adrenal PER2::LUC Rhythms Across Hormonal Manipulations

| Sex | Group | Weight, g | Peak1 Time, d | Period, h | Damping, d | Precision | n |
|---|---|---|---|---|---|---|---|
| Male | Sham | 1.4 ± 0.1[a,b,c] | 43.2 ± 0.4[a] | 22.4 ± 0.1[a] | 1.6 ± 0.1[a] | 1.8 ± 0.4[a] | 16 |
| Male | GDX-Control | 1.2 ± 0.1[b,c] | 43.2 ± 0.4[a] | 22.4 ± 0.2[a] | 1.3 ± 0.1[a] | 1.1 ± 0.2[a,b] | 16 |
| Male | GDX + T | 1.1 ± 0.1[c] | 43.2 ± 0.3[a] | 22.7 ± 0.1[a] | 1.5 ± 0.1[a] | 1.4 ± 0.2[a,b] | 16 |
| Male | GDX + E | 1.6 ± 0.2[a,b] | 43.2 ± 0.9[a] | 22.3 ± 0.2[a] | 1.2 ± 0.3[a] | 0.5 ± 0.1[b] | 8 |
| Female | Control | 1.9 ± 0.2[a] | 45.6 ± 0.3[a] | 22.9 ± 0.3[a] | 1.2 ± 0.1[a] | 0.9 ± 0.2[a,b] | 8 |
| Female | Testosterone | 1.2 ± 0.1[b,c] | 43.2 ± 0.2[a] | 23.0 ± 0.2[a] | 1.5 ± 0.1[a] | 1.5 ± 0.2[a,b] | 8 |

[a,b,c] Groups that do not share the same letter differ from one another (Tukey's HSD, $P < .005$).

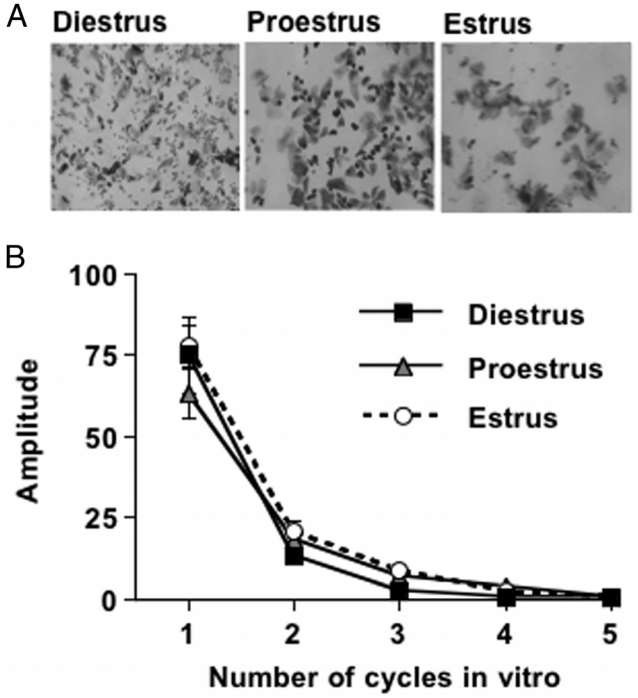


**Figure 4.** Estrus does not influence amplitude of adrenal PER2::LUC rhythms in female mice. A, Representative images of vaginal cytology during diestrus, proestrus, and estrus. Note that no samples were obtained from metaestrus. B, PER2::LUC amplitude did not fluctuate over the estrous cycle (repeated measures ANOVA: group, $F[2,31] = 1.23$, $P > .3$; time in vitro, $F[4,28] = 55.26$, $P < .0001$; group × time in vitro, $F[4,29] = 1.55$, $P > .2$). Number of cultures is 10–14 per group (see Table 3).

tured from 9- to 15-week-old male and female PER2::LUC mice for 9 days before a medium exchange with or without 2 $\mu$M T (Figure 5A). As expected, the sex difference in PER2::LUC amplitude was evident on each of the first four cycles in vitro (Figure 5B; repeated measures ANOVA, $P < .0001$). Also, consistent with previous work using other types of cells (34), medium exchange reinitiated PER2::LUC rhythms in adrenal glands (Figure 5A). Overall, the amplitude of the restored PER2::LUC rhythms differed by sex, with higher amplitude in male adrenals than female adrenals (Figure 5C; repeated measures ANOVA, $P < .0001$). Lastly, T influenced PER2::LUC amplitude in a manner that depended on sex (*Figure 5C*, within subject, repeated measures ANOVA, $P < .005$). In male adrenals, T increased PER2::LUC amplitude of rhythms on the first two cycles following medium exchange (Figure 5C). In contrast, PER2::LUC rhythms in female adrenals were not affected by T in vitro (Figure 5C). Collectively these results confirm the sex difference in intrinsic function of the adrenal clock and indicate that T can directly influence the adrenal clock in a sexually diergic manner.

## Discussion

The circadian clock is an important molecular mechanism regulating glucocorticoid release. Here we confirm the pronounced sex difference in adrenal clock function first described in an earlier report (25) and extend those findings by charting the developmental time course over which this sex difference emerges. These results provide suggestive evidence that T plays a role in driving this sex difference in adrenal clock function. When tested directly, it is apparent that activational effects of T in vivo markedly increase the amplitude of the PER2::LUC rhythms displayed by the adrenal clock in vitro. In contrast, we find little evidence for a strong modulatory role of estrogen in castrated males or naturally cycling females. The consequences of sex differences in intrinsic adrenal clock function warrant further investigation, given the important role of the adrenal circadian clock in regulating glucocorticoid release and the role of glucocorticoids in setting the phase of other tissue clocks in the circadian system (5). Collectively our results using the PER2::LUC assay reveal a novel hormone sensitivity in the intrinsic function of the adrenal clock. Insight into intrinsic clock function is important, given the role of local circadian mechanisms in regulating tissue physiology over the course of the day. It will be of interest to compare these results with adrenal rhythm in vivo, given that the adrenal clock of males and females would be expected to receive differential input from higher-order structures that are also sexually dimorphic (21, 22). Additional work exploring adrenal rhythms in both males and females may shed new light on the basis of sex differences in stress responses and stress-related illnesses.

We demonstrate here that the adrenal clock is markedly sensitive to activational effects of T. Androgen-induced changes in adrenal clock function are consistent with the ability of T to masculinize physiological and behavioral

**Table 3.** Parameters of Adrenal PER2::LUC Rhythms Across the Estrous Cycle

| Group | Weight, g | Peak1 Time, d | Period, h | Damping, d | Precision | n |
|---|---|---|---|---|---|---|
| Diestrus | 1.7 ± 0.1[a] | 43.2 ± 0.5[a] | 24.4 ± 1.1[a] | 0.8 ± 0.1[a] | 0.3 ± 0.1[a] | 10 |
| Proestrus | 2.1 ± 0.2[a] | 43.2 ± 0.4[a] | 22.6 ± 0.5[a] | 1.1 ± 0.2[a] | 0.4 ± 0.1[a] | 10 |
| Estrus | 2.0 ± 0.2[a] | 43.2 ± 0.3[a] | 23.4 ± 0.5[a] | 1.0 ± 0.1[a] | 0.5 ± 0.1[a] | 14 |

[a] Groups that do not share the same letter differ from one another (Tukey's HSD, $P < .02$).

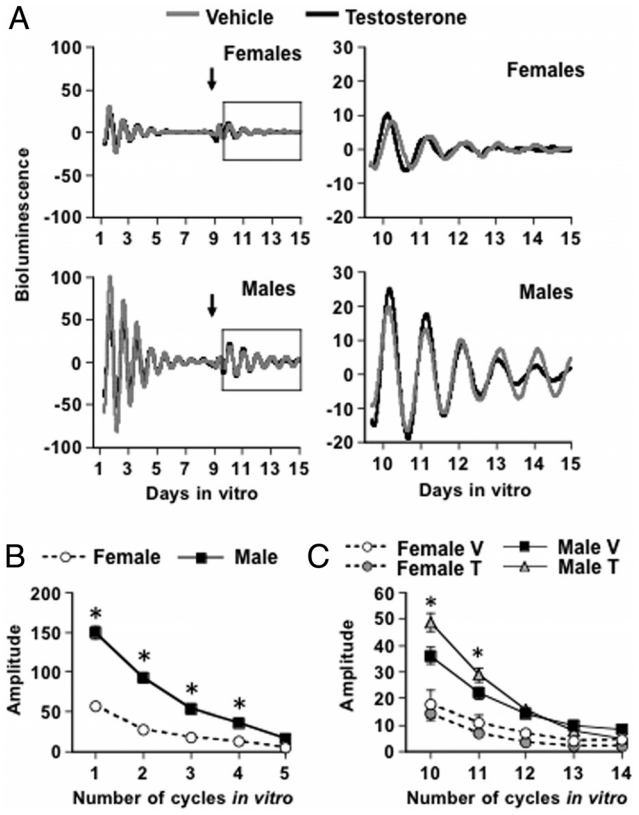


**Figure 5.** T exposure in vitro can influence adrenal clock function. A, Representative PER2::LUC bioluminescence rhythms from adrenal glands collected from female and male mice for 9 days and then exposed to a medium exchange with either T or vehicle. The arrow indicates the day of medium exchange with or without T. The boxed portion of the upper graph after the medium exchange is represented in the right graph for each sex. B, Adrenal amplitude on the first five cycles in vitro differed by sex (repeated measures ANOVA: sex, $F[1,38] = 104.63$, $P < .0001$; time in vitro, $F[4,35] = 140.39$, $P < .0001$; sex × time in vitro, $F[4,35] = 29.57$, $P < .0001$). Number of cultures is 16–22 per sex. C, T potentiated PER2::LUC amplitude in a sex-dependent manner (within subjects, repeated measures ANOVA: sex, $F[1,68] = 47.74$, $P < .0001$; medium, $F[1,68] = 0.05$, $P = .82$; time in vitro, $F[4,68] = 116.15$, $P < .0001$; sex × time in vitro, $F[4,68] = 23.83$, $P < .0001$; sex × medium, $F[1,68] = 3.40$, $P = .08$; medium × time in vitro, $F[4,68] = 3.56$, $P < .05$; sex × medium × time in vitro, $F[4,68] = 5.14$, $P < .005$). When divided by sex, T potentiated PER2::LUC amplitude in male adrenals but not female adrenals (within subjects, repeated measures ANOVA: males, medium, $F[1,28] = 11.32$, $P < .05$; time in vitro, $F[4,28] = 120.78$, $P < .0001$; medium × time in vitro, $F[4,28] = 13.58$, $P < .0001$; females, medium, $F[1,40] = 1,.57$, $P = .24$; time in vitro, $F[4,40] = 19.26$, $P < .0001$; medium × time in vitro, $F[4,40] = 0.13$, $P = .97$). *, Male T differs from male vehicle, $P < .05$. V, vehicle. Number of cultures is 8–11 per group.

stress responses in male (35) and female rodents (36). Given that estrogen failed to mimic the restorative effects of T in castrated male mice, this effect is likely mediated by androgen receptor signaling. Further evidence for activational effects of T were provided by the developmental maturation of the sex difference in adrenal clock function, although organizational effects of gonadal hormones may be involved as well. The finding that the decline in the magnitude of the sex difference precedes the age-related decline in T production (37) indicates that factors other than levels of circulating T may also influence adrenal clock function (37, 38). The persistence of the sex difference in adrenal clock function in vitro suggests that T alters the intrinsic function of the adrenal clock itself. Androgen receptors are located in many areas of the brain and body, including the hypothalamus, pituitary, and adrenal (21). For the most part, previous work exploring the effects of gonadal hormones on glucocorticoid release has used in vivo manipulations and in vivo measurements of HPA function, so it has been difficult to localize the epicenter of these effects. We find that T in vitro is able to increase the amplitude of PER2::LUC rhythms restored by medium exchange. This demonstrates that T can influence the adrenal gland directly to modulate circadian clock function. However, in contrast to the effect observed after a 4-week treatment in vivo, the effect of T in vitro was smaller in magnitude, shorter lived, and sexually dimorphic. The discrepancy between effects of T in vivo and in vitro results may reflect that longer-term treatment is required for the full effect to manifest. It is also possible that T in vivo potently influences adrenal PER2::LUC rhythms due to effects on higher-order structures that provide input to the adrenal gland. Given the sexually diergic response to T in vitro but not in vivo, this suggests that there is a sex difference in androgen sensitivity and/or site of action. Future studies using tissue-specific manipulations of androgen receptor signaling may prove useful for investigating the nature of this sex difference further.

Here we have interrogated adrenal clock function with hemisected tissue samples, which contain both the cortical and medullary compartments. Both the adrenal cortex and medulla display daily rhythms in circadian clock gene expression, although compartmental differences have been reported in male mice and rats (39–41). This previous work suggests that *Per2* expression is higher in the cortex than in the medulla, which would suggest that most of the PER2::LUC signal detected here derives from the cortex, at least in the male mice. Although it remains unclear whether this likewise applies to female rodents, it is known that adrenal glands from females are larger due to a greater number and/or volume of adrenocortical cells (30, 42, 43). Consistent with these previous studies, we find that adrenals are larger in female mice at 5 weeks of age and older. Because we detected the sex difference in adrenal clock function at 3–4 weeks of age, this suggests that sexual differentiation of adrenal clock function precedes the onset of sex differences in adrenal morphology and glucocorticoid release. Although we find little evidence that sexual differentiation of the adrenal clock is due to differences

in sample mass, it will be of interest to explore how this relates to sex differences in adrenal morphology (30, 44–47).

In addition, our results indicate that the function of the adrenal clock is not sensitive to ovarian hormones. First, across the reproductive life span, we found little evidence for changes in the adrenal rhythms of females but pronounced changes in males. Furthermore, estrogen was unable to restore the amplitude of adrenal PER2::LUC rhythms in castrated male mice. Lastly, adrenal PER2::LUC rhythms were not influenced by the estrous phase in naturally cycling female mice. The lack of an influence of estrogen was unexpected, given previous work in rats suggesting that estrogen alters basal and stress-induced corticosterone levels (18, 35, 36, 48–50). Furthermore, glucocorticoid production in cultured adrenocortical cells is enhanced by estrogen (24, 51–53). Because the vast majority of this previous work was conducted in rats, it remains unclear whether estrogen directly influences adrenal function in mice (22, 54, 55). Notably, interactions between ovarian hormones and circadian processes have been described in a variety of rodent species (56).

An important remaining question is whether the sex difference in adrenal clock function is related directly to sexual diergism in stress responses. The sex difference in adrenal clock function may prove influential, given that the adrenal clock regulates cholesterol transport, steroidogenesis, and ACTH responses (6, 14, 40) and inhibits glucocorticoid release (10). Based on this work, it is possible that lower amplitude clock protein expression in females may result in a perpetually derepressed state that contributes to their greater capacity to mount a glucocorticoid response (21, 22). This hypothesis may be tested by assessing molecular rhythms in both sexes. Circadian rhythms in molecular components of the glucocorticoid synthesis pathway have been assessed in males but not females despite the long-standing knowledge that intrinsic glucocorticoid release in vitro is sexually dimorphic (24). Future work could address this gap by assessing adrenal molecular rhythms in both sexes using in vivo and in vitro assays. On the other hand, it is possible that there is an opposite relationship, with high glucocorticoid release damping adrenal clock function. Higher glucocorticoid release in the female adrenal may act to suppress clock function, given there is a glucocorticoid response element within the *Per2* promoter (57).

Some insight into this question may be provided by our developmental time course. Previous work indicates that the sex difference in corticosterone levels appears in mice after 5 weeks of age (30), which is an age at which we detect a fairly robust sex difference in adrenal clock function. Nevertheless, there is also a suggestion that glucocorticoids may influence adrenal clock function. In particular, PER2::LUC amplitude on the first cycle in vitro was higher in 12-week-old males (Figures 1B and 5B) than in 12-week-old Sham males, and 26- or 52-week-old males (Figure 2). Although it is difficult to speculate on the cause of this difference, it may be that PER2::LUC amplitude is influenced by the stress of surgery, single housing, and/or aging. Future work exploring the relationship between adrenal clock function, glucocorticoid production, and stress is warranted.

Clock-regulated glucocorticoid release may have a profound influence on a wide range of biological process by acting on a variety of targets. The role of the clock in regulating daily release of glucocorticoids is well established and represents the proactive marshaling of energy during the active phase. In addition, the glucocorticoid rhythm produced by the adrenal gland is important for transducing suprachiasmatic nucleus output and drives molecular rhythms in a variety of peripheral and central targets, such as the liver, muscle, adipocytes, and the limbic forebrain. Lastly, the circadian clock can buffer the effects of glucocorticoids by limiting the induction of glucocorticoid-responsive genetic programs (10, 58). Within this context, pronounced sex differences in clock function may have important implications for both circadian and noncircadian processes, including stress-related mental health disorders.

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