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Circadian *Clock* Mutation Disrupts Estrous Cyclicity and Maintenance of Pregnancy

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Summary

Classic experiments have shown that ovulation and estrous cyclicity are under circadian control, and surgical ablation of the suprachiasmatic nuclei (SCN) results in estrous acyclicity in rats [1-3]. Here, we characterized reproductive function in the circadian *Clock* mutant mouse,[4, 5] and found that the circadian *Clock* mutation both disrupts estrous cyclicity and interferes with the maintenance of pregnancy. *Clock* mutant females have extended and irregular estrous cycles, lack a coordinated LH surge on the day of proestrus, exhibit increased fetal reabsorption during pregnancy, and have a high rate of full-term pregnancy failure. *Clock* mutants also show an unexpected decline in progesterone levels at mid-pregnancy and a shortened duration of pseudopregnancy, suggesting that maternal prolactin release may be abnormal. In a second set of experiments, we interrogated the function of each level of the hypothalamic-pituitary-gonadal (HPG) axis in order to determine how the *Clock* mutation disrupts estrous cyclicity. We report that *Clock* mutants fail to show an LH surge following estradiol priming, in spite of the fact that hypothalamic levels of gonadotropin-releasing hormone (GnRH), pituitary release of luteinizing hormone (LH), and serum levels of estradiol and progesterone are all normal in *Clock*/*Clock* females. These data suggest that *Clock* mutants lack an appropriate circadian daily timing signal required to coordinate hypothalamic hormone secretion. Defining the mechanisms by which the *Clock* mutation disrupts reproductive function offers a model for understanding how circadian genes affect complex physiological systems.

Results and Discussion

Clock mutant females exhibit defects in estrous cyclicity and proestrus LH release

The suprachiasmatic nuclei (SCN) of the hypothalamus coordinate circadian physiology and behavior by functioning as the master pacemaker in a hierarchical system of multiple circadian oscillators: the SCN receive photic input from retinal ganglion cells and subsequently phase-coordinate the activity of tissue-specific oscillators via neuronal and humoral output [6]. Circadian output from the SCN plays a major role in the regulation of female reproduction. Previous studies have shown that, in rats, disruption of communication between the SCN and the GnRH neurons responsible for regulating reproductive function—either by ablating the SCN or by severing the neuronal pathways between the SCN and the preoptic area—results in estrous acyclicity and infertility [2, 3]. Circadian output from the

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SCN can also be altered by disrupting core gene components of the molecular pacemaker, including *Clock* [4], *Bmal1* [7], *mPer1* and *mPer2* [8, 9] and *mCry1* and *mCry2* [10, 11]. Therefore, analysis of mice with 'clock gene' mutations provides a way to dissect genetically the role of circadian rhythms in reproduction. Here, we characterized reproductive function in the female *Clock* mutant mouse, which carries a 51-amino acid deletion in the transcriptional activation domain of the CLOCK protein [5, 12]. Wildtype, *Clock/+*, and *Clock/Clock* females were examined daily for the onset of vaginal opening and, upon reaching 10 weeks of age, were monitored by vaginal lavage to record estrous cyclicity (see Supplemental Data for a detailed description of all Materials and Methods). Although the timing of vaginal opening was normal (Supplemental Table 1), there were significant differences in the characteristics of estrous cycles among genotypes (Figure 1A): while wildtype females and the majority of *Clock*/+ females exhibited multiple consecutive 4-5 day long cycles, *Clock/Clock* females had prolonged and irregular cycles characterized by significantly fewer days of proestrus and more days of estrus (Figure 1B, Supplemental Figure 1).

Abnormal estrous cycles could be due to either central or peripheral defects. Therefore, we began by examining ovarian function in intact cycling females. Wildtype, *Clock/+*, and *Clock/ Clock* females were sacrificed on the afternoon of diestrus or proestrus, serum levels of estradiol and progesterone were measured by radioimmunoassay (RIA), and ovaries were fixed for histological analysis. In all three genotypes, estradiol (Supplemental Figure 2A) and progesterone (Supplemental Figure 2B) levels were low on diestrus and significantly elevated on the afternoon of proestrus, and histological analysis of ovarian tissue from proestrus *Clock/Clock* mice showed no gross morphological abnormalities (Supplemental Figure 3, Supplemental Table 2). Follicles at all stages of development were present, and both corpora lutea and Graafian follicles were present in numbers comparable to those observed in wildtype tissue. These results suggest that the estrous cycle irregularities we observed in *Clock/Clock* females do not stem from an effect of the *Clock* mutation acting at the level of the ovary.

We then investigated the timing and amplitude of the LH surge by collecting serial blood samples from ovary-intact wildtype and *Clock/Clock* mice on the morning, afternoon, and evening of proestrus, as defined by a nucleated vaginal smear. The proportions of wildtype (50%) and *Clock/Clock* (0%) mice exhibiting an LH peak of 10 ng/ml or greater differed significantly (chi square, $p < 0.01$, Figure 2A). None of the *Clock/Clock* females exhibited LH concentrations exceeding 1.8 ng/ml, and concentrations in the majority of mutants never varied from baseline (0.2 ng/ml). Maximum serum LH concentrations were significantly lower in *Clock/*Clock than wildtype females (Student's t-test. $p < 0.05$, Figure 2B).

It is possible that *Clock* mutants have a normal LH surge that is not temporally associated with a nucleated smear, but the hormonal profiles we observed make this unlikely. A minimum of 30 hours of exposure to estradiol is required to induce GnRH release [13]; both vaginal cytology and serum sampling show that *Clock* mutants have low estradiol levels prior to the day of a nucleated smear. Therefore, a large LH surge is probably not occurring before the initial sampling time (ZT5). It is also unlikely that a surge is occurring long after the morning of a nucleated smear, as extended elevation of progesterone results in inhibition of LH release [14], and *Clock* mutants exhibit elevated progesterone coincident with a nucleated smear. Thus, the most parsimonious explanation is that the small elevations in LH observed throughout the day of proestrus in individual *Clock* mutants are sufficient to induce ovulation.

Clock mutant females exhibit elevated rates of fetal reabsorption and pregnancy failure

To our knowledge, circadian rhythm defects have never been implicated in the abnormal progession of pregnancy, although some researchers have suggested a connection [15], and anecdotal evidence from our laboratory suggested that *Clock* mutant females regularly failed to produce offspring. To verify and characterize this defect, wildtype and *Clock/Clock* females were mated with wildtype males, and the initiation and progression of pregnancy was measured by observing fetal reabsorption at 11, 14, and 17 days post copulation (dpc), and at full-term. Although the early stages of pregnancy were normal in *Clock* mutants, *Clock/Clock* females showed an increased rate of midgestation fetal reabsorption, and pregnancy failure at full-term, compared to wildtype females. The percentage of *Clock/Clock* pregnancies showing signs of fetal reabsorption was significantly different from wildtype pregnancies at two time points, dpc 14 and full-term, and the percentage of fetuses being reabsorbed by *Clock* mutant dams was elevated at dpc 14 and full-term (chi square. $p < 0.05$. Figure 3A, Supplemental Figure 4). All of the wildtype females and 57% (4 of 7) of the *Clock/Clock* females allowed to carry their pregnancies to full term delivered live litters on dpc 20. However, 43% (3 of 7) of the *Clock/Clock* females either went into an extended but non-productive labor or failed to enter labor and instead fully reabsorbed the full-term fetuses.

Estradiol and progesterone are vital for maintaining uterine receptivity to developing fetuses during pregnancy and promoting parturition [16]. Therefore, we measured estradiol (Figure 3B) and progesterone (Figure 3C) levels at dpc 11, 14, 17, and 19 (the day before expected parturition) to determine whether altered ovarian hormone levels could explain the pregnancy phenotype in *Clock/Clock* females. *Clock* mutants exhibited significantly lower estradiol levels compared to wildtypes throughout pregnancy, although both genotypes displayed an increase in estradiol levels from mid-pregnancy (dpc 11, dpc 14) to late pregnancy (dpc 17, dpc 19). At full-term, estradiol in *Clock* mutants was only one-third the level of estradiol in wildtype females. Because estradiol is important for enhancing uterine contractility [17], it is likely that low estradiol levels were at least partially responsible for the failure of some *Clock/Clock* females to initiate labor.

Progesterone levels were also reduced in *Clock* mutants, most notably at dpc 11. Mid-gestational levels of progesterone are particularly important for maintaining blood flow to developing fetuses [18], and previous studies have shown a quantitative relationship between progesterone levels and maintenance of pregnancy [19]. Therefore, the reduced progesterone levels observed in *Clock* mutant dams at dpc 11 may explain the fetal reabsorption observed by dpc 14. By dpc 14, progesterone levels in *Clock* mutants had risen, and the rate of fetal reabsorption in *Clock/Clock* females remained similar to that in wildtypes until full-term.

The duration of pseudopregnancy is shortened in *Clock* mutant mice

We hypothesized that the observed abnormalities in fetal reabsorption and ovarian hormones in pregnant *Clock/Clock* females could be explained by abnormal prolactin release during early and/or mid-pregnancy. Prolactin release is induced by the copulatory stimulus, and thereafter occurs in two daily surges that are roughly coordinated to lights-on and lights-off, suggesting that release is under circadian control and is therefore vulnerable to gene mutations that alter the circadian pacemaker [20, 21]. Prolactin is the only factor required to rescue and maintain the ovarian corpora lutea (CL), which produce the elevated serum progesterone levels characteristic of and necessary for pregnancy and pseudopregnancy [22]. At mid-gestation, around dpc 10, maternal prolactin release tapers off and the CL are supported for the remainder of pregnancy by the prolactin-like placental lactogen (PL-1) produced by fetuses [23]. If the mating was infertile, there is no PL-1 to rescue the CLs, and

the female resumes estrous cyclicity. Thus, the termination of pseudopregnancy is a reliable indicator of the termination of mating-induced PRL release. Therefore, in order to determine whether a defect in maternal prolactin release might be responsible for low *Clock/Clock* progesterone levels at dpc 11 and subsequent elevated fetal reabsorption at dpc 14, we measured the duration of pseudopregnancy. This functional assessment was used rather than measurement of PRL levels because the expected variability in the timing of PRL secretion and prevalence of PRL release in response to stress would have made interpretation of the results difficult.

Pseudopregnancy was significantly shortened in *Clock/Clock* females compared to wildtype controls (Figure 3D), suggesting that PRL secretion may cease earlier in *Clock/Clock* mice than in wildtype females. The functional consequences of premature cessation of prolactin release are partial or complete CL regression, a drop in progesterone levels, and either abortion or an increase in fetal reabsorption [18]. These consequences are consistent with our observations. Although it is possible that there is a failure on the part of the *Clock/+* and *Clock/Clock* fetuses to begin to support the CLs at the appropriate developmental stage, the cessation of pseudopregnancy in mutant females often occurs several days prior to the onset of fetal PL-1 production. We have also observed that, when a litter of *Clock/Clock* embryos is transplanted into a pseudopregnant wildtype female, the pregnancy progresses normally, indicating that *Clock* mutant fetuses are capable of sustaining pregnancy in normal females (BHM, personal observation). Therefore, at least some of the *Clock* mutant pregnancy abnormalities are likely due to abnormal circadian control of maternal prolactin release. Our hypothesis is consistent with the findings of other groups that have described a link between the circadian system and prolactin secretion [24].

Abnormal LH release in *Clock* mutants is due to a hypothalamic defect

The role of circadian rhythms in LH release and the estrous cycle is more thoroughly described than in pregnancy. Therefore, we focused on the estrous cycle to determine how the *Clock* mutation could result in altered reproductive physiology. We hypothesized that *Clock/Clock* females display irregular estrous cycles and fail to have a coordinated LH surge due to a disruption of the daily timing signal from the SCN to the GnRH neurons, rather than as a result of pituitary defects or inappropriate feedback from ovarian hormones.

In rodents, coordinated GnRH release on the afternoon of proestrus requires both a daily timing signal originating in the SCN and permissive levels of estrogen and progesterone [25]. To determine whether the observed defect in LH release in *Clock* mutants was due to inappropriate steroid feedback, ovariectomized mutant and wildtype mice received low-dose estradiol capsules followed by an injection of estradiol benzoate; a paradigm that induces an LH surge during the circadian-timed window. Estradiol capsules alone resulted in baseline levels of LH in both genotypes due to negative feedback on GnRH neurons (Figure 4A). Injection with estradiol benzoate resulted in positive feedback and a subsequent LH surge in wildtype mice, but failed to induce an LH surge in *Clock/Clock* mice. These data are consistent with the hypothesis that *Clock* mutants lack a coordinated daily timing signal triggering GnRH release, and rules out the possibility that improper levels of ovarian hormones prevent ovary-intact *Clock* mutants from mounting a coordinated LH surge.

Several studies show that GnRH transcription is under circadian control, and the *Clock* mutation results in the down-regulation of transcription of a number of genes in addition to the core clock genes, introducing the possibility that the *Clock* mutation alters GnRH synthesis [26, 27]. To test this hypothesis, we measured GnRH by RIA in hypothalami from wildtype, *Clock/+*, and *Clock/Clock* mice on the afternoon of proestrus. Hypothalamic GnRH content was normal in *Clock/+* and *Clock/Clock* females (Figure 4B); thus, the

mutation does not interfere with the production of GnRH, and *Clock* mutants have an adequate supply of the peptide available for release on proestrus.

Finally, we evaluated pituitary function *in vivo* by measuring serum LH following treatment with GnRH in wildtype and mutant females. Expression of the core clock gene *Period2* is rhythmic in pituitaries in culture, raising the possibility that the *Clock* might interfere directly with either LH production or release at the level of the pituitary [28]. However, pituitary release of LH following a GnRH challenge was identical in wildtype and *Clock/Clock* females (Figure 4C), indicating that pituitary function is normal in *Clock* mutants.

Since all aspects of the HPG axis other than GnRH/LH release are normal in *Clock* mutants, estrous cycle defects in the *Clock* mutants appear to result from a disruption of the timing and/or coordination of GnRH release on proestrus. This disruption may be due to either altered output from the SCN to GnRH neurons or an effect of the *Clock* mutation on GnRH neurons themselves. Several groups have shown that core clock genes, including *Bmal1* and *Per1*, are expressed and cycle in an immortalized GnRH cell line (GT1-7), suggesting that intracellular pacemakers within GnRH neurons may be important for the circadian regulation of GnRH release [29, 30]. It is possible that expression of the *Clock* mutation within GnRH neurons may disrupt the expression of other proteins necessary for GnRH release, such as hormone or ion channel receptors. In support of this, Chappell and colleagues found that transfection of GT1-7 cells with the CLOCK-Δ19 dominant negative mutation decreases mean GnRH pulse frequency, whereas overexpression of *Cry1*, which inhibits CLOCK-induced transcription, increases GnRH pulse frequency [29]. However, the ultimate relationship between GnRH neuron pulsatility and the regulation of proestrus GnRH release remains undefined. It is therefore unclear whether the extent of the disruption of GnRH pulsatility induced by the *Clock* mutation in GT1-7 cells would be sufficient to cause the defects in LH release and estrous cyclicity that we observed in *Clock/Clock* mice.

Although a direct effect of the *Clock* mutation on GnRH neurons *in vivo* remains to be examined, we hypothesize that *Clock* mutants fail to have an LH surge because the SCN in mutant animals does not provide a coordinated time-of-day signal to GnRH neurons. Likely candidates for conveying the SCN-GnRH signal are the neuropeptides vasopressin and vasoactive intestinal peptide (VIP), both of which are expressed in neurons projecting from the SCN to the preoptic area [31]. VIP-containing fibers make direct projections to the population of GnRH neurons that expresses *c-fos* on the afternoon of proestrus [32, 33], and suppression of VIP synthesis in the SCN attenuates peak LH levels during estradiol-induced surges [34]. Vasopressin-containing neurons project to interneurons adjacent to GnRH fibers [35], and, in rats, inhibition of vasopressin signaling on the morning of a hormone-induced surge significantly attenuates LH release [36]. Furthermore, vasopressin expression is rhythmically controlled by CLOCK-induced transcription, and vasopressin content in the SCN is drastically reduced in *Clock* mutants, suggesting a molecular mechanism for the missing time-of-day signal in *Clock/Clock* females [37]. Further studies are necessary to refine our understanding of the roles these peptides play in controlling GnRH release. More generally, characterization of possible reproductive defects in other circadian mutants will help to clarify the role of the *Clock* gene specifically, as opposed to the role of the molecular pacemaker, in the circadian control of reproductive function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

SCN	suprachiasmatic nucleus
GnRH	gonadotropin-releasing hormone
HPG	hypothalamic-pituitary-gonadal
LH	luteinizing hormone
FSH	follicle stimulating hormone
CL	corpus luteum
DPC	days post copulation
RIA	radioimmunoassay
VIP	vasoactive intestinal peptide

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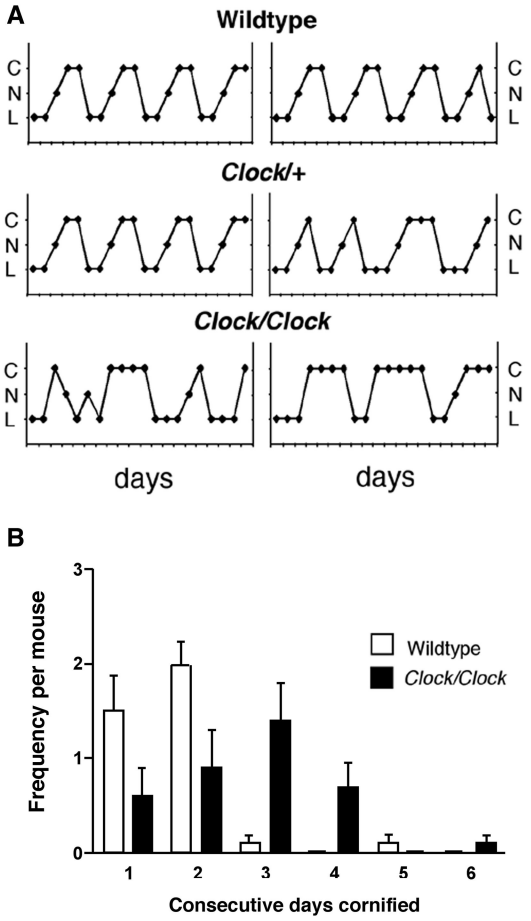

Figure 1. *Clock/Clock* females display lengthened and irregular estrous cycles

A, Representative estrous cycles as measured by vaginal cytology in wildtype (top), *Clock*/+ (middle), and *Clock/Clock* (bottom) females. C = cornified, N = nucleated, L = leukocytic. *B, Clock/Clock* females have significantly more days of cornified smears compared to wildtype females, as determined by an unpaired t-test for the number of consecutive days cornified (* indicates $p < 0.05$, ** indicates $p < 0.01$).

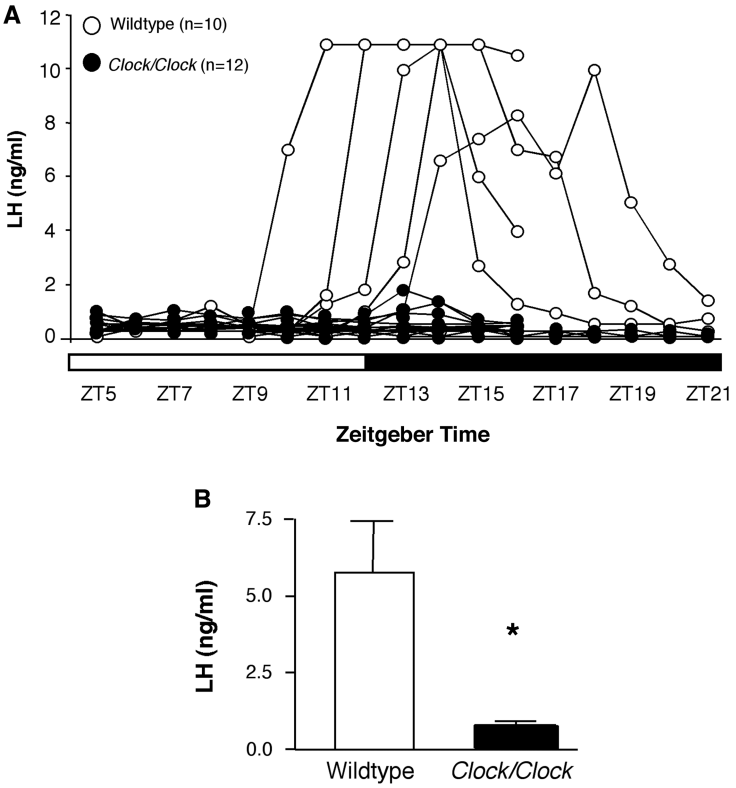

Figure 2. *Clock/Clock* mutants fail to have a coordinated LH surge on the day of proestrus

A, Individual LH traces from all wildtype (open circles) and *Clock/Clock* (closed circles) females sampled from either ZT5-16 or ZT9-21. *B*, Individual peak LH values in serum obtained from serially sampled mice. Peak *Clock/Clock* LH values were compared using an unpaired t-test and found to be significantly lower than peak wildtype values (** indicates $p < 0.01$). Due to the limited sample volume collected, samples with LH values exceeding the range of the RIA could not be re-assayed at lower sample concentrations, preventing absolute measurements of serum concentrations above 10 ng/ml. Therefore, for analysis and presentation purposes, these values were set at 10 ng/ml, and we defined the minimum "surge" value as 14% of the maximum, or 1.4 ng/ml, as previous studies in rats have shown that 14 percent of the peak surge is the minimum value of LH required for ovulation [38]. Using these criteria, only one *Clock* mutant reached the minimum surge level at any time point.

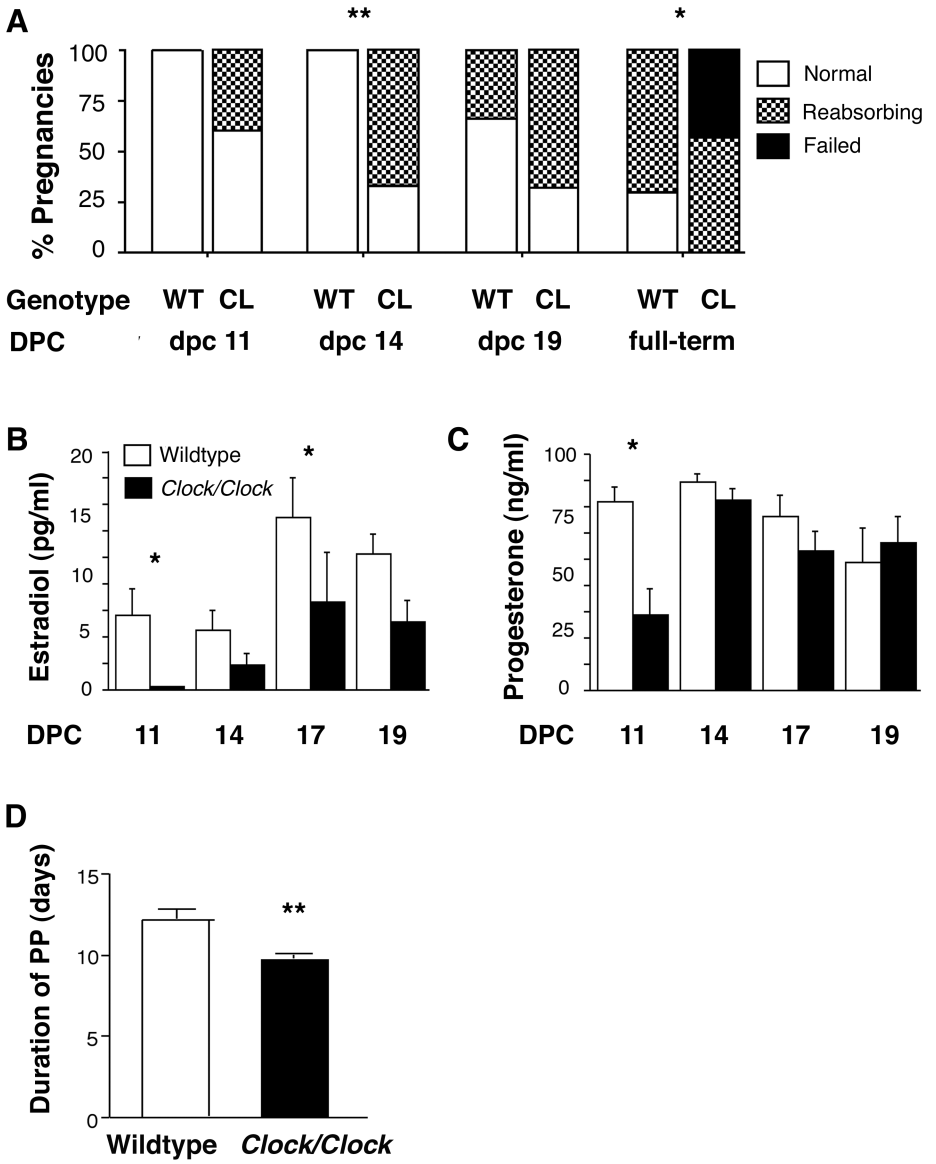

Figure 3. *Clock/Clock* pregnancies are characterized by increased rates of fetal reabsorption, reduced serum levels of estradiol and progesterone, and possible dysregulation of prolactin release

A, The percentage of *Clock* mutant dam pregnancies showing any signs of fetal reabsorption is significantly greater than wildtype at dpc 14 (** indicates $p < 0.01$) and full-term (* indicates $p \leq 0.05$), as determined by chi square analysis. In many cases, both wildtype and *Clock* pregnancies showed some signs of reabsorption by full-term, but reabsorbing fetuses occurred more frequently in *Clock* pregnancies. While all wildtype pregnancies delivered normally, 40% of *Clock* mutant dams carried to full-term but failed to deliver. Importantly, *Clock* mutant females exhibited copulatory plugs as frequently as wildtype females, indicating that mating behavior was intact in the mutants. Additionally, the average number of fetuses per genotype at dpc 11 was almost identical between genotypes (7.6 fetuses per wildtype dam, 7.75 fetuses per *Clock/Clock* dam), indicating that the early stages of pregnancy, including ovulation, fertilization, implantation, and early fetal development, occur normally in *Clock* mutants. WT = wildtype, CL = *Clock/Clock*, DPC = days post copulation. *B*, Estradiol levels are significantly reduced in *Clock/Clock* (closed circles) mice

compared to wildtypes (open circles) ($p < 0.001$). Estradiol in both genotypes is elevated in late pregnancy (dpc 17, 19) compared to mid-pregnancy (dpc 11, 14) ($p < 0.01$). *C*, Progesterone is significantly lower in *Clock/Clock* females compared to wildtypes ($p < 0.05$) due to very low progesterone levels in *Clock/Clock* females at dpc 11. In both genotypes, progesterone levels at dpc 14 are elevated compared to dpc 11 and dpc 19 values ($p \leq 0.01$). For both *B* and *C*, * indicates $p < 0.05$. Prior to testing the hypotheses, data were checked to verify that they met the assumptions of normality and equality of variance required for analysis of variance. Because the data violated one or more of the assumptions, data were transformed using logarithms and statistical analysis (two-way ANOVA and Fisher's LSD) was performed on the log transformed data. *D,* The duration of pseudopregnancy is significantly shorter in *Clock/Clock* females compared to wildtype controls as determined by t-test (** indicates $p \leq 0.001$). Wildtype ($n = 10$) and *Clock/Clock* ($n = 26$) females were mated with vasectomized CD-1 males and the length of pseudopregnancy, as indicated by leukocytic vaginal cytology, was measured by vaginal lavage. The presence of a copulatory plug was designated as dpc 1, and the final day of leukocytic smears was designated as the last day of pseudopregnancy.

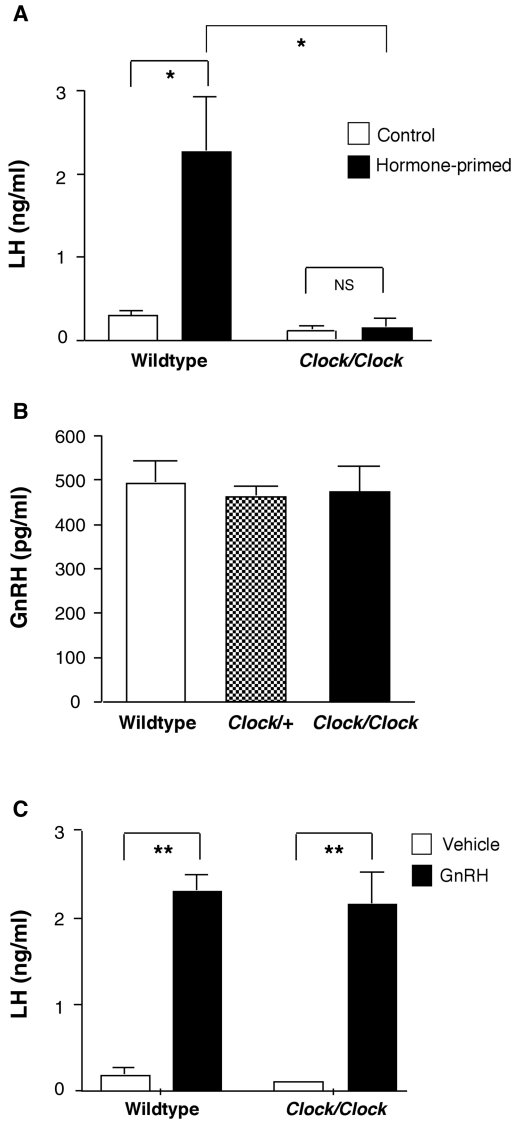

Figure 4. Hypothalamic-pituitary gonadal axis function in *Clock* mutants

A, Estradiol benzoate treatment resulted in a significant elevation in serum LH in wildtype ovariectomized females (* indicates $p < 0.01$), but did not produce elevated LH levels in *Clock/Clock* females. NS = not significant. Two-way ANOVA. Treatment ($F = 8.9$, df (1,24) $p < 0.01$); Genotype ($F = 11.8$, df (1,24), $p < 0.01$). Mice were ovariectomized and implanted with estradiol capsules. Six days following implantation, mice received an injection of either estradiol benzoate or sesame oil at 0800h, and samples were collected the following evening at ZT13. *B,* There is no difference in hypothalamic GnRH peptide content among wildtype, *Clock*/+, and *Clock/Clock* females on the afternoon of proestrus. One-way ANOVA ($F = 0.1$, df (2,11), $p = 0.9$). *C,* GnRH treatment (400 ng/kg, sc) resulted in significantly increased serum LH compared to control levels in both wildtype and *Clock/Clock* females that had been hormone primed as described above (** indicates $p < 0.001$).